UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

TESIS DOCTORAL

Biomarcadores del virus del papiloma humano (VPH) en el cáncer de cérvix

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Rosa Montero Macías

DIRECTORES

Pluvio Coronado Martín
Hélène Péré

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA
Programa de Doctorado en
Ciencias Médico-Quirúrgicas.

TESIS DOCTORAL

BIOMARCADORES DEL VIRUS DEL PAPILOMA HUMANO (VPH) EN EL CANCER DE CÉRVIX

HUMAN PAPILLOMAVIRUS (HPV) BIOMARKERS IN CERVICAL CANCER

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADO POR

Rosa María MONTERO MACÍAS

DIRECTORES

Pluvio Jesús CORONADO MARTÍN y Hélène PÉRÉ

BIOMARCADORES DEL VIRUS DEL PAPILOMA HUMANO (VPH) EN EL CANCER DE CÉRVIX

HUMAN PAPILLOMAVIRUS (HPV) BIOMARKERS IN CERVICAL CANCER

Rosa MONTERO MACÍAS

Conducted by:

Dr Pluvio CORONADO MARTIN

Women's Health Institute José Botella Llusiá, Hospital Clínico San Carlos, Fundación de Investigación del Hospital Clínico San Carlos (IdISSC), Facultad de Medicina. Universidad Complutense, Madrid, Spain

Dr Hélène PERE
- Virology Unit, European Georges Pompidou Hospital.

- INSERM, Functional Genomics of Solid Tumors (FunGeST), Centre de Recherche des Cordeliers, Paris Cité University and Sorbonne

FACULTAD DE MEDICINA, Programa de Doctorado en Ciencias Médico-Quirúrgicas.
Proyecto realizado en colaboración con la Université de Paris y el Hospital Europeo George Pompidou (Paris)

UNIVERSIDAD COMPLUTENSE MADRID

Université de Paris

To all the women suffering cervical cancer around the world.

Acknowledgements

The contribution of many people, in the professional or personal way, made this thesis possible. I wish to thanks everyone sincerely and express gratitude to the following people.

To patients who agreed to participate in these studies and for their collaboration in the research.

À mes directeurs : À Hélène Péré, tu es au centre de ce projet, cela n´aurait jamais été possible sans toi. Merci de m´avoir guidé durant ces 5 années, merci également pour ta patience et ta persévérance. A Pluvio Coronado, por haber aceptado dirigir mi tesis sin conocerme y guiarme en la distancia.

À l´équipe du Département de Virologie de l´Hôpital Européen George Pompidou (HEGP) qui ont contribué à ce projet ces 5 dernières années. Merci pour le temps qu´ils ont consacré aux manips, analyses, publications : Marion Mandavit, Nicolas Robillard, Thomas Bruneau, Julien Puech, Mélanie Boulhic, Laurent Bélec et David Veyer, merci à tous. Aussi à Valérie Taly du CRC.

À Marie-Aude Le Frére Belda, Cécile Badoual et Ivanna Stankovic du Département d´Anatomopathologie de l´HEGP pour le temps dédié à ce projet et de m´avoir introduit dans les caves d´anapth, les blocs, les lames et les copeaux.

Á l´équipe du Service de Chirurgie cancérologique de l´HEGP qui m´a formée et tellement appris dans la chirurgie oncologique et la recherche: Charlotte Ngô, Pr. Anne-Sophie Bats, Meriem Koual, Hélène Bonsang, Marie Gosset. Aux collègues qui ont facilité mes premiers pas parisiens et m´ont aidé pendant mes premières années à l´hôpital en France (Cynthia Lecurieux-Lafayette, Alice Bialot, Vanille Simon).

Et spécialement au Professor Fabrice Lecuru de m´avoir accepté dans son service en 2018, d´avoir été mon menteur en Gyneco Onco et de m´avoir proposé de participer à ce projet qui est ensuite devenu mon PhD.

À mon actuel chef de service (Vincent Villefranque) et mes collègues de l´Hôpital Simone Veil qui m´ont supportée et aidée ces deux dernières années, spécialement Roxana Sanfranciscus qui fait aujourd´hui partie de ma famille. Merci Elie Mikhael pour les corrections de l'anglais dans les publications. Á mon « équipe Gyneco » David Krief et Alex Boyer. À tous mes internes qui travaillent dure et rendent mes gardes beaucoup plus faciles.

À Pascal Rigolet pour ton énorme aide dans le nébuleux monde de la statistique, ta patiente et ta motivation. Merci Benjamin Seban de m´avoir mis en contact avec Pascal.

A la Fundación Martín Escudero por concederme la beca que me ha permitido venir a Francia a realizar este proyecto, formarme en cirugía oncológica y dar los primeros pasos en esta ciudad que es ahora mi casa.

A Martina Angeles por ayudarme con los datos clínicos y por tu disponibilidad.

A mis compañeros (y amigos) del Hospital de Fuenlabrada, algunos adjuntos primeros y compañeros después (Silvia Pérez, Silvia González, Natalia Pagola, Larraitz Ibañez, María Luque, Lola Ruíz, Carmen Sabadell, Javi Vega, Amparo Moreno,…). Por ser "casa" durante 7 años e introducirme en la especialidad. A Santiago Alonso por enseñarme tanto de patología cervical, VPH y mostrarme los primeros pasos en el mundo quirúrgico.

Al equipo de EnganCHADos que pasaron de ser compañeros de hospital a ser amigos (David Montes (y Vane!), Alfonso Antequera, Daniel Huerga,…).

À Elisabeth Raule de m'avoir montrée la motivation maximale en tant que chirurgienne et que les limites n'existent pas.

To all the professionals and patients with whom I shared my time in Blantyre (Malawi) in the MSF cervical cancer project. I learned a lot and saw cervical cancer from another perspective that makes me continue to motivate myself in this profession and especially in research to improve prevention, diagnosis and prognosis of our patients.

A mis amigos y compañeros de la Facultad de Sevilla, por compartir conmigo los primeros años de Medicina y entusiasmarnos juntos en este camino sin fin! Inma Medrano, Isaac Peña, Jesús Álvarez (Poke), Cris Ortiz, Javi León y Juanma Castillo (por desearme siempre Fuerza y honor en la cirugía, la investigación y la vida).

A mi "familia" de Madrid, por apoyarme en cada nuevo proyecto y locura, incluso en la distancia. A Rebeca Diéguez por tantos días y noches de escuchar mis historias de hospital y mis clases de la Universidad, además por ser tan buena amiga. Patricia Rodríguez, Alejandra Moore, Lissette González, por estar siempre ahí y darme amor. A Iván Brondino por enseñarme lo que es pasar un PhD, que es largo pero que ¡¡se puede!!.

A Alicia Castro, Ana Calvo, Mathieu Bonilla, y Carlos Soto que me han acompañado y apoyado en estos años parisinos y que me han hecho sentir en "familia" en Paris. Gracias por los domingos en familia. Gracias por hacerme reír y hablar "muy alto" en los momentos duros.

To Aqeel, Anis and Mursal, because you have taught me a lot about life, culture and you supported me in this last year.

A mis padres, José Antonio Montero y Chari Macías, y mi hermana Silvia, por acompañarme toda mi vida, siendo el mayor apoyo. Por haberme inculcado la fuerza y voluntad para realizar proyectos como este. Os quiero.

Gracias papá por enseñarme en silencio el camino de la medicina desde pequeña, compartir conmigo la motivación de esta profesión desde que empecé medicina y ser el modelo a seguir de "médico de vocación". Eres la única persona que en todos estos años jamás he visto quejarse después de una dura guardia o por tener mucho trabajo. Tu ilusión al contarme cada caso clínico me hacen ver a lo que me gustaría parecerme.

Gracias mamá por cuidarme incluso en la distancia. Por haber dedicado todo tu tiempo en nosotras y permitirnos ser lo que somos.

Gracias hermana por estar siempre ahí, por los años de infancia compartidos, los años de medicina viviendo y estudiando juntas. Espero que siempre compartamos esta pasión por la medicina. A Sofía y Alessandro Paolini por aumentar el amor en nuestra familia.

A toda mi familia, tíos, primos y abuelos (que ya no están con nosotros). Gracias por enseñarme la humildad y por hacerme seguir sintiendo que Andalucía es mi tierra.

À Walid, mon amour. Pour me soutenir, m´aider, m'aimer et faire partie de tous mes projets. Tu rends tout facile et me fais sentir encore plus forte. J'espère que c'est l'un des premiers projets que nous terminons ensemble mais qu'il sera suivi par bien d'autres dans le futur. TQMM. Aussi à la famille Gharbi Naciri/Présent de m´avoir accueillie et de m´avoir fait sentir comme à la maison depuis le premier jour.

Contents

Figures

Tables

Abbreviations

BMI: body mass index

CIN: cervical intraepithelial neoplasia

ctDNA: Circulating tumoral DNA

cfDNA: Circulating free DNA

CPP: Comité de protection de personnes

CCR: chemoradiotherapy

CRT: chemoradiotherapy

CTCEA: Common Terminology Criteria for Adverse Events

CT: Computed Tomography

DNA: Deoxyribonucleic acid

ddPCR: Droplet Digital Polymerase Chain Reaction

DFS: Disease-Free Survival

dNTP: Deoxynucleotide triphosphates

DSS: Disease-Specific survival

EBRT: external beam radiation therapy

EBV: Epstein-Barr Virus

ECC: Early Cervical Cancer

ESGO: European Society Gynaecological Oncology

ESMO: European Society Medical Oncology

FFPE: Formalin Fixed Paraffin Embedded

FIGO: Federation Internationale de Gynécologie et d'Obstetrique

HC: Hybrid Capture

HCT: Hybrid Capture Tube

HEGP: Hôpital Européen George Pompidou.

HES: Hematoxylin Eosin Safran

HPV: Human Papillomavirus

HR: Hazard Ratio

HSIL: high-grade squamous intraepithelial lesion

IARC : International Agency for Research on Cancer

ICG: Indocianine green

ITCs: Isolated tumor cells

LCR: long control region

LN: Lymph Nodes

LSIL: low-grade squamous intraepithelial lesion

LVSI: Lymphovascular Space Invasion

MCM: Minichromosome maintenance

MIC: Micrometastasis

MIS: minimally-invasive surgery

MRI: Magnetic resonance imaging

mRNA: messenger RNA

NA: Not available

NPV: negative predictive value

NGS: Next-Generation Sequencing

NS: not significant

N0: Nodes negatives

N+: Nodes positives.

ORFs : open-reading frames

OS: Overall Survival

PALND: para-aortic lymph node dissection

Pap: Papanicolaou

PCR: Polymerase Chain Reaction

PET-CT: positron emission tomography/computed tomography

PFS: Progression-frree survival

PLND: pelvic lymph node dissection

qPCR/ RT-PCR: Real-time quantitative PCR

RLU: relative light units

RNA: Ribonucleic Acid

RT: radiotherapy

SCC: squamous cell carcinoma

SCJ: squamocolumnar junction

SD: Standard deviation

SLN: Sentinel Lymph Node

TNM: Tumor, Nodes and Metastasis Staging

TZ: tranformation zone

VLPs: Virus-like particles

WHO: World Health Organization

Abstract

Human Papilloma Virus (HPV) persistent infection causes 99% of cervical cancer. Molecular detection of HPV is used nowadays mainly in cervical cancer screening, but the use of HPV detection as a prognosis factor or helping cervical cancer diagnosis is not generalised. In our project, we have evaluated the used of HPV related molecular biomarkers for the assessment of prognosis in cervical cancer patients without pelvic lymph node invasion (N0) and for the differential diagnosis of distant lesions.
In the Study I and II, we evaluated the use of new HPV biomarkers as prognosis factor in patients with ECC and negative lymph pelvic nodes in Sentinel Lymph Node (SLN) biopsy. It is known that N0 patients have a better prognosis than patients with lymph nodes invasion (N+), however 15 % of N0 patients have recurrences. In this context, we decided to evaluate the value of HPV biomarkers to classify N0 patients according to the risk of recurrence or death.

In the Study I, we analysed HPV in the ***primary tumors***. We identified specific molecular signatures of HPV by Capture technique coupled with NGS (Next-Generation Sequencing) in 40 tumors samples of ECC patients without histologically mestastasis in SLN biopsies and correlated with prognosis. HPV 16 was the most frequent genotype (55% of the patients). Different molecular statuses of the HPV viral genome were identified: Episomal (viral genome is fully covered), integrated (total loss of viral framed genomic coverage over a defined area framed on either side by chromosomal marks confirms complete integration of the HPV genome into the human genome) and Mixed (the genome is both present in episomal and integrated form). For the HPV16 genotype (22 patients), in 100% of the cases we found the episomal form (20 pure episomal (90.9%) and 2 mixed (9.1%)). However, HPV18 genotype (9 cases) was most often found in integrated status (5 cases pure (55.6%) and 2 mixte (22.2%)). Only 2 patients (22.2%) was pure episomal status for HPV18. This dependence between the molecular status and the virus genotype is highly significant (p=0.00002). Correlating with clinical data, 7 recurrence and 3 deaths were documented between the 40 patients. In 100% of these cases, HPV16 genotype was found in episomal form. We found significant differences for the risk of recurrences in relation to the presence or not of HPV16 in the episomal state (p=0.007).

In the Study II, we researched HPV tumoral DNA (HPVtDNA) in pelvic ***Sentinel Lymph Nodes*** (SLN) biopsy using ultrasensitive droplet-based digital PCR (ddPCR) to detect occult metastasis. More than half (51.7 %) of the patients finally showed HPVtDNA positivity in SLN initially diagnosed negative by histology. Two patients with negative HPVtDNA SLN and 6 with positive HPVtDNA SLN group presented recurrence (p=0.15). Moreover, all of the 4 deaths listed in our study occurred in the positive HPVtDNA SLN group. Lastly, we observed significant difference between HPVtDNA-positive and HPVtDNA-negative SLN groups in term of DSS (Disease Specific Survival) (p=0.040) but not for progression-free survival (p=0.101).

Finally, in the study III, we evaluated the use of molecular commercial assay for HPV detection based on Real Time (rt) PCR in patients with cervical cancer antecedent and a new ***distant lesion.*** The objective was to clarify the differential diagnosis between cervical cancer metastasis and other primary/metastasis of other tumor for this second lesion. We reported eight cases of patients with these criteria. Results from rt-PCR showed HPV-16 DNA in 6 cases and HPV-18 DNA in 1 case in the distant lesion confirming its cervical origin. Finally, a HPV negative distant lesion completed our serie confirming histological diagnosis of new primary independent cancerous lesion.

In conclusion, HPV biomarkers can be useful in clinical practice in the personalisation of the prognosis and to help of diagnosis in doubtful situations in cervical cancer patients.

Abstract in Spanish

La infección persistente por el virus del papiloma humano (VPH) causa el 99% de los cánceres de cuello de útero. La detección molecular del VPH se utiliza hoy en día principalmente en el cribado del cáncer de cervix, pero el uso de la detección del VPH como factor de pronóstico o de ayuda al diagnóstico del cáncer de cuello de útero no está generalizado. En nuestro proyecto, hemos evaluado el uso de biomarcadores moleculares relacionados con el VPH para la evaluación del pronóstico en pacientes con cáncer de cérvix sin invasión de los ganglios linfáticos pélvicos (N0) y para el diagnóstico diferencial de las lesiones a distancia.

En el Estudio I y II, se evaluó el uso de nuevos biomarcadores del VPH como factor de pronóstico en pacientes con cancer de cervix precoz (CCP) y ganglios linfáticos pélvicos negativos en la biopsia del ganglio linfático centinela (GLC). Se sabe que las pacientes N0 tienen un mejor pronóstico que las pacientes con invasión de los ganglios linfáticos (N+), sin embargo el 15% de las pacientes N0 tienen recidivas. En este contexto, decidimos evaluar el valor de los biomarcadores del VPH para clasificar a las pacientes N0 según el riesgo de recurrencia o muerte.

En el Estudio I, analizamos el VPH en los ***tumores primarios***. Identificamos firmas moleculares específicas del VPH mediante la técnica de captura acoplada a la NGS (Next-Generation Sequencing) en 40 muestras de tumores de pacientes con CCP sin mestástasis histológica en biopsias de GLC y las correlacionamos con el pronóstico. El VPH 16 fue el genotipo más frecuente (55% de los pacientes). Se identificaron diferentes estados moleculares del genoma viral del VPH: Episomal (el genoma viral está totalmente cubierto), integrado (la pérdida total de la cobertura genómica enmarcada por el virus en un área definida enmarcada a ambos lados por marcas cromosómicas confirma la integración completa del genoma del VPH en el genoma humano) y Mixto (el genoma está presente tanto en forma episomal como integrada). En cuanto al genotipo del VPH16 (22 pacientes), en el 100% de los casos se encontró la forma episomal (20 episomales puros (90,9%) y 2 mixtos (9,1%)). Sin embargo, el genotipo del VPH18 (9 casos) se encontró con mayor frecuencia en estado integrado (5 casos puros (55,6%) y 2 mixtos (22,2%)). Sólo 2 pacientes (22,2%) tenían un estado episomal puro para el VPH18. Esta dependencia entre el estado molecular y el genotipo del virus es altamente significativa (p=0,00002). En correlación con los datos clínicos, se documentaron 7 recidivas y 3 muertes entre las 40 pacientes. En 100% de estos casos, el genotipo del VPH16 se encontró en forma episomal. En nuestro studio, nosotros encontramos diferencias significativas respecto al riesgo de recurrencias en relación con la presencia o no de VPH16 en estado de episomal (p=0.007).

En el Estudio II, investigamos el ADN tumoral del VPH (ADNt del VPH) en la biopsia de los ***GLC pélvicos*** utilizando una PCR digital en gotas (ddPCR) ultrasensible para detectar metástasis ocultas. Más de la mitad (51,7%) de las pacientes mostraron finalmente positividad para el HPVtDNA en el GLC inicialmente diagnosticado como negativo por la histología. Dos pacientes con GLC VPHtADN negativo y 6 con GLC VPHtADN positivo presentaron recidiva (p=0,15). Además, todas las 4 muertes registradas en nuestro estudio se produjeron en el grupo de GLC con ADNt del VPH positivo. Por último, observamos una diferencia significativa entre los grupos de GLC con ADN del VPH positivo y con ADN del VPH negativo en lo que respecta a la DSS (Disease Specific Survival) (p=0,040), pero no en lo que respecta a la Supervivencia Sin Progression (SSP) (p=0,101).

Por último, en el estudio III, se evaluó el uso de una técnica molecular comercial para la detección del VPH basado en la PCR en tiempo real (rt) en pacientes con antecedentes de cáncer cervical y una nueva ***lesión a distancia***. El objetivo era aclarar el diagnóstico diferencial entre la metástasis del cáncer de cérvix y otro origen tumoral primario/metástasis para esta segunda lesión. Reportamos ocho casos de pacientes con estos criterios. Los resultados de la rt-PCR mostraron ADN del VPH-16 en 6 casos y ADN del VPH-18 en 1 caso en la lesión a distancia, confirmando su origen cervical. Finalmente, una lesión a distancia VPH negativa completó nuestra serie confirmando el diagnóstico histológico de una nueva lesión cancerosa primaria independiente.

En conclusión, los biomarcadores del VPH pueden ser útiles en la práctica clínica para personalizar el pronóstico y ayudar al diagnóstico en situaciones dudosas en pacientes con cáncer de cérvix.

Abstract in French

L'infection persistante par le papillomavirus humain (HPV) est à l'origine de 99 % des cancers du col de l'utérus. La détection moléculaire du virus HPV est aujourd'hui utilisée dans le dépistage du cancer du col de l'utérus, mais l'utilisation de la détection du HPV comme facteur de pronostic ou d'aide au diagnostic du cancer du col de l'utérus n'est pas généralisée. Dans notre projet, nous avons évalué l'utilisation de biomarqueurs moléculaires liés au HPV pour l'évaluation du pronostic chez les patientes atteintes d'un cancer du col de l'utérus sans envahissement ganglionnaire pelvien (N0) et pour le diagnostic différentiel des lésions à distance.

Dans les études I et II, nous avons évalué l'utilisation de nouveaux biomarqueurs HPV comme facteur de pronostic chez les patientes atteintes d'un cancer du col précoce (CCP) et présentant des ganglions pelviens négatifs lors de la biopsie du ganglion lymphatique sentinelle (GLS). Il est connu que les patients N0 ont un meilleur pronostic que les patients avec un envahissement ganglionnaire (N+), cependant 15 % des patients N0 ont des récidives. Dans ce contexte, nous avons décidé d'évaluer la valeur des biomarqueurs HPV pour classer les patients N0 en fonction du risque de récidive ou de décès.

Dans l'étude I, nous avons analysé le HPV dans les tumeurs primaires. Nous avons identifié des signatures moléculaires spécifiques du HPV par la technique de capture couplée au NGS (Next-Generation Sequencing) dans 40 échantillons de tumeurs de patients atteints de CCP sans mestastase histologique dans des biopsies GLS et corrélées avec le pronostic. Le HPV 16 était le génotype le plus fréquent (55% des patients). Différents statuts moléculaires du génome viral du HPV ont été décrits: statut épisomal (le génome viral est entièrement recouvert), statut intégré (la perte totale de la couverture génomique de l'ADN viral sur une zone définie et encadrée de part et d'autre par des marques chromosomiques confirmant l'intégration complète du génome HPV dans le génome humain) et statut mixte (le génome est à la fois présent sous forme épisomale et intégrée). Pour le génotype HPV16 (22 patients), dans 100% des cas nous avons retrouvé la forme épisomal (20 épisomaux purs (90,9%) et 2 mixtes (9,1%)). Par contre, le génotype HPV18 (9 cas) était le plus souvent retrouvé sous forme intégrée (5 cas purs (55,6%) et 2 mixtes (22,2%)). Seuls 2 patients (22,2 %) avaient un statut épisomal pur pour le HPV18. Cette dépendance entre le statut moléculaire et le génotype du virus est hautement significative (p=0,00002). En corrélation avec les données cliniques, 7 récidives et 3 décès ont été documentés parmi les 40 patients. Dans 100% de ces cas, le génotype HPV16 était trouvé sous forme épisomale. Dans notre étude, nous avons trouvé des différences significatives concernant le risque de récidives en fonction de la présence ou non de HPV16 à l'état épisomal(p=0,007).

Dans l'étude II, nous avons recherché l'ADN tumoral HPV (HPVtDNA) dans les biopsies de GLS pelviens de ces mêmes patientes en utilisant la PCR digitale en microgouttelettes (ddPCR) ultrasensible pour détecter les métastases occultes. Plus de la moitié (51,7 %) des patients ont finalement montré une positivité du HPVtDNA dans les GLS initialement diagnostiqués négatifs par histologie. Deux patientes avec GLS HPVtDNA négatif et 6 avec un GLS HPVtDNA positif ont présenté une récidive (p=0,15). De plus, les 4 décès répertoriés dans notre étude sont tous survenus dans le groupe GLS HPVtDNA positif. Enfin, nous avons observé une différence significative entre les groupes GLS HPVtDNA-positifs et HPVtDNA-négatifs en terme de Disease Specific Survival (DSS) (p=0.040) mais pas pour la survie sans progression (p=0.101).

Enfin, dans l'étude III, nous avons évalué l'utilisation d'un test commercial moléculaire pour la détection du HPV basé sur la PCR en temps réel (rt) chez les patientes présentant un antécédent de cancer du col de l'utérus et une nouvelle lésion distante. L'objectif était de clarifier le diagnostic différentiel entre une métastase du cancer du col de l'utérus et une autre tumeur primaire/métastase d'une autre tumeur pour cette deuxième lésion. Nous avons rapporté huit cas de patients répondant à ces critères. Les résultats de la rt-PCR ont montré la présence d'ADN de HPV-16 dans 6 cas et d'ADN de HPV-18 dans 1 cas dans la lésion distante confirmant son origine cervicale. Enfin, une lésion distante HPV négative a complété notre série confirmant le diagnostic histologique de nouvelle lésion cancéreuse primaire indépendante.

En conclusion, les biomarqueurs HPV peuvent être utiles en pratique clinique dans la personnalisation du pronostic et pour aider au diagnostic dans les situations douteuses chez les patientes atteintes de cancer du col de l'utérus.

Original studies

This thesis is based on following original studies, referred to in the text by their Roman numerals. The articles already published are appended at the end of the thesis.

I. Identification of specific molecular signatures of HPV virus in early cervical cancer without pelvis nodes metastasis by HPV Capture technique coupled with NGS (Next-Generation Sequencing) and correlation with a different prognosis- article is in writing process.

II. TRANSLACOL Project: Nodal Human Papillomavirus tumoral DNA detection by ddPCR for survival prediction in early cervical cancer patients without pelvic lymph node invasion- article accepted in Journal of Clinical Virology on February 18th 2023, in proofreading process.

III. HPV molecular genotyping as a differential diagnosis tool in cervical cancer metastasis- article published.
Reference: Montero-Macías R, Coronado PJ, Robillard N, Veyer D, Villefranque V, Le Frére-Belda M-A, Auberger E, Bitolog P, Stankovic I, Bélec L, Bats A-S, Lécuru F, Péré H. HPV Molecular Genotyping as a Differential Diagnosis Tool in Cervical Cancer Metastasis. *Journal of Personalized Medicine*. 2023; 13(2):177. https://doi.org/10.3390/jpm13020177

Thesis at a glance

Study	Aim	Results	Conclusion
Study I: **Identification of specific molecular signatures of HPV virus in early cervical cancer (ECC) without pelvis nodes metastasis by HPV Capture technique coupled with NGS (Next-Generation Sequencing) and correlation with a different prognosis.**	Identify specific molecular signatures of HPV virus by HPV Capture technique coupled with NGS (Next-Generation Sequencing) in ECC patients without histologically lymph node metastasis and correlate them with prognosis.	100% of the cases the HPV16 genotype were found the episomal form: 90.9% pure episomal and 9.1% mixte. However, HPV18 genotype was most often found in integrated status: 55.6% cases intregrated pure and 22.2% mixte. This dependence between the molecular status and the genotype is highly significant (p=0.00002). In 100% of cases of recurrence HPV 16 episomal was present. We found significant differences for the risk of recurrences in relation to the presence or not of HPV16 in the episomal state (p=0.007).	HPV18 integrates more easily into the human genome than the HPV16 genome. The most virulent molecular status of HPV16 seems to be associated with an episomal form of this genotype.
Study II: **TRANSLACOL Project: Nodal Human Papillomavirus tumoral DNA detection by ddPCR for survival prediction in early cervical cancer patients without pelvic lymph node invasion (N0)**	Research HPV tumoral DNA (HPVtDNA) in pelvic Sentinel Lymph Nodes (SLN) biopsies using ultrasensitive droplet-based digital PCR (ddPCR) as a prognosis marker in ECC patients without histologically lymph nodes metastasis(N0).	We observed significant difference between HPVtDNA-positive and HPVtDNA-negative SLN groups in term of Disease-specific survival (DSS).	The use of ultrasensitive ddPCR to detect HPVtDNA in SLN could allow the identification of two subgroups of N0 early cervical cancer patients that may have different prognosis and outcomes.
Study III: **HPV molecular genotyping as a differential diagnosis tool in cervical cancer metastasis**	Identify if an easy-to-use HPV molecular genotyping assay would allow differentiating HPV tumor secondary metastasis from a new independent primary non-HPV induced tumor.	In seven cases, we found oncogenic HPV in the biopsy of the distant lesion, which confirmed the diagnosis of cervical cancer metastasis. In the remaining case, no HPV was detected in the secondary lung biopsy, confirming the diagnosis of new primary lung cancer	HPV molecular genotyping can be use in newly diagnosed distant lesions in patients with a history of HPV cervical neoplasia to complete the clinical and histologic diagnosis in ambiguous situations.

Introduction

Cervical cancer remains worldwide the fourth most frequently diagnosed cancer and the fourth cause of cancer death in women (1)

99% of cervical cancer are caused for Human Papilloma Virus (HPV) persistence infection (2).
Besides for diagnosis and screening, the HPV molecular characteristics can be useful to determinate the cancer prognosis and to be predictive recurrence factor.

Our main aim is the identification of HPV related biomarkers (molecular and histopathological) that may be useful in the clinic: for the differential diagnosis of distant lesions and for the assessment of prognosis in cervical cancer patients without nodular invasion (N-).

Background

Cervical cancer

Anatomy

The cervix is a fibromuscular organ that links the uterine cavity to the vagina of approximately 4 cm in length and 3 cm in diameter. The cervix has several different linings. The endocervical canal is lined with glandular (columnar) epithelium, and the ectocervix is lined with squamous epithelium. The squamous epithelium meets the glandular epithelium at the squamocolumnar junction (SCJ). The SCJ is dynamic and moves during early adolescence and during a first pregnancy. The epithelium between these two SCJs (original and new) is the TZ or tranformation zone. Squamous cervical cancer accounts for the majority of cervical cancer and originates in the TZ. Glandular cervical cancer originates in either the TZ or the glandular epithelium above the TZ. (3)

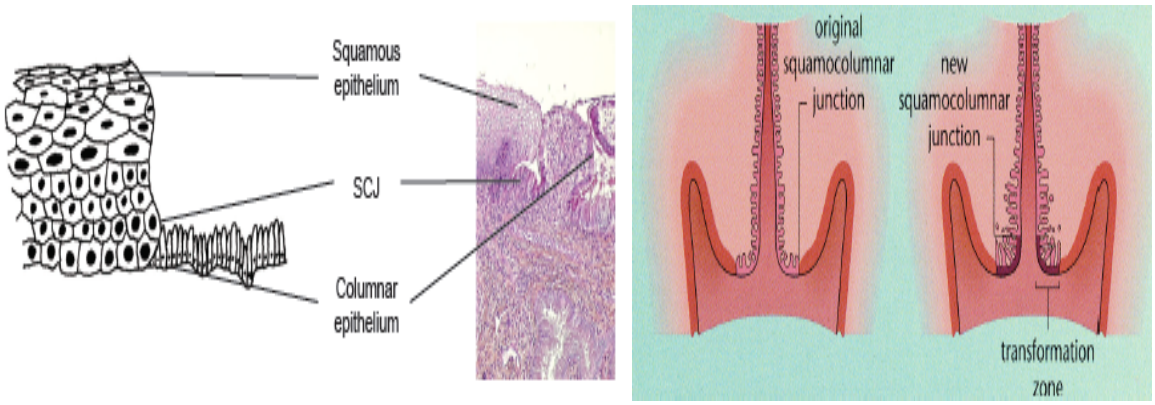


Figure 1- Anatomy and histology of squamocolumnar junction (SCJ) (3). Reproduced with the kind permission of IARC publications.

Epidemiology and aetiology

Cervical cancer is the 2nd most common female cancer in women aged 15 to 44 years and is the 4th most common cancer among women worldwide. The incidence reach around 40% in Easter Africa (4). Age-standardised mortality rate is around 7.3% and is the third leading cause of cancer death worldwide in women (5).

HPV has been found in 99.7% of cervical cancers, suggesting that HPV is a necessary cause of invasive cervical cancer (6). Others host factors including other sexually transmitted infections, patterns of sexual behaviour and parity, smoking and immune factors have also been related but with lesser significance.

Area	N Cases	Uncertainty intervals of new cancer cases [95% UI]	Crude rate[b]	ASR[b]	Cumulative risk (%) ages 0-74 years[a]	Ranking	
						All women	Women 15-44 years
World	604,127	[582,030.8-627,062.1]	15.6	13.3	1.39	4	2
High income	71,624	[69,708.5-73,592.1]	11.6	8.40	0.80	14	3
Low and middle income	532,239	[240,358.8-255,554.1]	16.4	14.8	1.57	2	2
Upper middle income	247,840	[240,358.8-255,554.1]	17.1	12.8	1.31	5	3
Lower middle income	236,828	[224,446-249,893]	15.9	16.9	1.87	2	2
Low income	47,571	[42,451.1-53,308.4]	15.8	23.8	2.59	2	2
Africa	117,316	[105,998.6-129,841.8]	17.5	25.6	2.82	2	2
Eastern Africa	54,560	[48,276.6-61,661.3]	24.3	40.1	4.46	1	1
Middle Africa	15,646	[13,437.4-18,217.6]	17.4	31.6	3.56	2	2
Northern Africa	6,971	[6,061.2-8,017.3]	5.69	6.25	0.72	4	5
Southern Africa	12,333	[11,952-12,726.2]	36.0	36.4	3.70	2	1
Western Africa	27,806	[23,307.1-33,173.3]	13.9	22.9	2.48	2	2
Americas	74,410	[56,232-62,828.9]	14.3	11.3	1.13	6	3
Caribbean	3,857	[3,427-4,341]	17.5	13.7	1.37	5	2
Central America	13,848	[13,283.5-14,436.5]	15.1	13.8	1.39	2	2
Northern America	14,971	[14,703.2-15,243.7]	8.04	6.15	0.59	14	4
South America	41,734	[38,925.2-44,745.5]	19.1	15.4	1.59	3	3
Asia	351,720	[339,675-364,192.1]	15.5	12.7	1.35	4	3
Central Asia	4,945	[1,677-1,882.9]	13.2	12.7	1.32	2	2
Eastern Asia	129,567	[126,381.1-132,833.1]	15.8	10.8	1.08	7	3
South-Eastern Asia	68,623	[64,656.6-72,832.8]	20.5	17.8	1.91	2	2
Southern Asia	143,183	[883-1,630.8]	15.2	15.4	1.72	2	2
Western Asia	5,402	[4,559-6,400.8]	4.07	4.14	0.45	12	6
Europe	58,169	[56,344.7-60,052.4]	15.0	10.7	1.03	9	3
Eastern Europe	32,348	[31,583.5-33,131]	20.8	14.5	1.42	5	2
Northern Europe	6,666	[6,414.5-6,927.3]	12.4	10.4	0.90	12	2
Southern Europe	9,053	[8,181.3-10,017.5]	11.5	7.72	0.76	14	4
Western Europe	10,102	[9,650.9-10,574.2]	10.1	7.03	0.67	14	4
Oceania	2,512	[2,299.4-2,744.2]	11.8	10.1	0.94	8	3
Australia & New Zealand	1,094	[1,021.2-1,172]	7.17	5.63	0.52	13	5
Melanesia	1,330	[975.5-1,813.4]	24.4	28.3	2.64	2	2
Micronesia	53	[33.3-84.4]	19.5	18.7	1.97	3	2
Polynesia	35	[20.2-60.7]	10.4	9.70	1.06	6	3

Data accessed on 27 Jan 2021

Table 1- Incidence of cervical cancer by World region and sub regions (estimates for 2020) (7) a) Cumulative risk (incidence). B) Rates per 100000 women per year. **Data Sources:** Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [27 January 2021]. Reproduced with the kind permission of IARC Publications.

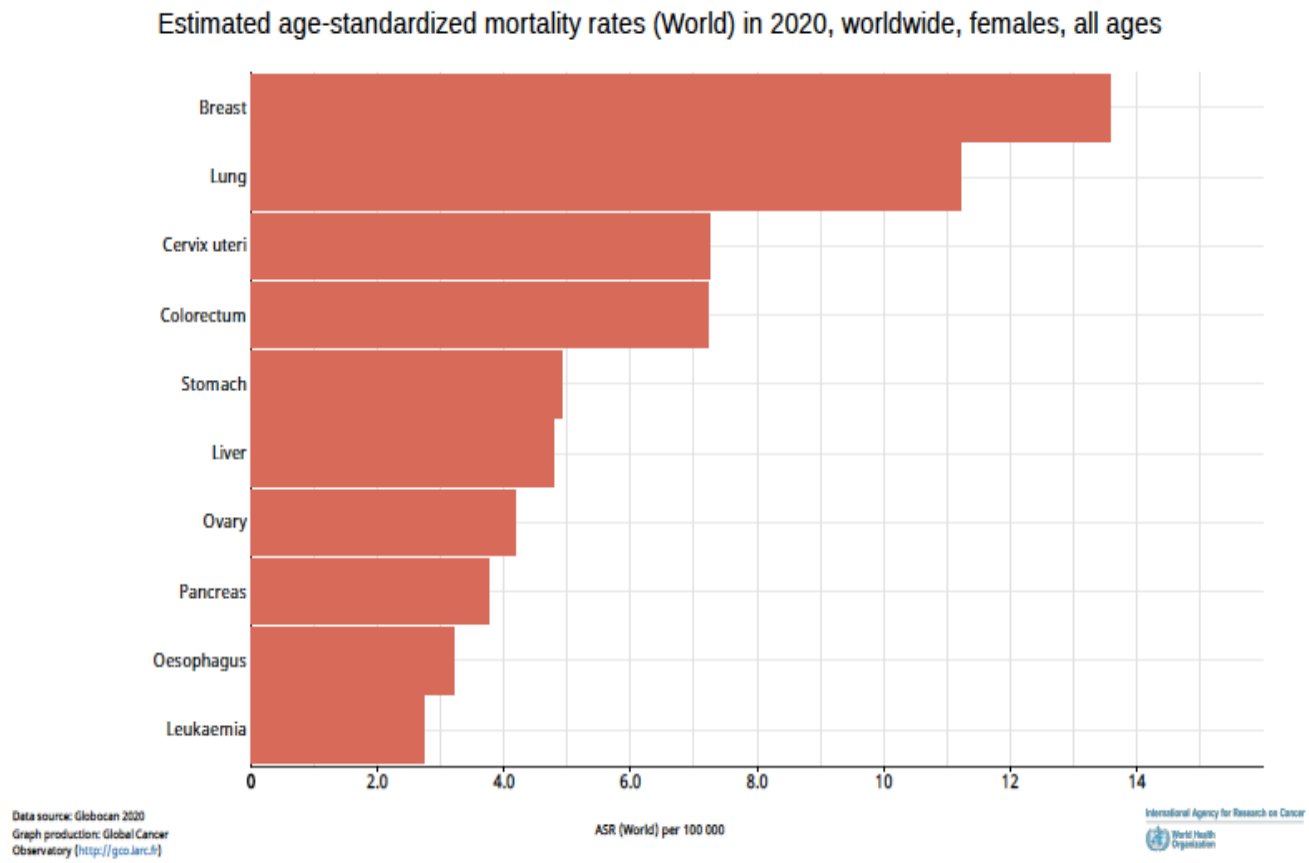


Figure 2- Estimated age-standardized mortality rated foe cervical cancer in 2020, worldwide. (1). Reproduced with the kind permission of John Wiley and Sons.

Screening

The Papanicolaou (Pap) or cytology-based test have been the standard method for cervical cancer screening during last decades, reducing the incidence by 60%–90% and the death rate by 90%. However, the limitations of this Pap test are the sensitivity (around 50%), subjective interpretation of morphological alterations and significant proportion of inadequate specimens. As HPV deoxyribonucleic acid (DNA) is present in almost all cervical cancers, HPV molecular detection has been introduced recently as a screening tool and demonstrated higher sensitivity for high grade cervical intraepithelial neoplasia (CIN 2-3) than that achieved by Pap test in several studies. The International HPV screening working group published a pooled analysis of four randomised controlled trials of HPV-based cervical screening versus conventional cytology showed that HPV-based screening provides 60-70% greater protection against invasive cervical carcinomas compared with cytology. Data of large-scale randomised trials support initiation of HPV-based screening from age 30 years and extension of screening intervals to at least 5 years. (8).

Symptoms and diagnosis

Cervical cancer can be asymptomatic in early stages. Screening programmes, installed mainly in most of the developed countries of the world, have radically decreased the proportion of advanced cancers at diagnosis. More of half of the cervical cancers in these countries are diagnosed at an early stage.

In advanced stages, symptoms may include bleeding, haematuria, hydronephrosis, lymphoedema or pain secondary to invasion of nearby structures.

In early stages, diagnosis is based in detection of precancerous lesions, leading to colposcopy, biopsies and conization that could diagnose invasive cancer.
In advanced stages, symptoms lead to cervical biopsies or imaging tests that guide the diagnosis: pelvic magnetic resonance imaging (MRI) and /or Endovaginal/transrectal ultrasound is an option if performed by a properly trained sonographer.

Magnetic resonance imaging (MRI) can determine tumor size, degree of stromal penetrations, parametrial involvement, vaginal extension and corpus extension with high accuracy (9).
Positron emission tomography (PET) has showed the potential to accurately delineate the extent of disease, particularly in lymph nodes that are not macroscopically enlarged and in distant sites, with high sensitivity and specificity. In early-stage disease, PET/CT has a sensitivity of 53%–73% and specificity of 90%–97% for the detection of lymph node involvement, while in more advanced stages the sensitivity for detecting the involvement of para-aortic nodes increases to 75% with 95% specificity (10).

Cystoscopy or rectoscopy may be considered to provide a biopsy if suspicious lesions in the urinary bladder or rectum are documented on MRI or ultrasound (11).

Pathology

Items to be included on pathology report of carcinomas of the cervix

Clinical/Surgical	Macroscopic	Microscopic
Specimen(s) submitted	**Specimen dimensions** • No. tissue pieces (for loops/cones) • Tissue piece dimensions (for loops/cones) ○ Diameter of ectocervix (2 measurements) ○ Depth of specimen • Vaginal cuff ○ Minimum length ○ Maximum length • Size of parametria in 2 dimensions (vertical and horizontal) **Macroscopic tumor site(s)**	**Tumor dimensions*** • Horizontal extent (2 measurements) • Depth of invasion or thickness **Histological tumor type** **Histological tumor grade** **LVSI** **Coexisting pathology** • Squamous intraepithelial lesion/cervical intraepithelial neoplasia • Adenocarcinoma in situ • Stratified mucin-producing intraepithelial lesion **Minimum distance of uninvolved cervical stroma** **Margin status (invasive and preinvasive diseases). Specify the margin(s)** **Lymph node status (sentinel lymph node status, number involved/number retrieved and presence of extranodal extension)** **Pathologically confirmed distant metastases** **Pathological staging pretumor board/multidisciplinary team meeting (TNM category)**

*Tumor dimension should be based on a correlation of the gross and histological features.

Table 2- Items to be included on pathology report of carcinoma of the cervix (11).
Reproduced with the kind permission of BMJ Publishing Group Ltd.

Histopathology (12)

Squamous Cell carcinoma

It accounts for more than 70% of cervical cancers. As it originates from the exocervix the macroscopic appearance is exophytic. Microscopically are composed of cells that are recognisably squamous but vary in either growth pattern or cytological morphology.
Various subtypes have been described: keratinising (characterised by the presence of keratin Pearls and mitoses are not frequent); non-keratinising (carcinomas do not form keratin pearls by definition, but may show individual cell keratinisation.); basaloid ("CINlike"- an aggressive high grade tumor); verrucous (not HPV-related - bulbous squamous pegs with a pushing border and minimal atypia); "warty" (associated with high-risk HPV); papillary; lymphoepithelioma-like (not EBV-driven unlike other lymphoepithelial cancers except in Asian populations); squamotransitional.

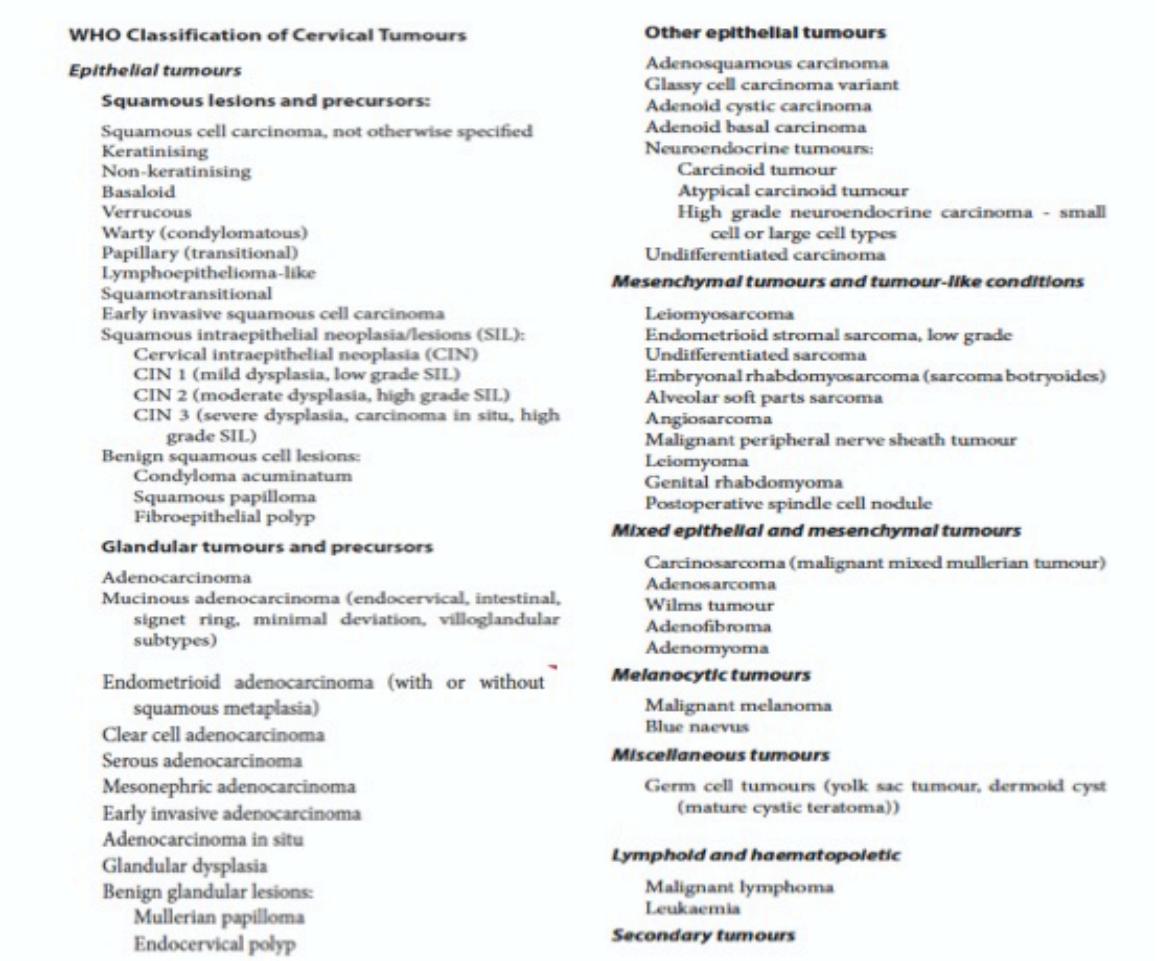
Adenocarcinoma

It is the second most frequent. The incidence of cervical adenocarcinoma is increasing in comparison to the more common squamous cell carcinoma (adenocarcinoma currently accounting for over 25% of cervical cancers). This is due probably to the success of screening programmes in detecting precursors of squamous cell carcinoma leading to a real decrease in invasive squamous cell carcinomas coupled with the fact that it is difficult to diagnose precancerous glandular lesions, so that adenocarcinomas are diagnosed more at advanced stages.

Adenocarcinoma tends to be associated with HPV18, compared with squamous carcinoma more associated with HPV16. The most of cervical adenocarcinoma are usual (HPV-relate) type, bur there is also a group of non-HPV-related adenocarcinoma, including gastric-type, clear cell and mesonephric, which tend to be more aggressive. 70% of adenocarcinomas are endocervical type making it difficult to diagnostic and accurate measurements.

Other cervical carcinoma

Neuroendocrine tumors include carcinoids, atypical carcinoids and neuroendocrine carcinomas. Diagnosis is histological and can be confirmed by neuroendocrine markers.



WHO Classification of Cervical Tumours

Epithelial tumours

Squamous lesions and precursors:

Squamous cell carcinoma, not otherwise specified
Keratinising
Non-keratinising
Basaloid
Verrucous
Warty (condylomatous)
Papillary (transitional)
Lymphoepithelioma-like
Squamotransitional
Early invasive squamous cell carcinoma
Squamous intraepithelial neoplasia/lesions (SIL):
- Cervical intraepithelial neoplasia (CIN)
- CIN 1 (mild dysplasia, low grade SIL)
- CIN 2 (moderate dysplasia, high grade SIL)
- CIN 3 (severe dysplasia, carcinoma in situ, high grade SIL)

Benign squamous cell lesions:
- Condyloma acuminatum
- Squamous papilloma
- Fibroepithelial polyp

Glandular tumours and precursors

Adenocarcinoma
Mucinous adenocarcinoma (endocervical, intestinal, signet ring, minimal deviation, villoglandular subtypes)
Endometrioid adenocarcinoma (with or without squamous metaplasia)
Clear cell adenocarcinoma
Serous adenocarcinoma
Mesonephric adenocarcinoma
Early invasive adenocarcinoma
Adenocarcinoma in situ
Glandular dysplasia
Benign glandular lesions:
- Mullerian papilloma
- Endocervical polyp

Other epithelial tumours

Adenosquamous carcinoma
Glassy cell carcinoma variant
Adenoid cystic carcinoma
Adenoid basal carcinoma
Neuroendocrine tumours:
- Carcinoid tumour
- Atypical carcinoid tumour
- High grade neuroendocrine carcinoma - small cell or large cell types

Undifferentiated carcinoma

Mesenchymal tumours and tumour-like conditions

Leiomyosarcoma
Endometrioid stromal sarcoma, low grade
Undifferentiated sarcoma
Embryonal rhabdomyosarcoma (sarcoma botryoides)
Alveolar soft parts sarcoma
Angiosarcoma
Malignant peripheral nerve sheath tumour
Leiomyoma
Genital rhabdomyoma
Postoperative spindle cell nodule

Mixed epithelial and mesenchymal tumours

Carcinosarcoma (malignant mixed mullerian tumour)
Adenosarcoma
Wilms tumour
Adenofibroma
Adenomyoma

Melanocytic tumours

Malignant melanoma
Blue naevus

Miscellaneous tumours

Germ cell tumours (yolk sac tumour, dermoid cyst (mature cystic teratoma))

Lymphoid and haematopoietic

Malignant lymphoma
Leukaemia

Secondary tumours

Figure 3- WHO Classifications of Cervical Tumors. (*WHO Histological Classification of Tumors of the Uterine Cervix*, n.d.). Reproduced with permission of IARC Publications.

Tumor dimensions

- Depth of invasion or thickness
- Horizontal extent (two measurements)

Tumor volume has been shown to predict prognosis more reliably than one or two only dimensions. This is one of the main reasons for recommending that three tumor dimensions: two of horizontal extent and one of thickness (13).

Grade

Cervical cancer grade can be classified from 1 to 3. A lower number means the cancer is a lower grade.

Low-grade cancers have cancer cells that are well differentiated.

High-grade cancers have cancer cells that are poorly differentiated or undifferentiated. The cells don't look like normal cells and are arranged very differently. Higher grade cancers tend to grow more quickly and are more likely to spread than low-grade cancers.

Lymphovascular space invasion (LVSI)

LVSI does not affect FIGO or TNM staging but should be clearly documented in the pathology report. Satellite LVSI is the most important factor predicting DFS and OS in early stage cervical cancer, especially when lymph nodes are negative (14).

Lymph node status

Lymph node status includes the total number of nodes found and the number of positive nodes and the presence of extranodal extension.

The risk of pelvic lymph node invasion in early cervical cancer is around 15% (15) and this nodes invasion is the most important prognosis factor in cervical cancer (16).

Nodal metastasis can be considered:
-Macrometastasis: greater than 2 mm.
-Micrometastasis (MIC). Greater than 0.2 mm and up to 2 mm. Reported pN1mi.
-Isolated tumor cells (ITCs no greater than 0.2 mm in regional nodes. Reported pN0 (i+).

FIGO Classification

Former classification 2009 FIGO staging was based mainly on clinical examination. In 2018, this has been revised by the FIGO Gynecologic Oncology Committee to allow imaging, pathological findings and nodal status, where available, to assign the stage.
This classification makes difference between tumors less than 2 cm (IB2) and more than 2 cm (IB3).

Also, the FIGO 2018 classification integrates nodal spread in the classification. Patients with nodal metastases are now considered as stage IIIc1 or IIIc2 whatever the tumor size (17).

Stage	Description
I	The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded)
IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion <5 mm[a]
IA1	Measured stromal invasion <3 mm in depth
IA2	Measured stromal invasion ≥3 mm and <5 mm in depth
IB	Invasive carcinoma with measured deepest invasion ≥5 mm (greater than Stage IA), lesion limited to the cervix uteri[b]
IB1	Invasive carcinoma ≥5 mm depth of stromal invasion, and <2 cm in greatest dimension
IB2	Invasive carcinoma ≥2 cm and <4 cm in greatest dimension
IB3	Invasive carcinoma ≥4 cm in greatest dimension
II	The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
IIA	Involvement limited to the upper two-thirds of the vagina without parametrial involvement
IIA1	Invasive carcinoma <4 cm in greatest dimension
IIA2	Invasive carcinoma ≥4 cm in greatest dimension
IIB	With parametrial involvement but not up to the pelvic wall
III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes[c]
IIIA	The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause)
IIIC	Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent (with r and p notations)[c]
IIIC1	Pelvic lymph node metastasis only
IIIC2	Para-aortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. (A bullous edema, as such, does not permit a case to be allotted to Stage IV)
IVA	Spread to adjacent pelvic organs
IVB	Spread to distant organs

When in doubt, the lower staging should be assigned.
[a]Imaging and pathology can be used, where available, to supplement clinical findings with respect to tumor size and extent, in all stages.
[b]The involvement of vascular/lymphatic spaces does not change the staging. The lateral extent of the lesion is no longer considered.
[c]Adding notation of r (imaging) and p (pathology) to indicate the findings that are used to allocate the case to Stage IIIC. Example: If imaging indicates pelvic lymph node metastasis, the stage allocation would be Stage IIIC1r, and if confirmed by pathologic findings, it would be Stage IIIC1p. The type of imaging modality or pathology technique used should always be documented.

Table 3- FIGO staging of cancer of the cervix (2018). (17) Reproduced with the kind permission of John Wiley and Sons.

Prognosis factors

The principal prognostic factors include (11):
-TNM and FIGO stage, including a maximum tumor size and volume.
-Extracervical tumor extension (parametrial invasion).
-Nodal involvement (number, size, location), the main prognostic factor.
-Pathological tumor type and grade.
-Depth of cervical stromal invasion and a minimum thickness of uninvolved cervical stroma.
-Presence or absence of LVSI.
-Presence or absence of distant metastases.

These factors have been also considered like criteria to decide the adjuvant treatment. The Sedlis group studied clinic-pathological risk factors like criteria to choose the patients (tumor size >4 cm, positive LVSI and deep stromal invasion) whom adjuvant radiation after radical surgery could decrease local recurrence. However, patients with tumor size less than 2 centimetres (cm), no LVSI, and depth of stromal invasion (DSI) less than 10 millimetres (mm) are classified as low-risk patients and radiation do not change the prognosis (18).

In early stages, nodal metastasis is considered as the most important prognostic factor. Kim et al, showed that in stages IB–IIA, the 5-year survival rates without lymph node metastasis and with lymph node metastasis are 88%–95% and 51%– 78%, respectively (19). With similar results, the FIGO annual report (2009) described that 5-year survival was over 96% for stage Ib1 N- cancers and 79% for Ib1 N+ (20).

Treatment

A general recommendation is that treatment strategy should aim for the avoidance of combining radical surgery and radiotherapy because of the highest morbidity after combined treatment.

Surgical treatment (11)

Uterus and ovarian treatment

- Conization or simple hysterectomy: can be considered a definitive treatment for stage IA1 and IA2 as parametrial resection is not indicated in this case.

- Radical hysterectomy: indicated in IB1- IIA1 stages. Retrospective studies showed that minimally invasive hysterectomy can be associated with lower blood loss, decreased rate of post-operative complications, faster recovery, and shorter inpatient hospital stay. However, the data from the prospective multicentre LACC trial published in 2018 by Ramirez et al showed that MIS radical hysterectomy was associated with lower rates of disease-free survival and overall survival than open abdominal radical hysterectomy among women with early-stage cervical cancer (21). According to the Update in April 2020 of ESMO guideline about cervical cancer Radical hysterectomy performed by laparoscopy or robot-assisted

surgery cannot be regarded as the preferred treatment in comparison with open surgery in patients with FIGO stage IA2, IB and IIA. Patients should be counselled about the risks and benefits of the different types of surgery (22)(23).

The type of radical hysterectomy (extent of parametrial resection, type A-C2 to the Querleu-Morrow classification) should be based on the presence of prognostic risk factors identified preoperatively.

Risk Group	Tumor Size	LVSI	Stromal Invasion	Type of Radical Hysterectomy*
Low risk	<2 cm	Negative	Inner 1/3	B1 (A)
Intermediate risk	≥2 cm	Negative	Any	B2 (C1)
	<2 cm	Positive	Any	
High risk	≥2 cm	Positive	Any	C1 (C2)

Table 4- Risk groups according to prognostic factors: suggested types of radical hysterectomy (11). Reproduced with the kind permission of BMJ Publishing Group Ltd.
*According to the Querleu-Morrow classification.

Type of Radical Hysterectomy	Paracervix or Lateral Parametrium	Ventral Parametrium	Dorsal Parametrium
Type A	Halfway between the cervix and ureter (medial to the ureter, ureter identified but not mobilized)	Minimal excision	Minimal excision
Type B1	At the ureter (at the level of the ureteral bed, ureter is mobilized from the cervix and lateral parametrium)	Partial excision of the vesicouterine ligament	Partial resection of the rectouterine/rectovaginal ligament and uterosacral peritoneal fold
Type B2	Identical to B1 plus paracervical lymphadenectomy without resection of vascular/nerve structures	Partial excision of the vesicouterine ligament	Partial resection of the rectouterine-rectovaginal ligament and uterosacral fold
Type C1	At the iliac vessels transversally, caudal part is preserved	Excision of the vesicouterine ligament (cranial to the ureter) at the bladder. Proximal part of the vesicovaginal ligament (bladder nerves are dissected and spared)	At the rectum (hypogastric nerve is dissected and spared)
Type C2	At the level of the medial aspect of iliac vessels completely (including the caudal part)	At the bladder (bladder nerves are sacrificed)	At the sacrum (hypogastric nerve is sacrificed)
Type D	At the pelvic wall, including resection of the internal iliac vessels and/or components of the pelvic sidewall	At the bladder. Not applicable if part of exenteration	At the sacrum. Not applicable if part of exenteration

*Querleu D, Cibula D, Abu-Rustum NR. 2017 Update on the Querleu-Morrow classification of radical hysterectomy. *Ann Surg Oncol* 2017;24:3406–3412.

Table 5- Querleu-Morrow classification. (11) Reproduced with the kind permission of BMJ Publishing Group Ltd.

Negative Lymph Nodes on Radiological staging is mandatory before performing uterus or pelvis lymph nodes surgery in early cervical cancer.

Ovarian preservation should be offered to premenopausal patients with squamous cell carcinoma and usual-type (human papillomavirus [HPV] related) adenocarcinoma. Bilateral salpingectomy should be considered.

Lymph nodes treatment

Techniques:

- Systematic pelvic lymphadenectomy is the standard lymph node staging procedure: include the removal of lymphatic tissue from regions with the most frequent occurrence of positive lymph nodes including obturator fossa, external iliac regions, common iliac regions bilaterally, and presacral region. Distal external iliac lymph nodes (circumflex iliac lymph nodes) should be spared if they are not macroscopically suspicious.

- Sentinel lymph node (SLN) biopsy (see below)

- Para-aortic lymph node dissection, at least up to inferior mesenteric artery, may be considered for staging purposes in advances stages.

Indications:

- Stage IA1: LN staging not indicated IA1 LVSI negative but can be considered if LVSI is positive. SLN biopsy alone is accepted.

- Stage IA2: LN staging can be considered IA2 LVSI negative but should be performed if LVSI is positive. SLN biopsy alone is accepted.

- Stages IB1-IIA1: The standard lymph node staging procedure is systematic pelvic lymphadenectomy. Sentinel node biopsy before pelvic lymphadenectomy is strongly recommended.

If lymph node involvement is detected intraoperatively including macrometastases or micrometastases, further pelvic lymph node dissection and radical hysterectomy should be avoided. Patients should be referred for definitive chemoradiotherapy.

- Stages > IIA1: para-aortic lymphadenectomy for staging purposes.

Sentinel lymph node biopsy

The current trend in gynaecological cancers is to reduce lymph node samples, mainly with Sentinel lymph node (SLN) technique (targeted lymph node sampling). The objectives with SLN biopsy are to limit the morbidity of the dissection, highlight aberrant drainage areas and performed ultra-staging nodes analysis to detect low-volume node metastasis. In the last decade the sentinel lymph node (SLN) biopsy has been validated in different studies in cervical cancer patients. In the SENTICOL I trial this technique was associated with high rates of SLN detection and with high sensitivity and negative predictive value (NPV) for metastasis detection when SLNs were detected bilaterally (24).

The results are promising and guidelines recommend performing SLN biopsy as the first step of the primary surgical management in early stages ≥IB1 of cervical cancer, but pelvic lymphadenectomy remains the gold standard and there are still no strong recommendations to avoid it (11). Lymphadenectomy in gynaecological cancer is nevertheless responsible for lymphatic complications: lymphocele in 34% of patients and lymphedema uni or bilateral in 11% which are the chronic complication, undermining the quality of life (25). In this sense, the trial SENTICOL 2 showed than SLN biopsy alone in patients with early cervical cancer induced less surgical morbidity, less lymphedema, and better quality of life than full pelvic lymph node dissection (26).

Other contribution of SLN technique is ultrastaging, that combining the use of serial sections with immuno-histochemistry allows the diagnosis of tumor cells (less than 0.2 mm) and micrometastases (0.2-2 mm), more rarely found by routine techniques involving a single slice per lymph node and standard staining. The sensitivity of the SLN biopsy ultrastaging combination is therefore superior to techniques, allowing the diagnosis of approximately 15% more patients with lymph node involvement, especially when the sentinel node detection was bilateral (27). Micrometastases in SLN is associated with decreased survival equivalent to macrometastases and the presence of MIC in LN is currently broadly accepted as an indication for adjuvant radiotherapy. Ultrastaging allows the detection of micrometastatic sentinel nodes which may have been overlooked resulting in under-diagnosis of N+ status and depriving patients of appropriate adjuvant management. The risk of micrometastases in pelvic LN in cases with negative SLN and the risk associated with ITC will remain unknown (28).

At the moment, no prospective studies have analysed the difference in survival between these two staging techniques. SENTICOL III is a prospective, International, randomized, multicentre, single-blind trial designed to compare 3-year disease-free survival (DFS), overall survival (OS) and health-related quality of life after SLN biopsy or SLN biopsy + pelvic lymphadenectomy in early cervical cancer (29). The Study completion data is estimated for 2026.

In the last ESGO guidelines (2018), the recommended technique in SLN biopsy in cervical cancer are combination of blue dye with radiocolloid or use of indocyanine Green (ICG) alone.(11). But in the prospective randomise study FILM, comparing ICG to blue in cervical and endometrial cancers, ICG detected more sentinel lymph nodes than blue alone (97% vs. 47%, $p<0.0001$) (30).

The IGC has many practical and logistical advantages: It allows avoid exposure of staff and patients to radioactive materials, coordination with a nuclear medicine department and painful injection before surgery. Moreover, Blue exposes the theoretical risk of anaphylaxis is higher. In terms of cost, the fluorescent method requires the injection of a single inexpensive agent intracervically at the time of surgery.

Adjuvant treatment (22)(11)

- *Radiotherapy:*

Brachytherapy or pelvic external beam radiation therapy (EBRT) are options in cervical cancer.

For preoperative high and intermediate risk patients, brachytherapy followed by surgery (type A) is used in a limited number of centres.

Adjuvant radiotherapy after surgery should be considered in the presence of combination of risk factors at final pathology such as tumor size, LVSI, and depth of stromal invasion (18). Information on para-aortic nodal status is essential for treatment planning, particularly in determining the superior extent of the external beam RT portal.

- *Radiochemotherapy:*

In patients with unequivocally involved pelvic lymph nodes on imaging or with histological proved, definitive chemoradiotherapy is recommended without radical surgery. Also, in advance cervical cancer or if a combination of risk factors is known at diagnosis, which would require an adjuvant treatment, definitive radiochemotherapy and brachytherapy can be considered without previous radical pelvic surgery.

In ESGO 2018 guidelines adjuvant chemoradiotherapy after primary radical surgery, is indicated in the following groups of patients (11):

*metastatic involvement of pelvic lymph nodes, including the presence of macrometastases pN1 or micrometastases pN1(mi) in either sentinel node or any other pelvic lymph nodes detected by intraoperative or final pathologic assessment: only chemoradiotherapy

*positive surgical margins (vagina/parametria) : chemoradiotherapy, brachytherapy boost may be considered

*parametrial involvement : only chemoradiotherapy.

The Intergroup 0107 trial compared concurrent chemotherapy (4 cycles of cisplatin and 5-flurouracil) and pelvic radiation therapy with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high risk group (positive surgical margin, parametrial involvement and lymph node metastasis), showing a improve of survival with combination treatment (31) . The most commonly used regimen is weekly cisplatin 40 mg/m2, although the meta-analysis also reported significant benefits with non-platinum agents.

- *Chemotherapy alone:*

Even if some studies have showed higher local control in patients treated with Neoadjuvant chemotherapy followed by surgery, in the last ESGO guidelines in 2018 this option is not recommended.

Recurrent, persistent or metastatic disease should be treated with Paclitaxel and cisplatin combined with bevacizumab in first-line (32) . Cisplatin-based doublets with topotecan or paclitaxel have demonstrated superiority to cisplatin monotherapy in terms of response rate and PFS. The combination of paclitaxel and carboplatin could be considered an alternative for patients that are not candidates for cisplatin. Palliative chemotherapy with the aim of relieving symptoms and improving quality of life is indicated if the patient has a Performance Status< 2 and no formal contraindications (22).

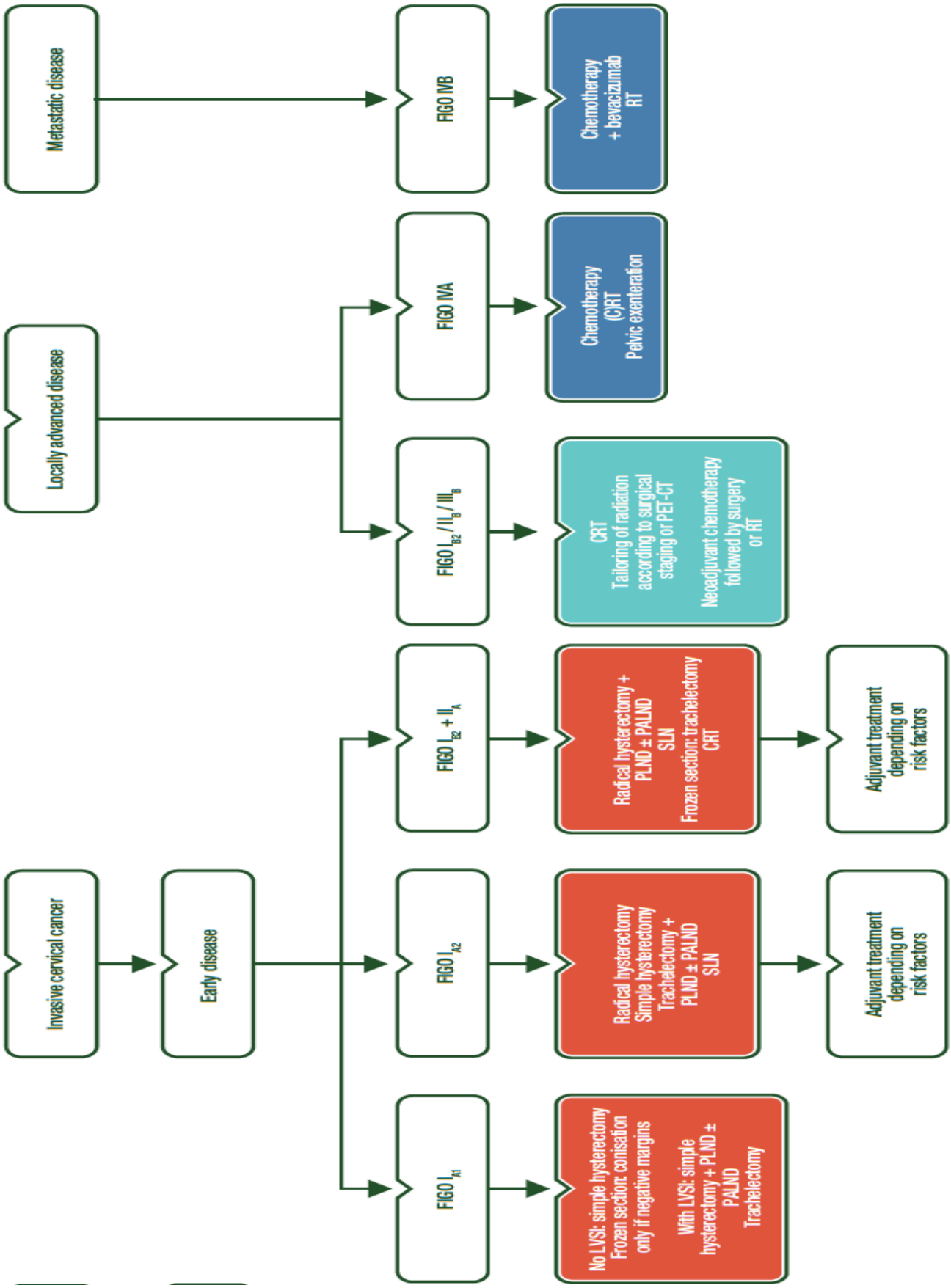


Figure 4- ESMO Treatment algorithm for cervical cancer.(22). Reproduced with the kind permission of Elsevier.

Papillomavirus

Viral structure

Papillomaviruses are small, epitheliotropic, double-stranded DNA viruses with a non-enveloped icosahedral capsid that infect mucosal and cutaneous epithelia. The genomes of all HPV types contain approximately eight open-reading frames (ORFs) that are all transcribed from a single DNA strand. The ORF can be divided into three functional parts: the early (E) region that encodes proteins (E1–E7) necessary for viral replication; the late (L) region that encodes the structural proteins (L1–L2) that are required for virion assembly; and a largely non-coding part that is referred to as the long control region (LCR), which contains *cis* elements that are necessary for the replication and transcription of viral DNA. The viral E proteins are transcribed from the early promoter whereas the L proteins are transcribed principally from the late promoter.

The two late (L) structural proteins are in the capsid: L1 (80% of total viral protein) and L2, both virally encoded. Virus-like particles (VLPs) can be produced by the expression of L1, alone or in combination with L2. The E1 and E2 proteins of HPV act as factors that recognize the origin of replication; E2 protein is also the main regulator of viral gene transcription. E4, despite its name, is believed to be involved in the late stages of the life cycle of the virus and E5 may function during both early and late phases. The E6 and E7 proteins target a number of negative regulators of the cell cycle, primarily p105Rb and p53, respectively. During the viral life cycle, E6 and E7 facilitate stable maintenance of viral episomes and stimulate differentiating cells to re-enter the S phase. The L1 and L2 proteins assemble in capsomers, which form icosahedral capsids around the viral genome during the generation of progeny virions (33).

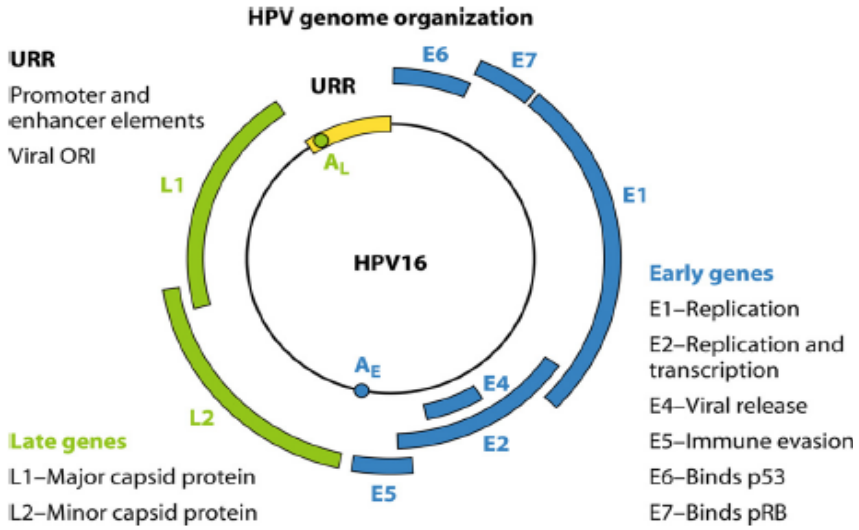


Figure 5- Illustrating the genomic organization of a typical mucosal high-risk HPV.(34). Reproduced with the kind permission of American Society for Microbiology.

HPV and the genesis of cervical cancer

More than 200 types of human papillomaviruses (HPVs) have been identified. Many genotypes of HPV have been detected in cervical cancers, anogenital (anus, vulva, vagina and penis) as well as oropharyngeal cancers. There are genotypes of HPV have been found in cancers and other not, which gives rise to the nomenclature of 'high-risk' (or oncogenic) and 'low-risk' HPVs. (35) HPV is detected in 99% of cervical tumors, particularly the oncogenic genotypes such as HPV 16 and 18 (70%) (36).
Although cervical cancer is always associated with oncogenic HPV, oncogenic HPV infection is a normal and usually transient infection that most healthy sexually active women will encounter in early reproductive life. Most women infected with oncogenic HPV will clear the infection without any increased risk of cervical cancer. In a small percentage of women, the infection persists, the HPV is introduced into the epithelial cell nuclei and changes from a latent to a transforming infection. It is in those cases that the risk of progression is high. For this reason, although oncogenic HPV tests will detect the presence of viral DNA it does not always mean that a lesion is present (3).

For a precancerous lesion to develop into an invasive cancer, persistent High-risk HPV infection is therefore required. These precancerous lesions are classified according to the Bethesda classification. A distinction is made between low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and invasive carcinoma: invasion of the tumor cells after they have crossed the basement membrane (3).

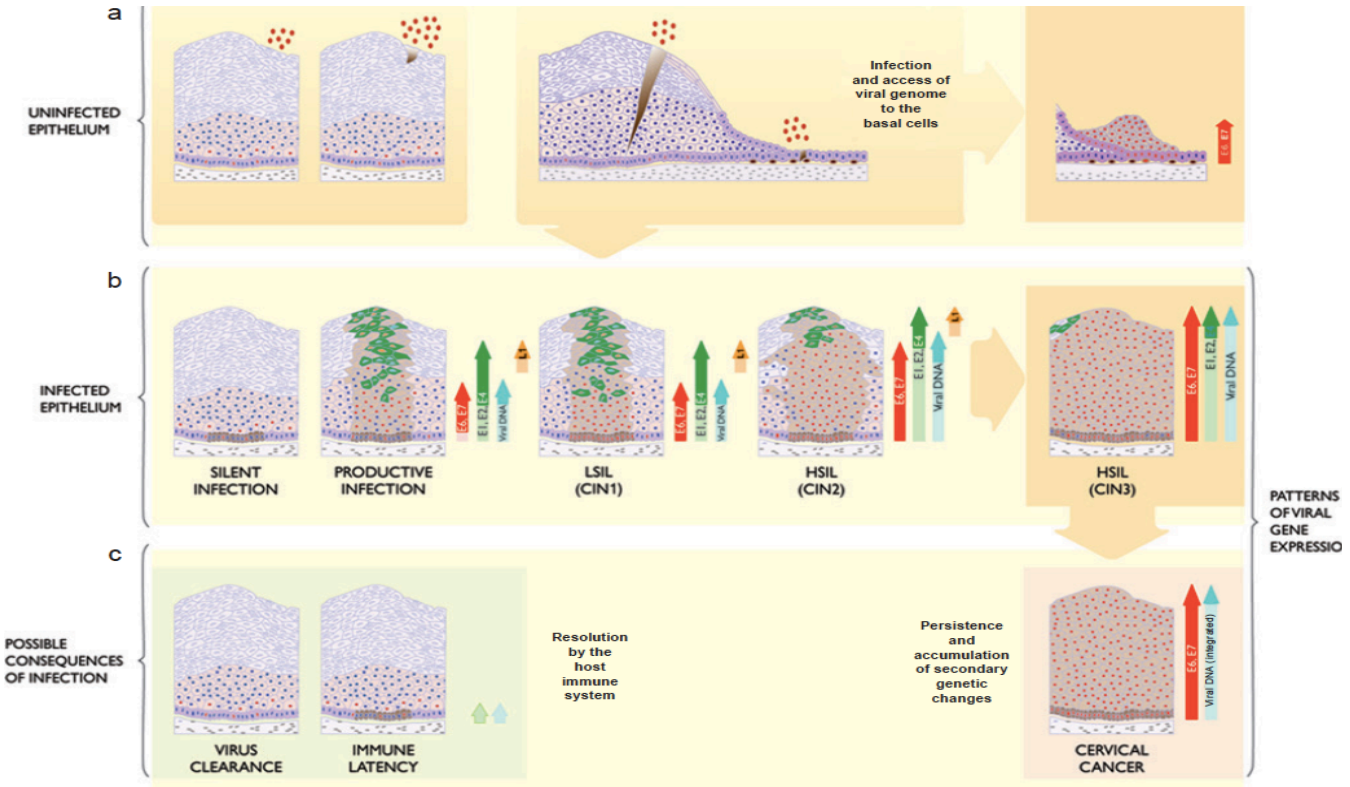


Figure 6- HPV expression in the epithelium. (3). Reproduced with the kind permission of IARC publications.

The HPV cycle is initiated by the infection of basal stratified epithelia cells of the skin, the anogenital and the oral mucosa, presumably at sites of injury, in which the viral genome is established. In the cervix, the cells are actively replicating in SCJ, making them susceptible to HPV infection, which can then reach their target cells either via the SCJ or via micro-lesions in the ectocervix. When basal cells undergo cell division, the daughter cell that loses contact with the basement membrane and migrates into the suprabasal compartment withdraws from the cell cycle and initiates a programme of terminal differentiation. The E6 and E7 viral proteins are expressed in the lower layers of the epithelium, and they reflect an initiation of the cell cycle. The basal cells proliferate, and viral copy numbers increase. The E6 and E7 proteins are expressed at very low levels, but fortunately surrogate markers, such as Minichromosome maintenance (MCM) and Ki-67, are identifiable, and they faithfully reflect the presence of E6 and E7. With an LSIL, there is early deregulation of the cell cycle. There are increased levels of E6 and E7 in the basal and parabasal layers, and therefore MCM is present.

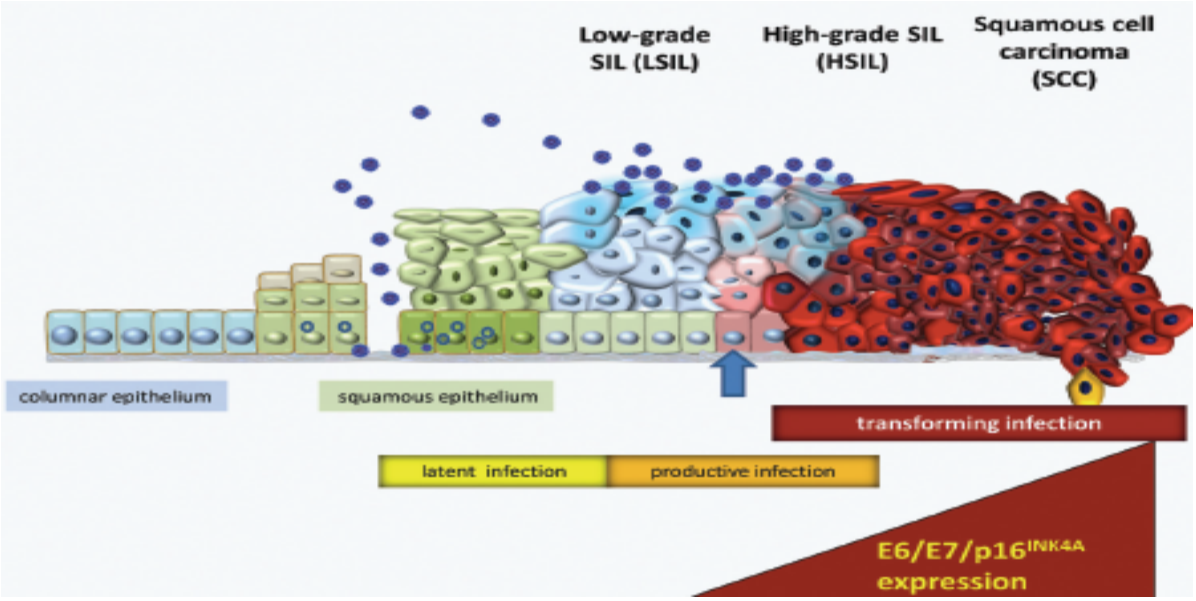


Figure 7 - Schematic representation of HPV infection stages in the cervical epithelium. Via minor lacerations the virus enters its host cell in the basal or para-basal epithelium. Differentiation of infected host cells goes along with replication of the viral genome and subsequent release of new viral particles from the superficial differentiated cells (productive infection), which is cytologically/histologically characterized as low grade squamous intraepithelial lesion (LSIL). Incident overexpression of the viral oncogenes E6/E7 (transforming infection) disrupts cell cycle control and may lead to high grade squamous intraepithelial lesion (HSIL) or subsequent squamous cell carcinoma (SCC). Transforming HPV infections are characterized by overexpression of p16INK4a. Modified from von Knebel Doeberitz and Vinokurova, 2009 (37). Reproduced with the kind permission of John Wiley and Sons.

The regulatory function of the E2 protein is crucial to prevent cell transformation, however, during persistent oncogenic HPV infection, the viral genome may **integrate** into the host cell DNA. Upon integration, part of the viral genome will be deleted, particularly at E1 and E2 region, resulting in loss of negative feedback of E6 and E7 oncogene expression normally regulated by E2 protein. This is the case in half of all cervical cancers. In the other half of cases, the HPV genome persists as an extrachromosomal **episomal** form. In these cases, the E2 gene is conserved. However, epigenetic modifications at the E2 binding sites have been identified that prevent E2 inhibitory regulation of the E6 and E7 oncogenic proteins by E2 by preventing its binding. [(38) These epigenetic modifications are based on methylation of the CpG islands of the E2BS binding sites, notably E2BS3 and 4, leading to the E6 and E7 oncogenic proteins (38) (39).

In all these cases, the overexpressed E6 and E7 proteins stimulate the cell cycle and lead to active cell proliferation. This will lead to the appearance of a dysfunction of these infected cells, leading them to a cancerous cell phenotype. The affinity of the E6 and E7 oncoproteins with their respective targets varies according to the HPV genotype, thus conditioning the high or low risk potential of the virus (39).

It has also been shown that the presence of E6 and E7 oncoproteins in cells is essential for maintaining the tumor phenotype (40). Thus, the viral DNA systematically present in transformed cells represents DNA from the tumor cell and therefore tumor DNA.

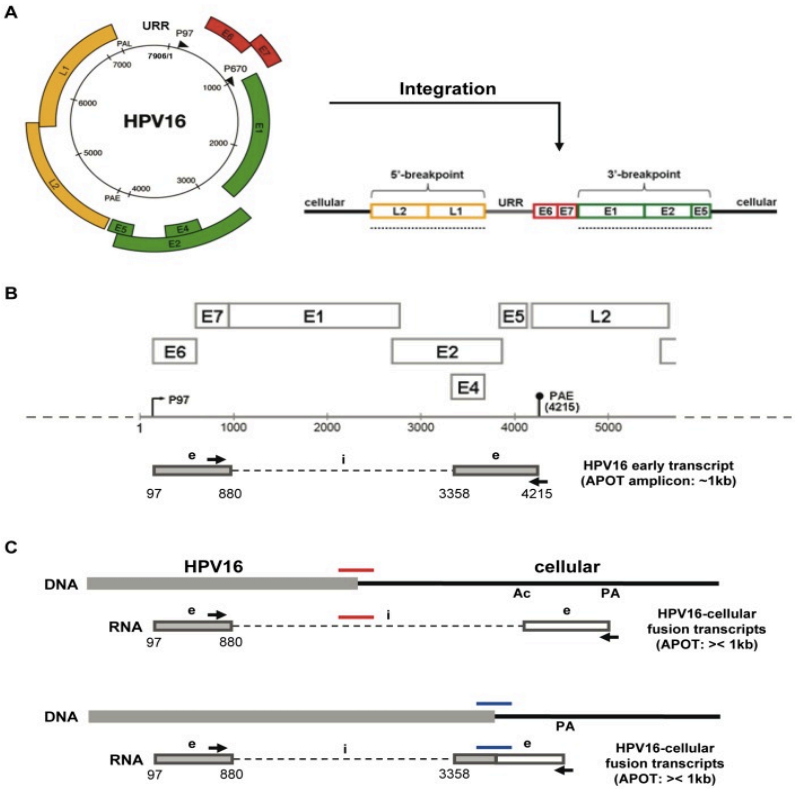


Figure 8 - Genomic structure and transcription of episomal and integrated HPV16 DNA (41) . Reproduced with the kind permission of PLOS

Biomarkers

An increasing number of molecular biological markers have enabled a better understanding of the pathway that progressive lesions take. Different measurable viral or cellular products are produced at different stages of cell transformation, depending on whether it is a productive or transforming infection. Protein biomarkers (secondary biomarkers) produced at different biological stages of viral activity, determine the virus activity which reflects the risk of progression to cervical cancer. These markers include HPV proteins, surrogate markers (e.g. p16), and methylation patterns.

The biomarker **p16** begins to appear in the lower epithelial layers, for two reasons: because of E6 and E7 deregulation, but also because p16 is a marker for oncogenic HPV activity, especially activity of HPV type 16. In a case of HSIL-CIN3, cellular activity is completely disordered and the abnormal basal and parabasal cells get pushed up through the cellular layers. At the same time, E4 (which reflects cell-cycle completion) appears higher and higher up the cellular layers and eventually disappears. p16 will be present and detectable in the histological section (3).

p16 is the only biomarker that the consensus document considered had sufficient evidence to recommend its routine use in histological specimens in defined cases (42). But p16 on its own is not currently considered to be a useful discriminator in cytology. P16 is expressed in about 40% of low-grade lesion smears in the lower epithelial levels. In cytology, the cellular architecture and site of staining cannot be recognized, and p16 is therefore less valuable.

Ki-67 is a proliferative marker and is expressed in proliferating cells within the nucleus of parabasal cells of normal epithelium. When Ki-67 is overexpressed, it indicates a proliferative cellular state.

The combination of p16 and Ki- 67 (known as dual testing) is useful in cytology. An international multicentre study of the utility of p16 and Ki-67 in cytology produced good evidence that this combination will confirm that there is a proliferative cellular state and a transforming infection, thereby indicating the likelihood of genuine cervical precancer, i.e. a high-grade lesion. The p16/Ki-67 dual-stained cytology combines superior sensitivity and non-inferior [similar] specificity over Pap cytology for detecting CIN2+. It suggests a potential role of dual-stained cytology in screening, especially in younger women where HPV testing has its limitations (43).

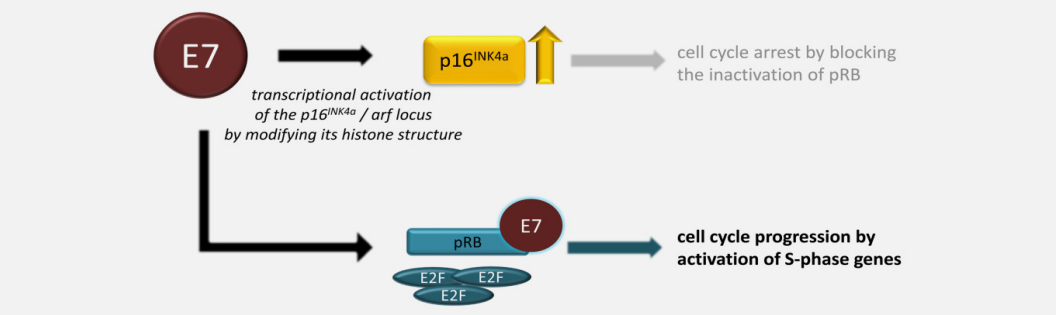


Figure 9- Scheme of HPV E7 effects on $p16^{INK4a}$ expression levels and cell cycle regulation (black arrows). E7 mediates $p16^{INK4a}$ overexpression *via* transcriptional activation. High $p16^{INK4a}$ levels would normally result in cell cycle arrest (grey arrow and text in figure). However, at the same time E7 disrupts pRB and thereby cell cycle progression is triggered (lower part of the figure) despite high $p16^{INK4a}$ levels. Reproduced with the kind permission of John Wiley and Sons (37).

Methods for detection of HPV genomes (used in our project)

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique that consists to the identification and quantification of variations of DNA or RNA amplifying millions to billions of copies of a specific segment of DNA or RNA, which can then be studied in greater detail. PCR involves using short synthetic DNA fragments called primers to select a segment of the genome to be amplified, and then multiple rounds of DNA synthesis to amplify that segment (44).

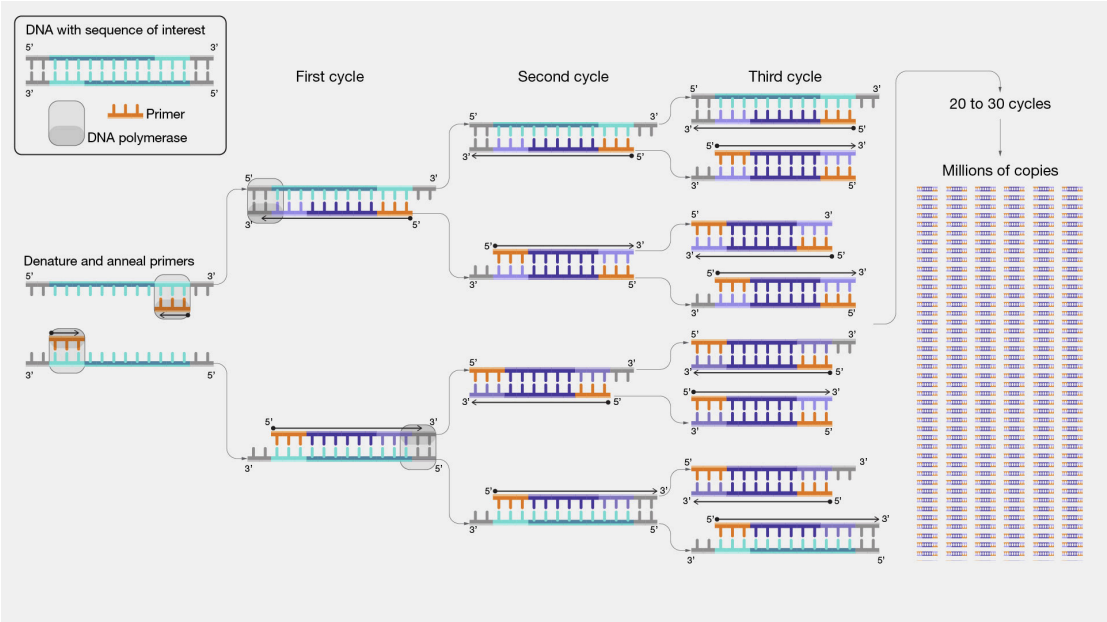


Figure 10- PCR mechanism. (44)**.** Courtesy: National Human Genome Research Institute

Real-time quantitative PCR (qPCR/rtPCR)

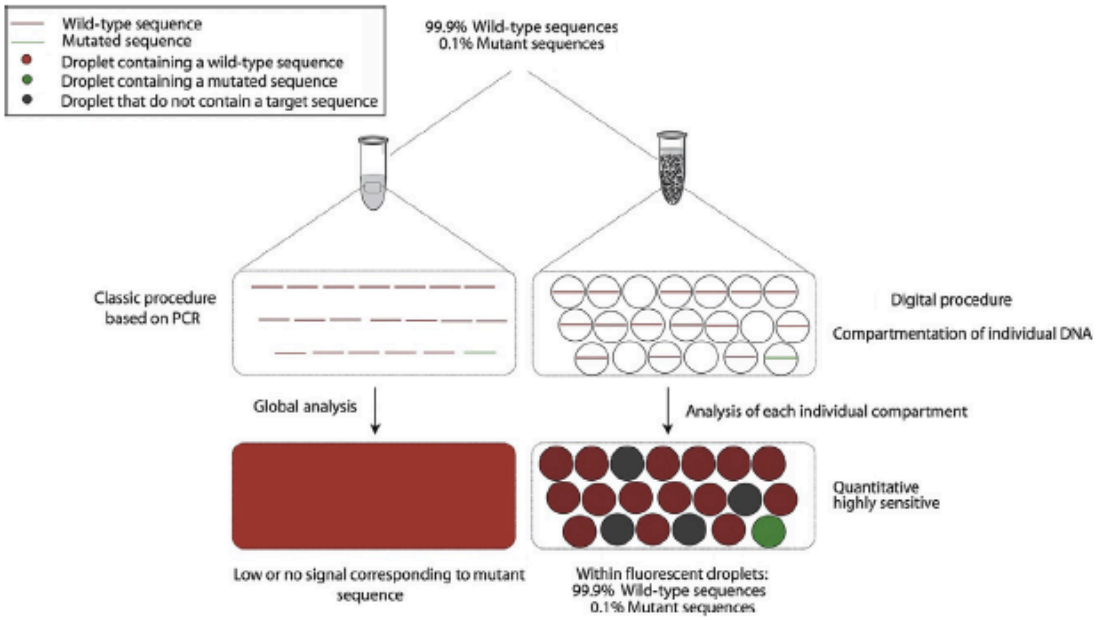
This method is base in the amplifications of DNA molecules within a sample and follow up of this amplification in real-time. In qPCR, DNA is copied until it produces a certain level of signal; the number of amplification cycles needed to reach this point is then used to calculate how many DNA molecules with the particular sequence were originally present relative to other DNA molecules in the sample (45).

Specifically for HPV genotyping, Seegene commercialized the real-time PCR Anyplex II HPV28 kit (H28). H28 is aimed at genotyping 28 HPV in only 2 PCR wells per sample. This system therefore has the potential to greatly simplify HPV genotyping (46). More details about this technique are done in Material and Methods section.

However, classical rtPCR has difficulty in detecting low abundance or poorly represented sequences and analysis of a complex DNA mixture by bulk PCR-based technologies leads to an averaged signal showing a very low sensitivity for the detection of rare sequences

Such limitations could be overcome with the use of digital PCR (ddPCR) where each target DNA molecule is isolated within individual compartment prior to PCR amplification (47).

Figure 11- Comparison between conventional PCR procedures and digital PCR.



Example of the analysis of a sample containing 0.1% of mutated sequences. The PCR experiment is realized with a mixture of probes specific of the mutated sequences (in green) and normal sequence (in red). The conventional qPCR (left) will amplify all target molecules present in the sample. Obtained signal will thus represent an averaged signal corresponding to the different DNA present in the sample; the signal resulting from under represented sequence could thus be hidden by highly represented sequences. The digital PCR (right) in contrast can amplify each target DNA within an independent compartment. The analysis of the fluorescence of each independent compartment allows detecting and quantifying the one containing mutated sequences. The procedure is now quantitative and its sensitivity depends on the number of compartments that can be analyzed. Modified from K. Perez-Toralla, D. Pekin, J.F. Bartolo, F. Garlan, P. Nizard, P. Laurent-Puig, J.C. Baret, V. Taly, Digital PCR compartmentalization I. Single molecule detection of rare mutations, Med. Sci. (Paris) 31 (2015) 84–92; V. Taly, D. Pekin, A.E. Abed, P. Laurent-Puig, Detecting biomarkers with microdroplet technology, Trends Mol. Med. 18 (2012) 405–416, with permission from Trends in Molecular Medicine. (47). Reproduce with the kind permission of Elsevier.

Digital PCR uses the same primers and probes as qPCR but is able of higher sensitivity and precision.

Detection methods	Sanger sequencing	Pyrosequencing	High resolution melting (HRM)	Next generation sequencing (NGS)	Real-time qPCR (Taqman)	Optimized real-time qPCR (CastPCR, ARMS)	Digital PCR in microcompartments
Sample types	Tumor tissue	Tumor tissue	Tumor tissue	Tumor tissue	Tumor tissue	Qualitative follow-up of tumor DNA, detection of rare variants within tissues	Quantitative follow-up of circulating tumor DNA (ctDNA), detection and quantification of rare variants within tissues
Fraction of detectable tumor DNA (sensitivity)	>10 %	5–10%	5%	2%	1–10%	0.1–0.01%	<0.001%

Table 6 - Potential methods used in clinic for detection of genetic alterations in cancer. The sensitivity of real-time qPCR is dependent of the type of assays (47). Reproduce with the kind permission of Elsevier

Droplet-based digital PCR (ddPCR)

This method based on the realization of thousand of single-molecule PCRs in parallel in independent compartment (here droplets of an emulsion) reflect more closely the original composition of nucleic acid mixtures than qPCR (48) (49) (47). Target DNA molecules contained in a tested sample are compartmentalized within aqueous independent droplets of a thermostable emulsion. In each droplet there is one (and sometimes several if there is co-encapsulation) strand of DNA, as well as the probes, dNTPs and polymerase necessary to perform a PCR. Thanks to this, each droplet can be considered as an independent "bioreactor" in which a PCR reaction is performed independently of the others. The number of PCRs is equal to the number of droplets, which gives a very high precision for each sample due to the large number of events.

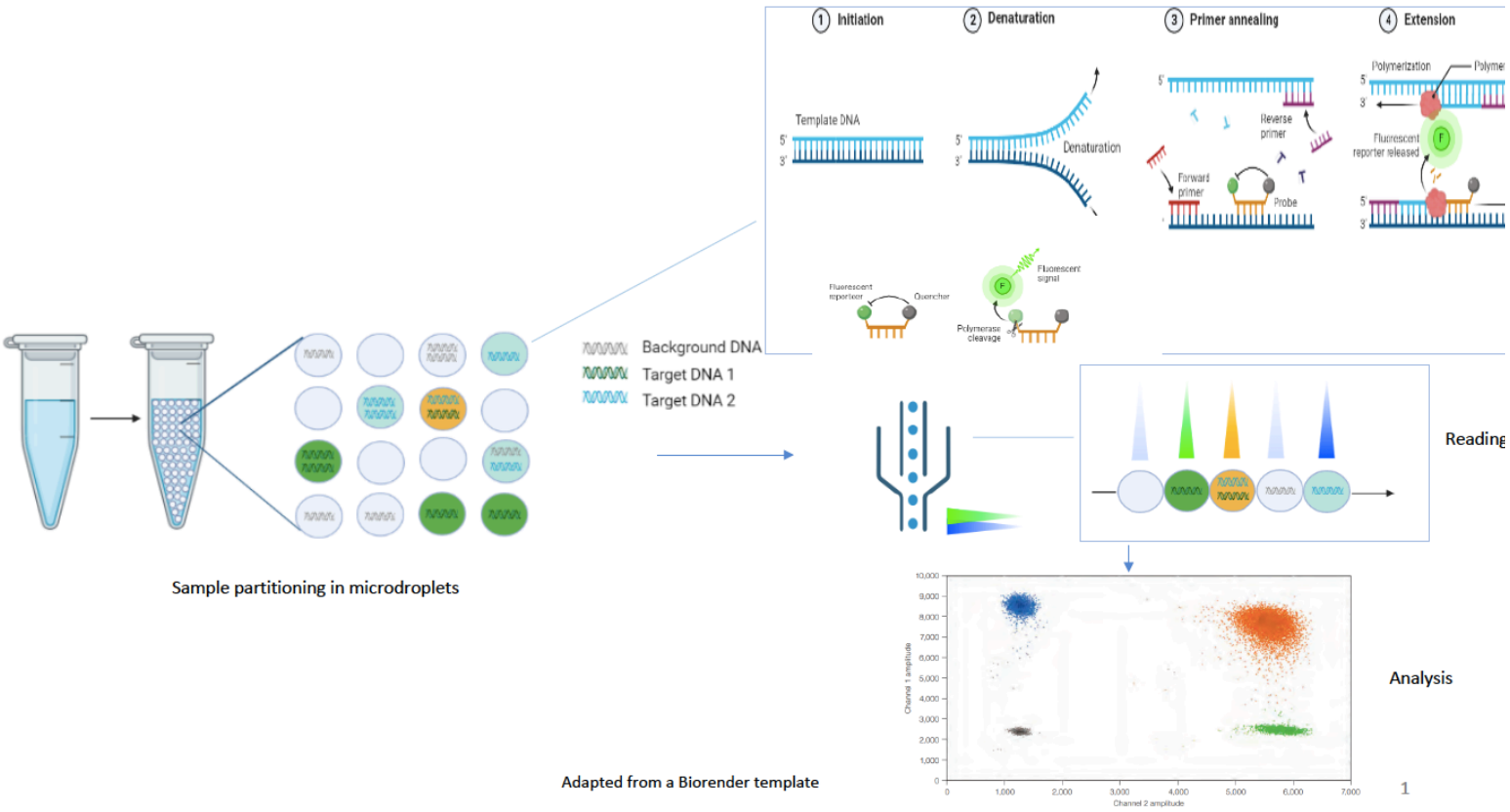


Figure 12- ddPCR mechanism. Figured created by Thomas Bruneau adapted from Biorender template.

Supplier	Instrument	Number of compartments	Process	Multiplexing capabilities	References
Bio-Rad	**QX200 System** (2 machines: droplets maker and droplets analyzer)	20,000 droplets of 1nL volume (8 samples in parallel)	(1) Samples loading in the chip (2) The chip is transfered in the droplet maker (3) Droplets are pipeted and transfered to the thermocycler (4) Fluorescence detection in the analyzer	2 colors/ 2 targets	Hindson et al., 2011
RainDance Technologies	**RainDrop Digital PCR** (2 machines: droplets maker and droplets analyzer):	5 – 10 millions droplets of 5 pL volume (8 samples in parallel)	(1) Samples are loaded in the chip (2) Chip is transfered to the droplet maker (Source) (3) The tubes containing the droplets are thermocycled (4) Detection of droplet fluorescence with the analyzer (Sense)	2 colors/ 10 targets	Pekin et al., 2011 Zhong et al., 2011
Stilla Technologies	**Naica Platform** (2 machines: droplets maker/thermocycler and droplets analyzer)	30,000 droplets of 0.5 nL volume (12 samples in parallel)	(1) Samples are loaded in the chip and chip sealed (2) Chip is transfered to the Geode for droplet generation and thermocycling (3) Scan chip with the Prism 3	3 colors/ 3 targets	Dangla et al., Proc Natl Acad Sci USA. 2013*

Figure 13- Commercial droplet-based digital PCR microfluidic platforms (47).

* This publication refers to the platform principle and not to its application to digital PCR. The newly developed platform needs to be validated for biological sample analysis. Modified from K. Perez-Toralla, D. Pekin, J.F. Bartolo, F. Garlan, P. Nizard, P. Laurent-Puig, J.C. Baret, V. Taly, Digital PCR compartmentalization I. Single-molecule detection of rare mutations, Med. Sci. (Paris) 31 (2015) 84–92; M. Baker, Digital PCR hits its stride, Nat. Methods 9 (2012) 541–544, with permission from Nature Methods. Reproduce with the kind permission of Elsevier.

This technique has a great reproducibility, even between different laboratories and different machines, great sensitivity and precision, thanks to the large number of events (one droplet = one PCR independent of the others) and it is more sensitive to PCR inhibitors.

Capture technique coupled with NGS (Next-Generation Sequencing)

Next generation sequencing (NGS) is a technology that enables parallel multiplexed analysis of DNA sequences on a massive scale—millions to billions of sequences from individual single strands of DNA analyzed separately, yet simultaneously.

With NGS, DNA from a single whole genome is fragmented and assembled into a single sequencing library (each patient has a unique index), and then sequenced in single run (50).

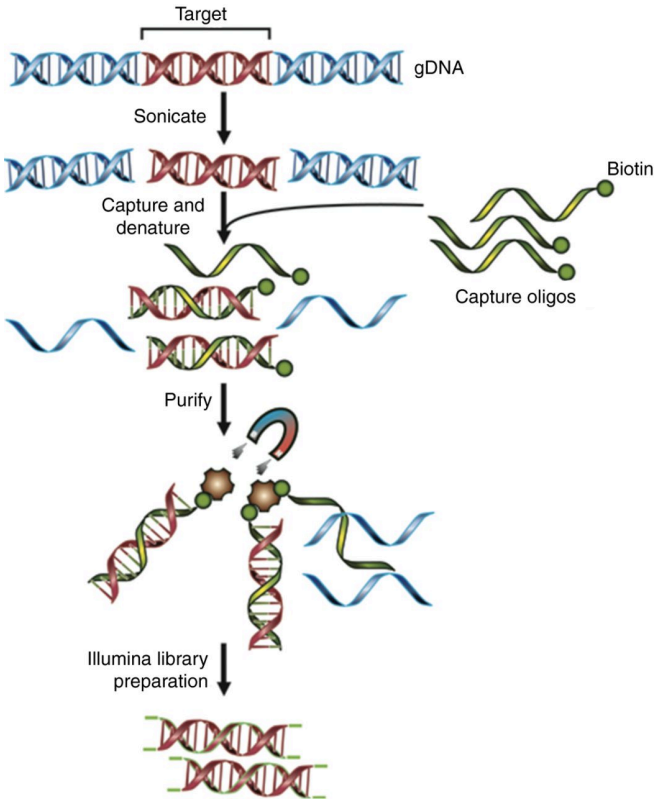


Figure 14- General scheme of DNA preparation for hybridization-based whole-exome capture and sequencing (51). In summary, genomic DNA is fragmented, denatured, and hybridized with capture oligos during library preparation for high-throughput sequencing. The captured sequences are then enriched with streptavidin-conjugated paramagnetic beads and further amplified before being subjected to Illumina sequencing. Diagrammed DNA sequences in red, on-target regions; sequences in blue, off-target regions; single-stranded oligos in green, capture probes labeled with biotin; brown particles, streptavidin-conjugated paramagnetic beads (Figure 14) (51). Reproduce with the kind permission of Cold Spring Harbor Laboratory Press.

In case of HPV detection, it is a generation of library hybridized with a biotinylated probe specific to 208 different HPVs (subtype included). These probes, linked to our HPV DNA, have an affinity form with streptavidin which covers the capture beads and therefore allows after washing to obtain only the specific HPV DNA(51) (52).

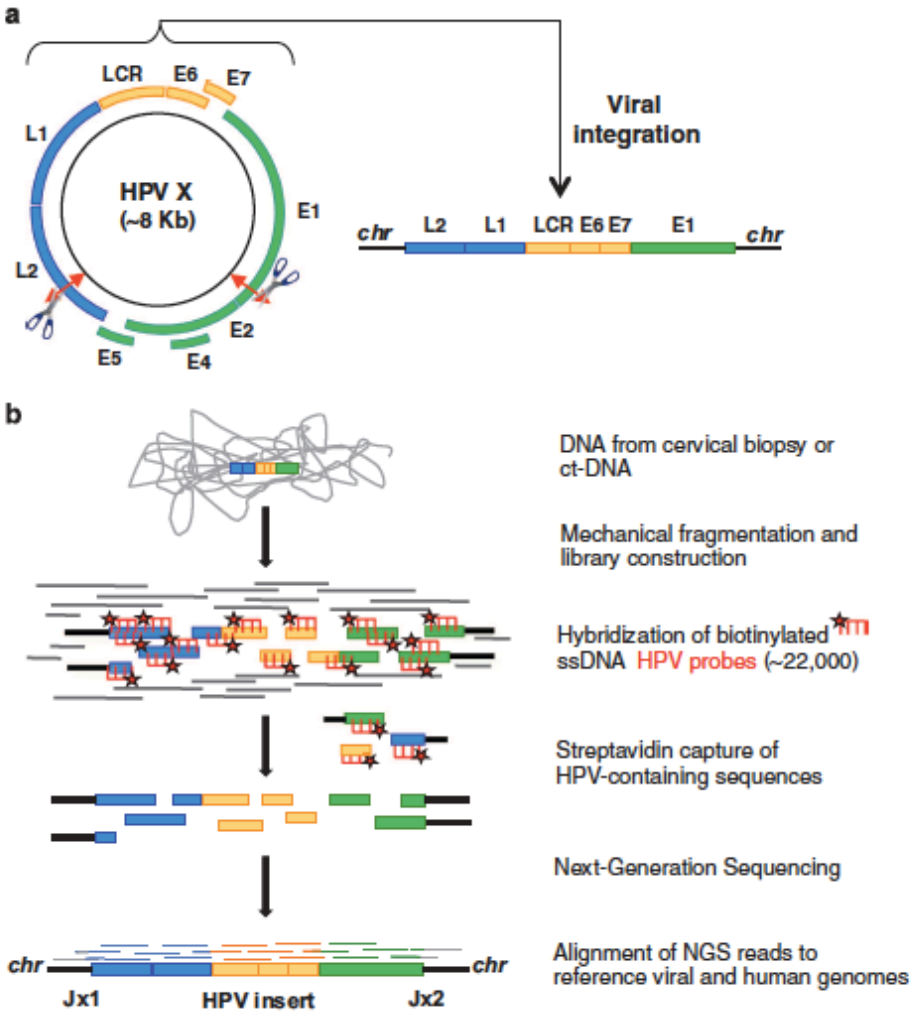


Figure 15- Capture-NGS method to identify HPV genotypes and integration sites.

(a) The HPV dsDNA circular genome contains early (E) and late (L) replicating genes, necessary for viral replication and capsid formation. Integration and disruption/loss of the E2 suppressor gene sequences may lead to overexpression of the E6 and E7 viral oncogenic sequences, as well as disruption of host genomic DNA sequences. (b) Capture- NGS of HPV-integrated viral–cellular junctions. Genomic DNA is mechanically and homogeneously nebulised, followed by dsDNA library preparation and annealing of adaptor barcodes. HPV-biotinylated single-stranded probes (~22,000) then bind and capture HPV-containing library sequences that are enriched on a streptavidin column. These enriched DNA products are sequenced by NGS whose read alignments permit identification of the genotype, as well as the HPV-cellular hybrid junctions and localisation of the HPV insert in the tumour genome (52). Reproduce with the kind permission of Springer Nature.

Circulating tumoral (ct)DNA and ddPCR

Circulating free (cf) DNA is used in oncology (the tumor fraction, ctDNA) and in non-invasive prenatal diagnosis (the fetal fraction is used). It is known that HPV DNA is systematically present in cancer cells in the case of HPV-induced cancers and that there is no detection of HPV DNA into the bloodstream when the infection is limited to the mucosal level (ref). Detection of HPV DNA in blood means detection of tumor DNA released from cancer cells

HPV-related cancers are known to be an ideal model to monitor circulating tumoral (ct)DNA by detecting HPV oncogenes E6 or E7 (53)(54)(55). Indeed, these HPV oncoproteins are responsible for cell transformation and the maintenance of tumor phenotype (56).

However, this DNA is only present in small quantities, so it is necessary to use an ultra-sensitive method such as digital PCR methods. The feasibility and interest of HPV ctDNA detection in the plasma in patients with HPV-related cervical cancer using new ultrasensitive molecular tools such as droplet-based digital PCR (ddPCR) assays have been recently demonstrated, particularly to monitor the treatment response (55). The growing interest of ddPCR for the detection and the monitoring of HPV ctDNA is based on its high-sensitivity, accuracy and inter- and intra-laboratories reproducibility as compared to classical PCR (57).

Context and Hypothesis

In HPV-related cancers, like cervical cancer, HPV DNA could be assimilated to tumoral DNA and represent an interesting marker of node metastasis (58). We proposed the used of HPV DNA as prognosis and diagnosis biomarker:

***In the tumor and in the Lymph nodes*:** In early stages of cervical cancer, nodal status is the cornerstone of treatment algorithm and patients with nodal metastasis (N+) present worse survival. However, 10 to 15% of patients without nodal metastasis (N0) present the same survival to patients with nodal metastasis (N+). No clinical, imaging or pathological risk-factor is today effective to select these patients for a different management. As HPV is the cause of almost 100% of cervical cancer, we hypothesize the use of the new viral molecular biomarkers for a more accurate classification of these early cervical cancers according to the risk of recurrence.

In the **Study I**, we studied the identification of specific molecular signatures of HPV in the *tumor* by HPV Capture technique coupled with NGS (Next-Generation Sequencing) that would allow the identification of different molecular status and possibly correlated with a different prognosis.

In the **Study II**, we explored the presence of tumoral HPV DNA in pelvic *lymph nodes* by new ultrasensitive droplet-based digital polymerase chain reaction (ddPCR) as a new additional prognosis biomarker correlated with an increased risk of recurrence in patients with negative lymph nodes in early cervical cancer as determined by classical clinical and histological parameters.

***In the metastasis*:** Patients with primary HPV-associated cervical cancer may have increased risk of developing secondary HPV-related malignancies given that HPV can cause precancerous lesions and invasive malignancy when it infects oro-genital mucosa and genital skin (59). Moreover, cervical cancer patients could present a distant lesion that may signify either secondary metastatic disease from the primary cervical cancer, a new primary tumor or metastasis of another form of cancer. Differentiating between these lesions can be difficult, but it is essential for patients' clinical care, treatment and prognosis.

In the **Study III**, we hypotize that HPV DNA detection in *metastatic* tissue with HPV genotyping real-time PCR in patients with cervical cancer could help in the differential diagnosis between cervical cancer metastasis and a secondary lesion.

Aims

General:

Validate HPV biomarkers as prognosis and diagnosis tools in patients with cervical cancer.

Specifics:

Study I: HPV Capture in early cervical cancer without sentinel biopsy lymph node involvement (N0)

- HPV whole genome sequencing (genotyping and variant determination).
- HPV signature: HPV status (integrated or episomal) and integration site in the virus and human genome
- To correlate recurrence/death with different genotypes and molecular status.

Study II: **Translacol study (HPV in lymph nodes by ddPCR)**

- **Primary Objectives:**
  - To assess the Progression-free survival (PFS) of N0 early cervical cancer patients by HPV tumoral DNA (positive or negative) status based on ultrasensitive droplet digital PCR detection.

- **Secondary objectives**
  - To assess the disease-specific survival (DSS) of N0 early cervical cancer patients by HPV tumoral DNA (positive or negative) status based on ultrasensitive droplet digital PCR detection.
  - To compare molecular parameters and histologic parameters to predict prognosis.

Study III: HPV in distant lesions.

- To highlight the interest of HPV molecular genotyping assay in differentiation between HPV tumor secondary metastasis and a new independent primary non-HPV induced tumor to improve care management of patients.

Material and methods

Common for *Studies I and II*

Patients selection

We included patients with histological confirmed cervical cancer with preoperative clinical and/or radiological diagnosis of early 2018 FIGO stage (tumor size less than 4 cm with or without upper 1/3 vaginal involvement without parametrial involvement) who underwent surgical treatment including pelvic SLN detection without metastasis detected in final histology after classical and ultrastaging examination [included macrometastasis, micrometastais or isolated tumor cells (ITCs)].

Exclusion criteria were: patients with missing or insufficient follow-up data (< 6 months), patients without nodal stage evaluation (patients with FIGO stage IA1 without lymphovascular space invasion (LVSI)), local advanced cervical cancer (tumor size > 4 cm, parametria involvement or inferior 1/3 of vagina), patients who received neoadjuvant radiochemotherapy or undergoing a para-aortic lymphadenectomy for set the radiation field and received radiochemotherapy as primary treatment.

We selected patients who participated in the Prospective multicentre studies SENTICOL I (139 patients) (2005-2007) (24) and SENTICOL II (267 patients) (2009-2012) (26), main investigator Fabrice Lecuru. Patients with diagnosis and treatment of early cervical cancer in the European Hospital George Pompidou, Paris (HEGP) (2004-2018), non-participating in SENTICOL, and who met the inclusion criteria were also included in this study.

Clinical data collected

We reported the main prognosis factors previously proved for recurrence (11) : tumor and node metastasis size and grade, 2018 FIGO stage (final histological stage), histology type, lymphovascular space involvement (LVSI), surgical-pathological results (parametrium, vaginal and margins status). Also, we collected the surgical approach, preoperative and adjuvant treatment. In preoperative treatment, we included only patients treated with brachytherapy (radiotherapy and chemotherapy in preoperative were the exclusion criteria).

In patients operated on before 2018, the FIGO stage was adapted from the 2009 classification to the 2018 classification in order to use the same classification in all patients.

The data collection was retrospective. The patients included were patients treated between 2005 and 2018. Follow-up data were obtained from the patient´s clinical records. The last contact was in September 2021. The data not available or unknown were not included in statistical analysis.

Treatment

Treatment of uterus consisted in total radical hysterectomy. Simple hysterectomy was performed in IA1 stages with LVSI. Simple or radical trachelectomy was performed in young patients for fertility sparing (tumor less than 2 cm without LVSI).

The surgical approach was surgeon preference: Mini-invasive (MIS) or open. Mainly the surgical criterion was performing a MIS approach since the cases were included before publication of LACC trial in 2018 (21).

Pelvic lymph node staging was evaluated with only SLN (Sentinel Lymph Node) or SLN biopsy + pelvic lymphadenectomy. In the second case we selected the SLN for analyse.

SLN biopsy was performed with combined technetium 99 and Patent Blue injection or technetium 99 (before 2017) and indocianine Green (IGC) (after 2017).

Ultrastaging technique in SLN was based on cut 5 sections spaced at 250-250 µm intervals with four sections stained with HES (Hematoxylin Eosin Safran) and one section used for immunohistochemistry with pancytokeratin antibodies (AE1/AE3).

The brachytherapy neo-adjuvant was an option in the patients with tumor size > 2 cm and < 4 cm according our centre recommendations.

The adjuvant treatment was performed according international guidelines (11) based on the presence of the following criteria in postoperative: vaginal/parametrial invasion, final tumor size larger than 4 cm or lymph node involvement. Adjuvant treatment consisted of concomitant chemotherapy and radiation with treatment fields adapted to the extension of the lymph node involvement. Patients with positive lymph-vascular invasion were postoperatively treated with brachytherapy.

Clinical and biological databases and biological resources

Updated clinical database of Senticol I and II were available in our institution (HEGP) and blocks of Formalin Fixed Paraffin Embedded (FFPE) tumors and associated pelvic SLN samples. We also obtained from one other centre (Oncopole Toulouse), which participated in these trials, clinical data, and paraffin blocks, which were returned and stocked in Biobanking Resource Centre of HEGP.

The patients and the hospital's ethics committee gave their consent (CPP Sud-Est III 20.02.11.68220-2).

Statistical analysis

Data were recorded in Excel files and statistical analyses were performed using SPSS 20.0 from the imported data files. For qualitative variables counts (n) and percentage (%) were considered whereas for quantitative data as mean and standard deviation (SD) were calculated. The chi- square test (or exact Fisher test when the sample size was too small) was used to evaluate the dependence between qualitative variables whereas a student-t test or a non-parametric Mann Whitney test (depending on the variable distribution) were used to compare the distributions of the quantitative variables. Survival curves were compared using Kaplan–Meier-curves and log-rank-test. For univariate analysis for survival Hazard ratio (HR) and 95% confidence interval (CI) were computed with a Cox regression model. Variables yielding p values lower than 0.2 in univariate analysis were systematically entered into a multivariate analysis in order to reveal variables independently associated to survival to HPV status in SLN. All the tests were performed as two-sided except the student and Mann-Whitney tests that were performed as one-sided tests. A p-value of 0.05 was considered unlikely under H0 and thus as the limit for significance in the statistical tests.

Specific for *Study I*: HPV Capture

In this retrospective study, the HPV-capture technique coupled with NGS was performed on DNA extracted from the tumor still available after HPV genotyping (n=64) in order to accurately describe the molecular HPV status in the tumors of these patients.

Technique

Preparation of DNA libraries.

DNA libraries were prepared using between 100 and 500ng of extracted tumoral DNA. Samples were fragmented to an average of 280bp by ultrasonic fragmentation using the following parameters: 30% duty cycle, 50W maximum incident power, 1000 cycles per burst and 90s duration (ME220 modele Covaris, Woburn, MA, USA,). Fragments sizes were controlled using the TapeStation 4200 (Agilent Technologies, Santa Clara, USA). 1µL of each sample and 3µL of D1000 buffer (Agilent technologies, Santa Clara, USA) were loaded onto a solid device comprising 16 nanocapillaries (D1000 ScreenTape, Agilent technologies, Santa Clara, USA) and analysed via the Tape Station 4200 (Agilent Technologies). The results were processed using Tape Station Analysis Software (Agilent Technologies, Santa Clara, USA).

End Repair and A-tailling consisting of repairing the 3' and 5' DNA ends in order to obtain clean ends were performed to facilitate the ligation of the KAPA Single-Indexed Adapter Kit specific to Illumina technology. There are 24 sequences of 6 nucleotides each allowing up to 24 samples to be indexed. After purification using Agencourt AMPure XP® beads, size selection was performed using different ratios of Agencourt AMPure XP® beads, followed by pre-capture PCR amplification from the indexes to increase the amount of DNA in the library.

HPV double capture method.

DNA libraries were multiplexed twice by 12 and hybridised for 18 hours at 47°C with HPV biotinylated oligonucleotide probes recognising 235 unique HPV types and variants containing over 84 different HPVs ranging from oncogenic safe to High-risk-HPV (SeqCap EZ Library, Roche NimbleGen Inc, Madison, WI, USA)(52) . DNA sequences were captured using capture beads (KAPA HyperCapture Bead Kit 24 rxn, Roche NimbleGen Inc, Madison, WI, USA), which have a streptavidin coating and then amplified. A second hybridisation for 18 hours at 47°C was performed as well as another step of DNA capture by the beads to improve specificity and efficiency. We then performed a 0.2N sodium hydroxide denaturation and load 600µL at 8pM into the cartridge (MiSeq Reagent Kit V2 300 cycles, Illumina, San Diego, CA, USA). We used Illumina MiSeq systems to sequence post-capture libraries in 150bp paired reads. The data were then analysed using a specific pipeline developed by the HEGP bioinformatics team (Dr Maxime Wack, Dr Bastien Rance) in collaboration with the virology team (Dr David Veyer, Dr Hélène Péré). The data obtained were the following:

- The complete sequences of the HPV genotypes present

- Molecular status ("episomal", "mixed" or "integrated") of the HPV genotypes identified

- Chromosomal and viral marks of integration in case of HPV integration.

Development and validation of the HPV-Capture technique coupled to NGS

The presence of 3 household genes (GAPDH (cellular marker), KLK3 (tumor cell marker) and RAB7A (cellular marker)), captured and sequenced at the same time as the HPV viral sequences potentially present, served as cellular controls validating from a technical point of view the results obtained upstream of the bioinformatic analysis.

This bioinformatic analysis was then based on different parameters:

- The corresponding HPV reference: Thanks to the HPV biotinylated oligonucleotide probes used in the HPV-capture method, of 75 nucleotides on average, which were designed to capture the complete sequence of 235 HPV genotypes and variants, we can know from the complete HPV viral genome sequence obtained, the precise reference of the HPV at the origin of the patient's tumor (52).

For example, the reference corresponding to the HPV tumoral DNA in Figure 16A is the variant HPV16_JP1773. Read depth represents the number of times a viral genomic sequence has been sequenced. This is an important parameter to take into account as the analysis will not be the same if the read depth is high compared to a low read depth result for which it will be more difficult to rule.

- Viral genomic coverage: It allows seeing if the whole genome has been captured and sequenced or if there are missing areas. From this coverage, we can determine whether the detected HPV has a circular complete/episomal, integrated or mixed status.

Different molecular statuses of the HPV viral genome were therefore be identified:

- Episomal: The viral genome is fully covered. Thus, in Figure 16, the HPV16 genome is identifiable over its entirety from 0 to 8000 bp with a homogeneous read depth covering for all the HPV genome.
- Integrated: Total loss of viral framed genomic coverage over a defined area framed on either side by chromosomal marks (defined later) confirms complete integration of the HPV genome into the human genome. For example, in Figure 16B, the HPV45 genome has lost part of its genome between 2500 bp and 3500 bp; the remaining viral genome having integrated into the chromosomes for which we find a right and left chromosome mark, in this example chromosome 10.
- Mixed: The genome is both present in episomal and integrated form. We can see a drastic decrease in the number of reads with a chromosomal mark (chromosome 9) on both sides but still with complete low-noise genomic coverage (Figure 16C).

Finally, for 'integrated' or 'mixed' molecular status, different parameters will be considered:

 Chromosomal-viral marker: Read consisting of both an HPV sequence and a human genomic sequence. It allows the determination of the precise chromosomal level coordinates of the HPV viral genome integration.

 Quality scores: these scores determine the relevance of the chromosomal integration coordinates in the human genome obtained. They are defined according to 3 scores H, C and T defined as follows:

o The H score validates the match with a human sequence in an unambiguous manner.

o The C score is based on the number of reads that will be composed of both HPV sequences and human genome.

o The T score validates the identification of chromosomal marks in the same human chromosome to the right and left of the viral genome integration.

The HCT score is therefore the best one.

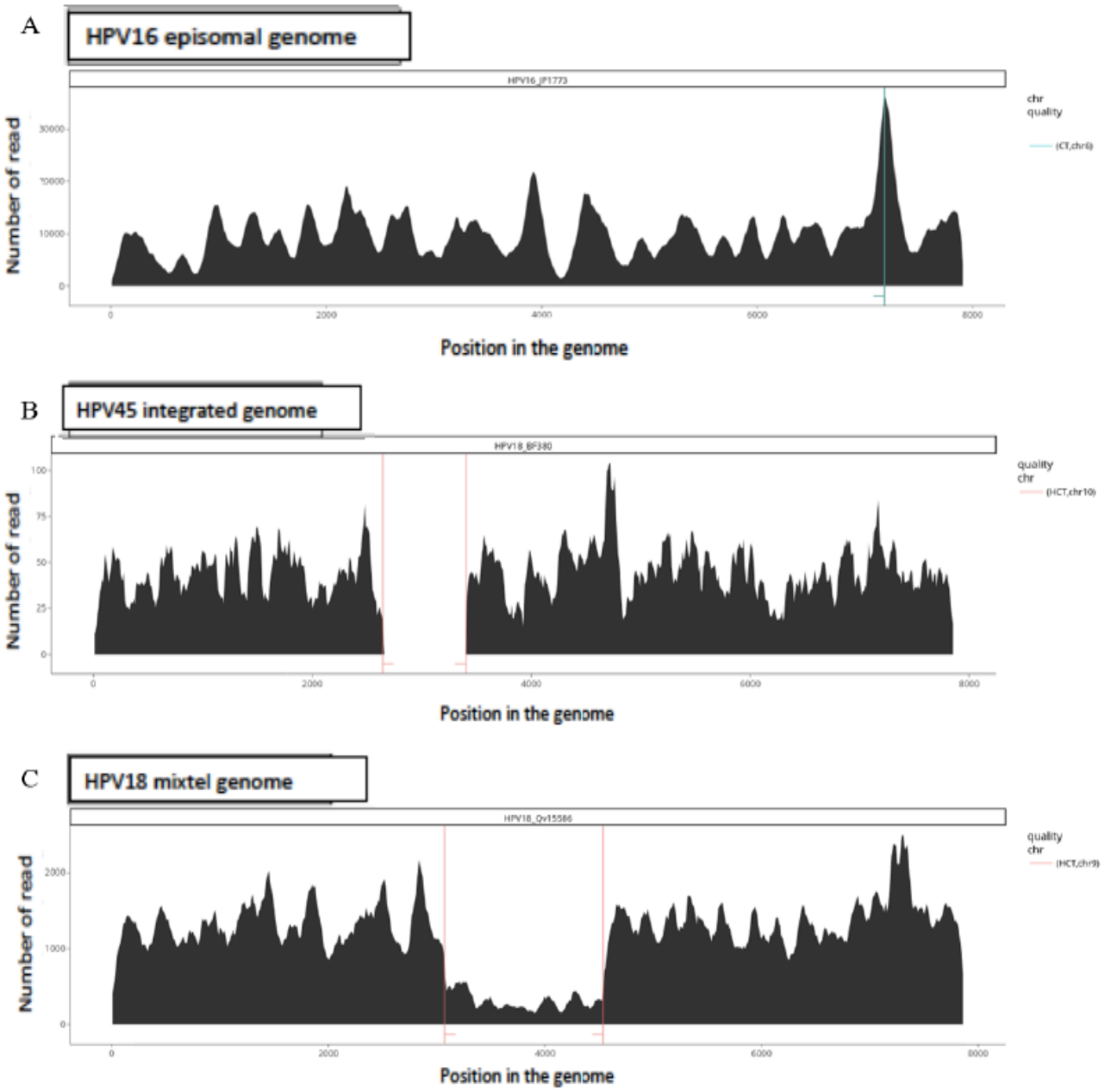


Figure 16- Example of results obtained in HPV-Capture coupled to NGS representing the number of reads of viral genomic sequences (ordinate) as a function of the coverage on the viral genome (abscissa). A: HPV16 Episomal genome. B: HPV45 Integrated genome. C: HPV18 Mixed genome.

Specific for *Study II*: Translacol study (HPV in lymph nodes by ddPCR)

Outcomes

The main endpoint of the study was PFS (Progression free-survival), defined as the time period between initial treatment and local/distant recurrence in the two defined groups as Positive HPV tumoral DNA or Negative HPV tumoral DNA by ddPCR in SLN.
The secondary endpoint of the study was the Disease-specific survival (DSS), defined as the time period between initial treatment and death for cervical cancer.
The disease-specific survival and OS was the same in our population because all the deaths were for cervical cancer. The mean time for death was 61.7 months; for this reason, we decided to analyse survival in 10 years and in addition to in 5 years

Techniques:

In primary tumors:

HPV-genotyping was performed using genomic DNA extracted from FFPE biopsy material obtained from primary tumors and based on commercial or in house PCR protocols, as previously described (60) .

DNA extraction procedures

Sections of FFPE biopsies were deparaffinised overnight at +56°C with 40 µL of proteinase K (Qiagen, Germany) and 360 µL of ATL buffer (Qiagen). Afterwards, 200 µL of ATL buffer was added and incubated for 10 min at +70°C. DNA was further extracted using QiaAmp DNA Mini Kit (Qiagen) and eluted in 50 µL of PCR-grade water.

HPV detection and genotyping by multiplex PCR

The AP28 assay that distinguishes 28 HPV genotypes, by amplifying 100–200 bp fragments of the L1 gene including 13 High Risk (oncogenics) types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), eight Low Risk types (HPV6, 11, 40, 42, 43, 44, 54, 61) and seven genotypes reported as possibly carcinogenic (HPV26, 53, 66, 69, 70, 73, 82)], and human gene β-globin in two different reactions, was used for multiplex HPV molecular testing. Melting curves were obtained at 30, 40, and 50 cycles. Results were first automatically analysed using the Seegene Viewer software, version 2.0 and raw data of results were checked by the virologist

We focused this first study only on patients with tumor induced by HPV 16, 18 or 33 because only type-specific primers and probes targeting HPV16, HPV18, and HPV33 oncogenes were designed. In HPV-positive tumor patients for one of these 3 genoytypes, we performed specific HPV ddPCR in associated pelvic lymph node to detect eventual HPV tumoral DNA.

In Sentinel lymph nodes:

DNA extraction.

Sections of FFPE biopsies were deparaffinised overnight at +56°C with 38 µL of proteinase K (Qiagen, Germany) and 500 µL of ATL buffer (Qiagen). Afterwards, 200 µL of ATL buffer was added and incubated for 10 min at +56°C. DNA was further extracted using QiaAmp DNA Mini Kit (Qiagen) and eluted in 60 µL of PCR-grade water.

Droplet-based digital PCR (ddPCR):

We used a Mix with ddPCR Supermix for probes (no dUTP, Bio-Rad, Hercules, CA, USA), the forward and reverse primers and Albumine probes (internal control for validate extraction and amplification), HPV 16 or l'HPV18 targeting HPV16 E6, HPV18 E7 and HPV33 E6 oncogenes were respectively described in Table 7 and H2O milliQ. The oncogenes were detected by ddPCR protocol adapted on the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA).

Droplets were generated from the reaction mix comprising 9µL of extract,13µL of mix and 70µL of droplet generation oil for probes unsing the QX100TM/200 droplet generator (Bio-Rad, Hercules, CA, USA) (Droplet Generation Oil for Probes, Bio-Rad, Hercules, CA, USA). The emulsions were carefully transferred to a 96-well PCR plate. Amplification was then performed in a conventional PCR thermal cycler with a ramp rate of 2°C/s. The enzyme was first activated for 10 min at 95°C, followed by 45 cycles of 30s denaturation at 94°C and 1min amplification at 55.6°C. The enzyme was then inactivated at 98°C for 10 min and the droplets were stabilised at 4°C for 30 min. The fluorescence obtained in the droplets was then read in a QX200 Droplet reader (Bio-Rad, Hercules, CA, USA) and the data is analysed with Quantasoft software version 1.7 (Bio-Rad, Hercules, CA, USA).

Before analysing the results, we performed a Limit of Blanck (LOB) with sixty negative samples to be able to determine the highest apparente HPV concentration expected to be found in negative samples. Here, the LOB was 2 droplets and it is therefore subtracted from each raw result corresponding to the absolute number of positive droplets obtained for each sample. If a sample had $\leq$ 2 Biorad HPV DNA positive droplets, it was therefore considered to be negative (Figure 17).

Simultaneously, the ubiquitous albumin gene was parallelly targeted and detected as an internal control for each sample (Figure 17).

We considered the sample *Not interpretable* when there were less than 100 Albumin droplets and zero droplets of HPV16/18/33 as we were not able to determine if the sample was negative or if it was positive but not detectable (false negative) because the quantity of tumor was very low and we had not enough material to detect HPV DNA (Figure 17).

Albumin	
	Sequences
Forward primer	GGG ATG GAA AGA ATC CTA TGC C
Reverse primer	GGA CAG GCT GAC CCC AAA TTC T
Probes (HEX)	AGG GTT CTC ATA ACC TA

HPV16 E6	
	Sequences
Forward primer	TTT TAT GCA CCA AAA GAG AAC TGC
Amorce anti-sens	AGC TCT GTG CAT AAC TGT GGT AAC TT
Probes (FAM)	ATG TTT CAG GAC CCA CAG GAG CGA CC

HPV18 E7	
	Sequences
Forward primer	GCA TTT AGA GCC CCA AAA TGA
Amorce anti-sens	CGT TTT CTT CCT CTG AGT CGC T
Probes (FAM)	TCC GGT TGA CCT TCT ATG TCA CGA GCA

HPV33 E6	
	Sequences
Forward primer	ATA TTT CGG GTC GTT GGG CA
Amorce anti-sens	CGT CAC AGT GCA GTT TCT CTA CGT
Probes (FAM)	CGC TGT GCG GTG TGT TGG AGG TCT

Table 7- Oligonucleotides used for amplification with ddPCR.

Assay mix HPV Biorad				
Reagent	Inital concentration (µM)	Final concentration (µM)	Volume (µL) for 1 reaction (1 well)	Concentration in 22µL of emulsion (1 well)
Primer FORWARD ALB	100	16	0,176	0,8
Primer REVERSE ALB	100	16	0,176	0,8
Probe ALB HEX	100	12	0,132	0,6
Primer FORWARD HPV	100	10	0,11	0,5
Primer REVERSE HPV	100	10	0,11	0,5
Probe HPV FAM	100	4	0,044	0,2
TRIS pH8 10mM/H2O	/	/	0,352	/
		Total	1,1	

Mix HPV Biorad			
Reagent	Dilution	Final concentration (µM)	Volume (µL) for 1 reaction (1 well)
Supermix	2	1	11
Assay Mix	20	1	1,1
H20	/	/	0
Volume total mix	22		12,1
Volume sample	/		9,9
Volume total assay /sample (µL)			22

PCR program

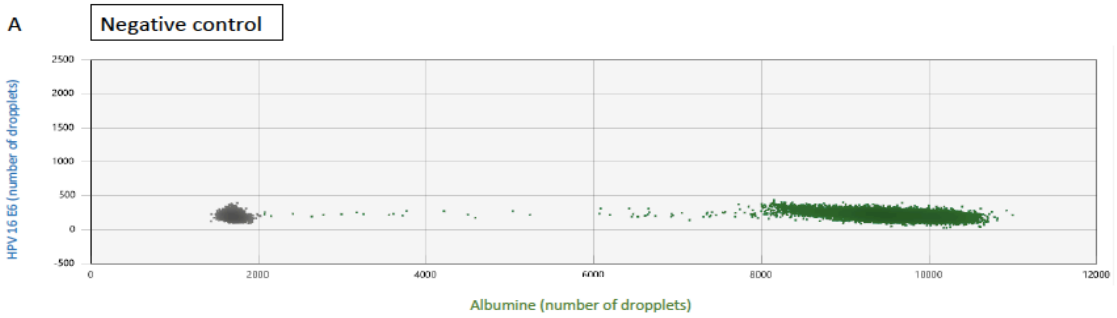
Step	T° (°C)	Time	Number of cycle	ramp (°C/s)
1	50	2:00	1	2
2	95	10:00	1	2
3	95	0:15	45	2
4	60	1:00		2
5	98	10:00	1	2
6	12	10:00	1	2

Référence

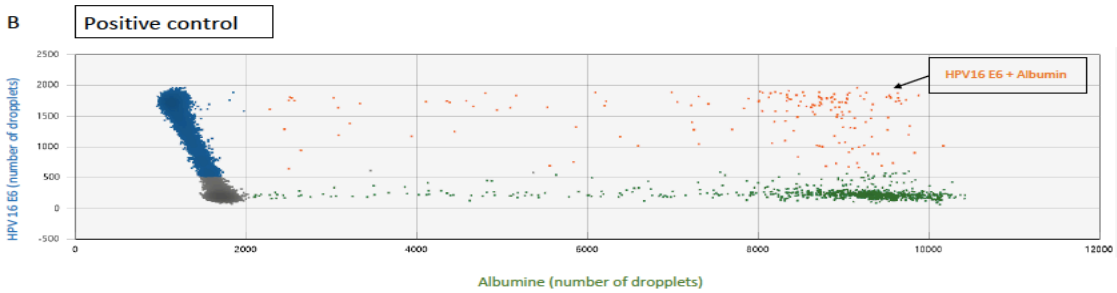
Supermix ddPCR supermix for probes by Bio-rad

Table 8- The concentrations used to prepared the PCR mix in every well with BioRAD.

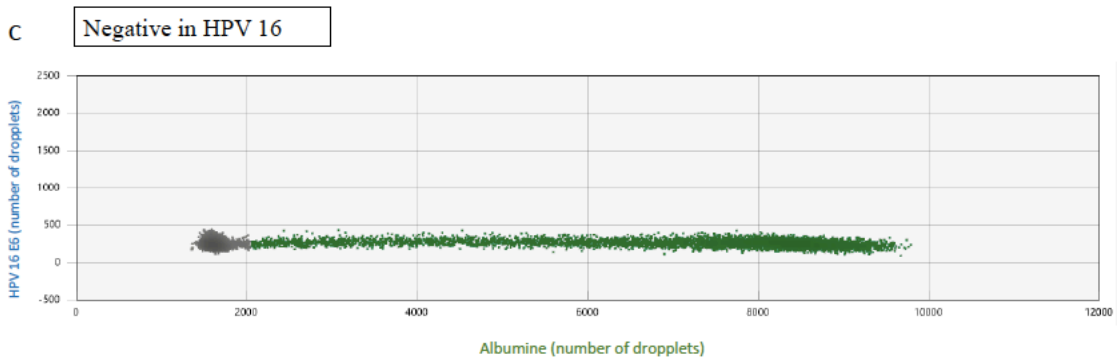
A


Negative control
HPV 16 E6 (number of dropplets)
Albumine (number of dropplets)

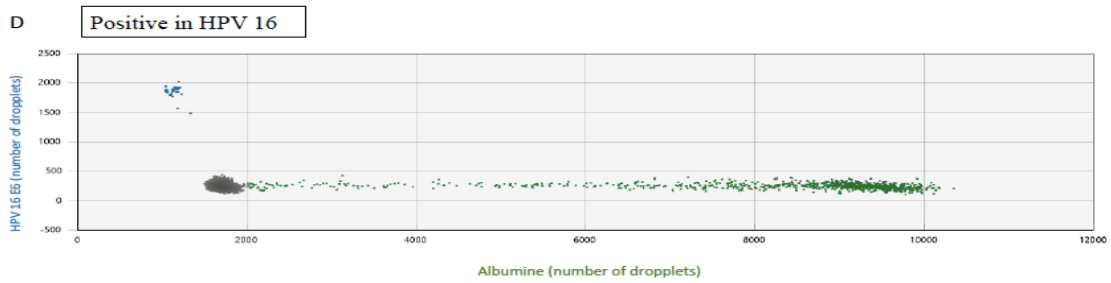
B


Positive control
HPV16 E6 + Albumin
HPV 16 E6 (number of dropplets)
Albumine (number of dropplets)

C


Negative in HPV 16
HPV 16 E6 (number of dropplets)
Albumine (number of dropplets)

D


Positive in HPV 16
HPV 16 E6 (number of dropplets)
Albumine (number of dropplets)

E

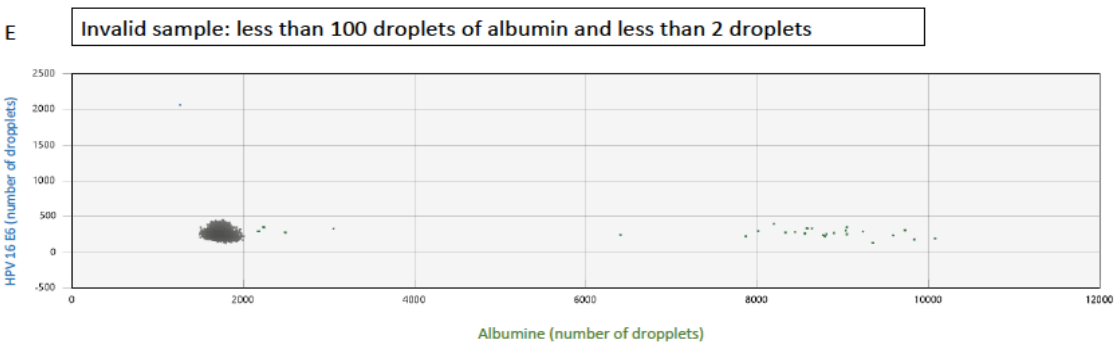

Invalid sample: less than 100 droplets of albumin and less than 2 droplets
HPV 16 E6 (number of dropplets)
Albumine (number of dropplets)

Figure 17- Example of raw ddPCR results showing the number of HPV16 positive droplets versus the number of Albumin positive droplets in a sentinel node biopsy with Biorad. A: Negative control used for each experiment. B: Positive control used for each experiment. C: HPV16 negative sample because once the LOB (2 droplets) is subtracted there are no HPV16 positive droplets. D: HPV16 positive sample. E: Invalid sample because the number of Albumin positive droplets is less than 100.

Study III: HPV in distant lesions.

Patients and data collection

A retrospective study was performed on all women diagnosed with cervical cancer who presented a new tumor for which uncertainty existed as to whether it was a metastasis or a new tumor. Patients were treated in two French medical centres (George Pompidou European Hospital and the Simone Veil Hospital) between 2010 and 2020.

We included women with a histologically confirmed primary cervical carcinoma treated with surgery and/or chemo-radiotherapy who also had another distant lesion. The lesion was suspect based on clinical symptoms or the results from imaging methods, namely Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET) or Computed Tomography Scan (CT). All new lesions were biopsied and assessed by pathologists.

Clinical and histologic differential diagnoses for the secondary lesion included metastatic cervical cancer screening in order to differentiate whether the lesion was caused by another primary cancer or by metastatic cancer from another site that was uncertain and should have been confirmed. In most cases, the histological type and immunohistochemistry were compatible with different origins (metastasis or other primary cancer) allowing for multiple diagnostic options.

The diagnosis of any secondary lesions occurred at the same time as that for primary cervical cancer (synchronous), or later on during follow-up. For any secondary lesion, we recorded the year of diagnosis, location of the lesion, histology type and the p16 expression status.

HPV status in primary tumors and distant lesions

DNA extraction

Formalin Fixed Paraffin Embedded (FFPE) biopsies of distant lesions and the correspondent available tumor were sent to the ISO 15189-accredited virology laboratory of the Georges Pompidou European Hospital (Paris, France) for DNA extraction as described by Steinau et al. (61). Sections of the FFPE biopsies were deparaffinized overnight at 56 °C with 360 µL of ATL buffer (Qiagen, Hilden, Germany) and then we added 40 µL of proteinase K. Afterwards, 200 µL of ATL buffer was added then incubated for 10 minutes at 70 °C. DNA was further extracted using QiaAmp DNA Mini Kit (Qiagen) and eluted in 50 µL of PCR-grade water.

HPV commercial molecular assay

HPV detection and genotyping was carried out in 5 µL of extracted DNA using the CE IVD-marked multiplex RT-PCR assay Anyplex™ II HPV28 (Seegene, Seoul, South Korea) (46)(62). The Anyplex ™ II HPV28 detection test distinguishes 28 HPV genotypes by amplifying 100–200 bp fragments of the L1 gene including 13 high-risk types (High-Risk-HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68), eight low-risk types (LR) (LR-HPV -6, -11, -40, -42, -43, -44, -54 and -70), then seven genotypes reported as possibly carcinogenic (HPV-26, -53, -61, -66, -69, -73 and -82) as well as the human gene β-globin in two different reactions (62). The DNA amplification and the genotyping process were carried out in two reactions performed on the CFX96™ real-time PCR instrument (Bio-Rad, Marnes-la-Coquette, France) (46). Melting curves were obtained at 30, 40 and 50 cycles. Data recording and interpretation were automated using Seegene Viewer software version 2.0 (Seegene) in accordance with the manufacturer's instructions. Raw data of the results were checked by a virologist. This molecular HPV genotyping assay has been found to be suitable for HPV detection and genotyping in cervical secretions (63)(64). Based on Seegene's proprietary DPO™ and MuDT™ technologies (65), this assay was conceived to avoid mismatch priming and to quantify each target in a single fluorescence channel, respectively. In addition, the Anyplex™ II HPV28 assay was shown to be suitable for an extended genotyping approach with a high sensitivity in FFPE specimens (64)(66).

Results

Study I: HPV Capture

Genotyping of HPV in tumor extracts

We performed HPV Capture coupled to NGS and analysed 64 cervical cancer tumor DNA extracts. Of these 64 captures, 46 had interpretable results, with an amount of DNA deemed analysable ([DNA] ≥ 5ng/µL) and/or an HCT score in the bioinformatics analysis.

Among the 46 patients, HPV-specific viral genotypes were found for 40 patients: HPV16 was the more found genotype with 22 patients (55%), followed by HPV18 with 9 patients (22.5%) and 3 patients for HPV45 (7.5%). Other HPV as 31, 33, 34, 39, 52, 56, 59, 66, 70, 73 and 82 were also detected, in 4 cases in co-infection: with HPV16 in 3 cases (2 HPV 73 and 1 HPV 70), with HPV18 mixte in 1 case (co-infection with HPV 39). Two patients were co-infected with HPV 16 and HPV 18.

Molecular status of detected HPV genomes

For the HPV16 genotype (22 patients), in 100% of the cases we found the episomal form (20 pure episomal (90.9%) and 2 mixed (9.1%)). However, HPV18 genotype (9 cases) was most often found in integrated status (5 cases pure (55.6%) and 2 mixte (22.2%)). Only 2 patients (22.2%) was pure episomal status for HPV18 (Table 9).

For the tumors where we found other HPV genotypes (16 cases), pure episomal form was detected in 50% of cases (n=8) (31 and 66 in co-infection, 33, 34, 52,56 and two 73), and pure integrated in the other 50% (n= 8) (39, 59, 70,73, 82, and the 3 HPV45).

This dependence between the molecular status and the virus genotype is highly significant (**p=0.00002**).

We found seven cases of co-infection so they are therefore counted twice in the table. Finally 47 molecular status were analysed.

Genotype / **Molecular status**	HPV16	HPV18	Other HPV	p value
Episomal	20 (90.9%)	2 (22.2%)	8 (50%)	**0.00002**
Integrated	0	5 (55.6%)	8 (50%)	
Mixte	2 (9.1%)	2 (22.2%)	0	
Total	22 (100%)	9 (100%)	16 (100%)	

Table 9- Summary table of the different HPV genotypes and their molecular status found in the tumors by the Capture-HPV method.

Chromosomes impact in case of integration

In total 17 integrations (pure + mixed integration) were found in 16 patients (1 co-infection): 2 for HPV16 (only associated with episomal virus and therefore in mixed form), 7 for HPV18, 3 for HPV 45, and one each for HPV 39, 59, 70,73 and 82. The E6 and E7 genes are systematically retained during these integrations. The breakpoints are from the E1 gene to the L1 gene (including E2) in the vast majority of cases, except for one HPV18 and one HPV82 where only the E1 gene is missing (Table 10).

Also we studied in which chromosome each of the 17 integrations identified was located in and whether some chromosomes were more impacted than others (Table 10). So far, we have been able to identify 4 integrations in chromosome 5 (two HPV18, one HPV45 and one HPV70), 2 integrations in chromosome 9 (one HPV18 and one HPV45), 2 in chromosome 2 (HPV18 and HPV16), 2 in chromosome 12 (HPV59 and HPV82) and finally one integration in chromosomes 3, 4, 10, 13, 15, 20 and 21.

Chromosome	HPV integreted	Genome Portion of viral deletion(pb)	Alteration of gen E2	Saize of genome deleted (pb)	Recurrence	Position	Quatity score
5	45	1800-4000	YES	2200	NOT	52015478-57731010	CT
	70	2000-4500	YES	2500	NOT	150952694-150952700	HCT
	18	1200-2000	NOT	800	NOT	57081498-57150682	HCT
	18	3000-6000	YES	3000	NOT	150948707*	H
9	45	2100-6000	YES	3900	NOT	139592736-139592748	HC/CT
	18	**3000-4500**	**YES**	**1500**	**NOT**	**114726375-114740712**	**HCT**
2	16	1500-3000	YES	1500	NOT	95717780-95717926	HCT
	18	3000-4000	YES	1000	NOT	146392215-146491431	HC/HT
12	59	2200-2900	YES	700	NOT	93421146*	HC
	82	2000-2800	NOT	800	NOT	50573876-96903890	CT
10	16	3000-4000	YES	1000	NOT	4703501-4718921	HCT
3	**39**	**2900-5700**	**YES**	**2800**	**NOT**	**78681701-78682500**	**HT**
4	45	2200-5000	YES	2800	NOT	74646349*	HC
13	18	3900-4000	YES	100	NOT	73977984-74027444	HC/CT
15	18	3000-4000	YES	1000	NOT	71811631-71951452	HCT
20	73	3700-5000	YES	1300	YES	60735346-60735352	HCT
21	18	1000-5000	YES	4000	YES	36206327-36218428	HCT

Table 10- Summary table of the different human chromosomes impacted, the position in the chromosome and the quality score assigned to the HPV integration. For each of the integrations, we have the genotype involved, the portion of the viral genome deleted, i.e. where the integration starts and ends, the total size of the HPV genome that is deleted, the chromosomes into which the HPV genomes have integrated, their positions in the chromosome, the quality score assigned to the validity of the integration and finally, whether or not the patient has relapsed from her disease. *A chromosomal marker is missing due to an insufficient number of reads. HPV18 and 39 in bold are a co-infection in the same patient.

Correlation of molecular status with clinical data (Table 11 and 12).

In the 40 patients analysed, we reported 7 recurrences (3 of them died).

100% of these patients with recurrence or death were infected for HPV16 in episomal form:

- 4 involved patients with a mono-infected HPV16 tumor in purely episomal form.

- 1 involved a patient co-infected with episomal HPV16 and HPV73 integrated in chromosome 20.

- 1 co-infection HPV 16 and 73 both episomal.

- 1 involved a patient co-infected with episomal HPV16 and mixed HPV18 part of which was integrated into chromosome 21.

For the two recurrences cases associated with integrated HPV, the E2 gene was deleted.

Regarding these results, we decide to compare the risk of recurrences between the patients with HPV 16 in episomal form and the rest of patients. In patients with co-infections, we decided to prioritize episomal HPV 16 since it is widely known in the literature that the oncogenic risk is much higher for HPV 16 and 18 (36) . The 2 patients with HPV 16 and 18 co-infections were excluded since it is not possible to determine which of the two genotypes is due to the risk of recurrence. So finally we analysed 38 patients.

6 recurrences were found in the HPV16 episomal group, we did not find recurrences in the patients without HPV16 episomal infection (p=0.007).

Recurrences	HPV16 episomal	Not HPV16 episomal	p value
Not	12 (66.7%)	20 (100%)	**0.007**
Yes	6 (33.3%)	0	
Total	18	20	38

Table 11- Difference of risk of recurrences in ECC patients regarding the presence of HPV16 in episomal status.

Kaplan Meyer curves could not be created to compared HPV16 episomal versus the other tumor because all the reccurrences were in HPV16 episomal group.

HPV genotype	Total patients	Molecular status	Chromosome	Recurrence	Death
16	n= 17	Episomal n =15		4	1
		Integrated n =0		0	0
		Mixte n= 2	2 10	0	0
18	n=6	Episomal n = 2		0	0
		Integrated n = 4	2 5 (Two cases) 13	0	0
		Mixte n =0		0	0
Others: 33,34,45,52,56, 59,73,82	n=10	Episomal n =5		0	0
		Integrated n= 5	4 5 9 12(Two cases)	0	0
		Mixte n=0		0	0
Co-infections: 16+ 70 16+73(2 cases) 16+18(2 cases) 18+39 31 + 66	n =7	Both Episomal= 2 31+ 66		0	0
		16 +73		1	0
		Combinations= 5 70 integrated + 16 episomal	5	0	0
		18 integrated + 16 episomal	15	0	0
		18 mixte + 16 episomal	21	1	1
		73 integrated + 16 episomal	20	1	1
		39 integrated + 18 mixte	3 9	0	0
Total	40	47	17	7	3

Table 12- Resume of all the data: HPV genotypes, molecular status, Chromosome (in cases of integration) and survival data.

Study II: Translacol study (HPV in lymph nodes by ddPCR)

In total, 109 patients were eligible for the study between January 2003 and December 2018. Forty-nine patients were excluded from the analysis: 15 with HPV negative primary tumor and 34 for insufficient SLN sample material (Not interpretable data). Analyses were carried out in a total of 60 patients without histological lymph nodes metastasis (Figure 18).

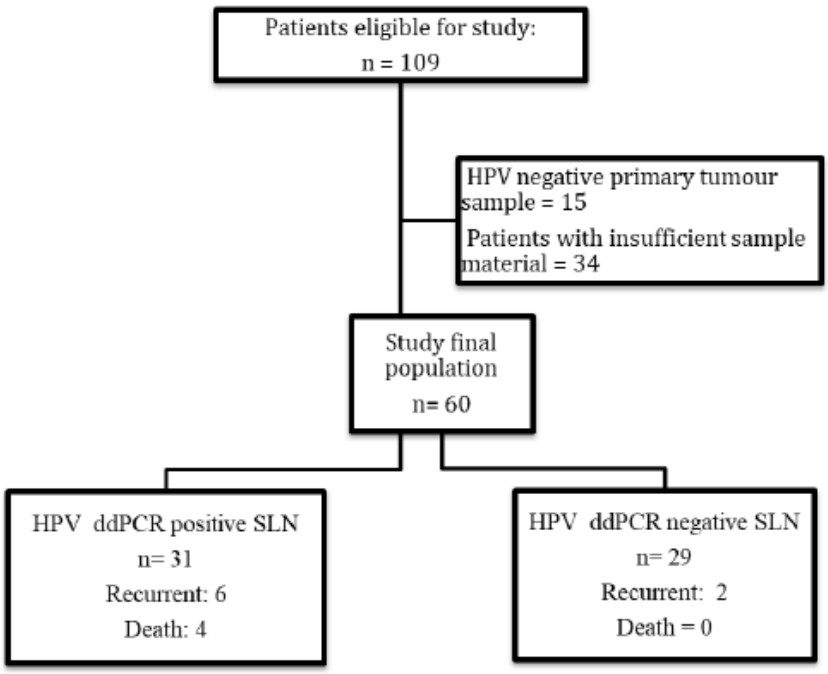


Figure 18- Flow chart of study inclusions and results of tumoral HPV DNA detection in pelvic Sentinel Lymph Nodes (SLN) using ddPCR.

Clinical and tumor characteristics (Table 13)

In our population, the mean age was 47.6 years. Most of patients were multiparous (61.7%). The tumors were most frequently IB1 FIGO stage (56.6%), squamous cell carcinoma (68.3%), grade 2 (51.2%) and without LVSI (74.6%). Finally, 66.1% of the tumor were <20 mm. Vaginal invasion and positive margins were rare (9.3 and 7.5% respectively).
HPV genotype more frequently found in our primary tumors was HPV16 (93.3%). HPV18 was detected in 5% and HPV33 in 1.7% of the patients.

Surgical and oncological treatment (Table 13)

Treatment of uterus consisted in total radical hysterectomy, simple hysterectomy and simple or radical trachelectomy.

The surgical approach was open in 1.7% or minimally-invasive surgery (MIS) in 98.3% of patients. Postoperative complications include CTCEA (Common Terminology Criteria for Adverse Events) (> 1 months) and Cliven-Dindo (<1 months) classifications.
The preoperative brachytherapy was an option in the patients with tumor size > 2 cm. This treatment was realised in 10% of patients.

The adjuvant treatment was performed according to international guidelines based on the presence of these criteria (11): positive nodes and margins/vaginal/parametrial invasion. Adjuvant treatment consisted of chemotherapy, EBRT (External Beam Radiation Therapy) with treatment fields adapted to the extension of the lymph node involvement) (6.7% of patients) or chemoradiotherapy (CCR) (3.3% of patients). Patients with positive lymphovascular invasion were postoperatively treated with brachytherapy (33.3% of patients).

Variables **Patient and tumor characteristics**		
Age (years)	**Mean ± SD** **(range)**	47.6 ± 13.2 (26-81)
BMI (kg/m^2)	**Mean ± SD** **(range)**	23.8 ± 4.8 (16 – 41)
Parity	**Nulliparous** **Multiparous** **Unknown**	15 (25.0%) 37 (61.7%) 8 (13.3%)
2018 pFIGO stage	**IA1 LVSI** **IA2** **IB1** **IB2** **IIA**	2 (3.3%) 4 (6.7%) 34 (56.6%) 17 (28.4%) 3 (5.0%)
HPV genotype	**16** **18** **33**	56 (93.3%) 3 (5%) 1 (1.7%)
Histology	**Squamous carcinoma** **Adenocarcinoma**	41 (68.3%) 19 (31.7%)
Grade	**1** **2** **3** **Unknown**	15 (38.5%) 20 (51.2%) 4 (10.3%) 21
Tumor size	**<20 mm** **≥ 20 mm** **Unknown**	39 (66.1%) 20 (33.9%) 1
LVSI	**None** **Yes** **Unknown**	44 (74.6%) 15 (25.4%) 1
Vaginal invasion	**None** **Yes** **Unknown**	49 (90.7%) 5 (9.3%) 6
Positive margin	**None** **Yes** **Unknown**	49 (92.5%) 4 (7.5%) 7
Surgical and oncological treatment		
Preoperative Brachytherapy	**None** **Yes**	54 (90.0%) 6 (10.0%)
Surgical approach	**MIS** **Open**	59 (98.3%) 1 (1.7%)
Adjuvant treatment (total)	**None** **Yes**	39 (65.0%) 21 (35.0%)
Adjuvant brachytherapy	**None** **Yes**	40 (66.7%) 20 (33.3%)
EBRT	**None** **Yes**	56 (93.3%) 4 (6.7%)
Chemoradiotherapy	**None** **Yes**	58 (96.7%) 2 (3.3%)

Table 13- Global characteristics of study population (n=60).
Abbreviation: BMI = body mass index, FIGO = international federation of gynecologic oncology, LVSI: Lymphovascular space invasion; MIS= micro-invasive surgery

HPV tumoral DNA (HPVtDNA) detection in SLN by ddPCR and correlation with clinical characteristics

All histologically positive SLN controls were positive for HPVtDNA by ddPCR, confirming that our technology works in tissue material.

In our study, we included 60 patients without metastasis in SLN (N-): 31 (51.7%) showed HPV16, 18 or 33 tumoral DNA detection in SLN, whereas 29 (48.3%) were negative by ddPCR in SLN.

The ddPCR results thus make it possible to categorize study patients with negative SLN into two groups: those with positive detection of cryptic HPVtDNA (group "HPV ddPCR positive SLN"), and those negative in histology and ultrasensitive molecular biology (group "HPV ddPCR negative SLN").

No statistical difference could be observed between HPVtDNA- positive and negative groups concerning their age (p=0.15), B.M.I. (p=0.2) or parity (p=0.48).

No statistical significant link was observed between the 2018 FIGO stage and the HPVtDNA status (positive or negative) (p=0.78); IB1 was the most frequent in both groups. Furthermore, no statistical differences could be observed between both groups regarding their HPV genotype in the cervical primary tumor, histological type, grade, LVSI, tumor size, vaginal invasion or positive margins. Data are detailed in the Table 14.

The difference in surgical approach was not statistically significant (p=0.32) between both groups. No differences could be observed between both groups regarding their complementary adjuvant treatment (p=0.12). Finally, no significant differences were revealed between both groups regarding the use of brachytherapy Preoperative or adjuvant, EBRT or chemoradiotherapy. These data are detailed in the Table 14.

Variables		HPV ddPCR negative SLN n= 29 (%)	HPV ddPCR positive SLN n= 31 (%)	p- value
Patient characteristics				
Age (years)	Mean ± SD	(49.3 ± 13.4)	(45.9 ± 12.9)	0.15
	(range)	(27 - 81)	(26 - 71)	
BMI (kg/m^2)	Mean ± SD	(24.3 ± 4.0)	(23.3 ± 5.5)	0.20
	(range)	(16 - 34)	(18 – 41)	
Parity	Nulliparous	7 (25.9%)	8 (32.0%)	0.48
	Multiparous	20 (74.1%)	17 (68.0%)	
	Unknown	2	6	
Tumor characteristics				
2018 pFIGO stage	IA1 LVSI	0	2 (6.5%)	0.78
	IA2	2 (6.9%)	2 (6.5%)	
	IB1	17 (58.6%)	17 (54.8%)	
	IB2	8 (27.6)	9 (29.0%)	
	IIA	2 (6.9%)	1 (3.2%)	
HPV genotype	16	28 (96.6%)	28 (90.3%)	0.53
	18	1 ((3.4%)	2 (6.5%)	
	33	0	1 (3.2%)	
Histology	SCC	20 (69.0%)	21 (76.7%)	0.91
	Adenocarcinoma	9 (31.0%)	10 (32.3%)	
Grade	1	6 (31.6%)	9 (45.0%)	0.12
	2	9 (47.4%)	11 (55.0%)	
	3	4 (21.1%)	0	
	Unknown	9	12	
Tumor size	<20 mm	20 (69.0%)	19 (63.3%)	0.65
	≥ 20 mm	9 (31.0%)	11 (36.7%)	
	Unknown		1	
LVSI	None	19 (67.9%)	25 (80.6%)	0.26
	Yes	9 (32.1%)	6 (40.0%)	
	Unknown	1		
Vaginal invasion	None	24 (85.7%)	25 (96.2%)	0.18
	Yes	4 (14.3%)	1 (3.8%)	
	Unknown	1	5	
Positive margin	None	26 (92.9%)	23 (92.0%)	0.65
	Yes	2 (7.1%)	2 (8.0%)	
	Unknown	1	6	
Treatment				
Preoperative Brachytherapy	None	25 (86.2%)	29 (93.5%)	0.51
	Yes	4 (13.8%)	2 (6.5%)	
Surgical approach	MIS	28 (96.6%)	31 (100%)	0.48
	Open	1 (3.4%)	0	
Adjuvant treatment (total)	None	16 (55.2%)	23 (74.2%)	0.12
	Yes	13 (44.8%)	8 (25.8%)	
Adjuvant brachytherapy	None	16 (55.2%)	24 (77.4%)	0.66
	Yes	13 (44.8%)	7 (22.6%)	
EBRT	None	27 (93.1%)	29 (93.5%)	0.67
	Yes	2 (6.9%)	2 (6.5%)	
Chemoradiotherapy	None	28 (96.6%)	30 (96.8%)	0.73
	Yes	1 (3.4%)	1 (3.2%)	

Table 14- Patient and tumor characteristics in two groups (HPV ddPCR positive or negative in SLN).

Abbreviation: BMI = body mass index, FIGO = international federation of gynecologic oncology, SCC= Squamous carcinoma**,** LVSI: Lymphovascular space invasion; MIS= micro-invasive surgery

Follow up and survival results

The mean follow-up was 52.2 ± 32.9 months (range, 1 – 129) in HPV ddPCR-negative SLN group and 48.1± 22.3 months (range, 7 – 99) in HPV ddPCR positive SLN group, without statistical difference (p=0.30). Concerning the cancer progression, 2 (6.9%) patients in HPV ddPCR-negative SLN group and 6 (19.3%) in HPV ddPCR positive SLN group developed recurrences (p=0.15).

The mean time of recurrences was 43.5 ± 36 months (range, 18 – 69) in HPV ddPCR-negative SLN group and 29.2 months ± 29.3 months (range, 2 – 64) in HPV ddPCR positive SLN group (p=0.22) Finally, regarding the DSS, 4 study patients died of cervical cancer, all belonging to the HPV ddPCR positive SLN positive group. These data are detailed in the Table 15.

Variables		HPV ddPCR negative SLN n= 29 (%)	HPV ddPCR positive SLN n= 31 (%)	p- value
Mean follow up (months)	Mean ± SD (range)	(52.2 ± 32.9) (1 - 129)	(48.1 ± 22.3) (7 -99)	0.30
Recurrent disease	None Yes	27 (93.1%) 2 (6.9%)	25 (80.6%) 6 (19.4%)	0.15
Mean time for recurrence (months)	Mean ± SD (range)	(43.5 ± 36) (18 - 69)	(29.2 ± 29.3) (2 - 64)	0.22
Death for cervical cancer	Alive Death	29 (100%) 0	27 (87.1%) 4 (12.9%)	0.07
Mean time for death (months)	Mean ± SD (range)		61.75 (38- 81)	

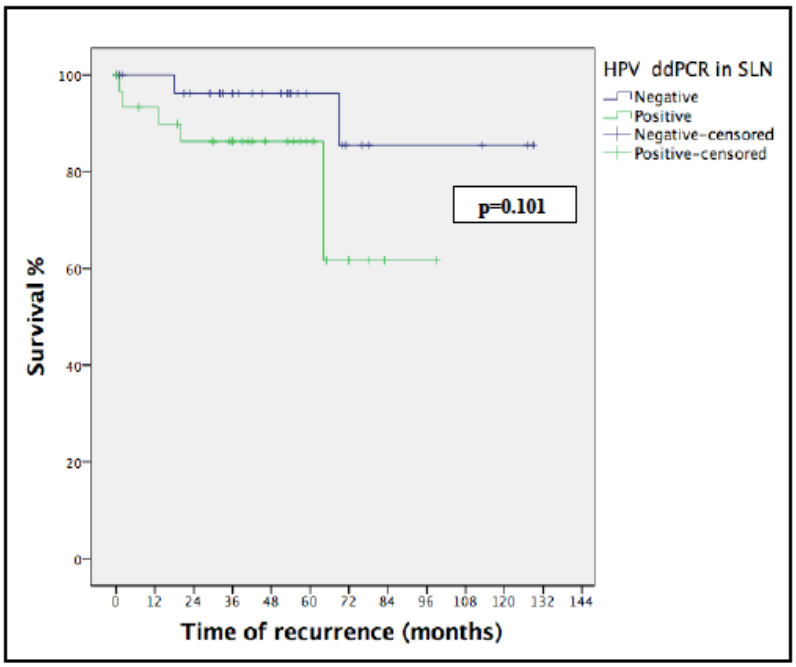
Table 15- Survival outcomes in two groups (HPV ddPCR positive or negative in SLN).

The Kaplan Meyer curves are shown in the Figure 19. The percentage of progression-free survival (PFS) to 5 years was 96.2% in the HPV ddPCR negative SLN group and 86.4% in the HPV ddPCR positive SLN group.
The 10 years percentage of progression-free survival (PFS) was 85.5% in the HPV ddPCR negative SLN group and 61.7% in the HPV ddPCR positive SLN group, without statistical difference (log rank test p=0.101) (Figure 19A).

The mean time for death was 61.7 months. The disease-specific survival to 5 years was slightly higher (100%) in the HPV ddPCR negative SLN group than that (95.5%) in the HPV ddPCR positive SLN group.The disease-specific survival to 10 years was slightly higher (100%) in the HPV ddPCR negative SLN group than that (47.7%) in the HPV ddPCR positive SLN group (log rank test **p=0.040**) (Figure 19B).

A. Progression-free survival analysis (p=0.101)

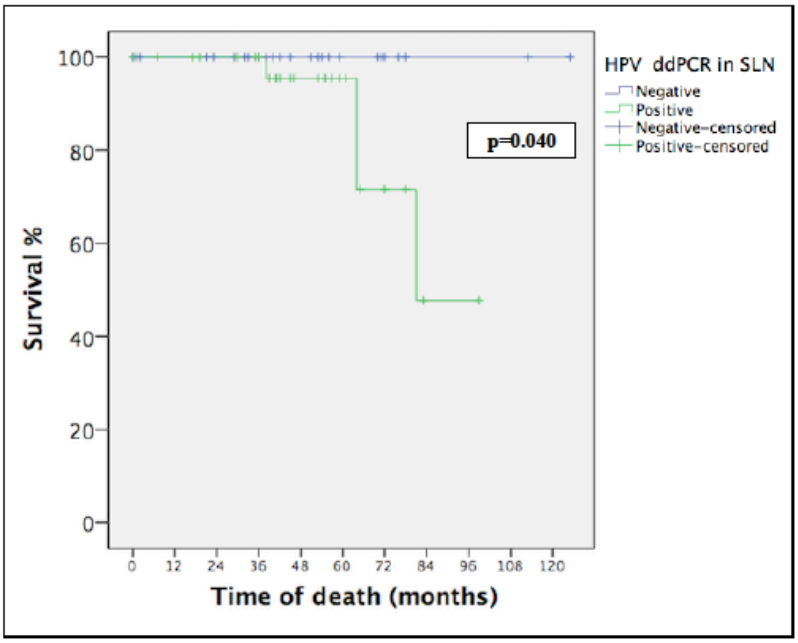
A



	Negative	Positive
PFS (%) to 5 years	96.2%	86.4%
PFS (%) to 10 years	85.5%	61.7%

B. Disease-specific survival analysis (p=0.040).

B



	Negative	Positive
DSS (%) to 5 years	100%	95.5%
DSS (%) to 10 years	100%	47.7%

Figure 19- Kaplan Meyer curves for PFS (A) and DSS (B) differences between the group of negative ddPCR HPVtDNA and the group of positive ddPCR HPVtDNA detection of cryptic in SLN biopsies previously negative in histology.

Univariate			
Variable	**HR**	**IC 95%**	**p**
Age			
≤45	1		
>45	1.608	0.499 –5.175	0.426
B.M.I			
≤25	1		
>25	5.832	0.057-598.463	0.455
Parity			
Nulliparous	1		
Multiparous	0.175	0.001–40.864	0.531
2018 pFIGO stage			
IA1 LVSI+	1		
IA2	0.006	0- 3.804	0.979
IB1	0.006	0– 6.398	0.956
IB2	13.576	0– 2.266	0.960
IIA	17.581	0– 2.953	0.956
HPV genotype			
HPV33/18	1		
HPV16	6.595	0.547-74.112	0.126
Tumor size			
< 20 mm	1		
≥ 20 mm	2.574	0.358- 18.517	0.348
Lymphovascular space involvement (LVSI)			
Non	1		
Yes	2.401	0.326– 17.652	0.390
Histology			
SCC	1		
Adenocarcinoma	1.529	0.565 – 4.190	0.399
HPV ddPCR in SLN			
Negative	1		
Positive	67.273	0.21-210496.208	0.305
Positive margin			
Negative	1		
Positive	0.999	0.015 –68.167	0.999
Parametrial invasion			
Negative	1		
Positive	1.238	0.399 – 3.841	0.712
Vaginal invasion			
Negative	1		
Positive	4.037	0.362 –45.033	0.257
Preoperative brachytherapy			
Non	1		
Yes	5.537	0.027–1120.03	0.528
Surgical approach			
Open	1		
MIS	4.924	0.001–17994.59	0.703
Adjuvant treatment (total)			
None	1		
Yes	0.953	0.305 –2.974	0.934
Adjuvant brachytherapy			
None	1		
Yes	5.513	0.036–834.544	0.505
EBRT			
None	1		
Yes	4.681	0 –268182.17	0.782
Chemoradiotherapy (CCR)			
None	1		
Yes	0.999	0.002-574.155	0.999

Table 16- Univariate analysis predicting Disease-specific survival using a Cox regression model. Multivariable was not done because no variable was significant in univariable analysis.
Abbreviation: BMI = body mass index, FIGO = international federation of gynecologic oncology, LVSI: Lymphovascular space invasion; MIS= micro-invasive surgery. HR = hazard ratio, IC 95% = confidence interval 95%

As we found significant statistical differences in DSS between the two groups, we decided to perform a univariable analysis for DSS (Table 16). No variables were significant for DSS, so we did not perform a multivariable analysis.

Study III: HPV in distant lesion

Clinical Characteristics and pathological findings of patients

Under our inclusion criteria, we identified eight women treated in two French comprehensive cancer centres between 2010 and 2020 (Table 17).

Case	Age (*year*)	Cervical cancer type	Multiplex rt-PCR**	New lesions			
				Location	Histology	p16(INK4a) expression*	Multiplex rt-PCR**
#A	79	Squamous cell carcinoma [stage IIB] *** in 2015	NA	Mediastinal adenopathies (2018)	Squamous cell carcinoma	+	HPV-16
#B	49	Adenocarcinoma [stage IIB] *** in 2017	NA	Lung, liver brain, retroperitoneum (2018)	Undifferenciated carcinoma	+	HPV-16
#C	53	Adenocarcinoma [stage IVA] *** in 2018	NA	Ovary (2018, synchronous)	Endometrioid Adenocarcinoma	ND	HPV-18
#D	42	Squamous cell carcinoma [stage IIB] *** in 2019	NA	Ovary (2019, synchronous)	Squamous cell carcinoma	+	HPV-18
#E	47	Squamous cell carcinoma [stage IIB] *** in 2016	HPV-16	Lung (2019)	Squamous cell carcinoma	ND	HPV-16
#F	40	Squamous cell carcinoma [stage IIB] *** in 2017	HPV-16	Lung (2018)	Squamous cell carcinoma	+	HPV- 16
#G	54	Squamous cell carcinoma [stage IIB] *** in 2010	HPV-16	Kidney (2012)	Squamous cell carcinoma	ND	HPV-16
#H	67	Squamous cell carcinoma [stage IVA] *** in 2016	HPV-16	Lung (2016)	Adenocarcinoma	ND	None

Table 17- Clinical and pathologic findings in patient cases of cervical cancer and possible metastatic disease.

* $p16^{(INK4a)}$ expression was assessed by immunohistochemistry using rabbit monoclonal anti-CDKN2A/$p16^{(INK4a)}$ antibody;
** Sections of formalin-fixed, paraffin-embedded (FFPE) biopsies were deparaffinized overnight at 56 °C with 40 µL c proteinase K (Qiagen, Hilden, Germany) and 360µL of ATL buffer (Qiagen), as described by Veyer, 2019. Afterwards, 20 µL of ATL buffer was added and incubated for 10 minutes at 70 °C. DNA was further extracted using QiaAmp DNA Mir Kit (Qiagen) and eluted in 50 µL of PCR-grade water. HPV detection and genotyping was carried out in 5 µL of extracte DNA using the CE IVD-marked multiplex rtRT-PCR assay Anyplex™ II HPV28 (Seegene, Seoul, South Korea) as describe by Estrade 2014 and Lillsunde Larsson 2015. The Anyplex ™ II HPV28 detection test distinguishes 28 genotypes of HPV by amplifying 100–200 bp fragments of the L1 gene including 13 high-risk types (High-Risk-HPV -16, -18, -31, -33, -35, -39 -45, -51, -52, -56, -58, -59 and -68), eight low-risk types (LR) (LR-HPV -6, -11, -40, -42, -43, -44, -54 and -70), then seve genotypes reported as possibly carcinogenic (HPV-26, -53, -61, -66, -69, -73 and -82) as well as the human gene β-globin i two different reactions (Lillsunde Larsson 2015). The DNA amplification and the genotyping process were carried out in tw reactions performed on the CFX96™ real-time PCR instrument (Bio-Rad, Marnes-la-Coquette, France) (Estrade 2014) Melting curves were obtained at 30, 40 and 50 cycles. Data recording and interpretation were automated using Seegen Viewer software version 2.0 (Seegene) in accordance with the manufacturer's instructions. Raw data of the results wer checked by a virologist. The virology laboratory was accredited in 2013 by the Comité Français d'Accréditation (COFRAC according to ISO 15189 norms for the biological markers "HPV detection" and "HPV genotyping".
*** According to the International Federation of Gynecology and Obstetrics (FIGO) classification.
Age is related to the distant lesion diagnosis. All patients were initially treated with radio-chemotherapy. Distant lesions v treated with chemotherapy: Carboplatin and Taxol in all the cases and adding Vinorelbine in case H (primary lung cancer).
ND; Not done, rt-PCR; real-time polymerase chain reaction, +; positive, NA; not available.

In these eight cases, the secondary lesion was difficult to identify as cervical tumor metastasis or secondary primary tumor.

The clinical and histological features of these women are described in Table 17. The mean age of the patients was 53.9 years (range 40-79). The most common histological type of tumor was squamous carcinoma (six cases). Two cases were adenocarcinoma. All cases were in advanced stages.

Two cases were synchronous with primary cervical cancer. The others were diagnosed later (the time of recurrence/metastasis was between one and three years). Moreover, except for the synchronous cases that were treated with surgery, all other patients received chemoradiotherapy as initial treatment. The most common site of the second tumor was the lung (four cases).

HPV detection and genotyping of distant lesions FFPE biopsies

In the distant lesion, results from RT-PCR showed HPV-16 DNA in six cases and HPV-18 DNA in one case thus completing the histological result from an anatomopathologist and confirming the cervical origin of the new lesion (Table 1). Lastly, a case with an HPV-negative distant lesion result completed our series confirming that the new primary independent cancerous lesion was not related to the HPV cervical primary cancer in this patient. HPV genotype was confirmed via biopsies of primary tumors in some of the cases (see #E, #F, #G and #H in Table 17).

Discussion

The therapeutic management of ECC remains challenging, mainly in patients with intermediate-risk factor tumors.

The surgical standard treatment in ECC is open radical hysterectomy with lymph node assessment. After surgery, taking into consideration the histopathological factors found in the specimen, the need for adjuvant treatment is determined. The European guidelines recommend adjuvant radiotherapy after radical surgery in patients with intermediate-risk factors: the presence of a combination of factors, such as tumor size, lymphovascular space invasion, and depth of stromal invasion at final pathology (11). Radiotherapy reduces the risk of local recurrence. But these 'intermediate risk' criteria for recurrence were stablished in a study was published in 1999 for Sedlis et al, showing a benefit in recurrence free rate in patients that received pelvic radiotherapy (18). A recent review article published for Rodríguez et al in 2022, evaluate and criticise the Sedlis Study and discuss several studies that have reconsidered the role of adjuvant therapy after radical hysterectomy in the presence of intermediate risk criteria. Considering the changes in FIGO Stage IB Classification in 2018, the evolution in treatment modalities in surgery and radiotherapy, and the improved identification of prognostic histopathological factors, the authors of this review proposed reconsideration of this factors (67).

Furthermore, the presence of nodal metastases constitutes the main prognostic factor in ECC patients (16). But 10 to 15% of patients without evidence of tumoral cells in their pelvic lymph nodes may develop recurrences, in association with poor prognostic (68).

For all these reasons, it is mandatory to define new markers to help clinicians to decide the more appropriate adjuvant treatment for the patient and evaluate the possibilities of fertility sparing surgery for the “low” risk patients.

In this context, as HPV infection is present in 99% of cervical cancer, we decide to use HPV detection as a prognostic biomarker in patients with EEC and negative SLN in histology.

HPV detection as prognosis factor

About HPV Capture (*Study I*)

In order to determine the value of HPV detection in the tumor as prognosis factor in these early stage tumors, particularly in terms of viral molecular status, 40 tumor extracts from patients were subjected to HPV capture coupled to NGS and revealed disparities in HPV molecular status.

HPV16 and HPV18, as already known in the literature (36), were often detected followed here by other genotypes less widespread but belonging in the majority of cases to oncogenic HPV.

Of the 22 HPV16 cases, 20 presented an exclusively episomal genome, i.e. 90% of cases, and 2 had a mixed genome (both episomal and integrated HPV DNA). For the HPV18 cases, out of the 9 cases, 2 are episomal (22.2%), 5 are integrated (55.6%) and 2 are mixed (22.2%). This suggests that HPV18 seems to integrate more easily into the human genome than the HPV16 genome. This correlation between genotype and molecular status was highly significant ($p=0.00002$) in our study. Similar data concerning the very high proportion of HPV18 integrated in cervical cancers have already been found in other whole genome sequencing studies (69) (70). In these same studies a significant proportion of HPV16 was also found in non-integrated form but without reaching the levels found in our study. Finally, for all HPV genotypes, we found integration (exclusive and mixed) into the human genome in 17 of the 40 patients.

When HPV genome is integrated, the E2 gene was no longer present in 89% of the cases, which confirms the hypothesis of oncogenesis caused by deletion of the E2 gene as a result of HPV DNA integration. However, for the 30 cases of exclusive episomal molecular status, as well as for the 2 patients for whom the viral E2 gene was conserved despite complete integration into the human genome, oncogenesis origin based on viral integration cannot be retained, epigenetic modifications, in particular methylation of the E2 binding sites (E2BS) on the viral genome, would be described in order to characterise the oncogenic HPV process induced in these tumors.

Regarding the prognosis value of molecurar status in HPV infection, in our study 100% of these patients with recurrence or death were infected for HPV16 in episomal form (pure or mixte) and we found significant differences in the risk of recurrence according to the presence or absence of HPV16 in the episomal state ($p=0.007$). These results suggest that the most virulent molecular status of HPV16 seems to be associated with an episomal form, which was recently reported by the team in the description of a very aggressive HPV16 episomal-induced anal canal cancer case report (71). However, the number of cases of tumors with non-episomal HPV16 is still too small to be able to make a definite statement on this point.

In a recent study published for Kamal et al, there was not significant correlation between the HPV integration signatures (EPI, 2J and MJ) and the PFS (70). Also in a study performed in patients with advanced (Squamous Cell Carcinoma of the Anal canal) SCCA associated with HPV infection and treated with Docetaxel, Cisplatin and 5-fluorouracil (DCF) found differents results of our study. Integrated/mixed forms were approximately twice more frequent (51% vs 28.6%) compared to episomal forms, i.e. 42.5% of all patients. Also, a better PFS was observed in case of patients with episomal DNA, raising the hypothesis of a predictive impact of HPV integration status (72).

Concerning the sites of viral integrations in the human genome, we observe a disparity in our study. For the same genotype, integrations do not occur at the same positions in the human genome. However, whatever the viral genotype, we observe chromosomes that are more susceptible to viral integration, such as chromosomes 5, 9, 2, 12 and 10. The correlation of these particular integrations with clinical data and the more precise analysis of the genes possibly impacted by these integrations will allow us to determine whether these occurrences may have a clinical significance or not, notably in terms of prognosis.

About Translacol study (HPV in Sentinel lymph nodes by ddPCR) (*Study II*)

In this study, we evaluated the prognosis value of HPV detection by ultrasensitive ddPCR in pelvic SLN. The hypothesis of this study was based in the possibility of the existence of "occult" metastases in SLN, not detected histologically, but identified by ultrasensitive molecular assay detecting HPV DNA (equivalent to tumor DNA).

In 2016, a study was designed to evaluate the feasibility and accuracy of a commercially available test to detect E6/E7 mRNA of 14 subtypes of high-risk HPVs (APTIMA; Hologic, Bedford, MA) in the sentinel lymph nodes of 54 cervical cancer patients laparoscopically operated. All the histologically confirmed metastatic lymph nodes were also HPV E6/E7 mRNA positive, resulting in a sensitivity of 100%. Four histologically free sentinel nodes were positive for HPV E6/E7 mRNA, resulting in a specificity of 96.4%. The authors concluded that the HPV E6/E7 mRNA assay in the SLNs of patients with cervical cancer is feasible and highly accurate. The detection of HPV mRNA in 4 women with negative SLNs could denote a shift from microscopic identification of metastasis to the molecular level. The prognostic value was not studied (73).

Recently, in 2020, a study has been carried out to compare the positive rate of SLNs metastasis detected by routine pathological examination, and investigated the value of HPV-DNA in the detection of ECC lymph node micrometastasis. Reverse transcription-quantitative PCR (RT-qPCR) was used in order to evaluate the HPV DNA detection. They concluded that the detection of HPV-DNA expression in pelvic lymph nodes of ECC may be used to improve the detection rate of micrometastasis, guide the postoperative adjuvant therapy more accurately and improve prognosis, but the autors did not realise survival analyses (74).

Moreover, Lee et al in 2007 published an study designed initially to examine whether the pathologic status of sentinel nodes in patients with cervical cancer represented metastatic disease in the lymph nodes and the sentinel-node HPV status plays a crucial role for predicting lymph node metastasis of the cancer. Secondary they also evaluated whether the HPV status in sentinel nodes might have a clinical value as a prognostic factor for the recurrence in cervical cancer patients. For HPV genotyping, they used a commercially available HPVDNAChip purchased from Mygene Co. Target HPV DNA was amplified with the polymerase chain reaction (PCR) using the primers (HPV and β-globin) and the PCR products were hybridized onto the chip.
HPV DNA was detected in sentinel nodes of 10 patients among 11 patients with lymph node metastasis what proves the direct relationship between the presence of cancer cells and HPV DNA. Moreover, disease recurred in five patients and, all of these patients had HPV in sentinel nodes, indicating a potential prognostic factor for survival in patients without histological detection of cells in SLN (75).

In the same year, a French group published a study designed to determine the frequency of HPV DNA detection by RT-PCR in sentinel lymph node (SLN), and its relation to the clinical characteristics and outcome of women with cervical cancer. HPV DNA was detected in the primary tumor and lymph node by E6-specific PCR. HPV DNA was more frequent in positive SLN than in negative SN ($P < 0.0001$). HPV DNA was detected in SLN in one (14%) of seven patients with recurrence and in nine (19.5%) of 46 patients without recurrence (P =NS). But in this survival analysis the authors do not differentiate between patients with presence or absence of cancer cells histologically in SLN. (76).

Dürst and colleagues recently used the detection of HPV-E6-E7-mRNA by classical RT-PCR in SLN as a predictor of recurrence in patients with N-early cervical cancer (77). A total of 189 early cervical cancer patients without histological probed SLN metastasis were included in a prospective, multicentre study. In patients with HPV-E6-E7-mRNA-negative SLN, the recurrence-free-survival was significantly longer (log rank test $p = 0.002$). The hazard ratio (95%CI) for disease-recurrence was 3.8 (1.5 – 9.3, $p = 0.004$) for HPV-mRNA-positive as compared to HPV-mRNA-negative patients (77).

The same team further compared ddPCR efficiency to classical real-time PCR in 10 sentinel lymph nodes with varying HPV transcript levels. For the analysis of both, clinical samples and serial dilution samples, ddPCR and qPCR showed comparable sensitivity. With regard to reproducibility, both methods differed considerably, especially for low template samples. The authors found with qPCR a mean variation coefficient of 126% whereas ddPCR enabled a significantly lower mean variation coefficient of 40% ($p = 0.01$) and senerally, they saw with ddPCR a substantial reduction of subsampling errors, which most likely reflects the large cDNA amounts available for analysis. So this study showed higher reliability of ddPCR to detect the presence of HPV mRNA in SLN of cervical cancer patients (78) . But this study did not provide survival results.

In the present study, we used the ultrasensitive ddPCR technology to evidence cryptic HPVtDNA in pelvic SLN negative for tumoral cells by histology in a retrospective series of 60 cases of early cervical cancer. 51.7 % of the patients showed positive HPV16, 18 or 33 tumoral DNA detection (regarding the HPV genotype found in their tumors) in SLN initially diagnosed as negative by histology, whereas the ddPCR confirmed the lack of HPVtDNA in SLN as previously detected by histology in 48.3% of patients. Our results showed significant worst DSS in patients with positive HPVtDNA in their SLN. Similar trend was observed for progression-free survival, although not significantly given the small size of our sample.

Taken together, these observations indicate that the use of ultrasensitive ddPCR to detect occult HPVtDNA in SLN could allow to better characterize N0 early cervical cancer in two subgroups of patients with different prognostic and likely care. These findings pose the rationale for the use of HPV DNA detection by ddPCR in addition to histology in SLN to reach optimized management of patients suffering from early cervical cancer. For more than two decades, evidences in the field of HPV DNA detection in LN in early cervical cancers with or without pelvic node metastasis ranged from simple epidemiological reports to use HPV detection as an additional test able to detect patients at higher risk of recurrence and so to determine the necessity of adjuvant treatment (79). However, this parameter should logically have an interest in the detection of LN micrometastasis missed through classical diagnostic procedures specifically in patients without histologically diagnosed nodal metastasis.

During cervical cancer development, the local immune response, implies the intervention of immune-competent phagocytes shown to possibly be able to transport the HPV-DNA from primary tumor cells to LNs (80). Therefore, it is not still evident if the HPV genome presence in pelvic SLNs is related to the ‘‘phagic’’ activity of immune cells or if it is related to the presence of real micrometastasis that could not have been detected through classical diagnostic procedures. However, the “phagic” hypothesis could not explain the poor outcomes observed in patients with positive HPV SLNs status by ddPCR since the histological investigation reporting the absence of metastasis.

To our knowledge, our study is the first one to evaluate the detection of HPVtDNA of different HPV genotypes in SLN in early cervical cancer using the new ultrasensitive technology of ddPCR as a prognosis biomarker in N- early cervical cancer. Therefore, the detection of HPVtDNA in SLN of patients histologically N- classified could be interesting to improve their medical care and likely to adapt the treatment of patients with positive HPVtDNA in their SLN by ddPCR. Another strength of our study concerns the choice of targeting HPV DNA oncogenes instead of RNA transcripts. Indeed, it is well known that DNA is more stable and resistant than RNA, particularly in FFPE samples. Thus, a molecular test for the detection of HPV DNA is likely more sensitive than a test for the detection of HPV mRNA, and in this respect will be associated with a better negative predictive value.

Our study has some limitations. Firstly, we used retrospective material, with the possibility of bias: some tumor and SLN blocks were up to 10 years old (study of patients since 2003). The age of the material added to the deparaffinization process makes 34 samples interpretable and invalid when performing the internal control with albumin.

HPV detection for diagnosis

About HPV in distant lesions (*study III):*

In this study, eight documented cases of women with cervical cancer showed that the use of routine HPV molecular detection and genotyping allowed the differentiation between metastatic cervical cancer disease and a new primary tumor.

We found HPV in all distant lesions except one case. This case, with negative HPV in the lesion, was considered as primary lung cancer and was treated with Navelbine with a fractionated dose of cisplatin (CDDP), unlike the cases diagnosed as metastatic cervical cancer which were treated with carboplatin and taxol.

The prognosis of patients with metastatic cervical cancer is poor (median survival time between 8 to 13 months). There are two types of metastasis in cervical cancer patients: hematogenous and lymphatic. Patients with hematogenous metastasis have a higher risk of mortality than those with lymphatic metastasis. In addition, the management of metastasis may be different depending on its origin and location. Surgery, radiotherapy, chemotherapy or combinations of several treatments are options (81).

In patients with HPV-related cancer and a distant lesion, the histologic differentiation of metastatic cervical cancer versus another primary tumor or metastasis from another cancer can be difficult, especially if the distant lesion shows similar histologic findings as the cervical cancer (82). However, this differential diagnosis is essential in view of its clinical, therapeutic and prognostic implications. In addition, patients with HPV-related cancer are at higher risk of having other carcinomas since smoking is a common risk factor. In our study, four of the distant lesions were pulmonary, which raised diagnostic doubts as to whether they were primary lung cancer or metastasis of cervical cancer. In both cases, smoking could have been a risk factor and therefore a confounding factor in the differential diagnosis.

Overexpression of the cyclin-dependent kinase inhibitor gene $p16^{(INK4a)}$ is a well-established surrogate marker in HPV-related malignancies (83). p16 expression detection could help in differential diagnosis but is not a specific marker. Data published in 2016 have shown that primary ovarian cancer and borderline ovarian lesions can also express p16 with diffuse, moderate-to-strong p16 immunoreactivity (84). Additionally, p16 expression has also been observed in different types of pulmonary carcinomas (85). This made difficult the diagnosis of our patients who had lung and ovarian lesions since p16 positivity did not allow us to make a clear diagnosis of metastatic cervical cancer.

Techniques such as *in situ* reverse transcriptase polymerase chain reaction (PCR) for human papillomavirus RNA and *in situ* hybridization have allowed the differentiation of metastatic cervical carcinoma from either a new primary tumor or metastasis from another primary tumor (82). Although *in situ* reverse transcriptase -PCR allows direct correlation of the viral signal with the histologic features in the tissue, RNA extraction from FFPE biopsies remains difficult and inefficient due to degradation or modification of the RNA. This renders the reverse transcriptase-PCR process on such samples delicate and relatively laborious (86). On the other hand, *in situ* hybridization is known to lack sensitivity compared to molecular techniques (82). Lastly, both of these techniques do not precisely identify detected HPV genotype.

Arfi et al. recently used the identification of the same HPV integration site in the paired DNA of endo-cervical and ovarian tumors as a proof of cervical metastasis in the ovaries (87). Nevertheless, this technique remains much more complex and expensive compared to easy-to-use commercial HPV genotyping kits. Moreover, as HPV infection and associated tumoral processes only occur in mucosal tissue, the confirmation of correspondent tumoral HPV presence by simple, specific and sensitive molecular techniques in non-mucosal tissue appears to be sufficient to confirm the metastatic status of a secondary diagnosed lesion after onset of primary mucosal HPV cancer.

Conversely, in the literature, we did not find any cases of HPV detection in distant lesions that were unrelated to cervical cancer, so the possibility of a false positive from this technique seems low.

In our cases we only identified HPV 16 or 18 in tumors and secondary lesions, however other studies have detected other types, most frequently HPV 45 (87).

The main limitations in our study was the small sample, to find patients with our inclusion criteria was difficult even if we opened the study to other centre. The second limitation was that our study was a retrospective analysis, so we only succeeded in achieving HPV genotyping in half of the initial cervical tumors, which allowed us to match HPV genotype from both, the initial and the distant lesion.

Finally, in view of our results, we suggest that HPV molecular genotyping of newly diagnosed distant lesions in patients with a history of cervical neoplasia could be helpful to improve the diagnosis and treatment of such new lesions in patients with doubtful distant tumors.

Conclusions

- HPV18 integrates more easily into the human genome than the HPV16 genome.

- When HPV is integrated, it founded more frequently integrations in chromosome 5.

- The most virulent molecular status of HPV16 seems to be associated with an episomal form because all 100% of patients with recurrences and deaths in our study were infected for episomal HPV16 and we also found significant differences in the risk of recurrence according to the presence or absence of HPV16 in the episomal state ($p=0.007$).

- We did not find differences in Progression Free Survival regarding HPVtDNA detection in Sentinel Lymph Nodes (SLN) with ultrasensitive droplet-based digital PCR (ddPCR).

- We found differences Disease Specific Survival regarding ultrasensitive ddPCR HPVtDNA status in SLN with worst prognosis associated to HPVtDNA positive detection.

- The detection of HPVtDNA with ultrasensitive ddPCR in SLN biopsies in patients early cervical cancer without lymph nodes invasion could be a prognosis factor to consider, since the positive HPVtDNA detection was associated to a higher mortality in these patients.

- HPV molecular genotyping can be use in cases of newly diagnosed distant lesions in patients with a history of HPV cervical neoplasia to complete the clinical and histologic differential diagnosis in ambiguous situations.

Future aspects

The main global perspective of our project is to generalise the use of these HPV biomarkers in clinical practice to allow personalized treatment in patients with cervical cancer.

The detection of the molecular status of HPV in tumor (*Study I*) or/and the presence of HPV in SLN (*Study II*) could allow reclassifying patients with early cervical cancers (ECC) in different groups according to risk of recurrence in poor or good prognostic patients. This impact is important to propose the more adapted treatment (chemo-radiation or surgery) as well as the fertility sparing treatment.

A new prospective clinical trial, SENTICOL III has started and aims to study ECC patient survival regarding nodes staging technique. SENTICOL III is a prospective randomized trial aiming to demonstrate a similar survival and a better quality of life after sentinel node biopsy vs systematic pelvic lymphadenectomy in ECC. Patients with <IIa2 cervical cancer will be included after informed consent. Patients with "optimal" sentinel node detection and negative nodes on frozen section will be randomized between a sentinel technique only (experimental arm) and a lymphadenectomy (experimental arm). Therefore, 950 patients will be randomized, in 15 countries (29). Two hundreds patients will be included in France. Each site will participate to a tumor and SLN samples. To complete the results of our study II, it will be possible to use these samples to check the presence of HPV DNA and mRNA by ddPCR in sentinel lymph nodes.

We hope that the data obtained from the SENTICOL 3 ancillary study will confirm the interest of HPV SLNs status by ddPCR for patients ultrastaging in terms of estimating risk of overall survival and recurrence and allow us to validate this additional new biomarker for a better medical management of early cervical cancer N- patients.

Other important perspective of our project in relation to the *Study III*, it also highlighted the interest of systematically performing HPV molecular genotyping in cervical cancer patients to optimize post-therapeutic medical care and monitoring of such patients, as for example in our study, in cases of distant lesions with uncertain origin.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209–49.

2. Bosch FX, Manos MM, Muñoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst. 1995 Jun 7;87(11):796–802.

3. Prendiville W, Sankaranarayanan R. Colposcopy and Treatment of Cervical Precancer [Internet]. [cited 2021 Nov 19]. Available from: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Technical-Publications/Colposcopy-And-Treatment-Of-Cervical-Precancer-2017

4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;68(6):394–424.

5. Cancer Today [Internet]. [cited 2021 Oct 30]. Available from: https://gco.iarc.fr/today/

6. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999 Sep;189(1):12–9.

7. Cancer today [Internet]. [cited 2021 Oct 30]. Available from: http://gco.iarc.fr/today/home

8. Ronco G, Dillner J, Elfström KM, Tunesi S, Snijders PJF, Arbyn M, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. Lancet Lond Engl. 2014 Feb 8;383(9916):524–32.

9. Wagenaar HC, Trimbos JB, Postema S, Anastasopoulou A, van der Geest RJ, Reiber JH, et al. Tumor diameter and volume assessed by magnetic resonance imaging in the prediction of outcome for invasive cervical cancer. Gynecol Oncol. 2001 Sep;82(3):474–82.

10. Patel CN, Nazir SA, Khan Z, Gleeson FV, Bradley KM. 18F-FDG PET/CT of cervical carcinoma. AJR Am J Roentgenol. 2011 May;196(5):1225–33.

11. Cibula D, Pötter R, Planchamp F, Avall-Lundqvist E, Fischerova D, Meder CH, et al. The European Society of Gynaecological Oncology/European Society for Radiotherapy and Oncology/European Society of Pathology Guidelines for the Management of Patients With Cervical Cancer. Int J Gynecol Cancer [Internet]. 2018 May 1 [cited 2021 Nov 16];28(4). Available from: https://ijgc.bmj.com/content/28/4/641

12. WHO histological classification of tumours of the uterine cervix [Internet]. [cited 2021 Nov 16]. Available from: https://screening.iarc.fr/atlasclassifwho.php

13. Trattner M, Graf AH, Lax S, Forstner R, Dandachi N, Haas J, et al. Prognostic Factors in Surgically Treated Stage Ib–IIb Cervical Carcinomas with Special Emphasis on the Importance of Tumor Volume. Gynecol Oncol. 2001 Jul 1;82(1):11–6.

14. Pol FJM, Zusterzeel PLM, van Ham MAPC, Kuijpers DAT, Bulten J, Massuger LFAG. Satellite lymphovascular space invasion: An independent risk factor in early stage cervical cancer. Gynecol Oncol. 2015 Sep;138(3):579–84.

15. Look KY, Brunetto VL, Clarke-Pearson DL, Averette HE, Major FJ, Alvarez RD, et al. An analysis of cell type in patients with surgically staged stage IB carcinoma of the cervix: a Gynecologic Oncology Group study. Gynecol Oncol. 1996 Dec;63(3):304–11.

16. Delgado G, Bundy BN, Fowler WC, Stehman FB, Sevin B, Creasman WT, et al. A prospective surgical pathological study of stage I squamous carcinoma of the cervix: a Gynecologic Oncology Group Study. Gynecol Oncol. 1989 Dec;35(3):314–20.

17. Bhatla N, Aoki D, Sharma DN, Sankaranarayanan R. Cancer of the cervix uteri. Int J Gynecol Obstet. 2018 Oct;143:22–36.

18. Sedlis A, Bundy BN, Rotman MZ, Lentz SS, Muderspach LI, Zaino RJ. A randomized trial of pelvic radiation therapy versus no further therapy in selected patients with stage IB carcinoma of the cervix after radical hysterectomy and pelvic lymphadenectomy: A Gynecologic Oncology Group Study. Gynecol Oncol. 1999 May;73(2):177–83.

19. Kim SM, Choi HS, Byun JS. Overall 5-year survival rate and prognostic factors in patients with stage IB and IIA cervical cancer treated by radical hysterectomy and pelvic lymph node dissection. Int J Gynecol Cancer Off J Int Gynecol Cancer Soc. 2000 Jul;10(4):305–12.

20. Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet. 2009 May;105(2):103–4.

21. Ramirez PT, Frumovitz M, Pareja R, Lopez A, Vieira M, Ribeiro R, et al. Minimally Invasive versus Abdominal Radical Hysterectomy for Cervical Cancer. N Engl J Med. 2018 Nov 15;379(20):1895–904.

22. Marth C, Landoni F, Mahner S, McCormack M, Gonzalez-Martin A, Colombo N, et al. Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol Off J Eur Soc Med Oncol. 2017 Jul 1;28(suppl_4):iv72–83.

23. eUpdate – Cervical Cancer Treatment Recommendations [Internet]. [cited 2021 Nov 18]. Available from: https://www.esmo.org/guidelines/gynaecological-cancers/cervical-cancer/eupdate-cervical-cancer-treatment-recommendations

24. Lécuru F, Mathevet P, Querleu D, Leblanc E, Morice P, Daraï E, et al. Bilateral negative sentinel nodes accurately predict absence of lymph node metastasis in early cervical cancer: results of the SENTICOL study. J Clin Oncol Off J Am Soc Clin Oncol. 2011 May 1;29(13):1686–91.

25. Kim SI, Lim MC, Lee JS, Lee Y, Park K, Joo J, et al. Impact of lower limb lymphedema on quality of life in gynecologic cancer survivors after pelvic lymph node dissection. Eur J Obstet Gynecol Reprod Biol. 2015 Sep;192:31–6.

26. Mathevet P, Lecuru F, Magaud L, Bouttitie F. Sentinel lymph node biopsy for early cervical cancer: Results of a randomized prospective, multicenter study (Senticol 2) comparing adding pelvic lymph node dissection vs sentinel node biopsy only. Gynecol Oncol. 2017 Jun 1;145:2–3.

27. Cibula D, Abu-Rustum NR, Dusek L, Slama J, Zikán M, Zaal A, et al. Bilateral ultrastaging of sentinel lymph node in cervical cancer: Lowering the false-negative rate and improving the detection of micrometastasis. Gynecol Oncol. 2012 Dec;127(3):462–6.

28. Cibula D, McCluggage WG. Sentinel lymph node (SLN) concept in cervical cancer: Current limitations and unanswered questions. Gynecol Oncol. 2019 Jan;152(1):202–7.

29. Lecuru FR, McCormack M, Hillemanns P, Anota A, Leitao M, Mathevet P, et al. SENTICOL III: an international validation study of sentinel node biopsy in early cervical cancer. A GINECO, ENGOT, GCIG and multicenter study. Int J Gynecol Cancer Off J Int Gynecol Cancer Soc. 2019 May;29(4):829–34.

30. Frumovitz M, Plante M, Lee PS, Sandadi S, Lilja JF, Escobar PF, et al. Near-infrared fluorescence for detection of sentinel lymph nodes in women with cervical and uterine cancers (FILM): a randomised, phase 3, multicentre, non-inferiority trial. Lancet Oncol. 2018 Oct;19(10):1394–403.

31. Peters WA, Liu PY, Barrett RJ, Stock RJ, Monk BJ, Berek JS, et al. Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. J Clin Oncol Off J Am Soc Clin Oncol. 2000 Apr;18(8):1606–13.

32. Tewari KS, Sill MW, Long HJ, Penson RT, Huang H, Ramondetta LM, et al. Improved survival with bevacizumab in advanced cervical cancer. N Engl J Med. 2014 Feb 20;370(8):734–43.

33. Fehrmann F, Laimins LA. Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. Oncogene. 2003 Aug 11;22(33):5201–7.

34. Stanley MA. Epithelial cell responses to infection with human papillomavirus. Clin Microbiol Rev. 2012 Apr;25(2):215–22.

35. Cogliano V, Baan R, Straif K, Grosse Y, Secretan B, Ghissassi FE. Carcinogenicity of human papillomaviruses. Lancet Oncol. 2005 Apr 1;6(4):204.

36. Bruni L, Albero G, Serrano B, Mena M, Collado JJ, Gómez D, Muñoz J, Bosch FX, de Sanjosé S. ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 22 October 2021.

37. Bergeron C, Ronco G, Reuschenbach M, Wentzensen N, Arbyn M, Stoler M, et al. The clinical impact of using p16(INK4a) immunochemistry in cervical histopathology and cytology: an update of recent developments. Int J Cancer. 2015 Jun 15;136(12):2741–51.

38. Chaiwongkot A, Vinokurova S, Pientong C, Ekalaksananan T, Kongyingyoes B, Kleebkaow P, et al. Differential methylation of E2 binding sites in episomal and integrated HPV 16 genomes in preinvasive and invasive cervical lesions. Int J Cancer. 2013 May 1;132(9):2087–94.

39. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57–70.

40. Ganguly N, Parihar SP. Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis. J Biosci. 2009 Mar;34(1):113–23.

41. Xu B, Chotewutmontri S, Wolf S, Klos U, Schmitz M, Dürst M, et al. Multiplex Identification of Human Papillomavirus 16 DNA Integration Sites in Cervical Carcinomas. PloS One. 2013;8(6):e66693.

42. Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, et al. The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. Arch Pathol Lab Med. 2012 Oct;136(10):1266–97.

43. Ikenberg H, Bergeron C, Schmidt D, Griesser H, Alameda F, Angeloni C, et al. Screening for cervical cancer precursors with p16/Ki-67 dual-stained cytology: results of the PALMS study. J Natl Cancer Inst. 2013 Oct 16;105(20):1550–7.

44. Polymerase Chain Reaction (PCR) [Internet]. Genome.gov. [cited 2022 Oct 26]. Available from: https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction

45. Baker M. Digital PCR hits its stride. Nat Methods. 2012 Jun;9(6):541–4.

46. Estrade C, Sahli R. Comparison of Seegene Anyplex II HPV28 with the PGMY-CHUV assay for human papillomavirus genotyping. J Clin Microbiol. 2014 Feb;52(2):607–12.

47. Perkins G, Lu H, Garlan F, Taly V. Droplet-Based Digital PCR: Application in Cancer Research. Adv Clin Chem. 2017;79:43–91.

48. Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Salem CB, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip. 2011 Jul 7;11(13):2156–66.

49. Taly V, Pekin D, El Abed A, Laurent-Puig P. Detecting biomarkers with microdroplet technology. Trends Mol Med. 2012 Jul;18(7):405–16.

50. Akagi K, Li J, Broutian TR, Padilla-Nash H, Xiao W, Jiang B, et al. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Res. 2014 Feb;24(2):185–99.

51. Chen R, Im H, Snyder M. Whole-Exome Enrichment with the Agilent SureSelect Human All Exon Platform. Cold Spring Harb Protoc. 2015 Mar 11;2015(7):626–33.

52. Holmes A, Lameiras S, Jeannot E, Marie Y, Castera L, Sastre-Garau X, et al. Mechanistic signatures of HPV insertions in cervical carcinomas. NPJ Genomic Med. 2016;1:16004.

53. Veyer D, Wack M, Mandavit M, Garrigou S, Hans S, Bonfils P, et al. HPV circulating tumoral DNA quantification by droplet-based digital PCR: A promising predictive and prognostic biomarker for HPV-associated oropharyngeal cancers. Int J Cancer. 2020 Aug 15;147(4):1222–7.

54. Veyer D, Pavie J, Pernot S, Mandavit M, Garrigou S, Lucas ML, et al. HPV-circulating tumoural DNA by droplet-based digital polymerase chain reaction, a new molecular tool for early detection of HPV metastatic anal cancer? A case report. Eur J Cancer Oxf Engl 1990. 2019 May;112:34–7.

55. Jeannot E, Latouche A, Bonneau C, Calméjane MA, Beaufort C, Ruigrok-Ritstier K, et al. Circulating HPV DNA as a Marker for Early Detection of Relapse in Patients with Cervical Cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2021 Nov 1;27(21):5869–77.

56. Alvarez-Salas LM, Cullinan AE, Siwkowski A, Hampel A, DiPaolo JA. Inhibition of HPV-16 E6/E7 immortalization of normal keratinocytes by hairpin ribozymes. Proc Natl Acad Sci U S A. 1998 Feb 3;95(3):1189–94.

57. Whale AS, Jones GM, Pavšič J, Dreo T, Redshaw N, Akyürek S, et al. Assessment of Digital PCR as a Primary Reference Measurement Procedure to Support Advances in Precision Medicine. Clin Chem. 2018 Sep;64(9):1296–307.

58. Hall AHS, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. J Virol. 2003 May;77(10):6066–9.

59. Gilbert DC, Wakeham K, Langley RE, Vale CL. Increased risk of second cancers at sites associated with HPV after a prior HPV-associated malignancy, a systematic review and meta-analysis. Br J Cancer. 2019 Jan;120(2):256–68.

60. Veyer D, Wack M, Grard O, Bonfils P, Hans S, Bélec L, et al. HPV detection and genotyping of head and neck cancer biopsies by molecular testing with regard to the new oropharyngeal squamous cell carcinoma classification based on HPV status. Pathology (Phila). 2019 Jun;51(4):421–5.

61. Steinau M, Patel SS, Unger ER. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. J Mol Diagn JMD. 2011 Jul;13(4):377–81.

62. Lillsunde Larsson G, Carlsson J, Karlsson MG, Helenius G. Evaluation of HPV Genotyping Assays for Archival Clinical Samples. J Mol Diagn JMD. 2015 May;17(3):293–301.

63. Kwon MJ, Roh KH, Park H, Woo HY. Comparison of the Anyplex II HPV28 assay with the Hybrid Capture 2 assay for the detection of HPV infection. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2014 Apr;59(4):246–9.

64. Latsuzbaia A, Tapp J, Nguyen T, Fischer M, Arbyn M, Weyers S, et al. Analytical performance evaluation of Anyplex II HPV28 and Euroarray HPV for genotyping of cervical samples. Diagn Microbiol Infect Dis. 2016 Jul;85(3):318–22.

65. Lee YJ, Kim D, Lee K, Chun JY. Single-channel multiplexing without melting curve analysis in real-time PCR. Sci Rep. 2014 Dec 11;4:7439.

66. Veyer D, Wack M, Grard O, Bonfils P, Hans S, Bélec L, et al. HPV detection and genotyping of head and neck cancer biopsies by molecular testing with regard to the new oropharyngeal squamous cell carcinoma classification based on HPV status. Pathology (Phila). 2019 Jun;51(4):421–5.

67. Rodriguez J, Viveros-Carreño D, Pareja R. Adjuvant treatment after radical surgery for cervical cancer with intermediate risk factors: is it time for an update? Int J Gynecol Cancer [Internet]. 2022 Oct 1 [cited 2022 Oct 12];32(10). Available from: https://ijgc.bmj.com/content/32/10/1219

68. Ho CM, Chien TY, Huang SH, Wu CJ, Shih BY, Chang SC. Multivariate analysis of the prognostic factors and outcomes in early cervical cancer patients undergoing radical hysterectomy. Gynecol Oncol. 2004 May;93(2):458–64.

69. Bodelon C, Untereiner ME, Machiela MJ, Vinokurova S, Wentzensen N. Genomic characterization of viral integration sites in HPV-related cancers. Int J Cancer. 2016 Nov 1;139(9):2001–11.

70. Kamal M, Lameiras S, Deloger M, Morel A, Vacher S, Lecerf C, et al. Human papilloma virus (HPV) integration signature in Cervical Cancer: identification of MACROD2 gene as HPV hot spot integration site. Br J Cancer. 2021 Feb;124(4):777–85.

71. Péré H, Vernet R, Pernot S, Pavie J, Robillard N, Puech J, et al. Episomal HPV16 responsible for aggressive and deadly metastatic anal squamous cell carcinoma evidenced in peripheral blood. Sci Rep. 2021 Feb 25;11(1):4633.

72. Debernardi A, Meurisse A, Prétet JL, Guenat D, Monnien F, Spehner L, et al. Prognostic role of HPV integration status and molecular profile in advanced anal carcinoma: An ancillary study to the epitopes-HPV02 trial. Front Oncol. 2022;12:941676.

73. Köhler C, Le X, Dogan NU, Pfiffer T, Schneider A, Marnitz S, et al. Molecular Diagnosis for Nodal Metastasis in Endoscopically Managed Cervical Cancer: The Accuracy of the APTIMA Test to Detect High-risk Human Papillomavirus Messenger RNA in Sentinel Lymph Nodes. J Minim Invasive Gynecol. 2016 Aug;23(5):748–52.

74. Lu Y, Xu X, Nong XH, Yao DS. Detection of high-risk human papillomavirus DNA in sentinel lymph nodes of patients with cervical cancer. Oncol Lett. 2020 Mar;19(3):2317–25.

75. Lee YS, Rhim CC, Lee HN, Lee KH, Park JS, Namkoong SE. HPV status in sentinel nodes might be a prognostic factor in cervical cancer. Gynecol Oncol. 2007 May;105(2):351–7.

76. Coutant C, Barranger E, Cortez A, Dabit D, Uzan S, Bernaudin JF, et al. Frequency and prognostic significance of HPV DNA in sentinel lymph nodes of patients with cervical cancer. Ann Oncol Off J Eur Soc Med Oncol. 2007 Sep;18(9):1513–7.

77. Dürst M, Hoyer H, Altgassen C, Greinke C, Häfner N, Fishta A, et al. Prognostic value of HPV-mRNA in sentinel lymph nodes of cervical cancer patients with pN0-status. Oncotarget. 2015 May 14;6(26):23015–25.

78. Carow K, Read C, Häfner N, Runnebaum IB, Corner A, Dürst M. A comparative study of digital PCR and real-time qPCR for the detection and quantification of HPV mRNA in sentinel lymph nodes of cervical cancer patients. BMC Res Notes. 2017 Oct 30;10(1):532.

79. Noventa M, Ancona E, Cosmi E, Saccardi C, Litta P, D'Antona D, et al. Usefulness, methods and rationale of lymph nodes HPV-DNA investigation in estimating risk of early stage cervical cancer recurrence: a systematic literature review. Clin Exp Metastasis. 2014 Oct;31(7):853–67.

80. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. Vaccine. 2012 Nov 20;30 Suppl 5:F55-70.

81. Li H, Wu X, Cheng X. Advances in diagnosis and treatment of metastatic cervical cancer. J Gynecol Oncol. 2016 Jul;27(4):e43.

82. Plaza JA, Ramirez NC, Nuovo GJ. Utility of HPV analysis for evaluation of possible metastatic disease in women with cervical cancer. Int J Gynecol Pathol Off J Int Soc Gynecol Pathol. 2004 Jan;23(1):7–12.

83. Ishikawa M, Fujii T, Saito M, Nindl I, Ono A, Kubushiro K, et al. Overexpression of p16 INK4a as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. Int J Gynecol Cancer Off J Int Gynecol Cancer Soc. 2006 Feb;16(1):347–53.

84. Yoon N, Yoon G, Park CK, Kim HS. Stromal p16 expression is significantly increased in malignant ovarian neoplasms. Oncotarget. 2016 Oct 4;7(40):64665–73.

85. Gazzeri S, Gouyer V, Vour'ch C, Brambilla C, Brambilla E. Mechanisms of p16INK4A inactivation in non small-cell lung cancers. Oncogene. 1998 Jan 29;16(4):497–504.

86. McKinney MD, Moon SJ, Kulesh DA, Larsen T, Schoepp RJ. Detection of viral RNA from paraffin-embedded tissues after prolonged formalin fixation. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2009 Jan;44(1):39–42.

87. Arfi A, Hequet D, Bataillon G, Tran-Perennou C, Farkhondeh F, Sastre-Garau X, et al. HPV DNA integration site as proof of the origin of ovarian metastasis from endocervical adenocarcinoma: three case reports. BMC Cancer. 2019 Apr 23;19(1):375.

Journal of *Personalized Medicine*

MDPI

Communication

HPV Molecular Genotyping as a Differential Diagnosis Tool in Cervical Cancer Metastasis

Rosa Montero-Macías [1,2], Pluvio J. Coronado [3], Nicolas Robillard [4], David Veyer [4,5], Vincent Villefranque [1], Marie-Aude Le Frére-Belda [6], Elisabeth Auberger [7], Pauline Bitolog [7], Ivana Stankovic [6], Laurent Bélec [4,8], Anne-Sophie Bats [8,9], Fabrice Lécuru [8,10] and Hélène Péré [4,8,*]

1 Obstetrics and Gynecology Department, Centre Hospitalier Simone Veil, 95602 Eaubonne, France
2 Complutense University of Madrid, 28223 Madrid, Spain
3 Women's Health Institute José Botella Llusiá, Fundación de Investigación del Hospital Clínico San Carlos (IdISSC), Universidad Complutense, 28040 Madrid, Spain
4 Virology Laboratory, Georges Pompidou European Hospital, 75015 Paris, France
5 INSERM, Functional Genomics of Solid Tumors (FunGeST), Centre de Recherche des Cordeliers, Université de Paris and Sorbonne Université, 75013 Paris, France
6 Pathology Department, Georges Pompidou European Hospital, 75015 Paris, France
7 Pathology Department, Simone Veil Hospital, 95600 Eaubonne, France
8 Faculty of Medicine, Paris University, 75015 Paris, France
9 Gynecologic and Breast Oncologic Surgery Department, Georges Pompidou European Hospital, 75015 Paris, France
10 Breast, Gynecology and Reconstructive Surgery Unit, Curie Institute, 75005 Paris, France
* Correspondence: helene.pere@aphp.fr

Abstract: Background: Differentiating metastatic cervical cancer from another primary tumor can be difficult in patients with a history of cervical cancer and a distant lesion. The use of routine HPV molecular detection and genotyping tests could help in these cases. The objective of this study was to identify if an easy-to-use HPV molecular genotyping assay would allow differentiating between HPV tumor metastasis and a new independent primary non-HPV-induced tumor. Materials and Methods: Between 2010 and 2020, we identified patients with a primary cervical carcinoma who also had another secondary lesion. This identification included a clinical and histologic differential diagnosis of metastatic cervical cancer versus a new primary cancer or metastatic cancer from another site. We used a routine multiplex real-time PCR (rt-PCR) Anyplex™ II HPV28 (Seegene, Seoul, Republic of Korea) to detect the high-risk (HR)-HPV genome in the distant lesions in these patients. Results: Eight cases of cervical cancer with a new secondary lesion were identified. In seven, HR-HPV DNA was detected in the biopsy of the distant lesion, which confirmed the diagnosis of cervical cancer metastasis. In the remaining case, no HPV was detected in the secondary lung biopsy, confirming the diagnosis of new primary lung cancer. Conclusion: Our results pave the way for HPV molecular genotyping use in cases of newly diagnosed distant lesions in patients with a history of HPV cervical neoplasia by using a routine diagnosis process to complete the clinical and histologic differential diagnosis when confronted with ambiguous situations.

Keywords: HPV; cervical cancer; metastasis; HPV commercial genotyping assays; differential diagnostic tool

Citation: Montero-Macías, R.; Coronado, P.J.; Robillard, N.; Veyer, D.; Villefranque, V.; Le Frére-Belda, M.-A.; Auberger, E.; Bitolog, P.; Stankovic, I.; Bélec, L.; et al. HPV Molecular Genotyping as a Differential Diagnosis Tool in Cervical Cancer Metastasis. *J. Pers. Med.* **2023**, *13*, 177. https://doi.org/10.3390/jpm13020177

Academic Editor: Salvatore Scacco

Received: 30 December 2022
Revised: 18 January 2023
Accepted: 18 January 2023
Published: 19 January 2023

1. Introduction

Cervical cancer induced by high risk (HR)-human papilloma virus (HPV) infection is the fourth most frequent cancer in women worldwide with 569,000 new cases each year [1]. Even in early clinical stages, cervical cancer can be complicated by lymph nodes metastasis or a hepatic, pulmonary or abdominal distant lesion implying a worse prognosis [2]. In addition, women with cervical cancer are at increased risk for other HPV-related carcinomas [3]. Patients with primary HPV-associated cervical cancer may have an increased risk

of developing secondary HPV-related malignancies given that HPV can cause precancerous lesions and invasive malignancy when it infects oro-genital mucosa and genital skin [4,5]. Thus, some cervical cancer patients can present a distant lesion that may signify either metastatic disease from the primary cervical cancer or a new primary tumor or metastasis of another form of cancer.

Lesions of unknown origin out of the genital tract could occur in cervical cancer patients. Differential diagnosis between these lesions in the pathological analysis can be difficult, even with the help of immunohistochemistry techniques (IHC). However, the right identification of their origin will be essential for patients' clinical care, treatment and prognosis.

Since HPV presence is required to maintain the tumoral phenotype, [6] HPV DNA could be assimilated to tumoral DNA and represent an interesting marker of the presence of tumor cells. HPV DNA should be detectable in metastatic tissue from primary tumors induced by HPV. Therefore, it prompts us to use our routine multiplex HPV genotyping real-time PCR (rt-PCR) Anyplex^{TM} II HPV28 (Seegene, Seoul, Republic of Korea) to study the HR-HPV genome in new distant lesions in cervical cancer patients. The objective of this study was to identify if such an easy-to-use HPV molecular genotyping assay would allow differentiating HPV tumor metastasis from a new independent primary non-HPV-induced tumor to improve care management of patients.

2. Material and Methods

2.1. Patients and Data Collection

We retrospectively included women with a histologically confirmed primary cervical carcinoma treated with surgery and/or chemoradiotherapy who also had another distant lesion. Patients were treated in two French medical centers (George Pompidou European Hospital and the Simone Veil Hospital) between 2010 and 2020. The lesion was suspected based on clinical symptoms or the results from imaging methods, namely magnetic resonance imaging (MRI), positron emission tomography (PET) or computed tomography scan (CT). All new lesions were biopsied and assessed by specialized pathologists in gynecological tumors.

Clinical and histologic differential diagnoses for the distant lesion were performed in order to differentiate whether the lesion was a metastasis from either the known cervical cancer or caused by another primary/metastatic cancer. Classical HE (Hematoxylin Eosin Safran) and IHC analyses were performed. In most cases, the histological type and IHC were compatible with different origins (metastasis or other primary cancer), allowing for multiple diagnostic options.

The diagnosis of any secondary lesions occurred at the same time as that for primary cervical cancer (synchronous), or later on during follow-up. For any secondary lesion, we recorded the year of diagnosis, location of the lesion, histology type and the p16 expression status (Table 1).

Table 1. Clinical and pathologic findings in patient cases of cervical cancer and possible metastatic disease.

Case	Age (Year)	Cervical Cancer Type	Multiplex rt-PCR**	New Lesions			
				Location	Histology	p16$^{(INK4a)}$ Expression *	Multiplex rt-PCR **
#A	79	Squamous cellcarcinoma [stage IIB] *** in 2015	NA	Mediastinal adenopathies (2018)	Squamous cell carcinoma	+	HPV-16
#B	49	Adenocarcinoma [stage IIB] *** in 2017	NA	Lung, liver, brain, retroperi-toneum (2018)	Undifferenciated carcinoma	+	HPV-16

Table 1. *Cont.*

Case	Age (Year)	Cervical Cancer Type	Multiplex rt-PCR**	New Lesions			
				Location	Histology	p16(INK4a) Expression *	Multiplex rt-PCR **
#C	53	Adenocarcinoma [stage IVA] *** in 2018	NA	Ovary (2018,synchronous)	Endometrioid Adenocarcinoma	ND	HPV-18
#D	42	Squamous cell carcinoma [stage IIB] *** in 2019	NA	Ovary (2019,synchronous)	Squamous cell carcinoma	+	HPV-18
#E	47	Squamous cell carcinoma [stage IIB] *** in 2016	HPV-16	Lung (2019)	Squamous cell carcinoma	ND	HPV-16
#F	40	Squamous cell carcinoma [stage IIB] *** in 2017	HPV-16	Lung (2018)	Squamous cell carcinoma	+	HPV-16
#G	54	Squamous cell carcinoma [stage IIB] *** in 2010	HPV-16	Kidney (2012)	Squamous cell carcinoma	ND	HPV-16
#H	67	Squamous cell carcinoma [stage IIB] *** in 2016	HPV-16	Lung (2016)	Adenocarcinoma	ND	None

* p16(INK4a) expression was assessed by immunohistochemistry using rabbit monoclonal anti-CDKN2A/p16(INK4a) antibody; ** Sections of formalin-fixed, paraffin-embedded (FFPE) biopsies were deparaffinized overnight at 56 °C with 40 µL of proteinase K (Qiagen, Hilden, Germany) and 360 µL of ATL buffer (Qiagen), as described by Veyer, 2019. Afterwards, 200 µL of ATL buffer was added and incubated for 10 min at 70 °C. DNA was further extracted using a QiaAmp DNA Mini Kit (Qiagen) and eluted in 50 µL of PCR-grade water. HPV detection and genotyping was carried out in 5 µL of extracted DNA using the CE IVD-marked multiplex rtRT-PCR assay Anyplex™ II HPV28 (Seegene, Seoul, Republic of Korea) as described by Estrade 2014 and Lillsunde Larsson, 2015. The Anyplex ™ II HPV28 detection test distinguishes 28 genotypes of HPV, by amplifying 100–200 bp fragments of the L1 gene including 13 high-risk types (HR-HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68), eight low-risk types (LR) (LR-HPV -6, -11, -40, -42, -43, -44, -54 and -70), seven genotypes reported as possibly carcinogenic (HPV-26, -53, -61, -66, -69, -73 and -82) as well as the human gene β-globin in two different reactions (Lillsunde Larsson 2015). The DNA amplification and the genotyping process were carried out in two reactions performed on the CFX96™ real-time PCR instrument (Bio-Rad, Marnes-la-Coquette, France) (Estrade 2014). Melting curves were obtained at 30, 40 and 50 cycles. Data recording and interpretation were automated using Seegene Viewer software version 2.0 (Seegene) in accordance with the manufacturer's instructions. Raw data of the results were checked by a virologist. The virology laboratory was accredited in 2013 by the Comité Français d'Accréditation (COFRAC) according to ISO 15189 norms for the biological markers "HPV detection" and "HPV genotyping". *** According to the International Federation of Gynecology and Obstetrics (FIGO) classification. Age is related to the distant lesion diagnosis. All patients were initially treated with radiochemotherapy. Distant lesions were treated with chemotherapy: carboplatin and taxol in all the cases and adding Vinorelbine in case H (primary lung cancer). ND, Not done; rt-PCR, real-time polymerase chain reaction; +, positive; NA, not available.

2.2. HPV Status in Primary Tumors and Distant Lesions

Formalin-fixed, paraffin-embedded (FFPE) biopsies of distant lesions and the correspondent available tumor (only available for 4/8 patients) were sent to the ISO 15189-accredited virology laboratory of the Georges Pompidou European Hospital (Paris, France) for DNA extraction as described by Steinau et al. [7] prior to HPV detection and genotyping using the Anyplex™ II HPV28 genotyping test (Seoul, Republic of Korea). Sections of the FFPE biopsies were deparaffinized overnight at 56 °C with 360 µL of ATL buffer (Qiagen, Hilden, Germany) and then we added 40 µL of proteinase K. Afterwards, 200 µL of ATL buffer was added then incubated for 10 min at 70 °C. DNA was further extracted using a QiaAmp DNA Mini Kit (Qiagen) and eluted in 50 µL of PCR-grade water. HPV detection and genotyping was carried out in 5 µL of extracted DNA using the CE IVD-marked multiplex rtRT-PCR assay Anyplex™ II HPV28 (Seegene, Seoul, Republic of Korea) [8,9]. The Anyplex ™ II HPV28 detection test distinguishes 28 HPV genotypes by amplifying

100–200 bp fragments of the L1 gene including 13 high-risk types (HR-HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68), eight low-risk types (LR) (LR-HPV -6, -11, -40, -42, -43, -44, -54 and -70), seven genotypes reported as possibly carcinogenic (HPV-26, -53, -61, -66, -69, -73 and -82) as well as the human gene β-globin in two different reactions [9]. The DNA amplification and the genotyping process were carried out in two reactions performed on the CFX96™ real-time PCR instrument (Bio-Rad, Marnes-la-Coquette, France) [8]. Melting curves were obtained at 30, 40 and 50 cycles. Data recording and interpretation were automated using Seegene Viewer software version 2.0 (Seegene) in accordance with the manufacturer's instructions. Raw data of the results were checked by a virologist. This molecular HPV genotyping assay has been found to be suitable for HPV detection and genotyping in cervical secretions [8–11]. Based on Seegene's proprietary DPO™ and MuDT™ technologies [12], this assay was conceived to avoid mismatch priming and to quantify each target in a single fluorescence channel, respectively. In addition, the Anyplex™ II HPV28 assay was shown to be suitable for an extended genotyping approach with a high sensitivity in FFPE specimens [11,13].

3. Results

3.1. Clinical Characteristics and Pathological Findings of Patients

Under our inclusion criteria, we identified eight women treated in two French comprehensive cancer centers between 2010 and 2020 (Table 1).

In these eight cases, the secondary lesion was difficult to identify as a cervical tumor metastasis or second primary tumor.

The clinical and histological features of these women are described in Table 1. The mean age of the patients was 53.9 years (range 40–79). The most common histological type of tumor was squamous carcinoma (six cases). Two cases were adenocarcinoma. All cases were diagnosed in advanced stages (IIb or IVa 2018 FIGO stage).

Two cases were synchronous with primary cervical cancer. The other cases were diagnosed later (the time of recurrence/metastasis was between one and three years). Moreover, except for the synchronous cases that were treated with surgery, all other patients received chemoradiotherapy as initial treatment after the tumors were biopsied. The most common site of the second tumor was the lung (four cases).

3.2. HPV Detection and Genotyping of Distant Lesions FFPE Biopsies

For the results interpretation, the quality of extracted DNA was confirmed by the systematic positivity of the internal control β-globin for all samples. Therefore, in the distant lesion, results from RT-PCR showed HPV-16 DNA in six cases and HPV-18 DNA in one case, thus completing the histological results and confirming the cervical origin of the new lesion (Table 1). Lastly, a case with an HPV-negative pulmonary lesion completed our series, confirming that this new primary independent cancerous lesion was not related to the primary HPV cervical cancer in this patient. The HPV genotype was confirmed via biopsies of primary tumors in some of the cases (see #E, #F, #G and #H in Table 1).

4. Discussion

In this study, eight documented cases of women with cervical cancer showed that the use of routine HPV molecular detection and genotyping allowed the differentiation between metastatic cervical cancer disease and a new primary tumor.

We found HPV in all distant lesions except one case. This case, with negative HPV in the lesion, was considered as primary lung cancer and was treated with Navelbine with a fractionated dose of cisplatin (CDDP), unlike the cases diagnosed as metastatic cervical cancer, which were treated with carboplatin and taxol.

The prognosis of patients with metastatic cervical cancer is poor (median survival time between 8 and 13 months). There are two types of metastases in cervical cancer patients: hematogenous and lymphatic. Patients with hematogenous metastasis have a higher risk of mortality than those with lymphatic metastasis. In addition, the management of metastasis

may be different depending on its origin and location. Surgery, radiotherapy, chemotherapy or combinations of several treatments are options [2].

In patients with HPV-related cancer and a distant lesion, the histologic differentiation of metastatic cervical cancer versus another primary tumor or metastasis from another cancer can be difficult, especially if the distant lesion shows similar histologic findings as the cervical cancer [14]. However, this differential diagnosis is essential in view of its clinical, therapeutic and prognostic implications. In addition, patients with HPV-related cancer are at higher risk of having other carcinomas since smoking is a common risk factor. In our study, four of the distant lesions were pulmonary, which raised diagnostic doubts as to whether they were primary lung cancer or metastasis of cervical cancer. In both cases, smoking could have been a risk factor and therefore a confounding factor in the differential diagnosis.

Overexpression of the cyclin-dependent kinase inhibitor gene $p16^{(INK4a)}$ is a well-established surrogate marker in HPV-related malignancies [15]. p16 expression detection could help in differential diagnosis but is not a specific marker. Data published in 2016 have shown that primary ovarian cancer and borderline ovarian lesions can also express p16 with diffuse, moderate-to-strong p16 immunoreactivity [16]. Additionally, p16 expression has also been observed in different types of pulmonary carcinomas [17]. This made the diagnosis of our patients who had lung and ovarian lesions difficult since p16 positivity did not allow us to make a clear diagnosis of metastatic cervical cancer.

Techniques such as in situ reverse transcriptase polymerase chain reaction (RT-PCR) for human papillomavirus RNA and in situ hybridization have allowed the differentiation of metastatic cervical carcinoma from either a new primary tumor or metastasis from another primary tumor [14]. Although in situ RT-PCR allows direct correlation of the viral signal with the histologic features in the tissue, RNA extraction from FFPE biopsies remains difficult and inefficient due to degradation or modification of the RNA. This renders the RT-PCR process on such samples delicate and relatively laborious [18]. On the other hand, in situ hybridization is known to lack sensitivity compared to molecular techniques [14]. Lastly, both of these techniques do not precisely identify the detected HPV genotype.

Arfi et al. recently used the identification of the same HPV integration site in the paired DNA of endo-cervical and ovarian tumors as a proof of cervical metastasis in the ovaries [19]. Nevertheless, this technique remains much more complex and expensive compared to easy-to-use commercial HPV genotyping kits. Moreover, as HPV infection and associated tumoral processes only occur in mucosal tissue, the confirmation of correspondent tumoral HPV presence by simple, specific and sensitive molecular techniques in non-mucosal tissue appears to be sufficient to confirm the metastatic status of a secondary diagnosed lesion after onset of primary mucosal HPV cancer.

Conversely, in the literature, we did not find any cases of HPV detection in distant lesions that were unrelated to cervical cancer, so the possibility of a false positive from this technique seems low.

In our cases, we only identified HPV 16 or 18 in tumors and secondary lesions; however, other studies have detected other types, most frequently HPV 45 [19].

As our study was a retrospective analysis, the sample size presented is low and represents a clear limitation of our study. Indeed, we only succeeded in achieving HPV genotyping in half of the initial cervical tumors, which allowed us to match the HPV genotype from both the initial and the distant lesion, due, in some cases, to the poor preservation of DNA in old FFPE samples. It also highlighted the interest of systematically performing HPV molecular genotyping of cervical cancer to optimize post-therapeutic medical care and monitoring of such patients. In view of our results, we suggest that HPV molecular genotyping of newly diagnosed distant lesions in patients with a history of cervical neoplasia could be helpful to improve the diagnosis and treatment of such new lesions in patients with doubtful distant tumors.

Author Contributions: Conceptualization: R.M.-M., P.J.C., E.A. and H.P.; Validation: R.M.-M., D.V., V.V., P.B. and H.P.; Writing: R.M.-M., H.P. and D.V. Writing—Review and Editing: N.R., V.V., M.-A.L.F.-B., I.S., L.B., A.-S.B. and F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study has been ethically approved by the "Comité de Protection des Personnes Sud-Est III" (20.02.11.68220-2).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is unavailable due to privacy or ethical restrictions.

Acknowledgments: Rosa Montero-Macías acknowledges the grant support from Alfonso Martín Escudero Foundation, Madrid (Spain). The authors acknowledge the technical team from the virology laboratory of the Georges Pompidou European Hospital and the pathology department of the Simone Veil Hospital. The authors would also like to thank AcaciaTools for their proofreading services.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Arbyn, M.; Weiderpass, E.; Bruni, L.; de Sanjosé, S.; Saraiya, M.; Ferlay, J.; Bray, F. Estimates of incidence and mortality of cervical cancer in 2018: A worldwide analysis. *Lancet Glob. Health* **2020**, *8*, e191–e203. [CrossRef] [PubMed]
2. Li, H.; Wu, X.; Cheng, X. Advances in diagnosis and treatment of metastatic cervical cancer. *J. Gynecol. Oncol.* **2016**, *27*, e43. [CrossRef] [PubMed]
3. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef] [PubMed]
4. Gilbert, D.C.; Wakeham, K.; Langley, R.E.; Vale, C.L. Increased risk of second cancers at sites associated with HPV after a prior HPV-associated malignancy, a systematic review and meta-analysis. *Br. J. Cancer* **2018**, *120*, 256–268. [CrossRef] [PubMed]
5. Péré, H.; Pavie, J.; Pernot, S.; Veyer, D.; Bertaud, D.; Hurel, S.; Bélec, L.; Hans, S.; Ménard, M.; Cochand-Priollet, B.; et al. Comment on "Increased risk of second cancers at sites associated with HPV after a prior HPV-associated malignancy, a systematic review and meta-analysis". *Br. J. Cancer* **2019**, *120*, 952–953. [CrossRef] [PubMed]
6. Allison, H.; Kenneth, A. RNA Interference of Human Papillomavirus Type 18 E6 and E7 Induces Senescence in HeLa Cells. *J. Virol.* **2003**, *77*, 6066–6069.
7. Steinau, M.; Patel, S.S.; Unger, E.R. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* **2011**, *13*, 377–381. [CrossRef] [PubMed]
8. Estrade, C.; Sahli, R. Comparison of Seegene Anyplex II HPV28 with the PGMY-CHUV assay for human papillomavirus genotyping. *J. Clin. Microbiol.* **2014**, *52*, 607–612. [CrossRef] [PubMed]
9. Lillsunde Larsson, G.; Carlsson, J.; Karlsson, M.G.; Helenius, G. Evaluation of HPV Genotyping Assays for Archival Clinical Samples. *J. Mol. Diagn.* **2015**, *17*, 293–301. [CrossRef] [PubMed]
10. Kwon, M.-J.; Roh, K.H.; Park, H.; Woo, H.-Y. Comparison of the Anyplex II HPV28 assay with the Hybrid Capture 2 assay for the detection of HPV infection. *J. Clin. Virol.* **2014**, *59*, 246–249. [CrossRef] [PubMed]
11. Latsuzbaia, A.; Tapp, J.; Nguyen, T.; Fischer, M.; Arbyn, M.; Weyers, S.; Mossong, J. Analytical performance evaluation of Anyplex II HPV28 and Euroarray HPV for genotyping of cervical samples. *Diagn. Microbiol. Infect. Dis.* **2016**, *85*, 318–322. [CrossRef] [PubMed]
12. Lee, Y.-J.; Kim, D.; Lee, K.; Chun, J.-Y. Single-channel multiplexing without melting curve analysis in real-time PCR. *Sci. Rep.* **2014**, *4*, 7439. [CrossRef] [PubMed]
13. Veyer, D.; Wack, M.; Grard, O.; Bonfils, P.; Hans, S.; Bélec, L.; Badoual, C.; Péré, H. HPV detection and genotyping of head and neck cancer biopsies by molecular testing with regard to the new oropharyngeal squamous cell carcinoma classification based on HPV status. *Pathology* **2019**, *51*, 421–425. [CrossRef] [PubMed]
14. Plaza, J.A.; Ramirez, N.C.; Nuovo, G.J. Utility of HPV analysis for evaluation of possible metastatic disease in women with cervical cancer. *Int. J. Gynecol. Pathol.* **2004**, *23*, 7–12. [CrossRef] [PubMed]
15. Ishikawa, M.; Fujii, T.; Saito, M.; Nindl, I.; Ono, A.; Kubushiro, K.; Tsukazaki, K.; Mukai, M.; Nozawa, S. Overexpression of p16 INK4a as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. *Int. J. Gynecol. Cancer* **2006**, *16*, 347–353. [CrossRef] [PubMed]
16. Yoon, N.; Yoon, G.; Park, C.K.; Kim, H.-S. Stromal p16 expression is significantly increased in malignant ovarian neoplasms. *Oncotarget* **2016**, *7*, 64665–64673. [CrossRef] [PubMed]

17. Gazzeri, S.; Gouyer, V.; Vour'Ch, C.; Brambilla, C.; Brambilla, E. Mechanisms of $p16^{INK4A}$ inactivation in non small-cell lung cancers. *Oncogene* **1998**, *16*, 497–504. [CrossRef] [PubMed]
18. McKinneyab, M.D.; Moonc, S.J.; Kuleshb, D.A.; Larsenc, T.; Schoeppb, R.J. Detection of viral RNA from paraffin-embedded tissues after prolonged formalin fixation. *J. Clin. Virol.* **2009**, *44*, 39–42. [CrossRef] [PubMed]
19. Arfi, A.; Hequet, D.; Bataillon, G.; Tran-Perennou, C.; Farkhondeh, F.; Sastre-Garau, X.; Fourchotte, V.; Rouzier, R.; Laas, E.; Pouget, N.; et al. HPV DNA integration site as proof of the origin of ovarian metastasis from endocervical adenocarcinoma: Three case reports. *BMC Cancer* **2019**, *19*, 375. [CrossRef] [PubMed]