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Molecular epidemiology of HPV and Chlamydia trachomatis pathogenic agents in a large cohort of women from a North-East Italian area: prevalence and distribution of HPV genotypes in the setting of co-infection with Chlamydia trachomatis.

Settore Scientifico Disciplinare BIO/12

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Non c' e' niente che sia per sempre

il tuo diploma in fallimento

e' una laurea per reagire

Non è sempre- AFTERHOURS

SUMMARY

INTRODUCTION

In the worldwide, sexual transmitted infections (STIs) represent an important public health problem with an estimated million of new cases of infections occurring each year. Young people account for only 25% of the sexually active population which accounts for almost 50% of newly acquired STIs (Siracusano et al., 2014). These consist in bacterial, fungal and protozoal infections that can be treated with appropriate chemotherapeutic agents or, as reported, can trigger viruses often leading incurable STIs (Gewirtzman et al., 2011).
Microorganisms causing chronic inflammatory diseases have become to be increasingly investigated in the last decade as possible cancer initiators/promoters (Idahl et al., 2011).
So far, the most common STIs are those caused by Human papillomavirus (HPV) and *Chlamydia trachomatis* (CT). However, epidemiological data on CT prevalence and CT/HPV co-infection are not yet well defined in Italy (Bellaminutti et al. 2014, Panatto et al 2014; Seraceni et al. 2014).

HUMAN PAPILLOMAVIRUS

Characteristics

Papillomavirus belong to the Papovaviridae family, which includes 16 different genera, five in humans (*Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus*, *Nupapillomavirus*), that have different types, life-cycle characteristics and disease associations, based on DNA sequence analysis. Of these, The Alpha genus contains the mucosal type viruses that cause genital warts or lesions associated with the development of cervical neoplasia and cancer at low-risk (LR-HPV) and high risk (HR-HPV), respectively. The Beta genus, instead, contains those genotypes that are associated with the development of cutaneous cancers. Their possible role in cancer progression in the general population is currently unresolved (Bernard et al., 2013) (Figure 1).

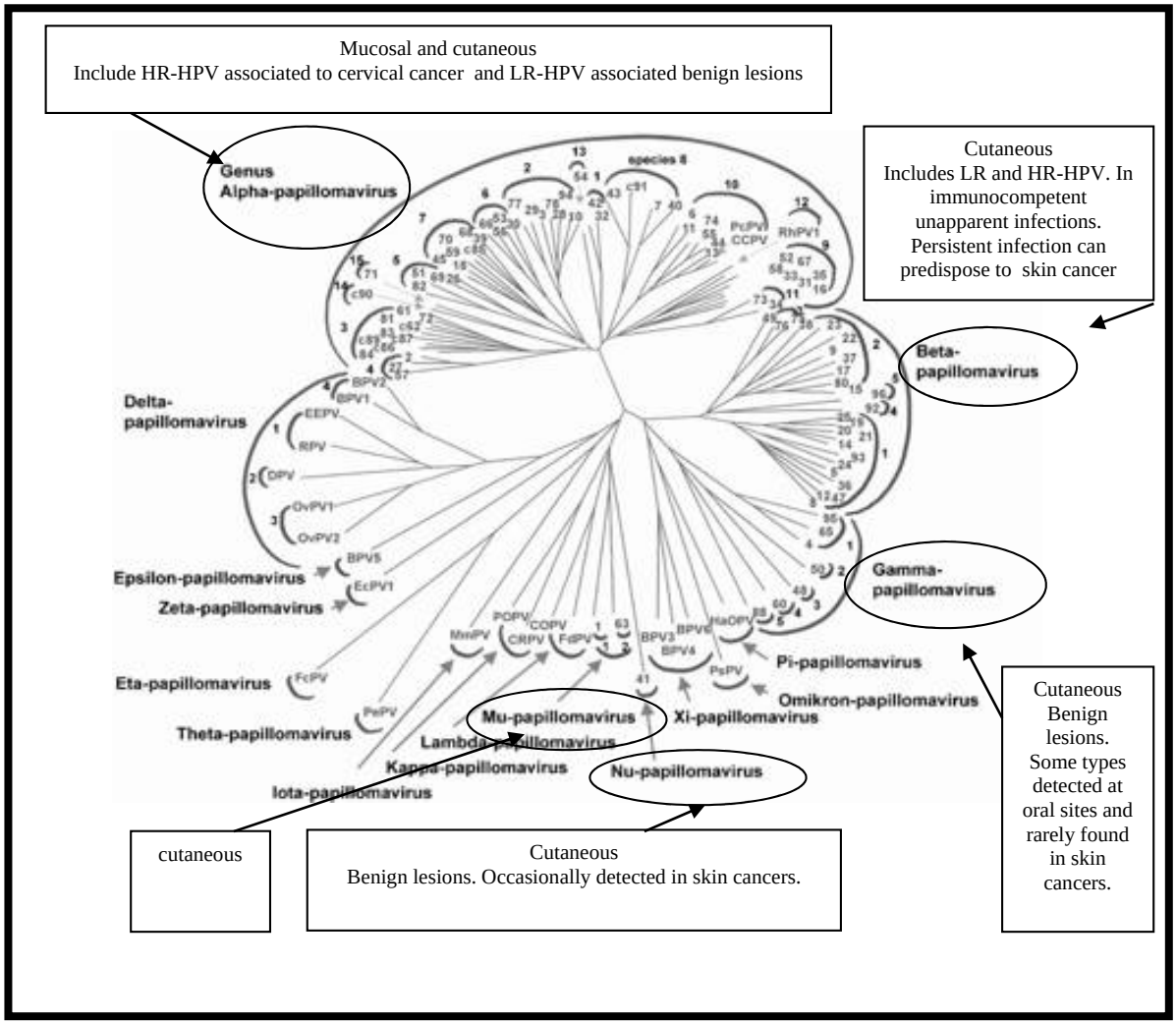


Fig. 1: Evolutionary Relationship between Human Papillomaviruses.

Human Papillomaviruses comprise five evolutionary groups with different epithelial tropisms and disease associations. (Modified and adapted from de Villers et al. 2004). http://www.bioafrica.net/rega-genotype/html/subtypeprocesshpv.html

These viruses contain a double strand DNA with 8000 pb approximately arranged in an 8 well defined genes. All members of the HPV family have a typical genomic organization with 8 or 9 open reading frames (ORFs) on the same DNA strand. The HPV genome is divided into three regions: six early genes (E) are involved in virus expression, replication and survival and two other genes called late genes (L), are involved in virus assembly; finally, the long control region (LCR), which is localized between ORFs L1 and E6, contains most of the regulatory elements involved in viral DNA replication and transcription. The designations E and L refer to the phase in the viral life cycle when these proteins are first expressed (Figure 2). E2 regulates early gene promoter and together E1 forms a heterodimer complex to control virus DNA replication. E4 may mediate the release of viral particles by destabilization of the cytokeratin network, whereas E5 stimulates mitogenic signals of growth factors. E6 and E7 are oncoproteines capable of deregulate

fundamental cellular events, such as cell cycle, DNA repair, senescence, apoptosis and differentiation, facilitating the accumulation of DNA damage and the progression towards malignancy. If the immune system can eliminate quickly the HPV-infected cells, E6 and E7 do not manage to accumulate chromosomal abnormalities and acquire a malignant phenotype, despite their transforming properties. Therefore, the establishment of a chronic infection is a fundamental and crucial event for the development of HPV-associated malignant diseases. Host and environmental factors significantly contribute to the chronicity of HPV infection, despite HR-HPV E6 and E7 target cellular pathways related to innate and adaptive immunity. L1 and L2 are major and minor capsid protein, respectively and L1 is the component of the HPV prophylactic vaccine.

HPV infects the epithelium of the cervix and their replication is closely linked to the differentiation of the epithelium (Doorbar et al., 2012; Tommasino, 2014).

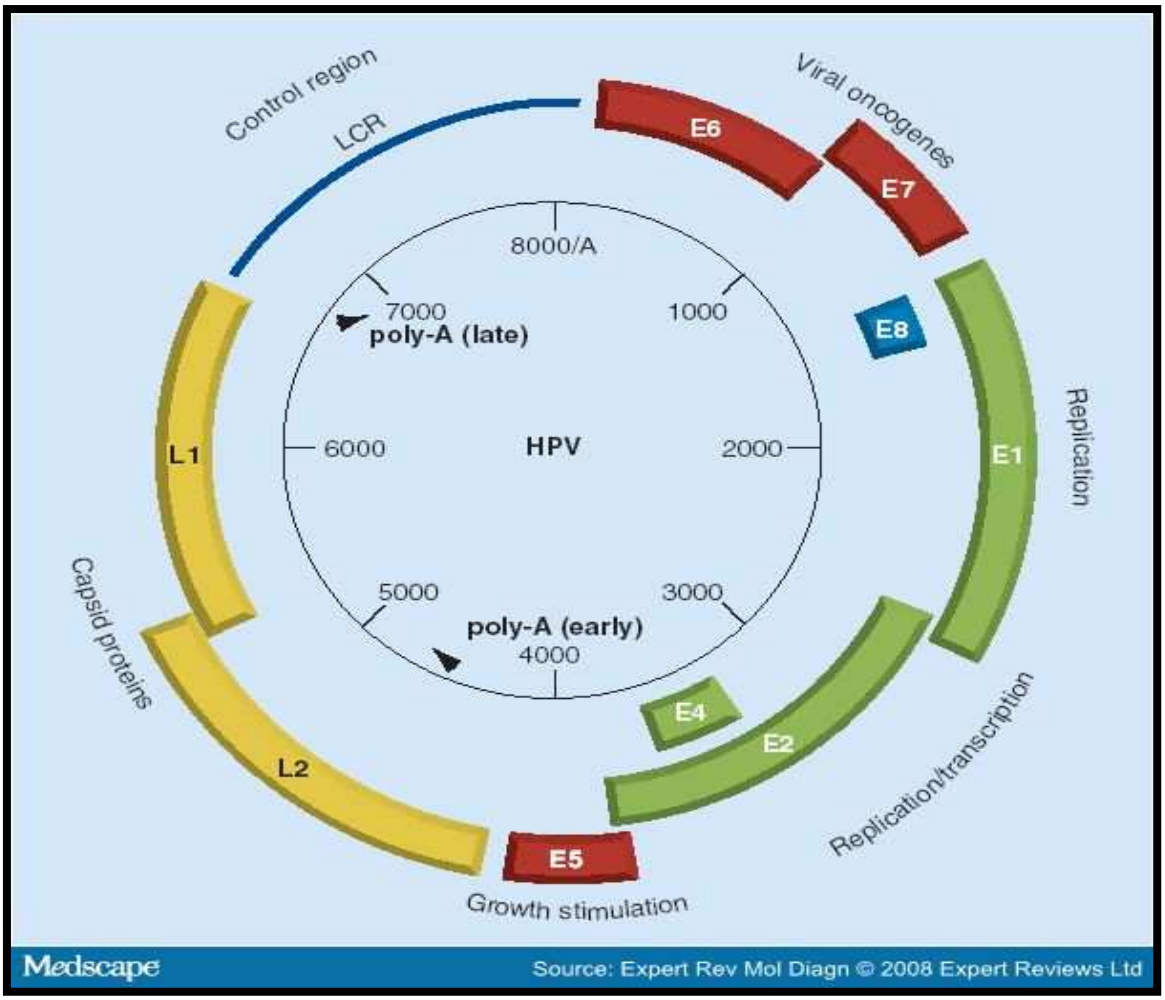
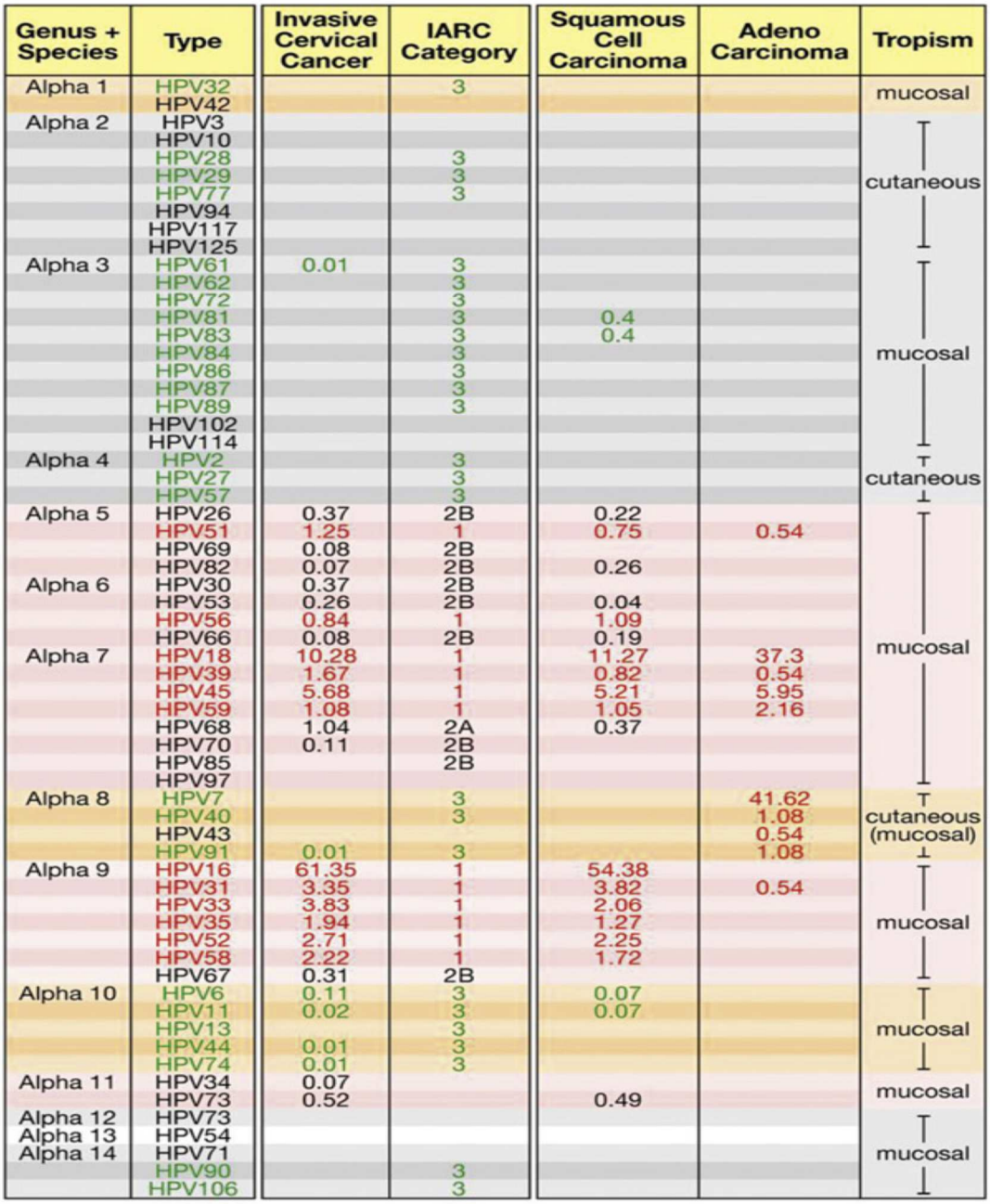


Fig. 2: The genome organization of HPV16 is typical of the high-risk Alphapapillomaviruses (Agnes and Gunnar, 2008). http://.medscape.com.

These virus, of which have been identified more than 150 HPV genotypes, have selective tropism for cutaneous or mucosal epithelia and are divided into two groups: LR-HPV and HR-HPV, as previously described. LR-HPV that cause benign lesions asymptomatic that may resolve spontaneously in 3-4 months, are named papillomas (small wart-like neoplasias), which are due to sexual transmission of the virus, occur in male and female genitals, urethra, anus and perianal area and rarely lead to cancer. HPV6 and HPV11, two among the most common LR-HPV, are associated with 90% of genital warts and recurrent respiratory papillomas. HR-HPV cause malignant cellular transformation and develop into large tumors, characterized by squamous intraepithelial lesion (SIL) that occur with rounded cells with nuclear and perinuclear (koilocytosis) atypia. Nuclear abnormalities, such as enlarged nuclei, hyperchromasia and mitotic cell features can also be found.

HPV infections are also common in intraepithelial cervical neoplasia (CIN); these are characterized by the presence of koilocytosis and are divided into low-grade (LSIL) and high-grade (HSIL). According to the histological classification there are three degrees of CIN: CIN 1 (mild), corresponds to LSIL, CIN2 and CIN3 correspond to moderate and severe HSIL, respectively. HPV infection is the major cause of the CIN development. Despite women's frequent exposure to HPV, the development of cervical cancer (CC) is relatively rare. Most low-grade cervical abnormalities, such as CIN1, are associated with benign viral replication, and spontaneously regress without requiring treatment (Martin et al., 2011). Studies in women have shown CIN1 regression rates of up to 70–80%; however, in adolescents and young women under 25 years, more than 90% show regression (Cox et al., 2003; Moscicki et al., 2004; 2010). In contrast, HSIL, specifically CIN3, has a much greater potential to progress to invasive cancer (progression rates of between 0.2–4% within 12 months) (Fearly et al., 2010). HR-HPV genotypes identified as causing CC belong to groups based on epidemiologic and mechanistic evidence of their carcinogenicity (Rosales and Rosales, 2014). Twelve HPV genotypes (HPV16,-18,-31,-33,-35,-39,-45,-51,-52,-56,-58 and-59) are classified as "carcinogenic to humans" (Group 1), HPV68 as "probably carcinogenic" (Group 2A) whilst others seven HPV genotypes, as "possibly carcinogenic" HPV26,-53,-66,-67,-70,-73,-82 (Group 2B) (IARC, 2012) (Figure 3).



Genus + Species	Type	Invasive Cervical Cancer	IARC Category	Squamous Cell Carcinoma	Adeno Carcinoma	Tropism
Alpha 1	HPV32		3			mucosal
	HPV42					
Alpha 2	HPV3					cutaneous
	HPV10					
	HPV28		3			
	HPV29		3			
	HPV77		3			
	HPV94					
	HPV117					
	HPV125					
Alpha 3	HPV61	0.01	3			mucosal
	HPV62		3			
	HPV72		3			
	HPV81		3	0.4		
	HPV83		3	0.4		
	HPV84		3			
	HPV86		3			
	HPV87		3			
	HPV89		3			
	HPV102					
	HPV114					
Alpha 4	HPV2		3			cutaneous
	HPV27		3			
	HPV57		3			
Alpha 5	HPV26	0.37	2B	0.22		mucosal
	HPV51	1.25	1	0.75	0.54	
	HPV69	0.08	2B			
	HPV82	0.07	2B	0.26		
Alpha 6	HPV30	0.37	2B			
	HPV53	0.26	2B	0.04		
	HPV56	0.84	1	1.09		
	HPV66	0.08	2B	0.19		
Alpha 7	HPV18	10.28	1	11.27	37.3	
	HPV39	1.67	1	0.82	0.54	
	HPV45	5.68	1	5.21	5.95	
	HPV59	1.08	1	1.05	2.16	
	HPV68	1.04	2A	0.37		
	HPV70	0.11	2B			
	HPV85		2B			
	HPV97					
Alpha 8	HPV7		3		41.62	cutaneous (mucosal)
	HPV40		3		1.08	
	HPV43				0.54	
	HPV91	0.01	3		1.08	
Alpha 9	HPV16	61.35	1	54.38		mucosal
	HPV31	3.35	1	3.82	0.54	
	HPV33	3.83	1	2.06		
	HPV35	1.94	1	1.27		
	HPV52	2.71	1	2.25		
	HPV58	2.22	1	1.72		
	HPV67	0.31	2B			
Alpha 10	HPV6	0.11	3	0.07		mucosal
	HPV11	0.02	3	0.07		
	HPV13		3			
	HPV44	0.01	3			
	HPV74	0.01	3			
Alpha 11	HPV34	0.07				mucosal
	HPV73	0.52		0.49		
Alpha 12	HPV73					mucosal
Alpha 13	HPV54					
Alpha 14	HPV71					
	HPV90		3			
	HPV106		3			

Fig. 3: The HR Alpha types have been clearly linked with the development of squamous cell carcinoma (SCC) and adenocarcinoma (AC) of the cervix. IARC category 1 and 2A HPV genotypes are classified (respectively) as carcinogenic and possibly-carcinogenic. Despite limited epidemiological data, the 2B classification is proposed for genotypes that are probably carcinogenic because of their close phylogenetic relationship with the established carcinogenic types. HPV genotypes in category 3 are considered non-carcinogenic (Doorbar et al., 2012).

HPV infection and Life Cycle

So far, most of the biological studies concerning the clear association of mucosal HR-HPV genotypes with human carcinogenesis (Zur Hausen, 2002), have been focused on these types. HPV infects the cells of the basal layer, where it is present at a relatively low copy number. The HR-HPV life cycle is tightly linked to the differentiation programme of stratified epithelia. HPV initiates the productive phase of its life cycle, that is characterized by vegetative viral DNA replication, when cells leave the basal layer of the epithelium. During this phase, the HPV genome is amplified to more than 1000 copies per cell and subsequently, the expression of late genes starts and viral particles are produced and released. In contrast to mucosal HPV types, nothing is known about the life cycle of the majority of HPV types that belong to the Beta and Gamma genera. Studies of mucosal HPV types have shown that the first step in HPV infection is the interaction of the viral capsid with the cytoplasmic membrane of cells at the basal layer of the epithelium (Tommasino, 2013). This event is mainly mediated by the major capsid protein, L1, which interacts with the cell surface via heparan sulfonated proteoglycan (HSPG) (Doobar et al., 2012). It is also possible that the viral particles bind to another component of the cellular membrane, the integrin α6, proposed as a secondary cellular receptor for HPV particles (Evander et al., 1997) even if, the precise nature of the entry receptor remains somewhat controversial (Doobar et a., 2012; Tommasino, 2013).

The internalization of HPV16 particles, after binding to the cellular membrane, is mediated by a clathrin-dependent endocytic pathway (Day et al., 2003). Additional findings indicate that other mucosal HPV types may use different endocytosis pathways (Bousarghin et al.,2003). It is also highly likely that the minor capsid protein, L2, plays a role in membrane binding and cellular internalization. In fact, in vitro assays anti-L2antibodies against specific linear epitopes are able to block the internalization of L1/L2 virus-like particles (Kawana et al., 2001**;** Gambhira et al., 2007) and the annexin A2 hetero tetramer contributes to HPV16 infection in an L2-dependent manner (Woodham et al., 2012). Recently, Surviladze and colleagues have presented evidence of a novel mechanism of viral entry, observing that HPV16 particles, after binding to the cell surface, are released as a soluble complex with HSPGs and growth factors. The growth factors mediate the interaction of the soluble complex with their cognate receptors, facilitating the internalization of the viral particles (Surviladze et al., 2012).

Natural history of HPV infection

The HPV lifecycle is closely linked to stratified epithelium differentiation (Pyeon et al., 2009). HPV virions infect the basal epithelium through micro-abrasions in the epidermis. The modality of virus invasion is still not fully understood, but several receptors, including heparan sulphate proteoglycans and alpha-6 integrin, have been associated with this process (Doobar, 2006). Upon migration to the basal cell nucleus, the viral genomes are established as episomes, the early promoter are activated and finally resulting in low levels of viral synthesis. During normal epithelium differentiation, the daughter cells migrate from the basal layer upwards and undergo terminal differentiation. Viruses finally reach the epithelial surface where they form a cornified layer of dead cells, which are eventually eliminated (Figure 4).

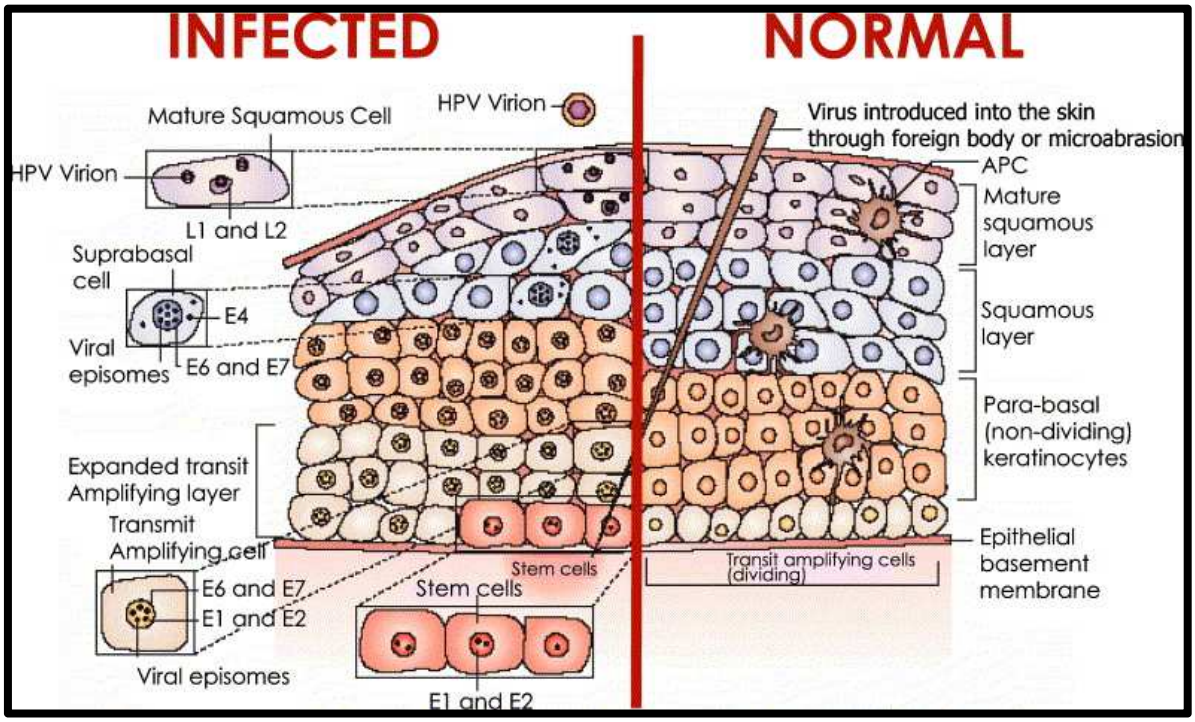


Fig 4: The location in the squamous epithelium of the main stages of the papillomavirus life cycle. Cervical stratified squamous epithelial cell architecture and the expression of HPV proteins after infection. Daughter cells of epithelial stem cells divide along the basement membrane and then mature vertically through the epithelium without further division (right side). **(**Muñoz et al, 2006) http://www.nature.com/nri/journal/v4/n1/fig_tab/nri1260_F2.html .

In HPV-infected differentiating cells, the late promoter is activated, leading to the vegetative state of the HPV lifecycle (Longworth et al., 2004). During this phase, high levels of viral DNA are replicated, enveloped into capsids and released from the cell. To maintain viral synthesis in the epithelium, the virus takes advantage of the host cell replication system. Consequently, the HPV oncoproteins E6 and E7 come into play, maintaining the cell cycle and preventing terminal differentiation. The HPV infected cells move up through the epithelium and the viral infected basal cell layer is maintained with a low level of viral DNA synthesis. This typically occurs in LSIL disease. HSIL lesions, such as CIN3, are typically associated with HPV DNA that has integrated into the host genome. Viral integration often occurs in the E1 and E2 regions downstream of the late genes. This can result in disruption and loss of these late genes, with subsequent loss of control of oncogene expression by the E2 viral gene (Woodman et al., 2007). To maintain the HPV infection, HR-HPV genotypes produce E6 and E7 oncogenes, which interfere with critical cell–cycle checkpoint pathways and proteins, namely p53 and retinoblastoma (Martin et al., 2011) (Fig. 5).

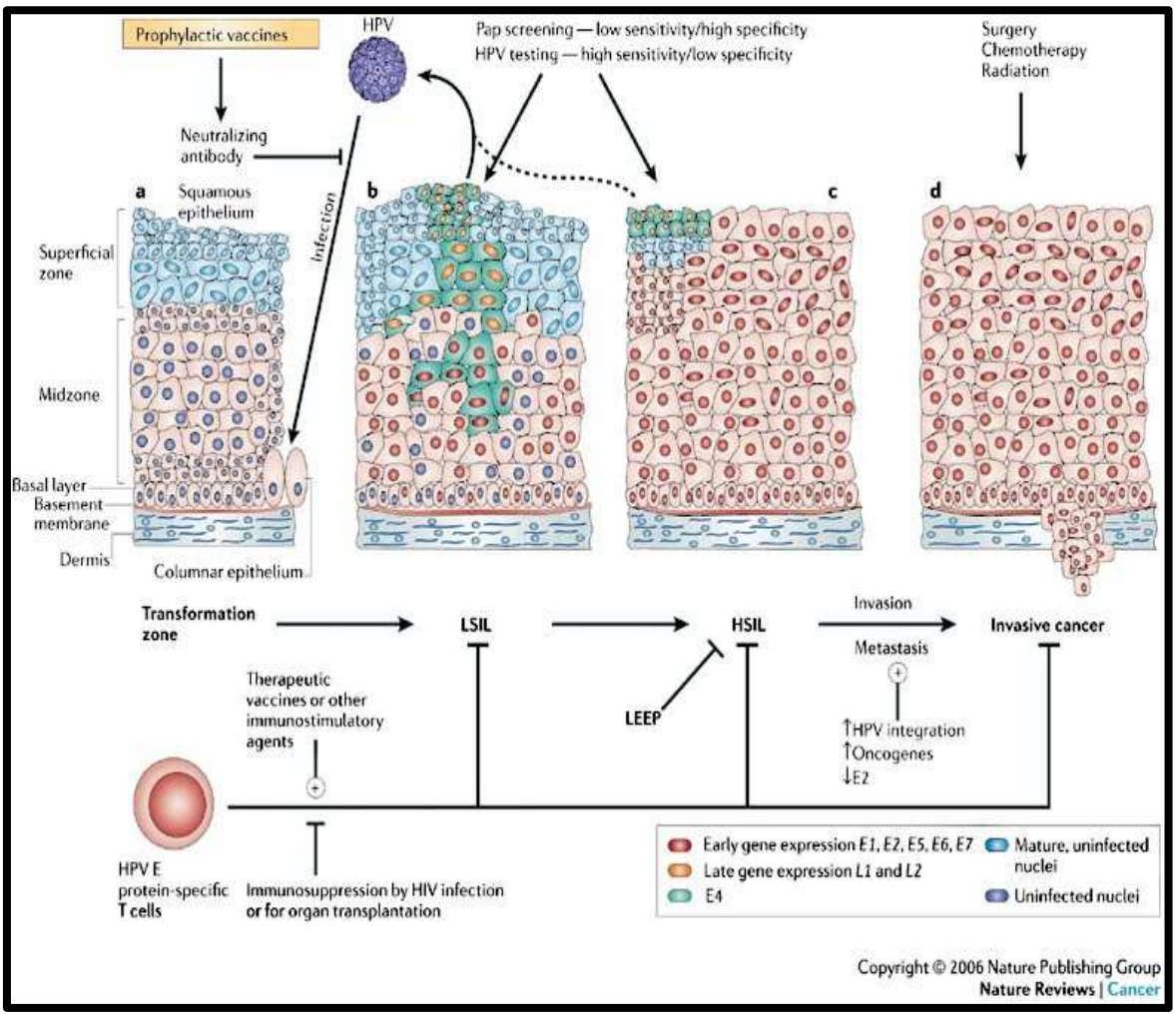


Fig. 5: A: The cervical squamocolumnar junction. The basal cells rest on the basement membrane, which is supported by the dermis. Normal squamous epithelium differentiates as shown. The transformation zone is the most common site for the development of CC. Prophylactic vaccines induce L1- or L2-specific antibodies that neutralize the virus. **B:** After the HPV infection of basal keratinocytes, the early HPV genes E1, E2, E5, E6 and E7 are expressed (red nuclei) and the viral DNA replicates. LSILs support productive viral replication. In the upper layers of epithelium the viral genome is replicated further, and E4 (green cytoplasm), L1 and L2 (orange nuclei) are expressed. L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus then re-initiates infection. **C:** A significant fraction of HR-HPV infections progress to HSILs, which show a lesser degree of differentiation. HSILs are effectively treated by loop electrosurgical excision (LEEP). Pap screening and HPV tests can be used to detect SILs. **D**:The progression of untreated lesions to micro invasive and frankly invasive cancer is associated with the integration of the HPV genome into the host chromosomes, loss of E2 and up-regulation of viral oncogene expression and genomic instability. These cancers are treated with surgery, chemotherapy or radiotherapy with limited success. Therapeutic vaccines and immune stimulants such as imiquimod can potentially induce an infiltration of T cells specific for the early viral antigens and clearance. (Roden and Wu, 2006).

Epidemiology

The worldwide prevalence of HPV infection in women with normal cytology is around 11–12%, with the majority prevalence in sub- Saharan Africa (24%), Eastern Europe (21%) and Latin America (16%) (Bruni et al, 2010). In women less than 25y, HPV prevalence has been observed highest, decreasing average with progression of age in many populations, some of which have a secondary peak in peri-menopausal or early menopausal time (Denis et al., 2008; Ali et al., 2013; Melo et al., 2014; Cuzicke et al., 2014). In China, the prevalence is instead relatively age independent. The explanation of these difference prevalence pattern and the clinical significance is not understood. Globally, the five most prevalent types are HPV16 (3.2%), HPV18 (1.4%), HPV52 (0.9%), HPV31 (0.8%) and HPV58 (0.7%) (Bruni et al, 2010). Prevalence increases in women with cytologic cervical pathology in direct proportion to the severity of the lesion, reaching around 90% in women with CIN3 and invasive CC. In fact, 100% of all CC has been found to be HPV positive. Of note, the proportion of HPV positive women in whom HPV16 is greatly detected, increases with lesion severity (Bosch et al., 2013).

Infection with HR-HPV types is recognized as one of the major causes of infection-related cancer worldwide. A strong evidence for a causal etiology with HPV has been stated by the IARC for cancers of the cervix uteri, penis, vulva, vagina, anus and oropharynx (including base of the tongue and tonsils), which has estimated the overall number of cancers attributed to HPV and classified by geographic region.

The prevalence of HPV infection with specific genotypes differ by age and area. In 2008, there were estimated in the world 12.7 million new cancers, of which 700,000 with an HPV-associated cancer site. 610,000 of these were attributable to HPV only (Ferlay et al., 2010) which alone represents 4.8% of the total burden of cancer worldwide that varies widely by geographic region, ranging from 1.2% in Australia and New Zealand to 14.2% in sub-Saharan Africa and 15.5% in India.

Of note, 80.6% of the total number of cases attributable to HPV occurred in underdeveloped countries (6.9%) compared with 2.1% in more developed countries. In the world, CC resulted the third most common female cancer, (approximately 86% of these cases occurred in underdeveloped country), with a strong association between CC incidence and level of development.

In underdeveloped countries, the incidence and mortality rates tend to be higher compared to developed and after 5-year relative survival, in these different conditions is observed a similar pattern 20% *vs* 65%, respectively. Global maps of CC rates show patterns of

variation largely consistent with level of develop. According to a meta-analysis regarding the prevalence of HPV genotypes in women with normal cytology, HR-HPV16, although high and variable across world regions, resulted the most prevalent genotype (22.5%). Others HPV genotypes highly prevalent resulted, HR-HPV18,-52-,31-58,-39,-51,-56 and LR-HPV6 (Bruni et al., 2010).

Italy, mirrors the world trend, with a strong variability of HPV prevalence by age and area (Agarossi et al., 2009; Giorgi Rossi et al., 2010, Giuffrè et al., 2010; Bellaminutti et al., 2014; Carrozzi et al., 2014). HPV16 resulted the most common genotype in our country (Giorgi et al., 2011; Bianchi et al., 2013; Carozzi et al., 2014).

Immunopathogenesis of HPV

The most frequent HPV infections recover spontaneously, within two years from infection and without any clinical manifestation by immune-competent individuals; consequently the immune system is able to effectively eliminate virus-infected cells. However, the natural immune response may be insufficient, not allowing the infection resolution. The virus does not cause viremia, or systemic infection. The virus absence in the blood and its characteristic intracellular replication and not accompanied by cell lysis, involves the inability of the immune system to mount a strong antibody response or to induce inflammation (Rosales and Rosales, 2014).

The initial inflammatory response induced by tissue damage leads to infiltration of immune cells mainly neutrophils, followed by macrophages and later lymphocytes, that recognize “danger” viral molecules detected by pattern recognition receptor (PRR), such as Toll like receptors (TLR) (Kawai and Akira, 2011). The immune response innate induces the lysis of the infected cells and the production of cytokines pro-inflammatory such as IL-1β, IL-6,IL-8, IL-12 and INF-α,-β,-γ, to activate natural killer cells (NK) and other immune cells (Woodworth, 2002).

T cells, activated by recognition of viral proteins, induce the growth and maturation of B cells. These cells, together with the cell-mediated response, are necessary to ensure an effective protection against the virus. The induction of neutralizing antibodies specific for L1 and L2 proteins viral capsid, is critical to prevent the onset of symptoms and the entrance of the virus into cell.

Infiltrations of $CD4^+$ (Helper) and $CD8^+$ (Cytotoxic) T cells that are absent in persistent lesions, has been detected in spontaneously regressing HPV-related lesions, indicating that the adaptive immune response against the virus is important and for the most cases

effective. This adaptive response comprises elements of humoral and cellular immunity (Stanley, 2006).

The humoral immune response, in the majority of patients with HPV infections, is characterized, in the first stage of infection, by antibodies against different HPV proteins such as L1, E2, and E4 proteins. Antibodies against E6 and E7 oncoproteins can be detected in low- and high-grade lesions, when viral DNA gets integrated into cellular genome. However, this antibody response is usually weak and variable. It does not seem to protect from future re-infections (Tjiong et al., 2001) and the seropositivity seems depending on the site of the cancer around the anogenital area, indicating that cancer development may lead to changes in antibody responses in a site-specific fashion (Carter, 2001). Thus, humoral responses are not enough efficient to eliminate established HPV lesions. In addition, antibody titers can persist for many years even after the virus is cleared, so seropositivity is a useful marker for past infection rather than current infection.

Cell-mediated immune responses are more important in clearing HPV-related lesions. T cells HPV-specific are generated to fight and eliminate infected cells; thus both, $CD4^+$ and $CD8^+$ T cells and also responses that include HPV specific regulatory T cells (Treg) that inhibit cytotoxic activity (Welters et al., 2003), are important for elimination of HPV infection. In fact, T cell responses to viral proteins are present in patients who successfully eliminated previous HPV16 infections. Moreover, T cells, with a predominance of Th1 cytokines, were observed in high ratio during regressing lesions. In contrast, in patients presenting CIN or CC, a deficient T cell response with a strong shift to Th-2 cytokine profile, are observed in persistent lesions. Thus, an efficient cytotoxic cell-mediated immune response is critical for elimination of HPV-related lesions. Unfortunately the virus has also evolved mechanisms to interfere with the immune response (Rosales and Rosales, 2014).

HPV Strategies for Evading Host Immune Response

HPV has evolved several mechanisms to evade the immune system. In the areas where HPV replication takes place, the immune surveillance is poor. In the stratified squamous epithelium of the uterine cervix, surveillance by dendritic cells (DCs) greatly declines towards the keratinized layers.

The keratinocytes infected by HPV express a low load of viral proteins and thus, do not induce their lysis. Expression of viral gene products up-regulates progressively with differentiation and upward migration of keratinocytes Moreover, HPV down-regulates, by E6 and E7 oncoproteins, the expression of major histocompatibility complex (MHC) class I molecules, TLR9, and cytokines such as, interferon and interleukin (IL-8) (Stanley, 2006).

In this way, HPV late proteins, which are the most immunogenic, are expressed at areas of poor immune surveillance (Figure 5). In addition, new virions are released through the normal rupture of surface epithelium, reducing any possible inflammatory response and avoiding uptake by DCs. Therefore, HPV replication is a local phenomenon with minimal activation of the immune system. Moreover, a reduced inflammation state is found in persistent lesions and in tumors. This condition correlates with a change in the cytokine profile produced at the site of infection.

A shift to Th2 cytokines is also common in persistent lesions (Bais et al., 2007; Rosenthal et al., 2012). This leads to an inhibitory state for helper $CD4^+$ T cells. In addition, Tregs have been found infiltrating tumors, especially in the early stage of tumor progression (Piersma et al., 2008). Thus, any therapeutic approach must be able to induce a strong HPV-specific immune cell response that involves $CD4^+$, $CD8^+$ cells, and Th1 type cytokines.

However, HPV has also evolved mechanisms to avoid both initial detection and to interfere with adaptive response, that allows the virus to persist and lesions to progress into cancer (Figure 6).

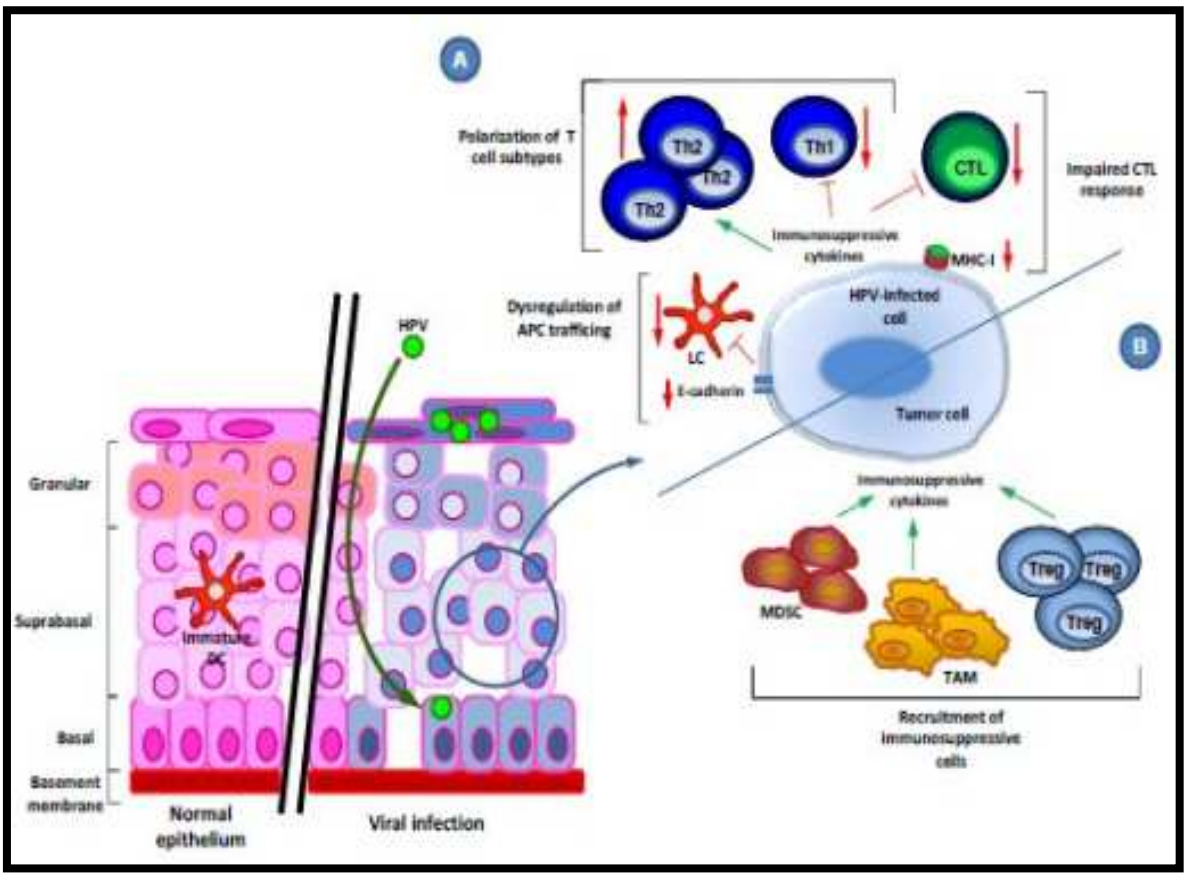


Fig. 6: HPV-mediated effects on the host immune response.

A: Immune evasion mechanisms employed by a HPV-infected cell are polarization of T cell subtypes, inhibition of the CTL ($C8^+$) response and modulation of APC trafficking.

B: Immune evasion mechanisms of HPV-driven malignantly transformed cells include recruitment of immunosuppressive cells, leading to immunosuppressive cytokine production. (Grabowska et a., 2012). http://openi.nlm.nih.gov/

HPV infection and Cervical Cancer

HPV is a sexually transmitted agent deemed a cause of CIN and invasive CC in worldwide. Currently it is widely accepted that specific genotypes of HPV are potentially oncogenic and are associated with virtually all cases of CC and, to a lesser extent, with cancers of the vagina, vulva, anus, penis, skin and oropharynx (Trottier et al., 2006; Parkin et al., 2006; Shukla et al., 2009; Moody et al., 2010).

Over than 100 HPV types classified into HR-HPV are linked to both to tumor precursor lesions and to the progression of invasive CC (Simonetti et al., 2009), the second most common cancer among women (Arbyn et al., 2011) and the seventh in the world (Forman et al.,2012). Persistent infection with one of the oncogenic HPV genotypes is required to cause CC (Walboomers et al. 1999; Bosch et al., 2002).

HPV must be persistent within the host epithelial cells as a preliminary step toward advanced neoplastic changes. This process takes years, if not decades, to occur after initial HPV infection. Persistent infection, with HPV genotypes with high oncogenic potential, increases the probability of ICC following an extended period of latency. The integrity of the cell-mediated immune response against HPV is an important factor for healing and prevention of the reactivation of the latent infection (Fernandes et al., 2013; Stanley, 2006).

Recent studies seem to suggest that these changes may develop more quickly than previously thought. Winer et al., followed women after initial HPV infection for the development of CIN 2/3 and approximately 27% of women with an initial HPV16 or 18 infection progressed to CIN 2/3 within 36 months (Winer et al., 2005). A second study, performed on a large health maintenance cohort, found that approximately 20% of women 30y or older, who were initially infected with HPV16, developed CIN3 or CC within 120 months, while women who had an initial HPV18 infection, had approximately a 15% risk (Khan et al., 2005).

The strong correlation between infection with HR-HPV and LSIL, HSIL, and CC suggests that HPV DNA testing would be an useful tool for the management of women with abnormal Pap test results, especially in the case of those with equivocal test results. In the case of an equivocal Pap test result, HPV DNA testing can help determine whether the individual should be referred for colposcopic assessment (Cox et a., 2003).

Accumulating evidence suggests that a combination of screening strategies is needed to detect as early as possible HPV induced lesions CC.

Epidemiological studies in HPV infected females have provided important clues about a spectrum of cofactors that can increase the carcinogenic HPV potential. Presumably, cervical infections with other pathogens, exposure to physical and chemical agents, hormonal factors and *Chlamydia trachomatis* (CT) itself, as previously demonstrated (Smith et al, 2002; de Abreu et al., 2012). Notably, the infections caused by these two pathogens are often associated with an intense chronic inflammatory response and ulcerations in the cervical epithelium (Scott et al., 1999; Adefuye and Sales, 2012, Silva et al., 2014).

CHLAMYDIA TRACHOMATIS

Characteristics

The *Chlamydiaceae* are a family of ubiquitous gram-negative, aerobic, obligate intracellular bacteria present in the environment including aquatic.

Once considered viruses, they grow in eukaryotic cells and are responsible of a wide range of diseases in human and animals. A recent revision has taxonomically re-classified the group in 4 distinct families (*Chlamydiaceae, Simkaniaceae, Parachlamydiaceae* and *Waddliaceae*) based on > 90% 16S rRNA identity and a single genus which include the different species. *C. pneumoniae* (CP) and *C. trachomatis* (CT) are common pathogens in humans, but the routes of transmission, susceptible populations and clinical presentations, differ markedly, although have a common developmental cycle (Contini and Seraceni, 2012). Currently, there have been described 11 species of Chlamydia. Most are able to infect several host species and anatomical sites. CT, the most medically significant chlamydial species, is a human-specific microorganism capable of produce pulmonary, ocular and genital pathologies in either neonates or adults (Bachmann et al., 2014) (Figure 7).

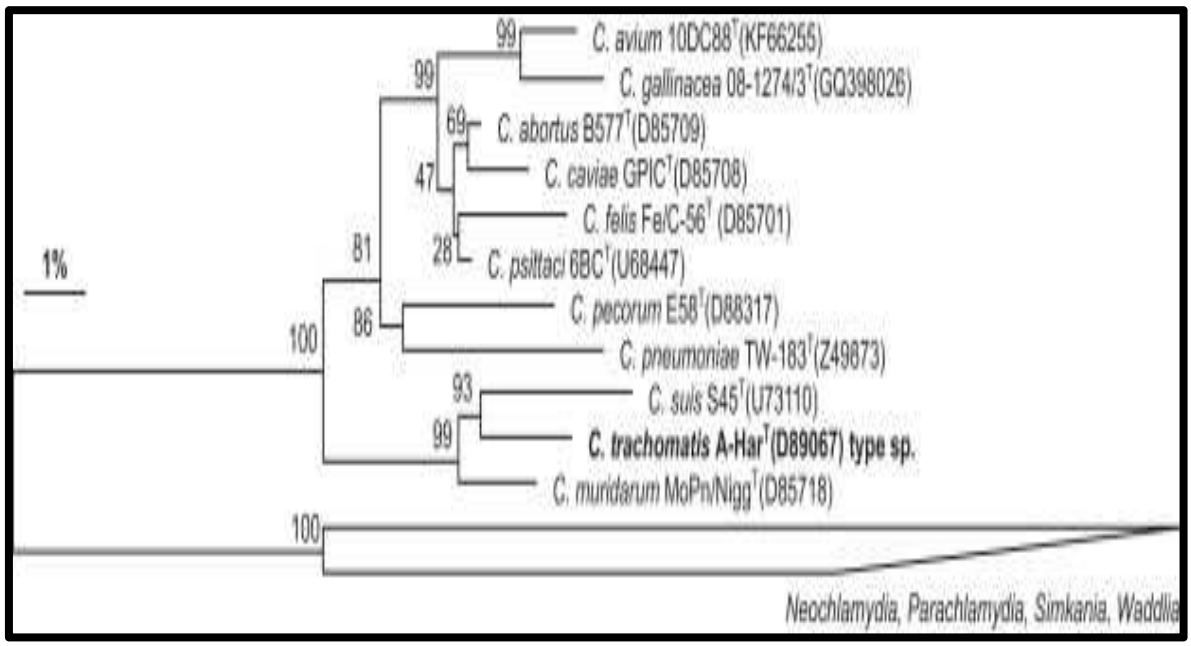


Fig. 7: Phylogenetic reconstruction based on almost complete 16S rRNA genes from type strains of established *Chlamydiaceae spp*., including the recently proposed new species *C. avium* and *C. gallinacea* (Sachse et al. Systematic and Applied Microbiology, 2015).

Ocular infections caused by CT are the leading cause of preventable blindness (trachoma) (Lu et al., 2013) or lymphomas (Contini et al., 2009; Contini et al., 2013) whereas genital infections are known to have adverse effects on female reproduction (Shao et al., 2013).

In women, due to the high frequency of the asymptomatic phase, CT can lead to pelvic inflammatory disease (PID), infertility, ectopic pregnancy and chronic pelvic pain. (Haggerty et al., 2010). Infections by CT are also known to facilitate rheumatic disease (Gerard et al., 2010; Carter et al., 2012; Zeidler and Hudson, 2014).

CP is the other human chlamydial pathogen prevalently associated to respiratory infections as community-acquired pneumonia worldwide (10–20%) and asthma (Blasi et al., 2004; Stocks et al., 2004; Hahn et al. 2012; Asner et al., 2014). Moreover, evidence seems to suggest that CP is also linked to cardiovascular, rheumatic and neurologic disease (Witkiewicz et al., 2005; Fernandez et al., 2005; Contini et al.; 2010; Contini et al., 2011).

The least five other Chlamydia species have a broad host-range in animals. *Chlamydia psittaci* is primarily a pathogen of avian species that causes respiratory diseases (interstitial pneumonia transmitted by birds) which usually occur through inhalation of the organism when it is dispersed in the air as fine droplets (aerosol) or dust particles (Moroney et al., 1998; Van Droogenbroeck et al., 2009).

CT have extremely small circular genome (1042 kbp), which contains also a cryptic plasmid (length 7500 bp) linked to virulence that can contribute, to the regulation of chlamydial chromosomal gene expression, by its transcriptional activity (Carlson et al., 2008). A detection of cryptic plasmid's nucleic acid, is utilized for diagnostic purposes (Ljubin-Sternak and Meštrović, 2014) and usually can detect all variants discovered such as Sweden mutation (Paavonen, 2012).

CT has tropism for conjunctival and genitals mucous membranes, where can lead to diseases with chronic inflammation.

According to different immunoreactivity, 19 human serotypes and related variants (A, B/Ba, C, D/Da, E, F, G, Ga, H, I/Ia, J, K, L1, L2, L2a and L3) have been identified by using mono or polyclonal antibodies directed against epitopes of the major outer protein membrane (MOMP). These serotypes are closely related to the genotype, which is based on the ompA gene (encoding the protein MOMP) (Wang et al., 1985; 1991; Hsu et al., 2006) and can be divided into three serogroups: the B group (serotypes B, Ba, D, Da, E, L1, L2 and L2a); the intermediate (I) group (serotypes F, G and Ga); and the C group (serotypes I, Ia, J, K, C, A, H and L3) (Bax et al., 2013). In contrast with the individual serotypes, that possess a certain correlation with the disease and the affected tissues, the serogroups do not correlate with either tissue tropism or the biological properties of the

organism. Therefore, based on the pathogenic potential, serotypes A, B, Ba and C are commonly associated with the development of trachoma, chronic eye condition, while D to K serotypes, are responsible of urogenital infections as well as neonatal conjunctivitis and pneumonia. The serotypes of lymphogranuloma venereum (LGV), L1 to L3 and L2a, are responsible of most invasive urogenital diseases (Dean, 1997; Gomes et al., 2006).
Additionally, it seems that the infections with CT serovars G, I and D are associated with cervical squamous cell carcinoma and chronic infections with serotype K, in women, have been recognized as a cause of infertility (Marrazzo & Stamm, 1998; Koskela et al., 2000; Morre et al., 2000; Anttila et al., 2001). The CT serotypes most commonly isolated from patients are: E (50%), F (20%) and D (10%). According to recent findings, F serotype seems be responsible of more severe infections, whilst E of asymptomatic infections (Choroszy-Król et al, 2012).

CT infection and Cell Cycle

The intracellular growth cycle of the *Chlamydiae* is complex and several growth options are possible, depending on the host-cell type, the particular environmental conditions in the host cell and the nature of tissue that is being affected.
Chlamydiae, have a characteristic biphasic growth cycle within a eukaryotic host cell, during which infectious, elementary bodies (EBs, 0.3–0.6 mm diameter) differentiate into the metabolically active but non infective reticulate bodies (RBs, 0.6–1 mm diameter), that divide by binary fission within the host, derived vacuoles named Chlamydial inclusions. After 48–72 h, RBs multiply by binary fission and reorganize into EB, which are released after host cell lysis. *In vitro*, this orderly alternation between EB and RB in life cycle development usually take place in 72 h, ranging from 36 to 96 h to complete, depending on each species and in the number of inclusions per host cell (from one in CT infected cell, to several inclusions for the others *Chlamydiae).* Under *in vitro* conditions with adverse factors, e.g., penicillins or INF-γ, RBs block division and maintain a stable association with the infected cell and become the aberrant or persistent bodies with enlarged forms, altered gene expression profile and multiple nucleoids, instead of undergoing rapid replication and differentiating into infectious EBs (Figure 8) (Contini and Seraceni, 2012). Although the life cycle of *Chlamydiae* is well characterized by microscopy, the signals that trigger interconversion of the morphologically distinct forms are not completely known (Beatty et al., 1994; Dautry-Varsat et al., 2005). However, EBs are no longer considered as inert organisms. The discovery that EBs can translocate stored proteins into the host under

distinct signaling pathways is further evidence that the entry process results from a dialogue between the bacteria and the host, although many features including EB protein attachment to target cells, remain to be clarified or discovered (Dautry-Varsat et al., 2005; Wuppermann et al., 2008). During chlamydial cell cycle, a stop in development may lead chronic infection, characterized by high transcriptional activity with aberrant bodies formation (Gerard et al., 2013). These events, reversible, constitute the basis of clinical persistence leading to chronic sequelae.

Moreover, aberrant forms of RBs, with reduced MOMP and lipopolysaccharide (LPS) antigens, persist with high production of chlamydial heat shock protein 60 (Hsp60) can induce inflammation and scarring, classic characteristics of chronic infection (Malhotra et al., 2013). The virulence of these microorganisms is principally due to components of the outer membrane, inclusion proteins and polymorphic membrane proteins, (pmp) related to the third type secretion system (TTS), to different secretory proteins (e.g. glycosyltransferase), chromosomally encoded and especially to extrachromosomal factors, specifically plasmids (Pawlikowska-Warych et al., 2015), capable to define the outcome of infection and disease severity. Multiple types of genetic variation are found in CT that impact variability and expression of virulence factors, such as high degree of variability in the exposed portions of MOMP, polymorphic TTS effectors, and amino acid substitutions in pmp autotransporters (Abdelsamed et al., 2103). These strategies have been demonstrated to foster chlamydial intracellular survival, aid in the evasion of the host immune system, and form the basis for distinct chlamydial disease variations in host tissue tropism (Byrne, 2010). Host genetics also play a role in the disease severity. For example, women who carry specific HLA DQ and IL-10 promoter alleles that modify host immune response were found to develop TFI more frequent than control group (Kinnunen et al., 2002).

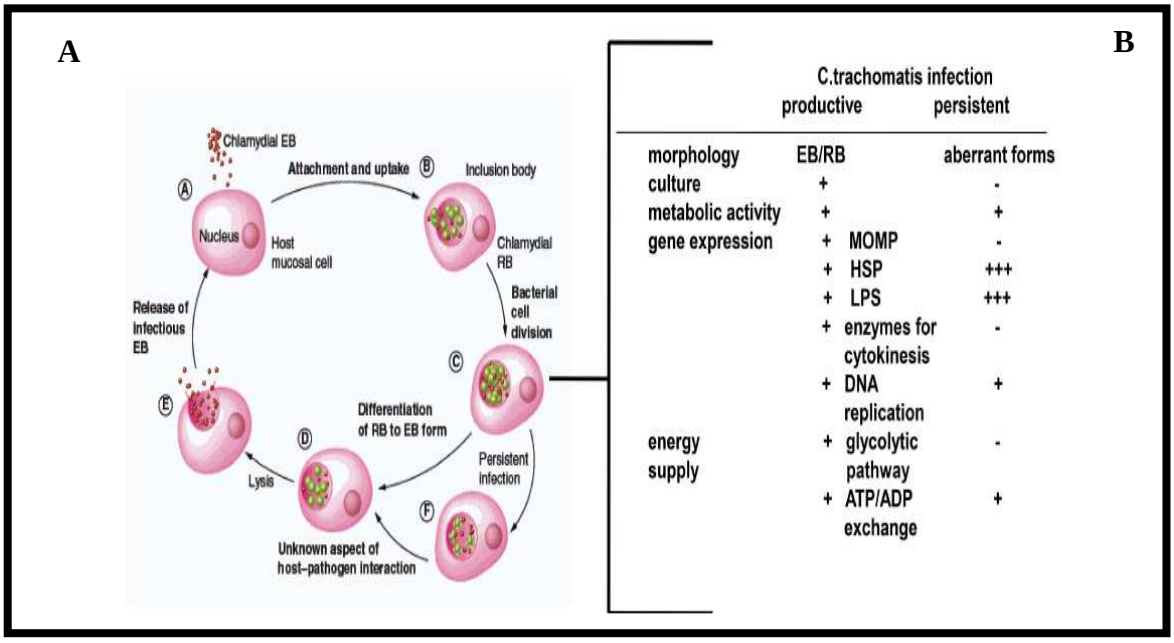


Fig. 8: Chlamydia life cycle with main morphological and metabolic features of normal productive *vs.* persistent CT infection.

A: schematic description of persistent life Chlamydia arrested at partially known state of the normal life cycle.

B: the morphologies, the results of culture-based detection of Chlamydia, their metabolic state, their gene expression profiles, and energy supply of Chlamydia during productive infection and in a persistent state.

+ indicates detection; −indicates lack of the corresponding messenger RNA (Zeidler and Hudson, 2013)

Epidemiology

The WHO estimates that, each year, CT infections are diagnosed over 90 million new cases (Kucinskiene et al., 2006). A 2012 report has revealed that in United States occur over 1 million new cases (rate 456.7 per 100,000 people) for year (CDC, 2012). In the developed countries, CT prevalence is high (3-6%) especially in health young heterosexual adults under 25y, especially those who are sexually active (Goulet et al., 2010; Eggleston et al., 2011). Urogenital chlamydial diseases are common in young population and tightly associated with sexual behavior. Other than age, other risk factors such as STDs co-infection, new o multiple sexual partners, oral contraceptive use, are important in the pathogenesis of this pathology. CT prevalence in the world varies among different types of persons and depending on laboratory techniques used for microorganism detection. These differences could represent real differences in sexual behaviours patterns and CT control efforts, but might also result from different study design and participation rates. Paavonen (2012), in a review reported that European prevalence among asymptomatic women varies

from 1.7% to 17%, while Ljubin-Sternak and Meštrović revealed a higher prevalence (35.3%), in symptomatic patients (18-25y) (Ljubin-Sternak and Meštrović .2014). A large meta-analysis from 11 EU/EEA countries and 14 studies from five other high income countries, reported on young women sexually active people (18–26y), a CT prevalence from 3.0–5.3% (Redmond et al., 2015).

In Italy, Italian Institute of Health reported an overall prevalence in women of 2.3% which was estimated approximately highest in subjects less 25y compared to over 25 (7.9% vs 2.5 %) (Salfa et al., 2014).

Immunopathogenesis of CT infection

CT causes clinically unapparent infections of the upper genital tract that may result in significant damage to the reproductive organs, such urethritis, mucopurulent cervicitis, plasma cell endometritis, salpingitis (Paaovonen, 2012). CT infections increase the risk for tubal factor infertility and can lead to pelvic inflammatory disease (PID), infertility, ectopic pregnancy and chronic pelvic pain (Haggerty et al., 2010) and have been also linked to other adverse pregnancy outcomes, including chorioamnionitis, placemtitis, premature rupture of membranes and preterm birth (Rours et al., 2011). Vertical transmission from the genital tract can cause conjunctivitis and pneumonitis in new-borns (Paaovonen, 2012).

The clinical course is usually subacute and poorly symptomatic, but the microorganisms are rarely detected in patients without clinical signs of infection. In fact, most CT infections are symptom-free or paucisymptomatic, remaining undetected and thus untreated for a prolonged period with the possibility of developing chronic infections because of spreading via monocytes and can cause local and systemic infections. CT is also a potent immunogen, stimulating the immune processes of microorganisms.

In the course of CT infection, the response mechanisms involved are: non-specific, specific, humoral and cellular. Chronic infection is characterized by maintenance of microorganisms in the host cell. Inflammation occurs in a less time period and with increased intensity and evokes a rapid immune response of lymphocytes, previously sensitized (Choroszy-Król et al., 2012) (Figure 9).

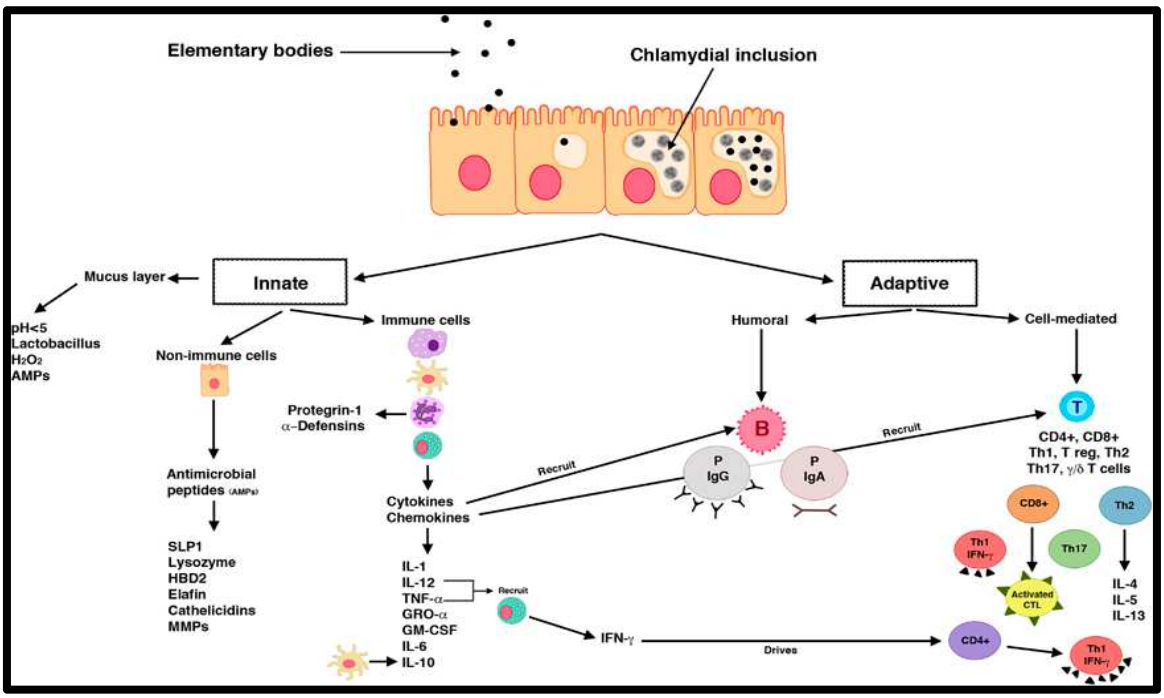


Fig 9: Immune protection against chlamydial infection in the female genital tract. Innate, humoral, and cell-mediated immunity act in concert to protect against CT infection of non-immune host genital epithelial cells and local innate immune cells in the female genital tract (FGT).

Innate: The epithelial barrier is relatively ineffective at protecting against *Chlamydia* as this mucosal pathogen has a myriad of mechanisms to evade barrier protection. A mucus layer containing a variety of antimicrobial factors and endogenous microbiota contributes towards regulating the pH of the FGT to protect against genital tract pathogens. Innate immune cells constitutively secrete an array of soluble antimicrobials, including secretory leukocyte protease inhibitor (SLPI), human β-defensin 2 (HBD2), lysozyme, lactoferrin, Elafin, cathelicidins. Chlamydial infection of columnar epithelial cells and local genital tract immune cells, including neutrophils, macrophages, and NK cells, produces soluble antimicrobials chemokines, and pro-inflammatory cytokines that selectively prevent bacterial infection of target host cells. (e.g., IL-1 released from infected epithelial cells promotes Th17 differentiation). Recruitment and activation of adaptive immune cells (T and B cells) are also orchestrated by the release of these secreted soluble antimicrobial factors from epithelial cells, dendritic cells, and macrophages.

Humoral: Antibodies potentially can prevent infection by *Chlamydia*. Immunoglobulin G (IgG) is the predominant antibody in the FGT. Antibodies released from plasma cells (IgG and IgA) inactivate extracellular chlamydial EB.

Cell-mediated: $CD4^+$, by IFN-γ production, contributes to host defence by inhibiting intracellular chlamydial replication, while $CD8^+$ induces apoptosis of infected cells.

CTL:$CD8^+$; GM-CSF: granulocyte-macrophages colony-stimulating factor; MMP: matrix metalloproteinase; GRO-α: growth related oncogene-α; TNF: tumor necrosis factor (Hafner et al., 2013). http://www.nature.com/mi/journal/v6/n5/full/mi201346a.html.

CT Strategies for Evading Host Immune Response

CT pathogenesis depends on the cell population invaded, the initiation of the replicative genetic state of the pathogen and the efficiency of the release of effector molecules into the host cell. A number of mechanisms can be considered to explain the evasion of host immune response. As many other intracellular bacteria (Brinkmann et al., 1987), endocystosed *Chlamydiae* are in fact sequestered within a host derived phagosome during the intracellular phase of developmental cycle. Their intracellular location largely protects them from antibody and complement attack. Cell mediated immunity is the predominant component in controlling CT infection, even if *Chlamydia* antibodies may play a significant role in controlling the infection at a later stage of the disease (Zhong, 2009). Moreover, studies using animal models have shown that both the IgA secreting B cells and IFN-γ producing $CD4^+$. Th1 T cells are the most important adaptive immunity mechanisms in course of infection, although other immune components also play some roles (Malhotra et al., 2013). Despite these powerful host defense mechanisms, acute infection (if not treated) can activate inflammation, inducing the production of a wide variety of inflammatory cytokines, (IL-1, IL-6, IL-8 and TN-α) and can persist in some infected hosts (Gottlieb et al, 2010). In fact, *Chlamydia* species have shown a tendency to cause persistent infections that may also play a role in oncogenesis. In this regard, the induced inflammatory responses cannot only fail to effectively clear the infection but also contribute to inflammatory pathologies (Stephens, 2003). The failure by the host to eradicate the disease involves the establishment of a state of chronic infection in which CT after internalization into mononuclear cells, enter into a state of quiescence (cryptic body) with intermittent periods of replication and characterized by antigenic variation, production of Hsps and pro-inflammatory cytokines (capable of evading host defenses) which trigger tissue damage (Stratton and Mitchell, 1997). In this regard, Hsp60, an ubiquitous and evolutionarily conserved chaperonin, normally sequestered inside the cell, particularly into mitochondria, can elicit an immune response in humans which although directed against the microbial molecule but also reacts with endogenous Hsp60 (Pockley, 2003). During cell stress conditions, as well as during carcinogenesis, this chaperonin becomes exposed on the cell surface and/or is secreted from cells into the extracellular space and circulation (Figure 10).

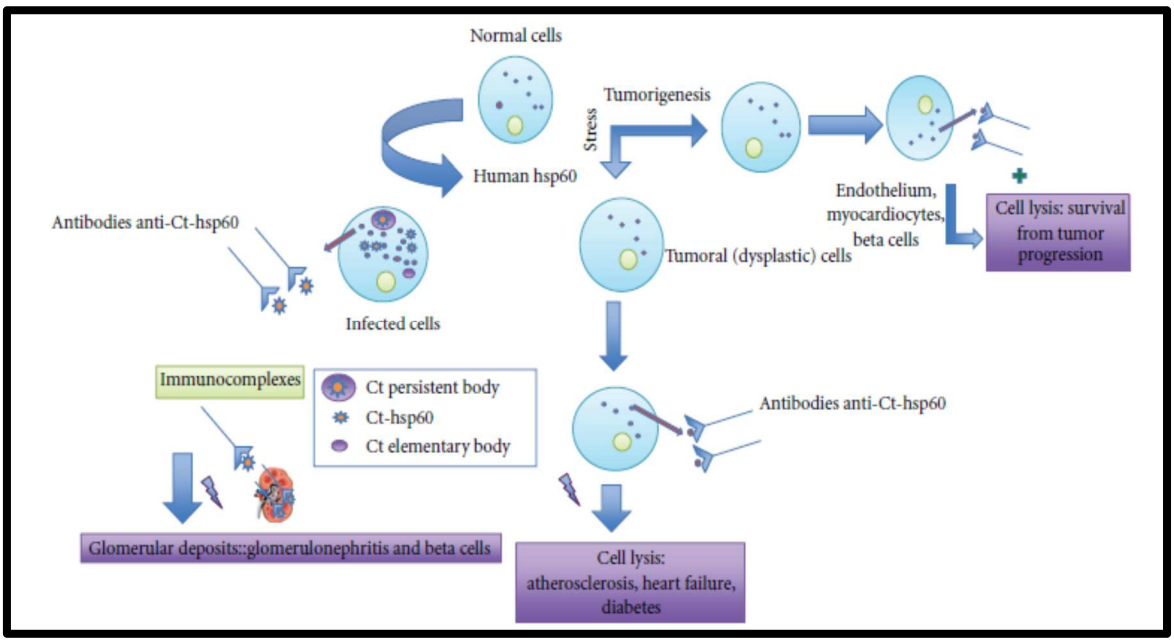


Fig. 10: Potential effects of anti-CT-Hsp60 antibodies. These antibodies recognize surface-Hsp60 onstressed or tumor cells, and consequently, they can lead to either damage and persistence of infection or cell lysis producing a regression of certain types of cancer. Immunocomplexes (CT-Hsp60 and anti-CT-Hsp60) can cause disease if they form deposits in the renal glomerulus (Mascellino et al., 2011). http://www.hindawi.com/journals/isrn/2011/436936/fig3/.

Quantification of circulating Hsp60 has recently become a potential useful marker of infection for clinicians in patients affected by a variety of diseases. However, interpretation of its values should be carefully evaluated, as a correlation between chaperonin levels and disease is difficult to establish. Hsp60 is also a ligand of TLR and its expression on cell membrane surface's correlates with apoptotic phenomena (Cappello et al., 2009). During CT invasion and intracellular growth, sensors of the host innate immunity (PRR) can detect the infection by recognizing microbial components (pathogen associated molecular patterns, PAMPs). *Chlamydia* PAMPs such as Hsp60 and Macrophage Infectivity Potentiator lipoprotein (MIP) are recognized by host PRR TLR4 and TLR2 respectively. These host receptors selectively recognize a broad spectrum of microbial components and endogenous molecules released by injured tissue (Bulut et al., 2002; Bas et al., 2008). In human cells, TLR4 recognizes CT LPS and Hsp60 and it is mainly expressed in the tubes and endometrium and little in endocervix, while TLR2 recognizes peptidoglycan and it is mainly expressed in the tubes and cervix (Mascellino et al., 2011) and appears to be the predominant receptor required for an inflammatory response to infection. Interestingly, TLR2 and its adaptor MyD88 localize to the periphery of the chlamydial inclusion during active infection, suggesting that may signal intracellularly during infection (Bastidas et al.,

2013). MIP or other lipoproteins could be released from EB surface and RBs, and retain inside tissues where they might activate resident cells and perpetuate inflammatory response even after the eradication of live bacteria with antibiotic therapy (Bas et al., 2008). In general, PRRs, upon ligand binding, can lead to activation of various inflammatory signaling pathways including NF-kB, NF-IL-6 and MAP kinases.

TTS apparatus is another mechanism which seems central to the biology of the *Chlamydiae*, as it mediates the translocation of bacterial toxins to the cytosol of infected cells. It is present in several important gram-negative bacterial pathogens (Peters et al., 2007). It consists in a molecular injection system protruding from the outer membrane, that appears to be expressed and functional in acute as well as in chronic infection and may represent a prominent virulence factor. A major role of T3S may also be involved, ensuring growth and development of the pathogen by modifying apoptosis signals or some other transcriptional regulation important for *Chlamydia* survival.

CT Persistence and Chronic Infection

The exact role of persistent stage in the CT developmental cycle as well as the molecular mechanisms allowing persistence remains to be elucidated. *In vivo* studies of microorganism persistence are hampered by genotype definition and viability of organism. However, characterization of *in vitro* persistent phase of pathogen and multiple lines of *in vivo* evidence, suggest that CT persists in an altered form during chronic disease (Hogan et al., 2004).

Persistence has long been recognized as a major factor in the pathogenesis of CT disease. It has been described as a viable but non-cultivable growth stage resulting in a long-term relationship with the infected host cell that may not necessarily manifest as clinically recognizable disease.

It is distinct from unapparent infections, which may or may not involve evident CT growth and refers to an atypical, intracellular and metabolically less active state that is difficult to resolve not only by the host-defense system, but also by antibiotic therapy.

Unlike the re-infections believed to be the result of exposure to a CT serotype different from the initial, persistent infections are due to the same type of pathogen genotype entered into a metabolic quiescent and non-infectious form and responsible of three to ten recurrences which can last many years (Dean and Powers, 2001).

In vitro studies have shown that several factors including nutrient depletion, cytokines, iron restriction, amino acids, Ca^{++} and certain antibiotics can favor the Chlamydial persistent stage (Beatty et al., 1994 ; Raulston ,1997; Dreses- Werringloer et al., 2000).

IFN-γ is considered a primary host protective cytokine against endocervical CT infections. This directly inhibits bacterial growth through the depletion of cellular tryptophan (TRP) by indolamine-2,3-dioxygenase (IDO) (Aiyar, 2014), which can stop the expression of late proteins, such as MOMP, that in turn stop the progress of RB division and RB conversion into EBs leading to aberrant Chlamydial RBs (Sardinia et al., 1988; Beatty et al., 1994).

A failed or weak Th1 response will allow CT RBs to respond to immune challenge by converting into a non-replicating but revivable persistent state (Debattista et al., 2003; Leonhardt et al., 2007). CT, in this persistent state, is able to survive and still allows for antigen-presentation (Rey-Ladino et al., 2007). A direct consequence of this prolonged infection is antibody or Th2-mediated hypersensitivity (Debattista et al., 2003).Moreover, an over-stimulated Th1 response will lead to delayed-type hypersensitivity and an increased risk of IFN-γ-mediated tissue damage, that is likely a consequence of an initially dominant Th2 response. Infected cells increase through new infections and decrease by cell death and clearance by host immune responses; nevertheless, antibiotic treatment, reduces the number of infected cells by eradicating CT; depletion of infected cells further, removes the antigen that is reflected on the immune system. As a direct result, the arrested immunity hypothesis underscores the importance of gaining a better understanding of the interplay between the immune biology of infection and the use of antibiotics (Gottlieb et al., 2010). This hypothesis suggests that early antibiotic treatment effectively attenuates the optimal development of protective immunity, leaving individuals as susceptible as before to reinfection with the same or a new serovar. Therefore the treatment may attenuate protective immunity in some persons and conversely that natural immunity may protect against reinfection (Marrazzo and Suchland, 2014).

In this context, a complete transcriptome analysis of CT serovar D growth in HeLa cells exposed to IFN-γ, demonstrated the up-regulation of many genes involved in active metabolic processes in the aberrant RBs, including those involved in DNA repair and recombination, protein translation, and phospholipid utilization (Belland et al. 2003).

Moreover, separate studies at transcriptional level have demonstrated a down-regulation of CT MOMP in HeLa cells and an up-regulation of CP MOMP in response to IFN-γ stimulation (Mathews et al. 2001; Molestina et al. 2002). This underlines the different roles played by MOMP in the two species. Also, Hsp60–1/groEL was found to be expressed predominantly during acute phase growth of CT serovar K and that the Hsp60-copy2/Ct-

604 gene transcript/protein was increased in iron-induced persistent cultures (Gérard et al. 2004).

Antibiotics such as penicillin and quinolones (such as ciprofloxacin and ofloxacin) have shown to favor persistence instead of resolving infection because of inducing aberrant but viable particles which may explain therapy failure (Dreses-Werringloer et al. 2000). CT exposure to penicillin leads to enlarged and aberrant RBs, the so called “penicillin forms” that return to normal growth after penicillin removal. On the other hand, although Chlamydial RBs are killed by macrolide treatment (azithromycin), residual antigens can persist for more than 28 days continuing to harbour inflammatory responses (Wyrick and Knight 2004).

In vivo studies have shown that the presence of Chlamydial antigens and nucleic acids even in absence of cultivable organisms is indicative of persisting organisms probably as result of immunologic stimulation during chronic disease. Chlamydia rRNA demonstration may provide evidence for in-apparent Chlamydial infections (Beagley et al., 2009).

All different species of *Chlamydia* have tendency to cause persistent infections that may play a role in chronic diseases (inflammation and scarring with significant damage to the host) and oncogenesis.

Previous studies have also revealed that in CT infection, the cytosolic levels of Hsp60 *in vivo* gradually increase during carcinogenetic steps, from normal tissue to dysplasia to fully developed carcinoma in various organs (Cappello et al., 2009).

CT and Apoptosis

Cell death by apoptosis is an active and important defense mechanism against invading pathogens. Apoptosis has a direct role in many infectious diseases, especially those caused by viruses, intracellular protozoans and intracellular bacteria (Byrne and Ojcius, 2004). For many of these pathogens, the apoptotic signaling starts from the pathogen and not by the host cell. In this regard, *Chlamydia* inhibits apoptotic signalling cascades during productive growth as part of its intracellular survival strategy (Miyairi and Byrne, 2006) in order to maintain the integrity of the host cell for the completion of its intracellular growth (Zhong, 2009). This is in part due to the proteolysis of host proteins for ensuring its own intracellular replication while maintaining the integrity of the infected host cells for long periods of time. CT also inhibits apoptosis during persistent growth or in phagocytes, but induces apoptosis in T cells, which suggests that apoptosis has an immunomodulatory role in Chlamydial infections. The anti-apoptotic activity has shown to be prolonged during CT

persistence. This strengthens the hypothesis that active CT metabolism maintains host cell integrity and contributes to intracellular survival (Bastidas et al., 2013).

The circumstances that dictate whether the *Chlamydiae* inhibit or activate host cell death reflect important pathogenic considerations, including whether if an acute or chronic infection is in progress and whether intracellular *Chlamydia* growth is programmed to go through a productive infectious cycle or is stalled under non-productive growth conditions. It is possible that apoptotic activity is controlled to some extent by the intracellular growth status of the *Chlamydiae,* which can be influenced by any or all of these considerations (Byrne and Ojcius 2004) and by strain. While for CP, active inhibition of apoptosis occurs in epithelial cells, macrophages and neutrophils, for CT and *C. psittaci,* the anti-apoptotic activity has been demonstrated mainly in epithelial cells later in their developmental cycle (Miyairi and Byrne, 2006; van Zandbergen et al., 2004). It is not known exactly how pro-apoptotic and antiapoptotic effects correlate with the wide spectrum of clinical manifestations and Chlamydial diseases. A *Chlamydia* induced apoptotic activity has been hypothesized during acute manifestation of disease, whereas inhibition of apoptosis, in chronic disease states (Byrne and Ojcius 2004). Chronic infection and clinical persistence are closely related. Inhibition of apoptosis could represent a mechanism that has evolved to establish a chronic infection. Several lines of evidence suggest that to provoke chronic infection, CT could adopt several strategies. One of these consists of being silent, resulting in asymptomatic infections that cannot be diagnosed at that time. This promotes bacterial progression, even to the most internal tissues. In addition, CT MOMP displays variable immunodominant antigenic epitopes. Variations in these epitopes explain the absence of strain specific immunity and multiple re-infections by different serovars or by the same mutated serovar are still possible (Millman et al. 2001). For these reasons, even if the initial infection is resolved, re-infections are possible and can lead to auto-pathological immune response induction (Beatty et al.1994). Although re-infections occur, the refinement of Chlamydial diagnostic methods will allow us to establish whether CT can persist.

Interaction between CT and HPV

Infectious agents play an important role in the aetiology of certain human malignancies, and are thought to be responsible for around 20% of the worldwide cancer burden (Parkin, 2001). Much of the burden of cancer incidence, morbidity, and mortality occurs in the developing world (up to 27%), with a large body of evidence regarding the role of viruses such as human papilloma virus (HPV), hepatitis B virus (HBV) and Epstein-Barr virus (EBV) in the complex processes of carcinogenesis of the cervix, stomach and liver (Jemal et al., 2010). In addition to viral agents implicated in carcinogenesis, a theory of possible association between bacterial infection and cancer has been proposed in early nineteenth century (Lax, 2005). Moreover, many bacteria that cause persistent infections produce toxins that disrupt cellular signaling, alter the regulation of cell growth, induce inflammation or directly damage DNA. Toxins may also mimic carcinogens and tumor promoters and might represent a paradigm for bacterially induced carcinogenesis. The question however remains quite controversial especially with regard to certain species of bacteria for oncogenic properties.

Some authors have suggested that exposure to CT-Hsp60 may be a risk factor for development of cancer (Di Felice et al., 2005), while the development of anti-CT-Hsp60 is also proposed to protect against malignancy (Cappello et al., 2009). Chronic persistent infection with CT to the upper genital tract is able to incur significant damage to the reproductive tract and proposed to induce ovarian cancer (Quirk & Kupinski, 2001).

Although epidemiological data have not yet provided consistent evidence about a real implication of CT in cervical cancer, the co-infection with Human papillomavirus (HPV), sharing the transmission route and the same risk factors, have been recently highlighted (Simonetti et al., 2009; Vaccarella et al.,2010; Paavonen, 2012). A role for CT as cofactor was suggested, since it seems to facilitate the penetration of HPV and the progress of cervical lesions interfering in the immunological response (Deluca et al., 2011). Moreover, some authors recently detected a high-risk for the development of cervical cancer in patients with HPV infection and history of CT (Jensen et al., 2014). Nevertheless, the prevalence and distribution of HPV genotypes associated to CT infection and its clinical persistence are poorly explored. On this basis, the characterization of HPV infection in women suffering from CT could be important in generating hypotheses regarding the possible synergism of these pathogens in cervical malignancy (Bathla et al., 2013; Silva et al., 2014; Tavares et al., 2014; Shew et al., 2013). Recently, a case-control study showed that CT is not able to modify the risk of progression to a high-grade lesion of HPV-positive

women but can increase the susceptibility of the cervical epithelium to further HPV infection and its persistence (Safaeian et al., 2010) Specifically young age (less than 25y) is related to an increased risk of both HPV and CT infection (Silva et al., 2013). Furthermore, the role of CT chronic infection in promoting HPV susceptibility has been evaluated. In some cases this infection has been associated with cervical atypia and/or metaplasia, which in turn, may increase the risk of neoplasia (Luostarinen et al., 2013).

In this setting, to investigate a pathogen as CT potentially implicated as oncogenic for its tendency to cause chronic and persistent infections, together with HPV co-infection, could have a great importance for the public health.

OBJECTIVES OF THE STUDY

As described previously, HPV and CT are STIs with significant implications for global health. In a large series (2009-2014) of women from the North-Eastern Italian area, we aimed to:

- highlight, by molecular techniques, the overall prevalence of HPV and CT infections in cervical swabs (CS) specimen of women that perform these types of investigations.
- highlight the overall prevalence of CT/HPV co-infections.
- highlight the prevalence of CT chronic infections and its performance in the context of HPV co-infections
- evaluate the distribution of HPV genotypes in CT/HPV co-infections, compared to women infected with HPV only
- examine the prevalence of single or multiple HPV infections in the setting of CT/HPV co-infections compared to women infected with HPV only.

MATERIALS AND METHODS

Clinical Specimens

A retrospective study was conducted on a large cohort of 7135 consecutive cytology samples collected starting January 2009 to December 2014 during gynecological health checks from immunocompetent women in Trieste, Italy.

Of this series, 6214 cervical swabs (CS) were collected from women at risk for *C. trachomatis* infection of which 5481 from Outpatients (mean age 35 ± 10y) asymptomatic women, 733 were from symptomatic women attending Sexually Transmitted Infection (STI) clinic (mean age 33 ± 10y) while 921 (mean age 43 ± 10y) samples were from women at risk for HPV infection attending as outpatients a second level centre for Cervical Cancer prevention.

CS were collected using a 200 mm polyethylene Cervex brush device (Rovers Medical Devices B.V., The Netherlands) and suspended in 1.5 ml of TE buffer. The top portion uses a soft, flexible brush to obtain cell samples, while the shape allows the top edges to follow the contours of the cervix. The longer middle bristles reach deep into the endocervical canal while the shorter bristles touch both the ectocervical area (external os) and the transformation zone (T-zone). The sample was divided into 3 aliquots of 500 µl each and stored at -80°C until analysis.

No informed consent or any action of the patient was required for this study because the anonymity of the patients was guaranteed. The analysis on this series of samples was conducted blinded.

Isolation of DNA from cervical cells

DNA isolation was performed within 24 hours after the collection of the samples. After specimen centrifugation, 500 µl of each sample was extracted using the NucliSENS® EasyMAG® automated system for total nucleic acid extraction (Biomérieux S.p.a. Florence, Italy), according to the manufacturer's instructions.

HPV detection and characterization

HPV was detected in CS samples by the bead-based Luminex suspension array technology (Luminex Corporation, Austin, TX). HPV genotyping was performed using the type specific E7 polymerase chain reaction bead-based multiplex assay (TS-E7-MPG, IARC, Lyon, France) able to identify 27 HPV types (HR-HPV types HPV16-18-31-33-35-39-45-51-52-56-58-59-66-68-73-cp108, pHR-HPV types HPV26-53-67-69-82 and LR-HPV types HPV6-11-55, -81, -83, -84 and the β-globin gene as internal positive control. Viral genomes detected by this assay ranged from 10 to 1,000 copies (Comar et al, 2012; Bellaminutti et al., 2014). Briefly, HPV genotypes were detected as the median fluorescence intensity (MFI) of at least 100 beads per bead set. The background value for each probe was considered the MFI value, resulted from hybridization mixture without the addition PCR product. The cut-off was computed by adding 5 MFI to 1.1 the median background value.

An additional set of HPV types including LR-HPV40,-42-43-44-54-61-70 was identified by the Anyplex™ II HPV Detection assay (Seegene Inc., Arrow diagnostics, Italy) using the CFX96 ™ Real-time PCR System (Bio-rad, France) as indicated by the supplier. This assay is based on a new developed TOCE™ technology, that can perform multiplex examination by either End point-CMTA (end point-Catcher Melting Temperature Analysis) or Cyclic-CMTA (Cyclic-Catcher Melting Temperature Analysis) method. Cyclic-CMTA method can discriminate major pathogens in the co-infected samples. It is a multiplex Rt-PCR assay that permits the simultaneous amplification, detection and differentiation of target DNA of 19 HR-HPV types and 9 LR-HPV types. A human housekeeping gene was utilized as an endogenous internal control ensuring DNA purification, PCR reaction and specimen quality .

CT detection

Real Time PCR (RT-PCR) for CT DNA detection was performed with a commercial kit (CTDNA, Dia.Pro, Italia) using the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Italy) following manufacturer’s protocol. The reproducibility detection limit of the assay was 5 copy/µl.

CT HSp60 Real Time PCR detection

Total RNA was extracted using 500 µl of a stored aliquot, by RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). In order to avoid DNA genomic contaminations, RNA final concentration (25 µg/ml) was treated with Dnase I (RNase-Free DNase Set, Qiagen GmbH, Hilden, Germany) and eluted in 50 µl of distilled water. cDNA was synthesized using the SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA).

A quantitative Real Time PCR (*q*PCR) was performed for the quantification of the transcription level of Hsp60 gene (Ct604) as previously described (Contini et al., 2012; Seraceni et al., 2014). Briefly, PCR reaction, in a final volume of 20 µl, contained: 5 µl of cDNA, 2 µl LC FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Germany), 5 mM $MgCl_2$ and 0.8 µM each primer. The thermocycling condition was: 95 °C at 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 63 °C. To avoid false positive results a serial standard curve, a negative control with PCR-grade water and a positive control (CT strain-TW-3) were included in the assay.

The concentration of unknown clinical samples based on their *C*t values was determined with analytical software (Software SDS 2.4; Applied Biosystems) (Brankatschk et al., 2012) while the specificity of the reaction was further confirmed by agarose gel electrophoresis analysis, which showed the expected amplification product of 161 bp in length.

The sensitivity of each run was determined to be the lowest dilution of DNA ($2\ 10^{-4}$ ng/ml corresponding to one genome copies/µl) that can be detected to 7900HT instrument (Applied Biosystems, Italy). The standard curve equations were used to calculate the absolute copy number of gene mRNA. Results were expressed as mRNA CT genome copies/µl found inside each clinical sample.

CT genotyping

OmpA gene was employed for CT genotyping; positive amplicons were purified, sequenced and analyzed with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as previously described (Contini et al., 2013).

Statistical Analysis

All statistical analyses were performed using the IBM SPSS Statistics 20 statistical program. Chi Square Test was used to compare frequencies of discrete variables. Fisher Exact Test was applied when necessary. P value ≤ 0.05 was considered as the threshold of statistical significance for all tests.

RESULTS

HPV infection

Table 1 described the characteristic of HPV infection stratified according to age (clinical recommended cut-off 25 years). The age, usually referred to a cut-off of 25y, represented the most important predictor factor for CT infection (Eggleston et al., 2011) and was included as a criterium in the prevention guidelines by the Centre for Disease Control and Prevention (Workowski and Berman, 2011). Health authorities in some European and other high income countries recommend screening in this age group to allow both early treatment of asymptomatic infection and the prevention of long term complications (National Chlamydia Screening Programme Standards, 2012) and half an HPV infections, who represent only 25% of the sexually experienced population, occur in young people (15-24y) (CDC, 2014).

In both groups the prevalence of HPV tested at 39% (59/153 ≤25y and 300/768 in > 25y). Among HPV infections, the 60% (216/921) was represented as single HPV genotype: 53% (31/59) in women ≤ 25y and 62% (185/300) in women > 25y. Multiple genotypes were detected in 40% (143/921) of the samples: 47% (28/153) in women aged ≤ 25y and 38% (115/300) in women > 25y. These data showed a high frequency of multiple infections in a cohort of younger women with a trend of prevalence similar to that recovered for single infection. All these women tested negative for co-infection with CT.

CT infection

In this series, the overall prevalence of CT infection was tested at 4%. A statistically significant high frequency of infection was found in women with ≤ 25 y, tested at 14% (127/885) ($p < 0.0001$), of specimens while in women > 25y, CT was detected in 2% (124/5329).

CT chronic infection, determined through the quantification of Hsp60 expression, showed a prevalence of 57% (144/251) with a higher frequency in younger women which was found in 69% (88/127) (Table 2).

CT and HPV co-infection and HPV genotypes distribution

CT/HPV co-infection was present in 58% (145/251) of samples resulted positive for CT. The distribution of co-infection was of 68% (86/127) in women with less than 25y and of 48% (59/124) in women over 25y. HPV was present as single or multiple genotypes in 32% and in 68% of the overall samples, respectively. Interesting, HPV multiple infections, according to age, were highly expressed in both cohorts, when compared to older women (Table 2). Figure 11, describes the overall view of the genotypes distribution in women with CT/HPV co-infection compared to women positive for HPV.

In CT/HPV co-infected women, the distribution of HPV as single and multiple genotypes accounted for 32% and of 68%, respectively, as for women exclusively infected by HPV only.

The overall genotypes distribution of HPV in women diagnosed with CT/HPV compared to women with single HPV infection is described in Figure 12. A large number of HPV genotypes including high, intermediate and low risk genotypes, has been detected at high frequency: HPV31 (28%), HPV42 (23%), HPV66 (18%), HPV51 (16%), HPV6,-56,-73 (15%) and HPV16 (14%). Of interest, the LR type, HPV44 (12%) was detected in this group of women only. Conversely, in women showing HPV only, the HR-HPV16 represented the one most frequently detected genotype (33%) followed by HPV31 (16%) and HPV62 (12%). Interesting, HPV55,-61,-62,-81,-82,-83,-84,-cp108 were exclusively detected in these women.

HPV genotypes distribution in single and multiple infections from CT/HPV and HPV women.

The distribution of HPV genotypes in single and multiple infections independently of the presence of CT is resumed in Figure 13. A different distribution pattern was particularly found in CT/HPV co-infected women where each genotype was detected with higher prevalence in multiple infections. HPV31 (23%), HPV 42 (19%), HPV56,-66 (15%), HPV6,-51 (14%), HPV73 (13%), HPV16,-59 (12%) were the main genotypes identified. In addition, HPV 31 (5%), HPV42 (4%), HPV39,-66 (3%) were the most prevalent genotypes detected as single infection in CT/HPV co-infected women.

In CT/HPV multiple infection, the association between specific HPV genotypes was highlighted. In fact HPV6,-51,-59,-66 were detected together in 19% of the samples while

HPV31-56 and HPV51-66 in 8% in 6%, respectively. Moreover, a high percentage of LR-HPV genotypes generally not detected in this area, has been recovered exclusively in CT/HPV multiple co-infection.

Of note, HPV16 was the most representative genotype (21%) in women with a single infection while in those with multiple HPV infections, the prevalence of HPV61-62, was higher (8% and 10%) with respect to the single infection (2%). HR-HPV52 (6%) was presented exclusively in this group.

CT Hsp60 chronic infection and HPV infections

CT chronic infection was diagnosed in 57% (144/251) of women. The mRNA expression of CT-Hsp60 gene was positively associated with HPV co-infection, with an overall frequency of 68% (98/144). Specifically, age distribution analysis showed that in young women the 72% (63/88) of CT/HPV co-infection was due to a chronic status (Table 3).

Regarding, HPV genotypes distribution, HPV single infection was represented in 31% (30/98) in contrast with multiple infection which accounted for 69% (68/98) of women. In particular, 78% (49/63) ($p < 0.0221$) of younger women presented multiple infections compared to 54% (19/35) of older women. On the contrary, in older women the prevalence of HPV single infection were higher 46% (16/35) than in younger women, 22% (14/63). The level of CT-Hsp60 expression was confirmed, significantly lower (± 805 copy/µl) in CT/HPV co-infected women compared to women with only CT infection (± 1993 copy/µl). CT serotyping confirmed the trend obtained in our previous study, with high frequency of F serotype.

Table 1: Total prevalence of HPV infection in women (2009-2014) according to age cohorts (clinical cut-off 25 years of age).

Age Samples n. (%)	HPV-	HPV+	Genotypes	
			Single	Multiple
≤ 25 153 (17)	94 (61)	59 (39)	31 (53)	28 (47)
>25 768 (83)	468 (61)	300 (39)	185 (62)	115 (38)
921	**564 (61)**	**359 (39)**	**216 (60)**	**143 (40)**

Table 2: Total prevalence of CT infection in women (2009-2014) according to age cohorts (clinical cut-off 25 years of age).

Age Samples n. (%)	CT-	CT+	CT+/HPV+	Genotypes		HSP60+
				Single	Multiple	
≤ 25 885 (14)	758 (86)	127 (14)	86 (68)	20 (23)	66 (77)	88 (69)
>25 5329 (86)	5205 (98)	124 (2)	59 (48)	27 (46)	32 (54)	56 (45)
6214	**5963 (96)**	**251 (4)**	**145 (58)**	**47 (32)**	**98 (68)**	**144 (57)**

Table 3: CT/HPV co-infection according to age and CT chronic infection.

HSP60- n.(%)					HSP60+ n. (%)				
Age Samples n. (%)	**CT+/HPV-**	**CT+/HPV+**	**HPV infection**		**Age Samples n. (%)**	**CT+/HPV-**	**CT+/HPV+**	**HPV infection**	
			Single	**Multiple**				**Single**	**Multiple**
≤ 25 39 (36)	16 (41)	23 (59)	6 (26)	17 (74)	**≤ 25** 88 (61)	25 (28)	63 (72)	14 (22)	49 (78)
>25 68 (64)	44 (65)	24 (35)	11 (46)	13 (54)	**>25** 56 (39)	21 (37)	35 (63)	16 (46)	19 (54)
107 (43)	**60 (56)**	**47 (44)**	**17 (36)**	**30 (64)**	**144 (57)**	**46 (32)**	**98 (68)**	**30 (31)**	**68 (69)**

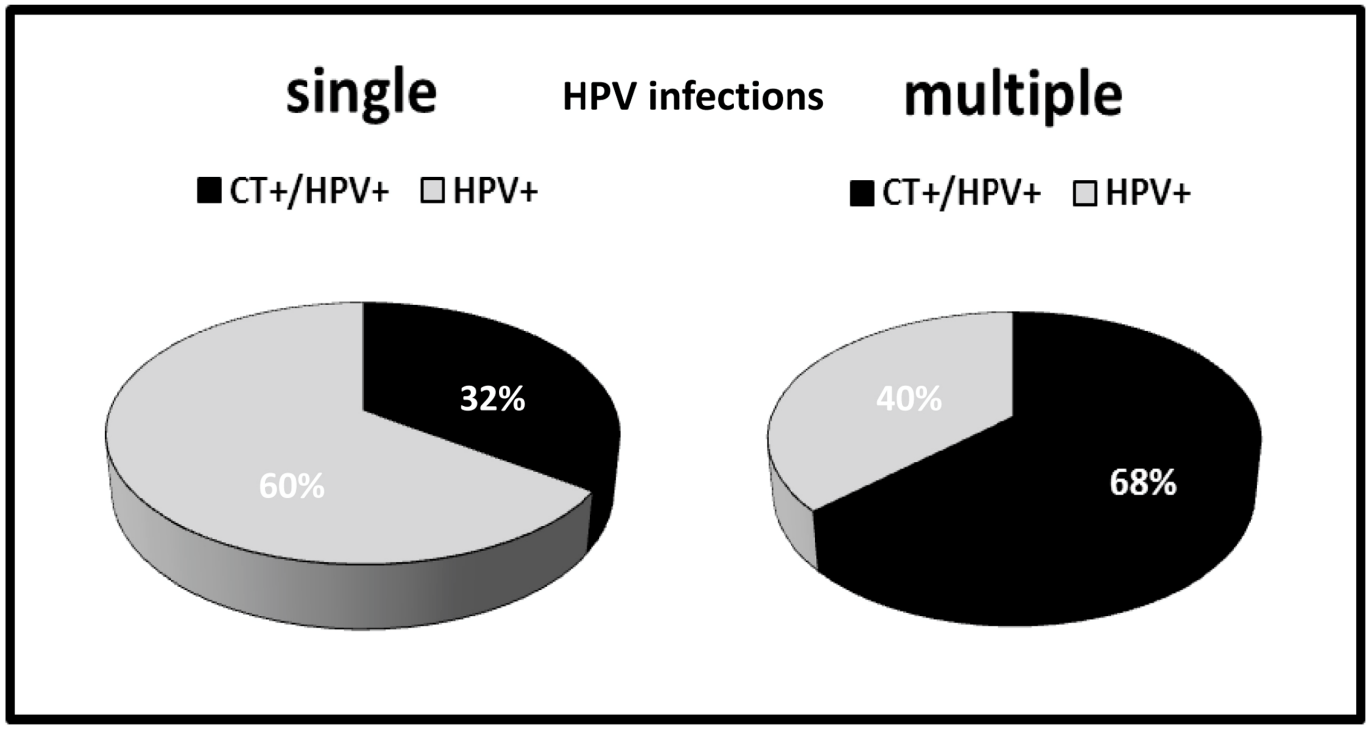


Fig. 11: HPV Single and Mutiple infections in CT/HPV co-infected compared to HPV infected women.

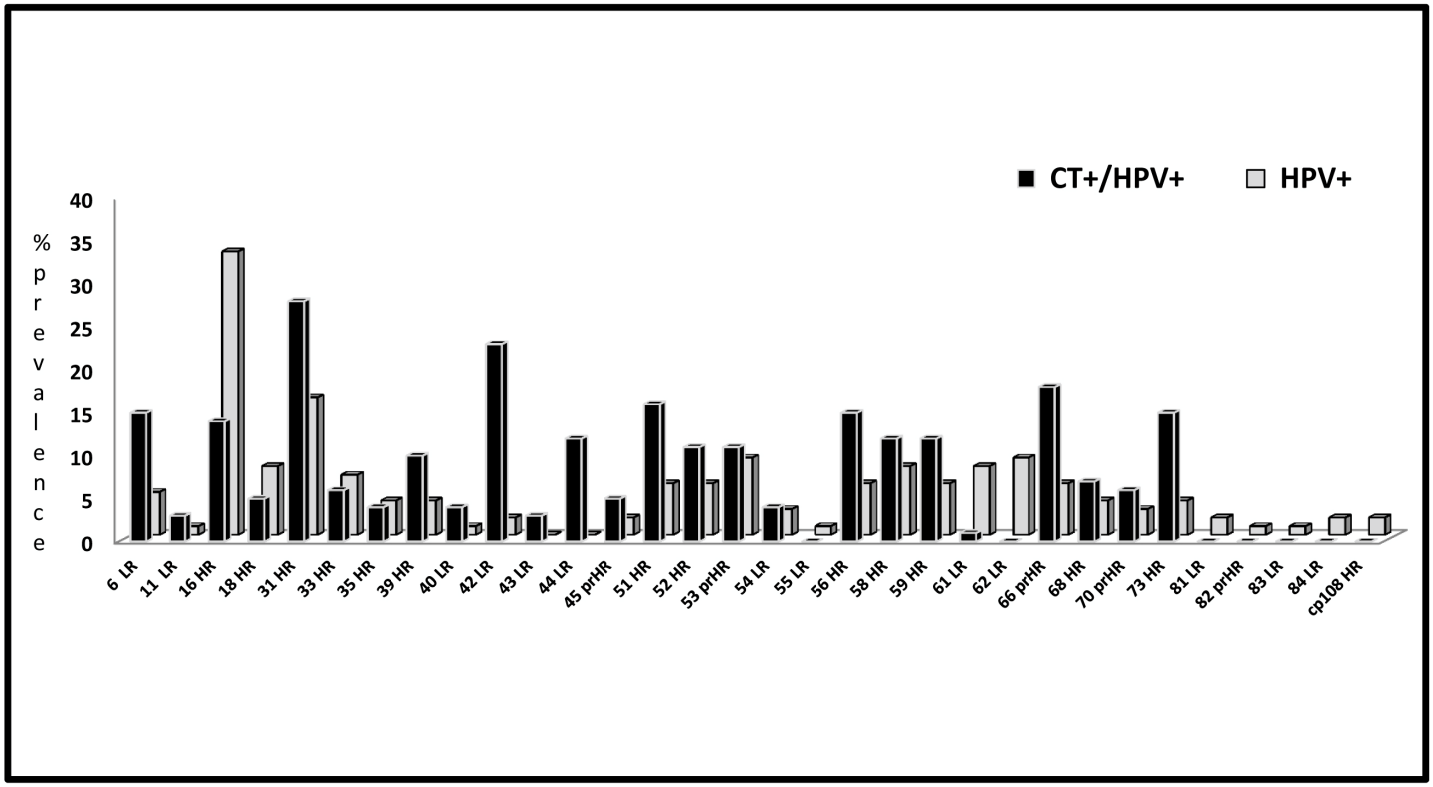


Fig. 12: Prevalence of HPV genotypes distribution in CT/HPV co-infected and HPV infected women. **LR**: low risk**; prHR**: presumable high risk; **HR:** high risk

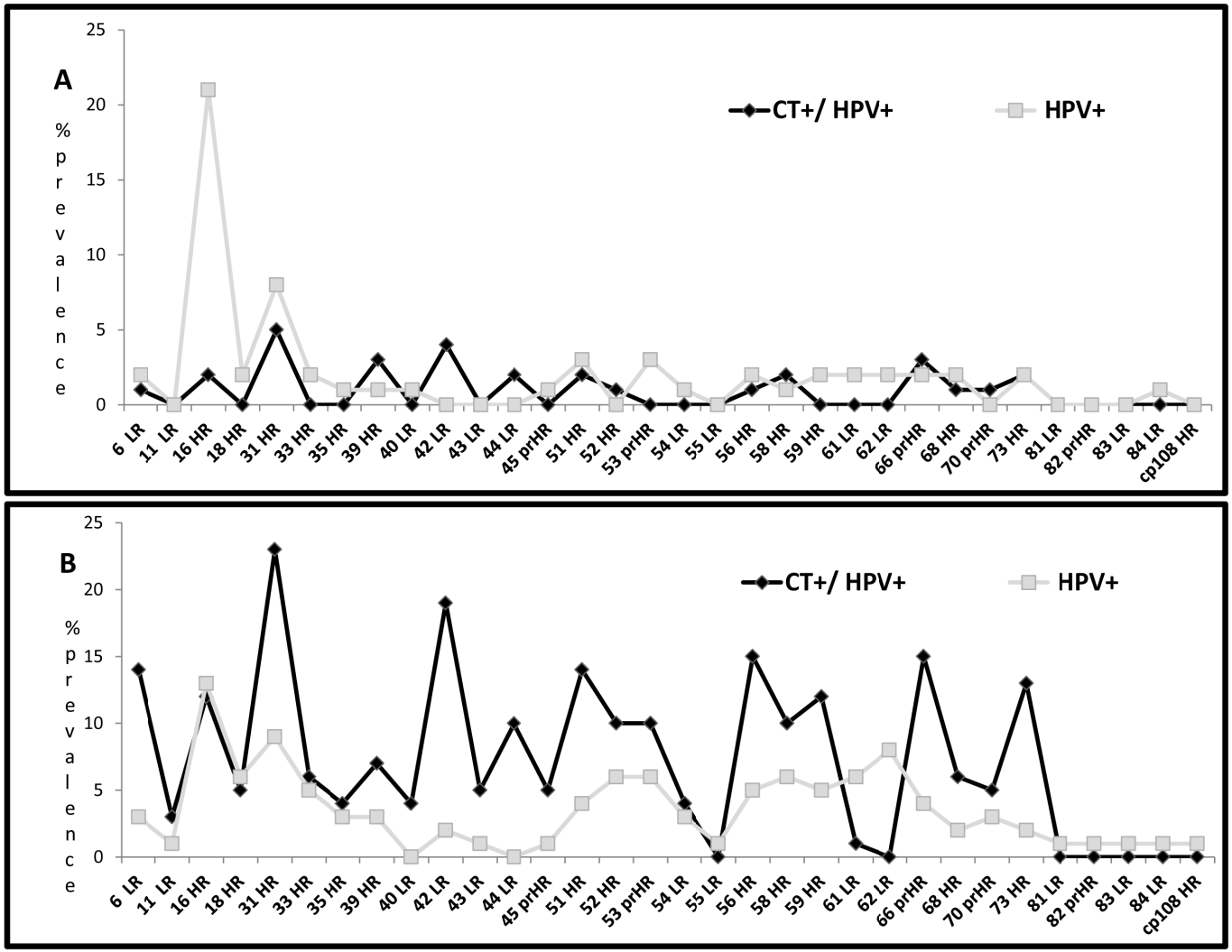


Fig. 13: Prevalence of HPV Single (A) and Multiple (B) infections in CT/HPV co-infected and HPV infected women. **LR**: low risk; **prHR**: presumable high risk; **HR:** high risk

DISCUSSION

In our previous pilot study conducted on CS from 1071 women, a high prevalence of CT/ HPV co-infection was documented in 60.4% of tested samples. Of interest, the 62.5% of co-infection included multiple HPV genotypes and was associated to a CT chronic status. ($p < 0.001$) (Seraceni et al., 2014).

In order to corroborate this result, we performed a large retrospective study on 7135 CS samples from females (range age from 14y to 85y) with particular attention to the association between CT positivity and the characteristic of HPV co-infection, including genotypes distribution in single and in multiple infection.

In the present study, HPV was present in 39% (359/921) of women (mean age 39y) with clinical indication for HPV infection, while CT infection was detected in 4% (251/6214) of women (mean age 29y) considered a risk for CT.

The CT chronic status, through Hsp60 expression, was documented in 57% (144/251) of CT positive women (mean age 26y). The CT/HPV co-infection tested at 58% (145/251) (mean age 28y) of which 68% was chronic infection.

As previously described, the age represents an important predictor factor in STIs infection. European survey, in asymptomatic women, reported a prevalence of CT infection ranging between 1.7% and 17% depending on social–economical context (Paavonen, 2012), while in a new systematic meta-analysis from 11 EU/EEA Member States, one non-EU/EEA European countries and four other high income countries, chlamydia prevalence resulted low in ≤ 26y people sexually active and very heterogeneous, compared to women ≤ 25y, ranging from 0.6-10.7% in (Redmond et al., 2015).

In the last report (2009-2012) from Italian Institute of Health, the overall CT prevalence resulted of more 3.2% than women (2.3%) (Salfa et al., 2014), placing Italy among the countries with a low endemicity for this infection (Marcone et al, 2012).

In contrast with the national data, in our series, the prevalence of CT infection confirmed to be at 4%, with the highest rate of infection (14%) in young women (≤25y) confirming age as an important risk factor. In addition, our large study showed a high prevalence of CT/HPV co-infection (58%) mostly in younger women (68%).

On the contrary, recent investigation in young women (16-26y) from different Italian areas reported a CT prevalence of 5.8% and a CT/HPV co-infection of 2.7% (Panatto et al., 2015). The above discordancy, compared to our data could be due to the molecular assay

typology used to test both CT and HPV, or to the different geographic areas (3 cities in northern Italy) or to the specific young women cohort examined (16-26y).

Of note, CT infection was associated to HPV multiple genotypes in 78% of the analyzed cases suggesting a strong susceptibility of these women in acquiring multiple HPV infections or, as recently demonstrated, a role of CT in type-specific HPV re-detection due to reactivation of a low level of persistent oncogenic HPV (Shew, 2013).

Several epidemiological data suggested that CT may play a role in cervical carcinogenesis (Sillins et al., 2005) although the question of how CT synergize with HPV in this transforming process is still debated (Idahl et al., 2010). CT/HPV co-presence was found in cervical precancerous lesions, and in HPV positive patients were recovered IgG antibodies or CT DNA (Paavonen, 2012; Silva et al., 2014; Tavares at al., 2014).

Regarding the characteristic of HPV infection among infected women, the presence of single or multiple infections showed an interesting distribution. HPV single infection was highly detected (60%) in women infected solely with HPV, while, in CT/HPV co-infected women**,** multiple infections were found prevalent (68%). Moreover, HPV genotypes distribution resulted extremely different. These findings seem to reinforce previous published data (Carozzi et al., 2014) which suggest that the HPV genotypes variability seems to depend on the local dynamics of transmission and local type mix, a phenomenon also reported in different Italian geographic area (Agarossi et al., 2009; Giorgi Rossi et al., 2010, Giuffrè et al., 2010; Bellaminutti et al. 2014).

In a worldwide meta-analysis on prevalence of HPV in women with normal cytology, although it was high and variable across world regions, HR-HPV16 (22.5%) followed by HR-HPV18,-52-,31-58,-39,-51,-56 and LR-HPV6, were the most common genotypes detected (Bruni et al., 2010). In Italy, various authors detected HPV16 (Giorgi et al., 2011; Bianchi et al., 2013; Carozzi et al., 2014) or HPV belonging to groups 1, 2A or 2B, among subject with CT/HPV co-infection, (Panatto et al., 2015) as the most prevalent types.

Interestingly, in our study, HPV16 resulted the most representative genotype in women infected by HPV only (overall 33%, 21% in single HPV infection), followed by HPV31 (17%). Moreover, some viral genotypes were recognized as the most representative genotypes exclusively in this group of women showing HPV61,-62 and HPV52.

While HPV61 and 62 were detected in Italy below 5% (Giuffrè et al., 2010), HPV52 seems one of the most common genotypes detected in Korea and Asian patients associated to invasive cancer and HSIL (Cho et al., 2015, Wang et al, 2015), whereas in Italy its prevalence was heterogenic between various centers (1.9-7.1%) (Carozzi et al., 2014).

Sammarco et al., 2013, found that almost half of women with HR-HPV present a persistent HPV31,-39,-73 infection, whereas the most frequently detected HPV genotypes were HPV16,-31 (common at the follow-up) and HPV52,-53. The authors in these women detected a low presence of CT and *Mycoplasma spp* infection, in agreement with our results whilst, in women infected with HPV, CT was not present, thus reinforcing our hypothesis that a primary HPV infection could reduce subsequent CT penetration and consequently the infection. Tavares at al., 2014, based on their collected data, have suggested that CT infection may play an important role in the natural history of HPV, thus suggesting that the HPV and CT association seems more related with potentiating mutual than with common way of transmission. Moreover, studies in vitro on CT immunological response have demonstrated that INF-γ can inhibit CT development (Shemer and Sarov, 1985; Wyrick, 2010), while in HPV infection, INF-γ increases its expression (Scott et al., 1999). In a recent study (Colín-Ferreyra et al., 2015) a high expression level of INF-γ was demonstrated in women co-infected with CT/HPV, promoting the CC development.

The association between CT and HPV genotypes at high risk of cancer development has been previously described. Panatto et al., 2015, by examining a regression analysis, found in co-infected women with CT a significantly higher odds of infection of single HR-HPV or combinations of multiple HPV genotypes with at least one HR-HPV type. This finding is of particular importance for the primary prevention of CC, since concurrent CT infection has been found to be associated with the persistence of HR and multiple HPV genotypes in female adolescents (Samoff et al., 2005). In turn, the persistence of HR-HPV genotypes among cytologically normal women leads to a greatly increased risk of CC (Chen et al., 2011). By contrast, the HPV pattern distribution that we observed in our study in CT/HPV co-infected women, demonstrated a high frequency of HPV genotypes usually lower, compared to HPV Italian screening (Giorgi et al., 2011; Carozzi et al., 2014). Among these, HPV31 and HPV42 were detected both in multiple (23%, 19%) and in single infections (5%, 4%) respectively. In CT/HPV co-infected women, the association between specific HPV genotypes was emphasized. HPV6,-51,-59,-66 were detected together in 19% of the samples, HPV31-56 in 8% and HPV51-66 in 6%.

In Italy, HPV56 was identified between the most frequent genotypes (Carozzi et al., 2014), associated to multiple infection together also to HSP51 (Agarossi et al. 2009), that in North Sardinia, which was unusually found high in invasive cervical neoplasia (Piana et al., 2013). Moreover, we recovered a high percentage of LR-HPV genotypes (HPV40,-42,-43,-

44) exclusively in CT/HPV multiple co-infections, which were not generally detected in this specific area.

An interesting finding of our study was the analysis of the presence of CT chronic infection. The CT-Hsp60 gene expression, linked to CT chronic status, was found positively associated with HPV co-infection in 68% of women and particularly in 72% of young women, confirming our previously data. Of note, 78% of younger women presented multiple infections compared to 54% of older women. On the contrary, in older women, the prevalence of HPV single infection was higher than that recovered in younger women (46% *vs* 22%). Moreover, the expression of Hsp60 gene has been found significantly lower in HPV co-infected women compared to women infected with CT only.

A discussion of this finding can be merely speculative, suggesting that the maintenance of a steady-state level of transcription of Hsp60 gene could favor a balance between the Hsp60 induced pro-inflammatory microenvironment and HPV coexistence (Silva et al., 2014). The chronic inflammation caused by CT increases oxidative stress proteins that seem to trigger HPV cell entrance and replication or enhance DNA breaks that may promote viral integration (Deluca et al., 2011; Silva et al., 2014; Tavares at al., 2014). Although it has been suggested that the concomitant presence of HPV viral oncoproteins during Hsp60 expression may lead to the ability to survive apoptotic stimuli, uncontrolled proliferation and, finally neoplastic transformation, in this study, specific HPV multiple genotypes were found associated to CT chronic status, independently of the risk attributed to each genotypes (Dean et al., 2008; Cappello et al., 2009).

CONCLUSIONS

The results of this large study confirmed that a high prevalence of multiple HPV infections has been associated with CT chronic infection in young women without cervical lesions. Of remarkable interest, specific HPV genotypes seem to be more frequently associated to CT co-infection. These data may deserve further consideration, owing to the accumulated evidence that the chlamydial chronic status could contribute to favor specific HPV genotypes representing possible implications for the prevention of cervical cancer.

Moreover, CT/HPV co-infection associated to young age support the possibility that CT could increase the risk for pre-cancer lesions in asymptomatic young women, in line with the active role of CT in favoring cells chromosome instability and cervical precancer (Grieshaber et al., 2006; Knowlton et al., 2011).

Even if CT is not present in cervical adenocarcinomas (Quint et al., 2009), its ability to cause a local inflammatory process in the upper genital tract facilitates HR-HPV cell transformation during carcinogenesis. It is worth nothing that CT/HPV co-infection, due to the same modality of transmission, is increasing in sexually active young women and therefore early diagnosis and treatment of infected individuals is required to prevent the spread of the disease and severe sequelae (Verteramo et al., 2009).

Collectively taken, data from this study emphasized the need of a screening program for CT in young women that could be associated to HPV. HPV testing can thus identify women at risk of cervical cancer, reducing cervical cancer incidence in a cost-effective manner in the developed world. HR-HPV types infection has been found in all cervical carcinomas and the persistent infection with the same genotype, strongly increases the risk of developing high-grade pre invasive disease. Although HPV infection is spread among young populations worldwide, the cervical cancer screening program in Italy, covered from Public Health System, is addressed to women over the age of 25 only, independently from their clinical history and the age of the first intercourse, in contrast with other European nations and the USA.

In conclusion this study adds new findings regarding the epidemiology of HPV and CT distribution in young asymptomatic women from North-Eastern Italian area and highlights both the high frequency of CT and CT/HPV co-infection detection in comparison to the National data. The high risk associated with concomitant CT and multiple HR-HPV infections in young women suggests that early prophylactic HPV vaccination and a screening program for CT/HPV co-infection could play a significant role in cervical cancer prevention.

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HPV and *Chlamydia trachomatis* Co-Detection in Young Asymptomatic Women from High Incidence Area for Cervical Cancer

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Chlamydia trachomatis causing chronic inflammatory diseases has investigated as possible human papillomavirus (HPV) cofactor in cervical cancer. The aim of this study is to evaluate the prevalence of *Chlamydia trachomatis* and HPV co-infection in different cohorts of asymptomatic women from a Northern Italy area at high incidence for cervical cancer. Cervical samples from 441 females were collected from Cervical Cancer Screening Program, Sexually Transmitted Infectious and Assisted Reproductive Technology centres. HPV and *Chlamydia trachomatis* were detected simultaneously and genotyped using a highly sensitive bead based assay. The overall prevalence of *Chlamydia trachomatis* was estimated 9.7%, in contrast with the reported national data of 2.3%, and co-infection with HPV was diagnosed in the 17% of the samples. In females $\leq$25 years of age, the infection reached a peak of 22% and co-infection with HPV of 45.8% ($P < 0.001$). Of note, in young females diagnosed with low grade cervical lesions, no significant difference between *Chlamydia trachomatis* and HPV distribution was observed, while differently, HPV co-infection was found significantly associated to the presence of intraepithelial lesions when compared to older females (20% vs. 1%; $P < 0.001$). In this study, the use of a high sensitive molecular technique exhibited higher analytical sensitivity than the referred assays for the diagnosis of *Chlamydia trachomatis* and HPV co-infection in asymptomatic females, leading to reduction of the potential to identify incorrectly the infection status. An active screening for timely treatment of *Chlamydia trachomatis* infection is suggested in young females to evaluate a possible decrease in incidence of pre-cancer intraepithelial lesions.

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KEY WORDS: *Chlamydia trachomatis*; human papillomavirus; cancer co-factors; cervical lesions; *Chlamydia trachomatis* screening

INTRODUCTION

Human Papillomavirus (HPV) and *Chlamydia trachomatis* are considered among the most common sexually transmitted infections worldwide [Bosch et al., 2012; Forman et al., 2012; Fernández-Benítez et al., 2013; Gottlieb et al., 2013].

A persistent infection with an oncogenic high risk-HPV type is recognized as a crucial event for cervical cancer development [Bosch et al., 2008]. Although only a small number of females infected with HPV develop cervical cancer, cervical precursor lesions designed as squamous intraepithelial lesions are frequently diagnosed, a process influenced highly by the HPV genotype and by other factors.

There is a great body of evidence showing that suboptimal host immune response could explain the inter-individual differences in the outcome of HPV

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infection. Most of HPV infections acquired during sexually active life are resolved, specifically in young female, after a median follow-up of 6 months whereas changes in the immunological response due to additional antigenic stimuli, such as a concurrent infection, may decrease the host ability to resolve HPV [Muñoz et al., 1996; Braaten and Laufer, 2008].

Microorganisms causing chronic inflammatory diseases, such as *Chlamydia trachomatis*, have been investigated in the last decade as associated possible risk factor for HPV transmission and persistence cooperating in the cervical carcinogenesis process [Samoff et al., 2005; Silins et al., 2005; Luostarinen et al., 2013; Shew et al., 2013].

Chlamydia trachomatis is a potent immunogen that highly stimulates the chronic inflammation pathway leading to a rapid immune response on the part of lymphocytes activated previously [Choroszy-Król et al., 2012]. The clinical course is usually subacute although, due to the high frequency of the asymptomatic phase, *Chlamydia trachomatis* may induce host chronic inflammation, epithelial tissue damage and pelvic inflammatory disease. In some cases, the infection has been associated with cervical atypia and/or metaplasia, which in turn, may increase the risk of neoplasia [Luostarinen et al., 2013]. European population-based studies reported that the prevalence of *Chlamydia trachomatis* in asymptomatic females ranges between 1.7% and 17% depending on social–economical context [Plummer et al., 2003; Cooksey et al., 2010], classifying Italy as a low endemic area with an overall prevalence of 2.3% [Wilson et al., 2002]. Many studies carried out on *Chlamydia trachomatis* demographic distribution in a wide range of different geographical settings suggested that age, referred usually to a cut-off of 25 years, represented the most important predictor factor, [Nelson and Helfand, 2001; Honey et al., 2002; Simonetti et al., 2009; Haggerty et al., 2010] recently included in the prevention guidelines by the Centre for Disease Control and Prevention [Workowski and Berman, 2011].

The aim of the present study was to assess the prevalence of *Chlamydia trachomatis* and HPV co-infection in different cohorts of asymptomatic females from a hyperendemic area for cervical cancer, in order to estimate the grade of spread of these infections and the association with cervical lesions. For this purpose, DNA extracted from cervical specimens was analysed for the simultaneous detection of *Chlamydia trachomatis* and HPV by a high sensitive bead-based assay supported by the Luminex technology.

MATERIALS AND METHODS

Specimens

Cervical samples were obtained over approximately 5 months in 2012, from a group of Italian females recruited as outpatients attending the prevention centre for Cervical Cancer Screening Program, the Sexually Transmitted Infections clinic and the Assisted Reproductive Technology centre. Cervical samples were collected using a 200 mm polyethylene Cervex brush device (Rovers Medical Devices B.V., Oss, The Netherlands) in 500 μl of TE buffer. The top portion uses a soft, flexible brush to obtain cell samples, while the shape allows the top edges to follow the contours of the cervix. The longer middle bristles reach deep into the endocervical canal while the shorter bristles touch both the ectocervical area (external os) and the transformation zone (T-zone). The cytopathological classification was performed in accordance with the diagnostic criteria for the Bethesda System 2001 [Solomon et al., 2002].

Specimens were collected anonymously, coded with indication of age and stored at −80°C at the Virology laboratory of the Institute for Maternal and Child Health (IRCCS) - "Burlo Garofolo" of Trieste, Italy. Among the 441 recruited females, 305 (mean age 36 ± 10 years) were included in group 1 (Cervical Cancer Screening Program) 85 (mean age 28 ± 10 years) in group 2 (Sexually Transmitted Infections) and 51 (mean age 37 ± 10 years) in group 3 (Assisted Reproductive Technology) respectively. All females were of Caucasian origin, living in the same geographic area, referred no previous history or symptoms of sexually transmitted infections and were asymptomatic for *Chlamydia trachomatis* or other genital infections at the time of sampling.

The study (R.C. n°02/11) was approved by the Institutional Scientific Board of the IRCCS "Burlo Garofolo"–Trieste, Italy and informed written consent was obtained from the participants.

HPV Type-specific E7 PCR Bead-Based Multiplex Genotyping and *Chlamydia Trachomatis* Detection

DNA was extracted from 500 μl of samples using a commercial kit (High Pure PCR Template preparation Kit, Roche Applied Science, Mannheim, Germany). The multiplex HPV type-specific E7 PCR (IARC, Lyon, France) utilizes HPV type-specific primers targeting the E7 region for the detection of 12 high risk-HPV types (HPV-16,-18,-31,-33,-35,-39,-45,-51,-52,-56,-58,-59), 7 possible or probable high risk-HPV types (HPV-26,-53,-66,-68 (a and b),-70,-3,-82), and 2 low risk-HPV types (HPV-6 and-11), with detection limits ranging from 10 to 1,000 copies of the viral genome. The amplicon size varies between 210 and 258 bp [Comar et al., 2012].

Two primers designed within a gene encoding for a conserved hypothetical virulence plasmid protein were additionally added for the amplification of *Chlamydia trachomatis*. Moreover, primers for amplification of the B-globin gene were also included as control for the quality of the template DNA. Following PCR amplification, 10 μl of each reaction mixture was analysed by MPG using the Luminex technology

(Luminex Corporation, Austin, TX) as described previously.

In brief, HPV genotypes were detected as the median fluorescence intensity (MFI) of at least 100 beads per bead set. The background value for each probe was considered the MFI value resulted from hybridization mixture without the addition PCR product. The cut-off was computed by adding 5 MFI to 1.1 the median background value. [Gheit et al., 2006; Schmitt et al., 2010].

Real Time PCR for *Chlamydia Trachomatis*

DNA samples were further investigated by Real Time PCR (RT-PCR) as confirmatory assay for *Chlamydia trachomatis*, detection limit of the test 10 copies/μl, using the commercial kit (CTDNA Dia.Pro, Milan, Italia) following recommended protocol. Amplification and PCR product detection were performed with the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Monza, Italy).

Statistical Analysis

Only cases with available and valid *Chlamydia trachomatis* and HPV DNA laboratory results were included in the present study. Statistical analyses were performed using the IBM SPSS Statistics 20 statistical program. Age was reported as mean ± standard deviation (σ) and discrete variables as number and percentages. The ANOVA (Analysis of variance) Test was performed to compare mean age values across groups and the Chi Square Test was calculated to compare frequencies of discrete variables. The Fisher Exact Test was applied when necessary. A value of $P \leq 0.05$ was considered statistically significant for all tests.

RESULTS

The analysis were restricted to cervical specimens, corresponding to 441 samples, tested positive for the globin gene using a bead-based multiplex PCR assay for *Chlamydia trachomatis*/HPV co-detection (Fig. 1). The performance of the assay has been evaluated by testing DNA in cervical samples. The agreement was almost perfect for both HPV (K coefficient: 0.71) [Comar et al., 2012] and *Chlamydia trachomatis* (K coefficient: 0.98) and the reference assays using for validation, Linear Array for HPV and RT-PCR for *Chlamydia trachomatis*, respectively.

The prevalence and distributions of *Chlamydia trachomatis* and HPV infections were reassumed in Table I. In brief, the overall prevalence of infections showed that the 9.7% (43/441) of the tested samples were positive for *Chlamydia trachomatis* and the 29% (127/441) for HPV as solitary infections, while co-infections with the two microorganisms were detected in 17% (75/441) of samples. Regarding the distribution of infections by clinical departments, the rate of *Chlamydia trachomatis* detection ranged from 6% to 26%, of HPV from 6% to 40% and of *Chlamydia trachomatis*/HPV co-infection from 10% to 42%. Specifically, the frequency of *Chlamydia trachomatis* infection was higher in samples from females attending the Sexually Transmitted Infections centre in comparison to those from the Cervical Cancer Screening Program and Assisted Reproductive Technology centres ($P < 0.001$). Conversely, HPV was found frequently in samples from Cervical Cancer Screening Program rather than in those from Sexually Transmitted Infections and Assisted Reproductive Technology ($P < 0.001$). In addition, the rate of co-infection was higher in samples from women attending the Sexually Transmitted Infections centre, showing a statistically significant difference ($P < 0.001$).

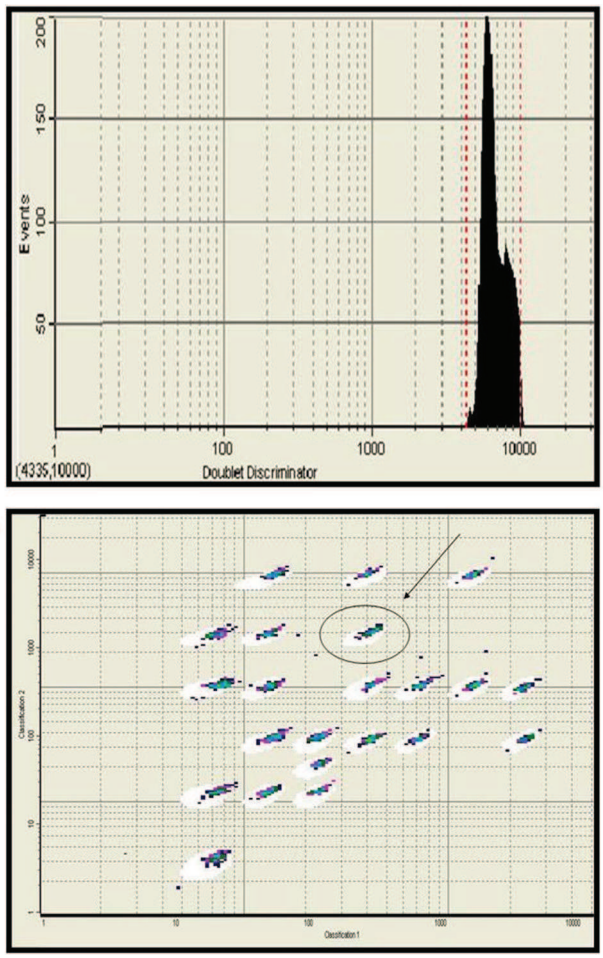

Fig. 1. The Bioplex results window: a) The histogram plots the number of events per channel number for the selected well, analyte(s), and channel type. An event is generated when particles such as a bead or aggregated beads pass through the path of the lasers. During a reading, a doublet discriminator (DD) channel measures the amount of light scatter from particles that flow past the red laser. The light scatter is directly proportional to particle size. b) Bead Map co-detection of HPV and Chlamydia trachomatis. The white areas on the map (arrow) indicate the expected regions of the selected analytes. Each cluster of dots that falls within a white region represents a unique bead set (analyte) within an assay.

TABLE I. Demographic Characteristics of Enrolled Females and Prevalence of CT, HPV and CT/HPV Infections

		Infections n°(%)			
Clinical Department	n° females	Mean Age ± σ	CT	HPV	CT/HPV
CCSP*	305	36±10	17 (6%)	122 (40%)	30 (10%)
STI**	85	28±10	22 (26%)	5 (6%)	36 (42%)
ART	51	37±10	4 (8%)	0 (0%)	9 (18%)
Total	441	33.8±10	43 (9.7%)	127 (29%)	75 (17%)

CCSP: Cervical Cancer ScreeningProgram; STI: Sexually Transmitted Infection; ART: Assisted Reproductive Technology; CT: *Chlamydia trachomatis*; HPV: Human Papillomavirus; CT/HPV: *Chlamydia trachomatis* and Human Papillomavirus co-infection.
STI** versus CCSP and versus ART considering CT $P < 0.001$.
STI** versus CCSP and versus ART considering CT/HPV $P < 0.001$.
*CCSP versus STI and versus ART considering HPV $P < 0.001$.

The age-standardize data (cut-off 25 years) referred to the prevalence of the two pathogens was shown in Table II. Although, the Assisted Reproductive Technology group has not been considered since no females aged less than 25 years attended the centre for infertility or reproduction defects, results from this analysis highlighted that *Chlamydia trachomatis* and *Chlamydia trachomatis* / HPV co-infection were associated significantly to young females (< 25 years), independently from clinical categories ($P = 0.02$; $P = 0.03$, respectively).

In these series, oncogenic high risk-HPV types were present in 100% of the infected females showing that 57.5% of the infections were attributed to HPV-16; 16.5% to HPV-31; 15% to HPV-33; 5.5% to HPV-18 and 5.5% to other genotypes. Interesting, a high rate of multiple HPV infections (2 to 5 HPV types) was observed, showing an overall prevalence of 75.6% (96/127) of which, 58.3% (56/96) was associated to *Chlamydia trachomatis* infection.

The cytopathological report was available for 92% (407/441) of the recruited females of which 43% (175/407) showed cytological alterations classified as low squamous intraepithelial lesions. Considering only females with low squamous intraepithelial lesions, a multivariate analysis including age (cut-off 25 years) was performed, which results have been reported in Table III. As expected, HPV was found associated to cervical lesions in 61% of women, independently of age (63% in females aged ≥ 25 years and 45% in females aged ≤ 25 years). HPV-16 genotype was the most prevalent (42%), found as solitary infection in 66.6% of cases.

In this group, *Chlamydia trachomatis* was found in 8% of the samples, which co-infection with HPV was detected more frequently in females aged ≤ 25 years (20% *vs*. 1%; $P < 0.001$).

DISCUSSION

In the developed world, HPV testing can identify females at risk for cervical cancer reducing cervical cancer incidence in a cost-effective manner. High risk-HPV types have been demonstrated in almost 100% of cervical carcinomas and persistent infection with the same genotype increases strongly the risk of developing high-grade pre-invasive disease.

Nevertheless, there is epidemiologic data suggesting that *Chlamydia trachomatis* may contribute in cervical carcinogenesis [Silins et al., 2005] although how it synergizes with HPV in the transforming process is still debated [Idahl et al., 2010]. It is worth noting that due to the same modality of transmission, *Chlamydia trachomatis* and HPV co-infection, is increasing in sexually active females and therefore early diagnosis and treatment of infected subjects is required to prevent the spread of the infections and possible sequelae [Verteramo et al., 2009]. In this context, the availability of a highly sensitive and specific molecular technique could improve the

TABLE II. Prevalence of HPV and CT Infections in Females According to Age Cohorts (Clinical Cut-off 25 Years of Age) and to Clinical Department

	Infections ≥ 25 years				Infections ≤ 25 years			
Clinical Dep	n° females	CT n°(%)	HPV n°(%)	CT/HPV n°(%)	n° females	CT n°(%)	HPV n°(%)	CT/HPV n°(%)
CCSP	254	8(3%)	109(43%)	10(4%)	51	9(18%)*	13(26%)	20(39%)*
STI	40	10(25%)	3(8%)	12(30%)	45	12(27%)	2(4%)	24(53%)**
ART	51	4(8%)	0(0%)	9(18%)	0	NA	NA	NA
Total	345	22(6.4%)	112(38%)	31(8.9%)	96	21(22%)	15(15.6%)	44(45.8%)

CCSP: Cervical Cancer Screening Program; STI: Sexually Transmitted Infection; ART: Assisted Reproductive Technology; CT: *Chlamydia trachomatis*; HPV: Human Papillomavirus; CT/HPV: *Chlamydia trachomatis* and Human Papillomavirus co-infection.
NA: not applicable.
*CCSP considering CT $P < 0.001$ and CT/HPV $P < 0.02$.
**STI considering CT/HPV $P < 0.03$.

TABLE III. Infection Frequency of HPV, CT, and CT/HPV Co-infection in Cytological Positive Samples According to Age Cohort (Cut-off 25 Years of Age)

	Positive cytological samples	Infection		
Age	n°	CT	HPV	CT/HPV
≥ 25 years old	155	4(3%)	98(63%)*	2(1%)
≤ 25 years old	20	4(20%)	9(45%)	4(20%)**
Total	175	8(5%)	107(61%)	6(3%)

CT: *Chlamydia trachomatis*; HPV: Human papillomavirus; CT/HPV: *Chlamydia trachomatis* and Human papillomavirus co-infection.
*HPV versus CT and CT/HPV $P < 0.001$.
**CT/HPV ≥ 25 years versus ≤ 25 years $P < 0.001$.

diagnosis of these pathogens, specifically when present at very low copies in asymptomatic high-risk females.

In this study, a better performance in the detection rate of *Chlamydia trachomatis* and HPV infection was obtained using a highly-sensitive bead-based multiplex PCR assay. In this cohort, the prevalence of *Chlamydia trachomatis* was of 9.7% in contrast with the national data indicating a percentage of 2.3% using routine molecular techniques [Marcone et al., 2012], suggesting that the prevalence of low-copy-number infections may be underestimated, specifically in asymptomatic subjects, by other assay.

In agreement, the highest prevalence of *Chlamydia trachomatis* was found in young females aged less than 25 years old (22%) although a peak of co-infection with HPV was reached in 45.8% of them, confirming "age" as an important demographic risk factor in the acquisition of these infections. Of note, *Chlamydia trachomatis* was found associated, in 58.3% of the cases, with a high risk-HPV genotype suggesting a role for *Chlamydia trachomatis* in type-specific HPV redetection, probably due to reactivation of a low level of persistent oncogenic HPV as recently reported [Shew et al., 2013]. Moreover, in young females with a referred low grade abnormal cytology, *Chlamydia trachomatis* or co-infection with HPV was found significantly associated to the cytological status independently of others risk factors. These findings seem to support a role for *Chlamydia trachomatis* in cell transformation, probably acting as additional risk factor, but in line with the exerted biological effect favouring damage of the mucosal barrier, interference in HPV viral clearance, cell chromosome instability and inflammatory process of the upper genital tract. [Grieshaber et al., 2006; Knowlton et al., 2011].

Given our results, although the highly sensitive multiplex assay used in this study appear to perform better at the analytical level than other molecular tests, the clinical benefit remains limited to *Chlamydia trachomatis* infection since it could contribute to prevention of sequelae and to a possible decrease of incidence of pre-cancer intraepithelial lesions.

In conclusion, this survey reported new findings about the epidemiology of *Chlamydia trachomatis* and HPV co-infection in young asymptomatic females from a hyperendemic area for cervical cancer, highlighting the high prevalence of infection and the significantly association with cytological abnormalities.

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We declare no conflict of interest.

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INFECTIOUS AGENTS AND CANCER

RESEARCH ARTICLE Open Access

High prevalence of hpv multiple genotypes in women with persistent chlamydia trachomatis infection

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Abstract

Background: *Chlamydia trachomatis* interaction with HR-HPV types has highlighted a central role in cervical cancer development. The aim of this study was to investigate HPV prevalence and genotypes distribution in women at risk for *C. trachomatis* infection and negative for intraepithelial lesion or malignancy.

Methods: 1071 cervical swabs were tested for *C. trachomatis* by Real Time PCR and genotyping by ompA gene sequencing. Additionally, a quantitative Real time-PCR was performed to assess the expression of the *C. trachomatis* Hsp60–encoding gene (Ct604 portion), linked to a persistent status of infection. HPV infection and genotypes was investigated in *C. trachomatis* positive women using Luminex technology.

Results: *C. trachomatis* infection was detected in 53 out of 1071 (4.5%) samples, of which the 53% resulted positive for Hsp60 gene expression. The overall prevalence of HPV infection in *C. trachomatis* positive samples was of 60.4% (32/53): in 37.5% of samples was present a single genotype, while multiple genotypes infections were found in the 62.5% of them. Among women with a *C. trachomatis* chronic infection, 68% were HPV co-infected and the 79% showed multiple genotypes. Should be noted that levels of *C. trachomatis* Hsp60 expression in HPV co-infected women were significantly lower compared to women infected only with *C. trachomatis*. The *C. trachomatis* serotype F was found in the majority of samples, independently of HPV infection.

Conclusions: A high prevalence of HPV multiple infections have been found in young women affected with a *C. trachomatis* chronic infection. These observations suggested that the expression of CHSP60-1, interfering with both apoptotic and cellular senescence pathways, may promote a favourable local microenvironment for HPV infection.

Keywords: Human papillomavirus, *Chlamydia trachomatis*, HPV multiple genotypes, Hsp60 RNA persistent infection

Background

Chlamydia trachomatis (*C. trachomatis*), an intracellular bacteria characterized by a unique biphasic developmental cycle, is the most common sexually transmitted pathogens in women. Although *C. trachomatis* can cause pelvic inflammatory disease (PID), infertility, ectopic pregnancy, the clinical course is usually sub-acute and poorly symptomatic and the microorganism is rarely detected in subjects without clinical signs of infection [1].

The ability of *C. trachomatis* to cause chronic persistent infection, characterized by the permanence of microorganisms in the host cells, can represent a common event [2]. During persistent status, *C. trachomatis* produces a large quantity of Heat shock protein 60 (Hsp60) exhibited on the host cell surface and released into the extracellular space and in the bloodstream. This protein is considered a useful marker during clinical complications since its expression induce host chronic inflammation response [1,3,4]. Thus, this microorganism is considered as a potent immunogen, stimulating a rapid and intense inflammatory response involving previously sensitized lymphocytes [5].

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Although epidemiological data have not yet provided consistent evidence about a real implication of *C. trachomatis* in cervical cancer, the co-infection with Human papillomavirus (HPV), sharing the transmission route and the same risk factors, have recently highlighted [6-8]. A role for *C. trachomatis* as cofactor was suggested, since it seems to facilitate the penetration of HPV and the progress of cervical lesions interfering in the immunological response [9]. Moreover, some authors recently detected a high-risk for the development of cervical cancer in patients with HPV infection and history of *C. trachomatis* [10]. Nevertheless, the prevalence and distribution of HPV genotypes associated to *C. trachomatis* infection and its clinical persistence are poorly explored.

HPVs are a family of DNA viruses that infect cutaneous epithelia, oral and genital mucosa. More than 100 different HPV types have been identified and characterized in two risk classes on the basis of their oncogenic potential: Low-risk (LR-HPV) types associated with benign genital warts and High-risk (HR-HPV) type considered the etiological agents of cervical cancer and other genital malignancies [11]. Approximately 15 HR-HPV genotypes are clearly associated with cervical cancer of which HPV16 and HPV18 are the most carcinogenic, since they are responsible for approximately 50% and 20% of all cervical cancers worldwide, respectively. Multiple human papillomavirus genotypes often coexist within cervical epithelia and are frequently detected together in women with precancer cervical lesions [12]. Nevertheless, although HPV is a prerequisite for cervical cancer only a small number of women exposed to this virus developed cancer, implying that other risk factors may be considered as cofactors rather than independent factors. On this basis, the characterization of HPV infection in women suffering from *C. trachomatis* could be important in generating hypotheses regarding the possible synergism of these pathogens in cervical malignancy [13-16].

The aim of this study was to investigate HPV genotypes distribution and the frequency of infection in Italian women considered at risk for *C. trachomatis* infection but negative for cervical lesions or malignancy. Furthermore, the role of *C. trachomatis* chronic infection in promoting HPV susceptibility has been evaluated.

Methods

Specimens

In 2013 year, cervical swabs (CS) specimen from 1071 women at risk for *C. trachomatis* infection were collected at the Virology laboratory of the IRCCS-Burlo Garofolo of Trieste, Italy, as part of *C. trachomatis* routine screening practices. Cervical samples were collected using a 200 mm polyethylene Cervex brush device (Rovers Medical Devices B.V., The Netherlands) in 500 μl of TE buffer. The study was approved by the Institutional Scientific Board of the Institute for Maternal and Child Health - IRCCS "Burlo Garofolo"–Trieste, Italy and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

C. trachomatis detection

Genomic DNA was extracted, after samples centrifugation, using the QIAamp DNA Blood miniKit (Qiagen, GmbH, Germany) as indicated by the supplier, and then stored at −80°C until analysis.

The presence of *C. trachomatis* DNA was detected by Real Time PCR (RT-PCR), using a commercial kit (CTDNA, Dia.Pro, Italia), detection limit of the assay was 1 copies/μl, to amplify a conserved region of the cryptic plasmid element of *C. trachomatis*, following recommended protocol. The amplification and PCR product detection were performed with the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Italy).

HSp60 gene expression

C. trachomatis -RNA extraction and cDNA synthesis

An aliquot, from each CS fresh specimen (1 ml), was centrifuged at 14.000 rpm for 15 min at 4°C and total RNA was extracted to the pellet obtained by RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) in according to the manufacturer's instructions. The RNA final concentration (25 μg/ml) eluted in 50 μl of distilled water, was treated during sample processing with Dnase I (RNase-Free DNase Set, Qiagen GmbH, Hilden, Germany), and subsequently stored at −80, in order to avoid DNA genomic contaminations.

cDNA synthesis was performed using kit SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Briefly, 14 μl of RNA were added to a mixture containing: 5X VILO™ Reaction Mix10, 10X Superscript Enzyme Mix and diethyl-pyro carbonate water (DEPC-treated) until a final volume of 20 μl and subsequently incubated at 42°C for 60 minutes and then at 85°C for 5 minutes. cDNA synthesized was employed for quantitative RT-PCR (*q*PCR).

C. trachomatis-Hsp60 qPCR

To quantify the transcript level for the *C. trachomatis* portion Hsp60 gene (Ct604) in specimen, a dedicated *q*PCR was used as previously described [17,18]. The sensitivity of each assay was determined to be the lowest dilution of DNA ($2\ 10^{-4}$ ng/ml corresponding to one genome copies/μl) and a standard curve equations were used to calculate the absolute copy number of gene mRNA.

In brief, the test included a serial standard curve, negative control with PCR-grade water and a positive control (*C. trachomatis* strain-TW-3). PCR reaction, in a final volume of 20 μl, contained: 5 μl of cDNA, 2 μl LC FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Germany), 5 mM $MgCl_2$ and 0.8 μM each primer. The thermocycling condition was: 95°C at 10 min followed by 40 cycles of 15 s at 95°C and 1 min at 63°C. The amplification was carried out in an ABI 7900HT Fast Real Time PCR System (Applied Biosystems, Italy). The concentration of unknown samples based on their *C*t values was determined with analytical software (Software SDS 2.4; Applied Biosystems) [19]. The specificity of *q*PCR was further confirmed by agarose gel electrophoresis analysis, which showed the expected amplification product of 161 bp in length.

C. trachomatis genotyping

C. trachomatis genotyping were performed by ompA gene primers; positive PCR amplification products were purified, sequenced and analysed with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as previously described [20].

HPV detection and characterization

HPV was detected in CS specimens using molecular assay supported by the Luminex technology (Luminex Corporation, Austin, TX). HPV genotyping was performed using the type specific E7 polymerase chain reaction bead-based multiplex assay (TS-E7-MPG, IARC, Lyon, France) as recently described [21]. The detection limits of the assay ranged from 10 to 1,000 copies of the viral genomes included in the analysis. In addition, the β-globin gene was included, as internal positive control [22]. To analyse a greater number of LR-HPV types, (HPV-6,-11,-40,-42,-43,-44,-54,-61,-70), the Anyplex™ II HPV Detection assay (Seegene Inc., Arrow diagnostics, Italy) was additionally used in according to manufacturer's instructions. A human housekeeping gene was used as an endogenous internal control, which can ensure DNA purification, PCR reaction and specimen quality (Anyplex™ user manual, Seegene 2012).

Statistical analysis

Chi Square Test was used to compare frequencies of discrete variables: Fisher Exact Test was applied when necessary. P value ≤ 0.05 was considered as the threshold of statistical significance for all tests.

Results

During 2013 year, 1071 cervical swabs from women at risk for *C. trachomatis* infection (mean age 35 ± 10 years; range: from 15 to 72 years) were analysed at the Virology laboratory of IRCCS-Burlo Garofolo, Trieste, Italy. Of these, 829 included Outpatients (mean age 35 ± 10 years), 161 patients from Sexually Transmitted Infection centre (STI) (mean age 30 ± 10 years) and 81 women from the Assisted Reproductive Technology (ART) (mean age 37 ± 10 years) clinic. All women were asymptomatic for *C. trachomatis* and other genital infections at the time of sampling, with the exception of STI women showing inflammatory symptoms. Moreover, all women were negative for cytological alterations, in accordance with Bethesda System 2001 diagnostic criteria [23].

Table 1 Prevalence of CT distribution in women at risk of infection by clinical department and mean age

Clinical Department	n° women	Mean Age ± σ	CT n° + (%)
Outpatients	829	35 ± 10	29 (3.5%)
STI	161	30 ± 10	20 (12.4%)
ART	81	37 ± 10	4 (4.9%)
Total	1071	35 ± 10	53 (4.5%)

STI: Sexually Transmitted Infection; **ART:** Assisted Reproductive Technology; **CT:** *C. trachomatis*.

In this study, as showed in Table 1, the overall prevalence of *C. trachomatis* infection was 4.5% (53/1071) (mean age 35 years). As expected, *C. trachomatis* prevalence, stratified by the different clinical departments and by age, was found statistically significant higher (12.4%) in symptomatic women attending the STI center, than in asymptomatic women from the other groups ($p < 0.001$).

In women with a *C. trachomatis* infection the overall prevalence of HPV was high, tested to 60.4% (32/53), as shown in Table 2. Regarding the distribution of HPV, the 37.5% (12/32) of the infections were constituted by a single genotype while the 62.5% (20/32) by multiple genotypes (from 2 to 8 types), recovered more frequently in younger women (mean age 24 years) (Figure 1).

The analysis of HPV genotypes, reassumed in the Figure 2, showed that HPV-42 and HPV-31 represented the most frequently detected genotypes, standing at 28% and 22%, respectively. Moreover, in these women, the

Table 2 HPV co-infection distribution in women with CT infection

CT POS	HPV	HPV single infection	HPV multiple infections*
N°	**N°POS/TOT**	**N°POS/TOT**	**N°POS/TOT**
	(%)	**(%)**	**(%)**
53	32/53	12/53	20/53
	(60.4)	(22.6)	(37.7)

*(from 2- to 8 genotypes); **CT:** *C. trachomatis;* **HPV:** Human papillomavirus.

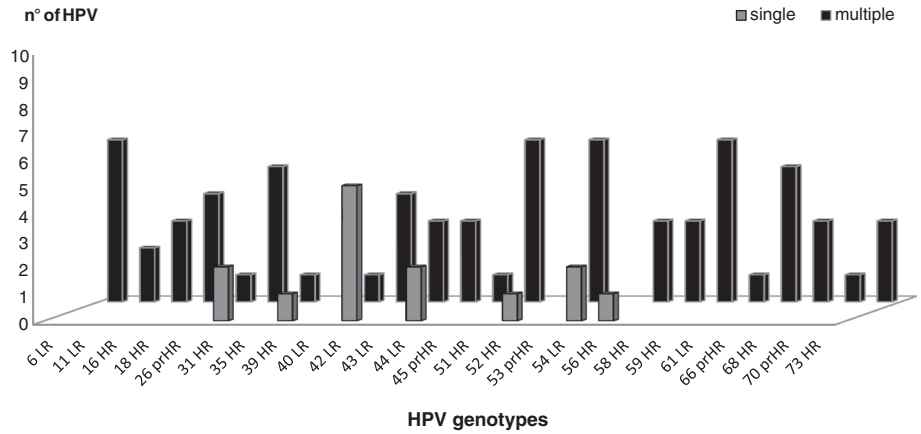

Figure 1 HPV genotypes distribution detected in *C. trachomatis* positive women, according to single versus multiple concurrent infection status. HR: High risk; prHR: Presumable High risk; LR: Low risk.

genotypes HPV-39,-53,-56,-58 were present only as single infection while the genotypes HPV-6-51-59-66 were detected together in 31% (10/32) of the recovered infections.

In order to characterize the chlamydial phase of infection, the mRNA expression of the Hsp60 gene showed that the 53% (28/53) of the women with *C. trachomatis* were suffering from a chronic infection (Table 3). Of these women (mean age 26 years), the 68% (19/28) resulted co-infected with HPV. In particular, the 79% (15/19) presented multiple infections and the 21% (4/19) single infections. Moreover, the level of Ct-Hsp60 expression was found significantly lower (±396 copy/μl) in women co-infected with HPV compared to women infected only with *C. trachomatis* (±862 copies/μl). The evidence for viable organisms and not just residual DNA from a previous infection was supported to high correlation between DNA and RNA results (data not shown), considering that, the expressed gene should be linked to the *C. trachomatis* persistence.

The classification of *C. trachomatis* serotypes through the ompA gene amplification and subsequent sequencing, performed in available samples, had revealed a high frequency of serotype F, independently of chlamydial status or HPV infection.

Discussion

Several epidemiological studies have stated a positive association between *C. trachomatis* and HPV-related cervical diseases. The co-presence of *C. trachomatis* and HPV was reported in cervical precancerous lesions, and high levels of specific IgG antibodies or DNA of *C. trachomatis* were recovered in HPV positive patients [6,14,15]. However, the exact relationship between *C. trachomatis* and HPV infection is still not completely understood.

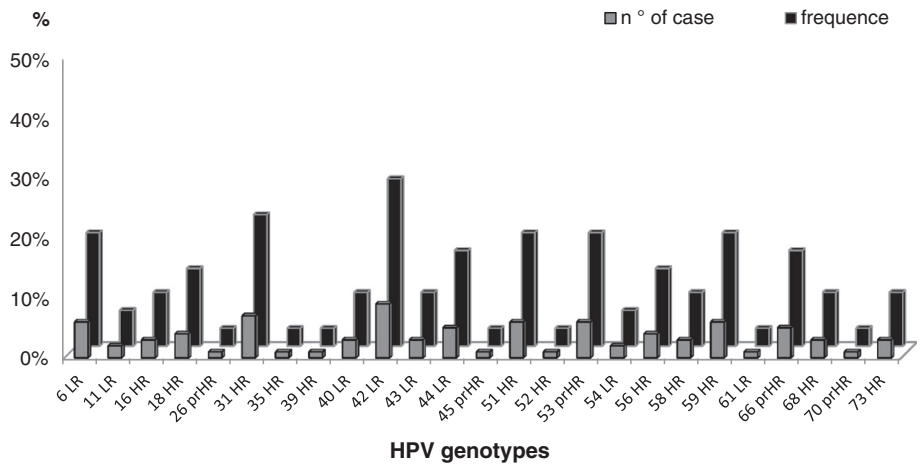

Figure 2 Prevalence of HPV genotypes detected in *C. trachomatis* co-infected women (n° and%). HR: High risk; prHR: Presumable High risk; LR: Low risk.

Table 3 RNA expression of the CT-Hsp60 gene in relation to the presence or absence of HPV infection

	CT-Hsp60 RNA	
	(+)	(–)
N°/TOT (%)	28/53 (53)	25/53 (47)
HPV + n° (%)	19/28 (68)*	13/25 (52)
HPV- n° (%)	9/28 (32)	12/25 (48)

*4/19 (21%) HPV single infection and 15/19 (79%) HPV multiple infections.
CT: *C. trachomatis*; Hsp60: Heat shock protein 60; HPV: Human papillomavirus.

In the present analysis, we evaluated the distributions of HPV DNA-positive infections analysing a large number of specimens collected as part of routine screening practices for *C. trachomatis* prevention. For the first time, estimates are provided on the prevalence of HPV infections and about genotype distribution in women with a chronic *C. trachomatis* infection; this work has never been assessed before. Data from our study showed that the 60.4% of women with a diagnosis of *C. trachomatis* infection and in absence of cervical lesions, resulted co-infected with one or more HPV genotypes. To note, the 53% of them showed a chronic infection and, HPV was found more frequently associated (68%) to this specific chlamydial status. Moreover, a consistent portion of these women (79%) resulted to be infected with multiple HPV genotypes. To note, HPV multiple infections including specific genotypes such as HPV-6-51-59-66, was reported in the 31% of these women supporting evidence that the presence of one HPV type does not increase the likelihood of acquiring further infections, but that, HPV multiple infections might be the result of the local immune system impairment [8,24-31]. In our screening population, the median age of women with 2 to 8 HPV genotypes was 24 years, according to recent data [32,33].

In our series, HPV-31 and HPV-42 represented the genotypes more frequently detected, testing at 28% and 22%, respectively. The overall prevalence of these genotypes, with a smaller estimated oncogenic potentials than HPV-16, were usually lower in HPV screening Italian women (13.8% and 0.3%,) [34,35]. While, conversely, HPV-16 (30.7%) [34] was found only in the 9%.

In the natural history of *C. trachomatis*, a chronic infection is referred as a stop in development of chlamydial cycle, with aberrant bodies formation, and this state is characterized by high transcriptional activity [36].

In this study, the expression of Hsp60 gene, a marker of chronic infection, has been found significantly lower in HPV co-infected women compared to women infected only with *C. trachomatis.* Discussion on this finding can be merely speculative, suggesting that the maintenance of a steady-state level of transcription of Hsp60 gene could favour a balance between the Hsp60 induced pro-inflammatory microenvironment and HPV coexistence [14].

The chronic inflammation caused by *C. trachomatis* increases oxidative stress proteins that seem to trigger HPV cell entrance, and replication or enhance DNA breaks that may promote viral integration [9,14,16]. Although it has been suggested that the concomitant presence of HPV viral oncoproteins during Hsp60 expression may lead to the ability to survive apoptotic stimuli, uncontrolled proliferation and, finally neoplastic transformation, in this study specific HPV multiple genotypes were found associated to *C. trachomatis* chronic status, independently of the genotypes risk [37,38].

Conclusions

In conclusion, the results of the present investigation provide evidence for the notion that a high prevalence of multiple HPV infections has been associated with *C. trachomatis* chronic infection in young women without cervical lesions. In addition, specific HPV genotypes seem to be more frequently associated to *C. trachomatis.* This data may deserve further consideration, owing to accumulate evidence that the chlamydial chronic status could contribute to favour specific HPV genotypes representing possible implications for the prevention of cervical cancer.

Abbreviations

HPV: Human papilloma virus; LR-HPV: Low risk-HPV; HR-HPV: High risk-HPV; C. trachomatis: Chlamydia trachomatis; CIN: Cervical intraepithelial neoplasia; ICC: Invasive cervical carcinoma; PID: Pelvic inflammatory disease; Hsp60: Heat shock protein 60; CS: Cervical swabs.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

SS contributed to: study concept and design, acquisition of data (Chlamydia molecular and CT-Hsp60), drafting of the manuscript. FDS and CC contributed to: collection of clinical specimens and demographic data. RDS and VZ contributed to: technical assistance in molecular HPV analysis. GP contributed to: data analysis. PDA contributed to: data analysis. CC contributed to: drafting of the manuscript. MC contributed to: study concept and design, interpretation of data, and revising the manuscript. All authors read and approved the final manuscript.

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