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Overview on Molecular Markers to Implement Cervical Cancer Prevention: Challenges and Perspectives

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1. Introduction

A decline in the incidence and mortality of cervical cancer has been observed in most Western countries since the first third of the past century. This may be mainly attributed to the introduction of Pap test, that is considered one of the milestones in medicine. Nevertheless, worldwide burden of cervicocarcinoma is still enormous. This fact is well known among the scientific community, and has led to an extensive search for optional screening tools and predictive markers, currently under testing in different countries.

2. A brief history

Modern research on Papillomavirus and Papillomaviruses began more than 150 years ago. In a famous paper, the Italian physician Rigoni-Stern analyzed death certificates of woman in Verona, during the period 1760-1839. He noted that cervical cancer was common among prostitutes, married women and widows but rare in virgins and nuns. He concluded that the development of this cancer should be related to sexual contacts. Later, in 1898, McFadyean and Hobday successfully transmitted canine oral papillomatosis, while Codeac transmitted warts from horse to horse, in 1901. In spite of a limited number of studies on Papillomavirus in subsequent decades, it was almost 80 more years later when this area of research engendered broad interest. In 1934 Rous and Beard noted that papillomas of domestic rabbits frequently converted to squamous-cell carcinomas. Although Rous conceptually preceded his contemporaries by several decades, the importance of his ponderous work was only acknowledged in 1966, when he received the Nobel Prize. Rous's research was not specifically driven towards the agent causing rabbit papillomas, but using the frequent progression of rabbit papillomas to squamous-cell carcinomas, he provided an universal model to analyse cervical cancer development. From this point, a number of anecdotal reports of the malignant conversion of genital warts appeared in the medical literature and resulted in a persistent interest about the possible role of HPV infection in cervical cancer. This interest began more concrete with the failures in finding traces of Herpes simplex virus type 2 (HSV2) DNA in cervical cancer biopsies. These fails prompted the search for other potential infectious etiology. In the mild-1970s, within precancerous

cervical lesions, Meisels and Fortin firstly observed areas of koilocytic atypia, which they considered to be the cytopathic effect of HPV infection (Meisels & Fortin, 1976).

In 1978, Della Torre et al., in Italy, and Laverty et al. in Australia, firstly demonstrated the presence of HPV virions within dense bodies of koilocytes, but deeper investigations were limited by the inability to propagate HPV in cultured cells or in simple animal models. By the end of 1970s, the revolutionary advent of recombinant DNA technologies and molecular cloning techniques, provided the key for advanced investigation on HPV biology, in order to confirm the role of Papillomavirus in cervical cancer etiology (Zur Hausen et al., 1977). Topically, in 1983, Harald zur Hausen et al., for the first time isolated HPV-16 from a cervical cancer biopsy and cloned its genome. Using HPV-16 sequence and Southern blotting technology, zur Hausen detected this genotype in about one-half of cervicocarcinoma biopsies (Boshart et al., 1984) and demonstrated the selective transcription of viral E6 and E7 oncoproteins in cervical cancer derived cell lines (Schwartz et al, 1985). zur Hausen's findings indirectly demonstrated the heterogeneity of the Human Papillomavirus family; this fact consequently led to the classification of genital HPVs into low-risk and high-risk oncogenic groups, in dependence of the ability to induce cancer. In 2008, Dr. zur Hausen will receive the Nobel prize for medicine. Actually, there is uniform agreement regarding the central role of high-risk HPV-infection in cervical cancer and the necessary but far from sufficient etiopathogenetic role of the virus in causing cervicocarcinomas (Solomon, 2003).

The practical implication of this long series of studies is evident: an enormous increase in the quality of diagnostic approach of precursor lesions of cervical cancer and the development of preventive vaccine (Zur Hausen, 2002).

3. HPV and its life cycle

Papillomavirus are small, non-enveloped, epitheliotropic, double-stranded DNA viruses that can infect basal epithelial cells of the skin or inner lining of tissue and induce cellular proliferation in a specie-specific manner. More than 100 genotypes of Papillomavirus have been isolated and branded molecularly; they were categorized as cutaneous or mucosal type.

Basing on their association with cervical cancer and precursor lesions, HPVs can be grouped in: "high risk" (HR) or "oncogenic" types (16, 18, 26, 31, 33, 35, 39, 45, 51, 53, 56, 58, 59, 66, 68, 73, 82, and "low-risk" (LR) or "non-oncogenic" types (6, 11, 40, 42, 44, 54, 61, 70, 72, 81, 89), related to benign epithelial proliferation but not with cancer (Munõz et al., 2004, 2006). Among the nineteen oncogenic HPV types, five are most often found to be associated with cervical cancer; particularly, virus 16 is accountable for about half of cervicocarcinoma cases discovered in Northern America, Europe and Australia; viruses 18, 31, 33 and 45 are responsible for additional 30% of cases (Cliffort et al., 2003; De Sanjosè et al., 2007; Kraus et al., 2006). All papillomaviruses show a common genetic structure: a single molecule of double-stranded circular DNA of about 7900 base-pairs genome, enclosed in a non-evenloped icosahedral capsid. HPV-DNA encodes for eight open-reading frames (ORFs) which are transcribed from the single encoding DNA strand. The genome is functionally divided into three regions. The first is a non-coding one, referred as Long Control Region (LCR) or Upper Regolatory Region (URR). This section of HPV's genome includes enhancer

and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. The second is the Early (E) Region, consisting of ORFs E1, E2, E4, E5, E6 and E7, which are implicated in viral replication and oncogenesis. The third is the Late (L) region, that encodes the L1 and L2 structural proteins.

4. A silent but epidemic virus

The global burden of HPV-associated disease is very high. At present, HPV infections represent the most common sexually transmitted disease (SMT) in the world (Snijders et al., 2006). In the United States alone, it was estimated that 6.2 million of new infections occur annually, with an approximately prevalence of 20 million (Clifford et al., 2005).

Within population, the prevalence of HPV infection in asymptomatic women is estimated to range from 2% to 44%; the highest peak was reported in women younger than 25 years of age.

The vast majority of HPV infection is transient and resolves within 2 years without necessarily led to clinically significant cervical lesions. Only 10% of infected women remain HPV-positive within 5 years. In 2008 (the most recent year for which statistics are available), 12,280 women in the United States had cervical cancer, and 4,021 died from the disease (Arbyn M et al., 2011). Rates of infection appears actually to be in incessant and rapidly increasing.

Interest in HPV and cervical pathology is primarily justified by the morbidity and mortality caused by cervical epithelial cancer: cervicocarcinomas represents the second most common cancer among women worldwide. Its worldwide burden is enormous, with over 500,000 new cases of cervical cancer diagnosed each year, and 280,000 deaths recorded (Jemal A et al., 2010; Parkin D et al., 2002). While in developed Western countries screening programmes have significantly reduced the incidence of disease, about 80% of cervicocarcinomas cases still occur in underdeveloped countries.

The natural history of cervical cancer is characterized by a well-defined pre-malignant phases. These pre-malignant changes represent a spectrum of abnormalities ranging from Low-grade Squamous Intraepithelial Lesion (LSIL) to High-grade Squamous intraepithelial Lesions (HSIL). In the United States, about five hundred thousand high-grade precursors and 2-3 milion cases of low-grade lesions are referred for colposcopic assessment each year. The attendant costs for the management of these lesions was estimated in 3-6 billion dollars (Stoler, 2004).

Prospective studies showed that infection with oncogenic HPVs precedes the development of cervical neoplasia, and that it's necessary, even if not sufficient, to have HR-HPV infection to develop squamous intraepithelial lesion (SIL) or invasive cervical cancer (ICC). Virtually all cervical cancers test positive for HR-HPV DNA. Surprisingly, the etiopathogenetic role of HPV for cervical cancer is higher than smoke is for lung cancer and than Human Epatitis B (HBV) is for liver cancer (Carter JR et al., 2011).

HR-HPVs increased the risk of developing cervical cancer in the order of 50-300 fold. A meta-analysis of Smith et al. shows that the overall prevalence of HR-HPVs in ICC is on the order of 87%, ranging from 86% to 94% by region. Overall, HR-HPVs prevalence in HSIL ranges from 78% in Asia to 88% in Europe (Smth et al., 2007).

There is a broad variation between the most prevalent HR-HPV types around the world. However, types 16 and 18 remain the most common in cervical lesions, causing 60-80% of all cervical cancers. HPV-16 ranges from 52% in Asia to 58% in Europe; HPV-18 ranges from 13% in South-Central America to 22% in North America (Smith et al., 2007).

It was originally believed that there was an irreversible progression of cervical disease from low-grade to high-grade lesions and then invasive cervical cancer. However, through a much greater understanding of the natural history of HPV infection, we actually know that the majority of low-grade lesions would regress without treatment. To the necessary activity of HR-HPV, other co-factors such as smoking, long-term oral contraceptive pill use, human immunodeficiency virus (HIV) co-infection, high parity, Chlamydia Trachomatis and HSV infections, immune suppression as well as nutritional and dietary factors may be associated to induce cervical cancer. Generally, the vast majority of these condition adversely affects antigen-processing Langerhans cells, needed for cell-mediated immunity, or enhances HPV gene expression in the cervix, thus promoting the integration of the virus into the host genome.

5. Molecular basis of the transformation: Knowledge is power

HPV life cycle is linked to the differentiation of the infected epithelial cell; it starts with the penetration of virions in the basal cells layers (stratum germinativum), through micro-abrasions of the transformation zone of the cervix. The ability of HPVs to establish their genome in basal cells relies mainly upon E1, E2, E6 and E7 genes. Normally, when basal cells undergo cell division , it loses contact with the basement membrane and migrates into the suprabasal compartment, where it withdraws from the cell cycle and initiates a programme of terminal differentiation. In HPV infection, suprabasal cells fail to withdraw from the cell cycle.

During acute infection, to replicate its genome and successfully produce infectious virions, HPV switches to amplify its DNA at high copy number, synthesises capsid protein and causes viral assembly, within the differentiated keratinocytes of the suprabasal layers of cervical epithelium. In this context many viral gene products are implicated. E7 has been shown to be necessary to induce suprabasal DNA synthesis, assisted by E5. E4 gene is essential for HPV-DNA replication and for the expression of L1 and L2 genes. E4 also interrupts cytoplasmic cytokeratin network, causing condentation of tonofilaments at the cell periphery, and perinuclear cytoplasmic halo; the results is the koilocyte. Encapsidation of viral DNA, to generate viral progeny into differentiated cellular compartment, is qualitatively dependent upon L2 gene. L2 may be also play a role during intracellular transport of virions, and in the localization of viral DNA within host nucleus.

During latent infection, HPV stays as non-productive within the basal layer of the epithelium and establish itself as a low copy number episome, usually once molecule/cell cycle (Doorbar, 2005). At the basal and parabasal cellular levels only very little, if any, gene expression of the virus can be observed. Conversely, expression of early E1, E2, E4, E5, E6 and E7 genes is evident within differentiated cells of the upper layers of the epithelium, which have lost the capacity to replicate their genome and are at no further risk of acquiring functional mutations. The above mentioned mechanism is a meticulous HPV strategy that allows maximal production of virions, causing almost no injury to infected host. The

situation changes radically if regulatory intracellular mechanisms driving the control of the viral are disturbed and deregulated. In this case, the expression of genes involved in the replication of viral genome occurs also in epithelial stem cells, which lost their capacity to differentiate. The inhibition of the differentiation process leads to a cellular state that cannot support the full viral life cycle. Interference of viral genes with cellular pathways that control replication and life cycle of epithelial cell might result in chromosomal instability. In epithelial stem cells with chromosomal instability, HPV DNA integrates into the host genome. HPV integration sites are randomly distributed over the whole genome, with a clear predilection for fragile sites (Wentzensen et al., 2004). Whether any property of the virus drives this integration event or whether it reflects random recombination events remain still unclear; however, two consequences of integration are certain: the loss of E2 regulatory gene and the selective up-regulation of viral E6 and E7 oncogenes. Disruption or deletion of E2 gene, occurring during HPV-DNA integration, interferes with down-regulation of E6/E7 transcription genes and leads to an increased and selective expression of E6/E7 oncoproteins, in replicating epithelial stem cells. As result, E6/E7 proteins bind to cell cycle regulators, stimulate cell-cycle progression and induce cellular instability (Doorbar, 2005). When E6 gene product binds to p53, physiological activities of p53, which governs G1 arrest and DNA repair or apoptosis, are abrogated (Doorbar, 2006; Tang et al., 2006). E6 protein of non-oncogenic HPV types does not bind p53 at detectable levels and has no effect on p53 stability, *in vivo*. When E7 gene product binds to the hypophosphorilated form of the pRb, the result is the disruption of pRb-cellular transcription factor E2F-1 complex, the release of E2F-1 and the transcription of genes whose products are required to enter in phase S of cellular cell cycle. Non-oncogenic E7 protein binds to pRb with a decreased affinity (Litchtig, 2006). Together E6/E7 proteins deregulate cell cycle checkpoints G1/S and G2/M.

Host cells showing viral E6/E7 expression may acquire defects in differentiations, immortal phenotype, chromosomal instability and an increased probability of mutation allowing to invasion. Such described are obviously extraordinary molecular accidents occurring during HPV cell cycle and, in view of many HPV-infected cells, are extremely rare events. On the other hand, the continuous and deregulated E6/E7 activity in cervical stem cells compartments, enhances the selection and the growth of mutated cell clones. Then persistence represents the essential and indispensable requisite to develop cervical cancer.

Numerous studies concerning HPV infection in immunocompromised population, reported that E6/E7 would also play an important role in the inhibition of the host cell immune response (Kraus et al., 2006), particularly by down-regulating the expression of Interleukin-8 (IL-8). IL-8 is a T-cell chemoattractive molecule (Guess & Mc Cance, 2005), that also suppresses the expression of the Monocyte Chemoattractant Protein 1 (MCP-1) in epithelial cells of female genital tract (Biswas & Sodhi, 2002). E6/E7 would also favour the evasion of infected cells from the antiviral and antiproliferative activities of Tumor Necrosis Factor Alpha (TNF-α) (Scott et al., 2001).

6. The ancient Pap test

The concept of utilizing exfoliative cytology to identify women with cervical cancer was introduced by Papanicolaou and Babes, in 1920s. Next, Papanicolaou refined the technique

and demonstrated that conventional cytology could also be used to identify precancerous lesions of the cervix. The shift from using cytology as a way to identify cases of invasive cervical cancer to using it to identify women with pre-neoplastic lesions was extremely significant. It meant that cervical cytology could be used to prevent cervical cancer, over than to identify cases in early phases.

In the 1960s, cervical cytology began to be extensively used. In about 30 years, cytology-based screening reduced the incidence of cervicocarcinomas by up to 75% in countries that have been able to realize quality-controlled screening programs (Arbyn et al., 2011). No improvements or modifications of the Pap test were instituted during this time, as there was no doubt that well-organized screening programs, realizing high compliance and good quality control, were effectual in saving lives.

The process of change began when the article "Lax Laboratories" was published in the Wall Street Journal in 1987 (November, 2), by Bogdanich. The phrase "The Pap Test Misses Much Cervical Cancer Through Labs Errors", implying that false negative Pap tests resulted largely from carelessness of doctors, alerted the public. The accuracy of cervical cytology began to be questioned. The article led to increased people awareness, and climaxed with the introduction, in 1988, of the governmental regulatory document known as CLIA 88 (Clinical Laboratory Improvement Act). CLIA 88 enforced regulations to ensure high quality testing

At the same time, in 1988, the National Cancer Institute sponsored a workshop to address the standardization of diagnosis in cervical cytology. The workshop introduced a new classification system designated as "The Bethesda System" (TBS). TBS, further revised in 1991 and 2001, provided a uniform format and offered a standardized terminology, specifically emphasizing communication of clinically relevant information (National Cancer Institute Group, 1989). TBS has been widely accepted and is endorsed by both Pathology and Gynecological Societies.

In the 1990s, it was realized that the efficacy of Pap test have reached a nadir, and meta-analyses indicated that both sensitivity and specificity of a single Pap test in detecting cervical intraepithelial neoplasia or invasive cancer is in the order of 80-85% and 76%, respectively (Stoler et al., 2001). A review of the UK program found that, within women developing invasive cervical cancer, 47% had an apparently adequate screening history during the previous 5 years (Sasieni et al., 1996); some of these patients also had a history of negative smear results. These considerations suggested that the frontiers of effectiveness of conventional cytology have been reached and that was imperative to improve the diagnostic assessment of the Pap test. This fact has been well recognized among the scientific community that emphasize the necessity to find other solutions to cope with this increasing problem.

Prerequisites for an effective screening program is a high quality in sampling technique, in processing and in reporting. Basing on these considerations, technology entered in cytology practice with a variety of optional screening tolls to replace or complement conventional Pap test; among these, monolayer cytology and computer-assisted cytology .

7. Challenges with conventional screening methods

7.1 Monolayer cytology

The low sensitivity of a single cervical smear is due to a variety of factors, including: incorrect or inadequate sampling of cervix; poor transfer of cells to the glass slide; non-

representative sample placed on the slide; poor fixation. Particularly, sampling and preparing are together guilty for about two-third of false-negative tests (Cibas et al., 2008).

To minimize false-negative cytology results and to improve the diagnostic accuracy of cervical cytology, some programs proposed to repeat the Pap test every year, to balance the relative limited sensitivity of conventional cytology; however, this conduct would compromise cost-efficacy of Pap test and the possibility to use an algorithm with greater screening intervals and similar safety. New methods of collection and processing would need to surmount all these problems.

Liquid-based cytology (LBC) was introduced in the mid-1990s, as a way to improve the performances of Pap test. By this technology, clinician does not prepare the specimen at the bedside by spreading the exfoliated cells onto a glass slide, but the cervical sampling device (i.e. spatula or brush) is rinsed in a vial containing a fixative-transport medium. In laboratory, slides are prepared using an instrument that mixes the specimen and transfers an exact number of cells onto a filter membrane, with minimal cellular overlap. Then, the filter with the cells are transferred onto a slide and stained using the Pap stain (Abulafia et al., 2003). The automated process of such slides preparation prevents drying artefacts (very common in conventional Pap slides) and eliminates non-diagnostic debris, such as blood, mucus and inflammatory cells. Background material such as inflammatory exudates, cytolysis, microorganisms and tumor diathesis can still be identified but it does not obscure the epithelial cells (Davey et al., 2006).

To date, there are three currently FDA-approved LBC techniques, These include: SurePath™ System (TriPath Imaging Inc., Burlington, NC, USA), ThinPrep® System (Hologic, Crawley, UK) and MonoPrep System (MonoGen, Lincolnshire, IL) (Cibas et al., 2008).

ThinPrep and SurePath methods are the most widely studied technologies in literature; their underlying principles are similar, the only difference being that ThinPrep collects samples into methanol-based preservative solution, while SurePath dispenses cells into ethanol-based fluids.

More than forty publications promote the use of these preparation methods. In particular, all the authors show statistically significant improvement (about 10% or more) of the diagnostic sensitivity of conventional cytology in all categories of cervical disease (Bernstein et al., 2001; Davey at al., 2007; Nance et al, 2007; Papillo et al., 2008.).

Currently, LBC constitutes over 80% of cervical screening tests in USA. In 2003, the UK National Institute for Clinical Excellence (NICE) recommended the introduction of LBC as primary way to process samples in cervical cancer screening programs (Stoykova et al., 2008). The National Health Service of the United Kingdom agreed to introduce LBC throughout the country, in view of the reduction of inadequate specimens from 9% with conventional cytology to 1-2% with LBC (Nance et al., 2007).

LBC techniques improved the quality of the smear, being cytological evaluation and interpretation facilitated by the thin layer of evenly distributed cells. Abnormal cells are not hidden in thick areas of the slide; Then, will follow an increased detection of the lesions (such as HSIL lesions), a reduction of the number of false-negative diagnosis and the diminution of unsatisfactory specimens. Moreover, the availability of residual cellular material, preserved for several week at room temperature, is usefulness for additional

investigations, such as immunocytochemistry or molecular procedures. In the USA, the Consensus Guidelines for the Management of Women with Cytologic Abnormalities considered HPV-DNA testing on residual LBC specimen to be the preferred approach to managing women with ASC-US cytological results. This suggestion was based on the grounds that HPV-DNA reflex testing could offer the advantage that women do not need to return to additional clinical examination (Wright at al., 2002).

7.2 Computer-assisted cytology

Screening of Pap smears is monotonous. It was recognised that the monotony of screening large numbers of normal slides promotes periods of lack of attention during which abnormal cells may be disregarded. Among women who have cervical cancer and have been screened, 14% to 33% of the cases represent failure to detect abnormalities. It is therefore not surprising that great efforts have been made to automate also this aspect of cervical cancer screening. The aim of computer-assisted reading of cervical smears is to increase the sensitivity of cytological testing by finding cells known to be very difficult to detect in conventional slides. This technology should also increase productivity by excluding normal slides or part of the slides from manual screening and by selecting most atypical images to be checked by cytologists (Dunton, 2000).

In the mid-1990s two automated devices, based on traditional computer image technology and neural network software, have been subjected to extensive multicentre trials and subsequently approved by the American Food and Drug Agency for screening cervical smears: PAPNET system (Neuromedical Sciences Inc, Amsterdam) and AutoPap 300 (NeoPath Inc, Redmond Washington USA). Both are interactive systems, which select smears for manual review by the screener. They were initially approved for quality control or supplementary screening of cervical smears. Subsequently they were admitted for primary screening.

PAPNET was introduced as a pre-screening method of conventional Pap smears. The smears were analysed using a combination of algorithmic and neural network programs; then, 128 images of the most abnormal looking cells or cell groups were selected for inspection by the screener.

The images were stored on compact disc and viewed by the screener on a video monitor placed in the laboratory. The screener triages the images and decides whether the slide is negative or requires manual review. Those slides which were triaged negative were not subjected to manual microscopic review.

AutoPap was also designed to also pre-screen conventional slides. This technology uses a computer algorithm method to classify and score glass slides to overall level of abnormality. All slide were processed through the device and then, on the basis of "abnormality index", they were ranked in descending order of potential abnormality and broken into quintiles. This system, now manufactured by TriPath inc. and called FocalPoint®, was designed to look for abnormalities slide by slide and to rule out the 25% of slides with the lowest risk. These slides were automatically excluded from the list of those requiring manual microscopic review thus reducing the screener's workload by 25%. Slides with the most severe abnormalities were completely reviewed by scientists.

FocalPoint is intended to be used on both conventionally-prepared and SurePath™ cervical cytology slides. This system analyses the samples using a series of algorithms and assigns a score to the sample. The sample is then graded into a group called "No Further Review" (NFR) or into one of 5 risk categories. The purpose of this assignation is to make unnecessary to look at the NFR category. Cytoscreeners can instead concentrate themselves on looking at the slides graded as abnormal. The operator is guided to the areas containing the cells of interest (Fields of View/FOV) which have been detected by the system.

Recently, Cytic Corporation developed its computer assisted system, the ThinPrep Imager®, which received FDA approval for use with ThinPrep slides. A bench-op image processor analyses ThinPrep slides, which were reviewed by cytotechnologists, by using a microscope with a motorized stage. Special software drives the reviewer to the 22 most abnormal fields on the slides. Full manual review is required only if any of the 22 fields contain a suspicious or abnormal cell.

Devices of computer-assisted screening were tested in extensive multicentre trials comparing automated and manual screening of the same slides. All trials found that automated systems were at least as sensitive as manual screening; however, in automation more smears could be analysed per unit of time. On the other hand, due to high development costs, these systems were not found to be cost effective for the use in laboratories processing less than 50,000 smears per annum; this fact excluded many laboratories in the USA and Europe.

8. The revolution: Molecular diagnosis

The awareness of the viral origin of cervicocarcinoma, the refinement of the techniques for cytological diagnosis and the introduction of liquid-based medium for collection of cytological specimens has open new and interesting options to improve cervical cancer screening programs.

The consciousness that cervical cancer is a multistep process and that it occurs in women who have been infected with oncogenic HPVs, led to the development of molecular techniques able to identifying carcinogenic Papillomavirus in cervical sample (Bosch et al., 2002). The era of diagnosis based on aetiology was beginning.

Serological assays to detect antibodies against HPV capsid or against functional protein received attention as investigational tools, both in epidemiological and clinical studies (Jochmus-Kudielka et al, 1989; Galloway, 1992). However, serology detected humoral immune response to HPV antigens, which may reflect cumulative exposure to HPV infection acquired in mucosal sites other than genital; moreover, it was unreliable to determine whether an HPV infection was present or past (Dillner J, 1999), by using serological tests. So, an accurate diagnosis of HPV infection could only be based on the detection of viral nucleic acid.

Testing for Human Papillomavirus by different molecular tools has been proposed as an adjunct or as an independent screening tool, with several potential advantages. Testing for the etiological agent of cervical cancer offers the opportunity to detect women at increased risk of cervical cancer at the stage of latent and subclinical infection, preceding by several months to years the clinical stages detectable by the Pap test.

Wide range of methods to detect HPV-DNA in cytological specimens are available. They evolved considerably in the last 25 years. Initial methods were based on direct probe hybridation technology, such as dot blot and Southern blot. Besides being labor-intensive and time-consuming, these techniques showed low sensitivity and required large amounts of DNA in clinical samples. So they have largely been superseded by amplification technology. Amplification techniques can be further classified into two separate categories:(i) target amplification assays (i.e., PCR, in which a target nucleic acid is amplified) and (ii) signal amplification assays (in which the signal generated from each probe is increased by a compound-probe or branched-probe technology). To date, all the above methodologies (Figure 1) have been applied to HPV detection field (Zappacosta et al., 2008).

Fig. 1. Technology assessment in cervical cancer screening

8.1 Southern blot

The first method for HPV detection was the Southern blot (Sb). Sb utilizes enzymes to break HPV-DNA chain extracted from the specimen. The product, integrated into a gel, is subjected to electrophoresis, that separates viral DNA basing on the size of each fragment. DNA fragments are next transferred to a nitrocellulose membrane and hybridized with HPV genomic probes, which are labelled with radioisotopes. In addition to poor labour applicability (reliance on radiolabelled probes) and high time-consuming, Sb procedures showed low sensitivity, mainly due to the need of large amounts of DNA in clinical samples. For all these reasons, Sb technique has now largely been superseded by amplification technologies.

8.2 Direct hybridization

In situ hybridization (ISH) is a direct probe method that assess the presence of a target nucleic acid or gene expression within either paraffin-embedded tissue or cervical smear. The nucleic acid probes used in ISH are derivatized, typically with biotin, in multiple sites. Detection is frequently achieved employing a sandwich approach, involving streptavidin-chromogen complexes. Improvements in sensitivity of ISH have been reached with fluorescent probe (FISH) utilization, in order to add a further amplification of the signal. The major advantages of ISH/FISH techniques is that HPV-DNA can be identified inside

specific cells (normal, koilocytes, neoplastic) and that viral physical status may also be determined (integration *versus* episomal) (Hopman et al., 2005). Low sensitivity and specificity (30% and 72%, respectively), nucleic acid degradation during sample processing, and high time-consuming (due to multiple assays which must be carried out for HPV genotyping), are the main factors that make these techniques troublesome in its performances (Seedlacek, 1999).

Laboratories using molecular assays for detection of infectious organisms should use standardized tools. In this context, the World Health Organization (WHO) has initiated an International Collaborative Study enrolling several laboratories worldwide (Pagliusi & Garland, 2007). The aim of developing HPV international standard reagents is to capacitate diagnostic laboratories to be able to validate their own assays and to determine their analytical sensitivity. Within surveillance studies, this standardization will allow comparison of HPV-DNA detection between different geographic localization, populations and anatomical sites over time. Standardization is particularly important in view of post-vaccine population responses data.

High-throughput assays suitable for large-scale cervical cancer screening are currently based on two different amplification technologies: Polymerase Chain Reaction (PCR) and hybridization-based assays (i.e., Hybrid Capture 2 assay). The advantages and disadvantages of these basically different assays will be extensively discussed below together with the analysis of several recent studies comparing the performances of both techniques.

8.3 Polymerase chain reaction

PCR is a selective target amplification assay capable of exponential and reproducible increase in the HPV sequences present in biological specimens. The amplification process can theoretically produce one billion HPV-DNA copies from a single double-stranded molecule. after 30 cycles of amplification, For this reason, PCR has very high level of molecular sensitivity and permits the detection of less than 10 copies of HPV-DNA in a mixture

There are two main approaches to detect HPV-DNA by PCR: type-specific PCR and consensus PCR. The latter are able to amplify sequences from several different HPV types, because they target conserved DNA regions in the HPV genome. The most extensively used PCR assay utilizes consensus primers that target a highly conserved region of HPV L1 genome, thus amplifying a vast spectrum of HPV types in one reaction. Initially, most laboratories used PCR assay with degenerated primers pair MY09/11. These primers are now been replaced by a new set of oligonucleotides pool: GP5/6 and modified GP5+/GP6+, PGMY09/11 (modified MY09/11), SPF1/2, the last one especially appropriated for formalin-fixed paraffin-embedded tissue samples, which often offer a small amount of amplifiable DNA (Boulet et al., 2008; Perrons et al., 2005). Amplification with each of these primers provides different size amplification products, resulting in varying sensitivity for HPV-DNA detection. Although discrimination of sequence homology is better for longer sequences, and would theoretically permit improved HPV types resolution, shorter fragments tend to confer better sensitivity when potentially degraded specimens, such as paraffin-embedded tumor tissue, are used.

Up to date literature reports clinical sensitivity of PCR protocols varying from 75% to 95% (Kulusingam et al., 2002), with a median of 82%. Of interest is the PCR versus HC2 data

obtained from ALTS study: on 278 cases of CIN3/cancer, PCR test employing the PGMY09/11 primers achieved clinical sensitivity and specificity of 87.4% and 55.6% respectively, while the corresponding value for HC2 test were 95.5 and 51.1% (Belinson et al., 2001; Zappacosta et al., 2010.

The commercial assay by Roche Diagnostics, Amplicor® HPV test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) has been recently released, although not yet FDA approved. The test is designed to amplify HPV DNA from 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), by using biotinylated primers to define a sequence of approximately 165 bp in length within the polymorphic L1 region of the HPV genome, and to simultaneously assess the presence of human β-globin gene as a positive control. The test do not discriminate HPV genotypes specifically. In the International Papillomavirus Congress which took place in Mexico City during 2004, several studies showed data related to the utilization of Amplicor® test in two different LBC media (ThinPrep® and Surepath™) and illustrated the performance of the test in different clinical settings. Particularly, van den Brule at al., (Hessenlink et al., 2005) compared Amplicor® test with the GP5+/GP6+ PCR assay in cervical samples collected in PreservCyt® medium. The two tests gave similar results, but the sensitivity of Amplicor® appeared slightly higher. Roche assay and HC2 test gave identical results in detecting high-grade CIN (Monsonego J et al., 2005).

The scenario changes when HPV genotyping is needed. HPV genotyping is now considered a relevant tool for women management, in order to identify persistent type-specific oncogenic HPV infection, and for the stratification of cancer risk. Among HPV-positive women, 20% to 40% harbour at least two genotypes (Mendez et al., 2005). Interest in multiple HPV infections has recently increased as prophylactic vaccines against HPV have been introduced (Jenkins, 2008). Moreover, the correct profiling of HPV types in patients with multiple infections is important to learn more about the natural history of cervical cancer. Constant progress in HPV typing based on PCR methods has been made over the past few years. The majority of available protocols uses degenerate and/or consensus primers, followed by the examination of the generated PCR product through sequence analysis, restriction fragments length polymorphisms analysis or hybridization with type-specific probes in different formats (such as the reverse line blot assay [van den Brule et al., 2002] or bead-based multiplex HPV genotyping method [Schmitt et al., 2006]). The use of these technologies offers the advantage of detecting a large spectrum of HPV types by a single PCR. However, they may be less efficient in detecting specific HPV types, in cases of multiple infections (Schmitt et al., 2010).

Generally, amplification-based methods, mainly PCR, are currently the most sensitive methods for the detection of HPV-DNA. They are ideal instruments for research and epidemiological purposes, since they allow the detection of low viral load infections, also minimizing the risk of misclassification of HPV infection status. However, due to the frequent contamination problems and consequent false-positive results as well as to the costs which are still too high, they are not routinely applicable in diagnostic laboratories. In order to overcome these problems, Digene Diagnostics developed Hybrid Capture System DNA detection.

8.4 Hybrid capture 2

Hybrid Capture 2 (HC2, Qiagen, Valencia, CA) is a simple, high-throughput, semi-automated HPV-DNA test, operating on the principle of signal amplification. HC2 is the only HPV test

currently approved by the US FDA. The method utilizes long (> 1 kb) single-strained RNA probes which are complementary to the entire HPV-DNA genomic sequence. DNA is firstly denatured and subsequently mixed with RNA probe pool in a buffered solution. Two RNA probe pool are used. The test can be performed using both probe pool together or separately. Probe A recognizes non-oncogenic HPV types (6, 11, 42, 43, and 44); pool B identifies oncogenic HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). DNA-RNA complexes are immobilized onto microplates which has been coated with polyclonal IgG antibody that recognize specific DNA-RNA hybrids, at room temperature. The immobilized hybrids are then identified by a second DNA-RNA antibody, conjugated to alkaline phosphatise and bounded to a chemiluminescent substrate, CDP Star (Figure 2). Microplates are then transferred into a software program where results are analysed; in particular, CDP Star ligh is measured by a luminometer. The intensity of the light emitted by each specimen is expressed on a scale as RLUs (Relative Light Units), relative to the average reactivity measured in triplicate wells with a positive control containing 1.0 pg of HPV-16 DNA/ml.

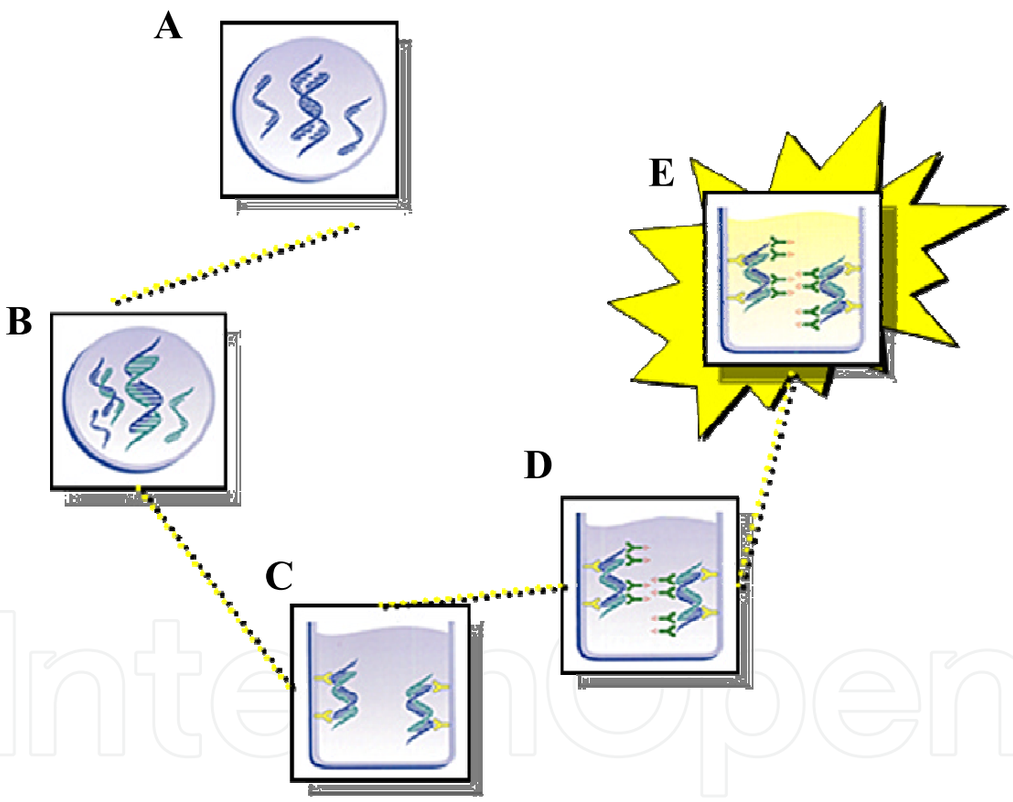

Fig. 2. Hybrid Capture 2 Technology. A - HPV-DNA denaturation; B – single-stranded RNA probes recognizes oncogenic HPV-DNA; C - DNA-RNA complexes are immobilized onto microplates coated with polyclonal IgG antibody; D – identification of immobilized hybrids by a second DNA-RNA alkaline phosphatise conjugated antibody, bounded to CDP star; E – a luminometer measures light emitted by the specimen (Digene-Qiagen website, modified)

The first generation of HC assay (HC1) was a tube-based detection system and probed for only nine HR-HPV types. The analytical sensitivity of the assay was 50,000 copies of HPV-16 DNA.

Several studies evaluated the clinical sensitivity and specificity of this first generation assay mainly against PCR and histology (Cavuslu et al., 1996; Cope et al., 1997; Shah et al., 1997). As expected, HC1 was less sensitive than PCR and other application techniques, but its specificity and positive predictive value (PPV) were higher than those of PCR. To improve the sensitivity of HC1, the Digene Corporation modified the first generation assay. The improved second-generation assay (Hybrid capture 2) is commercially available from the beginning of 1998. In this technology, the analytical sensitivity was increased to 1,000 HPV-DNA copies. Studies comparing first and second generation of Hybrid Capture technology shoved concordance in 96.8%. Interestingly, in all discordant samples containing HPV-DNA types covered by the probes included in both assay generation, the RLU/cutoff value ratios obtained in the second-generation assay were very low, which would indicate that low HPV viral load was the reason that produced false-negative results in the first-generation assay. HC2 assay is actually considered more sensitive than HC1, as well as more rapid, easier to perform and thus more appropriate for the routinary detection of HPV infection. In addition, as RLU signal is proportional to the amount of HPV-DNA present in the specimen, HC2 assay could be occasionally used to deduce viral load, on a semi-quantitative basis. The main advantage of HC2 test is the high sensitivity and the abundant clinical data which allowed to its FDA approval. Moreover, since HC2 test is less affected by cross-specimen contamination than PCR, it don't need for special laboratory (Carozzi et al., 2005; Cubie et al., 2005). On the other hand, there are many limitation to this techniques which have to be taken into account. First, the test cannot determine the specific HPV type present in the sample; this fact limits the recognition of persistent infection by the same viral type, which is the most important risk factor that induce cervical lesion to progress to invasive cancer. Second, at the standard FDA-approved cut-off of 1 pg/ml (RLU ≥1.0) and even at higher discriminating levels, RNA probes suffers from a cross-reactivity with non-oncogenic viral types and with certain oncogenic untargeted HPV types (53, 66, 67, 73) (Howard et al., 2004). Cross-reactivity with other oncogenic-HPV types might be beneficial for test sensitivity. On the other hand, cross-reactivity with non-cancer-causing types would have an adverse impact on clinical specificity and positive predictive value of the test, especially in population with a high prevalence of non-oncogenic types (Castle et al., 2003).

During 2003, a prototype version of Hybrid Capture 3® (HC3), possibly the next generation of HC2, has been evaluated for the detection of CIN3 and cancer. HC3 was designed to minimize cross-reactivity with untargeted HPV types. HC3 showed slightly higher sensitivity that HC2 for the detection of CIN2+ lesions but equal specificity. Actually, HC3 has not been marketed yet.

HC2 technology is amenable to automation, in case of high-volume screening use. The next-generation Hybrid Capture® (NextGen, QIAensemble™, Qiagen Inc., Gaithesburg, MD), consists of a fully automated platform (JE2000™) which utilizes a reengineered HC2 test. The implementation of analytical specificity, the maintenance of comparable levels of analytical sensitivity, the longer on-instrument stability of reagents, the detection of HPV-16 and 18 at lower copy number, the reduction of assay time (< 2.5 hrs compared to up to 5 hrs for HC2), as well as the reduction of the cross-reactivity with non-oncogenic HPV types (Eder et al., 2009), are the main advantages of NextGen. To validate the use of NextGen within screening context, pre-clinical studies using specimens from patients with histologically confirmed CIN2+ lesions are needed.

9. New paradigms in cervical cancer screening

Detection of oncogenic-HPV DNA is considered to be potentially useful in three clinical application: in population screening, as a primary test or in combination with cytology to detect cervical cancer precursors; in triaging, to select which cytological lesions must to be referred for colposcopy; in follow-up of women treated for high-grade intraepithelial lesion, to accurately identify patients with residual or recurrent lesion.

In adjunct to cytology, the purpose of HPV-DNA assay is the detection of latent or subclinical infection among symptoms-free women. In 2001, Belinson et al., performed a large cross-sectional study (1997 women ageing 35-45) in order to compare the sensitivity of LBC and HC2 test for the detection of CIN2+ lesions (Belinson et al., 2001). Essentially, the rationale of the use of adjunctive HPV-DNA testing is based on the accepted concept of necessary causality of HPV in determining cervical cancer and on the basis of the very high negative predictive value (90-100%) of the combination HPV-DNA test/LBC. Combining HPV-DNA test with LBC improves the performance of Pap test alone, especially when cervical cytology is ambiguous (*i.e.*, ASC-US and LSIL lesions). Using mathematical models to evaluate clinical and economics outcomes. Goldie at al., concluded that using HPV-DNA test plus cytology in women ≥ 30 years of age were more effective in reducing cancer incidence. Combining molecular biology and LBC could also result in increasing the screening interval for women testing negative at both cytology and DNA testing. Longer screening intervals with more sensitive tests would be the strategy providing the most advantageous balance between benefits and costs (Goldie et al., 2004).

In triage, the goal is to guide the management of patients with borderline or mildly diskaryotic smears (ASC-US and LSIL, respectively). ASC-US/LSIL Triage Study, a multicenter and randomized clinical trial sponsored by National Cancer Institute (NCI), evaluated three management strategies for women with ASC-US and LSIL cytological results: (i) immediate colposcopy; (ii) repeated cytology with referral to colposcopy if cytological findings showed HSIL lesion; (iii) HPV triage, with referral to colposcopy in case of HPV-DNA positivity (Rodriguez et al., 2008; Solomon et al., 2001).

ALTS established that: (a) HPV-DNA triage is as sensitive as immediate colposcopy in HSIL+ detection; however, molecular testing would spare all HPV-negative women from emotional and financial weight of colposcopy. (b) repeating cytology, would refer more than two-third of ASC-US/LSIL abnormalities to colposcopy. ALTS study attested that HPV-DNA method represents the best triage (Munoz et al., 2003). In the USA, HPV-DNA testing has been definitively integrated into cervical cancer screening programs and has become the standard of care in the triage of women over the age of 30, having minor cytological abnormalities (Wright et al., 2002); the rationale is the high proportion of HPV-DNA negative women (about 50%) among ASC-US group and the consequent extremely low risk of developing high-grade lesions. In this context, the role of DNA testing is to focus on those women ASC-US/DNA-positive in which colposcopic assessment is justified.

Following treatment with cryosurgery, laser ablation or LEEP for HSIL cervical lesion, 5-25% of patients may develop residual or recurrent high-grade disease (Kocken et al., 2011). Standard of care has been close cytological and colposcopic follow-up at 6, 12 and 24 months after treatment. However, since follow-up Pap test has a low specificity in detecting residual HSIL and given that HPV is cleared from the cervix following adequate treatment, DNA

testing has been evaluated to predict the presence of residual dysplasia (Nam et al., 2009). In a recent meta-analysis, Zielinski et al. considered 11 studies evaluating HR-HPV DNA testing in monitoring women after treatment of CIN2+ lesions. It has been shown that the association HPV-DNA testing/cytology reached a NPV of 99% (Zielinski et al., 2004). The adoption of such algorithm of surveillance would mean to focus colposcopy only on women positive for both Pap and DNA test.

The consciousness of the necessary causality of HPV in determining cervical cancer defined new possible approaches to screen and prevent cervical cancer. In population screening, as a primary test, the purpose of HPV-DNA assay is the detection of latent or subclinical infection among symptom-free women (Rebolj et al., 2011).

Most authors evaluated the performances of cytology (conventional or LBC) and HPV testing (PCR or HC2) in detecting CIN2+ lesions or cervical cancer in several populations. Despite various study design, various ranges of age, various HPV detection techniques, various cervical lesions prevalence rates, the global survey of results leads to three main conclusions: for CIN2+ or greater (CIN2+), HPV testing is more sensitive (88-98% *versus* 51-86%) and has an higher negative predictive value (NPV) than cytology; specificity of DNA testing is lower than that of cytology (83-94% *versus* 92-99%); The sensitive and NPV of combined testing is near to 100%. The objective is then to start the primary screening by the most sensitive and automated test, HPV testing, and in second time to use the best specificity of cytology for diagnosis and triage (Arbyn et al., 2009). Women with abnormal smears would be immediately refer to colposcopy. Since a single negative HPV-DNA test reliably predicts a low risk of subsequent CIN2+, it would be justified to extend screening intervals. (Bulkmans et al., 2007).

Current European Guidelines for quality assurance in cervical cancer screening recommend implementation of pilot program with a validated HPV-DNA test within national organized cervical cancer screening programs and, if effective, permanent implementation of such programs (Arbyn et al., 2008). Longitudinal studies assessing the incidence of CIN2+ lesions are then essential to define the role of HPV testing in cervical cancer screening policies (Leinonen et al., 2009). Actually only baseline results of large randomized controlled trials are available.

Anttila et al., evaluated the impact of primary HPV-DNA screening plus conventional cytology triage of HPV-DNA positive women and compared this cohort with cytology-based screening cohoort. The study incorporated the population enrolled in organised screening programme for cervical cancer in Finland (Anttila et al., 2010). The evaluation was based on the total number of CIN3+ cases detected within five years after the invitation. The study, adding longitudinal information based on cancer registry files, showed that a single round of HPV primary test has been able to reduce the number of cases of invasive cervical cancer. These data suggest that using HPV DNA testing in primary screening, followed by cytology to triage HPV-DNA positive women, would represent an approach more sensitive than that based on cytology alone, in identifying CIN2+ lesions.

In Sweden Naucler et al., explored the efficacy of 11 different screening strategies based on HPV DNA testing alone, cytology alone, and HPV DNA testing combined with cytology (Naucler et al., 2009). They showed that using HPV DNA testing as primary screening followed by cytological triage and repeating HPV DNA testing on DNA-positive/ cytology

negative women after at least 1 year would be a feasible strategy in primary cervical screening.

The Italian NTCC (New Technology for Cervical Cancer) randomized controlled trial compared Human Papillomavirus testing (HC2) alone with conventional cytology as the primary cervical cancer screening test, in a total of about 49,200 having a median age of 42 years (Ronco et al., 2008). Among women aged 35-60 years, HPV testing did not show a statistically significant extent *vs* conventional cytology for the detection of CIN2+ lesion. Vice versa, increasing the cut-off for HPV-DNA positive results from 1 to 2 pg/ml, molecular testing arm showed a statistically significant increase in sensitivity, associated to a non statistically significant reduction of PPV. The sensitivity of HPV testing compared with conventional cytology was much larger among women aging 25-34 than among older women. Ronco et al., recommended the use of HPV testing as primary screening in women older than 35 years of age and a preferable 2 pg/ml cut-off for HC2. In women aged less than 35 years, to avoid colposcopic overtreatment in HPV-positive/cytology negative patients, NTCC suggested 1 year molecular retesting (Figure 3).

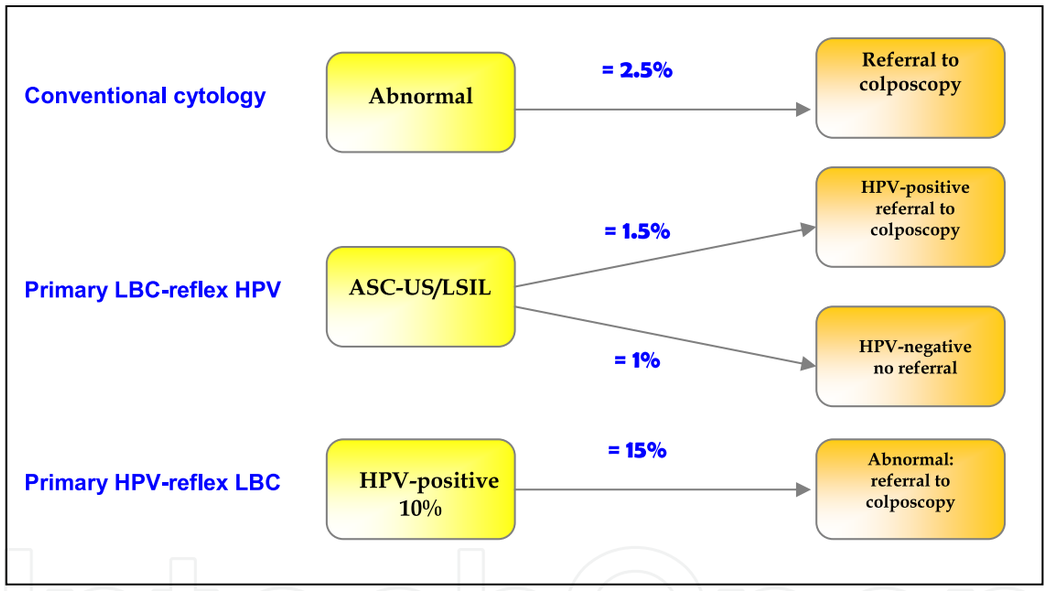

Fig. 3. Three different approaches to cervical cancer screening (Dillner J et al., BMJ 2008, modified)

The consultation of the Medline database by formulating the queries "HPV testing", "cervical cancer screening", "cervical cancer", "HPV testing in primary screening", "HPV-DNA screening test" allows to the following consideration:

1. all the trials (except for NTCC) found that sensitivity of HPV-DNA testing was higher than that of cytology, in detecting CIN2+ lesion;
2. all the trials state that NPP is higher for HPV testing than that for cytology. On the other hand, DNA testing is less specific than Pap test (89% *vs* 95%);
3. HPV testing alone might induce an overtreatment, especially in the youngest population. The specificity for both HPV testing and cytology are clearly lower below 30 years of age;

4. several studies showed that HPV-negativity alone or in combination with negative cytology triage would signify a longer disease interval against CIN2+ lesions than that being for cytology alone. In this context, it seems that a screening interval of five years, would be best choice. After five years, women testing DNA-negative would have a considerable lower risk for CIN2+ (0.25%) than woman having a normal Pap test (0.83%);
5. cytology triage of HPV-DNA positive women, would be the best strategy for referring to colposcopy patients who were DNA-positive/cytology positive;
6. it's meaningless to perform cytology on DNA-negative women; in this case, double testing adds negligible protection compared to HPV testing alone;
7. the better protection but the lower specificity of DNA testing implies that it should only be used in well-organized screening programs, in which the test is not used too frequently, or at too younger age (Dillner et al., 2008; Naucler et al., 2007; Naucler et al., 2009).

It's our opinion that further evaluation of the risk of CIN2+ lesions with different combination of test and in relation to women's age, would be the next step; then, it's important to choose the best primary screening tool, taking in account costs and logistics of the single countries.

The next step in research could be to improve the specificity of HPV-DNA testing by validating adjunctive markers, predictive of HPV persistence, in liquid-based cytology biobanks.

10. New ways to use existing technologies

From the meta-analyses summarised above, it is clear that HPV-DNA testing is substantially more sensitive than cytology at detecting CIN2+ lesions. However, molecular testing is rather less specific than cytology. The main problem with DNA testing is the high prevalence of HPV-DNA positivity among female population, compared with the low number of women with transient infection which spontaneously would regress. These considerations suggest that the more sensitive test should be applied firstly (i.e. DNA testing), while the more specific test should be used only in HPV-positive women, in order to establish the correct management strategy.

The approach of using HPV-DNA test as the sole primary screening modality has several advantages: HPV-DNA detection assays is an objective and automatable test with a dichotomous result; his allows for better quality of screening. Cytology can thus to be reserved for the 5-15% of women who are DNA-positive. This protocol would obtain cost savings through reductions in staff numbers, would reduce turnaround time of diagnosis, would avoid overtreatment and could permit a longer screening interval. For DNA testing, the requirement for adequate specimen sampling is less rigorous, when compared to cytology. Several studies evaluated the diagnostic accuracy of self-collected cervico-vaginal specimens (Ogilvie et al., 2005). With an overall sensitivity of 74% and specificity of 84%, self-taken sampling appears to be favourable in settings where sensitivity of cytology is typically less than 70%. Göh M et al., found that women prefer self-sampling to a clinician taken-sampling (Gök et al., 2010). These results suggest that self-sampling for HPV-DNA testing could be a valuable screening method to recruit women who refuse to attend clinician-based screening, and to improve population coverage of screening.

11. Screening in low-resource settings: changing the paradigm of cervical cancer prevention and control

In developing countries, it seems unrealistic to introduce cytological screening and histopathological follow-up because of financial, technical and human limitations. Alternative methods of screening that would surmount barriers consisting in the "three-visit cytology-based approach" are needed which accurately predict the presence of cervical cancer or precursors (Table). Three requisites are essential in effective screening program: screening diagnosis and treatment should be provided on-site or in clinics accessible to the vast majority of women at risk of developing cancer; reproducible, validated, low-cost screening test should be available; screening should ensure high participation of women at risk for cervical cancer, by using appropriate educational programmes direct towards both health workers and population. While the first two conditions are essential in low-resource settings, the third is an universal requirement.

In low-resource settings, a wide number of tests have been investigated over the years, as alternative screening tests to cytology. The four most widely studied alternative approaches are VIA (visual inspection with acetic acid), VILI (visual inspection with Lugol's iodine), self-sampling and HPV testing.

Screening test	Sensitivity*	Specificity*	Characteristics
Conventional cytology	44%-78%	91%-96%	Requires adequate laboratory-based healthcare infrastructure, stringent training and quality control
HPV testing	66%-100%	61%-96%	High throughput, objective, reproducible and robust but currently expensive
VIA	62%-80%	77%-84%	Low cost, strict linkage to immediate treatment
VILI	92%	85%	Low cost, strict linkage to immediate treatment
Colposcopy	44%-77%	85%-90%	Expensive, inappropriate for low-resource settings

Table. Ranges of sensitivity and specificity and characteristics of some screening methods. VIA: visual inspection with acetic acid; VILI: visual inspection with Lugol's iodine

11.1 Visual inspection with acetic acid

This technique involves the examination of the cervix with the naked eye and a bright light source, one minute after the application of 3-5% diluted acetic acid. Detection of well-defined aceto-white areas close to the squamocolonnar junction, indicates a positive test. CIN and microinvasive cancer switch-on white following acetic acid application. Aceto-whitening is essentially due to a reversible coagulation of intracellular proteins. The high concentration of intracellular proteins in neoplasia led to the dense aceto-whitening appearance. The main advantage of VIA is that it yields an immediate result, thus making it possible for treatment of abnormalities at the same visit ("screen-and-treat"approach). This method is also inexpensive and can be carried out by using modest equipments, without

the need for laboratory infrastructures. Moreover, health workers can be rapidly trained to perform VIA (about ten days of courses duration). Several cross-sectional studies evaluated the accuracy of VIA in developing countries. Pooled estimates of the sensitivity of the test in detecting CIN2+ lesions vary from 62 to 80%, and the specificity from 77 to 84% (Arbyn et al., 2008; Gaffikin et al., 2008; Hovland et al., 2010). The greatest reduction in incidence and mortality rates are observed for the 30-39 years of age group.

11.2 Visual inspection with Lugol's iodine

VILI involves the examination of the cervix the naked eye, in order to identify yellow areas after the application of Lugol's iodine. A multi-centre study conducted in Africa and India showed a pooled sensitivity and specificity to detect CIN2+ lesions of 92% and 85% respectively, thus indicating a higher sensitivity than VIA but a similar specificity (Sarian et al., 2005).

11.3 Self-sampling

Self-sampling method using self-collected vaginal samples is another alternative approach for primary screening in developing countries. The aim would be to try to increase the coverage of population when women do not undergo a gynaecological examination and when cytology screening is not available. Prevalence of oncogenic HPV types on self-sampled vaginal material is about 5-10%, lower than for cervical smear; sensitivity for detecting CIN is also decreased in respect to cytology (Bekkers et al., 2006). However, for women not participating in programs of screening, vaginal self-sampling could be a good alternative and could reduce the risk of cervical cancer.

11.4 HPV-DNA testing

Screening must be linked to treatment to ensure its efficacy. This can be done using the traditional approach (screen, diagnosis, confirm and treat), intermediate approach (screen, diagnose and treat, with post-treatment biopsy confirmation) or screen-and-treat approach (treatment is based on the result of screening alone). A number of studies have investigated the screen-and-treat approach, and its safety and feasibility has been always confirmed. Basing on these studies, primary screening with HPV testing was considered an attractive approach.

The use of HPV-DNA testing may prove more practical, especially when incorporated into strategies less dependent on existing laboratory infrastructure, such as low-resource setting and developing countries. This "single-visit HPV-DNA testing strategy" requires screening sites to run the test on the day in which the sample is received and to allow for treatment of cervical lesion during the same visit (Levin et al., 2010). "See and treat' approach would reduce the number of non-compliance to treatment and improve the efficiency of the program.

No HPV-DNA existing test was deemed appropriate for the use in low-resources settings. For this reason, PATH (Seattle, WA, USA) and Qiagen entered into collaborative agreement and developed a new rapid, simple and affordable HPV-DNA test, specifically designed for developing countries. CareHPV™ is a signal-amplification assay that detects 14 different carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in 2-5-hrs

processing time. The assay needs only a footprint, no mains electricity or running water. The short time-consuming would permit a same-day diagnostic evaluation, with treatment in a second visit, or even screening and treatment in the same day. CareHPV can be performed by inexperienced, newly trained and minimally educated technicians, under suboptimal condition of temperature, humidity, lightning and space. CareHPV use would be associated to fewer visits and transportation costs, and would reduce loss to follow-up. The rapid HPV-DNA, with its lower cost per test and greater sensitivity than conventional cytology now dominates other screening methods in developing countries. Levin CE et al., showed that accuracy for the detection of CIN2+ lesions was higher for careHPV test than for VIA. No differences has been detected between CareHPV and HC2, when screening women 30 years of age and older (Levin et al., 2010).

With the availability of vaccine against HPV-16 and 18, there has been enthusiasm for vaccination of young adolescent girls, also in disadvantage settings. Although preadolescent vaccination offers great hope for future generation, there exist a number of uncertainties that will persist for the next decades, including the need for drastically lower vaccine prices and creative financing sources to support vaccination costs. For the older women, the best option to reduce deaths from cervical cancer will still be the secondary prevention. Rapid HPV-DNA testing, 3 time per lifetime, would have excellent potential to be and effective and cost-effective strategy. Particularly, the strategy of vaccinating targeting young adolescent and screening and treat older women, could address social, economic and political organization to contribute in reducing cervical cancer incidence and mortality in developing countries (Tsu et al., 2008).

12. Molecular surrogate markers

The sensitivity of HPV-DNA assays for the detection of CIN2+ is high, although they cannot discriminate transient infections from persistent infections, thus lacking in specificity. Consequently, there is motivation to evaluate more disease-specific biomarkers, able to identify women susceptible to progression. Ideally the candidate marker, defining the different stages (clearance, persistence, progression) of cellular changes associated with HPV infection, should give a high positive predictive value in predicting progression to cervical cancer. Recently, guidelines have been proposed for the development of biomarker-based screening toll for early detection of cancer, which can be categorized into five phases: 1) preclinical exploratory studies for marker discovery; 2) clinical assay development and validation; 3) retrospective longitudinal repository studies; 4) prospective screening; 5) cancer control studies, including cost-benefit analysis.

Potential markers of progression include HPV viral load, DNA methylation, HPV-DNA sequences integrated into the host genome, over-expression of cell-cycle regulator proteins or proliferation markers, telomerase and messenger RNA for E6 and E7 oncoproteins. Here, we will restrict the discussion to the markers which have been evaluated in large clinical trials and reached some level of clinical applicability.

12.1 HPV Viral load

HR-HPV viral load has been suggested to be a useful marker in distinguishing infection of clinical relevance (48, 49). Among women with HPV-DNA positivity, cytological abnormalities are more common in those having high viral load (Lilli et al., 2005; Xi et al., 2011).

However, it is now clear that the liaison between viral load and cervical disease is more complex that it was previously thought. (De Oer et al., 2007). Many cross-sectional studies reported an increased viral load with growing disease severity, but others found no association (50). There are some probable reasons for these contradictions: HPV integrated status increases with enhancing of disease severity; integration status is characterized by a declined viral replication. Thus paradoxically, the risk of disease progression is not associated to the rate of viral replication but is sometimes just the opposite! (Denis et al., 2008). In our retrospective longitudinal analysis, we did not observe a significant association between semi-quantitative value of viral load and low-grade cervical lesions outcome (Zappacosta R et al, data not shown). In addition, the relationship between viral load and cervical disease varies among oncogenic HPV types. Studies using quantitative type-specific PCR for HR-HPV 16, 18, 31, 33 and 45 and LR-HPV 6 and 11 showed that HPV-16 can reach a much higher viral load than the other above listed types and that only HPV-16 viral load might correlate with increased severity of cervical disease (Saunier et al., 2008). Then, all oncogenic HPV types but 16 are able to provoke cervical cancer, even when they are present at low levels (Boulet et al., 2008).

12.2 DNA methylation markers

More recently, epigenetic alteration of genome of HPV-infected cells have been considered as diagnostic marker for cervical cancer. Aberrant presence of CpG-rich DNA sequences (the so-called CpG islands) in the promoter region of tumor suppressor genes, represents one of the several epigenetic changes that contribute to carcinogenesis (Esteller et al., 2002). DNA methylation involves the covalent addition of a methyl (-CH3) group at the carbon-5 position of a cytosine that precedes a guanosine. Usually, DNA methylation plays a role in maintaining genome stability and in regulating gene expression (Jung et al., 2011). However, global hypermethylation of CpG clusters located in the promoter region of multiples genes have been associated with malignancy (Ehrlich, 2002). Numerous clinical studies demonstrated that these epigenetic methylation changes are often present in a variety of cancer. In this framework, silencing of the Tumor Suppressor Lung Cancer 1 (TSLC1) gene by promoter hypermethylation may be a valuable biomarker to detect cervical lesions with high malignancy potential. TSLC1 was found to be silenced in 91% of cervical cancer cell lines, primarily resulting from promoter hypermethylation (Yang et al., 2006). Moreover, such hypermethylation was detected in 58% of cervical carcinomas and in 35% of CIN2+ lesions, but not in low-grade CIN or in normal cervix (Fenq et al., 2005). The high frequency of TSLC1 methylationin in cervical cancer was confirmed by studies of Li et al., (Li et al., 2005) and Gustafson et al. (Gustafson et al., 2004). These data suggest that the analysis of methylation patterns of TSLC1 gene maight be a valuable tool in forthcoming screening programs; however, they appear more likely to play a role in detecting cervical cancer cell clones rather than cells in early initiating events of cervical carcinogenesis.

12.3 Markers of viral DNA integration

Viral integration often occurs at the E2 gene of the HPV genome. Disruption of the E2 gene is believed to result in more intensive transcription of the oncogenes E6 and E7. In the episomal state, E2 and E6 DNA are present in equal amounts, while in the integrated form, less intact E2 is present (zur Hausen, 2002). Then, a decrease in E2/E6 DNA ratio assessed with real-time PCR would be a valuable potential progression marker.

12.4 Ki-67 and other markers of proliferation or regulation of cell cycle

Expression of Ki-67 protein occurs in proliferating cells and its presence is normally confined to the basal or suprabasal epithelial cells layers. Expression of Ki-67 allows distinction of negative atrophic cells and positive neoplastic cells in menopausal women. Expression beyond the inner third of the cervical epithelium is observed in case of CIN and cancer. Several authors have found a significant correlation between the presence/intensity of Ki-67 and the severity of cytological abnormality in cytological preparation (Luzzatto et al., 2004; Sahebali et al., 2003). However, deeper analysis did not confirm the initially hopeful results and showed that these markers did not have adequate sensitivity and specificity to supply the request of additive prognostic markers, in cytological screening (Wentzensen & von Knebel Doeberitz, 2007).

Several other proteins are overexpressed in proliferating cells. So, certain cell progression regulators have been proposed as potential markers for cervical neoplasia: Proliferating Cell Nuclear Antigen (PCNA), Mcm5, Cdc6 and Cyclin E. Proliferation markers are physiologically present in basal and parabasal cells, and are an objective indicator of neoplasia when observed beyond the lower cell layers. In cervical smears, lacking architectural information, the presence of proliferation markers is less informative and can easily yield false-positive results.

12.5 Telomerase

Telomerase are repeated arrays of six nucleotides (TTAGGG) at the chromosome ends that protects chromosomes against degradation, aberrant fusion or recombination. They become progressively shorter as cells multiply, resulting in chromosomal instability and senescence when a critical short length is reached. The enzyme telomerase is a ribo-nucleoprotein composed of two parts: an RNA part (hRT) and a catalytic part (hTERT), which controls telomere length. hTERT is believed to play a role in cells immortalization. Its activity in increased in CIN and cancer (Barbosa et al., 2011). The intensity of telomerase activity is reported to be correlated with the severity of the abnormality, both in cervical biopsies and cytology. However, reliable detection of hTR, hTERT and telomerase activity is still limited by analytical deficiencies (Xiang et al., 2011).

12.6 p16 ^{INK4a}

Cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors are key molecules that control the cell cycle and coordinate DNA synthesis, chromosome separation and cell division. The cyclin-dependent kinase inhibitor p16 ^{INK4a} prevent the CDK4/6 interaction with cyclin D1, thus stopping progression of cell cycle through the G1/S checkpoint. Usually, HPV-E7 oncoprotein is expressed only in terminally differentiated superficial cell layers of the cervical epithelium, Several factors, such as loss of cellular inhibiting factors and integration of HPV genome into the host genome, can lead to the expression of E7 in basal cell layers. The result is the cellular transformation and the massive accumulation of p16 ^{ink4A} into cells. Accumulation of p16 mRNA and related protein has been reported in response to inactivation of the retinoblastoma gene product (pRb), through binding with viral E7 oncoprotein. p16 is then overexpressed at very low levels in normal cells, while it is strongly over-expressed in cervical cancer cell lines, in which pRB has been inactivated by E7 (Lambert et al., 2006).

The usefulness of immunocytochemical detection of p16 overexpression in cervical samples has been shown in many trials and has been widely accepted, especially as reflex test for the triage of equivocal or mildly abnormal Pap cytologic results. In triaging ASC-US and LSIL cases, it has been shown that p16 immunocytochemical analyses might provide similar sensitivity for the detection of underlying CIN2+ lesions as HPV testing, but at significantly higher specificity level (Denton et al., 2010). A recent meta-analysis (Tsoumpou et al., 2009), showed a substantial variability in the literature regarding the cut-off for positive p16 results; this variability was probably due to the wide range of colorbased qualitative (nuclear or cytoplamatic positivity, staining intensity, etc.) and quantitative parameters (ie, number of immunoreactive cells), which singly or in combinations could be use for the evaluation of results. The purely colour-based approaches to identify abnormal cells in cervical smears using $p16^{INK4a}$, is hampered by the fact that some normal endocervical, endometrial, intercalated, squamous metaplastic or atrophic cells may also display p16-immunoreactivity. For this reason, it was proposed the evaluation of a score based on a four-tiered classification of nuclear abnormalities of p16-positive cells, to discriminate between no abnormal nuclei (score 1), slightly abnormal nuclei (score 2), clearly abnormal nuclei (score 3), and severely abnormal nuclei (score 4). Nuclei from normal-intermediate cells or polymorphonuclear granulocytes were used as an internal size, shape, staining, and texture standard (Wentzenses et al., 2005). Despite NS classification, there are still disagreement in literature regarding the utility of $p16^{INK4a}$ tool, both as locator of high-grade cervical cells-positive cell as well as interpreter, for the predictive assessment of potential abnormal cells. Disagreements are based on the common conviction about the poor feasibility of $p16^{INK4a}$ immunocytochemical assay.

13. Novel approaches with prognostic significance in cervical cancer prevention

13.1 *p16/Ki-67* dual stain

Under physiological conditions, the simultaneous expression of a protein with a tumor-suppressive function (p16) and a proliferation marker (Ki-67) mutually excludes each other. Vice versa, simultaneous detection of p16 overexpression and expression of proliferation marker K1-67 within the same cervical epithelial cell indicates deregulation of the cell cycle. In this context, it has been proposed the use of the immunocytochemical evaluation of p16-Ki-67 coexpression to identify esocervical cells with deregulated cell cycle, independently from morphology-based interpretation parameters (Galgano et al., 2010). The presence of one or more double-immunoreactive cell may be considered positive and indicative of underlying CIN2+ lesion (Schmidt D et al., 2011). The European Equivocal or Mildly Abnormal Papanicolaou Cytology Study (EEMAPS) evaluated the performance of the new immunocytochemical p16/Ki-67 dual-stain protocol (CINtec+ assay, mtm laboratories AG, Heidelberg, Germany) in the triage of ASC-US and LSIL lesions. Results from this study showed a high sensitivity of CINtec+ test for the detection of underlying CIN2+ lesions in women with ASC-US and LSIL cytology, comparable to sensitivity showed by HPV testing and p16 single-stain cytology. Particularly, Schmidt et al., showed that, in ASC-US triage, p16/Ki-67 dual-stain identified the same proportion of underlying CIN2+ lesions as HPV testing, but significantly reducing the number of women which would need referral to colposcopy, especially in younger population. Regarding CINtec+ dual-stain specificity, this was significantly improved in comparison with that of p16 single-stain approach.

In a recent retrospective analysis, conducted within the regional organized screening program of Abruzzo region (Italy), in which Hybrid Capture 2 test is used in primary screening, we analyzed the diagnostic performances of $p16^{INK4a}$/Ki67 dual-stained cytology in identifying CIN2+ lesion in 372 HPV-DNA positive women triaged for LSIL-or-worse (LSIL+) Pap cytology (unpublished data). Preliminary results showed that reflex CINtec+ test improved significantly sensitivity and NPV of cytology alone in triaging LSIL+ lesions (90.8% *vs* 62% and 77.2% *vs* 88.3%, respectively), but reduced triage cytology specificity and PPV (42.6% *vs* 79.6% and 49.4% *vs* 65.2%, respectively). In this context we believe that p16/Ki67 cytology, as a reflex test, may efficiently complement HPV-based screening programs to prevent cervical cancer, but follow-up studies are needed to assess its effective value in terms of predictive marker.

13.2 Direct detection of cervical carcinogenesis: mRNA markers

As previously described, one of the key consequences of HPV-induced chromosomal instability is the integration of HPV genome into the host-cell genome together with the continuous and deregulated expression of E6/E7 viral oncogenes. Then persistent expression of E6/E7 is a necessary step for HPV-induced carcinogenesis.

E6 expression is regulated at transcriptional or post-transcriptional level. HPV-16 E6 ORF encodes for three different variants of E6 protein, which may have dissimilar roles in the viral cell cycle. These transcripts are either unspliced (full length-FL- E6 transcripts) or spliced (Figure 4). Interestingly, only FL E6 protein has been found to be powerfully bound to p53, thus promoting its degradation. Moreover, only the unspliced E6 form was found to be more strongly associated with tumorigenicity. Studies carried out on cervical cancer samples show that FL transcripts are always present (Wise-Draper, & Wells, 2008). These studies indicate FL transcripts as being the most important biomarkers for the carcinogenic process (Asadurian et al., 2007). Then, tests for the detection of E6/E7 mRNA seems to be promising in increasing HPV testing specificity.

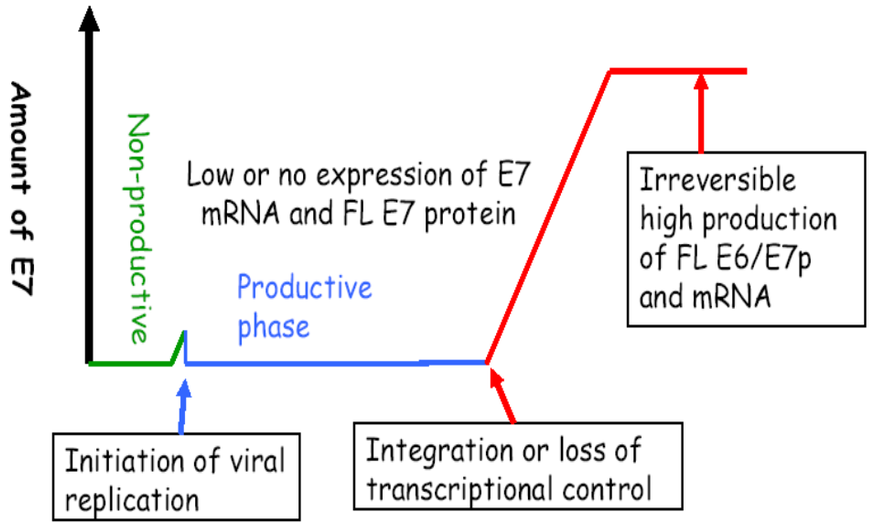

Fig. 4. Full length E7 transcripts in relation to HPV life cycle

13.2.1 Nuclisens® EasyQ HPV mRNA test and PreTect™ HPV- Proofer mRNA test

Various in-house assays for E6/E7 mRNA detection have been described, most of which have employed a reverse transcription PCR approach, focusing on HPV-16 and -18. Now, two commercial RNA assays are available: the Nuclisens EasyQ HPV assay (biomérieux S.A., France), in certain countries distribuited as the PreTect HPV-Proofer® assay (Norchip AS, Klokkarstua, Noway), and the Aptima® HPV Assay (Gen-Probe Incorporated, San Diego, CA) .

Both assays are compatible with samples collected in cytology media.

Nuclisens EasyQ HPV assay is a multiplex nucleic acid sequence amplification (NASBA) method, detecting FL transcripts of E6/E7 oncoproteins in a DNA background is commercially available from two companies; they are the and (Boulet et al., 2008). The test is based on the molecular beacon probe technology and on the real-time detection of five oncogenic HPV types (16, 18, 31, 33 and 45). Molecular beacons are oligonucleotide probes consisting of stem-loop structure and of two regions: loop region, is a single-stranded 18-30 base pair sequence that is complementary to the target sequence; stem region, typically consists of 5-7 base pair long double-stranded sequences that lie on both arms of loop region; 5′ sequences are labeled with a fluorescent dye (fluorophore) while 3′ sequences are covalently labeled with a non-fluorescent quencher. In absence of a complementary target sequence, molecular beacon remains closed and in a nonhybridized state; in this situation, the quencher captures the fluorescent signal. When beacon unfolds the presence of the complementary target (E6/E7 mRNA), loop region hybridizes with this sequence, fluorophore separates itself from the quencher, and the fluorescent signal is transmitted (Figure 5).

In Nuclisens procedure, two different labeled molecular beacon probes for each multiplex reaction are used. Fluorescein (FAM) is used as fluorophore for the detection of HPV-16, 31, and 33; Texas Red (TxR) as fluorophore for the detection of U1A gene, HPV-18, and HPV-45.

U1A is a small nuclear specific ribonucleoprotein A included in HPV-Proofer kit to avoid false-negative results, and to monitor sample mRNA integrity. NASBA amplification is achieved through coordinated activities of three enzymes (Avian Myeloblastosis Virus Reverse Transcriptase, *E.Coli* Rnase H and T7 RNA polymerase) and two DNA oligonucleotide primers that are specific for the target sequence of interest. RNA amplification is performed for one hundred and fifty minutes at isothermal temperature of 41 ^{o}C. In presence of the target sequences, a fluorescent signal is observed. A fluorescent analyzer measures, in real-time, the emitted fluorescence from molecular beacon hybridized with amplified mRNA (Varnai et al., 2008).

Cuschieri et al., carried out a follow-up study on 54 HPV-DNA positive samples obtained from 3,444 cytologically normal women. Samples which were PreTect HPV-Proofer-positive over 9 months (persistence), were proven to have CIN3 in most cases (Cuschieri et al., 2004). This study showed the strict correlation between E6/E7 mRNA expression and oncogenic HPV-DNA persistence, moreover detecting a mRNA test specificity higher (81%) than DNA-based methods (44%). Cuschieri concluded that mRNA test (PreTech or Nuclisens) would find persistent HPV infection and would reduce the need for follow-up or repeated test, that will be sometime necessary if DNA-based technology is used.

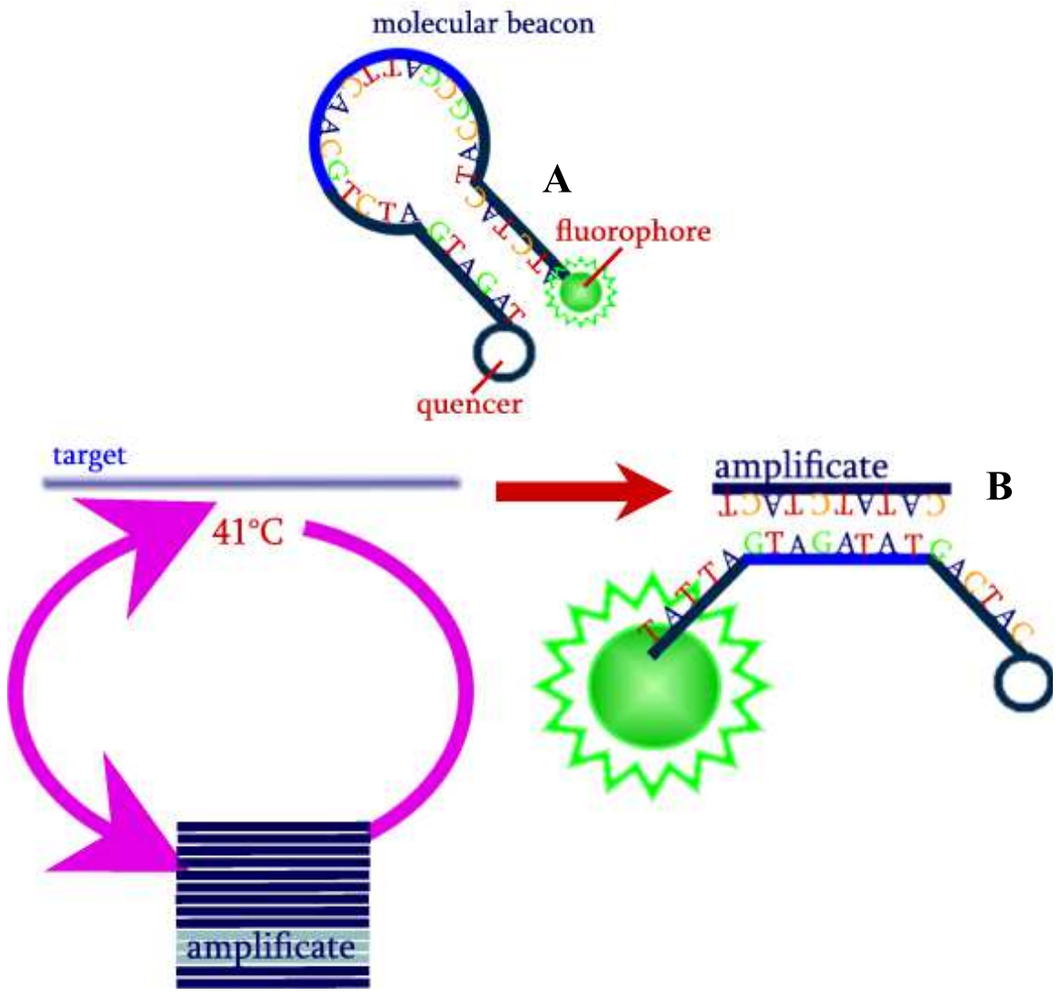

Fig. 5. NASBA technology. A – molecular beacon in absence of target sequence; B – molecular beacon unfolds the presence of the complementary target and transmits fluorescent

The high clinical accuracy of E6/E7 mRNA test has been confirmed by a large cross-sectional study, carried out on about 4,000 women older than 30 years assessed longitudinally for a minimum of 2 years, based on detection of HPV-DNA and E6/E7 mRNA. Cytological and histological data related to follow-up were also included (Molden et al., 2005). DNA and RNA test showed identical sensitivity to detect CIN2+ lesion, but the specificity for RNA and DNA test was 85% and 50% respectively. Benevolo et al carried out a retrospective study to evaluate the performance of mRNA test as a triage test for cytology and HPV DNA testing. The study analyzed 1,201 women, 688 of whom had a colposcopy follow-up and 195 of whom had histology-confirmed CIN2+ lesion. Diagnostic accuracy for CIN2+ were determined for mRNA test in comparison to HPV-DNA test and cytology. Stratifying by cytological grades, mRNA test sensitivity ranged from 62% to 83%. The corresponding figures for DNA testing ranged from 91% to 96%. Specificity values for mRNA test and DNA test ranged from 45% to 82% and from 4% to 29%, respectively. Used as a triage test for ASC-US and L-SIL, mRNA test reduced colposcopies by 69-79%, while

DNA testing reduced colposcopies by 15-38%. As a HPV-DNA positivity triage, mRNA test reduced colposcopies by 63%, whereas cytology at the ASC-US+ threshold reduced colposcopies by 23% (Benevolo et al., 2011).

These data, combined with others 15 extensive studies carried out in many countries, show that mRNA test might reduce the number of ASCUS and LSIL cases to be followed by colposcopy-directed biopsies by more than 70% (Nakagawa et al., 2000). Then, overexpression of E6/E7 for carcinogenic HPV types might prove a specific and predictive marker of precancerous lesions that need clinical attention. At present, only the above listed five oncogenic HPV types are detectable by Nuclisens technology; at the first sight this may be considered a disadvantage in comparison with the available DNA genotyping strategies. However, studies carried out to find E6/E7 mRNA in cervical smears of women with cervical cancer, demonstrated a 100% coverage by these genotypes (Kraus et al., 2006; Skomedal et al., 2006).

13.2.2 Aptima® HPV mRNA test

APTIMA® HPV Assay (Gen-Probe Incorporated, San Diego, CA) is a target amplification nucleic acid probe test for the in vitro qualitative detection of E6/E7 mRNA from 14 oncogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), in LBC specimens. The assay provides a qualitative result (positive/negative) for the presence/absence of these oncogenic HPV types, but does not determine the specific HPV type present in the specimen.This technology amplifies target mRNAs using transcription-mediated amplification (TMA) and detects the amplification products using nucleic acid probes without specific distinction among the specific HPV types implicated.

APTIMA HPV Assay involves three main steps, which take place in a single tube: capture, amplification and detection. To capture the target mRNA, HPV-specific capture oligomers and magnetic microparticles are used. Target mRNA is amplified by transcription-mediated amplifications (TMA) and using two enzymes: Moloney murine leukemia virus reverse transcriptase and T7 RNA polymerase. Targeted amplification is achieved using primers that hybridize to conserved regions of oncogenic E6/E7 mRNA. The detection of the amplification products (amplicons) is carried out through dual kinetic assay . Specifically, analyte amplicons are detected using 2-methyl acridinium ester-labeled probes, which hybridized to conserved region of carcinogenic HPV types. An fluoro-acridium ester-labeled probe is used to detect internal control amplicon added to each reaction to verify the performance of each step of the assay.

Positive and negative calibrators are used to determine the validity of the run and to establish the assay cut-off values for the internal control and analyte signals. Chemiluminescent signal observed for each reaction is measured using a luminometer, and compared to the cut-off values. Specimens with cut-off (S/CO) ratio ≥1.00 are considered positive. Samples with S/CO ratio <1.00 must have an internal control signal greater than or equal to the internal control cut-off value and may be considered a valid negative result.

FASE (French APTIMA Screening Evaluation) study was the first trial comparing Aptima mRNA test with LBC and HC2 (Monsonego et al., 2011). Data from about 4,500 Parisian women have been analyzed. Results showed that the sensitivity of Aptima mRNA test for

detecting CIN2+ lesions was similar to that offered by HC2 test but is significantly higher than that of LBC. In addition, the specificity of Aptima was significantly higher than that of both HC2 and cytology.

Actually, for its characteristics, Aptima test is considered to be the ideal assay for primary cervical cancer screening, offering good specificity without losing sensitivity. The use of APTIMA HPV Assay would reduce the number of false positive results compared to DNA assays, helping limit the number false positive results leading to inappropriate and costly diagnostic procedures, over-treatment and needless anxiety in women.

On the other hand, PreTect HPV-Proofer/Nuclisens EasyQ HPV mRNA tests with their high specificity and PPV, is considered helpful in the clinical work-up of DNA-positive women, particularly of those with ASC-US/LSIL cervical abnormalities, also in consideration of its flexibility when used in LBC samples, stored at room temperature in cytological biobanks.

Basing on these consideration, mRNA-based technology would certainly increase the diagnostic accuracy of cervical abnormalities, through a better identification of HPV infection which are more likely to persist and induce CIN2+ lesions in future, and by reducing psychological distress and costs for women who only have a transient infection.

14. Conclusions

Persistent HPV infection has proven to be important in predicting cell abnormalities. Hence, a supplementary method should reveal HPV persistence and preferentially give additional information about the outcome of the disease. The ideal test should then reveal optimal analytical and clinical accuracies. Analytical accuracy of a test merely refers to the ability of detection of an endpoint. Clinical accuracy is a parameter which is more related to medical practice: it expresses the ability of the test to detect a relevant phase of disease.

Studies summarized above resulted in a long list of candidate tools which may improve Pap test in the early detection of cervical cancer. Most of these markers have not yet passed the first phases of validation but surely, their number is expected to expand, as more genomic and proteomic studies will appear in the near future.

Before integrating the ideal marker into clinical practice, deeper clinical validation studies are needed, particularly longitudinal assessment to prospectively evaluate its clinical performances. At the moment, it is difficult to predict which of those markers or marker panels are ultimately the most promising candidates, also considering the shift to primary HPV screening.

Currently, most national vaccination program are primarily aimed to preadolescents and adolescents. It is an extremely positive fact to hope in use of a vaccine capable to prevent a neoplasia with so strong social impact such as cervicocarcinoma. However, there are several issues that still need to be addressed before the fully appreciation of HPV vaccination in matter of overall potential and impact for public health (Psirry & Di Maio, 2008; Stanley et al., 2006). First, the duration of protection is unknown. Second, bivalent vaccination will only protect against HPV-16 and 18. Third, prophylactic vaccines are likely to provide limited benefits to women previously infected with oncogenic HPVs. Fourth, vaccines are relatively expensive and vaccine delivery in developing world is more difficult. Fifth, the

effects of vaccination on the female psychology could be dangerous: if vaccinated women will believe to be at no further risk of developing HPV-induced cancer and will leave screening programs, the last impact of vaccination on the incidence of cervicocarcinoma will be invalided (Welters et al., 2008).

It is therefore important that both, women and healthcare professionals, do not perceive HPV vaccination as an immediate alternative to cervical cancer screening, because only integrating HPV vaccination into screening programs will maximize the benefits offered by vaccine and will lead to a greater reduction of cervical cancer prevalence, incidence, and mortality. It was recently reported that , if both HPV testing and vaccination are performed, the total number of annual number of Pap tests is predicted to be reduced by 43% (Logatto-Filho & Schmitt, 2007). In this context, the nature of the screening and the management of women must to be adapted to the new technologies.

In conclusion, moving the diagnostics from the cellular level into the molecular level allows not only to better identify cervical precancerous states, but also to prevent cervical pathology in the stage of molecular changes. In this context, the management of women with HPV infection would be based on risk categories rather than on specific assay results. This tailored cervical cancer risk assessment give hope for the improvement of effectiveness of cervical cancer prevention and for a significant reduction of screening costs.

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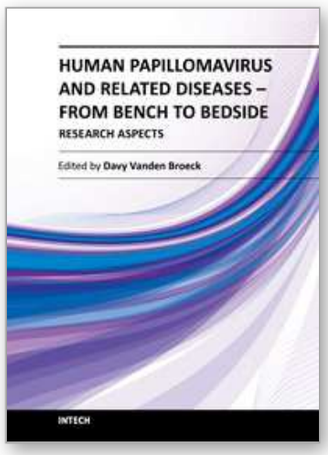

Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on epidemiological and fundamental research aspects in the area of HPV, and it will update those working in this fast-progressing field with the latest information.

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