UNIVERSITAT DE BARCELONA

Cervical cancer and pre-cancer in the EPIC cohort: the role of environmental cofactors

El càncer de coll uterí i els seus precursos en la cohort EPIC: el rol dels cofactors ambientals

Esther Roura Fornells

TESI DOCTORAL

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CONTENTS

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
CI	Confidence Interval
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma In Situ
cLIA	Competitive Luminex based Immunoassay
CT	*Chlamydia Trachomatis*
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EPIC	European Prospective Investigation into Cancer and Nutrition
FDA	Food and Drug Administration
GST	Glutathione S-Transferase
HC2	Hybrid Capture 2
HHV-2	Human Herpes Virus 2
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
HPV	Human Papillomavirus
HR	Hazard Ratio
HT	Hormone Therapy
HSIL	High-grade Squamous Intraepithelial Lesion
IARC	International Agency for Research on Cancer
ICC	Invasive Cervical Cancer
ICO	Institut Català d'Oncologia
ICD-10	International Classification of Diseases 10th revision
IgG	Immunoglobulin G
IUD	Intrauterine Device

LSIL	Low-grade Squamous Intraepithelial Lesion
OC	Oral Contraceptive
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PVs	Papillomaviruses
RR	Relative Risk
SCC	Squamous Cell Carcinoma
STI	Sexually Transmitted Infection
URR	Upstream Regulatory Region
VLP	Virus-Like Particle

INTRODUCTION

INTRODUCTION

In 2012, the International Agency for Research on Cancer (IARC) classified 11 infectious agents as established carcinogenic agents for humans, namely Epstein-Barr virus, hepatitis B virus, hepatitis C virus, Kaposi sarcoma herpesvirus, human immunodeficiency virus type 1, human papillomaviruses, human T-cell lymphotropic virus type 1, *Opisthorchis viverrini* and *Clonorchis sinensis*, *Schistosoma haematobium*, and *Helicobacter pylori* [1]. Of the estimated 14 million new annual cancer cases worldwide, 15.4%, corresponding to 2,2 million cases, were attributable to infection [2]. Attributable fractions varied among regions, ranging from 4.0% in North America to 31.3% in Sub-Saharan Africa, with highest estimations in low-income countries and lowest estimations in high-income countries. Most of the global burden of infection-attributable cancer occurs in less developed countries, and *Helicobacter pylori*, human papillomaviruses, and Hepatitis B and C viruses account for 90% of cases.

Human papillomaviruses (HPVs) were responsible for approximately 640,000 new cancer cases worldwide in 2012, accounting for 30% of infection-attributable cancers worldwide, being the second contributor after *Helicobacter pylori* [2]. HPV is the cause of virtually all cervical cancers and a fraction of cancers from the vulva, vagina, anus, penis, and oropharynx [3–6]. Cervical cancer is the most frequent HPV-related cancer, accounting for 90% of HPV-cancers. HPVs are characterized by genotype and numbered by order of discovery. From the more than 200 HPV types identified, only a few are classified as high-risk or carcinogenic, namely HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 [7–9].

Although most of sexually active women will acquire a cervical HPV infection during their lifetime, most of these infections will clear without any clinical significance. Only a small fraction of HPV infections persist and eventually progress to cervical cancer [10,11]. HPV is the necessary cause of cervical cancer but not a sufficient cause; other factors are involved in the progression of HPV infection and cervical carcinogenesis. This thesis focuses on the environmental factors that may play a role in the disease process once an HPV infection is established, specifically tobacco smoking, oral contraceptives, parity, other hormonal and contraceptive factors, and infection with other sexually transmitted infections.

1. Cervical cancer

1.1. Burden of disease

Cervical cancer is the fourth most common cancer in women worldwide, after breast, colorectal and lung cancers, with an estimation of 527,624 new cases in 2012, representing 7.9% of all female cancers [12]. The age-standardized incidence rate is 14.0 per 100,000 women. Nevertheless, among women aged 15-59 years, cervical cancer is the second most common cancer in women worldwide. As with the rest of infection-attributable cancers, almost 85% of cervical cancer occurs in less developed regions. In these regions, cervical cancer ranks the second most frequent cancer among women with an estimated incidence rate of 15.7 per 100,000 women, whereas in more developed regions ranks the 11th (9.9 per 100,000). Regions with highest rates of cervical cancer include Eastern Africa (age-standardized incidence rate of 42.7 per 100,000), Melanesia (33.3), Southern Africa (31.5) and Middle Africa (30.6), while rates are lowest in Australia/New Zealand (5.5) and Western Asia (4.4) (Figure 1).

In terms of mortality, cervical cancer is the fourth most common cause of female death from cancer worldwide with an estimation of 265,672 deaths in 2012, the 7.5% of total female cancer deaths. The ratio between mortality and incidence is 52%. The age-standardized mortality rate is 6.8 per 100,000 women. Again, as with the incidence, around 87% of deaths occur in less developed regions with an age-standardized mortality rate of 8.3 per 100,000 women in comparison with 3.3 per 100,000 women in more developed regions. Mortality rate for cervical cancer is the third and seventh cause among less and more developed regions respectively. Figure 2 presents differences between regions around the world, showing Middle and Eastern Africa with highest estimations and Australia and New Zealand, Western Europe and Western Asia with lowest rates.

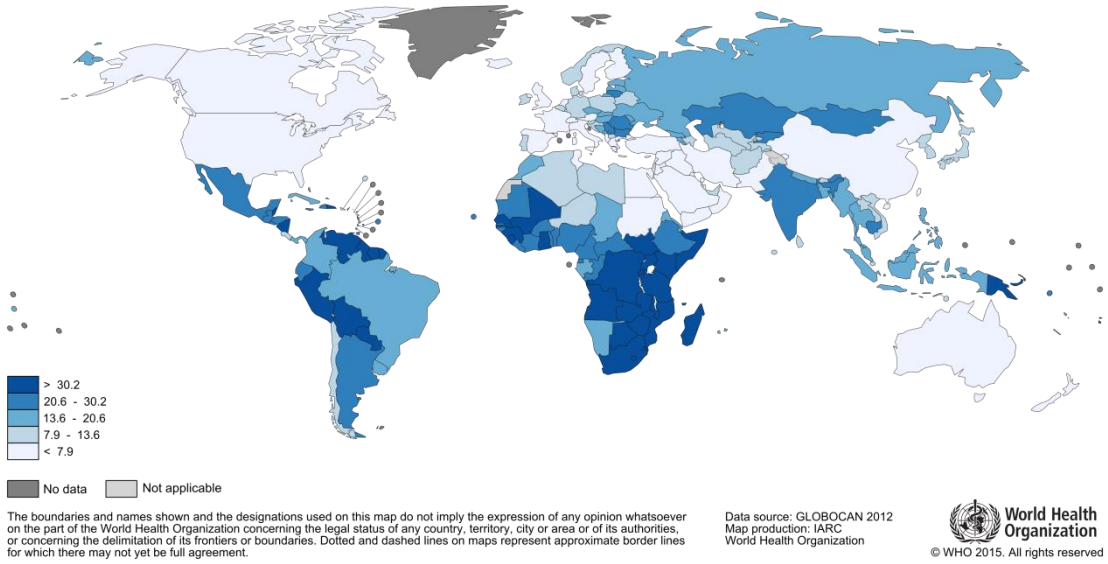

Figure 1. Estimated cervical cancer incidence worldwide in 2012. Extracted from GLOBOCAN 2012 [13].

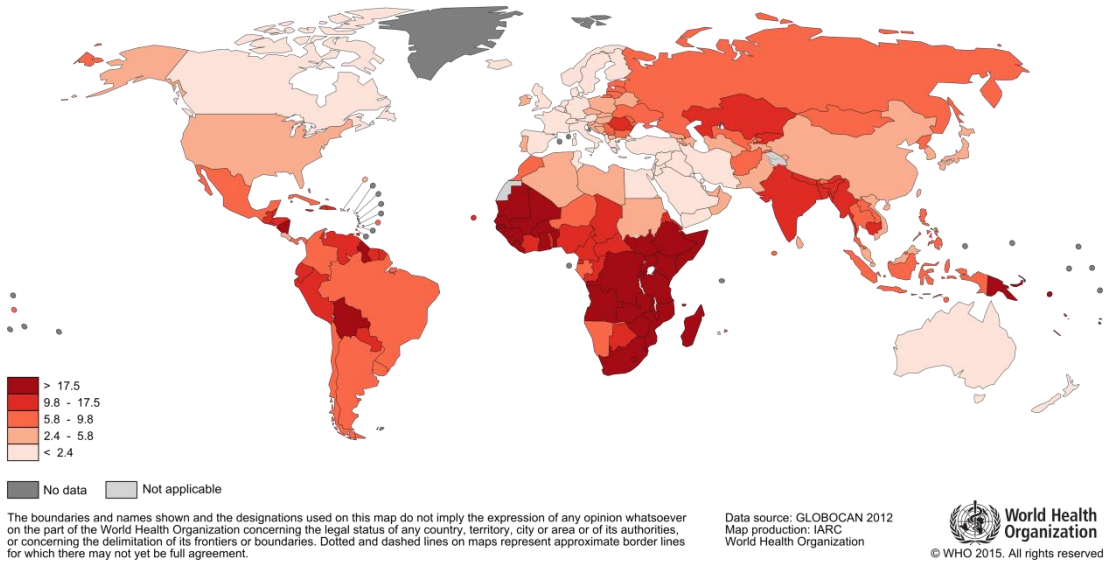

Figure 2. Estimated cervical cancer mortality worldwide in 2012. Extracted from GLOBOCAN 2012 [13].

Focusing in Europe, cervical cancer is the sixth most common cancer among females, accounting for 58,373 new cases in 2012, and the seventh cause of cancer mortality, with 24,385 deaths. These estimations correspond to an age-standardized incidence rate of 11.4

per 100,000 women and an age-standardized mortality rate of 3.8 per 100,000 women. However, it is the second most common cancer among women aged 15-49 years and the second cause of death among women aged 15-44 years. Central and Eastern Europe is the region with highest incidence and mortality rates (16.3 and 6.2 per 100,000 respectively), and Western Europe is the region with lowest rates (7.3 and 1.8 per 100,000 women respectively). Northern and Southern Europe have similar rates to those of Western Europe (8.7/2.2 and 8.5/2.4 per 100,000 women respectively). There are also variations between countries, ranging from Romania with the highest rates (28.6/10.8 per 100,000 women) or Lithuania (26.1/7.5 per 100,000 women) to Malta (3.8/0.8 per 100,000 women) or Switzerland (3.6/1.1 per 100,000 women) (Figures 1, 2, and 3).

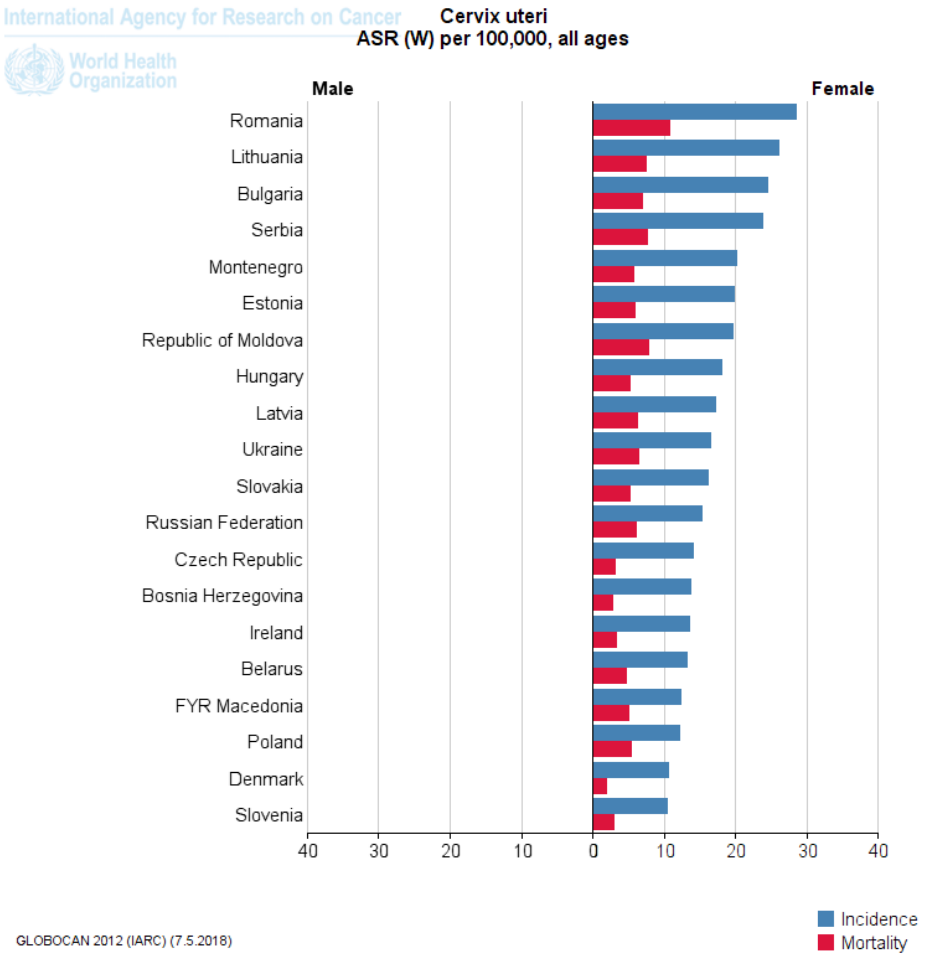

Figure 3. Estimated age-standardized incidence and mortality rates of cervical cancer in the 20 European countries with highest rates in 2012. Extracted from GLOBOCAN 2012 [13].

1.2. Cervical cancer prevention

Differences in cervical cancer burden among populations are mainly the result of historical disparities in cervical cancer prevention efforts and health care resources between developed and developing countries. The discovery of the central role of HPV infection in the etiology of cervical cancer has been of great importance for the development of new prevention tools. These new strategies include HPV vaccination of pre-adolescent and young women for primary prevention, and cervical cancer screening programs through HPV-based technologies for secondary prevention.

1.2.1. Primary prevention

There are currently three HPV prophylactic vaccines licensed by several regulatory agencies such as the European Medicines Agency (EMA) or the United States Food and Drug Administration (FDA). These HPV vaccines are identified as Cervarix® (GlaxoSmithKline Biologicals, Reixensart, Belgium), also referred to as the bivalent vaccine against high-risk HPV types 16 and 18, responsible for approximately 70% of cervical cancer cases; Gardasil® (Merck and Co., Inc., Whitehouse Station, New Jersey, United States), also referred to as the tetravalent vaccine that includes 4 types, HPVs 16 and 18, and low-risk HPV types 6 and 11, responsible for around 90% of genital warts; and Gardasil®9, also referred to as the 9-valent vaccine that contains the 4 HPV types already included in the tetravalent vaccine and the other 5 most common high-risk HPV types in cervical cancer, including HPVs 31, 33, 45, 52, and 58 [14,15]. The three HPV vaccines are composed primarily of virus-like particles (VLPs). The VLPs resemble L1, the major structural protein of the virion, but are completely non-infectious and non-oncogenic, since they do not contain the viral DNA genome.

Several clinical trials have confirmed that HPV vaccines are safe and highly efficacious against cervical, vulvar, vaginal, and anal infections caused by the HPV vaccine types, precancerous cervical, vulvar, vaginal, and anal lesions, and genital warts if the tetravalent or the 9-valent HPV vaccine is used [15,16]. Since the approval of the first HPV vaccines, they have already demonstrated their effectiveness and impact against HPV infection, genital warts and high-grade cervical lesions at population-level [17]. In addition, via “herd protection effect” in settings with high HPV vaccine coverage, it also reduces the prevalence of infection and disease in non-vaccinated girls and boys [17]. These solid and consistent results strongly support the potential

value and impact of the vaccines as high value public health interventions, and justify their broad implementation to prevent anogenital HPV infections and their associated neoplasia [18].

1.2.2. Secondary prevention

Cervical cytology or Papanicolaou test has been the fundamental test of cervical cancer screening until recently. Cervical cytology is a highly specific test that allows the early diagnosis of cervical precancerous lesions and, together with colposcopy, it made possible the establishment of screening programs from the 1950s. In the last decades, organized cytology-based screening programs have been shown to reduce the incidence and mortality of cervical cancer by up to 80% in developed regions such as Western Europe, North America, Japan, Australia and New Zealand [19]. However, cytology is a subjective test that involves a demanding technical training and a quality control and, due to its low sensitivity (62.5%) and despite being highly specific (96.6%), it must be repeated frequently [20]. Currently, the recommended cytological screening interval is to repeat the cytology every 3-5 years. Nevertheless, deficiencies in the quality of cytology and subsequent follow-up and treatment, a poor organization and an insufficient coverage have led to a lack of success of many cytology-based screening programs in countries with fewer resources [21,22].

In the last ten years, the use of HPV tests for cervical cancer screening has spread. Initially, they were introduced for its use with cytology for the management of cytological abnormalities, and recently they were already approved as a primary screening test due to their greater sensitivity and negative predictive value compared to conventional cervical cytology [23,24]. Gains in sensitivity are of the order of 50% and 5% in losses of specificity compared to cytology [20]. Large randomized clinical trials have shown that detection of HPV from age 30 as a primary test provides 60-70% greater protection against invasive cancer compared to cytology [25]. HPV tests are high throughput, objective, and highly reproducible.

Currently, most screening programs are still based on cytology and are facing a period of transformation to adopt HPV screening as a primary test and to anticipate a future where part of the population will be vaccinated and therefore with smaller screening requirements. However, the availability of multiple HPV detection technologies and multiple vaccination options also has a greater complexity in making decisions about optimal prevention strategies.

2. The role of HPV infection in the etiology of cervical cancer

Since HPV infection is the necessary cause of invasive cervical cancer, it is important to understand the biology and diversity of HPVs and the natural history of cervical cancer.

2.1. Human Papillomaviruses

2.1.1. HPV genome organization

Papillomaviruses (PVs) are small non-encapsulated viruses that contain a circular double-stranded DNA genome of approximately 8,000 base pairs [26]. The viral genome is divided into three major regions: an upstream regulatory region (URR), an early region, and a late region [27]. The URR is a non-coding region that harbors transcription factor-binding sites and controls gene expression. The early region encodes for six genes, the nonstructural early proteins E1, E2, E4, E5, E6, and E7, involved in multiple functions such as viral replication, viral transcription, gene expression and cell transformation. The late region encodes two genes, the structural L1 and L2 capsid proteins, which self-assemble to yield the virion.

2.1.2. Classification of HPVs

To date more than 200 different papillomaviruses types have been indentified in humans [28]. HPVs are divided into five genera: Alpha, Beta, Gamma, Nu and Mu-papillomavirus. Alpha-papillomavirus includes mucosal and cutaneous types, while the other HPV groups include only cutaneous types. The HPVs phylogenetic classification is represented in Figure 4.

Focusing on Alpha-papillomavirus and particularly on mucosal HPV types, they are known to infect the anogenital tract and the oral cavity. The IARC has classified the mucosal HPV types as high-risk or low-risk according to their carcinogenicity in humans [29]. Group 1, defined as carcinogenic to humans, includes HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 as the established high-risk types. HPV 16 is the most carcinogenic HPV type, and known to cause cancer at several sites, including cervix, vagina, vulva, anus, penis, oral cavity, oropharynx, and larynx. For the other HPV types, there is sufficient evidence to establish their carcinogenic role for cervical cancer. Group 2A, defined as probably carcinogenic to humans, includes HPV 68, which presents limited evidence in humans but strong mechanistic evidence for cervical

cancer. Group 2B, defined as possibly carcinogenic to humans, includes HPVs 26, 30, 34, 53, 66, 67, 70, 73, 82, 85 and 97, manifesting limited evidence in humans for cervical cancer. Group 3, defined as not classifiable as to its carcinogenicity to humans, includes HPVs 6 and 11, HPV types established as low-risk that were mainly associated with benign lesions such as genital warts and recurrent respiratory papillomatosis.

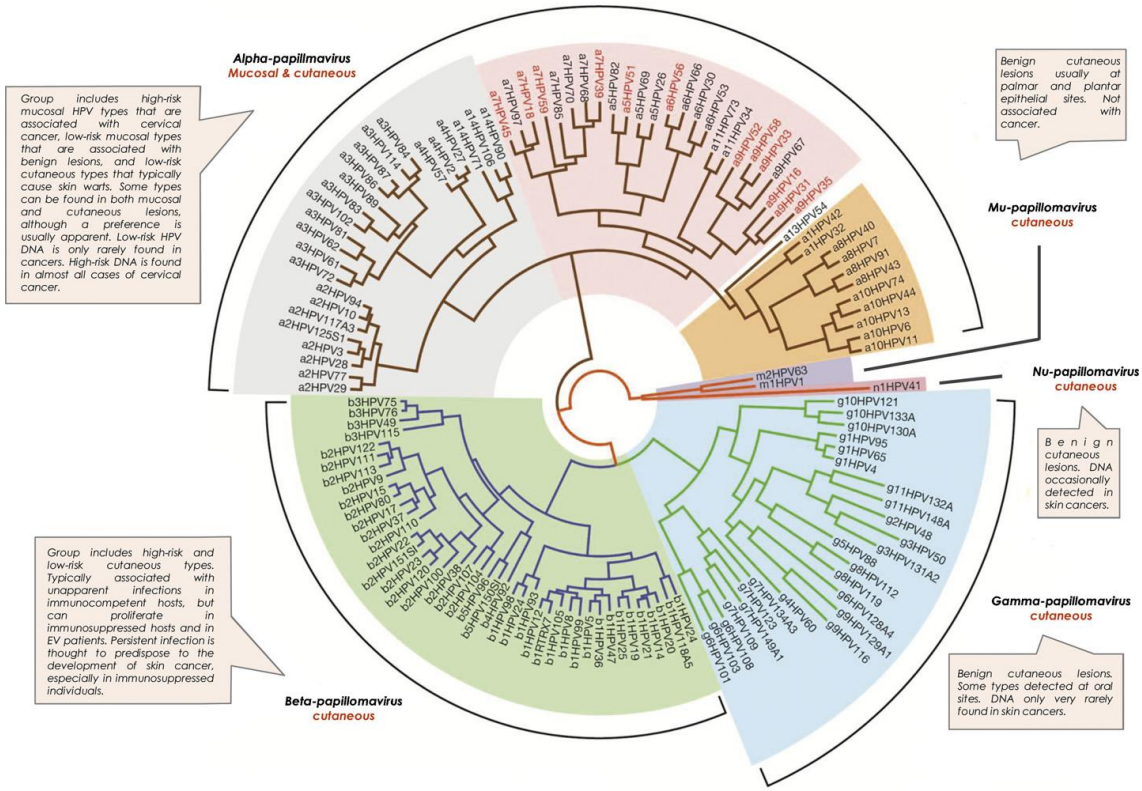

Figure 4. HPVs phylogenetic tree [30].

2.1.3. Attributable fractions and relative contributions in HPV-related cancer sites

Attributable fraction of genital HPV infection in invasive cervical cancer cases is almost 100%, and as such, HPV is being considered the necessary cause. Globally relative contributions of the most common high-risk HPV types in cervical cancer are, by order, HPV 16 (61%), HPV 18 (10%), HPV 45 (6%), HPV 33 (4%) and HPV 31 (4%) (Figure 5) [31]. HPV 16 is the most frequent type, and HPV types 16 and 18 are detected in 71% of the invasive cervical cancer cases.

Concerning the rest of HPV-related cancer sites, most cancers of the anus and vagina are likewise linked to HPV, with attributable fractions of 88% and 74% respectively, as it is a lower

fraction of cancers of the penis, vulva, oropharynx, oral cavity, and larynx (attributable fractions of 33%, 29%, 25%, 7%, and 6% respectively) (Figure 5) [32–36]. Attributable fractions of HPVs 16 and 18 are especially high in oropharyngeal and anal cancer sites, mostly due to HPV 16, by far the most frequently detected genotype across all anatomical sites.

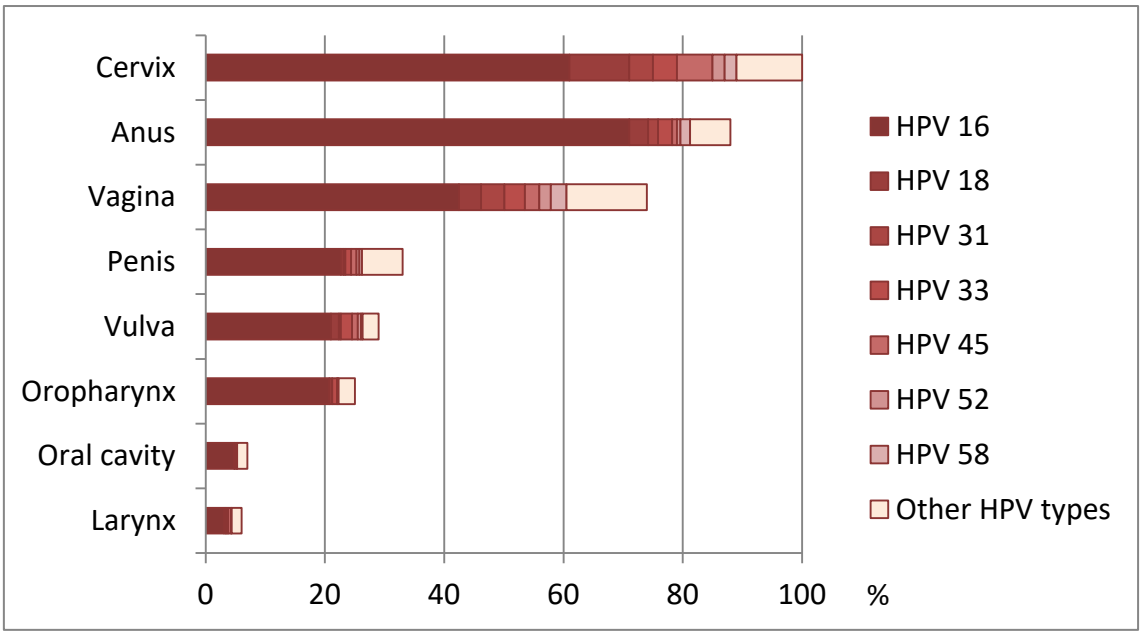

Figure 5. Worldwide attributable fraction and relative contribution of HPVs 16, 18, 31, 33, 45, 52, and 58 in HPV-related cancer sites. Data extracted from [31–36].

2.2. Natural history of cervical cancer from genital HPV infection

Genital HPV infections are frequently acquired in young men and women within weeks or months after the onset of their sexual activity. It is estimated that most of sexually active women will be infected with HPV during their lifetime (>70%) [10]. However, the majority of genital HPV infections (90%) are asymptomatic and clear spontaneously within 2 years [37]. Hence, only a small fraction of these persistent HPV infections progress to precancerous lesions. If these lesions are not treated, they may eventually progress to invasive cervical cancer [38].

Figure 6 shows the natural history of cervical cancer from genital HPV infection. The three major steps in cervical carcinogenesis after infection by a high-risk HPV include: HPV persistence, progression to cervical precancerous lesions, and invasive cervical cancer [38,39].

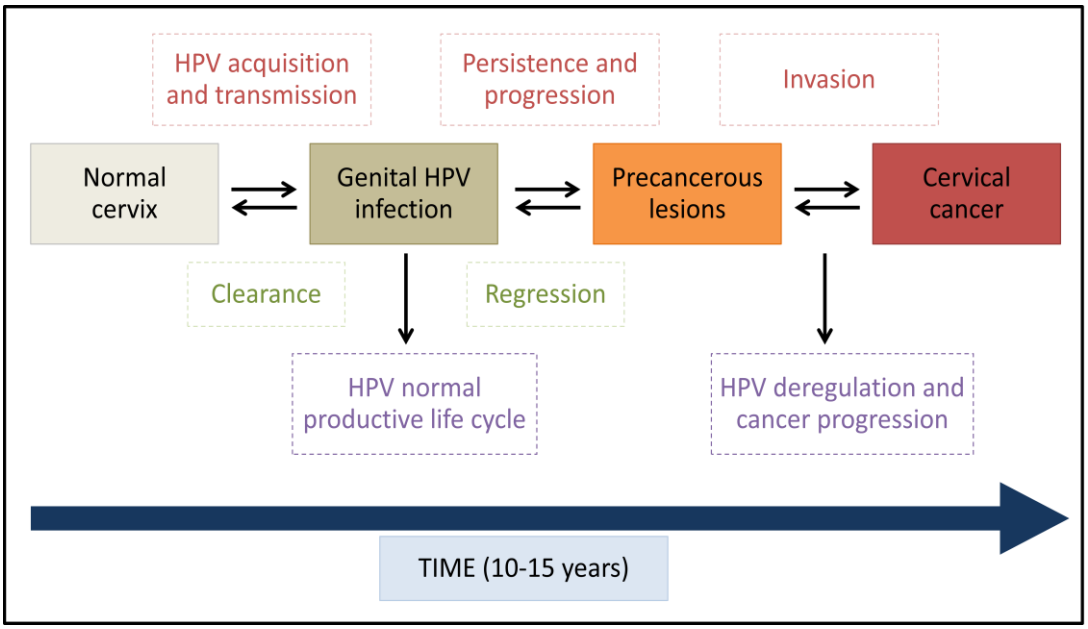

Figure 6. Natural history of cervical cancer from genital HPV infection.

2.2.1. HPV transmission and acquisition

Genital HPV infections are amongst the most common sexually transmitted infections worldwide, with a global prevalence of 11.7% among women without disease [40]. HPV transmission is primarily through sexual intercourse including both vaginal and anal sex [38,41,42]. There are additional mechanisms of transmission other than sexual intercourse that are less common but also plausible such as non-penetrative sexual contact (skin-to-skin or mucosa-to-mucosa contact) and mother-to-child transmission, but their association with cervical cancer is minimal [41–43]. Oral sex is another route of HPV transmission [44].

The acquisition rate of HPV may be as high as 40% in the following two years of initiating sexual activity [45]. More than 80% of sexually active women and men are estimated to acquire at least an HPV infection by age 45 years [46]. Risk factors associated with acquisition of HPV infection are related with patterns of sexual behavior, mainly number of new and recent sexual partners of both women and men [41,42,47,48]. Population surveys show heterogeneity in the number of lifetime and recent sexual partners, with a majority having none or one partner, and a minority having multiple partners. Furthermore, higher number of sexual partners is more often reported among men than women, and among younger cohorts than older ones. Additionally, an early sexual intercourse may be a marker for risky sexual behavior, such as greater lifetime number of partners and concurrent partnerships, but it is not consistently

associated with HPV acquisition. In contrast, condom use and male circumcision may partially protect from HPV acquisition [37,49].

2.2.2. Persistence and clearance of HPV infection

It is considered that a period of two years distinguish between transient and persistent HPV infections, and persistence after 24 months is very uncommon [50,51]. About 10% of infections do not clear spontaneously and are persistent. Globally, the median duration of any HPV detection has been reported to be 9.8 months, being more persistent for high-risk types (9.3 months) than for low-risk types (8.4 months) [52]. As expected, the most persistent HPV types tend to be the most common ones, considering prevalence as the result from the combined effect of acquisition and duration of clearance/persistence. HPV 16, the most common high-risk HPV type, has an average length of persistence that is longer than most other high-risk HPV types (12.4 months) [30,52]. However, persistence is not necessarily associated with carcinogenicity of HPV types considering that persistence of low-risk types can also be long (i.e. HPV 61). Several major determinants of HPV persistence have been described, including HPV type, viral load, and HPV variant; multiple HPV infection is a more controversial risk factor. HPV persistence also depends on the host immune response [39].

Most HPV infections do not cause symptoms or disease and are resolved within 1-2 years after infection, largely as a result of a cell-mediated immune response. However, it is not clear whether HPV infections are resolved by complete viral clearance, or whether there is a period of viral latency in which the virus replicates at undetectable extremely low levels [38,39,53]. If viral latency occurs, a woman who appears to have cleared her infection between two follow-up visits would still be at risk for development of HPV-associated diseases. Similarly, it is unclear whether HPV infections that are detectable at two different points in lifetime are the result of persistent HPV infection or HPV clearance and re-infection. It seems that reactivation of an infection may occur under immunosuppressive situations. Concerning HPV genotypes, HPV 16 has a particularly long time to clearance as compared to other HPV types.

2.2.3. Progression to precancerous cervical lesions

Persistent HPV infections are associated with progression to cervical precancerous lesions [54]. Within the high-risk types involved in the cervical carcinogenic process, persistent infection

with HPV 16 shows a faster progression to cervical lesions and invasive cervical cancer [53]. The time between HPV infection and detection of pre-cancer could be short, often within 5 years [38]. The median age of women with diagnosis of pre-cancer is estimated between 25-30 years, although this estimation depends on the sexual behavior of the population and on the active search of precancerous lesions [38].

In terms of histology, pre-cancer includes diagnosis of cervical intraepithelial neoplasia (CIN) grade 3 (CIN3) or carcinoma in situ (CIS) (Figure 7) [38,51]. CIN2 is a heterogeneous intermediate diagnosis, more likely to regress than CIN3, but also at an increased risk of progression to cervical cancer. CIN1, a low-grade or middle dysplasia, is usually associated with HPV infection, and tends to regress spontaneously.

In the same line, studies on natural history have concluded that low- and high-grade cervical lesions are distinct processes of HPV infection [39]. In terms of cytology, low-grade squamous intraepithelial lesions (LSIL) are similar to CIN1, and appear to represent a transient infection, showing minor cellular abnormalities. Both oncogenic and non-oncogenic types can cause low-grade lesions. In contrast, high-grade squamous intraepithelial lesions (HSIL) are comparable to CIN2 or CIN3; replication of immature cells and accumulation of cellular abnormalities occur. Most high-grade lesions, as well as carcinoma in situ and invasive cancer, are positive for oncogenic HPV types.

Histology		Cytology			Molecular
CIN	LAST	Pap	WHO	Bethesda	
Normal	Normal	I	Negative	NILM	Normal cervix
		II	Squamous atypia	ASC-US	
CIN1	LSIL	III	Mild	LSIL	HPV infection
		IIID			
CIN2 CIN3	HSIL	IV	Moderate Severe	HSIL	Precancer
Cancer	Cancer	V	Cancer	Cancer	Cancer

Figure 7. Nomenclature and classifications of histology, cytology and molecular systems for diagnosis, screening, and HPV natural history and carcinogenesis [51].

2.2.4. Invasion to cervical cancer

Cervical cancer is the end stage of an unresolved HPV infection followed by a succession of events that can close with cervical cancer and takes around 10-15 years or more (Figure 6). It is suggested that women who developed cervical cancer always progressed though distinct stages from low to moderate to high-grade intraepithelial lesions [39]. Compared with its immediate precursor, there are many more pre-cancers than cancers, suggesting that only a minority of them invade. It is clear that women with untreated CIN3 are at high risk of cervical cancer [55]. The probability that a particular pre-cancer will invade, if pre-cancer was not treated, will remain unknown because actually the treatment of a precancerous lesion is mandatory when it is diagnosed, and therefore it is not ethical to let pre-cancer evolve to invasive cancer [38,51]. Initial studies suggested that between one- and two-thirds of women with high-grade lesions will develop invasive cancer if left untreated, with a 20-30% risk of invasion over a 5-10 years, and around 50% within 30 years [38,39,55]. The average duration of a precancerous lesion that grows and leads to invasion seems to be much longer than the average time between HPV infection and pre-cancer detection [38]. The mean age of women with invasive cervical cancer is approximately 50 years, while the mean age of women with pre-cancer is approximately 25-30 years, also suggesting a long precancerous state [39].

In terms of histology, squamous cell carcinoma (SCC) is the most common subtype, accounting for about 80-85% of cases of cervical cancer [56]. Adenocarcinoma, rising from the glandular epithelial cells, is the second most common histological subtype, accounting for around 15-20% of all cervical cancer cases.

2.3. Molecular biology and life cycle of HPV infection and cervical cancer

Following the natural history scheme from Figure 6 at a viral level, we can define three main status of the HPV: the infection of the host epithelial cells with a normal productive life cycle, the viral latency, and the interaction with the host cell cycle producing deregulation and cancer progression.

2.3.1. HPV normal productive life cycle

Figure 8 summarizes the different stages of the life cycle of high-risk HPV and its possible consequences [26,27,30,53]. Classically described, HPV life cycle begins with the infection of the

basal layer of the epithelium that becomes exposed through microwounds during sexual intercourse. In the columnar cell layers, infection is thought to be facilitated by the proximity of the target cell to the epithelial surface, which may allow the virus to access a cell type that is unable to support the full productive life cycle. Infection is localized to a few cells surrounding the wound and it is not lytic, but it is stealthy and durable.

Following infection, the virus enters the basal cells and, in the infected host basal cells, the viral genome is maintained at low copy episomes [28,30]. In the initial stages of productive viral cell cycle, viral proteins E1 and E2 are responsible for viral genome replication and maintenance. This is mediated by the viral E6, E7, and E5 proteins. In the mid layers, these cells express the viral E4 protein, pushing differentiating cells into S or G2 phases of the cell cycle, and allowing viral genome amplification to occur. Following cellular differentiation, the expression of E6 and E7 is replaced by expression of viral proteins E1, E2, E4 and E5. In the upper layers, the virus replicates to a high copy number, and L1 and L2 capsid proteins are expressed in a subset of the cells that contain amplified viral DNA. They ensemble and produce daughter cells that migrate away from the basal layer, and they are released from the epithelial surface.

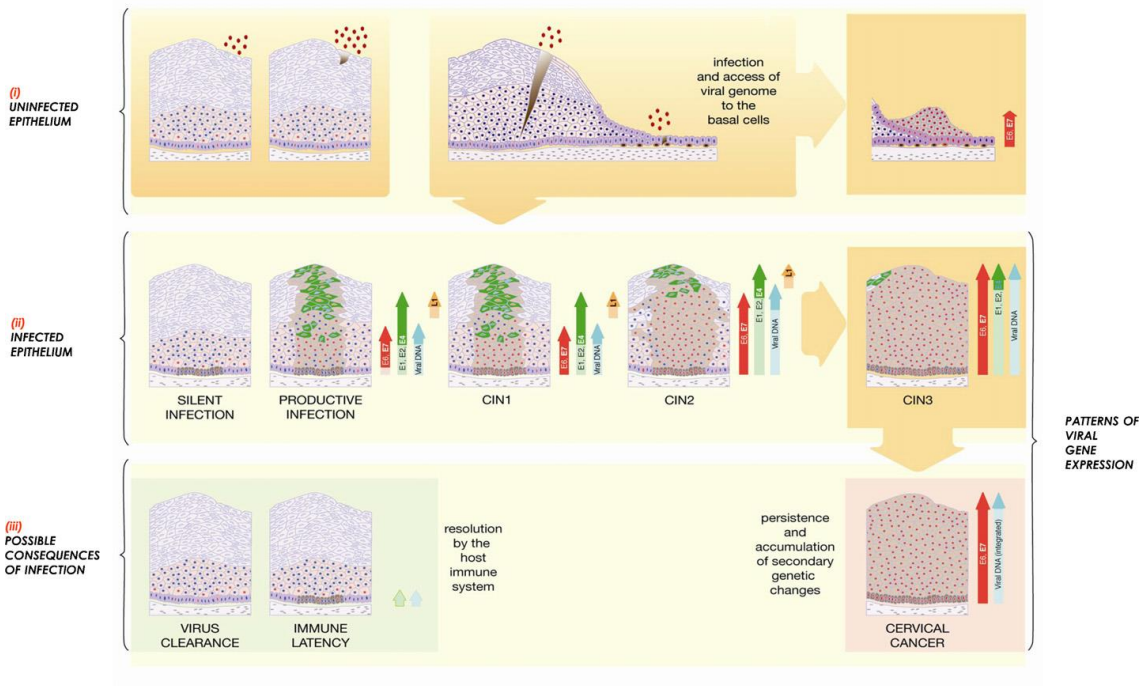

Figure 8. High-risk HPV infection and its possible consequences [30].

2.3.2. HPV latency

Viral latency could be defined as persistence of viral episomes in the epithelial basal layer without life cycle completion [26,30]. Latent infection is thought to require the expression of the viral E1 and E2 proteins necessary for genome maintenance in the basal layer, with the E6 and E7 genes not being required. However, viral gene expression patterns during latency are still poorly understood.

2.3.3. HPV deregulation and cancer progression

Cancer progression can occur when HPV interacts with the host cell cycle and produces deregulation of viral gene expression [26,28]. In cervical epithelium infected by high-risk HPV types, the regulation of proteins necessary for cell proliferation is altered. The increased oncogenic capacity of the high-risk types, and particularly the HPV 16 type, resides in the activity of the E6 and E7 oncoproteins. E6 and E7 interact with cellular proteins such as p53 and pRb (retinoblastoma protein) respectively, central molecules in the cell cycle control. Progression through the cell cycle is stimulated by the E7 protein, which binds and degrades pRb and activates the expression of proteins necessary for DNA replication. In addition, the E6 protein stimulates the degradation of the p53 protein that prevents growth arrest and/or apoptosis.

2.4. Viral factors

Several characteristics of the virus are essential in the progression from HPV infection to precancerous lesions and invasive cervical cancer.

2.4.1. HPV genotypes

The type of HPV is one of the most important determinants of viral persistence and progression to precancerous lesions and cervical cancer. In particular, HPV 16 is the most potent carcinogenic type, with a higher capacity of transmission, persistence, and progression to cervical lesions [29,57]. As previously mentioned, HPV 16 is involved in 61% of all cervical cancers [31]. The second most common HPV type found in cases of cervical cancer is HPV 18,

accounting for around 10%. Other high-risk HPV types, including HPV 45, 31 and 33, are also commonly detected in cervical cancer cases although in a less magnitude than HPVs 16 and 18.

2.4.2. Co-infection with other HPVs

Infections with more than one HPV type (i.e. multiple infections) have been found in around 30% of infected women [58]. However, it is important to note that the detection of concomitant HPV infections depends on the sensitivity of the HPV test used [57]. The presence of multiple high-risk HPV types was associated with a higher risk of cervical intraepithelial neoplasia, but there is still a debate about whether co-infections were associated with different grades of dysplasia and with cancer [58,59].

2.4.3. HPV variants

Some viral types have shown different variants with different oncogenic potential [53,60]. For example, HPV 16 can be divided into four main variant lineages (A, B, C, D) and nine sublineages, differing in L1 sequence by less than 10% for main variants and as little as 0.5% for sublineages [60]. Specific HPV 16 variants corresponding to the non-European lineages (B/C/D) strongly influence the risk of developing cervical cancer and pre-cancer in comparison to isolates from the European lineage (A) [60,61]. For other oncogenic HPV types (i.e. HPVs 18, 31, 52, 58), there is a lack of evidence for the role of HPV variants in the different stages of the cervical pathogenesis.

2.4.4. Viral load

Viral load is defined as the amount of virus present in the cervix, although its interpretation could be ambiguous. HPV infections are mainly transient, and viral load estimates may be similar for long-term active HPV infections and for recent infections, making it difficult to distinguish between them. Viral load measurements are not clinically useful, and their potential utility as an etiologic risk factor or as a diagnostic tool for cervical cancer is still on debate [62]. Much of the uncertainty is due to the different methods used to measure the HPV viral load, i.e. polymerase chain reaction (PCR) versus Hybrid Capture 2 (HC2).

Several reports indicated that a higher risk for cervical cancer was associated with higher viral loads of high-risk HPV types, in particular for HPV 16 [38,57,62]. Viral load of HPV 16 may also be a predictor of the future risk of developing high-grade dysplasia and cervical carcinoma in situ [59]. Furthermore, viral load of newly detected HPV 16 infections and changes in viral load could also predict persistence and progression to CIN3, suggesting a potential role of viral load in defining the course of the infection [63]. Other high-risk HPV types were less explored and did not show clear associations between viral load and persistence, progression and risk of precancerous lesions and cervical cancer.

2.4.5. Viral integration

Viral integration is considered to be an important molecular event in HPV-induced carcinogenesis. HPV integration can drive oncogenesis by deregulating expression of the E6 and E7 viral oncogenes, leading to inactivation of the E2 expression and increasing genetic instability in the host [53,64]. In this manner, the integration of the high-risk HPV genome into the host genome is associated with invasive cervical cancer and with premalignant lesions, particularly with CIN2/3 [38,57]. HPV integration might be an important biomarker distinguishing HPV infection from pre-cancer. Moreover, the frequency of HPV integration seems to increase with the degree of disease severity, which represents a significant event in the pathogenesis of cervical cancer and, therefore, potentially correlates with progression from pre-neoplastic lesions to cervical cancer [62,65].

3. The role of cofactors in the etiology of cervical cancer

Not all women infected by HPV will develop precancerous lesions and cervical cancer. In the process of cervical carcinogenesis, in addition to cervical HPV infection and viral factors, other cofactors may be involved. These potential cofactors could be classified into two groups: host-related cofactors, including endogenous hormones, genetic factors, and immune response; and environmental cofactors, including tobacco smoking, hormonal contraceptives, parity, infection with other sexually transmitted diseases, and nutritional factors (Figure 9) [3,53,57,59]. The different groups of cofactors are detailed below.

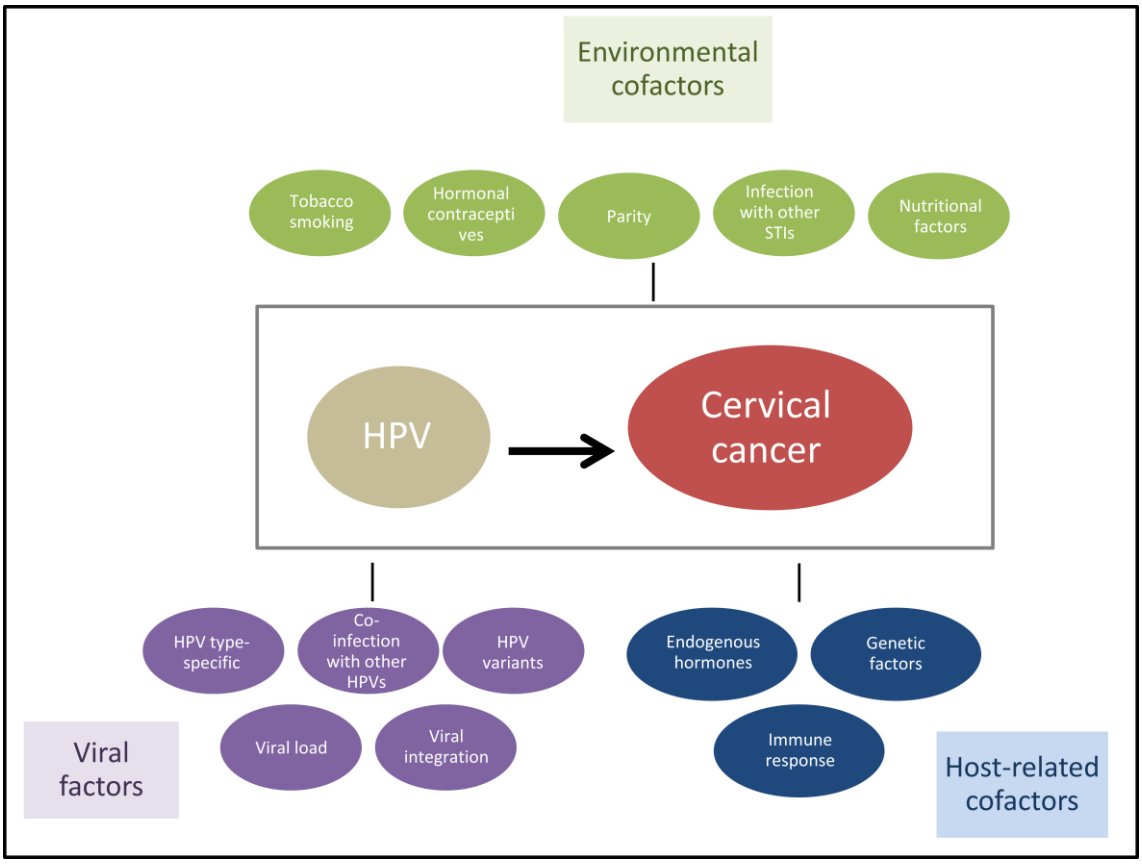

Figure 9. Factors related to cervical cancer.

3.1. Host-related cofactors

3.1.1. Endogenous hormones

The endocrine system is likely to play an important role in the process of cervical carcinogenesis. In particular, estrogen may be relevant in the early stages of several infections as it stimulates antibody- and cell-mediated immune responses, and could have a protective or damaging effect depending on the infected organism and the stage of the infection or disease [59]. Focusing on HPV infection, estrogen is a possible cofactor to the oncogenic effect of HPV. Previous studies found that estrogen could reduce susceptibility to primary HPV infection, but for persistent HPV infection, estrogen, and in particular estradiol, was likely to be associated with progression to cervical cancer [66–68].

3.1.2. Genetic factors

Inherited genetic susceptibility

Familial clustering of cervical cancer has been explored as a potential marker of inherited genetic susceptibility. Several reports have consistently suggested a familial risk in the development of cervical cancer [57]. In particular, having a sister or a mother with cervical cancer doubles a woman's risk of cervical cancer [62]. Therefore, heritability might explain some of the variation in cervical cancer risk. However, familial studies are limited in their capacity to separate completely the effects that can be attributed to genetic susceptibility from those that are related to environmental and behavioral characteristics.

Genetic susceptibility

Polymorphisms or genetic variations between individuals in immune-related genes might also be of interest in the process of cervical carcinogenesis. To date, human leukocyte antigens (HLAs) have been the most widely studied immune-related genes, suggesting strong associations of cervical neoplasia with risk and protective HLA alleles [69,70].

3.1.3. Natural immune response

As previously described, most cervical HPV infections are transient and resolved naturally within 1 to 2 years in 90% of cases. This implies that the host immune system, both humoral and cellular, play an important role in the prevention, control and elimination of HPV infection of the cervix [62,71]. The vast majority of infected women mount an effective immune response, becoming HPV DNA negative and being protected against HPV associated diseases [72]. Biologically, neutralizing antibodies are believed to be the main effectors of protection against infection, preventing the initial entry of the virus [73].

Effective immunity consists of a cell-mediated response to the early proteins, followed by seroconversion that results in detectable serum neutralizing antibodies to the major capsid protein L1 [71]. Nevertheless this response is slow and weak; the average time to seroconversion after the first detection of HPV DNA is about 8-9 months, with low levels of L1 antibodies. In addition, only 50-70% of women with incident HPV infection seroconvert [74,75]. However, antibodies to HPV L1 have stable titers that persist along time and can be detected decades after the infection. HPV L1 antibodies have also been shown to be type-specific, although some cross-reactivity with closely related types was also reported.

HPV L1 specific antibodies have been considered as markers for past and current infection, i.e. serology may be used as a proxy for lifetime cumulative HPV exposure [73,74]. In fact, HPV L1 antibodies are more frequently detected in subjects with persistent infections and precancerous cervical lesions. However, the role of natural antibodies in protection against subsequent infection or reactivation of the same HPV type is still unclear. While earlier studies did not find a protective role for natural antibody against subsequent infection and disease, more recent studies suggest that natural antibody levels mediate protection against HPV 16 and HPV 18 re-infection or reactivation, especially at higher titers [76]. Discrepancies among studies may be due to several factors, including the assays used for detection, definitions of seropositivity, study design, or data analysis. Larger longitudinal studies using the same methods are needed for a more comprehensive understanding of the role of antibody in the natural history of infection.

3.2. Environmental cofactors

3.2.1. Tobacco smoking

The role of tobacco smoking in the etiology of cervical cancer has been discussed since the late 1970s. In 1990, Winkelstein et al published an extensive review focusing on 15 epidemiological studies of cervical cancer and tobacco smoking reporting significant positive associations of different magnitudes in 11 of the studies [77]. However, at that moment most studies did not measure HPV exposure or control for sexual behavior, so the evidence was insufficient to rule out confounding by these factors. Since then, most case-control and cohort studies have adjusted for HPV infection or have restricted their analyses to HPV positive women, finding consistently increasing risks of cervical cancer and its precursor lesions among tobacco smokers. This evidence has been accurately summarized in several reviews [57,78–80] and pooled analyses conducted by the IARC [81,82], with magnitudes of the risk around 2-fold among smokers.

The most recently pooled analysis including 13,541 cervical cancer cases and 23,017 controls from 23 epidemiological studies was published in 2006 by Appleby et al [82]. In this reanalysis, current smokers had a significantly increased risk of squamous cell carcinoma compared to never smokers (RR=1.6), as well as for invasive cervical cancer and CIN3/CIS separately. Risks for past smokers were globally lower (RR=1.1), although there was no clear trend with time since stopping smoking (p-trend=0.6). Furthermore, among current smokers, the risk estimates of squamous cell carcinoma increased with increasing number of cigarettes smoked per day and with decreasing age at starting smoking (p-trend<0.001). However, there was no evidence of an association between risk of cervical carcinoma and duration of smoking (p-trend=0.3 for current smokers).

Only three cohort studies have published risk estimates for the association between tobacco smoking and cervical cancer risk restricting to HPV positive women. In 2000, Deacon et al carried out a nested case-control study within a prospective cohort accounting for more than 60,000 women in Manchester, United Kingdom [83]. Ever use of tobacco smoking was associated with a significant increase risk for CIN3 of more than 2-fold (Odds Ratio (OR)=2.2), as well as for intensity (≥17 cigarettes/day: OR=3.1) and duration of smoking (≥20 years: OR=3.1). In 2002, among participants of a prospective cohort of 1,812 women who tested positive for oncogenic HPV DNA in Portland, United States, Castle et al reported that former smokers,

women who current smoked less than one pack per day and one or more packs per day had consistently higher risks of CIN3 or cervical cancer than non-smokers (RR=2.1, 2.2 and 2.9 respectively, and OR=3.3, 2.9, and 4.3 respectively) [84]. Intensity of smoking was not associated with risk of CIN3 or more (CIN3+). In 2012, Jensen et al performed a prospective cohort study of 8,500 women in Copenhagen, Denmark [85]. Among high-risk HPV positive women, an increased risk for CIN3+ was associated with current (Hazard Ratio (HR)=1.4), long-term (≥10 years: HR=1.5) and heavy smoking (≥20 cigarettes/day: HR=1.5).

A meta-analysis published in 2012, based on 11 case-control studies including around 3,230 cases and 2,982 controls, evaluated the role of passive smoking in the etiology of cervical cancer risk [86]. The results showed that women who never smoked but were exposed to passive smoking had a 73% significant increase in risk of cervical cancer compared with non-exposed women.

Biological plausibility

Several biological mechanisms have been suggested as possible explanations of the increased cervical cancer risk related to smoking. Nicotine and its major metabolite cotinine, as well as the tobacco-specific carcinogen nitrosamine NNK, have been found in the cervical mucus of smoker [87,88]. These carcinogens could therefore induce direct smoking-related DNA damage of cervical epithelium. On the other hand, chemicals found in tobacco smoke include several groups of carcinogenic compounds, including polycyclic aromatic compounds [89]. Moreover, among smokers, the patterns of mutation of gen p53 show an exceptionally high prevalence of mutations at codons demonstrated to be sites of adduction of polycyclic aromatic compounds such as benzo[a]pyrene, one of the major carcinogens of tobacco smoke [90]. Interestingly, p53, one of the central proteins in the cell cycle control, is a target of the oncogenic viral proteins E6 and E7 [26,28]. This reinforces the hypothesis of an interaction between tobacco smoking and HPV for the development of precancerous lesions and cervical cancer. Some authors also theorize that tobacco exposure may affect the ability of the host to develop an effective immune response against viral infections. Smoking may reduce the number of Langerhans' cells, helper/inducer T lymphocytes and other markers of immune function in the squamous epithelia of the transformation zone of the uterine cervix, and may enhance the ability of HPV to evade the immune system [91,92].

Burden of tobacco smoking

Globally, tobacco smoking is responsible for the death of more than 7 million people every year: more than 6 million deaths result from direct tobacco use, and almost 1 million from exposure to second-hand smoke [93]. Even though tobacco use has declined in some countries and regions, population growth means that the absolute number of tobacco users is not yet decreasing. Figure 10 shows the prevalence of female smoking by country, indicating globally higher prevalence in developed countries than in developing countries [93,94]. In effect, in high income countries the mean female smoking prevalence is 17.2%, whereas in low income countries it is 3.1% [95]. Women in the developing world are therefore the largest potential new market for tobacco products. A substantial increase in smoking in this segment of the population would dramatically increase the global burden of smoking-related diseases.

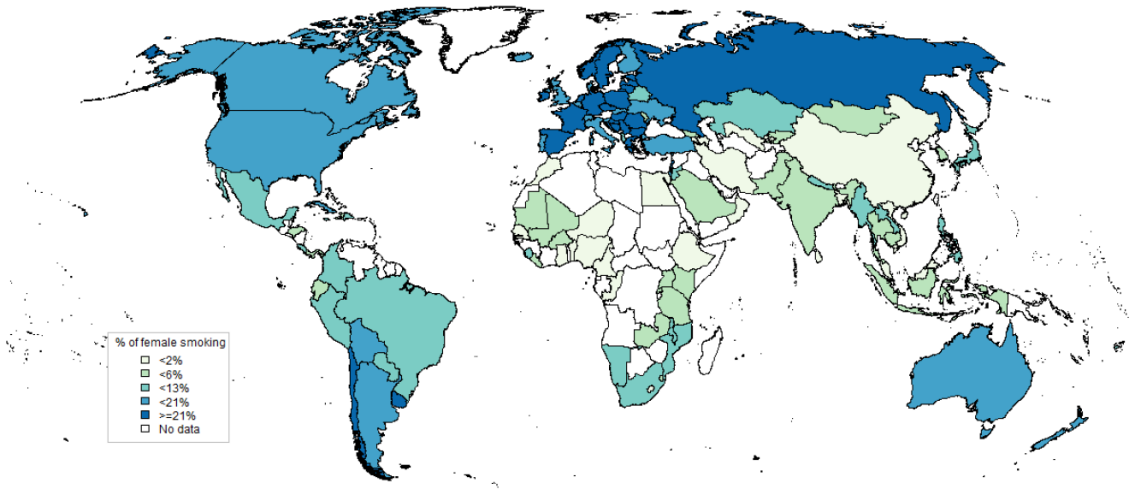

Figure 10. Prevalence of female smoking in the world. Extracted from [93,94].

3.2.2. Hormonal and reproductive factors

3.2.2.1. Hormonal contraceptive

The use of hormonal contraceptives, most commonly combined oral contraceptive (OC) formulations of estrogen and progestogen [96], has been hypothesized to be associated with development of pre-invasive and invasive cervical cancer [57,78,79,97]. In 1999, a first IARC

Monograph of oral use of combined hormonal contraceptives and cervical cancer, including 5 cohort and 16 case-control studies, showed a small increase in relative risk associated with long-term use of OCs [98]. However, some of the studies included did not take into account HPV status, or did not collect information on sexual behavior or Pap smear history, all of them potential confounders. Indeed, women who use oral contraceptives could be women whose sexual behavior is conductive to the acquisition of sexually transmitted agents (can start sexual intercourses at an earlier age, can have more sexual partners, and do not use barrier methods of contraception) [99], and also could be women who are more likely to have Pap smears. In this line, the Working Group concluded that biases related to sexual behavior and screening could not be ruled out as possible explanations of the observed associations.

Since then, two cohort and seven case-control studies, including two large pooled analyses [100,101], have provided new insights on cervical cancer and use of hormonal contraceptives, summarized in 2007 in an additional comprehensive review [97]. A collaborative pooled reanalysis including 16,573 cases and 35,509 controls was also published in 2007 [102]. The evidence indicated that the risk for cervical cancer significantly increased with increasing duration of use of combined oral contraceptives, with an overall magnitude of 2-fold (ratios between 1.4 and 4.0) for users for more than 10 years compared to never users (p-trend<0.0001); the risk decreased after stopping the use, returning to that of never users within 10-15 years (relative risks of 0.5 and 0.8 for past users for 15 or more years compared to non-users, p-trend<0.0001) [100–102]. The results were broadly similar for invasive and in situ cervical cancers, and regardless of adjustment for the presence of HPV DNA in cervical cells, the presence of serum antibodies against HPV, the number of sexual partners, cervical screening, smoking, and the use of other contraceptives; a similar pattern was found as well in analyses restricted to HPV positive women.

Only four prospective studies were identified in the literature, and they did not detect any significant association between the different oral contraceptive variables (i.e. status, duration, time since first use) and both CIN3 and cervical cancer risk among HPV positive cases and controls [83,84,103,104]. Regarding retrospective studies, the evidence for an association of cervical cancer with the use of oral contraceptives was inconsistent; while some studies found positive associations between OC use and cervical cancer [105,106], others did not find any association [107–113].

In addition to the drawbacks mentioned above, we must keep in mind the formulation and dose of the hormonal contraceptives used in the risk of cervical cancer. There is clinical

evidence that estrogens, and probably progestogens, may have a carcinogenic effect in HPV infected cervix [66–68,100]. However, whether the OC effect is mainly due to estrogen, progestogen, or both is still unclear.

Biological plausibility

Estrogens and progestogens, contraceptive hormones, are the two major groups of steroid hormones whose actions are mediated via specific intracellular receptors [114]. The plausibility of a role of contraceptive steroids on cervical cancer was enhanced by the discovery of hormone receptors in cervical tissue [115]. Studies on human cell lines support the hypothesis that mainly estrogens, and to a lesser extent progestogens, are the major sex steroid cofactors in cervical cancer [67]. Therefore, these hormones may affect cervical cells directly, promoting integration of HPV DNA into the host genome, stimulating HPV DNA transcription, and increasing cell proliferation [67,114]. They may increase the expression of HPV 16 E6 and E7 oncogenes, stimulating the degradation of p53 and pRb proteins, two tumor suppressor genes, leading to apoptotic failure and carcinogenesis [114,116]. These potential mechanisms are somewhat consistent with data from HPV transgenic mouse models suggesting that estrogens may promote cervical carcinogenesis, but there are not in line with those showing that progestogens may inhibit cervical cancer [117,118]. These hormones may also impact early upstream events in the natural history of HPV infection, enlarging the incidence of cervical ectropion, meaning that the squamocolumnar junction was more exposed to HPV and other potential carcinogens [116]. By another hand, they may also modulate the immune response, showing that progesterone may be associated with immune suppression, while estradiol seems to be associated with an increased immune defense [67,116].

Burden of hormonal contraceptives

Approximately 9% of women aged 15-49 years who are married or in union used the pill in 2015 [119]. Published studies show that contraceptive prevalence worldwide is projected to grow, mainly driven by increasing in developing countries [120]. Figure 11 shows the prevalence of hormonal contraceptive use among countries with available data [94,96]. Globally, the prevalence varies enormously by region, being higher among women in Northern and Western Europe, Australia and New Zealand, Northern Africa, Southern and Northern America, and South-Eastern Asia.

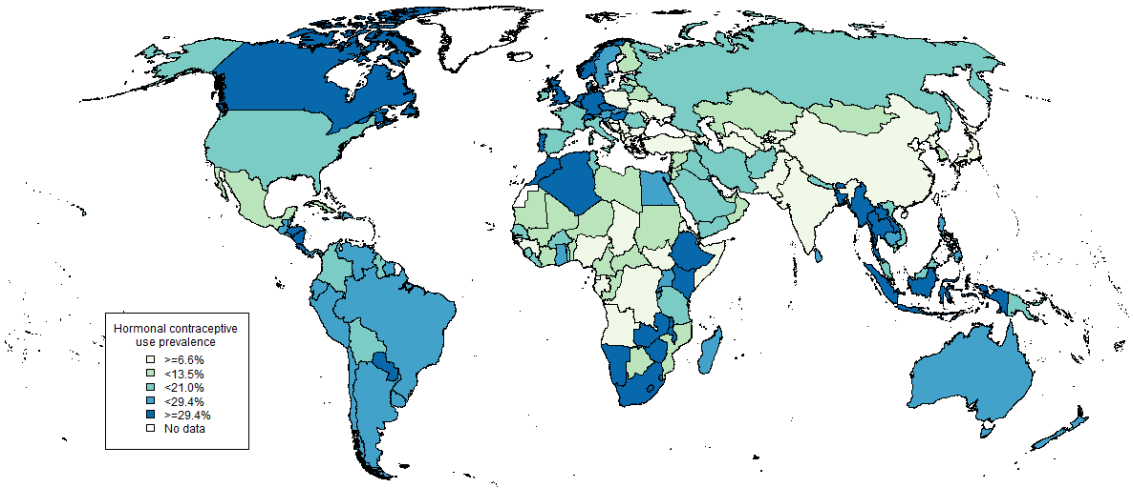

Figure 11. World prevalence of hormonal contraceptive use. Extracted from [94,96].

3.2.2.2. Intrauterine device

Intrauterine devices (IUDs) are one of the most common contraceptive methods considered worldwide [119]. One of the mechanisms by which IUDs prevent pregnancy is the creation of a sterile inflammatory response in the endometrium. Additionally, hormone-releasing IUDs or intrauterine systems release progestins or progesterone into the uterus. This may explain that most epidemiological studies have shown that IUD use reduces the risk of endometrial cancer [121–123]. However, the question of whether IUDs might also affect the risk of cervical cancer remains unanswered. Earlier clinical and epidemiological studies in several countries have reported inconsistent results [110,124–127], finding no associations in studies that did not account for HPV status in their analyses and reductions of risk in those that adjusted or restricted for HPV infection. In 2007, a systematic review of the use of any type of IUD and risk for neoplasia was conducted, observing no associations between IUD use and cervical cancer risk [123]. In 2011, Castellsagué et al conducted a pooled analysis of individual data from two large series by the IARC and ICO (Institut Català d'Oncologia) programs on HPV and cervical cancer, including HPV prevalence surveys and case-control studies, and reported a strong reduced risk by half among IUD users after adjusting for HPV DNA infection (OR=0.5, 95% Confidence Interval (CI): 0.4-0.7) [128]. In contrast, in a prospective population-based cohort conducted in Denmark, no association was seen between the development of CIN3+ and IUD use among women with high-risk HPV infection (HR=1.1, 95% CI: 0.8-1.5) [104].

Biological plausibility

One possible explanation for the potential protective effect of IUD on the risk of developing cervical cancer could be that the tissue trauma associated with loop insertion of IUD could induce a cellular immune response that might finally clear persistent HPV infections and pre-invasive lesions [129]. Another possible mechanism involving more chronic response to the presence of an IUD could be related to a repeated microtrauma and subsequent chronic mucosal inflammation processes induced by the device in the endocervix and cervix, through which IUDs can reduce the risk of cervical HPV progression, and consequently reducing the risk of cervical cancer [128].

Burden of intrauterine devices

The intrauterine device is the most commonly reversible contraceptive method used in the female population worldwide. Globally, 13.7% of women aged 15-49 years who are married or in union use IUD [119]. However, among women who use contraception, the percentage that use IUD varies greatly between continents and regions: from 1.1% in Oceania to 17.4% in Asia, reaching 40% in Central Asia.

3.2.2.3. Hormone therapy

Hormonal therapies (HT) were developed and firstly used in the 1930s to prevent menopausal symptoms, as well as increased rates of fracture and cardiovascular disease in post-menopausal women [97,98]. In the first decades, menopausal symptoms were treated mainly with estrogen alone. However, in 1975, results from studies linking post-menopausal estrogen therapy with increased rates of endometrial cancer led to the recommendation that progestogen had to be added to estrogen, at least among post-menopausal women with an intact uterus. In the early 1980s, the use of combined estrogen-progestogen became widespread worldwide, first in Europe and later in the United States [97,98].

The IARC monographs have assessed the potential role of estrogens and progestogens on cervical carcinogenesis on 1999 and 2007, detecting only two studies evaluating post-menopausal estrogen therapy only, and finding not significant reduced risks [97,98,130]. However, the investigators were unable to control for confounders, such as sexual behavior, cervical cancer screening practices or HPV infection. In 1997, Parazzini et al found a decreased risk of

invasive cervical cancer among ever users of post-menopausal estrogen therapy, after adjusting for confounding variables except HPV infection (OR=0.5, 95% CI: 0.3-0.8) [131]. Later on, few studies dealing with post-menopausal therapy and cervical cancer risk were identified, showing a non-significant decrease among ever users of hormone therapy, both with estrogens alone and with estrogens and progestogens [132,133]. In 2011, a review on hormone therapy and cervical cancer concluded that there was no association, although experimental data have shown that estradiol and progesterone can modulate the host immune response to HPV 16 [116].

Biological plausibility

The biological mechanisms that might explain the potential inverse associations between hormone therapy and invasive cervical cancer are still unknown. Previous studies in mice provide evidence that estrogens may promote cervical cancer in combination with HPV oncogenes, and progestogens may inhibit cervical carcinogenesis [117,118]. On the other hand, there seems to a consistent effect of OC use increasing cervical cancer risk. Although there are differences in formulation and doses, both HTs and OCs contain estrogen and progestogen hormones [134], and this opposite effects are not easy to understand. However, it must be taken into account that host factors interact with, or maybe drive, the action of these exogenous hormones. OCs are used before the menopause, by women with a functional ovary, while HTs are taken by post-menopausal women. Therefore, these exposures occur in women with a totally different background levels and types of sexual hormones.

3.2.2.4. Parity

For several decades, high parity has been suspected to increase the risk of both invasive cervical cancer and carcinoma in situ [57,78,79]. However, earlier studies did not measure HPV or did not control for HPV infection or other variables related to sexual habits that are potential confounders of the association. Consequently, they tended to consider that the association between reproductive factors and cervical cancer was chiefly accounted for by confounding from sexual habits. More recent studies included detailed information on sexual behavior and HPV infection, suggesting that the effect of reproductive factors was not totally explained by sexual habits or HPV infection [57,135,136]. Most of these major studies have reported an increased risk for cervical cancer and pre-cancer with increasing number of pregnancies.

In 2002, a large IARC study found that women with seven or more full-term pregnancies had a 4-fold increase in the risk of developing squamous cell carcinoma of the cervix as compared with nulliparous women (OR=3.8, 95% CI: 2.7-5.5), restricting the analysis to HPV positive women [135]. A reanalysis conducted in 2006 among parous women indicated an increased risk for invasive cervical cancer with number of full-term pregnancies (RR=1.8, 95% CI: 1.5-2.0 for seven or more full-term pregnancies compared with women with one or two) [136]. Early age at first full-term pregnancy was also associated with risk of both invasive and pre-invasive cancer (RR=2.6, 95% CI: 2.0-3.3 for age <16 years compared with ≥30 years, and RR=1.8, 95% CI: 1.3-2.4 for age <17 years compared with ≥25 years respectively). The findings were similar in analyses restricted to women who tested positive for high-risk HPV DNA infection.

Only three prospective studies have been identified, showing no association between reproductive variables (i.e. parity, number of full-term pregnancies, age at first full-term pregnancy) and cervical cancer risk among women HPV positive [83,84,104]. Regarding retrospective case-control studies, the results have been conflicting [105–110,137]; some studies found a strong link with multiparity, for at least four full-term pregnancies, although others did not find any association. A possible explanation of this discrepancy could be that these studies were mainly based on populations with low parity, and the positive associations were found in studies based on women with high parity. Regarding age at first full-term pregnancy, few studies have evaluated its association with cervical cancer. Some of them found an increased risk among women whose first full-term pregnancy occurred in younger ages, less than 17-20 years old, compared to women whose first full-term pregnancy was from 25 years old [106,137].

Biological plausibility

Concentrations of estrogens and progestogens in blood are known to increase progressively during pregnancy to reach the highest levels in the last weeks [135]. These hormonal changes are probably responsible for the alterations in the junction between the squamous and columnar epithelium (i.e. transformation zone) occurring during pregnancy [138,139]. The transformation zone is maintained on the exocervix for many years, facilitating the direct exposure to HPV and possibly other cofactors, contributing to HPV persistence and progression to cervical neoplasia and cancer. Hormonal changes induced by pregnancy, both estrogens and progestogens, may also modulate the cervical immune response to HPV [114,139]. Immunosuppression may favor the infection and the oncogenic potential of HPV, and thereby influence the risk of persistence or progression [135].

Burden of parity

Fertility is one of the major components of population growth and age structure change. The past few decades have observed an important decline in world fertility, with a broad diversity among regions, with levels of total fertility above 5.5 live births per woman in the period 1970-1975 compared to 2.3 births per woman in the period 2010-2015 [140]. Figure 12 shows fertility rates among almost all countries around the world [94,140]. Differences between developed and developing regions are clear, with higher prevalence in Africa, followed by South and Eastern Asia, and some Latin America countries.

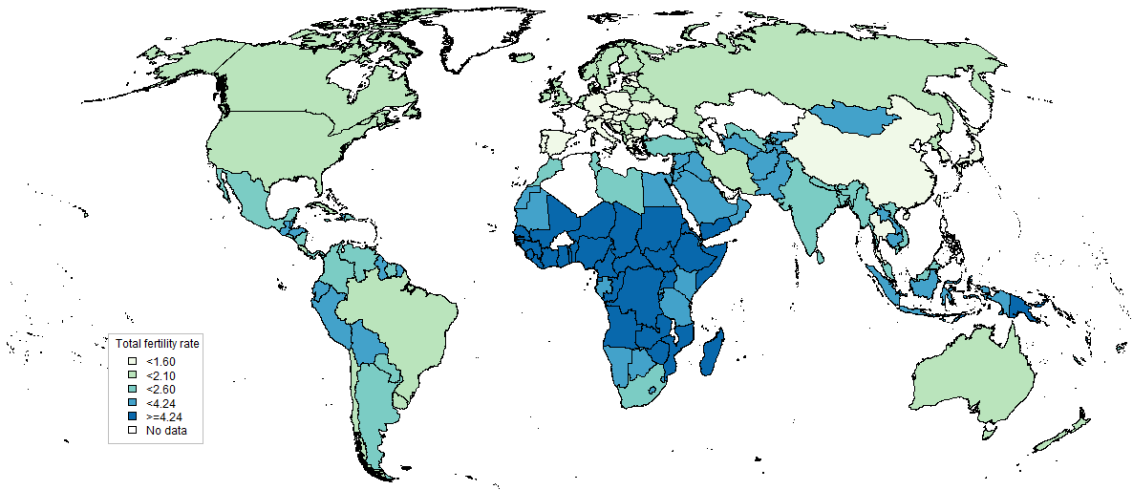

Figure 12. Fertility rates in the world. Extracted from [94,140].

3.2.3. Infection with other sexually transmitted infections

More than 30 different bacteria, viruses and parasites are known to be transmitted through sexual contact [141]. Eight of these pathogens are linked to the greatest incidence of sexually transmitted disease, among which four are currently curable: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Treponema pallidum subspecies pallidum* (syphilis). The other four are viral infections and are incurable: hepatitis B, herpes simplex virus, Human Immunodeficiency Virus (HIV), and HPV. Sexually transmitted infections are spread predominantly by sexual contact, including vaginal, anal and oral sex. More than 1 million sexually transmitted infections are acquired every day, having a deep impact on sexual and reproductive health worldwide.

HIV is a well-established cofactor of cervical cancer, being invasive cervical cancer an acquired immune deficiency syndrome (AIDS)-defining clinical condition. *Chlamydia trachomatis* and Herpes Simplex Virus have been associated with cervical cancer, although their role is less clear.

3.2.3.1. *Chlamydia trachomatis*

Chlamydia trachomatis (CT) is one of the four bacterial species in the genus *Chlamydia*. It is an obligate intracellular pathogen that infects human cells of the genital tract, as well as the ocular tissue. Based on the fact that CT and HPV infections are sexually transmitted and have similar behavioral risk factors, such as multiple sexual partners and younger age at first sexual intercourse, it seems obvious to evaluate the link between these two infections and its association with cervical lesions and cervical carcinogenesis. Many of previous studies on the role of CT and cervical cancer risk have adjusted for HPV DNA or serology, reducing the possibility of residual confounding by HPV in the risk estimates [97]. Adjustment for sexual behavior was also important because of its strong association with CT infection. Overall, the data reported provide initial evidence of a possible association between CT and cervical neoplasia. However, the exact stage of the multistage process of HPV-associated carcinogenesis that might be affected by CT has not been examined carefully and remains uncertain.

In 2000 and 2004, two large seroepidemiological studies showed highly significant associations between CT seropositivity and cervical cancer [142,143]. Koskela et al found an elevated risk for squamous cell carcinoma in women seropositive for CT at baseline compared with seronegative women, even after adjustment for HPV (OR=2.2, 95% CI: 1.3-3.5) [142]. In the IARC study conducted by Smith et al among HPV DNA positive cases and controls, serum antibodies to CT were also associated with an increased risk for squamous cell carcinoma (OR=1.8, 95% CI: 1.2-2.7) [143]. The effect of CT seropositivity on squamous cell carcinoma risk increased with increasing CT antibody titers (p-trend<0.001). Similar associations between antibodies to CT infection and cervical neoplasia were observed in several other seroepidemiological studies that controlled for HPV, with magnitudes around 2, and very few studies did not detect an association [97,144–147]. On the other hand, CT serotypes seem to be relevant regarding the association with cervical cancer, showing different risks depending on the serovar. Two previous studies observed that serum antibodies to CT serotypes G, I and E were significantly associated with highest risk of squamous cell carcinoma, and only one of them also found

increased risks for serotypes B, J, and D [144,148]. Few studies have evaluated the detection of CT DNA in the cervix in relation with the risk of neoplasia, finding controversial results [97,149,150].

Biological plausibility

CT infection may increase the risk of cervical cancer by increasing host susceptibility to HPV or enhancing the effects of HPV. Inflammation at the cervix resulting from chronic CT infection may facilitate the access of HPV to its host cells in the basal epithelium layer, increasing the risk of cervical cells transformation [151,152]. CT infection may also lead to reactive oxidative metabolites production and decrease in cell-mediated immunity, that can damage the DNA and DNA repair proteins and inhibit apoptosis, overexpress E6 and E7 oncogenes, and finally result in genetic instability [153,154]. Moreover, the inflammatory cells produce cytokines, chemokines, growth and angiogenic factors, which stimulate the tumor growth.

Burden of Chlamydia trachomatis

CT infection is the most commonly sexually transmitted bacterial infection [141]. Each year, there are estimated 131 million new cases of CT worldwide. Because 85-90% of CT infections are asymptomatic and remain undetected and untreated, it is likely that the true occurrence of new infections is even higher [97]. Furthermore, CT infections can persist for several months or even years, and may also recur or be reactivated. In 2012, among women aged 15-49 years, the estimated global prevalence of CT infection was 4.2% (95% CI: 3.7-4.7%), with regional values ranging from 1.8% in South-Eastern Asia to 7.6% in the Americas [155].

3.2.3.2. Human Herpes Virus 2

Herpes simplex virus 1 and 2, also known as human herpes virus 1 and 2 (HHV-1 and HHV-2), are two members of the herpesvirus family that infect humans. Before the establishment of the causal role of HPV in the development of cervical cancer, HHV-2 was itself regarded as a candidate etiological agent [156]. Although previous studies conducted during the 1970s demonstrated the carcinogenic potential of HHV-2, HHV-2 DNA was not consistently detected in cervical cancer specimens [156,157]. An increased understanding of the causal role of HPV and further studies on HHV-2 shifted the focus away from this sexually transmitted infection as an

etiological agent. However, epidemiological studies on HHV-2 and cervical cancer risk were still of interest taking into account its high frequency in the genital tract. Since the 1990s, the majority of seroepidemiological studies found moderate or even no associations between HHV-2 antibodies and cervical neoplasia [97]. When adjustment for HPV infection was take into account, no excess risk remains in most of the epidemiological studies.

In 2002, Lehtinen et al, using a Nordic cohort of more than half a million women, did not found differences between baseline seroprevalence of HHV-2 among cases and non-cases after adjustment of HPV serology (OR=1.0, 95% CI: 0.6-1.7) [158]. At the same time, Smith et al have reported results from a study conducted by the IARC that pooled data from seven case-control studies, finding a higher risk of squamous cell carcinoma among HHV-2 seropositive women than among seronegative women (OR=2.2, IC 95%: 1.4-3.4) when analyses were restricted to HPV DNA positive women [159]. Later on, a few additional investigations were published, reporting no significant associations of HHV-2 seropositivity with cervical cancer or pre-cancer risk [147]. A small number of studies tested for HHV-2 DNA in the cervix, giving conflicting results [97].

Biological plausibility

Although viral DNA of HHV-2 has not been consistently detected in cervical tumors [157], it is possible that HHV-2 may act on host cellular DNA by a hit-and-run mechanism. The mechanism by which HHV-2 induces transformation is not well understood. Various lines of investigation are developed, including that HHV-2 infection is able to cause mutations, induce gene integration and amplification, or activate cellular transcription [160]. HHV-2 infection, like HPV, can infect cervical squamous epithelial tissue in the squamocolumnar junction, where invasive cervical cancer arises. Lesions related to HHV-2 may facilitate the access of HPV to the basal cell layer [159]. Inflammatory responses induced by these lesions may interfere in the ability of a women to mount an effective immune response to HPV infection, which increases the likelihood of HPV-associated lesions progression [154]. The failure to detect viral DNA in a high proportion of human anogenital tumors made it difficult to implicate HHV-2 in the etiology of those neoplasias, but it is consistent, however, with the observations on the mode of HHV-2 transformation in vitro, and suggests that HHV-2 could be involved in a multistage process of oncogenic transformation.

Burden of Human Herpes Virus 2

HHV-2 is a sexually transmitted infection that produces most genital herpes, and it is ubiquitous and contagious. An estimated 417 million people aged 15-49 years worldwide have HHV-2 infection [141]. Differences related to HHV-2 prevalence were observed across regions, with higher estimations in Africa and South-Eastern Asia, with prevalences around 50% and 30% of the population respectively [161]. HHV-2 infection, as an incurable viral infection, is becoming more prevalent globally. Genital herpes infections often have no symptoms, and most infected people are unaware that they have the infection. Typically, about 10-20% of people with HHV-2 infection report a prior diagnosis of genital herpes.

3.2.3.3. Human Immunodeficiency Virus

It is well-established that Human Immunodeficiency Virus (HIV)-infected individuals are at substantially higher risk of cancer, mainly due to the combination of HIV-associated immunosuppression and co-infection with other carcinogenic infectious agents. Specifically, HIV-infected individuals are at 3-fold risk of cervical cancer, being invasive cervical cancer an AIDS-defining clinical condition since 1993 [162]. HIV and HPV interact at different levels of the natural history. Not only HIV positive women are at a statistically significant increased risk of CIN2, CIN3 and invasive cancer compared to HIV negative women[163–165], but HIV infection is strongly associated with an increased acquisition and persistence of HPV infection [166,167]; and the other way round, it has been also found that HPV infection may increase the risk of HIV acquisition [168]. Several reviews on the association between HIV and prevalence and natural history of HPV infection, as well as cervical neoplasia, have been published, including the IARC Monograph on HPVs [57,169,170].

However, as the information on HIV infection status was not available in our study, the European Prospective Investigation into Cancer and Nutrition (EPIC), this association is out of the scope of this thesis.

3.2.4. Nutritional factors: diet, body fatness, and physical activity

An update of the only randomized controlled trial assessing the effect of diet on cervical cancer, the Women's Health Initiative-Dietary Modification Trial, did not support a significant effect of low-fat dietary intervention on cervical cancer prevention [171]. A recent

comprehensive review of 32 prospective studies concluded that, in spite of limited results suggesting a protective effect for some dietary components, overall, the available evidence for an association between diet and cervical carcinogenesis is not convincing [172]. Most studies included in this review failed to take into account HPV infection. One of these studies was carried out in the framework of the EPIC project [173]; no significant associations were found for fruit or vegetable intake (overall or according to subgroups), for the intake of vitamin C, vitamin E, retinol, beta-carotene, vitamin D and folic acid in relation with cervical cancer risk (Annex).

Regarding the potential relationship between cervical cancer and overweight or obesity, in a new meta-analysis of nine studies, no significant linear dose-response relationship was observed between body mass index and cervical cancer risk or mortality [174]. Most studies adjusted for smoking, but none adjusted for screening practices or HPV infection. Moreover, the recent review by the IARC of the evidence of body fatness on the risk of cancer did not include cervical cancer among the thirteen tumor sites with sufficient evidence of a role of excess body fatness on cancer risk [175]. Therefore, in spite of some plausible mechanisms such as increased levels of circulating estrogen and androgen levels common in obese women, or obesity-induced changes in immune function that could affect clearance of HPV infection and elevated levels of inflammation, the current evidence does not support a link between excess body fatness and cervical cancer risk.

4. Rationale

Cervical cancer is an important disease among women worldwide, with a high burden that accounted for approximately 530,000 new cases and 265,000 deaths annually. Although it is well-established that persistent cervical high-risk HPV infection is the necessary cause of cervical cancer, it is not sufficient. Other factors, in conjunction with HPV infection, play a role in cervical carcinogenesis. There is evidence that cofactors, such as tobacco smoking, oral contraceptives use, and multiparity, may increase the risk of cervical cancer by around 2-fold. These cofactors are less relevant than viral factors in terms of magnitude and from a clinical perspective. However, due to their high prevalence, their impact in the global burden of cervical cancer may be substantial. Additional strategies related to these preventable cofactors, in addition to the established population-based prevention strategies (HPV vaccination and cervical cancer screening programs), may contribute in the reduction of the disease burden and mortality.

The contribution of these cofactors to cervical cancer risk is still not well-established for all of them, while for some a clear effect has been demonstrated but some features of the association remain to be elucidated. In Europe, we have the opportunity to quantify more precisely the estimations of the risks between environmental factors and cervical cancer using the EPIC study. Briefly, the EPIC is an ongoing multi-centre prospective cohort study designed to investigate the relationship between nutrition and cancer, and other chronic diseases [176]. The study currently includes 519,978 participants (366,521 women and 153,457 men, mostly aged 35-70 years) in 23 centers from 10 European countries, followed for cancer incidence and cause-specific mortality for several decades. The enrollment took place between 1992 and 2000 at each center, and questionnaires on dietary and lifestyle information were collected. Anthropometric measurements were also performed, and blood samples were taken at recruitment.

The main purpose of this thesis is to assess more accurately the potential associations between environmental HPV cofactors and risk of developing cervical cancer and pre-cancer using data from the EPIC study. EPIC represents the largest resource available today worldwide for prospective investigations on the etiology of cancers and other diseases that can integrate questionnaire data on lifestyle and diet, and biomarkers of measurable variables.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

There is a well-established relationship between tobacco smoking and cervical cancer risk. However, there are still some inconsistencies about some time-related variables of the habit (i.e. duration, age at start and time since quitting). The potential causal role of a factor is enhanced if a consistent dose-response relationship between exposure and risk is found, and when elimination of exposure is associated with a subsequent decrease in risk. Among that, we hypothesize that there is a significant trend to increased risk of cervical cancer with both intensity (amount daily smoked) and duration of the habit. On the other hand, we also hypothesize that, independently of intensity and duration of prior exposure, cessation of smoking habit involves a reduction in the risk of cervical cancer; specific time at which this decrease becomes significant remains to be elucidated. We further hypothesize that, in addition to active smoking, exposure to passive smoking among never smokers, in spite of the lower dose of exposure, could be related to a weak increase in the risk of cervical cancer. Finally, we hypothesize that all the mentioned effects apply to both pre-invasive (CIN3/CIS) and invasive cervical cancer (ICC), and that they remain after adjusting for HPV infection or when the analyses are restricted to women with HPV infection as determined by HPV serology.

Regarding parity, there seems to be a clear effect of increased risk of cervical cancer with the number of full-term pregnancies, although it is not totally clear whether this effect applies also to CIN3/CIS. There is also a well-established association of long-term use of oral contraceptives, while a suggested decreased risk after cessation of OC use is based on limited evidence. We first hypothesize that parity (number of full-term pregnancies) is associated with an increased risk of both pre-invasive and invasive cervical cancer. We also hypothesize that, as it happens for other hormone-dependent tumors, the increased risk of OC use is time dependent; specifically, it is associated with duration and it has a transient effect on both CIN3/CIS and ICC. Furthermore, in the same way as already proposed for tobacco smoking, our hypothesis is that the effects of parity and OC use remain after adjusting for HPV infection or when the analyses are restricted to women with HPV infection as determined by HPV serology.

An effect of intrauterine device use or hormone therapy in menopausal women has not been clearly elucidated yet. However, these factors share many characteristics with parity and OC use, including biological plausibility. Taking into account the literature, it is a reasonable hypothesis to be tested in our study that IUDs use and hormone therapies in menopausal women might have a protective effect on cervical cancer risk. If such an association is found,

we further explore all the mentioned effects apply both to CIN3/CIS and ICC, remain after adjusting for HPV infection or when the analyses are restricted to women with HPV infection as determined by HPV serology.

The role of specific infection agents in the pathogenesis of cervical cancer has been considered, and specifically, there seems to be an association with infection by *Chlamydia trachomatis* and Human Herpes Virus 2. However, most of the evidence comes from retrospective studies, and the specific role on either early stages of the disease (CIN3/CIS) or invasive cancer is not clearly disentangled. We hypothesize that there is an association between serological markers of CT and HHV-2 and cervical cancer risk, both for CIN3/CIS and ICC. Furthermore, we hypothesize that co-infection of HPV with CT and/or HHV-2 increased the risk of cervical cancer. Our hypothesis is that these are specific effects of CT and HHV-2 and therefore, no association would be found with serology of related infectious agents of non-sexually transmitted infections, such as *Chlamydia pneumonia*, cutaneous HPVs, or poliomaviruses.

Finally, we hypothesize that some serological markers of HPV may be suitable markers of either exposure to HPV or of cervical carcinogenesis. Specifically, our hypothesis is that (i) antibodies to L1 protein of HPV genotypes are valid markers for cumulative and past exposure to HPV, as shown by its association with increased risk of cervical cancer and pre-cancer; and (ii) antibodies against E6 and E7 oncoproteins of HPV types 16 and 18 could be strong markers of cervical cancer development, as shown by its specific association with invasive cervical cancer.

OBJECTIVES

1) To study prospectively the dose-response relationship between tobacco smoking and cervical cancer risk, including duration, intensity, and cessation, for pre-invasive and invasive cervical cancer, using two approaches: the analysis of the full EPIC cohort, and a nested case-control study within the cohort with available sera to test for serological markers of HPV infection to taking into account previous exposure.

2) To assess prospectively the association between hormonal and reproductive factors and the risk of cervical cancer, in particular, parity and multiparity, duration and cessation of oral contraceptives use, use of intrauterine devices, and use of menopausal hormone therapy, for pre-invasive and invasive cervical cancer, in the cohort and in the nested case-control study adjusting and restricting by HPV serology.

3) To evaluate prospectively the association between serological markers of *Chlamydia trachomatis* and Human Herpes Virus 2 and the risk of developing cervical cancer, including pre-invasive and invasive cervical cancer, in the nested case-control study adjusting by past exposure to HPV infection.

4) To assess the association of serological markers of HPV L1, including genotypes 11, 16, 18, 45, 31, 33, 35, 52 and 58, with the risk of cervical cancer, including pre-invasive and invasive cancer, in the nested case-control study.

5) To explore the association between E6 and E7 antibody oncoproteins of HPV types 16 and 18 and the risk of cervical cancer, for pre-invasive and invasive cervical cancer, in the nested case-control study.

ARTICLES

ARTICLE 1

Prospective seroepidemiologic study on the role of Human Papillomavirus and other infections in cervical carcinogenesis: Evidence from the EPIC cohort.

Castellsagué X, Pawlita M, **Roura E**, Margall N, Waterboer T, Bosch FX, de Sanjosé S, Gonzalez CA, Dillner J, Gram IT, Tjønneland A, Munk C, Pala V, Palli D, Khaw KT, Barnabas RV, Overvad K, Clavel-Chapelon F, Boutron-Ruault MC, Fagherazzi G, Kaaks R, Lukanova A, Steffen A, Trichopoulou A, Trichopoulos D, Klinaki E, Tumino R, Sacerdote C, Mattiello A, Bueno-de-Mesquita HB, Peeters PH, Lund E, Weiderpass E, Quirós JR, Sánchez MJ, Navarro C, Barricarte A, Larrañaga N, Ekström J, Hortlund M, Lindquist D, Wareham N, Travis RC, Rinaldi S, Tommasino M, Franceschi S, Riboli E.

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Prospective seroepidemiologic study on the role of Human Papillomavirus and other infections in cervical carcinogenesis: Evidence from the EPIC cohort

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Key words: cohort study, cervical cancer, HPV, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, human polyomaviruses, HHV-2, EPIC, STI, serology

Abbreviations: CC: cervical cancer; CI: confidence interval; CIN3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; CP: Chlamydia pneumonia; CT: Chlamydia trachomatis; EPIC: European Prospective Investigation into Cancer and Nutrition; GST: Glutathione S-transferase; HHV-2: Human Herpes Virus 2; HPV: Human Papillomavirus; HR: hazard ratios; IARC: International Agency for Research on Cancer; ICC: invasive cervical cancer; ICD-10: International Classification of Diseases, 10th revision; JCV: Human polyomavirus; KIV: Human polyomavirus; LPV: Human polyomavirus; MFI: median fluorescence intensity; MIF: microimmunofluorescence; OR: odds ratios; STI: sexually transmitted infection; WUV: Human polyomavirus.

Conflict of interest: Tim Waterboer is currently employed by F. Hoffman-La Roche Ltd. who had no influence on this manuscript. Joakim Dillner has received institutional research grants from Merck/SPMSD on the subject of long-term registry follow-up of vaccine impact in populations and HPV surveillance. Maria Hortlund has received institutional research grant from MSD/Merck and the affiliating institution has received financial support from Merck & Co., Inc. Christian Munk has received lecture fees and support for congress participation from SPMSD. The remaining authors declare no conflicts of interest that might bias the research reported in this manuscript.

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To evaluate prospectively the association between serological markers of selected infections, including HPV, and risk of developing cervical cancer (CC) and precancer, we performed a nested case–control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) study that included 184 cases of invasive CC (ICC), 425 cases of cervical intraepithelial neoplasia (CIN) grade 3 or carcinoma *in situ* (CIS), and 1,218 matched control women. At enrollment participants completed lifestyle questionnaires and provided sera. Subjects were followed-up for a median of 9 years. Immunoassays were used to detect serum antibodies to Human Herpes Virus 2 (HHV-2), *Chlamydia trachomatis* (CT), *Chlamydia pneumoniae*, L1 proteins of mucosal and cutaneous HPV types, E6/E7 proteins of HPV16/18, as well as to four polyomaviruses. Adjusted odds ratios (OR) [and 95% confidence intervals (CI)] for CIN3/CIS and ICC risk were respectively: 1.6 (1.2–2.0) and 1.8 (1.1–2.7) for L1 seropositivity to any mucosal HPV type, 1.0 (0.4–2.4) and 7.4 (2.8–19.7) for E6 seropositivity to HPV16/18, 1.3 (0.9–1.9) and 2.3

(1.3–4.1) for CT seropositivity, and 1.4 (1.0–2.0) and 1.5 (0.9–2.6) for HHV-2 seropositivity. The highest OR for ICC was observed for HPV16 E6 seropositivity [OR = 10.2 (3.3–31.1)]. Increasing number of sexually transmitted infections (STIs) was associated with increasing risk. Non-STIs were not associated with CC risk. In conclusion, this large prospective study confirms the important role of HPV and a possible contribution of CT and HHV-2 in cervical carcinogenesis. It further identifies HPV16 E6 seropositivity as the strongest marker to predict ICC well before disease development.

What's new?

Limited data are available from prospective studies concerning the role of past exposure to human papillomavirus (HPV) and other infections in cervical carcinogenesis. This study assessed associations between cervical cancer and pre-cancer and serological markers of exposure to mucosal and cutaneous HPVs, *Chlamydia trachomatis* (CT), *Chlamydia pneumonia*, human herpes virus-2 (HHV-2), and polyomaviruses using a nested case-control design within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Associations were found for mucosal HPVs, CT, and HHV-2. A greater number of sexually transmitted diseases further raised the risk of cervical cancer.

Persistent infection with high-risk Human Papillomavirus (HPV) genotypes is established as the necessary cause of CC. However, many studies have identified other sexually transmitted infections (STIs) and behavioral and lifestyle factors as possible cofactors involved in cervical carcinogenesis.[1] Furthermore, serological markers of HPV using a large panel of antigens have been little explored in prospective studies to assess the risk of cervical neoplasia and CC after seroconversion.

Serological markers of other STIs as measured years before cancer development are useful to assess the association of these infections with CC risk. In addition to HPV, associations have been found with *Chlamydia trachomatis* (CT) and Human Herpes Virus 2 (HHV-2).[1–6] However, most of the evidence comes from retrospective case–control studies and little data are available from prospective designs.

Few studies have taken into account comprehensive evaluation of past exposure to HPV by including several antigens (L1/E6/E7) and a large number of HPV types including cutaneous types as "control" non-STIs. Thus, further evidence from large prospective cohort studies taking into account HPV exposure is still needed to confirm the role of CT and HHV-2 in cervical carcinogenesis. Given its prospective design and size, the European Prospective Investigation into Cancer and Nutrition (EPIC) is a unique opportunity to assess these associations.[7]

The aim of this study is to estimate, prospectively, the association between serological markers of HPV infection, CT, and HHV-2, and the risk of developing CC and precancer. Serological markers of selected polyomaviruses, cutaneous HPVs and *Chlamydia pneumoniae* (CP) are also used to contrast associations between sexually and nonsexually transmitted infections.

Materials and Methods

The EPIC Cohort Study

The study has been described in detail previously.[7,8] Briefly, the European Prospective Investigation into Cancer and Nutrition (EPIC) is a large prospective cohort study composed of 521,448 participants recruited between 1992 and 2000 from 23 centers in 10 European countries: Denmark, France, Germany, Greece, Italy, The Netherlands, Norway, Spain, Sweden, and the United Kingdom. Of those, 367,993 were women mostly aged 35–70 years. All eligible subjects were invited to participate in the study, and those who accepted gave written informed consent, completed medical and lifestyle questionnaires, and were invited for blood collection.

Incident cases of CC and precancer among cohort members were identified using a record linkage with population-based cancer registries in Denmark, Italy, The Netherlands, Norway, Spain, Sweden, and the United Kingdom. In France, Germany and Greece, a combination of methods was used, including linkage to health insurance records, hospital-based cancer and pathology registries, and active follow-up of subjects. Data on vital status were obtained from regional and national mortality registries. The study only included CC cases with first primary incident cancer. For each EPIC center, time of follow-up was calculated between the date at recruitment and the date at diagnosis of CC or the date at censoring (death, loss of follow-up or end of follow-up on December 2006). A total of 2,775,235 person-years were followed-up, with a median of 9 years.

Study Population and Nested Case–Control Design

Out of the full EPIC cohort, we excluded all men ($n = 153,455$) and women who had prevalent cancer ($n = 22,180$), incomplete follow-up ($n = 2,295$), hysterectomy ($n = 34,973$), or no lifestyle questionnaire ($n = 509$). Among the total of 308,036 women included in the final cohort, 1,065 women with CC were identified: 261 with invasive CC (ICC) and 804 with high-grade cervical intraepithelial neoplasia or carcinoma *in situ* (CIN3/CIS). Out of this cohort, we conducted a nested case–control study. Thus, for each case of CC with available blood sample, a selection of two matched

control subjects was performed at random among all cancer-free cohort women who were at risk at the time of diagnosis of each corresponding case. Matching criteria included: Study center of enrolment, age at recruitment, menopausal status (pre-, postmenopausal), duration of follow-up time, date, time of day, and fasting status at blood collection and, among premenopausal women, phase of the menstrual cycle. A total of 609 cases (184 ICC and 425 CIN3/CIS) and 1,218 controls were included in the final analyses.

Multiplex Serology

Antibodies to the major capsid protein L1 of high-risk mucosal HPV types 16, 18, 31, 33, 35, 45, 52, and 58, and of low-risk mucosal HPV type 11, antibodies to proteins E6 and E7 of HPV types 16 and 18, antibodies to L1 of cutaneous types 1, 4, 8, and 77, and antibodies to VP1 of the polyomaviruses JCV, LPV, KIV, and WUV were tested for at the German Cancer Research Center, Heidelberg, Germany, between 2008 and 2011 using glutathione *S*-transferase (GST) and fluorescent bead-based multiplex serology.[9–12] The cutaneous HPV types and the polyomaviruses were used as specificity control (no association with cervical carcinoma expected). L1 proteins were expressed in *E. coli* bacteria as fusion proteins with N-terminal GST and a C-terminal tag epitope as described previously.[13] Fluorescence-labeled polystyrene beads (Luminex) were derivatized with glutathione casein.[14] GST-fusion proteins were specifically bound to glutathione-coated Luminex beads directly from the lysates. Differently colored beads were loaded with different fusion proteins. Human serum aliquots (1 μl) were reacted with a mixture of fusion protein-loaded beads (2,000 beads per color and fusion protein) in 100 μl of blocking buffer. Bound human antibodies were stained with biotinylated antihuman Ig followed by streptavidin-R-phycoerythrin. Beads were analyzed in a Luminex reader for bead color (fusion protein type) and quantity of bound human immunoglobulin (R-phycoerythrin fluorescence). The median fluorescence intensity (MFI) of at least 100 beads of each color was used as value indicating the amount of specifically bound human antibody. Background was determined on GST-tag loaded beads.

MFI values were dichotomized as antibody positive or antibody negative. L1 seropositivity cutoffs were HPV type specific and defined previously both for mucosal[15] and for cutaneous[11] HPV types. The cutoff for mucosal HPV was defined as 5 SDs above the mean of the final distribution of MFI values in a study investigating serum samples from HPV DNA-negative self-reported virgins from Korea.[15] To adjust the previously defined cutoffs for between-study variation, a subpanel of 188 Korean sera with known antibody prevalences was retested with all samples from the present study. Adjusted cutoff values for the present study were calculated to give the same seroprevalence in the Korean bridging panel as determined before. For HPV18 L1 the calculated cutoff was below 100 MFI and thus closes to the limit of detection of the assay, probably resulting in many false-positive reactions. Using a distribution-based method,[16,17] we therefore arbitrarily set the cutoff to 120 MFI to increase specificity. The distribution-based method was also applied to define cutoffs for the polyomaviruses. For E6/E7 serology cutoff values were calculated to give the same age-matched seroprevalence provided by a previous EPIC head and neck study.[12]

To assess reproducibility of the HPV assays, a random set of 186 samples was re-tested every assay day. The median correlation coefficient (R^2) of the MFI data across all HPV antigens was 0.92 (range 0.88–0.96). All assays were conducted without knowledge of case–control status or subjects' characteristics.

Chlamydial Serology

We used the commercially available Focus Diagnostics' Chlamydia micro-immunofluorescent (MIF) IgG assay (Focus Diagnostics™, Cypress, CA, USA) for the qualitative detection and semiquantitation of IgG antibodies. The MIF assay utilizes purified elementary bodies (EB) as the substrate to detect species and serovar-specific chlamydial antibody reactions. Semiquantitative endpoint titers are obtained by testing serial dilutions of positive specimens. The assay was used following manufacturer's recommendations.

A blinded reproducibility study was also conducted by retesting a random sample of specimens (about 10%) twice. Percent agreements in our laboratory for CT serovars A, BDE, CJHI, and FGK, and CP were 85.3, 86.2, 86.7, and 84.9% and 89.3%, respectively. When conflicting results, the original ones were used.

HHV-2 Serology

We used the commercially available Focus Diagnostics HerpeSelect®2 ELISA IgG assay. In this assay, the polystyrene microwells are coated with recombinant gG-2 antigen. Diluted serum samples and controls (1:101) are incubated in the wells to allow specific antibody present in the samples to react with the antigen. Nonspecific reactants are removed by washing, and peroxidase-conjugated antihuman IgG is added and reacts with specific IgG. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the stop reagent, the resultant color change is quantified by a spectrophotometric reading of optical density (OD). Sample optical density readings are compared with reference cutoff OD readings to determine results. An index value of >1.10 is presumptive for the presence of IgG antibodies to HHV-2. The commercially available assay *biokit* HSV-2 Rapid Test ("Biokit") was used to confirm all positive results. This is a rapid immunoassay that detects the presence of antibodies specific for HHV-2 in capillary whole blood or serum. The test was used following manufacturer's recommendations.

Statistical Analyses

Multivariate odds ratios (OR) and 95% CI for the association between serological markers and the two disease outcomes

Table 1. Baseline characteristics and prevalence of serological markers of selected infections in preinvasive and invasive cases and controls recruited in the nested case–control study

	CIN3/CIS			ICC		
Variables	**Controls (*N* = 850) *N* (%)**	**CIN3/CIS (*N* = 425) *N* (%)**	***p* of difference[1]**	**Controls (*N* = 368) *N* (%)**	**ICC (*N* = 184) *N* (%)**	***p* of difference[1]**
Country			Matched[2]			Matched[2]
France	12 (1.4)	6 (1.4)		2 (0.5)	1 (0.5)	
Italy	42 (4.9)	21 (4.9)		32 (8.7)	16 (8.7)	
Spain	44 (5.2)	22 (5.2)		44 (12.0)	22 (12.0)	
United Kingdom	156 (18.4)	78 (18.4)		34 (9.2)	17 (9.2)	
The Netherlands	6 (0.7)	3 (0.7)		28 (7.6)	14 (7.6)	
Greece	8 (0.9)	4 (0.9)		26 (7.1)	13 (7.1)	
Germany	98 (11.5)	49 (11.5)		46 (12.5)	23 (12.5)	
Sweden	328 (38.6)	164 (38.6)		86 (23.4)	43 (23.4)	
Denmark	128 (15.1)	64 (15.1)		66 (17.9)	33 (17.9)	
Norway	28 (3.3)	14 (3.3)		4 (1.1)	2 (1.1)	
Age at recruitment (years)			Matched[2]			Matched[2]
<30	50 (5.9)	22 (5.2)		9 (2.4)	4 (2.2)	
30–39	197 (23.2)	104 (24.5)		49 (13.3)	26 (14.1)	
40–49	278 (32.7)	140 (32.9)		121 (32.9)	63 (34.2)	
50–59	268 (31.5)	132 (31.1)		139 (37.8)	66 (35.9)	
≥60	57 (6.7)	27 (6.4)		50 (13.6)	25 (13.6)	
Mean age	45.2	45.2		49.5	49.4	
Mean years between blood collection and diagnosis (5th–95th percentile)	8.8 (0.003–12.8)	3.3 (0.3–7.9)		8.1 (0.003–12.1)	3.9 (0.3–8.9)	
Marital status			<0.001			<0.001
Single	77 (11.5)	48 (14.5)		15 (5.9)	18 (14.0)	
Married/living together	500 (75.0)	211 (63.6)		204 (79.7)	83 (64.3)	
Divorced/separated	73 (10.9)	66 (19.9)		26 (10.2)	25 (19.4)	
Widowed	17 (2.5)	7 (2.1)		11 (4.3)	3 (2.3)	
Missing[5]	183	95		112	55	
Smoking status			<0.001			<0.001
Never smokers	440 (52.5)	160 (38.2)		197 (54.3)	76 (41.3)	
Past smokers	212 (25.3)	96 (22.9)		82 (22.6)	45 (24.5)	
Current smokers	186 (22.2)	163 (38.9)		84 (23.1)	63 (34.2)	
Missing[5]	12	6		5	0	
OC use			0.12			0.175
Never users	225 (33.4)	99 (29.4)		139 (43.3)	56 (35.2)	
Past users	392 (58.2)	197 (58.5)		158 (49.2)	86 (54.1)	
Current users	57 (8.5)	41 (12.2)		24 (7.5)	17 (10.7)	
Missing[5]	176	88		47	25	
HPV L1 serology (9 mucosal types)[3]			<0.001			<0.001
Seronegative to all types	463 (54.8)	184 (43.6)		212 (57.6)	76 (41.8)	
Seropositive to any type	382 (45.2)	238 (56.4)		156 (42.4)	106 (58.2)	
Missing[5]	5	3		0	2	

Table 1. Baseline characteristics and prevalence of serological markers of selected infections in preinvasive and invasive cases and controls recruited in the nested case–control study (Continued)

	CIN3/CIS			ICC		
Variables	**Controls (*N* = 850) *N* (%)**	**CIN3/CIS (*N* = 425) *N* (%)**	***p* of difference[1]**	**Controls (*N* = 368) *N* (%)**	**ICC (*N* = 184) *N* (%)**	***p* of difference[1]**
HPV E6 serology (HPV16 and 18)			0.94			<0.001
Seronegative to both types	826 (97.8)	413 (97.9)		360 (97.8)	160 (87.9)	
Seropositive to either/both types	19 (2.2)	9 (2.1)		8 (2.2)	22 (12.1)	
Missing[5]	5	3		0	2	
HPV E7 serology (HPV16 and 18)			0.08			0.006
Seronegative to both types	764 (90.4)	367 (87.0)		339 (92.1)	153 (84.1)	
Seropositive to either/both types	81 (9.6)	55 (13.0)		29 (7.9)	29 (15.9)	
Missing[5]	5	3		0	2	
HPV E6 and/or E7 serology (HPV16 and 18)			0.14			<0.001
Seronegative to both types	747 (88.4)	360 (85.3)		334 (90.8)	137 (75.3)	
Seropositive to either/both types	98 (11.6)	62 (14.7)		34 (9.2)	45 (24.7)	
Missing[5]	5	3		0	2	
HPV L1 serology (4 cutaneous types)[4]			0.11			0.57
Seronegative to all types	404 (47.8)	181 (42.9)		197 (53.5)	92 (50.5)	
Seropositive to any type	441 (52.2)	241 (57.1)		171 (46.5)	90 (49.5)	
Missing[5]	5	3		0	2	
CT serology			0.005			<0.001
Seronegative	743 (87.8)	345 (81.8)		338 (91.8)	142 (78.0)	
Seropositive	103 (12.2)	77 (18.2)		30 (8.2)	40 (22.0)	
Missing[5]	4	3		0	2	
CP serology			0.96			0.85
Seronegative	272 (32.3)	137 (32.6)		132 (36.1)	63 (34.8)	
Seropositive	570 (67.7)	283 (67.4)		234 (63.9)	118 (65.2)	
Missing[5]	8	5		2	3	
HHV-2 serology			0.01			0.002
Seronegative	734 (86.7)	343 (81.3)		319 (86.7)	138 (75.8)	
Seropositive	113 (13.3)	79 (18.7)		49 (13.3)	44 (24.2)	
Missing[5]	3	3		0	2	

OC: Oral contraceptive; HPV: Human Papillomavirus; CT: *Chlamydia trachomatis*; CP: *Chlamydia pneumoniae*; HHV-2: Human Herpes Virus 2.
[1]Chi-squared test was used to compare baseline characteristics between cases and controls.
[2]Variables used for matching.
[3]Includes mucosal HPV types 11, 16, 18, 45, 31, 33, 35, 52, and 58.
[4]Includes cutaneous HPV types 1, 4, 8, and 77.
[5]Not included in the percentage distribution of the variable.

(CIN3/CIS and ICC) were estimated using conditional logistic regression models adjusted by potential confounders. We assessed associations with several combinations of serological markers and HPV types: (1) By antigen and specific HPV genotype; (2) by combining all types within each antigen (L1, E6, and E7), but without mixing mucosal and cutaneous HPV types; (3) by combining E6 and E7 markers by HPV type and combining HPV16 and 18. L1 and E6/E7 serologies were never combined. Referent groups for all L1 analyses included women L1 seronegative to all mucosal HPV types for the mucosal analyses and to all cutaneous HPV types for the cutaneous analyses. For E6/E7 analyses, the referent group included women seronegative to HPV16 and/or 18 for E6 and/or E7 depending on whether the analysis combined the two types and the two markers. In addition to matching variables (study center, age, menopausal status, duration of follow-up time, date, time of day and fasting status at blood collection, and phase of the menstrual cycle among premenopausal women), models were adjusted by HPV L1, CT, and HHV-2 serostatus, level of education (none, primary school

Table 2. Multivariate odds ratios for the association between serological markers of HPV and human polyomaviruses and risk of CIN3/CIS and ICC

	CIN3/CIS		ICC	
Serological marker	***N* cases/ *N* controls**	**OR[1] (95% CI)**	***N* cases/ *N* controls**	**OR[1] (95% CI)**
HPV L1 (9 mucosal types)[2]				
Seronegative to all types	184/463	1 (reference)	76/212	1 (reference)
Seropositive to any type	238/382	**1.6 (1.2–2.0)**	106/156	**1.8 (1.1–2.7)**
Seropositive to any HR type	206/329	**1.6 (1.2–2.1)**	87/138	**1.6 (1.1–2.6)**
Seropositive to only one type	117/217	**1.4 (1.0–1.8)**	59/79	**2.0 (1.2–3.2)**
Seropositive to multiple types (≥2 types)	121/165	**1.9 (1.3–2.6)**	47/77	1.5 (0.8–2.6)
To 2 types	56/73	**1.9 (1.3–3.0)**	17/31	1.3 (0.6–2.8)
To ≥3 types	65/92	**1.8 (1.2–2.8)**	30/46	1.6 (0.8–3.1)
Trend across number of types (1, 2, ≥3)		***0.01***		***0.05***
HPV E6 (HPV16 and 18)				
Seronegative to both types	413/826	1 (reference)	160/360	1 (reference)
Seropositive to either or both types	9/19	1.0 (0.4–2.4)	22/8	**7.4 (2.8–19.7)**
HPV E7 (HPV16 and 18)				
Seronegative to both types	367/764	1 (reference)	153/339	1 (reference)
Seropositive to either or both types	55/81	1.3 (0.9–1.9)	29/29	**2.9 (1.5–5.6)**
HPV E6 and/or E7 (HPV16 and 18)				
Seronegative to both markers and both types	360/747	1 (reference)	137/334	1 (reference)
Seropositive to either marker for either type	62/98	1.3 (0.9–1.8)	45/34	**4.3 (2.4–7.7)**
Seropositive to both markers for both types	2/2	1.3 (0.1–13.0)	6/3	**6.4 (1.3–30.7)**
HPV L1 (4 cutaneous types)[3]				
Seronegative to all types	181/404	1 (reference)	92/197	1 (reference)
Seropositive to any type	241/441	1.2 (0.9–1.6)	90/171	1.1 (0.7–1.7)
JCV VP1				
Seronegative to JC VP1 polyoma	176/379	1 (reference)	77/144	1 (reference)
Seropositive to JC VP1 polyoma	246/466	1.2 (0.9–1.5)	105/224	0.9 (0.6–1.4)
LPV VP1[4]				
Seronegative to LPV VP1 polyoma	111/235	1 (reference)	63/106	1 (reference)
Seropositive to LPV VP1 polyoma	83/159	1.0 (0.7–1.6)	44/110	0.7 (0.4–1.3)
KI VP1[4]				
Seronegative to KI VP1 polyoma	17/30	1 (reference)	7/17	1 (reference)
Seropositive to KI VP1 polyoma	177/364	1.0 (0.5–2.1)	100/199	1.1 (0.4–3.6)
WU VP1[4]				
Seronegative to WU VP1 polyoma	2/3	1 (reference)	0/1	1 (reference)
Seropositive to WU VP1 polyoma	192/391	0.7 (0.1–5.1)	107/215	–

HPV: Human Papillomavirus; OR: Odds ratio; CI: Confidence interval.
[1]Conditional regression models were adjusted by CT serology, HHV-2 serology, marital status, education level, smoking habits, OC use and duration. For associations with cutaneous HPV types and Polyomaviruses, models were further adjusted by mucosal HPV L1 serology. See methods for a list matching of variables.
[2]Includes mucosal HPV types 11, 16, 18, 45, 31, 33, 35, 52, and 58.
[3]Includes cutaneous HPV types 1, 4, 8, and 77.
[4]Excludes Denmark and Sweden because these serological markers were not tested.
The number of cases and controls does not add up the total number because of missing values.
Bold font indicates statistical significance ($p < 0.05$).

completed, technical/professional school, secondary school, and university degree), marital status (single, married/cohabiting, divorced/separated, and widowed), smoking habits (never, past, and duration <15 years, past and duration ≥15 years, current, and intensity <10 cig/day, current and intensity ≥10 cig/day), and OC use and duration (never, past, and <10 years, past and ≥10 years, current and <10 years, current and ≥10 years). These variables were selected using a stepwise regression strategy that considered other potential confounders (Roura et al., unpublished data). Interactions or effect modification among serological variables were assessed using the likelihood ratio test. When applicable, variables included a missing or unknown category in order to avoid the exclusion of participants in the regression models. Statistical analyses were performed using the R programming language [R Development Core Team, 2005 (http://www.R-project.org)]. All statistical tests were two-tailed, and p values below 0.05 were considered statistically significant.

Results

Table 1 shows baseline characteristics for CC and precancer cases and their corresponding matched controls finally included in the nested case–control study. The majority of women with ICC and precancer were from Sweden (23.4 and 38.6%, respectively), Denmark (17.9 and 15.1%, respectively), the United Kingdom (9.2 and 18.4%, respectively), and Germany (12.5 and 11.5%, respectively). Younger women were more likely to be diagnosed with CIN3/CIS and older women with ICC. When compared with control women, CIN3/CIS and ICC cases were more likely at recruitment to be single or separated ($p < 0.001$) and current smokers ($p < 0.001$). The mean number of years between blood collection and disease diagnosis was 3.3 (5th–95th percentiles: 0.3–7.9) and 3.9 (0.3–8.9) for CIN3/CIS and ICC, respectively. Cases with CIN3/CIS were more likely than controls to be seropositive for mucosal HPV L1, CT, and HHV-2. Finally, cases with ICC were more likely than controls to be seropositive for mucosal HPV L1, E6, and E7, CT, and HHV-2.

Table 2 presents the results from multivariate analyses concerning associations between serological markers of mucosal HPV, cutaneous HPV, and human polyomaviruses and CIN3/CIS and ICC. L1 seropositivity to any mucosal HPV was associated with both CIN3/CIS and ICC. We found a statistically significant trend across the number of L1 seropositive HPV types (1, 2, and 3+) and risk of CIN3/CIS and ICC. HPV16/18 E6/E7 seropositivity was strongly associated with ICC, but not with CIN3/CIS. The strongest association for ICC was observed for seropositivity to E6 of HPV16 and/or 18 with a statistically significant OR of 7.4 (95% CI 2.8–19.7). In contrast, seropositivity to cutaneous HPV L1 or seropositivity to the four polyomaviruses (JCV, LPV, KIV, and WUV) was not associated with any of the two disease outcomes.

Table 3 shows associations between HPV type-specific serological markers and risk of CIN3/CIS and ICC. Only L1 serological markers of HPV11, 16, 18, and 45 were associated with the risk of CIN3/CIS. In contrast, for ICC the strongest association was found with HPV16 E6 serology (OR = 10.2, 95%CI 3.3–31.1). We could not assess in an adequate statistical manner whether the strength of this association was dependent on the follow-up time because virtually all (26 out of 27) HPV16 E6+ cases and none (0 out of 15) of the HPV16 E6+ controls were diagnosed (cases) or censored (controls) within the first tertile of total follow-up time (<6.7 years). It is interesting to note, however, that one CIN3/CIS case was positive 8.6 years before diagnosis, three cases (one CIN3/CIS and two ICC) 6–8 years before diagnosis, and four cases (one CIN3/CIS and three ICC) 4–6 years before diagnosis. In contrast, all 15 HPV16 E6+ controls were positive at least 7.5 years before censoring. HPV16 L1 and E7 seropositivity was also associated with ICC risk, but to a minor extent than HPV16 E6 serology. HPV11 was the only other type associated with ICC risk (OR = 3.0; 95% CI 1.3–7.0). None of the cutaneous types were associated with risk as assessed by L1 serology. All reported associations remained virtually the same when logistic regression models were not adjusted by CT and HHV-2 serostatus or when parity was added as an adjusting variable (data not shown).

Table 4 shows associations between CC risk and serological markers of CT, CP, and HHV-2. Previous exposure to CT was strongly associated with ICC (OR = 2.3; 95% CI 1.3–4.1), and to a lesser extent and marginally with CIN3/CIS (OR = 1.3; 95%CI 0.9–1.9). In contrast, detection of antibodies against CP, a nonsexually transmitted chlamydial species, was not associated with any of the two outcomes. HHV-2 seropositivity was marginally associated with CIN3/CIS (OR = 1.4; 95% CI 1.0–2.0) and with ICC (OR = 1.5; 95% CI 0.9–2.6).

Table 5 shows associations between combined serological results of HPV L1, CT, and HHV-2 and risk of CIN3/CIS and ICC. We assessed whether an increasing number of previous STIs, as indicated by serum antibodies, was associated with increasing risk. As shown in the table, we found a strong linear association between increasing number of past STIs and CIN3/CIS ($p = 0.01$), and with borderline statistical significance with ICC ($p = 0.08$). Thus, as compared to women that at recruitment tested negative for the three infections, women who tested positive to 1, 2, or 3 STIs had increasing ORs of 1.5, 2.2, and 2.4 for CIN3/CIS and of 2.0, 2.3, and 11.4 for ICC. The OR for ICC among women who tested positive for the three infections was much higher for ICC (OR = 11.4) than for CIN3/CIS (OR = 2.4). No statistically significant interactions were found between antibodies to HPV and antibodies to CT or HHV-2.

Exclusion of cases and corresponding controls that were diagnosed within one year after blood collection did not alter the reported associations shown in Tables 2, 3, 4, and 5.

Discussion

The EPIC cohort is one of the largest cohorts of women in Europe that allows for the prospective assessment of the risk

Table 3. Multivariate odds ratios for the association between HPV type-specific serological markers and risk of CIN3/CIS and ICC

	CIN2–3/CIS		ICC	
HPV type-specific serology	***N* cases/ *N* controls**	**OR[1] (95% CI)**	***N* cases/ *N* controls**	**OR[1] (95% CI)**
HPV11				
L1 seronegative to all mucosal types[2]	184/463	1 (reference)	76/212	1 (reference)
L1 seropositive (single or multiple)	101/148	**1.6 (1.1–2.3)**	50/63	**2.0 (1.2–3.6)**[4]
L1 seropositive (single only)	32/53	1.5 (0.9–2.5)	19/18	**3.0 (1.3–7.0)**[4]
HPV16				
L1 seronegative to all mucosal types[2]	184/463	1 (reference)	76/212	1 (reference)
L1 seropositive	128/151	**2.4 (1.7–3.4)**	63/70	**2.4 (1.4–4.1)**
E6 seronegative to HPV16 and 18	413/826	1 (reference)	160/360	1 (reference)
E6 seropositive to HPV16	7/10	1.6 (0.5–4.8)	20/5	**10.2 (3.3–31.1)**
E7 seronegative to HPV16 and 18	367/764	1 (reference)	153/339	1 (reference)
E7 seropositive to HPV16	49/71	1.3 (0.8–1.9)	25/27	**2.8 (1.5–5.5)**
E6 and E7 seronegative to HPV16 and 18	360/747	1 (reference)	137/334	1 (reference)
E6 and/or E7 seropositive to HPV16	54/80	1.3 (0.9–2.0)	39/29	**4.3 (2.3–7.9)**
E6 and E7 seropositive to HPV16	2/1	1.6 (0.1–21.0)	6/3	**6.4 (1.3–30.7)**
HPV18				
L1 seronegative to all mucosal types[2]	184/463	1 (reference)	76/212	1 (reference)
HPV18 L1 seropositive	60/87	**1.8 (1.2–2.7)**	29/44	1.5 (0.8–2.8)
E6 seronegative to HPV16 and 18	413/826	1 (reference)	160/360	1 (reference)
E6 seropositive to HPV18	5/11	1.2 (0.4–3.8)	5/4	3.2 (0.7–15.2)
E7 seronegative to HPV16 and 18	367/764	1 (reference)	153/339	1 (reference)
E7 seropositive to HPV18	8/12	1.8 (0.7–4.8)	5/3	2.4 (0.4–13.7)
E6 and E7 seronegative to HPV16 and 18	360/747	1 (reference)	137/334	1 (reference)
E6 and/or E7 seropositive to HPV18	12/22	1.4 (0.6–3.1)	10/7	**3.7 (1.1–12.4)**
E6 and E7 seropositive to HPV18	1/1	3.6 (0.2–76.3)	–	–
L1 seronegative to all mucosal types[2]	184/463	1 (reference)	76/212	1 (reference)
HPV31 L1 seropositive	44/76	1.5 (1.0–2.4)	22/36	1.6 (0.8–3.4)
HPV33 L1 seropositive	20/38	1.3 (0.7–2.4)	15/19	2.1 (0.9–4.9)
HPV35 L1 seropositive	56/111	1.3 (0.9–1.9)	23/46	1.2 (0.6–2.4)
HPV45 L1 seropositive	71/111	1.6 (1.1–2.3)	33/47	1.8 (1.0–3.3)
HPV52 L1 seropositive	34/61	1.4 (0.8–2.3)	21/24	2.1 (1.0–4.6)
HPV58 L1 seropositive	23/56	0.9 (0.5–1.7)	14/22	1.6 (0.7–3.8)
L1 seronegative to all cutaneous types[3]	181/404	1 (reference)	92/197	1 (reference)
HPV1 L1 seropositive	146/254	1.3 (1.0–1.8)	42/89	1.0 (0.6–1.6)
HPV4 L1 seropositive	145/248	1.3 (0.9–1.7)	51/106	1.1 (0.7–1.7)
HPV8 L1 seropositive	54/94	1.1 (0.7–1.6)	26/47	1.0 (0.5–1.9)
HPV77 L1 seropositive	46/80	1.3 (0.8–2.0)	11/38	0.4 (0.2–1.0)

HPV: Human Papillomavirus; OR: Odds ratio; CI: Confidence interval.
[1]Conditional regression models were adjusted by CT serology, HHV-2 serology, marital status, education level, smoking habits, OC use, and duration. For associations with cutaneous HPV types, models were further adjusted by mucosal HPV L1 serology; see "Methods" for a list matching of variables.
[2]Includes mucosal HPV types 11, 16, 18, 45, 31, 33, 35, 52, and 58. Includes single and multiple types.
[3]Includes cutaneous HPV types 1, 4, 8, and 77. Includes single and multiple types.
The number of cases and controls does not add up the total number because of missing values.
Bold font indicates statistical significance ($p < 0.05$).

Table 4. Multivariate odds ratios for the association between serological markers of *Chlamydia trachomatis* (CT), *Chlamydia pneumoniae* (CP), and Human Herpes Virus 2 (HHV-2) and risk of CIN3/CIS and ICC

	CIN3/CIS		ICC	
Risk factor	***N* cases/ *N* controls**	**OR[1] (95% CI)**	***N* cases/ *N* controls**	**OR[1] (95% CI)**
CT serology				
Seronegative	345/743	1 (reference)	142/338	1 (reference)
Seropositive	77/103	1.3 (0.9–1.9)	40/30	**2.3 (1.3–4.1)**
CP serology				
Seronegative	137/272	1 (reference)	63/132	1 (reference)
Seropositive	283/570	0.9 (0.7–1.2)	118/234	1.1 (0.7–1.7)
HHV–2 serology				
Seronegative	343/734	1 (reference)	138/319	1 (reference)
Seropositive	79/113	1.4 (1.0–2.0)	44/49	1.5 (0.9–2.6)

OR: Odds ratio; CI: Confidence interval.
[1]Conditional regression models were adjusted by mucosal HPV L1 serology, CT serology and HHV-2 serology when appropriate, and marital status, level education, smoking habits, OC use, and duration; see "Methods" for a list of matching variables.
The number of cases and controls does not add up the total number because of missing values.
Bold font indicates statistical significance ($p < 0.05$).

Table 5. Multivariate odds ratios for the association between concomitant serological status of mucosal HPV L1, *Chlamydia trachomatis* (CT) and Human Herpes Virus 2 (HHV-2) infections and risk of CIN3/CIS and ICC

			CIN3/CIS		ICC	
HPV L1 serology (mucosal types)	**CT serology**	**HHV-2 serology**	***N* cases/ *N* controls**	**OR[1] (95% CI)**	***N* cases/ *N* controls**	**OR[1] (95% CI)**
–	–	–	134/377	1 (reference)	52/182	1 (reference)
Seropositive to one infection			197/355	**1.5 (1.2–2.0)**	83/141	**2.0 (1.3–3.1)**
–	–	+	20/41	1.2 (0.6–2.2)	13/13	**3.2 (1.3–8.1)**
–	+	–	26/39	1.7 (1.0–3.1)	9/11	1.8 (0.6–5.0)
+	–	–	151/275	**1.6 (1.1–2.1)**	61/117	**1.8 (1.1–3.0)**
Seropositive to two infections			76/93	**2.2 (1.5–3.4)**	34/41	**2.3 (1.2–4.5)**
–	+	+	4/5	1.3 (0.3–5.8)	2/6	0.6 (0.1–4.0)
+	–	+	40/48	**2.7 (1.6–4.6)**	16/26	1.5 (0.6–3.5)
+	+	–	32/40	**2.0 (1.1–3.4)**	16/9	**5.2 (1.9–14.2)**
Seropositive to all three infections:			15/19	**2.4 (1.1–5.2)**	13/4	**11.4 (3.2–40.2)**
Trend across number of infections (1, 2, 3)				***0.01***		*0.08*

HPV: Human Papillomavirus; OR: Odds ratio; CI: Confidence interval.
[1]Conditional regression models were adjusted by marital status, level education, smoking habits, OC use and duration; see "Methods" for a list of matching variables.
The number of cases and controls does not add up the total number because of missing values.
Bold font indicates statistical significance ($p < 0.05$).

of CC and precancer in relation to past exposure to infections, such as HPV, CT, HHV-2, and polyomaviruses. The results derived from the nested case–control study within the EPIC cohort confirmed the expected important role of mucosal HPV types and identified a possible role of CT and HHV-2 in cervical carcinogenesis. In contrast, it also confirms the lack of associations with other infections that are not sexually transmitted such as cutaneous HPV, CP, and polyomaviruses.

When concerning HPV, we found positive associations with virtually all markers of mucosal HPV types including antibodies against L1 proteins of 9 types, and antibodies against E6/E7 proteins of types 16 and 18. In contrast, no associations were found with serological markers of cutaneous HPV types. The largest association was found with HPV16 E6 serology with regard to ICC risk with a statistically significant OR of 10. Other HPV types associated with ICC were HPV11 and HPV18. The association with HPV11 seropositivity and ICC risk was found even among subjects singly seropositive for HPV11 [*i.e.*, not concomitantly seropositive for other HPV types; OR = 3.0 (1.3–7.0)]. However, given the low sensitivity of the HPV serology assays this

finding should be cautiously interpreted, as we cannot rule out that these cases were indeed infected concomitantly or in the past by established oncogenic HPV types. For CIN3/CIS the largest association was found with HPV16 L1 serology with an OR of 2.4. Other HPV types associated with CIN3/CIS were HPV11, HPV18, and HPV45. These findings are largely consistent with those from several other prospective and retrospective studies associating serological markers of past HPV exposure with CC risk.[6,18–21] The contribution of our study with regard to HPV serology is, in addition to the large sample size and the prospective design, the use of a wide panel of type-specific HPV markers that includes L1, E6, and E7 proteins and a high number of HPV types: Nine mucosal and four cutaneous.

It is interesting to note that E6/E7 serology was exclusively associated with ICC whereas the L1 serology was associated with both ICC and CIN3/CIS. HPV L1 antibodies are regarded as markers of previous exposure to a mixed group of current and past infections and just in the subgroup of persons that seroconvert.[22,23] In contrast, E6/E7 antibodies are considered markers of current HPV-related malignancy.[12,21,24–28] It is thus relevant that HPV16 E6 serology is so strongly associated with ICC in asymptomatic women with detection of antibodies even up to 8.6 years before diagnosis. Other studies using other designs have also found associations between antibodies to HPV16/18 E6/E7 proteins and ICC showing however a range of generally low sensitivity values when compared with HPV DNA detection in the tumor.[21,24,25] Consistent with these results which concern cervical cancer, two recent studies, one using also the EPIC cohort[12] and the other one using a multicentric case–control design,[26] found strong associations between HPV16 E6 seropositivity and oropharyngeal cancer. In the EPIC study HPV16 E6 seropositivity was present more than 10 years before diagnosis of oropharyngeal cancer.

Past CT seropositivity was strongly associated with the risk of developing ICC (OR = 2.3, 95% CI 1.3–4.1). In contrast, moderate and borderline associations were found with CIN3/CIS. History of CT infection has also been associated with CC in several previous studies[2–4,6] and it is assumed it increases the probability that HPV infections will become persistent, increasing thus the risk of neoplasia.[29,30] In contrast, a large prospective study based on the placebo arms of two multinational clinical trials of HPV vaccination found a moderate association between CT PCR positivity and CIN2 but not with CIN3.[31]

Some studies have also reported HHV-2 as a co-factor[5] but the accumulated evidence is much less consistent and has not been confirmed in longitudinal studies.[6,32] It is likely that the increased cancer risk associated with these infections is, at least in part, the result of the inflammatory response which has been associated with the generation of free radicals and development of genetic instability.[33]

As with cutaneous HPV infections, and as expected, no associations were found between CC and precancer and detection of antibodies against CP or against polyomaviruses (both nonsexually transmitted infections). The recent discovery of new human polyomaviruses has brought new interest in assessing the potential role of this group of DNA tumor viruses in human carcinogenesis. However, only the Merkel cell polyomavirus has been linked to Merkel cell carcinoma and none of the others have been associated to cancer so far.[34–36]

We found that the risk of CIN3/CIS and ICC linearly increased with increasing number of STIs the woman had had, including HPV, CT, and HHV-2. We do not know whether exposure to CT and/or HHV-2 truly contribute with HPV in ICC causation, or different STIs tend to cluster because of the common transmission route (reflecting thus a high-risk sexual behavior by the subject or her sexual partners), or whether this increase in the risk of CC reflect a decreased immunologic response to clear STIs.

Our study has a number of limitations. Ascertainment of CIN3/CIS is less complete than ICC as it greatly depends on the population coverage of cervical screening programs in the EPIC countries, the degree of systematic reporting to the corresponding cancer registry, as well as the accuracy of the cytopathology records. All EPIC countries have in place some sort of cervical cancer screening programs with varying quality and population coverage. Unfortunately, the questionnaires used in EPIC did not include questions about screening practices limiting our ability to adjust by these. It is likely thus that in countries with limited coverage/quality of screening programs detection of CIN3/CIS cases would be limited and that some control women might indeed be cases. This "contamination" of the control group would result in an attenuation of true associations. In addition, not all cases of CIN3/CIS or ICC in the initial full EPIC cohort could be included in the nested case–control study, mostly due to the lack of sera availability. It is however unlikely that the availability of sera was related to a subject's serostatus or case–control status. Another limitation is that we could not take into account time-dependent information after recruitment, as there were no regular updates on any of the questionnaire variables or on the subsequent STI serostatus during follow-up. Thus, one would expect that some subjects might become exposed to one or several STIs after recruitment. This potential for nondifferential exposure misclassification (since it would be as likely to occur in potential cases as in controls) would have had underestimated the true association between STIs and CC risk. Another weakness of our study is the limitations imposed by the use of HPV L1 antibodies as markers of HPV infection. In contrast to the very good performance of serology to detect infection with CT and HHV-2, it is well known that not all women infected with HPV will eventually seroconvert. HPV L1 serology has a good specificity, but a limited sensitivity, and it has been estimated that between 50 and 70% of women with past HPV infections show seroconversion to HPV L1.[37] Hence, it is likely that an unknown proportion of women were misclassified as not having been

exposed to HPV; thus, leading to underestimate the association between these markers and risk of disease.[20] This problem makes it also difficult to compare the strength of the association of CIN3/CIS and ICC with HPV versus the other STIs considered in the present paper and for which the presented ORs are less prone to be decreased by lack of antibody detection. Seropositivity to the E6 oncogene of HPV16 showed by far the strongest association with ICC risk, in agreement with previous findings on ICC[21,24,25] and partly with cancer of the oropharynx.[12,26–28] However, although HPV16 is known to be involved in about 50% of ICC, HPV16 E6 antibodies were detected in only 11% of ICC pointing to the substantial lack of sensitivity not only of L1 serology, but also of E6 serology. In respect to HPV16 E7 antibodies, we confirm that they have substantially poorer specificity for ICC than HPV16 E6 antibodies also in agreement with findings from studies of oropharyngeal cancer.[12] It is worth noting that the E6 and E7 serology assays were only performed for HPV 16 and 18 types and not for the remaining high-risk types or the cutaneous types, and this might have resulted in differences.

By design, we were not able to test for HPV DNA type in the tumors, being somehow a limitation of the study, as type-specific associations would be expected to be even stronger if limited to cancers caused by the same type.

From the clinical point of view our findings add clinical relevance to the identification of women with a current or past history of STIs, such as CT or HHV-2. These women might be at a higher risk of HPV persistence and/or progression to cervical precancer and cancer and should be closely monitored in terms of cervical cancer screening.

In conclusion, this large prospective study in Europe confirms the important role of HPV and a possible contribution of CT and HHV-2 in cervical carcinogenesis. It further identifies HPV16 E6 seropositivity as the strongest marker to predict ICC well before disease development, and it corroborates the lack of role of polyomaviruses in the risk of developing CC and precancer.

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ARTICLE 2

Smoking as a major risk factor for cervical cancer and pre-cancer: results from the EPIC cohort.

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Smoking as a major risk factor for cervical cancer and pre-cancer: Results from the EPIC cohort

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Key words: cohort study, cervical cancer, smoking, Human Papillomavirus serology, EPIC

Abbreviations: CC: cervical cancer; CI: confidence interval; CIN3: cervical intraepithelial neoplasia of grade 3; CIS: carcinoma *in situ*; CT: *Chlamydia Trachomatis*; EPIC: European Prospective Investigation into Cancer and Nutrition; GST: glutathione *S*-transferase; HHV-2: Human Herpes Virus 2; HPV: human papillomavirus; ICC: invasive cervical cancer; HR: hazard ratio; IARC: International Agency for Research on Cancer; ICD-10: International Classification of Diseases, 10th revision; MFI: median fluorescence intensity; MIF: microimmunofluorescence; OC: oral contraceptive; OR: odds ratio; STI: sexually transmitted infection

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A total of 308,036 women were selected from the European Prospective Investigation into Cancer and Nutrition (EPIC) study to evaluate the association between tobacco smoking and the risk of cervical intraepithelial neoplasia of grade 3 (CIN3)/carcinoma *in situ* (CIS) and invasive cervical cancer (ICC). At baseline, participants completed a questionnaire and provided blood samples. During a mean follow-up time of 9 years, 261 ICC cases and 804 CIN3/CIS cases were reported. In a nested case–control study, the baseline sera from 609 cases and 1,218 matched controls were tested for L1 antibodies against HPV types 11, 16, 18, 31, 33, 35, 45, 52, 58, and antibodies against *Chlamydia trachomatis* (CT), and Human Herpes Virus 2 (HHV-2). Cervical samples were not available for HPV-DNA analysis in this study. Multivariate analyses were used to estimate associations between smoking and risk of CIN3/CIS and ICC in the cohort and the case–control studies. In the cohort analyses smoking status, duration and intensity showed a two-fold increased risk of CIN3/CIS and ICC, while time since quitting was associated with a two-fold reduced risk. In the nested case–control study, consistent associations were observed after adjustment for HPV, CT and HHV-2 serostatus, in both HPV seronegative and seropositive women. Results from this large prospective study confirm the role of tobacco smoking as an important risk factor for both CIN3/CIS and ICC, even after taking into account HPV exposure as determined by HPV serology. The strong beneficial effect of quitting smoking is an important finding that will further support public health policies for smoking cessation.

What's new?
Tobacco smoking is a cited cause of cervical cancer, but whether it causes cervical malignancy independent of human papillomavirus (HPV) infection is unclear. Here, strong associations were found between most measures of tobacco smoking and the risk of cervical intraepithelial neoplasia of grade 3/carcinoma in situ and invasive cervical cancer, after taking into account past exposure to HPV infection. Quitting smoking was associated with a 2-fold risk reduction. The findings confirm the role of tobacco smoking in cervical carcinogenesis and show that quitting the habit has important benefits for cancer protection.

It is well-established that persistent infection with high-risk HPV genotypes is the necessary cause of cervical cancer (CC) and its precursor lesions.[1,2] However, most infections usually clear within two years, and only a small proportion of infected women will progress to develop precancerous lesions and CC. Epidemiological studies have consistently found that exposure to tobacco smoking may influence the risk of progression from cervical HPV infection to cervical malignancy. In addition, in a comprehensive review of the existing evidence, the International Agency for Research on Cancer (IARC) classified tobacco smoking as a cause of CC.[3] However, the evidence of a causal association between tobacco smoking and CC, independently of HPV, is still unclear, as is mostly derived from retrospective case–control studies.[4] Thus, further evidence from large prospective cohort studies taking into account HPV exposure is still needed to confirm the role of smoking in cervical carcinogenesis.

The aim of this study is to evaluate the association between tobacco smoking and the risk of developing cervical intraepithelial neoplasia of grade 3 (CIN3) or carcinoma *in situ* (CIS) and invasive cervical cancer (ICC) in a large multicentre European prospective cohort study accounting for serological markers of HPV exposure and other (STIs) at recruitment. In the accompanying article we report primary associations between serology markers of HPV infection and other selected infections in the development of both CIN3/CIS and ICC (Castellsagué et al., this issue).

Material and Methods

The EPIC cohort study

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a large prospective cohort study composed of 521,448 participants, of whom 367,993 were women, mostly aged 35 to 70 years, recruited between 1992 and 2000 from 23 centres in 10 European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. The study procedures have been described in detail previously.[5] Briefly, most participants were recruited from the general population residing in a specific geographic area except in the French cohort, which was based on a health insurance scheme for school and university employees, the Utrecht (the Netherlands) and Florence (Italy) cohorts, which included women attending breast cancer screening programs, parts of the Italian and Spanish cohorts, which were recruited among blood donors, and the Oxford (England) cohort, which included vegetarian and health-conscious volunteers. All eligible subjects were invited to participate in the study, and those who accepted gave their written informed consent, completed medical and lifestyle questionnaires including smoking habits, and were invited for blood collection.

Follow-up and case ascertainment

Cases of CIN3/CIS and ICC among cohort members were identified using record linkage with population-based cancer registries in Denmark, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom. In France, Germany and Greece, a combination of methods was used, including linkage to health insurance records, hospital-based cancer and pathology registries, and active follow-up of subjects. Data on vital status were obtained from regional and national mortality registries. The study only included women with first primary incident cancers coded as C53 (cervix uteri) according to the International Classification of Diseases, Injuries and Causes of Death, 10th revision (ICD-10). Contrary to ICC, CIN3/CIS cases were not systematically collected and consistently reported by all cancer registries and centers in EPIC and are, therefore, fewer than expected. Countries with a nationwide population-based screening program (Italy, the United Kingdom, the Netherlands, Sweden, Denmark and Norway) tended to register more pre-invasive cases than those with a non population-based program (France, Spain, Greece and Germany). For each EPIC centre, time of follow-up was calculated between the date at recruitment and the date at diagnosis for cases or the date at censoring (death, loss of follow-up or end of follow-up) for non-cases. End of follow-up ranged from December 2003 to December 2006, depending on the center.

Study population

From the full EPIC cohort, we excluded men ($n = 153,455$) and women who had prevalent cancer ($n = 22,180$), incomplete follow-up ($n = 2,295$), hysterectomy ($n = 34,973$) or no full lifestyle questionnaire ($n = 509$). A total of 308,036 women were followed-up for a median of 9 years providing a total of 2,775,235 person-years. Among the 308,036 women included in the final analysis 1,065 cases were identified: 261 with ICC and 804 with CIN3/CIS.

Nested case–control study

In order to take into account HPV in the assessment of potential associations between tobacco smoking and the risk of CIN3/CIS and ICC, we conducted a nested case–control study within the EPIC cohort. Thus, for each case with available blood sample, two matched control subjects were randomly selected among all cancer-free cohort women who were at risk at the time of diagnosis of the corresponding case. Matching criteria included: study centre of enrolment, age at recruitment (five-year intervals), menopausal status (pre-, post-menopausal), follow-up time, date, time and fasting status at blood collection, and, among pre-menopausal women, phase of the menstrual cycle. A total of 609 cases (184 ICC and 425 CIN3/CIS) and 1,218 controls were included in the analyses.

Serological testing

HPV serology was performed at the German Cancer Research Centre in Heidelberg, Germany. Antibodies to the L1 capsid protein of high-risk mucosal HPV types 16, 18, 31, 33, 35, 45, 52, and 58, and of low-risk mucosal HPV type 11 were analyzed by glutathione *S*-transferase (GST) capture and fluorescent bead-based multiplex serology.[6] L1 proteins were expressed in *Escherichia coli* bacteria as fusion proteins with N-terminal GST and a C-terminal tag epitope as described previously.[7] Antibody reactivity was quantitatively expressed in Median Fluorescence Intensity (MFI) units, and MFI values were dichotomized as antibody-positive or antibody-negative, using seropositivity cutoffs which were HPV type-specific and defined previously.[8] For the nested case–control analyses, HPV L1 seropositivity refers to that for at least one of the nine HPV types analyzed, and high-risk HPV L1 seropositivity refers to positivity for at least one of the eight high-risk HPV types analyzed.

Serum antibodies against CT and HHV-2 were tested at the Hospital of Santa Creu i Sant Pau in Barcelona, Spain. Detection of IgG antibodies against CT were performed by microimmunofluorescence (MIF) using the commercial assay Chlamydia MIF IgG (Focus Diagnostics, Cypress, CA). Antibodies against HHV-2 were evaluated by an enzyme immunoassay (EIA) using the commercial kit HerpeSelect® 2 ELISA IgG (Focus Diagnostics, Cypress, CA). The positive results were confirmed by a membrane-based immunoassay with the biokit HSV-2 Rapid Test (Biokit USA, Lexington, MA).

All serological assays were performed blinded to the subject's case–control status.

Statistical analyses

Cox proportional hazard models were used to estimate hazard ratios (HR) and 95% confidence intervals (CI) for the association between tobacco smoking and CIN3/CIS and ICC in the full cohort analysis. Age was used as the underlying time scale in the Cox models. Multivariate models were stratified by age at recruitment (in one-year categories) and study centre. Tests based on weighted residuals were used to evaluate the proportional hazards assumption for the Cox regression models fit. Tests for linear trend were conducted by entering categorical variables as continuous terms in the models used.

Cox models were adjusted for potential confounders, including education level, used as a proxy for socioeconomic status (none, primary school completed, technical/professional school, secondary school, university degree), marital status (single, married/cohabiting, divorced/separated, widowed), body mass index (BMI, underweight ($<$18.5), normal (18.5–25), overweight (25–30), obese ($\geq$30)), physical activity (inactive, moderately inactive, moderately active, active using a validated Cambridge Physical Activity Index[9]), number of full-term pregnancies (0, 1, 2, 3, $\geq$4), and use and duration of oral contraceptives (never, past and $<$10 years, past and $\geq$10 years, current and $<$10 years, current and $\geq$10 years). These variables were selected using a stepwise regression strategy.

We evaluated different patterns of smoking use in the multivariate Cox regression models. Smoking status was defined using three categories: never, past, and current smoking. Among current smokers, duration, intensity and age at starting were assessed. Among past smokers, age at stopping smoking, and time since quitting were also analyzed. A new variable of total pack-years of smoking was created multiplying intensity (average lifetime number of cigarettes per day smoked/20) by duration of smoking (years). Some of the centers also collected information on filter use, cigarette inhalation and type of cigarettes (light versus normal). Other models were fitted in which duration and intensity of smoking were mutually adjusted for, and in which total pack-years was used as an adjusting covariate to reduce co-linearity among smoking-related variables.[10] Passive smoking variables were also evaluated among non-smokers using smoking habits of parents during childhood and environmental exposure at home and at work. A composite dichotomous variable coding ever *versus* never exposure to any kind of passive smoking was created and included in the analysis. This information was only available in 12 centres from 7 countries (France, Italy, the Netherlands, Germany, Sweden, Denmark, and Norway).

In the nested case–control analysis, multivariate odds ratios (OR) and 95% CI for the association between tobacco smoking and risk of CIN3/CIS and ICC were estimated using conditional logistic regression models adjusted for HPV L1, CT and HHV-2 serostatus and other potential confounding factors (education level, marital status, and oral contraceptive use and duration). Other factors assessed in the models but found to be not statistically significant were: parity, hormone replacement therapy, use of other contraception methods, BMI, and physical activity among others. Multiplicative interactions between smoking variables and other risk factors were evaluated. Separate unconditional logistic regression

analyses among HPV L1 seropositive and seronegative women were also performed, including using the matching variables to adjust the models. Multiplicative interactions between smoking variables and HPV L1 serostatus were assessed using the likelihood ratio test.

When applicable, variables included a missing or unknown category in order to avoid the exclusion of participants in the regression models. All statistical tests were two-tailed, and p values below 0.05 were considered statistically significant.

Statistical analyses were performed using the R programming language (R Development Core Team, 2005, http://www.R-project.org).

Results

Table 1 shows baseline characteristics for ICC cases, CIN3/CIS cases and non-cases included in the cohort analysis. Most women with CIN3/CIS were recruited from the United Kingdom (37.1%), Sweden (20.8%) and Norway (15.0%). The contribution of ICC cases across participating countries was more homogeneous (between 5.0% and 16.9%) than that for CIN3/CIS. Younger women were more likely to have been diagnosed with CIN3/CIS and older women with ICC. Compared to non-cases, CIN3/CIS cases were more likely to be single or separated, to have a high educational degree, to smoke, to be nulliparous, to have ever used oral contraceptives, and to be premenopausal, whereas ICC cases were more likely to be separated, to smoke, to have ever used oral contraceptives, and to be premenopausal.

Not all cases in the cohort could be included in the nested case–control analysis due to unavailability of blood samples. Thus 70% of ICC cases and 53% of CIN3/CIS cases were included in the nested case–control study. France, the United Kingdom and Norway provided proportionally fewer cases to the nested case–control study than to the full cohort. The CIN3/CIS cases selected for the nested case–control study were slightly older than those involved in the cohort (data not shown). Participation to the nested case–control study was not associated with any of the other characteristics evaluated such as marital status, years of education, smoking status, oral contraceptive use, number of full-term pregnancies or menopausal status.

All seroprevalence estimates for HPV L1, CT and HHV-2 in the nested case–control study were higher among cases than among control women (data not shown). In the accompanying article we report fully adjusted associations between a wide panel of HPV markers and risk of both CIN3/CIS and ICC (Castellsagué et al., unpublished data).

Table 2 presents multivariate HR for the association between smoking-related variables and risk of CIN3/CIS and ICC in the analysis of data from the full cohort. All measures of smoking were associated with both disease outcomes. The strongest associations were found for smoking status, duration, intensity, and pack-years, as well as with time since quitting the habit. We found statistically significant linear increases in the risk of disease with increasing years of smoking (for CIN3/CIS and borderline for ICC), smoking intensity (borderline for ICC), and pack-years of smoking (for CIN3/CIS and marginally for ICC). Age at starting smoking did not show any significant linear association among ever smokers. Regarding quitting, as compared to women who were current smokers, increasing years since stopping the habit was associated with a reduced risk of both CIN3/CIS and ICC. For CIN3/CIS the risk reduction was statistically significant after quitting the habit for at least 10 years as opposed to at least 20 years for ICC. Similarly, ex-smokers who quit for at least 20 years reduced their risk of CIN3/CIS or ICC to that among non-smokers (OR of 1.0 for CIN3/CIS and OR of 0.8 for ICC as compared to never smokers; data not shown). We also explored the effect of other smoking related variables such as filter use, inhalation, and type of tobacco (light versus normal), and found that none of them was statistically significantly associated with risk (data not shown). In a model restricted to smokers, and mutually adjusted for either smoking intensity and duration or for pack-years, no differences were observed in the risk estimates according to duration, intensity, age at starting and time since quitting (data not shown). Furthermore, years of smoking appeared to be more strongly associated with CIN3/CIS and ICC risk than number of cigarettes smoked. The analyses stratified by histological type showed strong associations of the main smoking related variables with squamous cell carcinoma but not with adenocarcinoma. The number of cases of the latter however was small ($n = 52$; data not shown).

Table 3 shows associations between passive smoking and risk of CIN3/CIS and ICC among all women in the cohort who never smoked. None of the six measures of passive smoking were associated with risk of developing CIN3/CIS or ICC as compared to never smokers not exposed to passive smoking.

Table 4 shows the multivariate ORs for the association between smoking-related variables and CIN3/CIS and ICC risk as derived from the nested case–control study. The main contribution of these models is that they are additionally adjusted by serological markers of HPV L1, CT and HHV-2 infections, which may be considered proxy markers of sexual behavior, an aspect we did not enquire about in the questionnaire. Adjusted OR and 95% CI for CIN3/CIS and ICC risk were respectively: 1.8 (1.1–2.7) and 1.6 (1.2–2.0) for L1 seropositivity to any HPV type, 2.3 (1.3–4.1) and 1.3 (0.9–1.9) for CT seropositivity, and 1.5 (0.9–2.6) and 1.4 (1.0–2.0) for HHV-2 seropositivity (Castellsagué et al, unpublished data). Globally, the results of the nested case–control analyses concerning smoking are consistent with those derived from the full cohort. Thus, smoking status, duration, intensity, pack-years, and, to a lesser extent and inversely years since quitting, were all associated with CIN3/CIS and ICC risk. A difference with regard to the cohort analyses is that none of the tests for trend with duration, intensity and quitting reached statistical significance. In both analyses, women who stopped

Table 1. Baseline characteristics of cases and non-cases in the cohort study

Characteristics of subjects in the cohort study	Non-cases ($n = 306,971$), *N* (%)	Cases	
		CIN3/CIS ($n = 804$), *N* (%)	ICC ($n = 261$), *N* (%)
Country			
France	62,479 (20.4)	51 (6.3)	30 (11.5)
Italy	28,279 (9.2)	22 (2.7)	18 (6.9)
Spain	23,190 (7.6)	22 (2.7)	23 (8.8)
United Kingdom	48,975 (16.0)	298 (37.1)	35 (13.4)
The Netherlands	22,427 (7.3)	3 (0.4)	19 (7.3)
Greece	14,031 (4.6)	4 (0.5)	13 (5.0)
Germany	24,148 (7.9)	52 (6.5)	25 (9.6)
Sweden	26,709 (8.7)	167 (20.8)	44 (16.9)
Denmark	24,893 (8.1)	64 (8.0)	33 (12.6)
Norway	31,840 (10.4)	112 (15.0)	21 (8.0)
Age at recruitment (yrs)			
<30	10,216 (3.3)	125 (15.5)	9 (3.4)
30–39	31,383 (10.2)	180 (22.4)	32 (12.3)
40–49	103,483 (33.7)	275 (34.2)	95 (36.4)
50–59	109,403 (35.6)	186 (23.1)	85 (32.6)
≥60	52,486 (17.1)	38 (4.7)	40 (15.3)
Mean age (5^{th}–95^{th} percentile)	50.3 (32.5–65.8)	42.4 (24.4–59.8)	49.3 (31.4–64.8)
Marital status			
Single	28,807 (11.6)	141 (21.0)	27 (13.8)
Married/living together	195,074 (78.3)	426 (63.6)	133 (68.2)
Divorced/separated	14,674 (5.9)	93 (13.9)	31 (15.9)
Widowed	10,559 (4.2)	10 (1.5)	4 (2.1)
Missing[1]	57,857	134	66
Highest school level			
None	12,664 (4.3)	4 (0.5)	16 (6.3)
Primary school	65,466 (22.2)	110 (14.4)	59 (23.4)
Technical/professional school	65,144 (22.1)	241 (31.5)	60 (23.8)
Secondary school	79,677 (27.1)	201 (26.3)	71 (28.2)
University degree	71,458 (24.3)	208 (27.2)	46 (18.3)
Missing[1]	15,562	40	9
Smoking status			
Never smokers	170,511 (57.0)	325 (41.0)	108 (41.7)
Past smokers	68,524 (22.9)	198 (25.0)	66 (25.5)
Current smokers	60,278 (20.1)	270 (34.0)	85 (32.8)
Missing[1]	7,658	11	2
No. full-term pregnancies			
0	45,228 (16.0)	191 (27.7)	45 (19.9)
1	44,007 (15.6)	116 (16.8)	36 (15.9)
2	114,956 (40.8)	237 (34.4)	82 (36.3)
3	54,107 (19.2)	94 (13.6)	40 (17.7)
≥4	23,545 (7.7)	51 (7.4)	23 (10.2)
Missing[1]	25,128	115	35

Table 1. Baseline characteristics of cases and non-cases in the cohort study (Continued)

Characteristics of subjects in the cohort study	Non-cases ($n = 306{,}971$), *N* (%)	Cases	
		CIN3/CIS ($n = 804$), *N* (%)	ICC ($n = 261$), *N* (%)
OC use			
Never users	121,117 (41.6)	169 (23.9)	76 (32.8)
Past users	152,658 (52.4)	411 (58.1)	134 (57.8)
Current users	17,384 (6.0)	127 (18.0)	22 (9.5)
Missing[1]	15,812	97	29
Menopausal status			
Premenopausal	116,583 (38.0)	490 (60.9)	117 (44.8)
Postmenopausal	137,293 (44.7)	191 (23.8)	102 (39.1)
Perimenopausal	53,095 (17.3)	123 (15.3)	42 (16.1)

[1]Not included in the percentage distribution of the variable.
Abbreviations: CIN 3: cervical intraepithelial neoplasia grade 3, CIS: carcinoma *in situ*, ICC: invasive cervical cancer, OC: oral contraceptive.

Table 2. Multivariate hazard ratios for the association between smoking-related variables and risk of CIN3/CIS and ICC in the cohort study

			CIN3/CIS			ICC		
Risk factor	Person-years	Non-cases	Cases	HR[1]	95% CI	Cases	HR[1]	95% CI
Smoking status								
Never	1,581,045	170,511	325	1.0	(ref)	108	1.0	(ref)
Ever	1,125,354	128,802	468	**1.8**	**1.5–2.0**	151	**1.7**	**1.3–2.2**
Past	608,927	68,524	198	**1.5**	**1.2–1.8**	66	**1.5**	**1.1–2.1**
Current	516,427	60,278	270	**2.1**	**1.8–2.5**	85	**1.9**	**1.4–2.5**
Age started smoking (yrs)								
Never	1,581,045	170,511	325	1.0	(ref)	108	1.0	(ref)
≥19	402,947	45,340	123	**1.6**	**1.3–2.0**	56	**1.7**	**1.2–2.3**
16–18	535,170	62,787	233	**1.8**	**1.5–2.1**	61	**1.5**	**1.1–2.1**
≤15	138,463	15,768	91	**1.9**	**1.5–2.4**	22	1.5	1.0–2.5
p for trend among ever smokers				0.2			0.9	
Smoking duration (yr)								
Never	1,581,045	170,511	325	1.0	(ref)	108	1.0	(ref)
<10	189,192	22,018	81	1.3	1.0–1.6	17	1.2	0.7–2.0
10–19	267,091	29,912	133	**1.8**	**1.5–2.3**	31	1.4	0.9–2.2
20–29	314,903	36,287	129	**2.0**	**1.6–2.5**	42	**1.5**	**1.0–2.2**
≥30	287,477	33,675	99	**2.3**	**1.8–3.0**	47	**2.1**	**1.4–3.1**
p for trend among ever smokers				**<0.001**			**0.08**	
Lifetime smoking intensity (cig/day)[2]								
Never	958,393	115,076	223	1.0	(ref)	74	1.0	(ref)
<10	315,724	38,788	113	**1.7**	**1.4–2.2**	34	1.4	0.9–2.1
10–19	298,539	38,054	149	**1.9**	**1.6–2.4**	50	**1.9**	**1.3–2.9**
≥20	91,056	11,085	52	**2.1**	**1.5–2.8**	16	**1.9**	**1.1–3.4**
p for trend among ever smokers				0.2			**0.07**	
Smoking pack-years[2]								
Never	958,393	115,076	223	1.0	(ref)	74	1.0	(ref)
<10	339,376	41,784	148	**1.7**	**1.4–2.1**	39	1.5	1.0–2.2
10–19	209,899	26,664	91	**1.8**	**1.4–2.4**	29	**1.7**	**1.1–2.7**

Table 2. Multivariate hazard ratios for the association between smoking-related variables and risk of CIN3/CIS and ICC in the cohort study (Continued)

			CIN3/CIS			ICC		
Risk factor	**Person-years**	**Non-cases**	**Cases**	**HR[1]**	**95% CI**	**Cases**	**HR[1]**	**95% CI**
≥20	156,044	19,479	75	**2.8**	**2.1–3.7**	32	**2.2**	**1.4–3.4**
p for trend among ever smokers				**0.001**			**0.07**	
Smoking intensity at recruitment (cig/day)								
Never	1,581,045	170,511	325	1.0	(ref)	108	1.0	(ref)
<10	134,744	15,206	62	**2.0**	**1.5–2.6**	18	1.7	1.0–2.8
10–19	210,339	25,080	103	**1.8**	**1.4–2.2**	30	**1.6**	**1.0–2.4**
≥20	150,312	17,700	90	**2.5**	**2.0–3.2**	36	**2.5**	**1.7–3.8**
p for trend among current smokers				0.1			0.06	
Time since quitting smoking (yrs)[3]								
Current smokers	516,427	60,278	270	1.0	(ref)	85	1.0	(ref)
≤4	111,304	12,663	60	0.8	0.6–1.1	18	1.2	0.7–2.0
5–9	101,317	11,535	54	1.0	0.7–1.3	13	0.9	0.5–1.7
10–19	191,385	21,141	46	**0.5**	**0.4–0.8**	21	0.8	0.5–1.3
≥20	180,295	20,387	28	**0.5**	**0.3–0.7**	10	**0.4**	**0.2–0.8**
p for trend among past smokers				**0.02**			**0.02**	

The number of cases does not add up the total number of cases because of missing values. Bold font indicates a statistically significant effect ($p < 0.05$).
[1]Cox regression models were adjusted for body mass index, marital status, education level, physical activity, number of full-term pregnancies and OC use and duration.
[2]Excludes France and Sweden because information was not collected for this variable.
[3]Excludes never smokers.
Abbreviations: CIN 3: cervical intraepithelial neoplasia grade 3, CIS: carcinoma in situ, ICC: invasive cervical cancer, HR: hazard ratio, CI: confidence interval, ref: reference.

smoking 20 or more years before recruitment showed a reduced risk of disease, but it did not reach statistical significance (OR = 0.5, 95% CI: 0.2–1.1, for CIN3/CIS; OR = 0.3, 95% CI: 0.1–1.2, for ICC cases). Similarly, quitting for at least 20 years was associated with a similar risk of disease to that of never smokers (OR of 0.9 for CIN3/CIS and OR of 0.8 for ICC; data not shown). Similar results were obtained when the analyses were adjusted for L1 antibodies to high-risk HPV types (data not shown). No statistically significant interactions were found between smoking characteristics and other potential risk factors.

Table 5 shows associations between tobacco smoking and risk of CIN3/CIS and ICC, stratified by HPV L1 serostatus in women included in the nested case–control study. For ICC, HPV seronegative *versus* seropositive women showed similar associations with smoking status, duration, intensity, pack-years and time since quitting. In contrast, for CIN3/CIS, for smoking status, duration, intensity and pack-years, while higher smoking exposures were associated with higher risks for both serology groups, lower smoking exposures were associated with increased risk only among seropositive women. In fact, we found statistically significant synergistic interactions for smoking status ($p = 0.005$) and smoking duration ($p < 0.001$) with HPV L1 serostatus. The risk reduction for CIN3/CIS linked to quitting was stronger in the seronegative group (test for interaction: $p = 0.02$). Consistent results but with slightly stronger associations were obtained when the analyses were stratified by high-risk HPV L1 serostatus or by HPV16 L1 serostatus (data not shown).

Discussion

Our longitudinal study is one of the largest multinational cohorts of women that allows for the prospective assessment of the role of tobacco smoking on the risk of developing ICC and pre-cancer. The results consistently indicate that tobacco smoking is a major risk factor for both CIN3/CIS and ICC. Thus, we found a positive association with past and current smoking and a statistically significant linear association with increasing years of smoking and increasing number of cigarettes smoked per day. We also found that, as compared to women who continued smoking, women who stopped the habit for at least 10 years had half the risk of developing CIN3/CIS and ICC. Passive smoking was not associated with increased risk of CIN3/CIS and ICC among never smokers. In the nested case–control study, allowing for additional adjustment for serological markers of HPV L1, CT and HHV-2, the findings were largely consistent with those found for all women in the cohort.

Almost all published epidemiological studies have found positive associations between smoking and CC risk, but most

Table 3. Multivariate hazard ratios for the association between passive smoking and risk of CIN3/CIS and ICC among never smokers in the cohort study

		CIN3/CIS			ICC		
Risk factor	**Non-cases**	**Cases**	**HR[1]**	**95% CI**	**Cases**	**HR[1]**	**95% CI**
Parents smoked in your childhood[2]							
No	27,719	37	1.0	(ref)	14	1.0	(ref)
Yes	56,197	62	0.74	0.48–1.12	26	0.85	0.43–1.66
Missing	5,855	9	–		1	–	
Spent time where smoking in childhood[3]							
Never	11,446	8	1.0	(ref)	3	1.0	(ref)
Seldom	19,037	13	1.01	0.40–2.52	7	1.63	0.40–6.64
Few times during a week	8,859	6	0.96	0.33–2.81	2	1.02	0.17–6.31
Daily (for few/many hours)	9,743	4	0.63	0.18–2.19	5	3.06	0.68–13.67
Missing	7,220	9	–		2	–	
Someone regularly smoke at home/work[4]							
No	35,006	34	1.0	(ref)	19	1.0	(ref)
Yes	47,093	57	1.05	0.66–1.66	25	1.06	0.55–2.04
Missing	15,008	24	–		6	–	
Someone regularly smoke at home[5]							
No	34,674	40	1.0	(ref)	19	1.0	(ref)
Yes	16,609	28	1.54	0.92–2.59	7	0.64	0.26–1.60
Missing	35,755	38	–		16	–	
Someone regularly smoke at work[6]							
No	6,342	9	1.0	(ref)	3	1.0	(ref)
Yes	34,306	41	0.88	0.42–1.85	20	1.13	0.33–3.93
Missing	37,249	30	–		16	–	
Passive smoking[7]							
No exposure	16,516	14	1.0	(ref)	7	1.0	(ref)
Any exposure	71,276	89	1.58	0.88–2.82	35	0.98	0.42–2.30
Missing	6,075	5	–		4	–	

The number of cases does not add up the total number of cases because of missing values.
[1]Model adjusted for body mass index, marital status, education level, physical activity, number of full-term pregnancies and OC use and duration.
[2]Excludes Spain, United Kingdom, Bilthoven, Greece, Germany and Umea because data was not collected.
[3]Includes only France and Italy because data was not collected for the other countries.
[4]Excludes Spain, United Kingdom, Utrecht, Greece, Heidelberg and Umea because data was not collected.
[5]Excludes Naples, Spain, United Kingdom, Utrecht, Greece, Germany and Umea because data was not collected.
[6]Excludes Spain, United Kingdom, Utrecht, Greece, Germany, Umea and Norway because data was not collected.
[7]Excludes Spain, United Kingdom, Greece, Germany and Umea because data was not collected.
Abbreviations: HR: hazard ratio, CI: confidence interval.

of them used case–control designs.[4] A few of these studies evaluated the association restricting their analysis to HPV DNA positive women[4,11–16] or used serological markers of HPV to account for past HPV exposure,[17–22] and only a few used a prospective study design.[11,23–25]

An increased risk between current smoking and cervical cancer has been reported in most previous studies,[4,26–29] either with CIN3/CIS or with ICC. In contrast, a significant association with former smoking has been reported in fewer of them.[4,27,28] In agreement with the largest pooled analysis ever done on the topic,[4] our results, derived from both the cohort and the nested case–control studies, are consistent with the evidence that current smokers are at higher risk of developing CIN3/CIS and ICC than women who used to smoke but quit the habit.

Most studies have also found significant associations with other measures of smoking habits such as intensity and duration of smoking.[4,26–28] In our analyses, and consistent with results from previous studies, the magnitudes of these risks were quite similar, around twofold higher as compared with non-smokers even after adjustment for HPV antibodies.

We also found that quitting the habit for long enough substantially reduced the risk of cervical cancer and pre-

Table 4. Multivariate odds ratios for the association between smoking-related variables and risk of CIN3/CIS and ICC in the nested case–control study

	CIN3/CIS			ICC		
Risk factor	***N* cases/*N* controls**	**OR[1]**	**95% CI**	***N* cases/*N* controls**	**OR[1]**	**95% CI**
Smoking status						
Never	160/440	1.0	(ref)	76/197	1.0	(ref)
Ever	259/398	**1.7**	**1.3–2.2**	108/166	**1.8**	**1.1–2.8**
Past	96/212	1.1	0.8–1.6	45/82	1.6	0.9–2.7
Current	163/186	**2.4**	**1.7–3.2**	63/84	**2.0**	**1.2–3.2**
Age at started smoking (yr)						
Never	160/440	1.0	(ref)	76/197	1.0	(ref)
≥19	95/165	**1.5**	**1.0–2.1**	43/67	**1.8**	**1.0–3.1**
16–18	105/150	**1.8**	**1.3–2.5**	40/66	**1.8**	**1.0–3.2**
≤15	55/73	**1.9**	**1.2–2.9**	18/30	1.6	0.7–3.2
p for trend among ever smokers		0.7			0.8	
Smoking duration (yrs)						
Never	160/440	1.0	(ref)	76/197	1.0	(ref)
<10	29/79	1.0	0.6–1.6	12/20	1.9	0.8–4.6
10–19	78/110	**1.8**	**1.2–2.6**	22/46	1.3	0.7–2.7
20–29	72/94	**2.1**	**1.4–3.2**	30/45	1.5	0.8–2.9
≥30	74/95	**2.1**	**1.4–3.1**	36/50	**2.3**	**1.2–4.6**
p for trend among ever smokers		0.1			0.6	
Lifetime smoking intensity (cig/day)[2]						
Never	88/260	1.0	(ref)	57/148	1.0	(ref)
<10	53/84	**1.7**	**1.1–2.8**	26/54	1.4	0.7–2.9
10–19	76/105	**2.1**	**1.4–3.3**	35/51	**2.3**	**1.1–4.5**
≥20	23/26	**2.5**	**1.3–4.8**	12/12	**3.4**	**1.2–9.8**
p for trend among ever smokers		0.3			0.3	
Smoking pack-years[2]						
Never	88/260	1.0	(ref)	57/148	1.0	(ref)
<10	64/103	**1.7**	**1.1–2.6**	28/54	1.6	0.8–3.2
10–19	39/60	**1.8**	**1.1–3.0**	22/32	2.0	0.9–4.5
≥20	49/52	**2.9**	**1.7–4.9**	23/31	**2.7**	**1.2–6.1**
p for trend among ever smokers		*0.1*			*0.1*	
Time since quitting smoking (yrs)[3]						
Current smokers	163/186	1.0	(ref)	63/84	1.0	(ref)
≤4	27/48	0.6	0.3–1.4	13/15	2.0	0.6–6.4
5–9	23/36	0.6	0.3–1.4	10/14	0.5	0.1–1.9
10–19	29/69	0.7	0.3–1.3	14/30	0.6	0.2–1.8
≥20	14/48	0.5	0.2–1.1	7/19	0.3	0.1–1.2
p for trend among past smokers		0.2			0.1	

The number of cases and controls does not add up the total number because of missing values. Bold font indicates a statistically significant effect ($p < 0.05$).
[1]Conditional regression models were adjusted for HPV L1 serology, *Chlamydia trachomatis* serology, Human Herpes Virus 2 serology, marital status, education level, OC use and duration. See methods for a list of matching variables.
[2]Excludes France and Sweden because information was not collected for this variable.
[3]Excludes never smokers.
Abbreviations: CIN 3: cervical intraepithelial neoplasia grade 3, CIS: carcinoma in situ, ICC: invasive cervical cancer, OR: Odds Ratio, CI: confidence interval, ref: reference.

Table 5. Multivariate odds ratios for the association between smoking-related variables and CIN3/CIS and ICC risk among HPV L1 seronegative and seropositive women in the nested case–control study

	CIN3/CIS						ICC					
	Among HPV L1 seronegative women			Among HPV L1 seropositive women			Among HPV L1 seronegative women			Among HPV L1 seropositive women		
Risk factor	*N* cases/ *N* controls (184/463)	OR[1]	95% CI	*N* cases/ *N* controls (238/382)	OR[1]	95% CI	*N* cases/ *N* controls (76/212)	OR[1]	95% CI	*N* cases/ *N* controls (106/156)	OR[1]	95% CI
Smoking status												
Never	76/240	1.0	(ref)	83/200	1.0	(ref)	31/116	1.0	(ref)	43/81	1.0	(ref)
Ever	104/214	1.3	0.9-2.0	153/179	**1.9**	**1.3-2.7**	45/94	**1.9**	**1.0–3.5**	63/72	1.5	0.8-2.7
Past	30/124	0.7	0.4-1.1	66/87	**1.7**	**1.1–2.6**	17/40	1.9	0.9-4.1	28/42	1.1	0.5-2.3
Current	74/90	**2.3**	**1.5-3.5**	87/92	**2.1**	**1.4-3.2**	28/54	1.9	0.9-4.0	35/30	2.0	1.0–4.2
Smoking duration (yrs)												
Never	76/240	1.0	(ref)	83/200	1.0	(ref)	31/116	1.0	(ref)	43/81	1.0	(ref)
<10	9/44	0.6	0.3-1.3	20/34	1.4	0.7-2.7	4/9	2.0	0.5–7.9	8/11	1.8	0.6-5.7
10–20	22/68	0.9	0.5-1.5	55/42	**2.8**	**1.7-4.7**	10/24	1.7	0.6-4.4	12/22	0.6	0.2-1.6
20–29	33/44	**2.4**	**1.4-4.2**	38/48	**1.7**	**1.0–3.0**	13/29	1.7	0.7-4.0	17/16	1.4	0.6-3.6
≥30	37/44	**2.7**	**1.5-5.1**	37/49	1.7	0.9-2.9	15/28	**2.6**	**1.0–6.6**	21/22	2.3	1.0–5.4
p for trend among ever smokers		**<0.001**			0.5			0.9			*0.1*	
Lifetime smoking intensity (cig/day)[2]												
Never	40/134	1.0	(ref)	47/126	1.0	(ref)	22/88	1.0	(ref)	34/60	1.0	(ref)
<10	18/42	1.3	0.6-2.6	35/42	**2.3**	**1.2–4.2**	10/29	1.7	0.6-4.9	16/25	1.0	0.4-2.6
10–19	30/53	1.6	0.9-3.0	46/50	**2.6**	**1.5-4.6**	12/28	1.8	0.6-5.0	23/23	1.9	0.8-4.8
≥20	10/16	2.1	0.8-5.5	13/10	**2.6**	**1.0–7.0**	5/6	**4.5**	**1.1–19.3**	7/6	3.0	0.7–14.2
p for trend among ever smokers		0.4			0.4			0.4			**0.04**	
Smoking pack-years[2]												
Never	40/134	1.0	(ref)	47/126	1.0	(ref)	22/88	1.0	(ref)	34/60	1.0	(ref)
<10	19/57	1.0	0.5-1.9	45/46	**2.7**	**1.5-4.8**	10/29	1.8	0.6-5.0	18/25	1.2	0.5-3.1
10–19	18/33	1.6	0.7-3.3	21/27	**2.1**	**1.0–4.4**	7/17	1.5	0.5-5.2	15/15	1.8	0.6-5.4
≥20	21/21	**3.6**	**1.6–8.2**	28/29	**2.4**	**1.2–4.9**	10/17	3.1	1.0–10.1	13/14	1.8	0.6-5.4
p for trend among ever smokers		**0.006**			0.2			0.6			0.3	
Time since quitting smoking (years)[3]												
Current smokers	74/90	1.0	(ref)	87/92	1.0	(ref)	28/54	1.0	(ref)	35/30	1.0	(ref)
≤4	12/30	0.5	0.2-1.0	15/18	0.8	0.4-1.9	8/9	3.1	0.8–11.6	5/6	0.6	0.1–3.0
5–9	5/19	**0.3**	**0.1-0.8**	18/17	0.8	0.4-1.9	2/7	1.0	0.2–7.0	8/7	0.9	0.2–3.2
10–19	7/36	**0.2**	**0.1-0.6**	22/32	0.6	0.3-1.2	5/14	0.7	0.1–3.4	9/16	0.6	0.2-2.1
≥20	4/31	**0.1**	**0.04-0.4**	10/17	0.7	0.3-1.9	2/7	0.9	0.1–7.2	5/12	0.4	0.1-1.5
p for trend among ever smokers		**0.04**			0.6			0.1			0.9	

The number of cases and controls does not add up the total number because of missing values. Bold font indicates a statistically significant effect ($p < 0.05$).

[1]Unconditional regression models were adjusted by age, country, menopausal status, *Chlamydia trachomatis* serology, Human Herpes Virus 2 serology, marital status, education level, OC use and duration.

[2]Excludes France and Sweden because information was not collected for this variable.

[3]Excludes never smokers.

Abbreviations: CIN: cervical intraepithelial neoplaisa; CIS: carcinoma *in situ*; OR: odds ratio; CI: confidence interval; ref: reference.

cancer. Thus, women who had stopped smoking for long periods of time (20 or more years) showed a statistically significant twofold decreased risk of disease as compared with current smokers, even after adjustment for HPV seropositivity. Similarly, no excess risk was observed among those women as compared with never smokers. In contrast, recent quitters (<10 years) showed a similar risk to that of women who were current smokers. In agreement with these results, the IARC pooled analysis[4] also showed that women who had stopped smoking for less than four years still had increased risks of CIN3/CIS and ICC as compared with non-smokers, although the trends in the risk reductions were not statistically significant (p for trend = 0.5 and 0.6, respectively). Similar results were obtained in another prospective study conducted in Sweden[30] between time since quitting smoking and CIS. Shields *et al.*[19] found a non-significant reduced risk of ICC in women who had quit the habit for more than 15 years as compared with women who had stopped smoking for less than five years. Taken together, these findings are of great importance from a public health perspective, as smoking is a modifiable risk factor that if successfully reduced may substantially decrease the risk of CC.

We did not find any association between passive smoking and CC risk. However, not all countries in the study contributed data on passive smoking, and those that did frequently had incomplete information. Our results are consistent with those from a recent study conducted by Louie *et al.*[31] concluding that passive smoking could not be detected as an independent risk factor of ICC in the absence of active smoking. However, in a prospective study performed in the US, Trimble *et al.* found an increased risk of cervical neoplasia among passive smokers living with smokers.[25] Furthermore, in a meta-analysis published in 2012, Zeng *et al.* showed that women who never smoked but were exposed to smoking had a 2.8-fold increased risk of CC as compared with non-exposed (OR = 2.8, 95% CI: 1.9–4.2).[32] The assessment of passive smoking is challenging as it is usually difficult to rule out residual confounding, mainly because it is possible that the smoking habits of a woman's male partner are correlated with his sexual behavior and, hence, to the probability of HPV transmission within the couple.

In the nested case–control study we were able to include, and thus stratify by or adjust for, serological markers of exposure to HPV L1, CT and HHV-2. This was especially important as serostatus for HPV L1, CT and HHV-2 may be regarded as markers of sexual behavior, an aspect that was poorly or not consistently evaluated in the EPIC questionnaires. Since smoking is associated with both high-risk sexual habits and current HPV infection,[33] it was important to assess associations with smoking after at least partially adjusting for sexual behavior. These analyses showed similar patterns of statistically significant associations between smoking factors and CIN3/CIS and ICC risk to those found in the full cohort analyses. The fact that the significant excess risk of CC associated with smoking was not reduced by adjustment for HPV L1 serostatus suggests, as other studies did,[3,12,21,22,34] that smoking may indeed have an independent role in cervical carcinogenesis. In contrast, two retrospective case–control studies and two pooled analyses suggested that this association may be due to residual confounding by HPV or sexual behavior as a surrogate of HPV exposure.[4,13,18,20]

We were able to explore these potential associations stratifying by HPV serostatus as a proxy measure of past/present, i.e. cumulative, HPV exposure. We found comparable associations with CIN3/CIS and ICC risk in the two serology groups, especially in the higher categories of smoking intensity and duration. However, it is interesting to note that an effect of smoking for less than 20 years for CIN3/CIS risk was observed among HPV seropositive women but not among seronegative women (Table 5). A possible interpretation is that in the absence of HPV exposure smoking is a long-term risk factor for pre-invasive cancer; however, in the presence of HPV exposure, smoking may require less time to induce CIN3/CIS, suggesting that HPV and smoking duration have a synergistic interaction. Consistent with this, quitting smoking for at least 5 years significantly reduced the risk of CIN3/CIS only among HPV seronegative women, suggesting that quitting smoking is less protective in women who have serological evidence of HPV exposure that in those that do not as measured by our serological assay. Other studies have assessed the interaction between HPV serology and smoking on the risk of CC. A study in Norway found a non-significant additive interaction between HPV16 antibodies and smoking status on the risk of developing CIN2–3.[20] Other studies conducted in the US and Sweden did not find a synergistic effect between smoking status and serological markers of HPV16 on the risk of CIN2–3 and ICC.[17,18,21] However, all of these studies found significantly increased risks of CIN2–3 and ICC in women who had ever smoked and were HPV seropositive as compared with women who were non-smokers and HPV seronegative (ORs between 5.2 and 7.2 for CIN2–3 and 15.3 for ICC). Stratification by HR HPV serology instead of any HPV serology yielded virtually the same associations.

Smoking could increase the risk of cervical neoplasia through several plausible biological mechanisms. One of these mechanisms is the induction of a local immunosuppressive effect caused by tobacco metabolites which could produce a detrimental effect on the ability of the host to develop an effective immune response against viral infections, increasing the risk of persistent infections in the cervix.[14,35,36] In addition, the chemicals found in cigarettes, such as nicotine and its metabolite cotinine, which can cause DNA damage in squamous epithelial cells, have also been found in the cervical mucus of female smokers.[22,37]

Our study has important strengths but also several limitations that need to be taken into account. The main strengths of our study include the multicentric and long-term

prospective design and the large number of enrolled women in 10 European countries. A valuable feature of the cohort is the collection of sera at the time of recruitment that allowed the testing of serological markers of HPV, CT and HHV-2 well before disease development. However, a limitation of this prospective study is that its design did not allow taking into account changes and events occurring from recruitment throughout diagnosis or follow-up closure such as discontinuation or initiation of smoking or development of new HPV infections, among others.

Another limitation is that CIN3/CIS cases were not systematically identified and consistently reported across cancer registries and EPIC centers. Clearly, countries with nationwide population-based screening programs (i.e. the United Kingdom, Sweden, Norway) yielded a higher number of CIN3/CIS cases than those with other screening strategies. Thus it is likely that our CIN3/CIS cases may not be fully representative of the true underlying population with this disease. Furthermore, EPIC did not collect information on screening attendance, and it has been reported that smoking is associated with less adherence to screening programs.[38] Thus, we cannot rule out that some residual confounding by screening may explain some of the associations with CIN3/CIS.

The lack of information about sexual habits is another limitation of the current study that did not allow us to completely elucidate whether the association found with smoking might be due to residual confounding by sexual behavior or is a true causal and independent association with preinvasive and invasive CC. The risks obtained could be overestimated because smokers are more likely to have high-risk sexual behaviors.[33] However, HPV serology and other sexually transmitted infections such as CT or HHV-2 are good proxy measures of sexual activity, and taking into account those, our results from the cohort and the nested case–control studies were substantially consistent. Thus, we can rule out quite confidently a large effect of residual confounding for the reported associations.

Regarding HPV detection, HPV capsid protein serology is a useful tool for the identification of women previously exposed to HPV. However, one limitation of these assays is that they show a low sensitivity, as only 50% to 70% of HPV-exposed women seroconvert.[39] This potential misclassification due to lack of seroconversion of infected women could lead to further exposure misclassification, and the associations found between smoking and CIN3/CIS or ICC risk might partially be due to residual confounding by HPV. We attempted to reduce this potential misclassification by restricting our analyses to CIN3/CIS or ICC cases but only including controls that were HPV seropositive (instead of all controls), as done in a previous study.[19] The results obtained from these analyses showed stronger dose-response associations with smoking duration, intensity and quitting than those obtained including all women (data not shown). This suggests that our reported associations might be actually underestimated.

In conclusion, our prospective study confirms the important role of smoking in the development of both ICC and pre-cancer. The strongest associations were found with smoking duration, and inversely with quitting, even after adjustment for markers of exposure to HPV and other STIs. The consistent and strong beneficial effect of quitting smoking in this prospective design provides a scientifically sound public health message about the importance of smoking prevention and cessation to reduce the risk of cervical cancer.

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ARTICLE 3

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The Influence of Hormonal Factors on the Risk of Developing Cervical Cancer and Pre-Cancer: Results from the EPIC Cohort

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Abbreviations: CC, Cervical Cancer; CI, Confidence Interval; CIN3, Cervical Intraepithelial Neoplasia grade 3; CIS, Carcinoma In Situ; CT, Chlamydia trachomatis; EPIC, European Prospective Investigation into Cancer and Nutrition; FTP, Full-term pregnancy; HHV-2, Human herpesvirus 2; HPV, Human Papillomavirus; HR, Hazard Ratio; HT, Hormone Therapy; IARC, International Agency for Research on Cancer; ICC, Invasive Cervical Cancer; IUD, Intrauterine Device; OR, Odds Ratio; OC, Oral Contraceptives; SCC, Squamous Cell Carcinoma; STIs, Sexually Transmitted Infections.

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Abstract

Background

In addition to HPV, high parity and hormonal contraceptives have been associated with cervical cancer (CC). However, most of the evidence comes from retrospective case-control studies. The aim of this study is to prospectively evaluate associations between hormonal factors and risk of developing cervical intraepithelial neoplasia grade 3 (CIN3)/carcinoma in situ (CIS) and invasive cervical cancer (ICC).

Methods and Findings

We followed a cohort of 308,036 women recruited in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study. At enrollment, participants completed a questionnaire and provided serum. After a 9-year median follow-up, 261 ICC and 804 CIN3/CIS cases were reported. In a nested case-control study, the sera from 609 cases and 1,218 matched controls were tested for L1 antibodies against HPV types 11,16,18,31,33,35,45, 52,58, and antibodies against *Chlamydia trachomatis* and *Human herpesvirus 2*. Multivariate analyses were performed to estimate hazard ratios (HR), odds ratios (OR) and corresponding 95% confidence intervals (CI). The cohort analysis showed that number of full-term pregnancies was positively associated with CIN3/CIS risk (p-trend = 0.03). Duration of oral contraceptives use was associated with a significantly increased risk of both CIN3/CIS and ICC (HR = 1.6 and HR = 1.8 respectively for $\geq$15 years *versus* never use). Ever use of menopausal hormone therapy was associated with a reduced risk of ICC (HR = 0.5, 95% CI: 0.4–0.8). A non-significant reduced risk of ICC with ever use of intrauterine devices (IUD) was found in the nested case-control analysis (OR = 0.6). Analyses restricted to all cases and HPV seropositive controls yielded similar results, revealing a significant inverse association with IUD for combined CIN3/CIS and ICC (OR = 0.7).

Conclusions

Even though HPV is the necessary cause of CC, our results suggest that several hormonal factors are risk factors for cervical carcinogenesis. Adherence to current cervical cancer screening guidelines should minimize the increased risk of CC associated with these hormonal risk factors.

Introduction

Cervical cancer (CC) is the fourth most common cancer among women worldwide with an estimated 528,000 new cases and the fourth most common cause of female death from cancer with an estimated 266,000 deaths in 2012 [1]. Human papillomavirus (HPV) is one of the most common sexually transmitted infections worldwide. In fact, most sexually active women will be infected with HPV during their lifetime, although the majority of HPV infections are cleared within 2 years [2,3]. HPV genotypes are classified as low-risk or high-risk based on their association with cervical cancer (CC) [4]. It is well established that persistent infection with high-risk HPV genotypes is the necessary although not sufficient cause of CC [5]. Thus, the involvement of other factors, in addition to HPV, is needed to induce cervical carcinogenesis. High parity and hormonal contraceptives have long been recognized as potential cofactors of CC [5]. A comprehensive review conducted by the International Agency for Research on Cancer (IARC) classified the use of combined oral contraceptives (OC) as carcinogenic to humans, and this was partly based on the reported associations with CC [6]. A collaborative pooled reanalysis evaluating CC, hormonal contraceptives and parity found an increased risk of CC in current and long-term OC users, a reduced risk after stopping these hormones [7], and positive associations with both number of full-term pregnancies (FTP) and an early age at first FTP [8]. In addition, results from the European Prospective Investigation into Cancer and Nutrition (EPIC) showed that circulating levels of sex steroid hormones testosterone and possibly estradiol were also positively involved in the etiology of CC [9]. However, even though these associations are generally consistent across studies, it must be noted that the evidence for a role of hormones in cervical carcinogenesis is mostly derived from retrospective case-control studies that did not always take into account HPV. Thus the aim of this study is to prospectively examine potential associations between hormonal factors and risk of developing cervical cancer and pre-cancer using data from a large prospective study that additionally uses serological markers of HPV exposure.

Materials and Methods

The EPIC cohort study

The EPIC study is a large prospective cohort study including 521,448 participants (367,993 women and 153,455 men) recruited between 1992 and 2000 through 23 centres in 10 European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. Most of the EPIC participants were between the ages of 35 and 70 years. The study procedures have been described in detail elsewhere [9,10]. At recruitment, participants gave their written informed consent and completed questionnaires on their diet, medical and lifestyle history. They were also invited to provide blood samples for future testing of markers of interest. The EPIC study was approved by the ethical review committees from each center.

Study population

Of the approximately 370,000 women enrolled in the study, women were not eligible for this analysis if they had prevalent cancer or pre-cancer (n = 22,180), incomplete follow-up (n = 2,295), hysterectomy (n = 34,973) or incomplete lifestyle questionnaire (n = 509) at baseline. This left a total of 308,036 women in these analyses.

Identification of cases and follow-up

Cases of cervical intraepithelial neoplasia grade 3 (CIN3)/carcinoma in situ (CIS) and invasive cervical cancer (ICC) were identified through several methods, including a record linkage with population-based cancer registries (Denmark, Italy, the Netherlands, Norway, Spain, Sweden

and the United Kingdom), health insurance records, hospital-based cancer and pathology registries and active follow-up of subjects (France, Germany and Greece). Data on vital status were obtained from mortality registries at regional and national level. Cervical cancer cases included only those women with first primary incident cancer according to the International Classification of Diseases, 10th revision (code C53: cervix uteri). Contrary to ICC, ascertainment of CIN3/CIS cases was not systematically done in all cancer registries and EPIC centers. Follow-up time was calculated between the date at recruitment and the date at diagnosis for cases or the date at censoring (death, loss of follow-up or end of follow-up) for non-cases. The end of follow-up ranged from December 2003 to December 2006, depending on the center. The median follow-up time in this cohort was around 9 years (25^{th}-75^{th} percentile: 7.5–10.8 years) contributing a total of 2,775,235 person-year. Among the 308,036 women included in the final analysis 1,065 cases were identified: 261 ICC cases and 804 CIN3/CIS cases. Detailed tumor histology was specified for 953 cases (89%), of which 901 (95%) were classified as squamous cell carcinoma (SCC, 712 in situ and 189 invasive) and 52 (5%) as adenocarcinoma (9 in situ and 43 invasive).

Nested case-control study

A nested case-control study within the EPIC cohort was conducted to allow for the adjustment by serological markers of HPV and other sexually transmitted infections (STIs), even though this strategy involves a lower number of subjects. For each case with available blood sample, two matched control subjects were randomly selected from the cancer-free cohort of women that were at risk at the time of diagnosis of the corresponding case. Matching criteria included: study center, age at recruitment (5 year intervals), menopausal status (pre-, peri- and postmenopausal), follow-up time, date, time and fasting status at blood collection, and, among premenopausal women, phase of the menstrual cycle [9]. Approximately 70% of ICC cases and 53% of CIN3/CIS cases identified in the cohort provided serum samples, yielding a total of 609 cases (184 ICC and 425 CIN3/CIS) and 1,218 controls for the analyses.

Serological testing

HPV serology was performed at the German Cancer Research Centre in Heidelberg, Germany. Assay procedures have been explained in detail previously [11,12]. Briefly, antibodies to the capsid protein L1 of high-risk mucosal HPV types 16, 18, 31, 33, 35, 45, 52, and 58, and of low-risk mucosal HPV type 11 were tested by Glutathione S-transferase capture and fluorescent bead-based multiplex serology [13–15]. For the nested case-control analyses, HPV L1 seropositivity refers to that for at least one of the nine HPV types analyzed, and high-risk HPV L1 seropositivity refers to positivity for at least one of the eight high-risk HPV types analyzed.

Serum antibodies against *Chlamydia trachomatis* (CT) and *Human herpesvirus 2* (HHV-2) were tested at the Hospital of Santa Creu i Sant Pau in Barcelona, Spain. We used the commercial assay Chlamydia MIF IgG (Focus Diagnostics, Cypress, CA, USA) for the detection of CT IgG serum antibodies performed by microimmunofluorescence, and the commercial kit HerpeSelect® 2 ELISA IgG (Focus Diagnostics, Cypress, CA, USA) for the detection of antibodies against HHV-2 evaluated by an enzyme immunoassay. The positive results of HHV-2 antibodies were confirmed by a membrane-based immunoassay with the **biokit**HSV-2 Rapid Test (Biokit USA, Lexington, MA, USA).

All serological assays were performed blinded to the subject's characteristics.

Statistical analyses

Multivariate hazard ratios (HR) and 95% confidence intervals (CI) were estimated to evaluate the risk of CIN3/CIS and ICC in relation to several hormonal risk factors using Cox

proportional hazard regression models. In all analyses, age was used as the underlying time variable, and the models were stratified by age at recruitment (in one-year categories) and study center. The proportional hazards assumption was evaluated for all models fitted using tests based on weighted residuals. Tests for linear trend were performed using continuous variables.

We estimated HR for variables related with hormonal factors collected at recruitment. The parity-related variables evaluated in these analyses were the following: ever FTP (never, ever), number of FTP (1, 2, 3, ≥4), age at first FTP (≥30, 25–29, 21–24, ≤20 years old), and number of induced abortions (1, ≥2) if any (never, ever). Self-reported baseline menopausal status was defined as postmenopausal (no menses in the last 12 months or bilateral ovariectomy), perimenopausal (<9 menses in the past 12 months), and premenopausal (regular menses in the past 12 months). Women with unknown menopausal status were classified as postmenopausal if they were 55 years old or more, perimenopausal if they were between 46 and 55 years of age and premenopausal when they were less than 46 years of age at recruitment. We estimated the cumulative years of menstrual cycling as the difference between the age at menopause (for postmenopausal women) or of the age at recruitment (for pre- and perimenopausal women) and the age at menarche minus the total time being pregnant (number of FTP x 9 months) and the total time using OCs; this variable was categorized in quintiles: ≤19.50, 19.51–26.76, 26.77–31.50, 31.51–35.50, ≥35.51 years. The exogenous hormone-related factors evaluated included: OC use (never, ever, past, current), duration of OC use (≤1, 2–4, 5–9, 10–14, ≥15 years), latency of OC use or time since first use (≤10, 11–20, ≥21 years), and recency of OC use or time since last use (≤5, 6–14, ≥15 years) among past OC users. Use of intrauterine device (IUD) was also analyzed and dichotomized as never and ever IUD use. Among post- and perimenopausal women, use of menopausal hormone therapy (HT) was evaluated as: HT use (never, ever, past, current), duration of HT use (≤1, 2–4, ≥5 years) and HT formulation (estrogen alone, progesterone alone, combination of estrogen/progesterone). Ovariectomy was also assessed (no, unilateral, bilateral). Combined variables using number of FTP and age at first FTP, duration and recency of OC use, and number of FTP and duration of OC use were also created and evaluated. The following variables were also analyzed but not included in the tables presented because they were either collinear with the final selected variables or not associated with risk in any of the analyses: time since first FTP, pregnancies, live births, stillbirths, miscarriages, breastfeeding, age started and stopped OC use, use of different contraceptives (condoms, spermicidal creams, tubal ligation, rhythm methods, diaphragm, vasectomy), age started HT use, type of HT (oral, injectable, topical), menopausal status, age at menarche, and age at menopause.

We used stepwise regression modeling to assess potential confounding by other variables such as: body mass index (BMI, underweight (<18.5), normal (18.5–25), overweight (25–30), obese (≥30)), marital status (single, married/cohabiting, divorced/separated, widowed),education level (none, primary school, technical/professional school, secondary school, university degree), physical activity (inactive, moderately inactive, moderately active, and active, using a validated Cambridge Physical Activity Index [16]), and smoking habits (never, former and duration <15 years, former and duration ≥15 years, current and intensity <10 cig/day, current and intensity ≥10 cig/day). Number of FTP (0, 1, 2, 3, ≥4), OC use and duration (never, past for <10 years, past for ≥10 years, current for <10 years, current for ≥10 years), and menopausal status with HT use (premenopausal, peri- and postmenopausal and non HT users, peri- and postmenopausal and HT users) were used as adjusting variables when appropriate.

In the nested case-control analysis, multivariate odds ratios (OR) and 95% CI were estimated to evaluate the risk of CIN3/CIS and ICC in relation to hormonal factors using conditional logistic regression models. Models were adjusted for HPV L1 serology, CT serology, HHV-2 serology, and other potential confounding factors (BMI, marital status, education

level, physical activity, smoking habits, OC use and duration, number of FTP and menopausal status with HT use (when appropriate)). Unconditional logistic regression analyses among all cases and HPV L1 seropositive controls were also performed, including the matching variables in the models as adjusting covariates. Tests for interaction among hormonal variables and other risk factors were based on the likelihood ratio test comparing the models with and without the interaction terms.

When applicable, variables included a missing or unknown category in order to avoid the exclusion of participants in the regression models. All statistical tests were 2-tailed, and p-values below 0.05 were considered statistically significant. Statistical analyses were performed using the R programing language (R Development Core Team, 2005, http://www.R-project.org).

Ethics statement

All participants gave written informed consent, and the study was approved by the local ethics committees in the participating countries (Athens: University of Athens Medical School; Cambridge: Norwich District Ethics Committee; Denmark (Aarhus, Copenhagen): The National Committee on Health Research Ethics; France (Paris): Comité de Protection des Personnes; Heidelberg: Ethics Committee of the Heidelberg University Medical School; International Agency for Research on Cancer: IARC Ethics Committee; Imperial College: Imperial College Research Ethics Committee [ICREC]; Italy (Florence, Milan, Naples, Ragusa, Turin): Comitato Etico Indipendente, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano; Florence: Comitato Etico Locale Azienda Sanitaria di Firenze; Malmo: Ethics Committee of Lundst University; Netherlands (Bilthoven and Utrecht): The Medical Ethical Committee (METC = Medisch Ethische Toetsingscommissie) of the University Medical Center Utrecht (UMCU), Utrecht, the Netherlands; Norway: Regional ethical committee for Northern Norway and the Norwegian Data Inspectorate; Oxford: Scotland A Research Ethics Committee; Potsdam: Ethikkommission der Landesärztekammer Brandenburg Cottbus, Deutschland; Spain (Asturias, Barcelona, Granada, Murcia, Navarre, San Sebastian): CEIC Comité de Ética de Investigación Clínica; Turin: Human Genetics Foundation Torino: Ethics Committee; Umea: Umea Regional Ethical Review Board) and the Internal Review Board of the International Agency for Research on Cancer.

Results

Baseline characteristics for cases and non-cases included in the cohort analysis have been reported previously (S1 Table) [11]. In brief, most CIN3/CIS cases were recruited from the United Kingdom (37.1%), Sweden (20.8%) and Norway (15.0%), and most of the ICC cases were from Sweden (16.9%), the United Kingdom (13.4%), Denmark (12.6%) and France (11.5%). CIN3/CIS cases were younger than ICC cases. As compared to non-cases, women with CIN3/CIS or ICC were more likely to be single or separated, to smoke, to have ever used OCs, and to be premenopausal. The characteristics of the women included in the nested case-control analysis have already been described in two previous articles [11,12].

Table 1 shows associations between factors related to endogenous hormones and risk of developing CIN3/CIS and ICC by study design (full cohort study and nested case-control study). In both analyses the risk of CIN3/CIS increased significantly with increasing number of FTP. In contrast, for ICC, a non significant decreased risk was observed among women who ever had a FTP. A decreased risk of CIN3/CIS and ICC was observed with decreasing age at first FTP, but only in the case-control study and this was only statistically significant for CIN3/CIS. In both study designs, a non significant decreased risk of CIN3/CIS was observed among women who had more than one induced abortion. However, for ICC an increased risk was

Table 1. Risk of CIN3/CIS and ICC of the cervix according to factors related with endogenous hormones.

Risk factor	Cohort study				Nested case-control study			
	CIN3/CIS		ICC		CIN3/CIS		ICC	
	Non-cases / Cases	HR (95% CI) [1]	Non-cases / Cases	HR (95% CI) [1]	Controls / Cases	OR (95% CI) [2]	Controls / Cases	OR (95% CI) [2]
Number of FTP								
Never	45,228 / 191	1.0 (ref)	45,419 / 45	1.0 (ref)	139 / 51	1.0 (ref)	43 / 30	1.0 (ref)
Ever	246,823 / 516	**1.5 (1.2–1.9)**	247,339 / 191	0.7 (0.5–1.0)	543 / 288	**2.0 (1.2–3.2)**	281 / 131	0.8 (0.4–1.5)
1	44,007 / 116	**1.4 (1.0–1.8)**	44,123 / 36	0.7 (0.5–1.2)	132 / 71	1.7 (1.0–2.9)	42 / 26	0.8 (0.3–2.0)
2	114,956 / 237	**1.4 (1.1–1.9)**	115,193 / 82	0.7 (0.4–1.0)	247 / 132	**2.1 (1.2–3.4)**	153 / 53	0.5 (0.2–1.1)
3	54,107 / 94	**1.4 (1.0–1.9)**	54,201 / 40	0.7 (0.4–1.2)	97 / 46	**2.1 (1.2–3.8)**	55 / 28	1.0 (0.4–2.3)
≥4	23,545 / 51	**2.3 (1.6–3.3)**	23,596 / 23	0.9 (0.5–1.6)	42 / 28	**2.6 (1.3–5.3)**	18 / 16	1.5 (0.5–4.4)
p for trend among ever ftp		***0.03***		*0.4*		*0.2*		***0.02***
Age at first FTP (years) [3]								
≥30	35,122 / 71	1.0 (ref)	35,193 / 22	1.0 (ref)	69 / 39	1.0 (ref)	22 / 13	1.0 (ref)
25–29	85,567 / 161	1.1 (0.8–1.4)	85,728 / 66	1.2 (0.7–2.0)	164 / 90	1.0 (0.6–1.8)	104 / 48	0.8 (0.2–2.9)
21–24	89,750 / 181	1.1 (0.8–1.5)	89,931 / 65	1.0 (0.6–1.6)	192 / 106	0.7 (0.4–1.3)	107 / 41	0.6 (0.2–1.9)
≤20	35,372 / 101	1.2 (0.9–1.7)	35,473 / 36	1.1 (0.6–2.0)	113 / 52	0.5 (0.3–1.1)	47 / 27	1.0 (0.2–4.3)
p for trend among ever ftp		*0.2*		*0.8*		***0.03***		*0.9*
Number of induced abortions [4]								
Never	157,566 / 278	1.0 (ref)	157,844 / 99	1.0 (ref)	295 / 166	1.0 (ref)	180 / 70	1.0 (ref)
Ever	41,079 / 77	0.9 (0.7–1.1)	41,156 / 53	**1.9 (1.3–2.7)**	84 / 45	0.6 (0.4–1.0)	57 / 41	1.7 (0.8–3.4)
1	28,058 / 63	1.0 (0.7–1.3)	28,121 / 36	**1.9 (1.3–2.8)**	63 / 36	0.6 (0.4–1.2)	38 / 26	1.4 (0.6–3.4)
≥2	12,722 / 13	0.6 (0.3–1.0)	12,735 / 17	**1.8 (1.0–3.2)**	21 / 9	0.5 (0.2–1.2)	18 / 15	2.1 (0.8–6.1)
Cumulative years of menstrual cycles without OCs (quintiles) [5]								
Quintile 1	47,020 / 270	1.0 (ref)	47,290 / 57	1.0(ref)	126 / 78	1.0 (ref)	43 / 26	1.0 (ref)
Quintile 2	46,953 / 118	0.9 (0.7–1.1)	47,071 / 29	**0.5 (0.3–0.8)**	124 / 64	1.0 (0.6–1.7)	39 / 28	1.6 (0.6–3.8)
Quintile 3	48,557 / 91	0.8 (0.6–1.1)	48,648 / 49	0.8 (0.5–1.3)	106 / 57	0.8 (0.5–1.4)	66 / 22	0.5 (0.2–1.2)
Quintile 4	47,855 / 63	**0.7 (0.5–1.0)**	47,918 / 36	0.6 (0.4–1.1)	111 / 43	**0.5 (0.3–0.9)**	60 / 39	1.0 (0.4–2.3)
Quintile 5	44,733 / 47	0.7 (0.5–1.1)	44,780 / 24	**0.4 (0.2–0.8)**	114 / 49	**0.5 (0.3–0.9)**	74 / 23	0.7 (0.3–1.7)
p for trend		***0.009***		***0.005***		***0.01***		*0.6*

CIN3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; ICC: invasive cervical cancer; HR: hazard ratio; OR: odds ratio; CI: confidence interval; FTP: full-term pregnancy; OC: oral contraceptives; HT: hormone therapy. The number of cases does not add up the total number of cases because of missing values. Bold font indicates a statistically significant effect ($p<0.05$).

[1] Models were adjusted by body mass index, marital status, level education, physical activity, smoking habits, OC use and duration and menopausal status with HT use.

[2] Conditional regression models were adjusted by HPV L1 serology, *Chlamydia trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits, OC use and duration and menopausal status with HT use. See methods for list matching variables.

[3] Among parous women.

[4] Excludes Bilthoven, Sweden and Norway because information was not collected for this variable.

[5] Model not adjusted by OC use and duration because of co-linearity with cumulative duration of menstrual cycles. For cohort study, quintiles correspond to: q1: ≤19.50; q2: 19.51–26.76; q3: 26.77–31.50; q4: 31.51–35.50; q5: ≥35.51. For nested case-control study, quintiles correspond to: q1: ≤14.66; q2: 14.67–24.23; q3: 24.24–29.17; q4: 29.18–33.75; q5: ≥33.76.

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found among women who had at least one induced abortion but the association was statistically significant only in the cohort study. When analyses were adjusted or stratified by number of FTP, the magnitude of the point estimates of the associations with both outcomes remained mostly unchanged (data not shown). Women with higher lifetime years of menstrual cycles had a lower risk of CIN3/CIS in both studies and of ICC in the cohort study.

When analyses were restricted to parous women and mutually adjusted for number of FTP and age at first FTP, the effect of number of FTP for CIN3/CIS risk was maintained in the full cohort analysis (HR = 1.6, 95% CI: 1.1–2.3), and was of borderline statistical significance in the nested case-control study (OR = 2.1, 95% CI: 1.0–4.6; data not shown). No associations were found for ICC risk.

Table 2 summarizes associations with factors related to exogenous hormones. Current use of OCs and increasing years of use were both associated with CIN3/CIS and ICC in the cohort study, even though the trend was only statistically significant for CIN3/CIS. As compared to current users, increasing years since last OC use was associated with a reduction in the risk of developing CIN3/CIS in the cohort study. In the case-control study associations were found in the same direction but did not reach statistical significance. Ever use of HT among peri- and postmenopausal women significantly decreased the risk of ICC in both studies. We found a decreased risk of ICC with duration of HT use but the trend did not reach statistical significance. In contrast, for CIN3/CIS we found a statistically significant reduced risk with years of HT in the case-control study. Similar results were obtained when analyses where restricted to postmenopausal women only (data not shown). We also assessed the effect of HT formulation finding a non significant increased risk of CIN3/CIS for users of menopausal estrogens alone (HR = 1.7, 95% CI: 0.9–3.1, for ever *versus* never users) and a lack of association for combined formulations (data not shown). The effect of progestin alone could not be assessed due to the low number of exposed subjects. For ICC, most exposed cases used some kind of combination of hormones showing a borderline inverse association (HR = 0.6, 95% CI: 0.3–1.0, for ever *versus* never users). There were not enough cases to assess the effect of estrogen or progestin alone (data not shown). Concerning IUD use, a non significant inverse association with both CIN3/CIS and ICC risk was observed in the nested case-control study.

When the combined effect of duration and recency of OC use was evaluated, the risk of CIN3/CIS declined progressively with increasing years since last use (data not shown). This pattern was not observed for ICC risk.

In both study designs, the combined effect of number of FTP and duration of OC use was analyzed, and showed a significant increased risk of CIN3/CIS with increasing number of FTP within each category of OC use (S2 Table). Women with 4 or more FTP had a four-fold risk within each category of OC use as compared to women who were nulliparous and never used OCs. The test of interaction between OC use and FTP reached statistical significance in the cohort study ($p = 0.004$). In contrast, for ICC we only found a marginal increased risk among multiparous women who used OCs for more than 5 years in the cohort study.

Concerning associations by histological type, the risk of SCC showed the same overall pattern: increased risk of CIS with number of FTP and years of OC use and decreased risk with years of HT use, and increased risk of invasive SCC with years of OC use and decreased risk with years of HT use (data not shown). Regarding adenocarcinomas, associations could not be evaluated accurately because of the small number of cases (52 cases in the cohort study and 33 cases in the nested case-control study for both in situ and invasive adenocarcinomas). Nevertheless, these analyses showed non significant and weak positive associations with number of FTP and OC use for invasive adenocarcinoma, and a non significant inverse association with HT use (data not shown).

Table 2. Risk of CIN3/CIS and ICC of the cervix according to factors related with exogenous hormones.

Risk factor	Cohort study				Nested case-control study			
	CIN3/CIS		ICC		CIN3/CIS		ICC	
	Non-cases / Cases	HR (95% CI) [1]	Non-cases / Cases	HR (95% CI) [1]	Controls / Cases	OR (95% CI) [2]	Controls / Cases	OR (95% CI) [2]
IUD use [3]								
Never	170,843 / 371	1.0 (ref)	171,214 / 144	1.0 (ref)	325 / 160	1.0 (ref)	194 / 106	1.0 (ref)
Ever	63,677 / 136	1.1 (0.9–1.4)	63,813 / 43	0.9 (0.6–1.3)	160 / 82	0.8 (0.5–1.3)	70 / 28	0.6 (0.3–1.2)
OC use								
Never	121,117 / 169	1.0 (ref)	121,286 / 76	1.0 (ref)	225 / 99	1.0 (ref)	139 / 56	1.0 (ref)
Ever	176,993 / 548	1.1 (0.9–1.3)	177,541 / 165	**1.6 (1.1–2.3)**	466 / 244	1.1 (0.8–1.5)	186 / 109	1.5 (0.8–2.6)
Past	152,658 / 411	1.0 (0.9–1.3)	153,069 / 134	**1.6 (1.1–2.2)**	392 / 197	1.0 (0.7–1.5)	158 / 86	1.3 (0.7–2.4)
Current	17,384 / 127	**1.8 (1.4–2.4)**	17,511 / 22	**2.2 (1.3–4.0)**	57 / 41	1.7 (0.9–3.1)	24 / 17	2.2 (0.7–6.7)
Duration of OC use (years)								
Never	121,117 / 169	1.0 (ref)	121,286 / 76	1.0 (ref)	225 / 99	1.0 (ref)	139 / 56	1.0 (ref)
≤1	31,867 / 78	1.0 (0.8–1.3)	31,945 / 27	1.5 (0.9–2.4)	180 / 82	1.0 (0.6–1.5)	76 / 36	1.1 (0.6–2.2)
2–4	40,168 / 127	1.1 (0.8–1.4)	40,295 / 27	1.3 (0.8–2.0)				
5–9	38,816 / 136	1.1 (0.9–1.4)	38,952 / 41	**2.0 (1.3–3.0)**	108 / 54	1.1 (0.7–1.7)	38 / 22	1.6 (0.7–3.7)
10–14	26,969 / 90	1.2 (0.9–1.6)	27,059 / 26	1.6 (1.0–2.6)	138 / 94	**1.6 (1.0–2.5)**	63 / 42	1.5 (0.7–3.1)
≥15	23,395 / 82	**1.6 (1.2–2.2)**	23,477 / 28	**1.8 (1.1–2.9)**				
p for trend among OC users		***0.01***		*0.2*		*0.2*		*0.9*
Recency of OC use (years) [4,5]								
Current	7,678 / 95	1.0 (ref)	7,773 / 8	1.0 (ref)	32 / 20	1.0 (ref)	10 / 12	1.0 (ref) [7]
≤5	9,662 / 79	**0.7 (0.5–1.0)**	9,741 / 11	1.1 (0.4–2.8)	30 / 21	1.0 (0.2–4.2)		
6–14	17,368 / 60	**0.6 (0.4–0.8)**	17,428 / 14	0.8 (0.3–2.0)	44 / 21	0.4 (0.1–1.9)	27 / 11	-
≥15	25,681 / 45	**0.6 (0.4–0.9)**	25,726 / 25	1.0 (0.4–2.5)	48 / 22	0.6 (0.1–5.0)	32 / 20	-
p for trend among past OC users		*0.2*		*0.7*		-		-
HT use [6]								
Never	114,271 / 149	1.0 (ref)	114,420 / 94	1.0 (ref)	170 / 83	1.0 (ref)	107 / 67	1.0 (ref)
Ever	63,839 / 131	1.2 (0.9–1.5)	63,970 / 31	**0.5 (0.4–0.8)**	149 / 84	0.9 (0.6–1.5)	72 / 18	**0.3 (0.1–0.7)**
Past	18,508 / 22	1.0 (0.6–1.5)	18,530 / 11	0.6 (0.3–1.2)	42 / 17	0.7 (0.3–1.4)	19 / 8	0.6 (0.2–2.0)
Current	43,110 / 102	1.3 (1.0–1.7)	43,212 / 19	**0.5 (0.3–0.8)**	102 / 61	1.1 (0.7–1.9)	51 / 10	**0.2 (0.1–0.5)**
Duration of HT use (years) [6]								
Never	114,271 / 149	1.0 (ref)	114,420 / 94	1.0 (ref)	170 / 83	1.0 (ref)	107 / 67	1.0 (ref)
≤1	22,819 / 43	1.3 (0.9–1.9)	22,862 / 12	0.7 (0.4–1.2)	42 / 28	1.2 (0.6–2.5)	39 / 11	**0.4 (0.1–1.0)**
2–4	19,032 / 32	1.0 (0.7–1.5)	19,064 / 9	0.6 (0.3–1.1)	44 / 15	0.5 (0.2–1.1)		
≥5	15,834 / 27	1.0 (0.7–1.6)	15,861 / 7	**0.4 (0.2–0.9)**	49 / 19	0.6 (0.3–1.4)	26 / 5	**0.1 (0.03–0.6)**
p for trend among menopausal hormones users		*0.2*		*0.4*		-		-

CIN 3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; ICC: invasive cervical cancer; HR: hazard ratio; OR: odds ratio; CI: confidence interval; IUD: intrauterine device; OC: oral contraceptives; HT: hormone therapy. The number of cases does not add up the total number of cases because of missing values. Bold font indicates a statistically significant effect ($p<0.05$).

[1] Models were adjusted by body mass index, marital status, level education, physical activity, smoking habits, number of full-term pregnancies and menopausal status with HT use.

[2] Conditional regression models were adjusted by HPV L1 serology, *Chlamydia trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits, number of full-term pregnancies and menopausal status with HT use. See methods for list matching variables.

[3] Excludes Bilthoven, Sweden and Norway because information was not collected for this variable.

[4] Among OC users (excluding non OC users).

[5] Excludes Bilthoven, France, Germany, Sweden, Denmark and Norway because information was not collected for this variable.

[6] Among peri and postmenopausal women (excluding premenopausal women) and models also adjusted by OC use and duration but not adjusted by menopausal status with HT use. [7] Risk estimates were not estimated due to lack of power in the model.

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Analyses restricted to countries with screening programs (United Kingdom, Sweden, Denmark, Norway) did not substantially change the magnitude of the associations for the several risk factors (data not shown).

Table 3 shows associations restricted to all cases and HPV L1 seropositive controls in the nested case-control study. In this analysis number of FTP was strongly associated with an increased risk of CIN3/CIS. For induced abortions we found opposite effects for CIN3/CIS *versus* ICC (OR = 0.5 and OR = 1.7 respectively, for ever *versus* never). Current use and duration of OCs increased the risk of both CIN3/CIS and ICC although the associations were not statistically significant. HT use and duration was inversely and significantly associated with ICC. Finally, IUD use was inversely associated with both CIN3/CIS and ICC risk without statistical significance, although when we combined CIN3/CIS and ICC the significance emerged (OR = 0.7, 95% CI: 0.5–0.96; data not shown). Associations were broadly similar in analyses restricted to all cases and high-risk HPV seropositive controls and in analyses including only HPV seropositive cases and controls (data not shown), revealing a significant inverse association between IUD use and ICC among HPV seropositive women (OR = 0.3, 95% CI: 0.1–0.9; data not shown).

Discussion

The results of this large prospective cohort study show that certain endogenous and exogenous hormonal factors appear to be related to cervical carcinogenesis. Thus the risk of cervical pre-cancer increased with increasing number of FTP and duration of OC use, and decreased with increasing years since last OC use among past users. For ICC, the risk increased with number of abortions and duration of OC use, and decreased with increasing duration of HT. A reduced risk of ICC was also observed among IUD users in the nested case-control study. Globally the associations were somewhat stronger in the cohort study than in the nested case-control study. The case-control study is useful to support the results obtained in the cohort study since it allowed for the additional adjustment of serological markers of HPV exposure and other STIs.

OC use

Consistent with results from previously published pooled analyses [7,17], our study highlighted strong and positive associations between OC use and risk of cervical cancer and pre-cancer; specifically, the risk increased with duration of use and decreased with cessation of use. While the IARC collaborative study found a relative risk of 1.6 for long-term OC users with a significant trend, Moreno *et al.* observed a stronger association (OR = 4.0). In line with our findings, these two studies also found a reduced risk of cervical cancer for users who ceased OCs (RR = 0.8 and OR = 0.5, respectively). Other prospective studies did not find associations between OCs and CIN3/CC risk [18–20]. Analyses combining duration and recency of use evaluated in our study found a trend of lower CIN3/CIS risk with cessation of use for both short and long-term OC users, reinforcing the null association among past users and the higher risk among current users. The IARC collaborative study has also analyzed these factors in combination, obtaining similar patterns of trend, but unlike our study, for both CIN3/CIS and ICC risk.

Since HPV is the necessary cause of CC, analyses including some measure of HPV infection are needed to assess potential residual confounding. In our nested case-control study we included the adjustment by HPV serology, and we evaluated OC use and CC risk among all cases and HPV seropositive controls. As already discussed, these results were broadly similar to those obtained in the cohort study, suggesting that we can reasonably rule out a large confounding effect due to HPV infection in our associations. In the last 10 years, most of the

Table 3. Multivariate odds ratios for the association between factors related to endogenous and exogenous hormones and CIN3/CIS and ICC cases among all cases and HPV L1 seropositive control women in the nested case-control study.

Risk factor	Among all cases and HPV L1 seropositive control women			
	CIN3/CIS		ICC	
	Controls / Cases	OR (95% CI)	Controls / Cases	OR (95% CI)
Number of FTP [1]				
Never	64 / 51	1.0 (ref)	16 / 30	1.0 (ref)
Ever	250 / 288	**2.0 (1.2–3.2)**	122 / 131	0.6 (0.3–1.4)
1	71 / 71	1.4 (0.8–2.6)	21 / 26	0.9 (0.3–2.6)
2	99 / 132	**2.3 (1.3–4.0)**	69 / 53	**0.4 (0.2–1.0)**
3	47 / 46	**2.0 (1.0–4.0)**	24 / 28	0.7 (0.3–2.0)
≥4	24 / 28	**2.6 (1.2–5.9)**	7 / 16	1.2 (0.3–4.6)
p for trend among ever ftp		*0.2*		*0.3*
Number of induced abortions [1,3]				
Never	127 / 166	1.0 (ref)	77 / 70	1.0 (ref)
Ever	58 / 45	**0.5 (0.3–0.8)**	29 / 41	1.7 (0.8–3.9)
1	42 / 36	**0.5 (0.3–0.9)**	18 / 26	2.0 (0.8–5.0)
≥2	16 / 9	**0.4 (0.1–0.9)**	11 / 15	1.4 (0.5–4.3)
IUD use [2,3]				
Never	143 / 160	1.0 (ref)	78 / 106	1.0 (ref)
Ever	86 / 82	0.7 (0.4–1.1)	37 / 28	0.6 (0.3–1.2)
OC use [2]				
Never	103 / 99	1.0 (ref)	59 / 56	1.0 (ref)
Ever	214 / 244	1.1 (0.7–1.6)	80 / 109	1.7 (0.9–3.3)
Past	182 / 197	1.1 (0.7–1.6)	70 / 86	1.6 (0.8–3.1)
Current	26 / 41	1.6 (0.8–3.2)	10 / 17	2.7 (0.8–9.1)
Duration of OC use (years) [2]				
Never	103 / 99	1.0 (ref)	59 / 56	1.0 (ref)
≤4	82 / 82	1.0 (0.7–1.7)	32 / 36	1.4 (0.6–3.0)
5–9	53 / 54	0.9 (0.5–1.6)	15 / 22	2.1 (0.8–5.5)
≥10	60 / 94	1.6 (0.9–2.6)	31 / 42	1.8 (0.8–4.2)
p for trend among OC users		*0.1*		*0.3*
HT use [4,5]				
Never	84 / 83	1.0 (ref)	54 / 67	1.0 (ref)
Ever	77 / 84	1.0 (0.6–1.7)	39 / 18	**0.3 (0.1–0.6)**
Past	23 / 17	0.6 (0.3–1.4)	10 / 8	0.5 (0.1–1.7)
Current	51 / 61	1.2 (0.7–2.2)	29 / 10	**0.2 (0.1–0.5)**
Duration of HT use (years) [4,5]				
Never	84 / 83	1.0 (ref)	54 / 67	1.0 (ref)
≤1	18 / 28	1.4 (0.6–3.0)	21 / 11	**0.3 (0.1–0.8)**
2–4	24 / 15	0.5 (0.2–1.2)		
≥5	28 / 19	0.7 (0.3–1.6)	14 / 5	**0.2 (0.05–0.8)**
p for trend among menopausal hormones users		*0.3*		-

CIN3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; ICC: invasive cervical cancer; OR: odds ratio; CI: confidence interval; FTP: full-term pregnancy; IUD: intrauterine device; OC: oral contraceptive; HT: hormone therapy. The number of cases and controls does not add up the total number because of missing values. Bold font indicates a statistically significant effect ($p<0.05$).

[1] Unconditional regression models were adjusted by age, country, *Chlamydia Trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits, OC use and duration and menopausal status with HT use.

[2] Unconditional regression models were adjusted by age, country, *Chlamydia Trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits, number of full-term pregnancies and menopausal status with HT use.

[3] Excludes Bilthoven, Sweden and Norway because information was not collected for this variable.

[4] Unconditional regression models were adjusted by age, country, *Chlamydia Trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits, OC use and duration and number of full-term pregnancies.

[5] Among post and perimenopausal women (excluding premenopausal women).

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published studies have also considered HPV infection in their analyses, and the results were globally comparable to those for all women. On the other hand, the very few studies that have used serology as HPV measurement found contradictory results [21–23]. Even though our study did not explicitly collect data related to sexual behavior we were able to adjust for serological markers of HPV, CT and HHV-2, as well as for marital status, which can all be considered good surrogate indicators of sexual behavior and risk of HPV exposure [24]. Since the results were very consistent with these adjustments, we were reassured that the potential effects of confounding by sexual behavior on our reported associations were minimal. Behavioral factors related to cervical cancer screening may also confound these associations. Again, even though the study did not collect individual data on screening practices, we systematically adjusted for surrogate markers of screening-related behavior such as number of pregnancies and variables related to contraceptive methods used in the past that may somewhat reduce the potential confounding effects due to screening practices. Also it is important to note that other studies that have adjusted for cervical screening did find positive associations with CC risk [7,17].

A possible mechanism to explain the associations between OC use and CC risk is that estrogens and progestogens may interact with hormone receptors, mainly progesterone, present in cervical tissue and influence the natural history of HPV infection. Specifically, sex steroid hormones are thought to enhance the expression of HPV 16 E6 and E7 oncogenes stimulating the degradation of p53 tumor suppressor genes and enhancing the ability of the viral DNA to transform cells and induce carcinogenesis [7,9,25,26]. These potential mechanisms are somewhat consistent with data from transgenic mouse models showing that estrogen and its nuclear receptor promote CC in combination with HPV oncogenes, but there are not in line with the observation that progesterone inhibits cervical carcinogenesis in mice [27,28].

Parity

We found contrasting results between parity and risk of CIN3/CIS and ICC. Thus, while women with high parity had a higher risk of CIN3/CIS than nulliparous women, no associations were found with ICC. We also found that this association with high parity was present in each level of OC duration with a synergistic effect between the two variables. These results are in concordance with results from some [20,23] but not all [8,18,19,29] studies. The IARC collaborative study [8] and the IARC multicenter study [29] consistently found a significantly higher risk of CIN3/CIS and ICC among women with high parity. In contrast, two prospective studies conducted by Castle *et al.* [18,19] and a case-control study done in the US [23] did not find a significant association between parity and risk of CIN3/ICC. The lack of association between high parity and ICC risk found in our study could be explained by screening practices related to parity since it is likely that nulliparous women tend to be less screened than parous women. Hence, screening may act as a negative confounder, reducing the association between FTP and ICC risk. Globally the literature supports for an association between high parity and cervical cancer and pre-cancer risk.

As discussed in relation to OC use, these associations may be confounded by a number of other factors. However, adjustment by proxy measures of HPV exposure and sexual behavior did not change our risk estimates. In addition, two pooled analyses that adjusted their models by cervical screening practices obtained also similar results [8,29].

A possible biological mechanism for these associations could be that the elevated levels of estrogen and especially progesterone during pregnancy are responsible for the alterations in the squamo-columnal junction occurring during pregnancy, maintaining the transformation zone on the exocervix for many years. This would facilitate the direct exposure to HPV

contributing to HPV persistence and progression to cervical neoplasia and cancer [8,20,29]. Another possible mechanism is immunosuppression linked to pregnancy which might enhance the role of HPV in cervical carcinogenesis [8].

HT use

Our findings provide evidence for a reduction in ICC risk among peri- and postmenopausal women using HT, an effect that was stronger with longer duration of use. We found very few studies evaluating HT and CC [26,30]. Overall, the literature rules out positive associations. If anything, most studies found weak inverse associations that rarely reached statistical significance. In assessing HT, however, it is important to consider the hormonal composition of these treatments. The IARC review reported an increased risk for breast and endometrial cancer in the 1970s among postmenopausal women using estrogen only therapy, and this triggered health authorities to modify the hormonal composition of HT [6]. Even though our data on HT formulation were very limited, menopausal estrogens alone were associated with an increased risk of CIN3/CIS and combined HT were inversely associated with ICC. Other studies found some evidence for a reduced risk of ICC with ever use of estrogen therapy as well as duration as compared with never use [30–32]. When HT also included progesterone, the inverse association with ICC risk still remained both in our study as well as in other studies [30,33].

As noted previously, one of the limitations of this study is the lack of accurate individual screening history that may indeed influence the effects of HT use on CC risk. Thus, the inverse associations found with ICC, an outcome more susceptible to screening bias, could be somewhat overestimated. One possible explanation of this effect is that women who take HT are more frequently screened than non users, being consequently diagnosed and treated earlier of pre-cancerous lesions. Nevertheless, when we stratified analyses by proxy measures of screening such as parity and OC use, the inverse association remained (data not shown). The intrinsic mechanisms that might explain the biology of these potential associations are currently unknown. Data from HPV transgenic mouse models suggest that estrogens promote cervical carcinogenesis and progesterone inhibits CC [27,28]. However, our results, limited by the low number of subjects, are only partially consistent with these findings.

Induced abortions

An increased risk of ICC and a reduced risk of CIN3/CIS were found among women reporting an induced abortion. Globally, the literature with regard to cervical cancer and pre-cancer risk and induced abortions is inconclusive and our results were in line with some [24,34–36] but not all [29,37,38] studies. More data are needed to better explore these potential associations.

Menstrual lifespan

We observed a decreased risk of both CIN3/CIS and ICC risk with increasing cumulative years of menstrual cycles. Longer menstrual lifespan is implicitly associated with shorter OC use duration and lower parity. However, the advantage of using menstrual lifespan is that includes in a single indicator combined information on the cumulative exposure to some endogenous and exogenous hormones taking into account the actual hormonal lifespan of the woman, providing thus more robustness in the risk estimates. It is interesting to note that even though this variable has been used in studies of other hormone-related cancers such as breast, ovarian and endometrial, no previous studies of CC have been identified in the literature.

IUD use

We found a non significant decreased risk of both CIN3/CIS and ICC among IUD users in the nested case-control study that reached statistical significance in the analyses restricted to all cases and HPV seropositive controls when combining CIN3/CIS and ICC cases (OR = 0.7, 95% CI: 0.5–0.96; data not shown) and in the analyses restricted to HPV seropositive women for ICC risk (OR = 0.3, 95% CI: 0.1–0.9; data not shown). The full case-control study and the different stratified analyses consistently found that IUD use was inversely associated with both CIN3/CIS and ICC risk. Few studies have evaluated associations between IUD use and CC risk with inconsistent results [20,23,37,39]. In a population-based study conducted in the US, Shields and colleagues found a protective effect between duration of IUD and ICC (OR = 0.3, 95% CI: 0.2–0.8, for $\geq$5 years of IUD use) [23]. In a large pooled analysis, Castellsagué and colleagues reported a strong inverse association between IUD use and ICC (OR = 0.5, 95% CI: 0.4–0.7) [39]. Other studies, however, have not confirmed these findings, although the use of a combined outcome (CIN3/CIS and ICC) or a low number of cases could explain these discrepancies [20,37]. Even though we adjusted for major risk factors such as past exposure to HPV, STIs and sexual behavior, it is true that we cannot completely rule out a residual confounding effect exerted by CC screening. It is important to note that the study by Castellsagué did adjust the analyses for cervical HPV DNA, age at first sex, and number of PAP smears, and still found a statistically significant inverse association. One possible mechanism that could explain this potential protective effect is through a device-related inflammatory reaction in the cervix and endocervix that could influence the subsequent likelihood of HPV persistence and/or progression to CC [39].

SCC versus adenocarcinoma

Globally, the associations found between both exogenous and endogenous hormones and SCC risk were comparable to those obtained in models evaluating CIN3/CIS and ICC risk (data not shown). Concerning the risk of invasive adenocarcinoma, even though there were very few cases, an association was found with increasing number of FTP, in addition to OC use and HT, in the cohort study (data not shown). In concordance with our study, the IARC collaborative reanalysis evaluating risk factors for SCC and adenocarcinoma obtained an increased risk for each histological type with increasing duration of OC use [40]. Contrary to this and to another study [41], our results did not indicate that high parity is a risk cofactor for developing SCC, probably due to screening bias. Concerning HT use and contrary to our findings, Lacey and colleagues found that exogenous estrogens, especially unopposed estrogens, were positively associated with adenocarcinomas [30]. However, since both studies accounted for small number of cases, these findings should be cautiously interpreted.

Strengths and limitations

Our study has several strengths including its prospective, population-based design in most of the countries, a large sample size that embraces various regions in Europe, and the inclusion of two disease outcomes: CIN3/CIS and ICC. In addition, our inclusion of a nested case-control study within the cohort allowed us to determine and take into account biomarkers of past exposure to STIs such as HPV, CT and HHV-2.

Nevertheless, our study design failed to consider direct markers of HPV infection, cervical cancer screening practices or sexual behavior, not only at recruitment but also during follow-up, as none of the assessed risk factors or potential confounding variables was reassessed after recruitment. Since these risk factors could and most likely did change in an unknown direction during follow-up, our associations may have led to under- or overestimation of the real effects on CC risk. Another limitation was the low sensitivity of the HPV serology technique, as only

half of infected women seroconvert, and a potential misclassification cannot be totally ruled out [12].

Conclusions

This study contributes strong evidence to consider high parity and long-term OC use important risk factors for cervical cancer and pre-cancer. These risk factors would act not independently but rather as cofactors that interact with HPV to induce cervical carcinogenesis. Our study also provides some evidence that IUD use and HT confer a reduced risk of cervical cancer, but these findings need further confirmation. Despite the clear involvement of hormones in cervical carcinogenesis, and from a public health point of view, adherence to current screening recommendations will certainly minimize the potential increased risk of CC associated with some of these hormonal risk factors.

Supporting Information

S1 Table. Baseline characteristics of cases and non-cases in the cohort study.
(DOCX)

S2 Table. Risk of CIN3/CIS and ICC of the cervix according to the combined effect of number of full-term pregnancies and duration of use of hormonal contraceptives.
(DOCX)

S1 Text. STROBE checklist.
(DOC)

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Author Contributions

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S1 Table. Baseline characteristics of cases and non-cases in the cohort study

Characteristics of subjects in the cohort study	Person-years	Non-cases (N=306,971)	Cases	
			CIN3/CIS (N=804)	ICC (N=261)
		N (%)	N (%)	N (%)
Country				
France	710,002	62,479 (20.4)	51 (6.3)	30 (11.5)
Italy	244,437	28,279 (9.2)	22 (2.7)	18 (6.9)
Spain	229,273	23,190 (7.6)	22 (2.7)	23 (8.8)
United Kingdom	420,545	48,975 (16.0)	298 (37.1)	35 (13.4)
The Netherlands	197,604	22,427 (7.3)	3 (0.4)	19 (7.3)
Greece	102,361	14,031 (4.6)	4 (0.5)	13 (5.0)
Germany	201,023	24,148 (7.9)	52 (6.5)	25 (9.6)
Sweden	285,082	26,709 (8.7)	167 (20.8)	44 (16.9)
Denmark	192,795	24,893 (8.1)	64 (8.0)	33 (12.6)
Norway	192,114	31,840 (10.4)	112 (15.0)	21 (8.0)
Age at recruitment (years)				
<30	87,347	10,216 (3.3)	125 (15.5)	9 (3.4)
30-39	275,337	31,383 (10.2)	180 (22.4)	32 (12.3)
40-49	942,999	103,483 (33.7)	275 (34.2)	95 (36.4)
50-59	989,738	109,403 (35.6)	186 (23.1)	85 (32.6)
≥60	479,814	52,486 (17.1)	38 (4.7)	40 (15.3)
Mean age (5th - 95th percentile)	-	50.3 (32.5-65.8)	42.4 (24.4-59.8)	49.3 (31.4-64.8)
Marital status				
Single	1,780,208	28,807 (11.6)	141 (21.0)	27 (13.8)
Married/living together	275,572	195,074 (78.3)	426 (63.6)	133 (68.2)
Divorced/separated	131,296	14,674 (5.9)	93 (13.9)	31 (15.9)

Widowed	90,828	10,559 (4.2)	10 (1.5)	4 (2.1)
Missing †	497,331	57,857	134	66
Smoking status				
Never smokers	1,581,045	170,511 (57.0)	325 (41.0)	108 (41.7)
Past smokers	608,927	68,524 (22.9)	198 (25.0)	66 (25.5)
Current smokers	516,427	60,278 (20.1)	270 (34.0)	85 (32.8)
Missing †	68,837	7,658	11	2
Number of FTP				
0	397,014	45,228 (16.0)	191 (27.7)	45 (19.9)
1	399,396	44,007 (15.6)	116 (16.8)	36 (15.9)
2	1,032,936	114,956 (10.8)	237 (34.4)	82 (36.3)
3	485,631	54,107 (19.2)	94 (13.6)	40 (17.7)
≥4	212,074	23,545 (8.4)	51 (7.4)	23 (10.2)
Missing †	248,184	25,128	115	35
OC use				
Never users	1,090,625	121,117 (41.6)	169 (23.9)	76 (32.8)
Past users	1,366,500	152,658 (52.4)	411 (58.1)	134 (57.8)
Current users	150,646	17,384 (6.0)	127 (18.0)	22 (9.5)
Missing †	167,464	15,812	97	29
Menopausal status				
Premenopausal	1,044,990	116,583 (38.0)	490 (60.9)	117 (44.8)
Perimenopausal	480,394	53,095 (17.3)	123 (15.3)	42 (16.1)
Postmenopausal	1,249,851	137,293 (44.7)	191 (23.8)	102 (39.1)

CIN3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; ICC: invasive cervical cancer; FTP: full-term pregnancy; OC: oral contraceptive.

† Not included in the percentage distribution of the variable.

S2 Table. Risk of CIN3/CIS and ICC of the cervix according to the combined effect of number of full-term pregnancies and duration of use of hormonal contraceptives

Risk factor	Cohort study				Nested case-control study			
	CIN3/CIS		ICC		CIN3/CIS		ICC	
	Non-cases / Cases	HR (95% CI) [1]	Non-cases / Cases	HR (95% CI) [1]	Controls / Cases	OR (95% CI) [2]	Controls / Cases	OR (95% CI) [2]
Never OC use								
Nulliparous	18,786 / 21	1.0 (ref)	18,807 / 14	1.0 (ref)	46 / 9	1.0 (ref))	19 / 11	1.0 (ref)
1 FTP	17,414 / 22	**2.1 (1.1-3.8)**	17,436 / 11	0.9 (0.4-1.9)	46 / 14	2.2 (0.8-6.5)	16 / 9	1.1 (0.3-4.6)
2-3 FTP	67,745 / 97	**2.9 (1.7-4.7)**	67,842 / 39	0.8 (0.4-1.4)	100 / 54	**4.7 (1.8-12.4)**	90 / 26	0.5 (0.2-1.5)
≥4 FTP	12,040 / 18	**4.0 (2.1-7.7)**	12,058 / 9	1.0 (0.4-2.3)	18 / 13	**6.0 (1.8-21.4)**	11 / 7	2.1 (0.5-8.4)
OC use < 5 years								
Nulliparous	10,132 / 64	**2.3 (1.4-3.8)**	10,196 / 9	1.4 (0.6-3.4)	38 / 16	1.8 (0.6-4.9)	11 / 6	1.1 (0.2-5.4)
1 FTP	10,022 / 26	**2.1 (1.2-3.8)**	10,048 / 5	0.7 (0.3-2.1)	31 / 15	**3.3 (1.1-9.9)**	8 / 4	0.6 (0.1-3.6)
2-3 FTP	43,114 / 96	**2.5 (1.5-4.0)**	43,210 / 31	1.1 (0.6-2.1)	96 / 44	**3.6 (1.4-9.5)**	50 / 20	0.9 (0.3-2.6)
≥4 FTP	5,560 / 17	**4.4 (2.3-8.5)**	5,577 / 5	1.1 (0.4-3.3)	13 / 7	**4.5 (1.2-16.4)**	3 / 4	2.4 (0.3-17.7)
OC use ≥ 5 years								
Nulliparous	14,056 / 94	**2.5 (1.6-4.1)**	14,150 / 18	1.9 (0.9-4.0)	46 / 24	**3.6 (1.3-10.1)**	10 / 11	1.6 (0.4-6.8)
1 FTP	14,458 / 65	**3.2 (1.9-5.4)**	14,523 / 19	1.7 (0.8-3.5)	53 / 42	**4.5 (1.8-13.5)**	17 / 12	1.3 (0.4-4.8)
2-3 FTP	50,117 / 125	**2.6 (1.6-4.2)**	50,242 / 47	1.3 (0.7-2.5)	134 / 73	**4.2 (1.7-10.6)**	67 / 34	1.1 (0.4-3.4)
≥4 FTP	4,863 / 15	**4.4 (2.2-8.6)**	4,878 / 9	**2.4 (1.0-5.8)**	10 / 8	**6.5 (1.7-24.8)**	3 / 5	1.3 (0.1-12.7)
P value [3]	**0.004**		0.9		0.2		0.8	

CIN3: cervical intraepithelial neoplasia grade 3; CIS: carcinoma in situ; ICC: invasive cervical cancer; HR: hazard ratio; OR: odds ratio; CI: confidence interval; FTP: full-term pregnancy; OC: oral contraceptives; HT: hormone therapy.

[1] Models were adjusted by body mass index, marital status, level education, physical activity, smoking habits and menopausal status with HT use. [2] Conditional regression models were adjusted by HPV L1 serology, *Chlamydia trachomatis* serology, *Human herpesvirus 2* serology, body mass index, marital status, level education, physical activity, smoking habits and menopausal status with HT use. See methods for list matching variables. [3] P value for interaction between number of FTP and duration of OC use.

The number of cases does not add up the total number of cases because of missing values.

Bold font signifies a statistically significant effect ($p<0.05$).

S1 - STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract **– ABSTRACT**
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found **– ABSTRACT**
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported **– INTRODUCTION**
Objectives	3	State specific objectives, including any prespecified hypotheses **– INTRODUCTION**
Methods		
Study design	4	Present key elements of study design early in the paper **– METHODS (The EPIC cohort study)**
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection **– METHODS (The EPIC cohort study, Identification of cases and follow-up)**
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up **– METHODS (Study population, Identification of cases and follow-up)** *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls **– METHODS (Study population, Identification of cases and follow-up, Nested case-control study)** *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants **– NOT APPLICABLE**
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed **– NOT APPLICABLE** *Case-control study*—For matched studies, give matching criteria and the number of controls per case **– METHODS (Nested case-control study)**
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. **– METHODS (Identification of cases and follow-up, Nested case-control study , Serological testing, Statistical analyses)** Give diagnostic criteria, if applicable **– METHODS (Identification of cases and follow-up)**
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). **– METHODS (Identification of cases and follow-up, Nested case-control study , Serological testing, Statistical analyses)** Describe comparability of assessment methods if there is more than one group **– NOT APPLICABLE**
Bias	9	Describe any efforts to address potential sources of bias **– METHODS (Statistical analyses), DISCUSSION**
Study size	10	Explain how the study size was arrived at **– METHODS (Study population, Identification of cases and follow-up, Nested case-control study)**

Quantitative variables		11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why **– METHODS (Statistical analyses)**
Statistical methods		12	(*a*) Describe all statistical methods, including those used to control for confounding **– METHODS (Statistical analyses)**
			(*b*) Describe any methods used to examine subgroups and interactions **– METHODS (Statistical analyses)**
			(*c*) Explain how missing data were addressed **– METHODS (Statistical analyses)**
			(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed **– METHODS (Identification of cases and follow-up)** *Case-control study*—If applicable, explain how matching of cases and controls was addressed **– METHODS (Nested case-control study)** *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy **– NOT APPLICABLE**
			(*e*) Describe any sensitivity analyses **– RESULTS, DISCUSSION (for HT use, Induced abortions, IUD use)**
Results			
Participants	13*		(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed **– METHODS (The EPIC cohort study, Study population, Identification of cases and follow-up, Nested case-control study)**
			(b) Give reasons for non-participation at each stage **– METHODS (Study population, Identification of cases and follow-up, Nested case-control study)**
			(c) Consider use of a flow diagram **– NOT APPLICABLE**
Descriptive data	14*		(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders – **RESULTS (TABLE 1)**
			(b) Indicate number of participants with missing data for each variable of interest **– RESULTS (TABLE 1)**
			(c) *Cohort study*—Summarise follow-up time (eg, average and total amount) **– METHODS (Identification of cases and follow-up), RESULTS (TABLE 1)**
Outcome data	15*		*Cohort study*—Report numbers of outcome events or summary measures over time **– RESULTS (TABLE 1)**
			Case-control study—Report numbers in each exposure category, or summary measures of exposure **– RESULTS (TABLES 2 AND 3)**
			Cross-sectional study—Report numbers of outcome events or summary measures **– NOT APPLICABLE**
Main results	16		(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included **– METHODS (Statistical analyses), RESULTS (TABLES 2 AND 3)**
			(*b*) Report category boundaries when continuous variables were categorized **– RESULTS (TABLES 1, 2, 3 AND 4)**
			(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period **– NOT RELEVANT GIVEN THE CONTEXT**
Other analyses	17		Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses **– RESULTS (TABLE 4, S1 TABLE)**
Discussion			

Key results	18	Summarise key results with reference to study objectives **– DISCUSSION (first paragraph)**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias **– DISCUSSION (OC use, Parity, HT use, IUD use, Strengths and limitations)**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence **– DISCUSSION (CONCLUSIONS)**
Generalisability	21	Discuss the generalisability (external validity) of the study results **– DISCUSSION (Strengths and limitations**
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based **– FUNDING**

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

SUMMARY OF MAIN FINDINGS

We followed a cohort of 308,036 women recruited in the EPIC study to prospectively evaluate associations between environmental factors and risk of developing invasive and pre-invasive cervical cancer. During a mean follow-up of 9 years, 261 invasive cervical cancer cases and 804 CIN3/CIS cases were reported. The hypothesized associations with environmental, hormonal and reproductive factors were assessed within the whole cohort. A nested case-control study within the EPIC cohort was also performed, including the sera from 184 invasive cases, 425 cases of CIN3/CIS, and 1,218 matched control women. They were tested for L1 antibodies against several HPV types and against *Chlamydia trachomatis* and Human Herpes Virus 2 to allow the adjustment for these variables in the statistical analyses and thus confirm associations obtained in the cohort study. Associations between serological markers of HPV infection, including L1, E6 and E7 antibodies, CT and HHV-2, and cervical cancer and pre-cancer risk were also assessed. Moreover, associations observed in the full cohort analysis were explored within the nested case-control, either adjusting by HPV infection as determined by HPV serology, or restricting the analysis to HPV seropositive women.

Article 1

The objective of this article was to estimate prospectively the association between serological markers of HPV, CT, and HHV-2 infections and the risk of developing cervical cancer and pre-cancer. Serological markers of four selected polyomaviruses, cutaneous HPVs and *Chlamydia Pneumoniae* were also used to contrast associations between sexually and non-sexually transmitted infections. Immunoassays were used to detect serum antibodies to HHV-2, CT, *Chlamydia Pneumoniae*, L1 proteins of mucosal and cutaneous HPV types, E6 and E7 oncoproteins of HPV types 16 and 18, and to four polyomaviruses.

L1 seropositivity to any mucosal HPV type was significantly associated with both CIN3/CIS and ICC. Associations with HPV types 16 and 18 E6 and E7 seropositivity were only significant for invasive cancer. By HPV type-specific serological markers, HPVs 11, 16, 18, and 45 L1 were associated with CIN3/CIS whereas only HPVs 11 and 16 L1 were significantly related to invasive cervical cancer risk. Furthermore, both HPVs 16 and 18 E6 and E7 serology were risk factors for invasive cancer, being the strongest association with HPV 16 E6 seropositivity (OR=10.2, 95% CI 3.3-31.1). Previous exposure to CT was strongly associated with ICC and weakly associated with CIN3/CIS, and HHV-2 seropositivity was marginally associated with both CIN3/CIS and ICC risk. Increasing number of sexually transmitted infections (HPV L1, CT and HHV-2) was associated with increasing both CIN3/CIS and ICC risk. In contrast, seropositivity to non-sexually transmitted infections, such as cutaneous HPVs L1, polyomaviruses, and *Chlamydia Pneumoniae*, was not associated with any of the two disease outcomes.

This large prospective study confirms a possible contribution of CT and HHV-2 in cervical carcinogenesis. It further identifies HPV 16 E6 seropositivity as a potential marker to predict invasive cervical cancer before the disease development.

Article 2

The aim of this article was to evaluate prospectively the association between tobacco smoking and the risk of pre-invasive and invasive cervical cancer.

In the cohort analyses, current smokers showed a two-fold increased risk of CIN3/CIS and ICC compared to never smokers. Smoking duration and intensity increased the risk of cervical cancer and pre-cancer, with a clear dose-response among ever smokers. Overall, smoking cessation was associated with a reduced by a half of the risk of both CIN3/CIS and ICC, although for pre-invasive cancer the risk reduction was statistically significant after quitting the habit for at least 10 years as opposed to at least 20 years for invasive cancer. Regarding passive smoking, none of the measures collected were associated with risk of developing cervical cancer and pre-cancer among never smokers.

In the nested case-control study, consistent associations were observed after adjustment for HPV L1, CT and HHV-2 serostatus, confirming the results obtained in the cohort. Increased risks of CIN3/CIS and ICC for current smoking, duration and intensity, and a reduced risk for quitting the habit were also found, although not statistically significant. Restricted analyses to HPV L1 seropositive women also showed similar associations for the different smoking variables.

Results from this large prospective study confirm the role of tobacco smoking as an important cofactor for both CIN3/CIS and ICC, even after taking into account HPV exposure as determined by HPV serology. The strong beneficial effect of quitting smoking is an important finding that will further support public health policies for smoking cessation.

Article 3

The purpose of this article was to prospectively evaluate associations between hormonal and reproductive factors and the risk of developing pre-invasive and invasive cervical cancer.

Being a parous woman was positively associated with CIN3/CIS risk, with magnitudes of two-fold, but not with ICC. The risk of pre-invasive cancer also increased with increasing number of full-term pregnancies. Duration of oral contraceptives use was associated with a significantly increased risk of CIN3/CIS and ICC, with relative risks of 1.6 and 1.8 respectively for more than 15 years of use compared to never use. Quitting the use for more than 5 years reduced the risk for CIN3/CIS to almost a half. Ever use of menopausal hormone therapy was associated with a significantly reduced risk of ICC (HR=0.5). No association was found between cervical cancer risk and ever use of IUD.

To confirm the associations obtained in the cohort study, analyses restricted to all cases and HPV seropositive controls were conducted, yielding similar results for parity, OC use and hormonal therapy use. Furthermore, a significant inverse association with IUD was observed for combined CIN3/CIS and ICC (OR=0.7).

Our results suggest that several hormonal and reproductive factors are cofactors for cervical carcinogenesis. Adherence to current cervical cancer screening guidelines should minimize the increased risk of cervical cancer associated with these hormonal risk factors.

DISCUSSION

DISCUSSION

In this section, the main results obtained from the three articles included in this thesis are being discussed. First, we present and discuss one by one the main epidemiological findings, including the risk and protective cofactors associated with cervical cancer and pre-cancer risk, the factors related to squamous cell carcinoma and adenocarcinoma separately, and the role of HPV serology in cervical carcinogenesis. Further, we discuss some methodological considerations, including potential strengths and limitations.

Environmental cofactors associated with invasive and pre-invasive cervical cancer risk

Table 1 summarizes the magnitude of risks of the multiple cofactors evaluated in this thesis at three different states: based on the evidence observed in the literature before the elaboration of this thesis, the contribution of our work, and the combined evidence to date. We display global averages of the relative risks and the direction of the associations, with minimum and maximum values observed from the different sources. Regarding our work, a global risk of the proposed cofactors is displayed, corresponding to the invasive cervical cancer outcome in the cohort analysis; when estimates of pre-invasive cancer or those obtained in the nested case-control study differed substantially, they are reported as well.

Cofactors	Evidence before this thesis	Evidence from the present work	Combined evidence to date
Tobacco smoking			
Current	↑2 (1.4-2.6)	↑1.9	↑2
Past	NA or ↑1.5 to 2 (1.3-3.3)	↑1.5	Suggestive of ↑1.5
Duration **(in years)**	↑2 to 3 (1.4-7.5) (for ≥5 to 20 years)	↑2.1 (for ≥30 years)	↑2 to 3 (for ≥10 to 20 years)
Intensity **(in cig/day)**	↑2 to 3 (1.5-6.5) (for ≥6 to 20 cig/day)	↑1.9 (for ≥20 cig/day)	↑2 to 3 (for ≥10 to 20 cig/day)
Early age at start	NA or ↑2	NA	NA
Smoking cessation **(in years)**	NA or ↓0.8 (for ≥15 years)	↓0.4 (for ≥20 years)	↓0.5 (for ≥20 years)
Passive smoking	NA or ↑2 (1.7-2.1)	NA	NA
OC use			
Current	NA or ↑1.5 to 2 (1.6-2.6)	↑2.2	↑2
Past	NA	↑1.6 (ICC); NA (CIS)	NA
Duration **(in years)**	NA or ↑2 to 3 (1.5-5.5) (for ≥5 to 15 years)	↑1.8 (for ≥15 years)	↑2 to 3 (for ≥10 years)
Cessation **(in years)**	NA or ↓ (for ≥15 years)	NA (ICC); ↓0.6 (CIS) (for ≥6 years)	Suggestive of a protective effect (≥ 10y)
Parity			
Number of FTP	NA or ↑2 (1.6-3.8) (for ≥4 FTP)	NA (ICC); ↑2.3 (CIS) (for ≥4 FTP)	↑2 (for ≥4 FTP)
IUD use			
Ever	NA or ↓0.5 (0.3-0.7)	↓0.7 (nested)	Limited evidence of a protective effect
HT use			
Ever	NA or ↓0.5 (ICC)	↓0.5 (ICC); NA (CIS)	Limited evidence of a protective effect (ICC)
Infection with CT			
Seropositivity	NA or ↑2 (1.5-5.0)	↑2.3 (ICC); NA (CIS)	↑2
Infection with HHV-2			
Seropositivity	NA or ↑ 1.5 (1.2-2.6)	NA	Limited evidence of a risk effect (ICC)

Abbreviations: NA: Not associated; ICC: invasive cervical cancer; CIS: carcinoma in situ, including CIN3; OC: oral contraceptives; FTP: full-term pregnancies; IUD: intrauterine device; HT: hormone therapy; CT: *Chlamydia trachomatis*; HHV-2: Human Herpes Virus 2.

Table 1. Summary of environmental cofactors associated with cervical cancer risk.

Tobacco smoking

- **We can conclude that current smoking increases the risk of invasive and pre-invasive cervical cancer 2-fold.**

An increased risk between current smoking and cervical cancer was found in our study, both with CIN3/CIS and ICC, showing magnitudes around 2-fold, slightly greater in precancerous lesions than in invasive cancer. This increased risk has been consistently reported in most previous studies, either in prospective or retrospective designs, with relative risks around 2, ranging from 1.4 to 2.6 [57,78,79,81,82,85,177,178]. Furthermore, associations were similar for analyses not adjusting, adjusting or restricting for HPV infection, either using HPV DNA or HPV serology.

- **However, associations between past smoking and risk of invasive and pre-invasive cervical cancer are inconsistent.**

A significant association of cervical cancer with past smoking has been shown in the cohort study of our article, with an increased risk of 50% for both CIN3/CIS and ICC. This association has been also reported before, but only in few studies and mainly for pre-invasive cervical cancer, with magnitudes globally less than 2 and ranging from 1.3 to 3.3 [57,79,82,177]. These lower values among past smokers compared to current smokers may point out to a protective effect of smoking cessation.

- **Importantly, cervical cancer risk increases with increasing duration and intensity of use 2- to 3-fold.**

In our analyses, smoking for more than 10-20 years and more than 10-20 cigarettes per day showed significant associations with pre-invasive and invasive cervical cancer risk. In particular, women need to have smoked for at least 10 years and one cigarette per day to have an increased risk of CIN3/CIS, while they need 20-30 years or 10 cigarettes per day to be at a higher risk of ICC. The magnitudes of these risks were around 2 to 3-fold higher compared to non-smokers, with a clear dose-response. Consistent with our results, nearly all previous studies have also found these solid associations with quite similar magnitudes [79,82,85,177,178].

Pack-years smoked is a variable created as a combination of duration and intensity of smoking. In our analyses, associations between smoking more than 10-20 pack-years and both CIN3/CIS and ICC were around 3-fold. A few of individual studies included in the reviews or pooled analyses have evaluated pack-years, finding quite similar associations [57,82]. It is expected that the combination of two risk factors will provide a higher magnitude of the effect than each

factor independently. However, methodological work has suggested that the use of intensity and duration as separate variables may lead to a better model fit than using pack-years [179].

- **Evidence on the effect of age at starting smoking is still inconclusive.**

Our analyses did not find an increasing risk of cervical cancer with decreasing age at initiation among smokers (p-trends not statistically significant). Consistent with our results, no associations were found in previous studies [81,85,178], except in the collaborative reanalysis conducted by the IARC that showed an increased risk among current smokers who started earlier compared to those who started later (p-trend<0.001) [82].

- **Quitting smoking reduces the risk of cervical cancer and pre-cancer at half.**

We found in our study that quitting smoking for long enough substantially reduced the risk of cervical cancer and pre-cancer. Women who had stopped smoking at least 10 years for CIN3/CIS and 20 years for ICC showed a statistically significant 2-fold decreased risk of disease compared to current smokers. In contrast, recent quitters for less than 10 years have only a slight reduction as compared to women who were current smokers. Few studies have evaluated time since quitting smoking in relation to cervical cancer risk, and, in agreement with our results, they found consistently risk reductions among women who had stopped smoking for more than 5 to 15 years, depending on the study, although statistically significance was only evident in some of them [82,105,110]. Taken together, these findings are of great importance from a public health perspective, as smoking is a modifiable risk factor that, if successfully avoided, may substantially decrease the risk of cervical cancer and pre-cancer.

- **Passive smoking seems not to be associated with pre-invasive and invasive cervical cancer risk.**

In our results, we did not find any association between passive smoking and cervical cancer risk. However, not all countries in the study contributed on passive smoking data, and those that did, frequently had incomplete information. Our results are consistent with those from a pooled analysis carried out of spouses of active smokers, concluding that passive smoking could not be detected as an independent risk factor of ICC in the absence of active smoking [180]. In contrast, a meta-analysis published in 2012, based on limited number of studies, showed that women who never smoked but were exposed to smoking had a significant 70% increased risk of cervical cancer compared to non-exposed women [86]. The assessment of passive smoking is challenging as it is usually difficult to rule out residual confounding, mainly because it is possible that the smoking habits of a woman's male partner are correlated with his sexual

behavior and, hence, related to HPV transmissibility. Moreover, there is no clear cut definition of lifetime never smokers.

Oral contraceptives use

- **Current users of OCs seem to have a 2-fold higher risk of cervical cancer and pre-cancer.**

Globally, our results, mainly from the cohort study, showed that current users of OCs had a higher risk of cervical cancer compared to non-users, with magnitudes around 2; lower associations were found for past and ever users. These results were in line with some previous studies, finding positive associations between current OC users and cervical cancer risk around 2 [102,105,106]. In contrast, other studies did not find any association, including the prospective cohort studies [83,84,103,109,111], and those adjusted or restricted by HPV serology [108,110]. The reasons of discrepancies found in the literature are not easy to elucidate, and could be related to the definition of current and past use, the study design, or the adjustment for confounding variables. Among past users, previous studies consistently did not find an association with cervical cancer and pre-cancer risk [83,103,105,109,111].

Taking into account that past and current users presented discordant risks of cervical cancer, the use of ever users to OCs in the analyses would not be the most appropriate variable. Indeed, previous studies found a lot of discrepancies in the associations between ever users and cervical cancer risk, with increasing risks in some studies and lack of association in others, for both ICC and CIN3/CIS [100,107,112,113,126,181–183]. However, some studies have only collected information on ever use of OCs, not being able to separate current from past users, and then the risk estimates would be difficult to explain and should be interpreted with caution.

- **Supporting evidence on a dose-response relationship increases the risk of cervical cancer and pre-cancer with duration of OC use.**

Our study found that risk of cervical cancer increased with duration of OC use, with magnitudes around 1.8 for users of more than 15 years, greater for invasive than for pre-invasive cancer. Our results were consistent with those published in pooled analyses and in almost all cohort studies (mostly retrospective), finding a range of relative risks of 1.5 to 5 for long-term OC users for more than around 10 years compared to non-users [100–102,105,126,181,184]. In contrast, the majority of case-control studies did not find any association with long-term duration of OC use [83,107,108,110–112]. A possible explanation of these discrepancies could be a

recall bias in case-control studies that leads to underestimate the relative risks. In this line, a recent meta-analysis, including 16 case-control studies, published by Peng et al in 2017 did not find any association for duration of 10 or more years of OC use compared to non-users [185]. This meta-analysis included only case-control studies, being heterogeneous in terms of population and outcome.

- **Effect of cessation of OC use is still inconclusive.**

Our results have shown a decreased risk to almost a half of developing CIN3/CIS for past users who quit taking OCs by more than 5 years compared to current users. This protective effect was not observed for invasive cervical cancer risk. Previous pooled reports and a case-control study conducted in South Africa showed a reduced risk of invasive cervical cancer among past users of OCs for more than 15 years [100–102,112]. The discrepancy between this finding and our results on invasive cancer risk could be related to a lack of power of our data, given that half of the countries did not collect that information and could not contribute to the corresponding analysis. The rest of previous studies, the majority of which did not take into account HPV status, have estimated the risks of cessation of OC exposure comparing past to never users instead of current, finding inconsistent results and being more difficult to evaluate and interpret [100,102,105,106,111,126,181–183].

- **There is not enough data to evaluate the combined effect of duration and cessation of OC use.**

Analyses combining duration and quitting of OC use were evaluated in our study, detecting a progressively decline risk of cervical cancer with increasing years since last use for both short and long-term OC users, more drastic for pre-invasive cases (data not shown in the article). Specifically, the estimated risks for the development of CIN3/CIS fell to values close to those seen in never users in women who had stopped taking OCs for at least 6 years irrespective of the duration of use (HR=1.2 and HR=1.3 for short and long-term OC users respectively). For ICC risk, in spite of the low number of cases, a continuous decline was also observed with increasing years since last use for both short and long-term OC users. These findings were more consistent in the cohort than in the nested case-control study. In the literature, previous pooled and multicentric studies found similar patterns of trend as those observed in our study [100–102]. Thus, it seems that the harmful effect due to the use of OCs in relation to cervical cancer was transient, and its cessation reduced the risk irrespective of the number of years used. However, the interpretation of these results must be taken with caution, since they were based on few cases and controls.

- **Findings related to association between cervical cancer and types of contraceptive composition are unclear.**

The available evidence indicates that sex steroid hormones, mainly estrogens and to a lesser extent progestogens, enhances the development of cervical cancer [67,68,114]. Specifically, a previous study conducted within the EPIC cohort showed that circulating levels of testosterone and possibly estradiol were positively involved in the etiology of invasive cervical cancer [68]. Unfortunately, in our cohort we were not able to evaluate these sex hormones as medications, because the questionnaires were not designed to gather enough detail about the type of hormonal contraceptives used, i.e. combined methods with estrogens and progestogens, and progestogen-only methods. Few studies have collected these data, finding no association for neither of the two OC types [83,112]. More studies including both types of hormonal contraceptives separately could be of interest to try to better elucidate the role of estrogens and progestogens in cervical carcinogenesis.

Parity

- **High number of full-term pregnancies (four or more) increases by 2-fold the risk of cervical cancer and pre-cancer.**

In our study, increasing number of full-term pregnancies augmented significantly the risk of CIN3/CIS for more than 2 times among women with 4 or more pregnancies compared with nulliparous women. In contrast, for ICC, no risk was observed among women who ever had full-term pregnancies. This result was quite unexpected, and could be partially explained by a detection bias due to differential screening practices related to parity. In effect, it is likely that nulliparous women tend to be less screened than parous women. In such case, nulliparous women would be less often detected in early stages of the disease, and thus would be more diagnosed of invasive cancer than parous women, increasing the number of invasive cases among nulliparous, and therefore reducing the association between multiparity and invasive cancer risk. The same rationale may have inflated the estimates of CIN3/CIS by increasing the number of pre-invasive cases among parous women because they would be more screened than nulliparous. The IARC studies have shown significantly higher risks for both pre-invasive and invasive cancer among women with high parity, at least more than 5 pregnancies, with magnitudes ranging from 1.6 to 3.8 [135,136,186]. These results are in agreement with some previous population-based case-control studies [106,107,109,110]. In contrast, prospective studies did

not find a significant association between high parity and risk of cervical cancer and pre-cancer [83,84,103,104]. The discrepancies found in the literature could be related to the different reproductive patterns across populations.

Intrauterine device use

- **Use of IUD seems to reduce the risk of invasive cervical cancer, although the evidence is limited.**

In our study, we found a non-significant decreased risk of CIN3/CIS and ICC among IUD users compared to non-users in the analysis adjusting for HPV serology, although when we combined CIN3/CIS and ICC the significance was reached (OR=0.7, 95% CI: 0.5-0.96). For ICC risk, a significant protective effect was also observed in analyses restricted to HPV seropositive women (OR=0.3, 95% CI: 0.1-0.9). Few studies have evaluated associations between IUD use and cervical cancer risk. Previous investigations that either adjusted for HPV infection or restricted the analyses to HPV positive women found a significant inverse association with risk of developing invasive cervical cancer, with estimations ranging from 0.3 to 0.7 [110,124,128]. Other studies, the majority of them without taking into account HPV infection, did find no significant association, although a moderate reduced risk was observed in mostly of them [104,123,125–127]. Cortessis et al have published in 2017 a meta-analysis showing a summary reduced risk of 0.6 (95% CI: 0.5-0.8) among women who used an IUD [187]. In 2018, Averbach et al published a case-control study, finding no associations between recent use of IUD (within 18 months) and risk of CIN3+ [188]. Possible explanations of discrepancies in the literature could be related to the adjustment or not by HPV infection, and to the use of separate or combined outcomes, including in situ and invasive cases. Taking into account previous as well as our own results, IUD use could act as a protective cofactor in cervical carcinogenesis, but at the current status of the evidence, it must be considered inconclusive.

Hormone therapy use

- **Evidence on the effect of hormone therapy use in cervical cancer is inconclusive, although a decreased risk for invasive cancer suggests a somewhat protective effect.**

In our study, the use of hormone therapy among peri- and postmenopausal women significantly decreased the risk of invasive cervical cancer at least at half, and the effect was

stronger with longer duration of use. No association was observed for pre-invasive cancer. Our findings were in line with the few previous published data [130–133]. Overall, the literature seems to rule out positive associations; if anything, a weak inverse association that rarely reached statistical significance was observed for invasive cervical cancer.

- **The effect of hormone therapy formulation in the risk of cervical cancer, including estrogen and/or progestogen hormones, is contradictory.**

In assessing hormone therapy, however, it is important to consider the hormonal composition of these treatments, taking into account the increased risk reported for endometrial cancer in the 1970s among postmenopausal women using estrogen therapy only. Studies that evaluated the composition of the therapy found some evidence for a reduced risk of invasive cervical cancer with ever use of estrogen therapy only as compared with never use [130–132]. When progestogen was included in the menopausal therapy, the inverse association with invasive cancer risk still remained [132,133]. Our data on hormonal therapy formulation was very limited since few centers collected this information; combined estrogen and progestogen therapy among menopausal women was inversely associated with invasive cervical cancer, while a positive association was found between use of estrogen alone and pre-invasive cancer. None of these associations were statistically significant. More evidence is needed on the hormone therapy formulation to clarify the role of estrogens and progestogens in the cervical carcinogenesis.

Infection with *Chlamydia trachomatis*

- ***Chlamydia trachomatis* seropositivity seems to increase the risk of invasive cervical cancer 2-fold.**

We found a positive association between CT seropositivity and risk of developing invasive cervical cancer, which is lower for pre-invasive cancer. This relationship was stronger for invasive cases in analyses restricted to HPV seropositive women (OR=4.3, 95% CI: 1.9-9.9; data not shown in the article). In agreement with our results, recent history of CT infection has also been associated with cervical cancer and pre-cancer risk in several previous studies, both using prospective and retrospective designs, as well as pooled analyses, with magnitudes near 2 [142–144,146–148,189,190]. The estimations were more clearly found for invasive than for pre-invasive lesions. In 2017, Zhu et al published a meta-analysis finding consistent 2-fold increased risk of

cervical cancer, including combined pre-invasive and invasive cases [191]. In contrast, a large prospective study conducted in Costa Rica did not find a significant association with incident CIN2+ cases [145], that could be explained by the assays used to determine CT status, that differed from those used in previous studies.

- **Co-seropositivity of HPV and *Chlamydia trachomatis* highly increases the risk of pre-invasive and invasive cervical cancer.**

When combined serological markers of HPV L1 and CT infections were evaluated in our study, a higher risk among women seropositive for both infections was observed for invasive (OR=5.7, 95% CI: 2.5-12.8; data not shown in the article) and pre-invasive cancer (OR=1.9, 95% CI: 1.2-3.1; data not shown in the article). Consistent with our results, previous studies also found that co-infection of HPV and CT had a higher risk of invasive cervical cancer and of combined pre-invasive and invasive cases, around 3- to 4-fold [191,192].

In the last 5 years, two new reviews on CT and its relation with HPV infection and cervical carcinogenesis have been published [193,194], suggesting that CT may increase the risk of HPV infection and persistence, being indirectly related to cervical lesions and reinforced by plausibility of biological mechanisms.

Infection with Human Herpes Virus 2

- **The effect of past exposure to HHV-2 on cervical cancer risk is unclear.**

In our study, HHV-2 seropositivity was marginally associated with CIN3/CIS and ICC, even among HPV seropositive women, with magnitudes around 1.5. These results were in concordance with many previous studies, including the most recent cohorts [147,158], finding not significant increased risks of cervical cancer among HHV-2 seropositive women. Only the pooled analysis published by Smith et al found significant results, which could be explained by the use of HPV DNA PCR-based assay instead of HPV serology, or by the large number of cases included (more than one thousand) [159]. Recently, a large meta-analysis of 20 observational studies reported a significant increase in risk of cervical cancer for case-control studies (n=14), while no association was found for the overall estimate of the six longitudinal studies [195]. A cross-sectional study based on data from 1999 to 2014 in the USA also reported a positive association between the prevalence of cervical cancer and HHV-2 seropositivity [196]. Despite

this, clear evidence supporting a risk effect of HHV-2 infection on cervical cancer was still insufficient.

- **Co-seropositivity of HPV and HHV-2 increases the risk of pre-invasive and invasive cervical cancer.**

The combination of serological markers of HPV L1 and HHV-2 infections were also evaluated in our study, and a significant higher risk among women seropositive for both infections was observed for pre-invasive and invasive cancer (OR=2.3, 95% CI: 1.5-3.8, and OR=2.4, 95% CI: 1.2-4.9 respectively; data not shown in the article). Co-infection of both viruses was also evaluated in Li et al, suggesting a higher risk for cervical cancer compared with seronegative women for both HPV and HHV-2 infections [196]. These results suggest a role of HHV-2 infection as a cofactor of HPV infection that increases the risk of cervical cancer, although more data is needed.

Co-infection with HPV, *Chlamydia trachomatis* and Human Herpes Virus 2

- **Co-seropositivity with HPV, *Chlamydia trachomatis* and HHV-2 increases the risk of cervical cancer and pre-cancer, but interaction effects are inconclusive.**

As described previously, the co-infection of CT and HHV-2 infections with HPV separately have shown increased risks of cervical cancer compared with seronegative women. In our study, we have also conducted analyses combining serological results of the three infections, obtaining a linear association between increasing number of sexually transmitted infections and pre-invasive and invasive cancer, stronger for CIN3/CIS. However, no statistically significant interactions were found between antibodies to HPV and antibodies to CT or HHV-2. Indeed, we do not know whether exposure to CT and HHV-2 truly contributes to the carcinogenic process with HPV as a cause of invasive cervical cancer, or whether the different infections tend to come together because of the common transmission route and thus reflecting a high-risk sexual behavior, or whether this increase in the risk of cervical cancer reflect a decrease of the immune response to clear infections. Further investigation is needed to better establish the role of the combined effects or the potential interactions between HPV and other sexually transmitted infections in cervical carcinogenesis.

Differences by cervical cancer histology (adenocarcinoma vs squamous cell carcinoma)

Cervical carcinomas can be divided according to their histological type: squamous cell carcinoma (SCC) account for approximately 80-85% of cases, adenocarcinoma of the cervix for around 15-20% of the cases, and minor subtypes account for less than 5% overall. In general, the proportion of adenocarcinoma cases is higher in developed countries with adequate cervical cancer screening programs, and thus with a global low incidence of cervical cancer [56]. This relatively high proportion of adenocarcinomas is due to the difficulty to detect precursors of these cancerous lesions by cytology, which occurs within the cervical canal. Furthermore, adenocarcinoma is associated with a poorer prognosis compared with SCC [197]. Relative and absolute incidences of adenocarcinoma cases, mainly in these regions, have risen in recent years [56].

Persistent high-risk HPV infection is the cause of both histological subtypes, although in squamous cell cases HPV 16 is the predominant type (55%), followed by HPV 18 (12%), whereas in adenocarcinoma cases HPV 18 is the most common type (37%), followed by HPV 16 (31%) [7]. Cofactors that can modulate the carcinogenesis of HPV infection may also differ among histology [186,198].

In our study, the majority of cervical cancer cases are squamous cell (n=901, 712 CIN3/CIS and 189 ICC), and the number of adenocarcinoma cases is small (n=52). Regarding the role of potential cofactors in cervical carcinogenesis, associations found and previously described with pre-invasive and invasive cervical cancer risk were globally comparable to those obtained in analyses evaluating pre-invasive and invasive SCC risk (data not shown). On the other hand, the small number of adenocarcinomas limited the analyses to assess the risks associated with these cofactors, and we were unable to separate in situ from invasive cases. Thus, we discuss below the main differences between cofactors associated with adenocarcinoma of the cervix and those obtained with SCC (Table 2). Globally, the principal differences appear to be related to smoking, and less consistently, to parity and past infection to *Chlamydia trachomatis*.

Cofactors	Squamous cell carcinoma	Adenocarcinoma
Tobacco smoking	↑	NA or ↓
OC use	↑	↑
Parity	↑	NA
IUD use	Limited evidence of a protective effect	Limited evidence of a protective effect
HT use	Limited evidence of a protective effect	NA
Infection with CT	↑	NA
Infection with HHV-2	Limited evidence of a risk effect	Limited evidence of a risk effect

Abbreviations: NA: Not associated; OC: oral contraceptives; IUD: intrauterine device; HT: hormone therapy; CT: *Chlamydia trachomatis*; HHV-2: Human Herpes Virus 2.

Table 2. Summary of cofactors associated with cervical squamous cell carcinoma and adenocarcinoma cases.

- **In contrast to SCC, smoking does not appear to be a risk factor for adenocarcinoma.**

Both the literature and our study did not find a relationship between smoking variables and cervical adenocarcinoma, even a somewhat inverse association was observed [82,106,186,198–201]. These results are opposed to those observed when evaluating squamous cell cases, both in our study and in the literature, finding consistent increased risks among smokers compared to non-smokers, stronger for long duration and high intensity [81,82,106,110,186,198,200]. Several large studies have performed a case-case analysis comparing the risks for SCC and for adenocarcinoma regarding tobacco smoking, and they found statistically significant differences (for current smoking: $p \leq 0.001$, and for past smoking: $p=0.01/0.04$) [82,106,186,198]. Some possible biological explanations could elucidate these differences. First of all, other epithelial cancers, such as those from the nasal cavity, the oesophagus and possibly the lung, also appear to show differences between SCC and adenocarcinoma in relation to smoking, with the effect of smoking being greater for squamous cell tumours [80]. In addition, it has been postulated that adenocarcioma of the cervix is etiologically related to adenocarcinoma of the endometrium [202]. In this sense, smoking, through a hypothesized anti-estrogenic action, decreases the risk of endometrial adenocarcinoma, which arises from estrogen excess [200].

- **Association of adenocarcinoma with parity is inconclusive, contrary to the increase risk observed for SCC.**

Our study was not able to estimate credible relative risks for cervical adenocarcinoma in relation to parity, owing the small number of cases. In the literature, inconsistent results were found among the most recent published studies. Although the majority of them support a moderate increased risk among multiparous women compared to nulliparous [106,135,137,186,198,199,203,204], only the pooled analyses reached the statistical significance. Regarding SCC, most previous studies have shown strong positive associations with multiparity, with generally significant trends [106,110,135,137,186,198,203]. Globally, there is some evidence that adenocarcinoma does not seem to be affected by a high number of full-term pregnancies to the same extent as squamous cell. When a direct case-case comparison between parity and risk of squamous cell and adenocarcinoma was conducted, the relative risks increased with number of full-term pregnancies for squamous cell compared to adenocarcinoma (relative risks about 1.3 and 1.5, with a statistically significance) [106,186,198].

- **Evidence is limited regarding association between adenocarcinoma and *Chlamydia trachomatis* infection, in contrast with the increased risk of SCC.**

Virtually no associations were found between adenocarcinoma cases and CT seropositivity, both in our study and in most previous ones [142–144,147,191,199,205], consistent with other studies that have evaluated CT DNA using PCR instead of serology [206,207]. The several studies that have evaluated the association between CT serology and SCC risk, including ours, found consistent higher risks among seropositive women [142–144,146–148,191,205]. Globally, the increased risk was specific for squamous cell and not for adenocarcinoma, something a little bit surprising since endocervical glandular cells are targets for *Chlamydia trachomatis* [142].

- **Similar associations are found between hormonal factors (OCs, IUDs, and HTs) and infection with HHV-2 in the risk of adenocarcinoma and SCC.**

Regarding other environmental cofactors, both histological subtypes seem to have similar patterns of risk. A higher risk of cervical adenocarcinoma among current and long-term users of OCs was found both in our study and in the literature, including several pooled analyses [106,111,186,198,199,208–210], and the associations were comparable to those obtained with SCC cases [100,106,110,111,186,198]. Although based on few studies, a decreased risk of cervical adenocarcinoma was observed among IUD users, both in our study and in the literature [128,199], similar to the virtually no increased risk, or even a possible protective effect, found for squamous cell cases

[127,128]. For HT use, there is very limited data for cervical adenocarcinoma risk, finding an increased risk observed in a previous study [132] as opposed to a protective effect obtained in our study; for squamous cell carcinomas, a protective effect among HT users was observed both in our study and in the previous ones [130–133]. Discrepant results were observed between the presence of HHV-2 antibodies and the risk of cervical adenocarcinomas [147,159,199], with higher risks mainly found in pooled analysis, and no associations observed in other studies; similar discrepancies were observed for HHV-2 seropositivity and risk of SCC [147,158,159].

- **Findings suggest that some of the cofactors that influence cervical adenocarcinoma are etiologically similar to those seen for endometrial adenocarcinoma.**

Taken together, parity was not clearly associated with risk of cervical adenocarcinoma, and an inverse relationship was found with smoking and IUD use, cofactors similar to those identified for endometrial adenocarcinoma [211,212]. In fact, endometrial adenocarcinoma is hypothesized to have an hormonal cause, in which any factor that increases exposure to estrogens (such as menopausal hormonal therapy, obesity, and irregular menstrual cycles) tends to increase the risk of the disease, while factors that decrease exposure to estrogens or increase progestogens levels (such as oral contraceptives or smoking) tend to be protective. However, contrary to endometrial cancer, other hormonal risk factors such as OC use are associated with an increased risk of cervical adenocarcinoma. Thus, it is still on debate weather risk factors associated with cervical adenocarcinomas are more similar to those established for cervical squamous cell cases or for endometrial adenocarcinoma.

Serological markers for HPV infection and invasive and pre-invasive cervical cancer risk

Mucosal HPV L1 types

- **HPV types 16 and 18 L1 are valid markers for past infection.**

In our study, positive associations were found for seroprevalence of L1 antibodies to HPV types 16 and 18 and invasive cervical cancer and pre-cancer risk, greater for HPV 16 than for HPV 18, around 2.5 and 1.5 respectively. These findings are consistent with those from previous prospective studies, mostly conducted in the Nordic countries, and from retrospective ones, with small variations in the magnitudes [146,147,189,190,213–217]; associations found with HPV 16 L1 ranged from 2.2 to 12.5, and for HPV 18 L1 varied from 1.5 to 2.8. After the publication of our work, two more recent studies conducted by Combes et al in 2014 and Kreimer et al 2015 published results on HPV antibodies and cervical cancer risk from a IARC multicentric case-control study and from a nested case-control design in the EPIC cohort, as our study [218,219]. In fact, this last study and ours share cases of cervical cancer. Briefly, Combes et al also found comparable results between serological markers of HPV 16 and 18 L1 and invasive cervical cancer [218]. Given that persistent high-risk HPV infection is the necessary cause of cervical cancer, the consistent association found between L1 seropositivity and cervical cancer risk is a strong indication that L1 antibodies against HPV types 16 and 18 are valid markers of past infection.

- **Seropositivity of HPV types L1, other than HPVs 16 and 18, are also potential markers of infection.**

Concerning other high-risk HPV types evaluated in our study, significant associations were found only for HPV 45 and risk of pre-invasive cancer. Little is known about the immune response to the L1 proteins of these high-risk HPV types, but statistical significant associations have been reported for HPV types 33, 31, 39, 58 and 59 with estimations between 1.5 and 3 depending on the subtype [189,215,217]. In our study, HPV types 31 and 58 were marginally associated with cervical cancer and pre-cancer risk. Globally, our associations were a little bit lower than those found in a previous nested case-control study conducted in Colombia [217]. Differences in the HPV assay, including the definition of several cut-offs, could explain these discrepancies.

With respect to HPV 11 L1, the only low-risk HPV type tested in our study, we obtained an increased risk with both pre-invasive and invasive cervical cancer. Previous studies have shown that HPV 6 and 11 correlated with seropositivity for oncogenic HPV types and with condyloma history [220]. Given the low sensitivity of the HPV serology assays, we cannot rule out that HPV 11 L1 positive cases in our study were indeed infected concomitantly or in the past by established high-risk HPV types. We could assume that HPV 11 L1 seropositivity is a marker of previous exposition to HPV and therefore of cancer risk, but in no case (neither for HPV 11 nor for the rest of the types tested by serology), cervical cancer or lesion attribution can be inferred from seropositivity.

- **In conclusion, mucosal HPV L1 types are solid markers of past infection.**

HPV L1 antibodies are therefore regarded as markers of previous exposure to both current and past infections, and so as markers of sexual behavior [74,221]. Of note, being negative for HPV L1 antibodies cannot be interpreted as absence of prior infection, as it is well-known that there is a proportion of infected women who do not seroconvert.

Furthermore, higher levels of naturally acquired antibodies detected using an L1-based VLP assay have shown a lower risk of a subsequent newly detected infection or cervical abnormality associated with the same HPV type, particularly with HPV 16 [76,222]. Type-specific seropositivity would be associated with a decreased risk of cancer and pre-cancer from the same type, but as a marker of exposure to HPV infection from any type, HPV seropositive individuals have globally a higher risk of disease.

Mucosal HPV E6 and E7 types

- **Pre-diagnostic seroprevalences of HPV types 16 and 18 E6 and E7 are significantly higher among invasive cervical cases than among controls.**

Baseline seroprevalences of antibodies against E6 and E7 proteins of HPV types 16 and 18 were estimated among cases and controls in our study, showing statistically significant differences only for invasive cervical cancer. Previous studies have also shown globally higher seroprevalences of HPVs 16 and 18 E6 and E7 antibodies among cases, accounting for 25 to 55% depending on the HPV serological assay [190,205,215,223–232]. These results, quite low, most likely indicate a lack of sensitivity of the HPV serological assays for proteins E6 and E7. Seroprevalences observed among controls were more similar across studies, including ours,

detecting globally less than 5-10% of seropositivity. The differences observed between studies might be partially a technical issue related to type-specific assay, or a more strict or lax threshold for positivity, in addition to the possibility of a biological principle of type-specific HPV immune response [223,233].

- **HPV 16 E6 seropositivity, and to a lesser extent HPV 16 E7, could be a good marker of development of invasive cervical cancer.**

We found that the strongest association was observed for HPV 16 E6 serology and invasive cervical cancer risk, with an OR of 10.2, and to a lesser extent with HPV 16 E7. No associations were found with pre-invasive cancer. Consistent with our results, seropositivity to the E6 oncogene of HPV 16 has been shown by far the largest association with invasive cancer risk, reaching an OR of 45.7 in a study conducted in Russia [190,205,218,219,224,232]. For HPV 16 E7, the magnitudes of risk were globally lower than those observed for E6, either in the literature and in our study, ranging from 1.6 to 17.5 [190,205,218,219,224,232]. Regarding the risk of pre-invasive cervical cancer, the associations found with E6 and E7 antibodies were less evaluated, finding moderate increased risks of about 1.4 to 1.8 [225,232].

- **The potential of seropositivity to HPV 18 E6 or E7 as a marker for cervical cancer risk is unclear.**

No statistically significant associations were found for HPV 18 E6 or E7 in cervical cancer risk in our study, probably due to the small number of cases, although estimations were globally high. In the same line, few studies have estimated associations with HPV 18 E6 and E7 alone, principally due to low number of cases [190,218,219]. Increased risks of cervical cancer among women seropositive for HPV 18 E6 were observed, with magnitudes around 2 to 6-fold [218,219]. Regarding HPV 18 E7 antibodies, Combes et al found a 12.2-fold higher risk of invasive cervical cancer [218], much higher and statistically significant than that found in our study and in a previous one [190].

- **In conclusion, HPV 16 E6, and to a lesser extent HPV 16 E7 and HPV 18 E6 and E7, could be a strong marker of invasive cervical cancer risk.**

Antibodies against E6 of HPV 16 have been found predominantly in patients with cervical cancer in comparison to controls, with estimations of risk statistically significant, suggestive of being therefore highly specific markers of cancer transformation [224,228,234]. The results were less consistent for antibodies against E7 of HPV 16 and against E6 and E7 of HPV 18. These findings suggest that screening for cervical cancer should be considered in the clinical examination of

patients who tested positive for HPV 16 E6, in particular in populations with a lack of adequate cervical screening. However, more data is needed to confirm the potential of HPV 16 E6 as a pre-diagnostic biomarker for cervical cancer, and more generally for HPV-driven cancers.

Methodological considerations

In this part, we discuss some methodological considerations concerning our study. We discuss the main strengths and limitations, and potential biases are also debated. The main characteristics of a cohort and a nested case-control design are presented, together with the convenience of the adjustment for infection by HPV using serology in the nested case-control study, and its relative value as compared with restriction by HPV infection, when assessing the role of cofactors found in the whole cohort analysis.

Strengths

Prospective design

The whole cohort approach

The EPIC study is a prospective cohort that included approximately half a million people enrolled in 10 European countries, which have been followed-up for an average of about fifteen years. This huge study provides the opportunity to evaluate a large number of cervical cancer and pre-cancer cases identified in the cohort, with enough power to detect associations with several environmental risk factors, including smoking, OC use and parity. The prospective design also makes it possible the assessment of exposure to these risk factors at different times before the diagnostic.

Bias, mainly selection and information bias, are a matter of concern in all observational studies. However, because of the prospective design, cohort studies are less prone to bias, mainly selection and information bias, and therefore they are less likely to affect the validity of the associations observed in the study. In the EPIC study, subjects were not randomly selected but they were samples of convenience of volunteers who agreed to participate. This means that EPIC is not a representative sample of the general population. However, this does not affect at all to comparison made within the cohort, for instance comparing the incidence of an outcome (i.e. cervical cancer) between smokers and non-smokers. Therefore, in spite of non-representativity, results on association based upon the cohort have internal validity. Recall bias, a specific case of the more general information bias, is often defined by the fact that

people with the outcome of interest are more likely to recall, minimize or magnify certain patterns or past exposures. It is not relevant in prospective cohorts because the information is collected at recruitment before the development of the disease. Therefore, even if some degree of measurement error is present in the assessment of the exposure, this misclassification is non-differential (i.e. affecting equally to cases and non-cases), and the expected effect is always shifting the association estimate to the null. Loss of follow-up is another type of bias, in this case, relevant in prospective studies. However, taking into account the huge number of participants included in the EPIC cohort, loss to follow-up has been so far minimal and should not have biased our results. Finally, detection bias could appear if the assessment of the outcome is somehow related to the exposure. In most EPIC centers, ascertainment of cancer cases is achieved by means of record-linkage with population cancer registries, making the procedure totally blind to the exposure status of participants.

The nested case-control approach

This type of design allows the assessment of risk factors that must be measured in biological samples. Instead of measuring markers of these factors in the whole cohort, the nested case-control design provides a cost-saving and efficient way to analyze the cohort. In this design, the biological samples used correspond to all cases and a sample of controls (typically one or two per case) sampled from the risk set, defined as the subjects who are alive and at risk of the disease at the time the case is diagnosed. Usually the controls are matched to cases by potential confounders. It must be kept in mind that, in spite of the name (case-control), this is a prospective design, retaining all the advantages of the cohort approach described above. Moreover, when properly analyzed by means of a conditional model taking into account the matching, the estimated odds ratio is an unbiased estimate of the incidence density ratio or the hazard ratio estimated in the whole cohort. In our study, blood samples of some cases and controls with available serum were collected at baseline, allowing the determination of serological markers of HPV, *Chlamydia trachomatis* and Human Herpes Virus 2 infections, several years before disease development. Furthermore, the evaluation of co-infection of these two sexually transmitted infections (CT and HHV-2) with HPV infection in the enhancement of cervical cancer development is also viable.

Results from the cohort and case-control studies

In addition to the specific objectives mentioned above, the inclusion of these biomarkers in the study is relevant because it allows a complementary approach to the cohort analysis. First, in the cohort, there is no information on the antecedent of HPV infection, the major determinant of cervical cancer, nor on sexual behavior that can be considered a proxy. In the nested case-control, there is possible to adjust for HPV seropositivity, that can be considered a marker or proxy of HPV infection, and there is possible as well further adjustment by past exposure to *Chlamydia trachomatis* and HHV-2 as proxy measures of sexual behavior. On the other hand, since persistent infection by HPV is the necessary cause of cervical cancer, the effect of some factors found in the cohort is interpreted as their contribution to cervical cancer risk in addition to the carcinogenic effect of HPV; this is why they are considered cofactors. The nested case-control allows to confirm or reinforce these results by restricting the analysis to HPV seropositive subjects, or alternatively to all cases (assumed have had previous HPV infection) and seropositive controls. In our study, results derived from the nested case-control study, that included the adjustment or restriction by HPV serostatus, globally confirm the results obtained in the cohort.

Limitations

Although our study has the strengths just mentioned above related to its prospective design, it has also some limitations. Since our main purpose is the evaluation of potential risk factors associated with cervical cancer risk, it would be necessary to include the status of HPV infection because of the evidence that HPV is a necessary factor in cervical cancer. However, we lack biological material to assess the most valid method to detect the infection by means of DNA. As already discussed, we only have serological markers of HPV infection. We did not collect any information on sexual behavior, although again, we may use data from the nested case-control of serology of different HPV markers as well as serology of *Chlamydia trachomatis* and HHV-2 as proxy measures. We lack also information on the individual screening practice and history of women included in the cohort. The potential relevance of this for the interpretation of our results will be discussed later on. Finally, in our study, the exposure information was collected at recruitment only; no repeated measurements were carried out during follow-up for all participants. We must assume that the baseline information remains unchanged during follow-up, and specifically for the cases, until the time of diagnosis. However, it must be acknowledged that some changes are possible, mainly for longer follow-

up periods. This would induce misclassification errors, which would be always non-differential, leading to a dilution or underestimation of the true effect. In the next section, we discuss with more detail specific methodological issues that might have affected our study.

Specific issues: analytic approach, and potential of bias and confounding

Analytical approach

As mentioned, there are two related questions: first we lack direct information of previous HPV infection; on the other hand, beyond the availability of such information, in a framework assuming that persistent infection by HPV is the necessary cause of cervical cancer, any other exposure found to be associated with cancer risk must be considered as a cofactor. Different strategies of analyses were implemented in our study to try to decide the best way to analyze our data taking into account the availability of only HPV serological data. The most rigorous way to deal with this would be restricting the analyses to HPV positive women. When statistical power is not enough to restrict the analyses to HPV positive women, adjustment by HPV could also be a valid approach.

Analyses adjusting by HPV L1 serostatus in our nested case-control study have shown similar associations between all cofactors analyzed and risk of CIN3/CIS and ICC to those found in the full cohort analyses, where HPV serostatus was not assessed. Globally, risk estimates were comparable although with wider confidence intervals in the case-control study, and thus statistical significance disappeared for some variables. These results reinforce the relevant associations found between different cofactors and cervical cancer risk in the cohort study. On the other hand, the adjustment by HPV L1 serology is necessary when assessing potential risk factors associated with cervical cancer risk, because it may somewhat reduce potential confounding effect due to HPV infection. However, we cannot totally rule out a residual confounding of HPV because of the low sensitivity of the HPV serology assays (50-70%), meaning that almost half of women were detected as HPV seronegative in our data when they actually have been infected.

Another approach to deal with this issue was to restrict the analyses to HPV seropositive women. Using this strategy, we found slightly weaker associations for the different cofactors evaluated and the risk of cervical cancer and pre-cancer when compared to those adjusting by HPV serology. In this analysis, lack of seroconversion of infected women could lead to loss of power, since some women (cases and controls) that were actually infected had a negative

serology. However, we can be confident that those women included in these analyses were certainly infected by HPV at some point during their lifetime. Therefore, in spite of some lower precision of estimates, they are valid estimates, or at least not affected by residual confounding due to HPV infection.

A similar approach was performed by restricting the analyses to all cervical cancer cases, assuming that virtually all of them were HPV positive, and HPV seropositive controls. The results obtained between potential cofactors and cervical cancer risk showed associations somewhat stronger, mainly with smoking indicators, than those obtained including only seropositive women. This suggests that the previous estimations might have been partially underestimated.

Having all the previous consideration in mind, we think that the best way to analyze cofactors related to cervical cancer risk in a nested case-control study is restriction to HPV seropositive women, either including only HPV seropositive cases or all cases. Indeed, because of the evidence that HPV is a necessary factor in cervical cancer, it should be an habitual approach to include analyses restricted to HPV positive women, either using DNA or serology, to assess accurately the contribution of additional factors to the risk of cervical cancer.

Classification of the disease

The diagnosis of both CIN3/CIS and ICC cases among the EPIC cohort is based on population cancer registries in seven countries (Denmark, Italy, The Netherlands, Norway, Spain, Sweden and the UK) and on a combination of methods including health insurance records, cancer and pathology registries, and on active follow-up through study subjects and their next-of-kin in three countries (France, Germany and Greece). The cases, including only women with first primary incident cancers, were classified as C53 (cervix uteri) according to the International Classification of Diseases, Injuries and Causes of Death, 10th revision (ICD-10). It is possible that some incident cervical cancer cases were not registered and thus not included as cases; this would lead to a loss of power of the study (most likely small); however, given the huge sample size of the cohort, the classification of a true case as a non-case would not affect the results.

Regarding pre-invasive cases, they were not systematically identified and consistently reported across cancer registries and EPIC centers. Clearly, ascertainment of pre-invasive cervical cancer cases is less complete than invasive cases, and greatly depends on the quality and population

coverage of cervical screening programs in the EPIC countries, the degree of systematic reporting to the corresponding cancer registry, and the accuracy of the cytopathology records. It is likely that reported pre-invasive cases are greater among countries with nationwide population-based screening programs, such as the UK, Sweden, and Norway, compared to other screening strategies. In countries with limited coverage and/or quality of screening programs, detection of pre-invasive cases could have been limited and some control women might indeed be cases, resulting in a potential attenuation of true associations. Probably our CIN3/CIS cases may not be fully representative of the true underlying population with this disease. In addition, not all cases of CIN3/CIS or ICC could be included in the nested case-control study, mostly due to the lack of sera availability. It is however unlikely that the availability of sera was related to a lifestyle habit or serostatus of subjects.

Residual confounding

In epidemiological studies, the term residual confusion generally refers to lack of complete control by all relevant confounders. However, it also must be applied to the incomplete control due to error measurements in some confounders. In prospective studies, the possible measurement error in the confounding variables is usually assumed to be non-differential with respect to the disease, meaning that the effect for these cofactors would be underestimated, but the potential effect on the exposure of interest depends on the direction and strength of the association of the (misclassified) confounder with both the exposure and the outcome (disease). In this project, we have not collected important variables to evaluate the risk of development of cervical cancer, such as HPV DNA infection, sexual behavior, or factors related to cervical cancer screening practices. Since HPV is the necessary cause of cervical cancer, analyses including some measure of HPV infection are needed to assess potential residual confounding. Furthermore, sexual behavior can be a confounding variable of cervical cancer taking into account the sexual transmissibility of the virus. These two factors are clearly associated with cervical cancer risk (in fact HPV infection is the major determinant), but they are most likely associated with cofactors of interest in our study, such as hormonal and reproductive factors and smoking habits. Factors related to cervical cancer screening could affect the detection of the outcome but could also be related with the reproductive history of the women, and may indeed influence the effects on cervical cancer risk. To try to minimize this problem, other variables have been used as adjustment or restriction instead of these ones.

Serological markers of HPV infection were analyzed among all cancer cases and matched controls with serum available in our nested case-control study. Antibodies of HPV L1 are known to be a marker of past and cumulative HPV exposure, so they could be considered a good surrogate indicator of sexual behavior, and even, of risk of HPV exposure [74]. Thus, HPV serostatus could be a good proxy of sexual activity, and we are quite confident that this indicator must be used in our analyses.

Regarding sexual behavior, even if our study did not explicitly collect data related to sexual habits (only available for France and partially for Spain), serological markers of HPV infection, as well as of *Chlamydia trachomatis* and HHV-2, may be regarded as good markers, considering the sexual route of transmission of these infections. Marital status, an adjusting variable of our analyses, could also be used as a proxy of sexual activity.

Regarding cervical cancer screening practices, although the study did not collect individual data on Pap smears, number of pregnancies and variables related to contraceptive methods used in the past can be used as surrogate markers of screening-related behavior. In the case of screening practices, in addition to the problem of residual confounding, there could be, at least in theory, a potential for bias (detection bias). It is reasonable to assume that women who are pregnant or those who use hormonal therapy (contraceptive or not contraceptive) are more likely to be screened. Thus, to the extent that identification of cases (detection) is related to an exposure of interest (i.e. parity, or OC or HT user), the misclassification is no longer non-differential. In our study this may have affected mostly to CIN3. Therefore, it could be that the increased risk of CIN3 estimated for parity or hormone use is overestimated. Moreover, by the same rationale, this could lead to an underestimation of the effect of these exposures in the risk of invasive cancers.

A final comment must be considered concerning the potential for residual confounding and bias that may have affected our study. It is important to note that most studies that have collected information on HPV infection, sexual behavior or screening practices accurately and have adjusted or even restricted for these variables reported consistent associations with cervical cancer risk, including tobacco smoking, OC use, parity, and to some extent IUD and HT use. Furthermore, there is also biological plausibility and mechanisms that have been proposed for all variables identified as risk or protective cofactors of cervical cancer, suggesting that associations found in our study were not entirely due to residual confounding or bias, and are quite reliable.

HPV serology: technical issues

HPV serology is a useful tool for the identification of women previously exposed to HPV infection. There are several valid laboratory methods to evaluate HPV type-specific antibody titers based on HPV capsids (also VLPs), and mainly focused on Immunoglobulin G (IgG) antibody concentrations [235]. The majority of serologic studies have used an enzyme-linked immunosorbent assay (ELISA) to measure serum antibody to capsid proteins; other studies have also used either in vitro neutralisation assays or a competitive Luminex based immunoassay (cLIA). In 2001, a new method for multiplex serology was developed, permitting antibody analyses of a large number of sera against up to 100 antigens in parallel, and using viral L1 proteins expressed as glutathione S-transferase (GST) fusion proteins as antigens instead of the use of VLPs [236–238]. It can also detect E6 and E7 proteins based on GST-glutathione interaction. HPV seropositivity depends on the assay used, and therefore serological data are not directly comparable between studies using different techniques, with different characteristics, sensitivities, and cut-offs [239]. However, most of these assays show a low sensitivity, as only 50 to 70% of women with past HPV infections show seroconversion [74,75]. Hence, it is likely that an unknown proportion of women were misclassified as not having been exposed to HPV, leading to underestimate the association between these markers and risk of disease. In addition, this low sensitivity may limit the usefulness of the serological assays as a diagnostic test for HPV infection.

Despite the difficulties to validate these assays, serological techniques have shown a good concordance with detection of viral DNA in the cervix [74,238]. We were unable to test for HPV DNA type either in cervical tumors or in controls in our study, being somehow a limitation of the study. However, it is worth to mention that most epidemiological studies test for HPV DNA but at only one time point, which favors the detection of HPV DNA in cases but not in controls, more likely to have a transient infection if they test positive just once. This could bias the measure for lifetime exposure, and thus the impact of HPV infection. In any case, the debate about which measure of HPV is more appropriate to use in different studies is still open.

CONCLUSIONS

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- Strong positive associations are found between risk of cervical cancer and pre-cancer and increasing levels of smoking duration and intensity, with a clear dose-response effect. These associations also remain after adjusting or restricting the analyses to those individuals with evidence of previous exposure to HPV by serology.
- Smoking cessation is associated with a 2-fold risk reduction of cervical cancer and pre-cancer. Again, this association is also evident after adjustment or restriction for markers of exposure to HPV.
- Four or more full-term pregnancies increase the risk of cervical cancer and pre-cancer. A dose-response relationship between cervical cancer and pre-cancer risk and duration of OC use is found, with a suggestive protective effect after cessation of use.
- IUD use and menopausal hormone therapy could confer a moderately reduced risk of cervical cancer, although these findings need further confirmation.
- Previous exposure to *Chlamydia trachomatis,* and to a lesser extent Human Herpes Virus 2, is associated with cervical cancer risk, both at pre-invasive and invasive stages. Co-seropositivity of HPV, *Chlamydia trachomatis* and Human Herpes Virus 2 increases the risk of both outcomes.
- No associations are found between non-sexually transmitted infections and risk of cervical cancer and pre-cancer.
- HPV L1 seropositivity to mucosal types seems to be a valid marker of past and cumulative HPV exposure.
- Seropositivity to HPVs 16 and 18 E6 and E7 is positively associated with invasive cervical cancer, but not with pre-invasive cancer. HPV 16 E6 seropositivity could be a good marker of invasive cervical cancer, and could be useful to predict cervical cancer well before disease development.

IMPLICATIONS IN PUBLIC HEALTH

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There are consistent and strong beneficial health effects of quitting smoking, mainly concerning heart diseases and cancer. The finding of a risk reduction of cervical cancer to about half for past users that quit the habit for almost 20 years compared to current smokers is important, and further supports public health policies for smoking cessation.

The findings of an increase in cervical cancer risk among long-term users of oral contraceptives are consistently found. However, this effect seems to be transient and probably limited to long-term users. The current evidence also recognizes significant associations with breast and liver cancers. In contrast, cancer risk seems to be reduced for endometrium and ovary sites. Globally, the benefits obtained by the use of oral contraceptives, including the unwanted pregnancy avoidance especially in developing countries, outbalance the increase in risk of some cancers, and should not require a major change in the current family planning strategies. Adherence to current screening recommendations will certainly minimize the potential increased risk of cervical cancer associated with oral contraceptives use.

Consistent results show that antibodies against oncoprotein E6 for HPV 16 could be specific markers for invasive cervical cancer. Other studies also show even higher associations for oropharyngeal and anal cancers. Patients with HPV 16 E6 seropositivity are at more than 132- to 200-fold and 76-fold increased risk of oropharyngeal and anal cancer respectively compared to seronegative patients [219,240]. These findings are of great importance for developing cancer screening tools and programs. It could be relevant to confirm HPV 16 E6 as a potential pre-diagnostic marker of HPV-related cancers.

RESUM EN CATALÀ

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INTRODUCCIÓ

El càncer de coll d'úter

El càncer de coll uterí és el quart càncer més freqüent entre les dones d'arreu del món, amb unes estimacions de 527.624 casos nous i 265.672 morts l'any 2012, el que correspon a gairebé un 8% del total de càncers incidents i de morts en les dones [12]. Prop del 85% dels casos es produeixen en països en vies de desenvolupament. Las taxes de incidència estandarditzades per edat més elevades (>30 per 100.000 dones) s'observen en països de l'Àfrica Sub-Sahariana i Melanèsia; en canvi les taxes més baixes (<6 per 100.000 dones) s'observen en països de l'oest d'Àsia, i de Australia i Nova Zelanda. A Europa, les taxes oscil·len entre el 3,6 per 100.000 dones a Suïssa i el 28,6 per 100.000 dones a Romania.

El rol del Virus del Papil·loma Humà (VPH) en la etiologia del càncer de coll d'úter

Fins ara, s'han identificat més de 200 tipus diferents del VPH, alguns infecten la pell i d'altres les mucoses [28]. Els tipus del VPH que infecten les mucoses es divideixen en tipus d'alt risc i tipus de baix risc segons la seva carcinogenicitat [29]. Els genotips del VPH identificats com a alt risc inclouen, entre d'altres, els tipus 16, 18, 31, 33, 35, 45, 52, i 58, responsables de aproximadament el 90% de tots els casos de càncer de coll uterí arreu del món [31]. El VPH 16 és el més freqüent en tot el món, responsable del 61% de tots els casos de càncer cervical, seguit del VPH 18, que representa un 10% del total de casos. Els genotips del VPH 6 i 11 s'han identificat com a tipus de baix risc, principalment associats a berrugues genitals.

El VPH és un virus de transmissió sexual molt comú entre els homes i les dones sexualment actius, principalment joves que acaben d'iniciar les seves relacions sexuals. Es calcula que més d'un 70% de les dones sexualment actives s'infectaran pel VPH en algun moment de les seves vides [10]. Ara bé, el 90% d'aquestes infeccions pel VPH són asimptomàtiques i transitòries, i desapareixen de forma espontània en els següents 2 anys [37]. Només una petita fracció de les infeccions persistents, principalment degudes a tipus d'alt risc, progressen a lesions precanceroses. Si aquestes lesions no es tracten, poden acabar progressant i convertir-se en un càncer cervical invasor [38].

En els darrers 40 anys, estudis epidemiològics relacionats amb el VPH han reconegut el paper central de la infecció pel VPH en la etiologia de pràcticament tots els càncers de coll d'úter [4–6]. Aquesta evidència epidemiològica ha permès considerar la infecció persistent per tipus oncogènics del VPH com la causa necessària del càncer de coll uterí.

El rol dels cofactors en la etiologia del càncer de coll d'úter

Tot i que el VPH és la causa necessària del càncer cervical, ja hem vist que únicament una petita fracció de les dones infectades desenvolupa lesions cervicals neoplàsiques, i eventualment un càncer cervical, pel que la infecció pel VPH és una causa necessària però no suficient del càncer de cèrvix. Per tant altres factors, juntament amb el VPH, intervenen en el procés patològic de la malaltia (Figura 6). Aquests cofactors podrien definir-se com aquelles exposicions que influeixen en el risc de progressió d'infecció pel VPH a malignitat. A part dels factors de risc propis de la infecció pel VPH (genotip, variants, càrrega viral, integració, infeccions múltiples), els cofactors candidats s'han classificat en dos grups: els ambientals i els propis de l'individu [3,53,57,59]. En aquesta tesi estudiarem principalment els factors ambientals, que inclouen el tabac, factors hormonals i reproductius (paritat o número d'embarassos, ús d'anticonceptius hormonals, dispositius intrauterins i teràpia hormonal substitutiva), i els agents infecciosos de transmissió sexual, com la *Chlamydia trachomatis* (CT) i el Virus Herpes Humà tipus 2 (HHV-2).

Les dades dels principals estudis sobre les lesions precanceroses i el càncer de coll uterí, que ajusten per infecció per VPH o inclouen únicament dones infectades pel VPH, aporten suficient evidència per concloure que l'ús del tabac [81,82], l'ús prolongat de contraceptius orals [100–102], i una alta paritat [135,136], augmenten el risc de progressió d'una infecció pel VPH a una lesió precancerosa o a un càncer cervical. Les associacions amb el tabac són les més fortes i coherents, amb estimacions del risc estadísticament significatives entre 1.5 i 7. Les associacions amb l'ús de contraceptius orals i una alta paritat oscil·len entre 1.5 i 5.5 segons la durada d'ús dels contraceptius (entre més de 5 i 15 anys d'ús), i entre 1.6 i 3.8 segons el nombre d'embarassos (a partir de 4 embarassos). Existeixen pocs estudis que hagin estudiat l'ús dels dispositius intrauterins [110,124–128] o de la teràpia hormonal substitutiva en dones menopàusiques [130–133] en relació al càncer cervical i als seus precursors, i suggereixen una disminució del risc d'un 50% en aquelles dones que utilitzen aquest tipus d'hormones comparat amb les que no les utilitzen; la significació estadística es dóna en pocs estudis. En quant a les dades dels principals estudis epidemiològics que han avaluat l'associació entre el risc de càncer o pre-càncer cervical i altres infeccions de transmissió sexual com la *Chlamydia*

trachomatis [142,143] i el Virus Herpes Humà tipus 2 [158,159], s'observa un augment de risc de aproximadament el doble en les dones infectades per CT i HHV-2 comparat amb les dones no infectades, associació més clara amb la infecció per *Chlamydia trachomatis*.

Justificació

El principal objectiu d'aquesta tesi és estimar de forma més acurada les associacions entre els cofactors ambientals i el risc de desenvolupar un càncer cervical invasor o pre-invasor utilitzant les dades de l'estudi EPIC, una cohort prospectiva que inclou més de mig milió d'individus reclutats en 10 països europeus i seguits durant gairebé 10 anys. Per tant l'EPIC representa un recurs únic a nivell mundial per a dur a terme investigacions prospectives sobre l'etiologia dels càncers i altres malalties, integrant dades de qüestionari sobre estils de vida i biomarcadors.

OBJECTIUS

Els objectius específics de la tesi són:

1) Estudiar prospectivament la relació dosi-resposta entre el tabac i el risc de desenvolupar un càncer cervical, incloent duració, intensitat, o deixar de fumar, per invasor i pre-invasor, utilitzant dues aproximacions: l'anàlisi de tota la cohort, i en el cas-control aniuat dins de la cohort que té en compte la infecció prèvia per VPH.

2) Avaluar de forma prospectiva l'associació entre diferents factors hormonals i reproductius i el risc de patir un càncer de cèrvix invasor o pre-invasor, en concret, l'alta paritat, l'ús d'anticonceptius orals, de dispositius intrauterins i de teràpia hormonal substitutiva en dones menopàusiques, tant en l'estudi de cohort com en el cas-control aniuat ajustant o restringint per la infecció prèvia al VPH.

3) Determinar prospectivament l'associació entre els marcadors serològics per infecció de *Chlamydia trachomatis* i el Virus Herpes Humà tipus 2 i el risc de desenvolupar un càncer o un pre-càncer cervical, utilitzant el cas-control aniuat que té en compte la infecció prèvia per VPH.

4) Avaluar les associacions entre els marcadors serològics del VPH per a la proteïna L1, incloent els tipus 11, 16, 18, 45, 31, 33, 35, 52 i 58, amb el risc de desenvolupar un càncer o un pre-càncer cervical, utilitzant el cas-control aniuat.

5) Explorar les associacions entre els marcadors serològics per a les oncoproteïnes del VPH E6 i E7 dels tipus 16 i 18 i el risc de desenvolupar un càncer de cèrvix invasor o pre-invasor, utilitzant el cas-control aniuat.

MÈTODES I RESULTATS PRINCIPALS

En el present treball, hem seguit una cohort de 308,036 dones reclutades dins l'estudi EPIC amb l'objectiu d'avaluar prospectivament les associacions entre diferents factors ambientals i el risc de desenvolupar un càncer cervical invasor o pre-invasor. Durant un període mitjà de 9 anys de seguiment, es van identificar 261 casos de càncer invasor (ICC) i 804 casos de càncer pre-invasor (CIN3/CIS). També es va dur a terme un estudi de casos i controls aniuat dins la cohort EPIC, incloent els sèrums de 184 casos invasors, 425 casos de CIN3/CIS i 1.218 controls aparellats. Es van testar anticossos contra la proteïna L1 pels tipus del VPH 11, 16, 18, 31, 33, 35, 45, 52 i 58, i contra les oncoproteïnes E6 i E7 pels tipus del VPH 16 i 18, així com anticossos contra la *Chlamydia trachomatis* i el Virus Herpes Humà tipus 2. L'objectiu d'aquest estudi cas-control va ser el de confirmar les associacions prèviament obtingudes en l'estudi de cohorts, així com estimar les possibles associacions entre els marcadors serològics d'infecció per VPH, CT i HHV-2 i el risc de patir un càncer de coll uterí o un pre-càncer.

L'article 1 va estimar prospectivament l'associació entre els marcadors serològics d'infecció per VPH, *Chlamydia trachomatis* i Virus Herpes Humà tipus 2 i el risc de desenvolupar un càncer de coll uterí o un pre-càncer. Els principals resultats van determinar que la seropositivitat per la proteïna L1 a qualsevol tipus de VPH mucós es va associar tant a CIN3/CIS com a ICC. Les associacions amb la seropositivitat per les oncoproteïnes E6 i E7 pels tipus del VPH 16 i 18 només van ser significatives pel càncer invasor. Segons els marcadors serològics específics per tipus, els VPH 11, 16, 18 i 45 per la proteïna L1 es van associar a pre-càncer, mentre que només els tipus del VPH 11 i 16 per la proteïna L1 es van relacionar de forma significativa amb el risc de càncer invasor. La serologia pel VPH 16 a les oncoproteïnes E6 i E7 es van identificar com a factors de risc pel càncer invasor, trobant la associació més forta amb la seropositivitat pel VPH 16 E6 (OR = 10.2). L'exposició passada a la *Chlamydia trachomatis* es va veure fortament associada al càncer invasor, i de forma més feble al càncer pre-invasor. La seropositivitat al Virus Herpes Humà tipus 2 es va associar de forma més marginal al risc de desenvolupar tant CIN3/CIS com ICC. Un major nombre d'infeccions de transmissió sexual, incloent VPH L1, CT i HHV-2, es va associar a un augment del risc de lesions precanceroses i de càncer cervical. En

canvi, la seropositivitat a infeccions de transmissió no sexual, com els VPHs cutanis, els poliomavirus i la *Chlamydia Pneumoniae*, no es van veure associats amb un major risc de cáncer o pre-càncer cervical. Per tant, aquest estudi prospectiu ens confirma una possible contribució de la *Chlamydia trachomatis* i del Virus Herpes Humà tipus 2 en la carcinogènesi cervical. A més, identifica la seropositivitat al VPH 16 per la oncoproteïna E6 com un possible marcador per a predir el càncer de coll uterí invasor abans del desenvolupament de la malaltia.

L'article 2 va avaluar de manera prospectiva les possibles associacions entre el tabac i el risc de desenvolupar un càncer cervical pre-invasor o invasor. En les anàlisis de l'estudi de cohorts, les dones que fumaven en el moment del reclutament van mostrar un augment de risc de aproximadament 2 cops de desenvolupar un CIN3/CIS o un ICC en comparació amb les que no van fumar mai. A més, fumar durant més de 20 anys o amb una intensitat de més de 10 cigarretes al dia va augmentar el risc de càncer cervical i pre-càncer en 2-3 vegades, amb una clara dosi-resposta entre les que havien fumat alguna vegada. El fet de deixar de fumar es va associar a una reducció del risc a la meitat pel desenvolupament de lesiones precanceroses i càncer, tot i que en el cas del càncers pre-invasors la reducció del risc va ser estadísticament significativa després d'abandonar l'hàbit de fumar durant almenys 10 anys, en canvi es necessitaven com a mínim 20 anys pel càncer invasor. Pel que fa al tabaquisme passiu, cap de les mesures recollides es va veure associada a un major risc de desenvolupar un càncer cervical o un pre-càncer entre les no fumadores. En l'estudi de casos i controls aniuat, es van observar associacions consistents després d'ajustar les anàlisis per l'estatus serològic de VPH, CT i HHV-2, confirmant els resultats obtinguts en la cohort. Les anàlisis restringides a les dones seropositives pel VPH per la proteïna L1 també van mostrar associacions similars per a les diferents variables de tabac. Per tant, els resultats d'aquest estudi prospectiu confirmen el paper del tabac com a cofactor important pel desenvolupament de les lesions cervicals precanceroses i canceroses, fins i tot després d'haver tingut en compte l'exposició al VPH a partir de la determinació amb serologia. A més, el gran efecte beneficiós de deixar de fumar és una troballa important que permetrà reforçar les polítiques de salut pública per deixar l'hàbit tabàquic.

L'article 3 va avaluar prospectivament les associacions entre diferents factors hormonals i reproductius i el risc de desenvolupar un càncer cervical invasor o un pre-càncer. Els principals resultats van mostrar que la paritat es va veure associada de forma positiva al risc de desenvolupar un CIN3/CIS, amb una magnitud de 2, però no al risc de patir un càncer invasor. L'augment en el nombre d'embarassos es va associar a un augment en el risc de tenir una lesió precancerosa. La durada en l'ús d'anticonceptius orals es va associar a un risc significativament

major de desenvolupar un CIN3/CIS o un ICC, amb riscos relatius al voltant de 1,6 i 1,8 respectivament durant més de 15 anys d'ús en comparació amb les dones que no n'havien utilitzat mai. Deixar d'utilitzar anticonceptius orals durant com a mínim 5 anys va reduir el risc de CIN3/CIS a gairebé la meitat. L'ús de la teràpia hormonal substitutiva es va associar a un menor risc de patir un càncer cervical invasor (HR = 0,5). No es va trobar cap associació significativa entre l'ús de dispositius intrauterins i el càncer de coll uterí. Per confirmar les associacions obtingudes en l'estudi de cohorts, es van dur a terme anàlisis restringides a tots els casos i als controls seropositius pel VPH, obtenint resultats similars. A més, es va trobar una associació inversa estadísticament significativa entre l'ús dels dispositius intrauterins i el risc de la combinació de càncer cervical invasor i pre-invasor (OR=0.7). Per tant els nostres resultats suggereixen que diversos factors hormonals actuen com a cofactors en la carcinogènesi cervical. L'adhesió a les pautes actuals del cribratge de càncer de coll uterí haurien de minimitzar l'augment de risc de càncer associat a aquests factors hormonals.

DISCUSSIÓ

De forma clara i consistent, fumar dobla el risc de desenvolupar un càncer de coll uterí invasor o pre-invasor. A més, aquest risc es veu augmentat quan s'incrementa la durada o la intensitat d'ús, de 2 a 3 vegades superior comparat amb els no fumadors. En canvi, les associacions observades entre haver estat fumador i el risc de tenir un càncer o un pre-càncer cervical són inconsistents; mentre alguns estudis troben un augment de risc, entre ells el nostre, d'altres no troben cap associació. En aquesta línia, el fet de deixar de fumar redueix el risc de patir un càncer cervical invasor o pre-invasor a la meitat. Aquesta troballa, observada tant en el nostre estudi com en treballs previs, mostren aquest efecte protector a partir dels 15 anys després d'haver deixat de fumar. En canvi, el tabaquisme passiu, caracteritzat per aquelles dones que no han fumat mai però que estan exposades al fum del tabac degut al seu entorn, no està associat a un major risc de càncer o pre-càncer cervical, tot i que és difícil poder avaluar de forma vàlida aquesta associació.

En quant als anticonceptius orals, les dones que els utilitzen sembla que tenen un risc de càncer o pre-càncer de coll uterí 2 vegades superior a les dones que no els utilitzen. Aquests resultats s'han observat en el nostre estudi i en alguns estudis previs, tot i que n'hi ha d'altres que no troben cap associació. Estudis de cohorts o estudis en els que s'han dut a terme anàlisis combinades troben que aquesta associació és més clara i consistent quan s'augmenta la

durada dels anticonceptius a 5-10 anys, sent de 2 a 3 vegades superior comparat amb les dones que no han pres mai aquests contraceptius. Quan mirem l'efecte de deixar els anticonceptius orals, els resultats no són concloents, tot i que suggereixen una reducció en el risc de càncer i pre-càncer cervical. Mirar aquest efecte segons la seva durada d'ús podria ser més interessant ja que són dues variables molt relacionades, i sembla que es troba una disminució progressiva del risc de càncer de coll uterí a mesura que augmenten els anys des del últim ús, tant per a usuàries de curta durada com de llarga durada, amb tendències més clares per a casos pre-invasors. Tot i això, no hi ha prou dades per avaluar correctament l'efecte combinat de la durada i de deixar d'utilitzar anticonceptius orals. Respecte a l'associació entre el càncer cervical i els tipus d'anticonceptius utilitzats, ja siguin estròtgens i progestàgens combinats o progestàgens sols, no hi ha prous estudis per aclarir el paper de les diferents hormones sexuals en la carcinogènesi cervical.

Tenir més fills augmenta el risc de càncer cervical invasor i pre-invasor, sent el doble entre les dones que han tingut quatre fills o més comparat amb les dones que no han tingut fills. Aquests resultats es troben en alguns estudis previs, principalment en els de casos i controls que inclouen poblacions amb una alta paritat; en canvi, altres estudis no troben cap relació amb la paritat. El nostre estudi troba un augment de risc de CIN3/CIS en les dones amb una alta paritat, però no de càncer invasor, possiblement degut a un biaix de detecció a causa de les pràctiques de cribratge diferencials segons la paritat.

L'ús d'altres mètodes anticonceptius com els dispositius intrauterins sembla que redueix a la meitat el risc de desenvolupar un càncer de coll uterí invasor, tot i que la evidència és limitada. Aquest efecte protector es troba tant en el nostre estudi com en estudis previs, tot i que la significació estadística s'assoleix en pocs d'aquests treballs, principalment als que han tingut en compte la infecció per VPH en les seves anàlisis.

L'evidència respecte al efecte de l'ús de les hormones de teràpia substitutiva en dones menopàusiques en el càncer de coll uterí no són concloents, tot i que els resultats suggereixen un cert efecte protector contra el càncer cervical invasor. L'efecte de la formulació de la teràpia substitutiva, tant si són estròtgens com si són progestàgens, dóna resultats poc clars.

En quant a la seropositivitat per altres infeccions de transmissió sexual, haver tingut una infecció per *Chlamydia trachomatis* dobla el risc de càncer de coll uterí, principalment invasor. Aquesta associació es troba també en la gran majoria d'estudis previs. Quan la infecció per *Chlamydia trachomatis* s'ajunta amb la infecció per VPH, el risc de càncer cervical és més gran, tot i que no hi ha una interacció estadística. L'efecte de l'exposició passada al Virus Herpes

Humà tipus 2 respecte al risc de càncer cervical no està clar, tot i que la majoria d'estudis, inclòs el nostre, troben un augment de risc moderat i no significatiu en molts casos, possiblement degut a una falta de poder estadístic. La combinació de les infeccions per VPH i Virus Herpes Humà tipus 2 fa augmentar el risc de càncer pre-invasor i invasor, esdevenint les associacions significatives en la majoria dels casos. La infecció pels tres virus de transmissió sexual estudiats augmenta el risc de càncer i pre-càncer cervical, tot i que els efectes d'una possible interacció no són concloents.

Pel que fa a la seropositivitat pel VPH, els tipus 16 i 18 per la proteïna L1 són, de forma molt consistent, marcadors vàlids d'infecció passada, sobretot el VPH 16, amb associacions positives de entre 2 i 12. La seropositivitat pels altres tipus del virus d'alt risc per la proteïna L1 que no són el 16 i 18 també es poden considerar bons marcadors d'infecció prèvia. L'efecte potencial del VPH 11, un tipus de baix risc, per la proteïna L1 com a marcador d'infecció passada no està clara. En resum, els tipus mucosos d'alt risc del VPH per la proteïna L1 són marcadors sòlids d'infecció passada al virus.

En quant a les seroprevalences basals del VPH pels tipus 16 i 18 per les oncoproteïnes E6 i E7, aquestes són significativament més elevades entre els casos invasors que entre els controls, resultats observats tant en el nostre estudis com en treballs previs. Si mirem la seropositivitat pel VPH tipus 16 per la oncoproteïna E6, trobem de forma consistent que podria ser un bon marcador de desenvolupament de càncer cervical invasor; en menor mesura, també s'observa pel VPH 16 per la oncoproteïna E7. Pel que fa a la seropositivitat pel VPH 18 per les oncoproteïnes E6 o E7, el potencial marcador de risc de càncer cervical no està del tot clar.

També és important discutir algunes consideracions metodològiques de l'estudi dutes a terme en les nostres anàlisis, incloent les fortaleses i limitacions del projecte. Com a principals fortaleses, destaquem el tipus de disseny prospectiu que inclou més de mig milió de participants, i que dóna suficient poder estadístic per detectar associacions entre el risc de càncer cervical i els diferents factors de risc ambientals avaluats. En l'estudi cas-control aniuat dins de la cohort prospectiva, incloem mostres de sang dels casos i dels controls, que ens permeten tenir en compte infeccions prèvies a VPH, *Chlamydia trachomatis* i Virus Herpes Humà tipus 2, i que per tant ens permeten ajustar o restringir millor les nostres anàlisis.

Respecte a les limitacions del projecte, en primer lloc, la identificació dels casos pre-invasors (CIN3/CIS) no s'ha fet de forma sistemàtica en tots els centres EPIC, i depenia molt de la qualitat i la cobertura poblacional dels programes de cribratge; països com el Regne Unit, Suècia o Noruega, amb programes de cribratge poblacional, van reportar una taxa més elevada

de casos pre-invasors que els altres països europeus. Aquesta mala classificació d'alguns controls que en realitat serien casos podria haver induït una atenuació de les associacions reals. Per una altra banda, com en la majoria d'estudis epidemiològics, hi pot haver certa confusió residual, que normalment es refereix a la falta de control total de les variables confusores, però que també pot ser degut a errors de classificació d'aquestes variables. En aquest projecte concret, no es va recollir informació de variables importants com la infecció pel VPH utilitzant ADN, el comportament sexual o factors relacionats amb les pràctiques de cribratge. Per minimitzar aquesta possible confusió, es van utilitzar variables proxies com a ajustament en els models, com podien ser l'ús de la serologia per VPH, *Chlamydia trachomatis* o Virus Herpes Humà tipus 2, l'estat civil, el número de fills o l'ús de mètodes anticonceptius. Pel que fa als factors relacionats amb les pràctiques de cribratge, sembla que la identificació dels casos podria estar relacionada amb l'exposició d'interès, com podien ser la paritat o l'ús d'anticonceptius orals, i per tant la mala classificació seria diferencial, i podríem estar sobreestimant les associacions trobades amb el càncer pre-invasor, o subestimant les associacions trobades amb càncer invasor. Ara bé, la majoria d'estudis previs que van recollir la informació correctament i van ajustar o fins i tot restringir les anàlisis per aquestes variables, van trobar associacions coherents amb les nostres, tant per tabaquisme, com per ús d'anticonceptius orals, paritat o, fins i tot, DIU i hormones substitutives. A més, existeix una plausibilitat biològica per a totes les variables identificades com a cofactors de risc o de protecció respecte al càncer cervical que explicaria les associacions trobades, la qual cosa suggereix que els nostres resultats no són totalment deguts a una confusió residual, i per tant són bastant fiables. Per una altra banda, l'ús de la serologia per detectar la infecció per VPH enlloc del ADN és important perquè únicament entre un 50 i un 70% de les dones infectades seroconverteixen, i per tant és probable que una proporció desconeguda de dones no s'hagi classificat correctament. Aquesta mala classificació suposaria probablement una subestimació de les associacions trobades entre aquests marcadors i el risc de patir el càncer cervical.

CONCLUSIONS

Les conclusions de la tesi són les següents:

- S'han trobat associacions fortes entre el risc de desenvolupar un càncer de coll uterí o un pre-càncer i un augment en la duració i la intensitat de fumar, amb una clara dosi-

resposta. Aquestes associacions també es mantenen després de tenir en compte l'exposició prèvia al VPH a partir de la determinació amb serologia.

- Deixar de fumar s'associa a una reducció a la meitat del risc de desenvolupar un càncer de coll uterí o un pre-càncer. Novament, aquestes associacions també es veuen després d'ajustar o restringir segons els marcadors d'exposició al VPH.
- Tenir quatre o més fills augmenta el risc de desenvolupar un càncer cervical invasor o pre-invasor. Es troba una dosi-resposta entre l'ús prolongat d'anticonceptius orals i el risc de tenir un càncer o un pre-càncer cervical, amb un efecte protector que sembla aparèixer uns anys després de deixar-ne l'ús.
- L'ús dels dispositius intrauterins i de la teràpia hormonal substitutiva en dones menopàusiques sembla que donin una certa protecció al risc de desenvolupar un càncer de coll uterí, tot i que aquests resultats necessiten una major confirmació.
- L'exposició prèvia a la *Chlamydia trachomatis* i, en menor mesura al Virus Herpes Humà tipus 2, s'associa al risc de patir una lesió precancerosa o un càncer cervical. La co-seropositivitat entre VPH, *Chlamydia trachomatis* i Virus Herpes Humà tipus 2 augmenta el risc de càncer i pre-càncer cervical.
- No es troben associacions entre les infeccions que són de transmissió no sexual i el risc de desenvolupar un càncer cervical invasor o pre-invasor.
- La seropositivitat per a tipus mucosos del VPH per la proteïna L1 sembla un marcador vàlid d'exposició acumulada i passada al VPH.
- La seropositivitat pels tipus 16 i 18 del VPH per les oncoproteïnes E6 i E7 s'associen positivament al càncer cervical invasor, però no a les lesions precanceroses. La seropositivitat pel VPH 16 per la oncoproteïna E6 podria ser un bon marcador de càncer cervical invasor que també fos útil per a predir el càncer cervical abans del desenvolupament de la malaltia.

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ANNEX

ANNEX

Dietary factors and in situ and invasive cervical cancer risk in the European prospective investigation into cancer and nutrition study.

González CA, Travier N, Luján-Barroso L, Castellsagué X, Bosch FX, **Roura E**, Bueno-de-Mesquita HB, Palli D, Boeing H, Pala V, Sacerdote C, Tumino R, Panico S, Manjer J, Dillner J, Hallmans G, Kjellberg L, Sanchez MJ, Altzibar JM, Barricarte A, Navarro C, Rodriguez L, Allen N, Key TJ, Kaaks R, Rohrmann S, Overvad K, Olsen A, Tjønneland A, Munk C, Kjaer SK, Peeters PH, van Duijnhoven FJ, Clavel-Chapelon F, Boutron-Ruault MC, Trichopoulou A, Benetou V, Naska A, Lund E, Engeset D, Skeie G, Franceschi S, Slimani N, Rinaldi S, Riboli E.

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Dietary factors and *in situ* and invasive cervical cancer risk in the European prospective investigation into cancer and nutrition study

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Some dietary factors could be involved as cofactors in cervical carcinogenesis, but evidence is inconclusive. There are no data about the effect of fruits and vegetables intake (F&V) on cervical cancer from cohort studies. We examined the association between the intake of F&V and selected nutrients and the incidence of carcinoma *in situ* (CIS) and invasive squamous cervical cancer (ISC) in a prospective study of 299,649 women, participating in the European Prospective Investigation into Cancer and Nutrition study. Cox proportional hazard models were used to estimate adjusted hazard ratios (HRs) and 95% confidence intervals (95% CI). A calibration study was used to control measurement errors in the dietary questionnaire. After a mean of 9 years of follow-up, 253 ISC and 817 CIS cases were diagnosed. In the calibrated model, we observed a statistically significant inverse association of ISC with a daily increase in intake of 100 g of total fruits (HR 0.83; 95% CI 0.72–0.98) and a statistically nonsignificant inverse association with a daily increase in intake of 100 g of total vegetables (HR 0.85: 95% CI 0.65–1.10). Statistically nonsignificant inverse associations were also observed for leafy vegetables, root vegetables, garlic and onions, citrus fruits, vitamin C, vitamin E and retinol for ISC. No association was found regarding beta-carotene, vitamin D and folic acid for ISC. None of the dietary factors examined was associated with CIS. Our study suggests a possible protective role of fruit intake and other dietary factors on ISC that need to be confirmed on a larger number of ISC cases.

Cervical cancer is the second most frequent cancer in women worldwide between the age of 15 and 44 years and the most common in developing countries.[1] Out of over 100 human papillomaviruses (HPV) types, Types 16, 18, 31, 33 and 45 account for up to 83% of all cervical cancers cases.[2] HPV is established as a necessary but not as a sufficient cause for cervical cancer.[3] Endogenous and exogenous cofactors might influence the risk of developing cervical cancer in combination with HPV.[4] Cofactors include long-term use of oral contraceptives, sexually transmitted infections such as human immunodeficiency virus, chlamydia and herpes simplex virus Type 2, high parity, smoking and diet.[5] Host and viral factors such as HPV genotype, HPV variant, coinfection of multiple HPVs, viral load and viral integration are other cofactors of cervical carcinogenesis.

A small number of infected women are unable to clear the HPV infection and will develop persistent HPV infection. Cofactors may function to increase viral persistence as well as acting in combination with viral persistence playing a role in the development of cervical intraepithelial neoplasia (CIN) and eventually invasive squamous cervical cancer (ISC). However, up to 55–60% of the CIN2-3 lesions experience spontaneous regression,[6] and most of the women infected with HPV will not develop cervical cancer.

Until now, only a small number of case–control and cohort studies looked at the role of diet intake as a cofactor for cervical cancer or as a risk factor for HPV persistence.[5,7] In a recent comprehensive review, an international expert committee concluded that there is limited evidence suggesting that carrot intake may protect against cervical cancer.[7] For other dietary factors such as vegetables, fruits, retinol, vitamin E, vitamin D, vitamin C, beta-carotene and folate, the data were either too sparse or too inconsistent to allow conclusions to be reached.

The aim of this work is to prospectively assess the association between intake of fruits, vegetables and other selected nutrients and the risk of developing carcinoma *in situ* (CIS) and ISC in the EPIC (European Prospective Investigation into Cancer and nutrition) study.

Material and Methods

Study participants

EPIC is a large prospective study carried out in 23 centers from ten European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom. The study has been fully described elsewhere.[8] The EPIC cohort consists of 521,448 subjects, mostly aged 35–70 years and recruited between 1992 and 1998.

Our study uses the data from female participants of the EPIC cohort, after *a priori* exclusion of women with prevalent cancer at any site during baseline examination. Most women were recruited from the general population residing in a specific geographical area, a town or province. Exceptions were the French cohort, based on members of the health insurance for employees; the Utrecht cohort from the Netherlands and the Florence cohort from Italy, both including women attending breast cancer screening; a part of the Italian and the Spanish cohorts including mostly members of local blood donor organizations and finally, most women of the Oxford cohort in England which included vegetarian and health-conscious volunteers. All eligible subjects were invited to participate in the study. Those who accepted gave

informed consent and completed questionnaires on diet, lifestyle and medical history and were invited to a center for blood collection and anthropometrical measurements including height and weight. The EPIC study was approved by the ethical review committees from participating centers.

Diet and lifestyle questionnaires

The usual diet over the previous 12 months was measured in all subjects by country-specific questionnaires.[8] Most centers adopted a self-administered questionnaire of 88–266 food items, whereas the centers in Greece, Ragusa (Italy) and Spain performed a face-to-face dietary interview. In France, Ragusa (Italy) and Spain, dietary questionnaire, structured by meals, was used. Questionnaires in France, Germany, Greece, Northern Italy, the Netherlands and Spain were quantitative, estimating individual average portion sizes systematically. Those in Denmark, Naples (Italy), Norway and Umea (Sweden) were semiquantitative, with the same standard portion assigned to all participants. In Malmo (Sweden), a modified diet history was used, combining 168-item questionnaire with a 7-day menu book and a structured interview. In Spain, a diet history method was used, through a computerized program. A food frequency questionnaire and a 7-day record were adopted in the United Kingdom. All dietary measurement instruments have been validated previously.[9]

Lifestyle questionnaires included questions on education, lifetime history of smoking and alcohol, occupation, reproductive history, use of hormones, history of previous illness and surgeries as well as physical activity.

Follow-up and end points

In Denmark, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom, follow-up was based on population cancer registries. In France, Germany and Greece, a combination of methods was used including health insurance records, cancer and pathology registries and active follow-up through study participants and their next-of-kin. Mortality data were obtained from mortality registries at regional or national levels. ISC included first primary incident cancers coded as C53 according to the International Classification of Diseases, Injuries and Causes of Death, 10th revision (ICD-10). CIS included cervical intraepithelial neoplasias (CIN 2 and CIN 3) and CIS, classified according to behavior and morphology (ICDO2) information. Follow-up was calculated until date of diagnosis of cervical cancer (CIS or ISC) or the date at which follow-up ended, defined as the last date at which follow-up data were judged to be complete or the last date of contact in the centers that used active follow-up (ranged from 2003 to 2006, depending on the center).

Statistical methods

The Cox proportional hazard regression method was used to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs). The analysis was stratified by center to control for potential center effects related to different follow-up procedures and questionnaire designs. Age was used as the primary time variable in all models. Entry time was defined as the woman's age at recruitment and exit time as age at diagnoses (cases) or age at censoring (at risk women). All models were adjusted for energy intake (continuous), body mass index ($<$18.5, 18.5–24.9, 25–29.9 and $>$30), number of full-time pregnancies (0, 1, 2, 3, 4 and $>$5), ever use of oral contraceptives taking into account duration in years (never, 1 year or less, 2–4 years, 5–9 years, 10–14 years, $>$15 years and missing), smoking status (never, former $<$ 15 years, former $>$ 15 years, current $<$ 10 cg/day, current $>$ 10 cg/day? and missing), education (none, primary school, technical/professional school, secondary school, university and missing), marital status as a proxy for multiple sexual contacts (single, divorced/separated, married/living together, widowed and missing), number of births (none, 1, 2, 3, 4 or more and missing), leisure and work physical activity (inactive, moderately inactive, moderately active, active and missing) and lifetime consumption of alcohol (never, former $<$ 12 g/day, former $>$ 12 g/day, 1–6 g/day, 7–18 g/day, 19–30 g/day, 31–60 g/day, $>$61 g/day and missing). The p value for trend across quartiles was calculated by assigning a score range from 1 to 4 according to their quartile of intake. This variable was entered as a continuous term in the Cox regression models.

Analysis of effect of fruits and vegetables (F&V) was based on estimated intake from the dietary questionnaires and calculated in grams per day and analyzed as quantitative and as categorical by quartiles. The total vegetables group consists of the following subgroups: leafy vegetables (except cabbages), fruiting vegetables, root vegetables, cabbages, mushrooms, grain and pod vegetables (peas and corn), onion and garlic, stalk vegetables and sprouts, mixed salads and mixed vegetables and unclassified vegetables. Potatoes and other tubers were not included. The subgroups leafy, fruiting, root, cabbages, mushrooms, onions and garlic are studied separately, whereas the remaining subgroups are not studied, because they only account for a small portion of total vegetables intake.[10] Total fruit consumption consisted mainly of all sorts of fresh fruits (90%) but also a small proportion of dried and canned fruits. Fruits are also studied for the subgroups of citrus fruits and hard fruits. Other subcategories as well as F&V juices are not studied because they only account for a small proportion of total F&V intake.[10]

Selected nutrients included in the analysis were intake of vitamin C, vitamin E, vitamin D, beta-carotene, retinol and folic acid, estimated from EPIC's questionnaire using the EPIC's food composition table.[11] Description of intake based on the 24-hr recall from the calibration study, used to compare the mean intake by country, did not include folate because it was not available in the EPIC's food composition table.

Calibration

We used a detailed computerized 24-hr diet recall (24-HR) method[12] administered once through a face-to-face interview

Table 1. Descriptives of fruit, vegetables and selected nutrients for countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort[1] (women)

Country	Cohort sample	Person-years	Cases cervical cancer[2]		Mean (SD) daily intake[3]						
			CIS	ISC	Total vegetables	Total fruits	Vitamin C	Vitamin D	Vitamin E	Beta-carotene	Retinol
Denmark	24,484	188818.9	62	33	142.95 (131.46)	180.04 (197.11)	107.31 (91.80)	3.89 (6.63)	8.47 (4.84)	3750.28 (6383.49)	732.34 (1230.41)
France	61,325	696248.4	50	29	208.29 (161.83)	235.69 (204.89)	112.73 (74.28)	2.69 (4.55)	10.97 (7.83)	4226.07 (4246.75)	644.01 (1455.02)
Germany	23,720	196870.6	50	24	170.86 (146.55)	219.90 (215.57)	123.74 (93.68)	3.16 (7.15)	13.57 (10.42)	3812.59 (5658.48)	743.49 (1807.08)
Greece	13,776	100,387	4	13	209.24 (173.23)	218.32 (216.08)	110.40 (81.83)	2.68 (3.61)	15.21 (8.70)	2491.80 (3474.70)	377.83 (2083.78)
Italy	27,744	239415.1	21	18	183.17 (157.37)	327.68 (258.08)	117.61 (91.20)	1.84 (3.26)	10.09 (5.66)	2387.74 (2481.00)	416.92 (2142.39)
Norway	31,343	188256.2	157	21	126.27 (125.20)	156.34 (164.69)	97.85 (75.48)	4.18 (5.81)	7.76 (4.19)	2436.42 (3938.92)	765.60 (918.43)
Spain	22,784	224772.9	22	23	198.24 (185.49)	344.69 (272.90)	140.09 (108.74)	3.82 (5.86)	13.19 (8.85)	2252.38 (2605.54)	433.06 (1464.29)
Sweden	26,381	279439.4	164	40	131.09 (114.44)	167.31 (166.46)	95.31 (71.47)	6.03 (4.62)	7.77 (3.67)	2063.01 (1561.74)	1200.67 (1561.74)
The Netherlands	21,917	192974.6	3	19	135.99 (116.7)	193.80 (187.08)	106.74 (80.86)	3.80 (4.56)	10.85 (6.84)	1986.53 (2770.01)	751.88 (1232.63)
United Kingdom	46,177	393483.4	284	33	145.70 (131.11)	172.28 (191.69)	116.96 (85.86)	3.25 (4.09)	10.10 (6.27)	3219.90 (4283.36)	489.64 (1081.66)
Total	29,9651	2,700,667	817	253	163.20 (145.72)	213.93 (211.41)	111.27 (84.43)	3.59 (5.20)	10.52 (7.25)	2922.74 (4031.94)	706.47 (1576.13)

[1]Study centers per country: France (North-East, North-West, South, South coast); Italy (Florence, Varese, Ragusa, Turin, Naples); Spain (Asturias, Granada, Murcia, Navarra, San Sebastian); United Kingdom (Cambridge, Oxford [general and health-conscious population]); The Netherlands (Bilthoven, Utrecht); Germany (Heidelberg, Potsdam); Greece; Sweden (Malmö, Umeå); Denmark (Aarhus, Copenhagen). [2]Cases uncertain whether benign or malignant ($n = 164$) were censored and those missing for behavior of the tumor ($n = 13$) were considered as noncases. [3]In g (vegetables and fruits), mg (vitC and vitE), and μg (vitD, beta carotene and retinol). Based on 24-hr recall dietary questionnaire of the calibration study participants (women = 23,440). This information was not available for folic acid.

Abbreviations: CIS: *in situ* cervical cancer (including CIN2 and CIN3); ISC: Invasive squamous carcinoma; SD: standard deviation.

Table 2. Baseline characteristics of the female participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort overall and according to quartiles of intake of total fruits and vegetables[1,2]

		Total observed vegetables intake, g/day[3]		Total observed fruits intake g/day[4]	
	Whole cohort (n = 299,651)	Quartile 1 [<117] (n = 74,912)	Quartile 4 [>286] (n = 74,913)	Quartile 1 [<125] (n = 74,912)	Quartile 4 [>337] (n = 74,913)
Mean age, years (SD)	50.24 (9.92)	49.82 (9.64)	50.56 (10.49)	48.67 (9.67)	51.19 (10.13)
Mean BMI, kg/m^2 (SD)	24.86 (4.41)	24.88 (4.31)	25.09 (4.68)	24.56 (4.30)	25.53 (4.68)
Ever tobacco smoking (%)	43	49.72	35.81	53.42	35.14
Mean number of cigarettes per day in current smokers (SD)	13.17 (7.94)	13.63 (7.58)	13.06 (9.0)	14.32 (7.79)	12.36 (8.59)
Mean years of time since quitting in former smokers (SD)	14.70 (9.88)	14.14 (9.71)	15.04 (10.06)	13.91 (9.68)	14.75 (9.94)
Secondary school or higher (%)	51.73	40.66	60.23	45.61	52.88
Mean energy intake, kcal/day (SD)	1,947 (542)	1,732 (478)	2,139 (568)	1,752 (495)	2,137 (566)
Mean number of full-term pregnancies (SD)	1.94 (1.24)	1.93 (1.22)	1.96 (1.28)	1.89 (1.22)	1.98 (1.28)
Marital status single (%)	11.56	9.92	13.07	11.93	11.13
Ever use of oral contraceptives (OC) (%)	59.65	62.37	53.44	68.50	47.83
Mean duration of OC in ever users (SD)	6.60 (5.02)	7.12 (5.24)	5.98 (4.72)	7.08 (5.14)	5.80 (4.80)
Mean physical activity (SD)	2.78 (1.23)	3.18 (1.41)	2.50 (0.94)	2.97 (1.42)	2.67 (1.01)
Median alcohol intake at recruitment, g/day (range)	3.42 (238.48)	2.38 (238.48)	3.58 (176.57)	4.02 (217.75)	2.32 (176.57)
Lifetime median alcohol intake, g/day (range)	4.07 (385.34)	3.92 (385.34)	3.49 (339.72)	5.30 (308.88)	2.72 (385.34)

[1]Intakes determined from the EPIC dietary questionnaire data. [2]For continuous variables, wilcoxon rank sum tests were used. For categorical variables, chi_square tests were used. [3]p value for trend <.0001 except for BMI (<0.01). [4]p value for trend <.0001 for all variables.
Abbreviations: SD: standard deviation; BMI: body mass index.

Table 3. Mean intake (range) of total vegetables and total fruits according to quartiles in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total vegetables (g/day)	115.0 (<117)	149.2 (117–185)	190.4 (186–285)	233.4 (>286)
Total fruits (g/day)	117.3 (<125)	190.5 (125–217)	247.6 (218–337)	330.3 (>337)
Vitamin C (mg/day)	78.66 (<86.37)	103.06 (86.37–118.32)	120.95 (118.33–163.53)	142.85 (>163.54)
Vitamin D (ug/day)	2.33 (<2.04)	2.96 (2.05–3.14)	3.85 (3.15–4.86)	5.11 (>4.87)
Vitamin E (mg/day)	8.91 (<7.20)	10.20 (7.21–9.72)	11.33 (9.73–13.38)	13.36 (>13.39)
Beta-carotene (ug/day)	1907.18 (<1892.52)	2627.76 (1892.53–2923.88)	3135.90 (2923.89–4306.75)	3960.06 (>4306.76)
Retinol (ug/day)	426.29 (<287.24)	647.68 (287.25–490.09)	840.77 (490.10–994.22)	949.40 (>994.23)

Ranges are based on the values reported on the food frequency questionnaires, and means were estimated from the 24-hr dietary recall data from the calibration study.
Information of folic acid is not available from the 24-hr recall.

to obtain a second dietary measurement from a random sample of the cohort (7.1% of total cohort; $n = 28{,}716$ women in our analysis) to correct for systematic over- or underestimation of dietary intakes.[13] Using these data, food intake estimated from the food frequency questionnaires can be transformed to a common scale, enabling comparisons of cancer risk in relation to food intake to be made across all EPIC centers as a whole. The 24-HR values were regressed on the intake values from the main dietary questionnaires. Weight, height, age at study recruitment and study center were included as additional covariates, and data were weighted by the day of the week and the season of the year in which the 24-HR diet recall data were collected. Cox regression models were then run using the predicted (calibrated) values of the variables of interest for each individual on a continuous scale. The standard error of the deattenuated coefficient was calculated with bootstrap sampling in the calibration and disease models consecutively.[14]

Results

The original number of women in the cohort was 343,518. We excluded from the analysis 509 women with no lifestyle data, 34,973 with previous hysterectomy and 8,385 women who were in the top or bottom 1% of energy intake. The remaining women used for the analysis were 299,651 (person years 2,700,667), and after a mean follow-up of 9 years, 253 ISC and 817 CIS cases were diagnosed and used in the analysis (Table 1). The high proportion of CIS cases from the United Kingdom, Sweden and Norway is explained by the routine and systematic registry of these cases in the country cancer registries. There were also 164 cases who were classified as uncertain benign *versus* malignant tumor behavior, and these were censored at date of diagnosis.

Table 1 also shows the mean intake of total vegetables, total fruits, vitamin C, vitamin E, vitamin D, beta-carotene and retinol by country, estimated using 24-HR recall data collected in the calibration study. Both fruit and vegetables consumption varied between countries by approximately two-fold, and there was also a great variation regarding nutrient intake. Baseline characteristics of the participants according to observed intake levels of F&V in quartiles are reported in Table 2 for the upper and lower quartile. Women with the highest intake of F&V were less likely to have ever smoked and ever used oral contraceptives than women in the lowest category. In addition, women in the highest quartile of F&V were older and had a higher BMI mean when compared to the lowest quartile of intake. Furthermore, they were more likely to have a higher mean energy intake.

Table 3 shows the daily intake levels of total vegetables, total fruits and selected nutrients within each quartile of intake. The mean intake of vegetables in the highest quartile was more than two times higher than in the lowest quartile. The mean intake of fruit in the highest quartile was approximately three times higher than in the lowest quartile.

Table 4 presents data on the estimated HRs for the risk of CIS and ISC associated with total vegetables and fruits intake and some subcategories of F&V. In the observed model, we found a nonsignificant association between ISC and total fruit intake. After correction for measurement error in the calibrated model, the association was statistically significant (HR = 0.83; 95% CI 0.72–0.98 for a daily increase of 100 g). We found a statistically significant inverse association between leafy vegetables intake and ISC (HR = 0.64; 95% CI 0.48–0.86 for a daily increase of 50 g) in the observed model with a significant dose response in the categorical analyses (p for trend 0.034). In the calibrated model, even though the point estimate of the HR is relatively similar (0.65), the CI became wider and was nonstatistically significant. We observed also in the calibrated model a nonsignificant inverse association between ISC and total vegetables, root vegetables, garlic and onions and citrus fruits. No associations were observed between F&V and CIS. Subsequent analyses were run adjusting F&V for each other, but no differences were observed. No interaction was found between total vegetables intake and smoking status (data not shown). In the categorical analysis, we found a positive and significant association with mushroom for ISC and for CIS. However, this association was not found in the observed or in the calibrated analysis.

Table 5 shows the HRs for selected nutrients. In the observed analysis, a significant inverse association between vitamin C

Table 4. Multivariable hazard ratio (HR) for CIS ($N - 817$), ISC ($N = 253$) and total cervical cancer ($N = 1,070$) (95% confidence intervals) for observed and calibrated intakes of total vegetables and fruits intake and for vegetables and fruits subgroups, in women from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort[1]

Cervical cancer behavior and type of vegetable/fruit	Categorical (observed quartile)[1]				Continuous	
	2	3	4	p for trend	Observed	Calibrated
Total vegetables						
CIS	1.00 (0.82–1.22)	1.18 (0.96–1.45)	0.98 (0.77–1.25)	0.718	1.00 (0.95–1.06)	1.06 (0.92–1.22)
ISC	1.06 (0.75–1.50)	1.14 (0.79–1.66)	0.73 (0.46–1.16)	0.389	0.92 (0.81–1.03)	0.85 (0.65–1.10)
CIS and ISC	1.02 (0.86–1.21)	1.17 (0.98–1.40)	0.92 (0.74–1.14)	0.937	0.98 (0.93–1.04)	1.02 (0.90–1.15)
Leafy vegetables[2]						
CIS	1.12 (0.88–1.42)	1.26 (0.97–1.65)	0.90 (0.61–1.33)	0.581	0.93 (0.77–1.12)	0.97 (0.68–1.34)
ISC	0.97 (0.63–1.51)	0.84 (0.51–1.38)	0.52 (0.29–0.95)	0.034	0.64 (0.48–0.86)	0.65 (0.37–1.14)
CIS and ISC	1.09 (0.88–1.34)	1.16 (0.91–1.46)	0.78 (0.56–1.08)	0.563	0.82 (0.70–0.96)	0.91 (0.68–1.21)
Fruiting vegetables						
CIS	1.03 (0.85–1.26)	1.06 (0.86–1.32)	0.94 (0.74–1.21)	0.788	1.00 (0.92–1.08)	1.03 (0.92–1.16)
ISC	1.22 (0.86–1.73)	0.94 (0.63–1.41)	0.84 (0.53–1.32)	0.315	0.89 (0.78–1.02)	0.88 (0.70–1.10)
CIS and ISC	1.07 (0.91–1.27)	1.04 (0.86–1.25)	0.92 (0.74–1.14)	0.483	0.96 (0.90–1.03)	0.99 (0.90–1.00)
Root vegetables						
CIS	0.95 (0.76–1.19)	0.99 (0.79–1.25)	1.03 (0.82–1.30)	0.585	1.01 (0.92–1.12)	1.08 (0.89–1.28)
ISC	0.78 (0.55–1.10)	0.88 (0.61–1.27)	0.71 (0.47–1.06)	0.157	0.96 (0.78–1.18)	0.77 (0.51–1.17)
CIS and ISC	0.89 (0.74–1.08)	0.95 (0.79–1.15)	0.95 (0.78–1.15)	0.858	1.00 (0.92–1.10)	1.01 (0.86–1.18)
Cabbages						
CIS	1.07 (0.84–1.36)	1.12 (0.87–1.44)	1.15 (0.89–1.50)	0.287	1.00 (0.91–1.09)	1.17 (0.92–1.49)
ISC	0.83 (0.57–1.21)	0.87 (0.58–1.30)	0.90 (0.57–1.43)	0.693	0.99 (0.79–1.24)	0.82 (0.50–1.33)
CIS and ISC	0.99 (0.81–1.21)	1.04 (0.84–1.29)	1.08 (0.86–1.35)	0.444	1.00 (0.92–1.09)	1.11 (0.90–1.36)
Mushrooms						
CIS	1.23 (0.95–1.60)	1.35 (1.04–1.75)	1.39 (1.05–1.85)	0.028	1.13 (0.80–1.59)	1.35 (0.65–2.83)
ISC	0.92 (0.59–1.42)	1.35 (0.89–2.06)	1.07 (0.67–1.72)	0.397	0.73 (0.28–1.91)	0.76 (0.13–4.26)
CIS and ISC	1.14 (0.91–1.42)	1.34 (1.07–1.67)	1.30 (1.03–1.66)	0.022	1.06 (0.76–1.47)	1.21 (0.63–1.36)
Garlic and onions						
CIS	0.85 (0.68–1.07)	1.01 (0.79–1.30)	1.07 (0.80–1.42)	0.454	1.14 (0.82–1.58)	0.87 (0.49–1.56)
ISC	1.17 (0.80–1.71)	1.04 (0.66–1.63)	1.13 (0.67–1.91)	0.714	0.73 (0.39–1.38)	0.53 (0.18–1.56)
CIS and ISC	0.93 (0.77–1.13)	1.03 (0.83–1.28)	1.09 (0.85–1.40)	0.408	1.03 (0.77–1.38)	0.74 (0.45–1.22)
Total fruits						
CIS	0.92 (0.76–1.10)	1.04 (0.86–1.27)	1.06 (0.85–1.31)	0.473	1.00 (0.96–1.05)	1.04 (0.94–1.14)
ISC	0.91 (0.65–1.27)	0.80 (0.55–1.15)	0.79 (0.53–1.18)	0.191	0.93 (0.86–1.01)	0.83 (0.72–0.98)
CIS and ISC	0.92 (0.78–1.08)	0.98 (0.83–1.17)	0.99 (0.82–1.20)	0.990	0.99 (0.95–1.03)	0.97 (0.90–1.06)
Citrus fruits						
CIS	0.95 (0.79–1.15)	0.90 (0.75–1.10)	1.00 (0.81–1.23)	0.754	1.00 (0.94–1.07)	1.00 (0.87–1.16)
ISC	0.86 (0.60–1.22)	0.81 (0.57–1.17)	0.75 (0.51–1.10)	0.139	0.90 (0.80–1.02)	0.85 (0.68–1.06)
CIS and ISC	0.93 (0.79–1.10)	0.89 (0.75–1.05)	0.94 (0.78–1.12)	0.333	0.98 (0.92–1.04)	0.96 (0.85–1.07)
Hard fruit						
CIS	0.88 (0.72–1.08)	0.96 (0.79–1.18)	0.94 (0.77–1.15)	0.731	1.00 (0.95–1.04)	1.04 (0.95–1.14)
ISC	0.80 (0.56–1.13)	0.74 (0.51–1.07)	0.77 (0.53–1.12)	0.156	0.93 (0.85–1.02)	0.91 (0.79–1.06)
CIS and ISC	0.87 (0.73–1.03)	0.91 (0.76–1.08)	0.90 (0.76–1.08)	0.348	0.98 (0.94–1.02)	1.00 (0.93–1.08)

Table 4. Multivariable hazard ratio (HR) for CIS ($N - 817$), ISC ($N = 253$) and total cervical cancer ($N = 1,070$) (95% confidence intervals) for observed and calibrated intakes of total vegetables and fruits intake and for vegetables and fruits subgroups, in women from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort[1] (Continued)

Cervical cancer behavior and type of vegetable/fruit	Categorical (observed quartile)[1]				Continuous	
	2	3	4	p for trend	Observed	Calibrated
Other fruits						
CIS	1.11 (0.91–1.34)	1.10 (0.90–1.34)	1.06 (0.86–1.31)	0.582	0.99 (0.94–1.04)	1.01 (0.89–1.15)
ISC	0.95 (0.67–1.34)	0.75 (0.50–1.10)	1.11 (0.76–1.64)	0.927	1.00 (0.92–1.09)	0.92 (0.73–1.15)
CIS and ISC	1.07 (0.90–1.27)	1.01 (0.84–1.21)	1.07 (0.89–1.29)	0.603	0.99 (0.95–1.04)	0.98 (0.88–1.09)

[1]Reference categories are the lowest quartile for quartile analysis. For continuous analysis, HRs are for daily increase in intake of 100 g for total vegetables and total fruits, the subcategories of fruit and vegetables are illustrated for daily intake increase of 50 g. The analysis was stratified by center and age at EPIC study entry and adjusted by BMI, education level, number of full-time pregnancies, births, physical activity, marital status, tobacco smoking, use of oral contraceptive, alcohol and energy intake. [2]Leafy vegetables, excluding cabbages.
Abbreviation: CIS: carcinoma *in situ*; ISC. invasive squamous carcinoma.

Table 5. Multivariable hazard ratio (HR) for CIS ($N = 817$), ISC ($N = 253$) and total cervical cancer ($N = 1,070$) (95% confidence intervals) for observed and calibrated intakes of selected nutrients in women from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort

Cervical cancer behavior and type of selected nutrients	Categorical (observed quartile)				Continuous	
	2	3	4	*p* for trend	Observed[1]	Calibrated[1]
Vitamin C						
CIS	1.15 (0.95–1.39)	1.09 (0.88–1.34)	1.14 (0.91–1.43)	0.319	1.02 (0.95–1.10)	1.10 (0.95–1.27)
ISC	0.73 (0.52–1.04)	0.91 (0.65–1.30)	0.59 (0.39–0.89)	0.047	0.85 (0.73–0.99)	0.81 (0.62–1.06)
CIS and ISC	1.03 (0.88–1.22)	1.05 (0.87–1.25)	0.98 (0.81–1.19)	0.925	0.98 (0.92–1.05)	1.03 (0.91–1.16)
Vitamin D[2]						
CIS	0.99 (0.79–1.24)	1.07 (0.85–1.34)	1.05 (0.82–1.35)	0.615	1.00 (0.97–1.03)	0.97 (0.88–1.07)
ISC	0.65 (0.44–0.95)	0.62 (0.42–0.93)	0.47 (0.30–0.76)	0.004	0.92 (0.85–1.00)	0.97 (0.82–1.15)
CIS and ISC	0.89 (0.74–1.08)	0.93 (0.77–1.14)	0.88 (0.70–1.09)	0.339	0.99 (0.96–1.02)	0.97 (0.89–1.06)
Vitamin E						
CIS	0.84 (0.68–1.04)	0.87 (0.69–1.11)	0.96 (0.73–1.27)	0.906	1.03 (0.93–1.14)	1.12 (0.83–1.52)
ISC	0.73 (0.51–1.05)	0.85 (0.57–1.26)	0.64 (0.38–1.06)	0.155	0.93 (0.75–1.15)	0.86 (0.53–1.38)
CIS and ISC	0.81 (0.67–0.97)	0.86 (0.70–1.06)	0.88 (0.69–1.12)	0.424	1.01 (0.92–1.11)	1.02 (0.79–1.32)
Beta-carotene						
CIS	0.99 (0.81–1.21)	1.17 (0.96–1.42)	0.94 (0.76–1.15)	0.957	1.00 (0.96–1.04)	1.02 (0.95–1.10)
ISC	0.87 (0.61–1.24)	0.97 (0.68–1.41)	0.86 (0.59–1.27)	0.578	0.99 (1.00–1.06)	0.91 (0.78–1.05)
CIS and ISC	0.97 (0.81–1.15)	1.12 (0.94–1.33)	0.92 (0.77–1.11)	0.770	0.99 (0.96–1.03)	1.00 (0.94–1.06)
Retinol[3]						
CIS	1.02 (0.83–1.24)	0.88 (0.71–1.10)	1.01 (0.81–1.27)	0.926	1.00 (0.98–1.02)	0.97 (0.92–1.03)
ISC	0.81 (0.56–1.16)	0.81 (0.55–1.18)	0.97 (0.67–1.41)	0.558	1.02 (0.99–1.05)	0.81 (0.62–1.06)
CIS and ISC	0.97 (0.81–1.15)	0.87 (0.72–1.05)	1.01 (0.83–1.22)	0.719	1.01 (0.99–1.02)	0.98 (0.93–1.02)
Folic acid[4]						
CIS	0.90 (0.67–1.22)	1.22 (0.89–1.66)	1.24 (0.90–1.71)	0.986	1.14 (0.98–1.32)	–
ISC	0.81 (0.49–1.35)	0.85 (0.48–1.52)	1.11 (0.61–2.04)	0.800	0.87 (0.62–1.27)	–
CIS and ISC	0.84 (0.67–1.12)	1.08 (0.83–1.42)	1.14 (0.86–1.50)	0.070	1.09 (0.95–1.25)	–

The analysis was stratified by center and age at EPIC study entry and adjusted by BMI, education level, number of full-time pregnancies, births, physical acitivity, marital status, tobacco smoking, we of oral contraceptive, alcohol and energy intake.
[1]For a daily increase of 65 mg for vit C, 1.5 mg. for vit D, 6 mg for vit E, 1,500 μg for beta-carotene, 200 μg for retinol and 170 μg for folic acid. [2]Information for 13,776 subjects was missing, including four missing for CIS, and 13 for ISC. [3]Information for 22,094 subjects was missing, including 111 missing for CIS, μg and 11 for ISC. [4]Information not available from the 24-hr recall.

intake (HR = 0.85; 95% CI 0.73–0.99 for 50 milligram per daily increase; p for trend 0.047) and ISC was found. The association was slightly stronger but not significant in the calibrated analysis (HR 0.81; 95% CI 0.62–1.06). In the calibrated analysis, an inverse association between ISC and vitamin E and retinol was also observed that was not significant. We observed a strong inverse association between ISC and vitamin D intake in the categorical analysis (p for trend 0.004), which was not confirmed in the calibrated analysis. No associations were observed between selected nutrients and CIS. There was no evidence of association between beta-carotene and folic acid intake and ISC or CIS. The effects of dietary intake after excluding cases diagnosed in the first 2 years of follow-up did not substantially alter the reported associations (data not shown).

Discussion

This is the first cohort study to examine the association between intake of F&V and the incidence of CIS and ISC. In this study, a significant inverse association between consumption of total fruit intake and the risk of ISC was found in the calibrated model. An inverse association although not significant was also observed for intake of citrus fruits. We also found a statistically significant inverse association between ISC and leafy vegetables intake in the observed model with a clear dose-response relationship. In the calibrated model, the strength of the association was the same, but the CIs were wider and did not reach statistical significance. An inverse, but nonsignificant association between ISC and total vegetables, root vegetables and garlic and onions intake in the calibrated model was also observed. No associations were observed between F&V intake and CIS.

In the categorical analysis, we found a significant positive association between mushroom intake and CIS that was not significant in the continuous observed and calibrated models. As far as we know, there is no evidence in the literature for an association between mushroom intake and cervical cancer risk. There are no identified compounds in mushrooms than can explain a role in cervical carcinogenesis, and therefore this result could be due to chance.

After calibration, none of the intakes of selected nutrients was statistically significantly related to risk of CIS or ISC. Overall, our findings show that there may be a role of dietary factors in the invasive cervical cancer but not in the *in situ* tumors (including also CIN 2 and CIN 3), thus suggesting that if there is any true effect, it would act in a late stage in the cancer process. This is consistent with the conclusion of the systematic review of the WRCF&AICR (2007): "overall, the results of numerous case–control studies on the relationship between F&V consumption with cervical neoplasia suggest a protective association for invasive cancer but no consistent association for noninvasive lesions."

There are only few studies on F&V intake in relation to cervical cancer risk.[7] There is no evidence from prospective studies on CIS or ISC risk, but there is a suggestion of a protective effect of some F&V against HPV persistence.[15–17] Some case–control studies support an inverse association of ISC and fresh fruits[18,19] or citrus fruits[20] but others do not.[21–24] Some case–control studies observed an inverse association between ISC and intake of total vegetables,[18,19,24] carrots,[19,21,24,25] cruciferous,[18,20,26] garlic[27] and leafy vegetables,[19,23,28] although results for these specific food items are not completely consistent across these studies.

A randomized clinical trial (RCT) of oral supplementation with vitamin C[29] found no effect with cervical cancer. Furthermore, there is no evidence from cohort studies on vitamin C intake and ISC risk. A nested case–control study[30] did not find any association between vitamin C intake and CIS. In a meta-analysis of case–control studies of cervical cancer with suitable continuous data[7] and after excluding one study with a very large standard deviation in the mean intake, a significant negative association was observed. Although case–control studies suggested an inverse association between vitamin E intake and invasive cervical cancer,[18,31,32] inconsistent results have been reported for noninvasive lesions.[30,33–37] Two prospective studies suggest a protective effect of dietary beta-carotene[15,16] against HPV persistence, but results from case–control studies on ISC are inconsistent,[22,26,31,38] and a meta-analysis of RCT[7] did not observe a beneficial effect. Folate intake on ISC has been assessed in some case–control studies[26,39] and results were also inconsistent, and a meta-analysis of RCT[7] did not observe an effect. However, prospective studies showed that higher circulating concentration of red blood cell folate is associated with lower risk of becoming positive for HPV and lower persistent HPV infection[40] and lower risk of CIN 2-3.[41] Retinol intake was not associated with ISC in one follow-up study,[42] while results from case–control studies are inconsistent.[18,22,26,32] A RCT showed an increase in the regression rates of CIN2[43,44] but not of CIN3. There is no published evidence about the potential effects of vitamin D on cervical cancer risk.[7]

There are plausible biologic mechanisms by which dietary factors may protect against cervical carcinogenesis. Vitamin C and E may inhibit carcinogenesis by enhancing mucosal immune response to infection or could act as efficient scavengers of free radicals and oxidants.[7] These radicals, which increase production during the inflammation process, could lead to extensive damage to DNA, proteins and lipids if not counteracted by antioxidant molecules.[45] Also, vitamin C and E could inhibit DNA adduct formation, which is induced by tobacco products.[46] On the other hand, antioxidant nutrients could modulate immune response and decrease viral replication and gene expression.[47] However, studies using biomarkers of oxidative damage to assess the effect of ascorbate supplement did not observe an effect, except perhaps in those with very low levels of intake.[48]

Our study has several potential limitations. Our results could be affected by measurement error in dietary intake, a common limitation of epidemiological studies. However, the wide range of dietary intake reported in the EPIC study thereby increasing the between-person variance in diet probably minimizes the impact of measurement error.[49] In addition, to adjust for possible systematic overestimation or

underestimation in dietary intake measurements, a calibration approach was used,[13] although the measurement error of the 24-hr recall is not independent of that of dietary questionnaires. Information about HPV status is lacking and therefore results are not adjusted by HPV infection. Nevertheless, results of studies on dietary factors and cervical cancer taking HPV infection into account do not differ substantially from those previous studies that did not control for it.[5] Another possible limitation of our study regarding CIS is that there are higher detection rates in some centers than in others because of country differences in the systematic reporting of CIS to the cancer registries. Differences in age at enrollment between centers could also have influenced the number of CIS cases. However, because all our analyses were stratified by center, it is unlikely that this would have biased our relative risk estimates as nondifferential disease misclassification can only underestimate the true relative risk.[50] Furthermore, in a sensitivity analysis, we compared results of CIS in centers with a higher detection rate and lower detection rates and we did not observe differences.

Finally, other potential limitation is the lack on available information on screening participation. Screening behavior may be associated with diet. Women participating in a screening program may have a healthy diet, increasing the likely of detecting a CIS and preventing progression to ISC. However, in the analysis of available data from the follow-up questionnaire in the EPIC center of Oxford, contrary to what they might have expected, there was no difference in the number of cervical smear between daily and not-daily eaters of fruit (T. Key and P. Appleby, personal communication).

In conclusion, our study has shown a significant inverse association between ISC and a daily intake of total fruits in the calibration model, although this study had no information on HPV status and cervical screening and therefore could have residual confounding. Our study also supports a possible protective role of citrus fruits, total vegetables, leafy vegetables, root vegetables, garlic and onions, vitamin C, vitamin E and retinol intake against ISC that need to be confirmed on a larger number of ISC cases. Our study found no associations between dietary factors and risk of CIS.

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