A NGS-based Blood Test For the Diagnosis of Invasive HPV-associated Carcinomas with Extensive Viral Genomic Characterization

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ABSTRACT

Purpose: Use of circulating tumor DNA (ctDNA) for diagnosis is limited regarding the low number of target molecules in early-stage tumors. Human papillomavirus (HPV)–associated carcinomas represent a privileged model using circulating viral DNA (ctHPV DNA) as a tumor marker. However, the plurality of HPV genotypes represents a challenge. The next-generation sequencing (NGS)-based CaptHPV approach is able to characterize any HPV DNA sequence. To assess the ability of this method to establish the diagnosis of HPV-associated cancer via a blood sample, we analyzed ctHPV DNA in HPV-positive or HPV-negative carcinomas.

Experimental Design: Patients (135) from France and Senegal with carcinoma developed in the uterine cervix (74), oropharynx (25), oral cavity (19), anus (12), and vulva (5) were prospectively registered. Matched tumor tissue and blood samples (10 mL) were taken before treatment and independently analyzed using the CaptHPV method.

Results: HPV prevalence in tumors was 60.0% (81/135; 15 different genotypes). Viral analysis of plasmas compared with tumors was available for 134 patients. In the group of 80 patients with HPV-positive tumors, 77 were also positive in plasma (sensitivity 95.0%); in the group of 54 patients with HPV-negative tumors, one was positive in plasma (specificity 98.1%). In most cases, the complete HPV pattern observed in tumors could be established from the analysis of ctHPV DNA.

Conclusions: In patients with carcinoma associated with any HPV genotype, a complete viral genome characterization can be obtained via the analysis of a standard blood sample. This should favor the development of noninvasive diagnostic tests providing the identification of personalized tumor markers.

See related commentary by Rostami et al., p. 5158

Introduction

Numerous applications that identify circulating tumor DNA (ctDNA) as a diagnostic marker in oncology are under development (1–3). The current sensitivity rate of ctDNA detection for localized tumors is between 59% and 73% (1, 2). However, these methods of detection have limitations due to their inability to detect low levels of ctDNA fragments harboring point mutations dispersed among germline DNA which affects the overall detection of early-stage tumors (4).

Tumors associated with human papillomaviruses (HPV) represent an ideal model for the detection of ctDNA. Most carcinomas (95%) developed in the uterine cervix (5) or the anal canal (6) and 20%–30% of the tumors developed in the oropharynx (7, 8) or the vulva (9, 10) are associated with specific HPV genotypes. The HPV genome, a 7.8 kbp-long circular DNA molecule, is present in the nucleus of tumor cells as free episomes and/or as an integrated form into the cell genome. The viral genome is identified in the host with copy numbers varying from a few to thousands per cell which means that HPV DNA, released in the blood from tumor cells, is identified as foreign DNA and is more readily detected than rare circulating genomic DNA fragments with point mutations. Therefore, using droplet-digital PCR (ddPCR) with primers specific for HPV16 or HPV18, circulating HPV DNA could be detected in 71.2% to 95.2% of patients with carcinomas associated with these viral genotypes (11–13) and up to 100% in locally advanced (14) or metastatic (15) cervical tumors. No circulating viral DNA was found in nontumor patients (13) or in patients with intraepithelial neoplasia (12), indicating that ctHPV DNA constitutes a specific form of ctDNA, referred to as ctHPV DNA, specifically associated with invasive HPV-associated carcinomas that can serve as a tumor marker for improved diagnosis, prognosis, and treatment monitoring (16, 17).

In spite of this favorable context, the use of HPV DNA as a diagnostic and/or predictive marker is not yet fully developed. This is related first to the large diversity of the HPV genotypes associated with tumors. Among the hundreds of HPV types identified so far (18), at least 13 genotypes, classified as "high-risk", including HPV16, 18, 31,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

There is a large heterogeneity of oncogenic HPV genotypes and the interaction between viral and cell genomes frequently leads to genomic alterations implied in oncogenesis. The CaptHPV NGS-based innovative approach allows the extensive molecular characterization of any HPV DNA, including its full sequence (genotype), integration pattern, and chromosomal insertion site. We show here, via a systematic prospective study, that the HPV DNA pattern characterizing tumor cells can be identified from a standard blood sample by the analysis of circulating viral tumor DNA using this test. This allows a highly sensitive and specific noninvasive procedure for the characterization of viral sequences in patients with HPV-associated cancer. In practice, this approach should facilitate the diagnosis of deeply located tumor relapse. Furthermore, the specific tumor markers obtained via this approach can be useful for the biological follow-up of patients, as circulating and dynamic surrogates of tumor response in innovative therapeutic approaches.

33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, cause virtually all cases of HPV-associated cancers (19). Therefore, a methodologic approach designed to allow the diagnosis of HPV-associated tumors using a blood sample should be able to detect all of these genotypes. In addition, the identification of specific tumor markers such as viral-host DNA sequences (20), detectable in the blood (21) and of potential clinical relevance (22, 23) requires the full characterization of the viral sequences.

We have described the NGS-based CaptHPV method developed for the capture and the extensive characterization of all HPV DNA sequences identified in DNA extracted from tumor tissues (24). In order to further investigate whether this original method was also able to detect and characterize ct-HPV DNA, we designed a prospective study aiming to, first, demonstrate that, without knowledge of the tumor HPV status, our CaptHPV NGS-based method is able to detect ct-HPV DNA related to any HPV genotype from a blood specimen taken from patients with different types of HPV-associated carcinomas, in European and African populations, and, secondly, to show that the CaptHPV technique can provide a full molecular characterization of the circulating viral DNA sequences, including a map of the entire viral genome and of flanking cellular sequences related to viral integration.

Materials and Methods

Study design

This CaptHPV (NCT02981862) prospective study was performed at the Institut de Cancérologie de Lorraine (ICL), Nancy, France, and at the Institut du Cancer Joliot Curie (ICJC), Centre Hospitalier Aristide Le Dantec, Dakar, Sénégal. Two populations of patients were included: a series of 100 consecutive patients treated at the ICL in Nancy, France, for a tumor developed in the uterine cervix, anal canal, vulva, oral cavity, or oropharynx and a series of 50 consecutive patients treated at the ICJC, Dakar, Senegal, for a tumor developed in the uterine cervix. We collected simultaneously a tumor tissue fragment for histologic and virological analyses and a blood sample for virological analysis. Fifteen patients were excluded regarding irrelevance in histology (8) or inclusion criteria (4), or due to lack of blood samples (2) or of patient's consent (1). Altogether, the study was based on 135 invasive carcinoma cases with available tumor and blood samples. The clinical data registered includes patients' ages, tumor localizations, tumor sizes, tumor–node–metastasis (TNM) and/or Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stages. Study was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from each subject. The protocols received agreements from the Comité de Protection des Personnes (CPP) EST III (N° 16.04.04) and from the Agence Nationale de Sécurité du Médicament et des produits de Santé (ANSM; N° 2016-A01085–46) for the patients treated in Nancy, France, and from the Comité National de la Recherche en Santé du Sénégal for the patients treated in Dakar (Sénégal).

Procedures and strategy for analysis

The viral analysis of tumor tissues combined three tests: p16 IHC, PCR and CaptHPV methods. The tumor viral status was classified as "HPV-positive" when at least two of these tests provided positive result. Among 12 HPV-positive tumors with only two positive tests, there were 10 p16+/PCR-/CaptHPV+ cases, one p16-/PCR+/CaptHPV+ and one p16+/PCR+/CaptHPV- case. When none or only one test was positive, the tumor was classified as "HPV-negative". The viral analysis of plasmas was performed using the CaptHPV method. The analyses were performed independently in three laboratories: (i) Histologic and immunophenotypic (p16) analyses were performed on formalin-fixed tissues in the Laboratory of Pathology (A. Leroux), ICL, Nancy, France; (ii) HPV DNA analyses on tumor tissue, using PCR and CaptHPV methods, were performed in the Tumor Biology Unit (J.-L. Merlin), ICL, Nancy; (iii) HPV DNA detection in cell-free DNA extracts from plasma specimens using the CaptHPV method were performed in the CERBA laboratory (J.-M. Costa), Saint-Ouen-L'Aumône, France. Results were blindly registered on a data base. Details on methods and regulatory aspects are provided in Supplementary Data I.

Statistical analyses

Statistical analyses were performed using SAS software, v9.4 (SAS Institute Inc., Cary, NC 25513). Qualitative parameters were described as frequency and percentage, quantitative parameters as mean and standard deviation or median and interquartile range according to the normality of the distribution assessed by the Shapiro–Wilk test. Comparisons according to HPV genotypes were performed with the χ^2 test or Fisher exact test. Sensitivity and specificity of the CaptHPV blood test were computed with tumor viral DNA status as the gold standard. The sensitivities of the CaptHPV blood tests were compared between clinical stages with the Mac Nemar test within the HPV positive cases in tumors. *P*-values <0.05 were considered statistically significant.

Results

Tumor localization and histology

This case panel is comprised of 135 invasive carcinomas, developed in the cervix (74 cases including 28 from ICL, Nancy and 46 from ICJC, Dakar), oropharynx (25 cases), oral cavity (19 cases), anus (12 cases), and vulva (5 cases; **Table 1**). Histologically, we observed 117 squamous cell carcinomas (SCC, 86.7%), 7 adenocarcinomas (5.2%), 4 undifferentiated carcinomas (3%), and 7 cases with other histologic types (5.2%), including three small cell carcinoma, two cases of adenosquamous carcinoma, one verrucous, and one glassy cell carcinoma. Clinical data are provided in **Table 1**.

HPV DNA detection in tumors

The prevalence of HPV DNA in tumors was 60.0% (81/135; **Table 1**). We observed HPV DNA in 85.1% (63/74) of cervical, 100% (12/12) of

Table 1. Clinical and virological data in invasive carcinomas.

Characteristics	ICL ($n = 89$)	ICJC ($n = 46$)	All cases
Patients' age	59.7 ± 11.1	52.0 ± 11.2	57.1 ± 11.7
Tumor localization			
Cervix	28 (31.5%)	46 (100%)	74 (54.8%)
Oropharynx	25 (28.1%)	–	25 (28.1%)
Oral cavity	19 (21.4%)	–	19 (21.4%)
Anal canal	12 (13.5%)	–	12 (13.5%)
Vulva	5 (5.6%)	–	5 (5.6%)
Clinical tumor size (mm)	32 [27–51]	47.5 [40–52]	42 [30–52]
Tumor stage (TNM)			
T1	4 (4.5%)	0 (0%)	4 (3.0%)
T2	16 (18.0%)	3 (6.5%)	19 (14.1%)
T3	38 (42.7%)	27 (58.7%)	65 (48.1%)
T4	16 (18.0%)	15 (32.6%)	31 (23.0%)
TX	15 (16.8%)	1 (2.2%)	16 (11.8%)
N0	47 (52.8%)	NA	47 (52.8%)
N1	17 (19.1%)	NA	17 (19.1%)
N2	18 (21.2%)	NA	18 (21.2%)
N3/N4	3 (3.5%)	NA	3 (3.5%)
M0	81 (94.2%)	46 (100%)	127 (96.2%)
M1	5 (5.8%)	0 (0%)	5 (5.8%)
Histology			
SCC	82 (92.1%)	35 (76.1%)	117 (86.7%)
ADC	3 (3.4%)	4 (8.7%)	7 (5.2%)
UC	1 (1.1%)	3 (6.5%)	4 (3.0%)
Others	3 (3.4%)	4 (8.7%)	7 (5.2%)
HPV-positive cases			
Cervix	24 (85.7%)	39 (84.8%)	63 (85.1%)
Oropharynx	3 (12.0%)	–	3 (12.0%)
Oral cavity	2 (10.5%)	–	2 (10.5%)
Anal canal	12 (100%)	–	12 (100%)
Vulva	1 (20.0%)	–	1 (20.0%)
HPV physical state			
Episomal (E)	10 (23.8%)	10 (25.6%)	20 (24.7%)
Integrated ± (E)	31 (73.8%)	24 (61.6%)	55 (67.9%)
NA	1 (2.4%)	5 (12.8%)	6 (7.4%)

Abbreviations: ADC, adenocarcinoma; NA, not available; UC, undifferentiated carcinoma.

anal, 20.0% (1/5) of vulvar, 12.0% (3/25) of oropharyngeal, and 10.5% (2/19) of oral tumors. One p16+/PCR+/CaptHPV- anal cancer case was classified as HPV positive. Sequence analysis provided by CaptHPV identified 15 different HPV genotypes: HPV16 (45 cases), HPV18 (11 cases), HPV45 (7 cases), HPV 51 (5 cases), HPV31 (3 cases), HPV33 (2 cases), and single cases of HPV11, 35, 39, 52, 53, 56, 59, 68, and 69 (**Table 2**). Two different HPV genotypes were identified in six cases. The viral genome existed in a nonintegrated episomal state in 24.7% of all cases and in an integrated state in the cell genome, with or without episomes, in 67.9% cases (**Table 1**). Viral DNA integration was observed in 70.8% (34/48) of the HPV16-associated cases, 76.5% (13/17) for HPV18/45, and 55.6% (10/18) for the other genotypes. When comparing the respective prevalence of the viral genotypes in cervical cancers in the French and Senegalese populations, HPV16 was more frequent in France (36/47 = 76.2%) than in Senegal (14/42 = 33.3%; $P = 0.001$) whereas the prevalence was higher in Senegal for HPV18 (8/4 = 19.1% vs. 3/47 = 6.4%; $P = 0.070$), HPV45 (6/42 = 14.3% vs. 1/47 = 2.1%; $P = 0.049$), and HPV51 (5/42 = 11.9% vs. 0%; $P = 0.020$; **Table 2**).

Comparison between HPV status in tumors and plasmas

The main objective of our project was to assess the sensitivity of the CaptHPV test for the detection of ctHPV DNA corresponding to various HPV genotypes in patients with different tumor localizations. Viral analysis of plasmas compared with tumors, available for 134 patients, showed that, in the group of 80 patients with HPV-positive tumors, 77 were also positive in plasma (sensitivity 95.0%; **Table 3**); in the group of 54 patients with HPV-negative tumors, one was positive in plasma (specificity 98.1%; **Table 3**). A positive relationship was found between clinical stage and sensitivity of the test which was 72.7% for T1 stage tumors, 95.9% for T2, and 100% for T3/T4 ($P = 0.012$; **Table 3**). All tumors with positive lymph node or metastasis were ctHPV DNA positive (sensitivity 100%, specificity 97.4%). We also identified the same genotypes within the tumor and plasma matched samples. Among the six tumors harboring two HPV genotypes, the analysis of matched plasmas found only one genotype in three and the two genotypes in three (**Table 2**).

HPV patterns identified from the analysis of plasma samples

The second aim of our study was to evaluate whether, from a blood sample analysis, CaptHPV could determine the HPV pattern and therefore identify specific tumor markers. The comparison between tumor and plasma HPV status could be performed in 76/80 cases. Analysis of the full viral sequences permitted identification of single-nucleotide polymorphism (SNP) and indels, a step allowing further characterization of each individual HPV strain and therefore preventing any false positive results (all SNPs and indels are presented in Supplementary Fig. S1). Regarding the integration status, viral genomes were detected in their full length corresponding to episomal form in both tumor and plasma in 8 cases (**Fig. 1A**; **Table 2**). In the other cases, hybrid viral-genome sequences, corresponding to an integrated HPV status with or without episomal forms, were detected in tumors and/or plasmas (**Table 2**). The case by case comparison showed that there was one single integration site in 34 cases. In 20 of them, the integration locus was identical in tumors and in plasmas (**Fig. 1B**), whereas in 14 cases, only the episomal form of the viral genome was detected in the matched specimen (**Fig. 1C**). Other cases harbored multiple integration sites, either clustered at the same locus (three cases; **Fig. 1D**) or scattered into different chromosome sites (31 cases; **Fig. 1E**). There were 58 hybrids identified in tumors and 60 in plasmas, among which 40 were commonly shared in the two types of specimens. In 4 cases, noncommon hybrids between tumor and blood sample were found (**Fig. 1F**). All integration patterns are provided in Supplementary Fig. S2 and molecular coordinates of the hybrid viral-genome sequences in tumors and plasmas are provided in Supplementary Table S1.

Annotations of the HPV insertions

In HPV-associated carcinoma, the hybrid viral-genome sequences map at different chromosomal loci. We identified HPV DNA insertion at 140 different chromosomal loci in tumors or in plasma samples (**Table 2** and **Fig. 1**). Viral sequences were found inserted within coding sequences in 93 loci (66.4%) or within 500 Kb from a known gene in 38 cases (27.1%). Recurrent integrations (two cases each) were found within *CASC21, INPP4B, MAPK10, VMP1, NFIA*, and near *PIBF1-KLF1-KLF12*. Interestingly, the two cases with viral insertions within *CASC21* corresponded to HPV18-associated small cell carcinoma of the cervix, a very rare histologic type. In one of these cases (02–028), viral-genome junctions were located at each extremity of the *CASC21* exon 2 which was deleted, and, in the second case, viral insertion occurred in the first intron of the gene (**Fig. 2**). Among the other genes directly targeted by HPV integration and that have not been reported thus far were *TEX41, RBM47, IGF1, RAD51L3, RPTOR, IL1RAPL1*.

Table 2. Detailed data for each patient included in the study.

Inclusion number	Patients' age	Tumor localization	Clinical stage	Tumor size (mm)	Tumor histology	HPV status in tumors			HPV status in plasma		
						HPV type	Chromosome locus	Target gene	HPV type	Chromosome locus	Target gene
01-057	50	Oral cavity	T2N2	45	UC	HPV16	10q23.1	**NRG3**	HPV16	7p14.2	**ELMO1**
01-060	54	Oral cavity	T2NX	32	SCC	HPV16	18q23	**PARD6G**	HPV16	13q12.12 17q25.3	**SGCG** **BAHCC1**
01-039	54	Oropharynx	T1N0	27	SCC	HPV16			HPV16		
01-051	54	Oropharynx	T2N2	NA	SCC	HPV16			HPV16		
01-073	51	Oropharynx	T2N1	31	SCC	HPV16	6q12 2q36.1	**ADGRB3** **LOC440934**	HPV16	2q31.3 2q24.1 2q24.1 5p14.3	GALNT5 GALNT5 GALNT5 **CDH12**
01-027	30	Vulva	T1N0	28	SCC	HPV16	12p12.1	**SOX5**	HPV16		
01-009	57	Anal canal	T2N0	24	SCC	HPV16	Xp11.21	WNK3...	HPV16		
01-016	72	Anal canal	T3N0	85	SCC	HPV16	9q22.1	SPATA31E1...	HPV16	9q22.1	SPATA31E1 ...
01-024	66	Anal canal	T2N0	30	SCC	HPV16	14q32.2	**BCL11B**	HPV16	14q32.2	**BCL11B**
01-025		Anal canal	T3N3	80		HPV16	9p24.1 12q23.2 2q24.1 9q21.13 4q28.3 9p22.2	**CD274** **IGF1** **CCDC148** **ABHD17B** LOC105377437 BNC2...	HPV16	9p24.1 12q23.2 2q24.1 9q21.13 1p34.1	**CD274** **IGF1** **CCDC148** **ABHD17B** **NASP**
01-026	69	Anal canal	T2N0	24	SCC	HPV16	17q25.3	**RPTOR**	HPV16	17q25.3	**RPTOR**
01-034	67	Anal canal	T2N1	42	SCC	HPV16 HPV11	9q21.13 21q21.3	LOC101927358 **GRIK1**	HPV16	9q21.13	LOC101927358
01-049	54	Anal canal	T2N2	31	SCC	HPV16	3p24.2	**NGLY1**	HPV16	3p24.2	**NGLY1**
01-084	81	Anal canal	T2N0	42	SCC	HPV16 HPV53	17q25.1 17q25.1 Yp11.2	**EXOC7** MYO15B... RPS4Y1	HPV16		
01-088	76	Anal canal	T2N0	44	SCC	HPV16	1q41	**SLC30A10**	HPV16		
01-089	68	Anal canal	TXN0	55	SCC	HPV16	2q22.3 2q22.3 11p15.4	**TEX41** **TEX41** **TRIM5,6,34**	HPV16	2q22.3	
01-093	65	Anal canal	T1N0	30	SCC	HPV16	11q23.1	**BTG4**	No HPV		
01-017	32	Cervix	T2N0	43	SCC	HPV16			HPV16	1q23.2	**ATP1A2**
01-018	63	Cervix	T2N1	40	SCC	HPV16			HPV16	12q12	**PDZRN4**
01-019	86	Cervix	T1N0	29	SCC	HPV68	10q26.3 1p31.3	MIR378C... **NFIA**	No HPV		
01-030	61	Cervix	T2N1	55	SCC	HPV16	17q25.3	**CCDC40**	HPV16	17q25.3	**CCD40**
01-031	51	Cervix	T2N0	69	SCC	HPV56	5q11.2	ITGA1...	HPV56		
01-033	47	Cervix	T1N0	19	SCC	HPV16	9q34.11	PKN3...	HPV16		
01-035	55	Cervix	T2N0	50	ADC	HPV16 HPV18	4q31.21 9q22.33	**INPP4B** **LOC101928438**	HPV16 HPV18		
01-036	40	Cervix	T2N0	34	ADC	HPV16	17q23.1	**VMP1**	HPV16		
01-042	56	Cervix	T2N0	31	SCC	HPV31 HPV16			HPV31 HPV16		
01-044	59	Cervix	T2N0	44	SCC	HPV16	18q21.33	**PHLPP1**	HPV16	18q21.33	**PHLPP1**
01-054	61	Cervix	T1N0	40	SCC	HPV18	9q22.32	**C9orf3**	HPV18	9q22.32	**C9orf3**
01-055	38	Cervix	T2N0	66	SCC	HPV31	4q21.3	**MAPK10**	HPV31	4q21.3	**MAPK10**
01-056	65	Cervix	T1N0	12	SCC	HPV45	13q14.2	**RB1**	HPV45	13q14.2	**RB1**
01-067	85	Cervix	T3N0	93	SCC	HPV39			HPV39		
01-068	50	Cervix	T2N0	32	ADC	HPV16			HPV16	2p24.1 3p26.1	**HS1BP3** **GRM7**
01-070	46	Cervix	T4N1	80	SCC	HPV16	7p22.2	**SDK1**	HPV16	7p22.2	**SDK1**
01-081	39	Cervix	T1N0	65	SCC	HPV16	5q33.1	LOC105378231	HPV16	5q33.1 4p15.2	LOC105378231 **PPARGC1A**
01-082	57	Cervix	T2N1	35	SCC	HPV16			HPV16		
01-083	52	Cervix	T2N0	12	SmCC	HPV18	5q35.1	**LINC01048**	HPV18	5q35.1	**LINC01048**
01-085	44	Cervix	T2N0	30	SCC	HPV16	12q24.31	**KNTC1, CLIP1**	HPV16		
01-086	62	Cervix	T4N1	73	SCC	HPV16			HPV16	6p25.3 16p12.1 20q11.22 6q21	**LOC102723944** **GSG1L** **PIGU** **LOC10192776, HS3ST5**

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Table 2. Detailed data for each patient included in the study. (Cont'd)

						HPV status in tumors			HPV status in plasma		
Inclusion number	Patients' age	Tumor localization	Clinical stage	Tumor size (mm)	Tumor histology	HPV type	Chromosome locus	Target gene	HPV type	Chromosome locus	Target gene
01-090	54	Cervix	T2N0	33	SCC	HPV16	19p12 6q23.2	**ZNF208** **SGK1**	HPV16		
01-091	34	Cervix	T2N0	NA	SCC	HPV16			HPV16	3p21.31	**CCDC12**
01-100	56	Cervix	T3N0	44	SCC	HPV16	4p16.3	**TACC3**	HPV16	12p13.33 4p16.3	**TACC3** MAEA
02-002	53	Cervix	T2NX	50	SCC	HPV16	13q22.1 2q34	PIBF1,KLF5,12 **IKZF2**	HPV16	13q22.1	PIBF1,KLF5, KLF12
02-003	34	Cervix	T2NX	40	SCC	HPV16	17q21.2	KRT42P	HPV16	17q21.2	KRT42P
02-004	42	Cervix	T2NX	30	ADS	HPV45	17q23.1	**VMP1**	HPV45 HPV16		
02-007	48	Cervix	T2NX	46	SCC	HPV16 HPV53			HPV16	15q22.2 6p21.1	FAM81A **LOC107986598**
02-008	60	Cervix	T3NX	58	SCC	HPV18	12q23.2 - 12q24.31	**SLC17A8,** **TMEM132B**	HPV18		
02-009	41	Cervix	T2NX	50	SCC	HPV16			HPV16	13q22.1 5q21.3 1q43	LINC01347 **FBXL17** **SDCCAG8**
02-010	44	Cervix	T2NX	57	SCC	HPV51			HPV51	3q26.31	**FNDC3B**
02-012	60	Cervix	T2NX	46	ADC	HPV45	3p14.2	**FHIT**	HPV45	3p14.2	**FHIT**
02-013	60	Cervix	T1NX	20	SCC	HPV31	5q32 5q32	**CAMK2A** **CDX1**	HPV31	5q32 5q32 5q32 5q32 11p13 1q32.1	**CAMK2A** **CDX1** **CSF1R** **SLC6A7** **DCDC1** **KDM5B**
02-014	40	Cervix	T2NX	40	ADC	HPV18	5p13.2 16q21	IL7R... **LOC105371308**	HPV18	5p13.2	IL7R
02-015	60	Cervix	T3NX	56		HPV45	1q21.1 18q11.2	**HYDIN2** CTAGE1...	HPV45	1q21.1 18q11.2	**HYDIN2** CTAGE1
02-016	76	Cervix	T3NX	60	SCC	HPV16	17p11.2	FAM106B...	HPV16	17p11.2	FAM106B...
02-019	53	Cervix	T3NX	57		HPV53			HPV53		
02-020	60	Cervix	T3NX	57	UC	HPV45			HPV45	1p22.1 1q32.2	BRDT, TGFBR3 LINC01696
02-022	45	Cervix	T2NX	52	SCC	HPV18			HPV18	13q22.1	MARK2P12
02-023	48	Cervix	T1NX	22	SCC	HPV69			HPV69		
02-024	45	Cervix	T3NX	55	SCC	HPV51			HPV51		
02-025	46	Cervix	T2NX	40	SCC	HPV45	4q21.3	**MAPK10**	HPV45	4q21.3	**MAPK10,** **PTPN13**
02-028	63	Cervix	T4NX	62	SmCC	HPV18	8q24.21 4q31.21 2q24.2 5q22.2	**CASC21,CASC8** **INPP4B** **BAZ2B** **EPB41L4A**	HPV18	8q24.21 4q21.31 2p15	**CASC21,CAS** **C8** **INPP4B** **WDPCP**
02-029	55	Cervix	T2NX	47	SCC	HPV51	Xp21.3	**IL1RAPL1**	HPV51	Xp21.3 8q24.3	**IL1RAPL1** **TSNARE1**
02-030	44	Cervix	T2NX	40	SmCC	HPV18	4p14 8q24.21 18q12.3	**RBM47** **CASC21** **SLC14A2**	HPV18	4p14 8q24.21 2q36.3	**RBM47** **CASC21** **COL4A4**
02-031	82	Cervix	T2NX	42	SCC	HPV59			HPV59	1p31.3 13q31.3	**NFIA** **GPC6**
02-032	47	Cervix	T2NX	36	SCC	HPV35	2q24.3 2q24.3	**FIGN** **FIGN**	HPV35	2q24.3	**FIGN**
02-033	57	Cervix	T3NX	52	SCC	HPV33			HPV33	11p12 21p11.2 10p12.31 8p11.23	HNRNPKP3, API5 CDC27P11 **MALRD1** **LETM2**
02-034	61	Cervix	T2NX	40	SCC	HPV16	2p12 3q11.1	LOC105374830 PROS1	HPV16	 12p13.33 1p36.31 20q13.12 7q36.1	 **CACNA1C** **PLEKHG5** **TOX2** **KMT2C**
02-035	62	Cervix	T2NX	40	SCC	HPV16 HPV52			HPV16 HPV52		
02-036	58	Cervix	T3NX	56	SCC	HPV18			HPV18	4q28.3	PCDH18
02-037	35	Cervix	T3NX	50	SCC	HPV16	9p23 9q31.1 10q24.31 n/a n/a	**LURAP1L-AS1** **ALDOB** **PAX2** **COX3, ND5** **ND4**	n/a		
02-038	58	Cervix	T2NX	43	SCC	HPV45			HPV45	16q23.2	CDYL2

(Continued on the following page)

Table 2. Detailed data for each patient included in the study. (Cont'd)

Inclusion number	Patients' age	Tumor localization	Clinical stage	Tumor size (mm)	Tumor histology	HPV status in tumors			HPV status in plasma		
						HPV type	Chromosome locus	Target gene	HPV type	Chromosome locus	Target gene
02-039	26	Cervix	T2NX	44	GCC	HPV16			No HPV		
02-042	50	Cervix	T2NX	48	SCC	HPV16	17q23.1 17q12 17q12	CCDC57... **ERBB2, PGAP3** **ERBB2**	HPV16	17q25.3	CCDC57
02-043	59	Cervix	T2NX	30	SCC	HPV18			HPV18	2q23.3	**LOC105373684**
02-044	50	Cervix	T2NX	42	SCC	HPV16	1q41	**SUSD4**	HPV16	1q41	**SUSD4**
02-045	41	Cervix	T4NX	40	SCC	HPV16	18q21.2 9p13.3	MAPK4... LOC107987019	HPV16	18q12.1	CCDC178,NOL4
02-046	62	Cervix	T3NX	48	SCC	HPV16	13q22.1 13q22.1 13q22.1 13q22.1	BORA,KLF5,KLF12 BORA,KLF5,KLF12 PIBF1,KLF5,KLF12 PIBF1,KLF5,KLF12	HPV16	13q22.1	BORA,KLF5,KLF12
02-047	33	Cervix	T3NX	50	SCC	HPV16	3q28 22q12.2	**TPRG1** **THOC5**	HPV16	3q28 3q21.3 19p12	LPP... **ISY1-RAB43, RAB43** ZNF728...
02-048	35	Cervix	T3NX	52	SCC	HPV33	18q21.33	**RNF152**	HPV33		
02-049	47	Cervix	T2NX	34	SCC	HPV51	17q12	**RAD51L3-RFFL**	HPV51	17q12	**RAD51L3-RFFL**
02-050	70	Cervix	T3NX	52	SCC	HPV51	1p34.3	**LOC105378657**	HPV51	1p34.3 3q13.2	**LOC105378657** **TMPRSS7**

Note: All genomic data, insertion locus, and target genes are presented. Insertions detected in both tissue and plasma are presented in red. Target genes indicated in bold when harboring viral DNA within the gene locus.

Discussion

The aims of our project were to demonstrate that, from the analysis of a standard blood sample, our original NGS-based CaptHPV method was able to detect ct-HPV DNA related to any HPV genotype and provide a full characterization of the circulating viral DNA. Our prospective study, including patients from France and Senegal, shows that, using this method, circulating viral DNA from 15 different HPV genotypes could be detected in patients with different types of HPV-associated carcinomas with a 95% rate of sensitivity and 98.1% of specificity. The approach further provided the full characterization of the viral pattern, allowing the identification of specific tumor markers, including cellular genes targeted by viral DNA insertion. These data should favor the development of noninvasive tests for the diagnosis of HPV-associated invasive cancers and for the identification of personalized tumor markers. These data constitute important steps for the clinical validation of the liquid biopsy approach to establish the diagnosis of invasive HPV-associated carcinoma. For instance, in the follow-up of patients previously treated for an HPV-associated carcinoma, the diagnosis of relapse may be difficult to obtain in cases of deeply located tumor growth that require micro-biopsies or fine needle aspirations, procedures that can lead to adverse events. In these circumstances, the "liquid biopsy" approach (25) may represent an attractive alternative. Regarding the use of specific tumor markers in the follow-up of patients with HPV-associated tumors, the differential diagnosis between a metastasis or a second primary tumor may be facilitated by the DNA sequence marker evidence showing identical HPV insertional signatures in both lesions (26).

Table 3. Sensitivity and specificity of CaptHPV blood test according to tumor localization and clinical stage.

Population	No. of cases	HPV-positive cases		Sensitivity	Specificity
		Tumors	Blood		
All cases	134	80	77	95.0% (76/80)	98.1% (53/54)
Cervix	73	62	60	96.8% (60/62)	100% (11/11)
Oropharynx	25	3	4	100% (3/3)	95.5% (21/22)
Oral cavity	19	2	2	100% (2/2)	100% (17/17)
Anal canal	12	12	10	83.3% (10/12)	–
Vulva	5	1	1	100% (1/1)	100% (4/4)
T1	19	11	8	72.7% (8/11)	100% (8/8)
T2	65	47	46	97.9% (46/47)	100% (18/18)
T3	30	18	19	100% (18/18)	91.7% (11/12)
T4	16	3	3	100% (3/3)	100% (13/13)
Tx	4	1	1	100% (1/1)	100% (3/3)
N0	47	29	26	89.7% (26/29)	100% (18/18)
N+	38	12	13	100% (12/12)	96.1% (25/26)
Nx	4	1	1	100% (1/1)	100% (3/3)
M0	126	79	76	94.9% (75/79)	97.9% (46/47)
M1	5	1	1	100% (1/1)	100% (4/4)
Mx	3	0	0	–	100% (3/3)

Our study has certain limitations: no plasma samples from patients without tumors or from patients with different types of intraepithelial neoplasias were included in our study. The analysis of such samples using the CaptHPV assay would have reinforced the specificity of this approach and confirmed that the presence of ct-HPV DNA is the surrogate of an invasive tissular lesion. In addition, the NGS-based approach is more complex and expensive than the ddPCR technique which has equivalent sensitivity (11–15). Unlike the NGS approaches, prior knowledge of the viral sequences to be detected is required for ddPCR and this method is also limited by the number of targets that can be simultaneously detected. Therefore, ddPCR approach is not appropriate to analyze ctDNA straightforwardly and in a single step when the personalized targets (e.g., hybrids viral/host DNA or rare HPV genotypes sequences) have not been previously identified. This

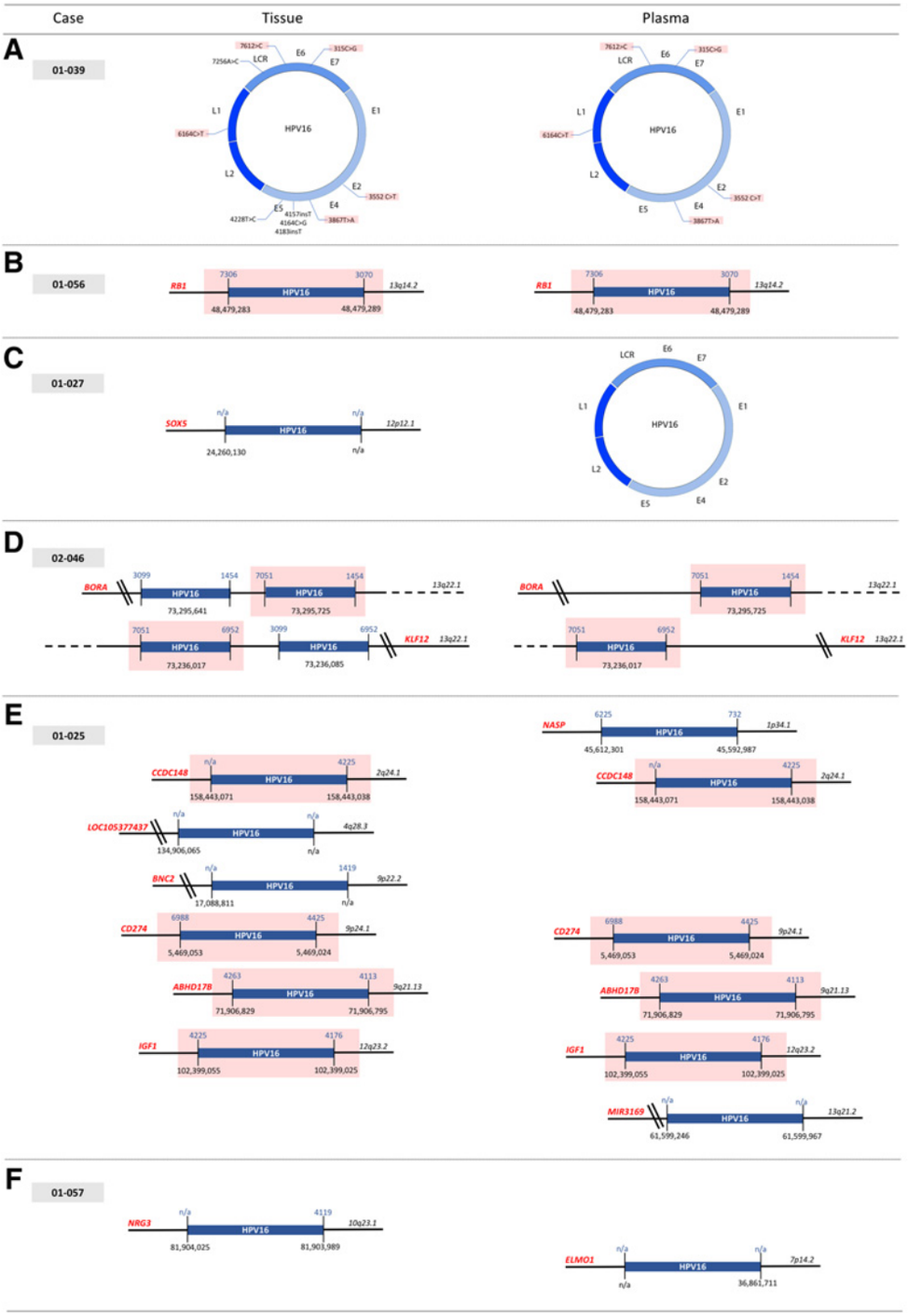

Figure 1.

Examples of the respective HPV status found in tumor and plasma DNA samples from the same patient, using the CaptHPV technique. **A,** Only episomal form of the viral genome with 50% common SNPs found in both tumor and plasma; **B,** Identical hybrid viral-genome sequences corresponding to a single HPV integration site found in the two specimens; **C,** Hybrid viral-genome detected in tumor DNA corresponding to a unique integration site, whereas only viral episomes found in plasma; **D,** Multiple viral-cell junctions clustered at the same chromosomal locus in tumor and plasma; **E,** Multiple hybrids corresponding to HPV integration loci scattered at different chromosome sites in tumor and plasma; **F,** Different hybrids corresponding to distinct viral integration sites detected in tumor and plasma. Names of target genes are in red. A double bar indicates that the HPV insertion is not within the gene locus and the closest gene is indicated in red. Hg38 reference genome has been used for the genomic coordinates indicated below the HPV box and the chromosomal loci. HPV breakpoints are reported above the HPV box. Hybrids or SNPs shared in common in tumor and plasma samples are indicated by a pink-colored box.

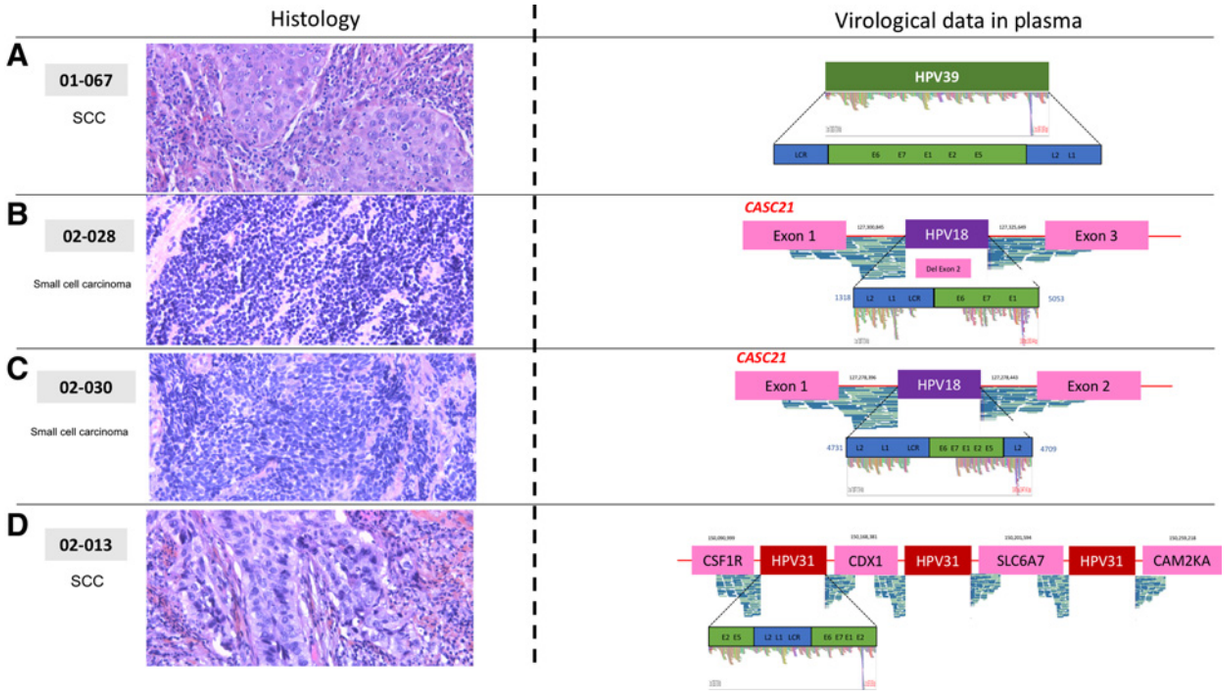

Figure 2.

Virologic data from plasma analyses (right) in comparison with tumor histology (left) in patients with cervical carcinoma. SCC (**A**) with circulating HPV39 DNA sequences covering the whole viral genome (episomal form). Two cases of small-cell carcinoma with circulating HPV18 DNA sequences showing viral DNA inserted within the *CASC21* gene, between exon 1 and exon 3 in **B** with gene disruption and loss of the exon 2, and between exon 1 and exon 2 in **C**. **D,** SCC with circulating HPV31 DNA sequences showing viral DNA inserted at the 5q32 chromosomal band with a complex pattern involving the *CAM2KA, SLC6A7, CSF1R*, and *CDX1* genes located at this locus.

limitation accounts for the fact that we have not been able to compare ddPCR and NGS in the present prospective analysis of plasma samples. Further study, including multiplex ddPCR assays, will be necessary to compare the ability of the two methods to detect circulating viral DNA from any HPV genotype, and to assess their respective sensitivity. There is currently growing evidence that analyses of ctHPV DNA at the end of treatment (27) or during patients' follow-up (28) may facilitate early initiation of salvage therapy for tumor relapse. Obviously, sequential analyses of plasma during the biological follow-up of patients should be based on simple and inexpensive techniques. However we show that the full characterization of the HPV status, including genotyping, complete sequence, and insertion pattern, obtained from the analysis of a mere blood specimen, may represent an important preliminary step to identify markers of clinical relevance (23) or to verify the adequacy of the primers to be used in sequential analyses. In addition, the identification of the cellular genes altered by integration may point to potential therapeutic targets. As an example, two cases of cervical carcinoma harboring HPV DNA sequences integrated at the *ERBB2* locus (29) and associated with *ERBB2* amplification (30) have been recently reported. A limitation in the identification of tumor markers lies in the fact that tumors solely harboring HPV episomes do not present specific viral genome rearrangements or hybrids that may be detected specifically in the tumor or in the blood. However, the HPV genotype constitutes *per se* a diagnostic signature (31), and, in addition, HPV polymorphism, that is, single nucleotide variants and insertion or deletions identified via the full sequencing of the viral DNA can accurately characterize a "personalized HPV strain" for each patient (32). Nevertheless, the same virus strain can lead to the development of distinct tumors in a patient. We must also emphasize that our study based on an extensive NGS-based analysis of tumors and blood samples found 14.8% of HPV-negative cervical cancers, a rate slightly higher than the 9.8% and 13.0% rates recently reported (14, 33). Tumors of nonsquamous histology were slightly overrepresented in our cases. Several parameters, such as methodologic limitations or the truncation of viral sequences have been hypothesized to account for false negative results in the detection of HPV-associated tumors (34). Our data do not support the hypothesis of false negativity related to structural alterations of the viral genome and cervical cancers, especially of the glandular phenotype, may develop independently of viral oncogenesis (33, 34). Large scale molecular analyses should help to identify the alterations that characterize HPV-negative cervical carcinomas and provide a more accurate assessment of the prevalence of these tumors, ruling out the possibility that false positive results might have led to overestimates of the rate of HPV-associated cervical cancers in some studies.

Using NGS-based approaches, several works have reported extensive characterization of tumor-associated HPV DNA (35–38) but data on the analysis of ct-HPV DNA are scarce. In their detection of circulating viral DNA in patients with HPV16-associated head and neck tumors, Lee and colleagues have described an original NGS approach based on the use of a panel of 39 amplicons covering thirty-four distinct regions of the HPV16 genome (39). A 100% rate of detection of ct-HPV DNA has been obtained in this series of advanced stage tumors. Such an approach is particularly well adapted to the monitoring of patients with head & neck tumors regarding the high

prevalence of HPV16 in this localization. However, this method does not allow the identification of viral integration loci and of other HPV genotypes, also observed in a limited number of head & neck tumors (40).

The systematic description of a detailed viral status in HPV-associated cancers should also increase our knowledge of HPV-related oncogenesis. We found one cervical cancer case associated with circulating HPV69 DNA, a genotype still classified as "potentially oncogenic in humans" (19). In our Senegalese population of patients with cervical cancer, we also observed three cases of small cell carcinomas, a rare histological type with severe outcome. Circulating HPV18 DNA was detected in these three cases, and, in two of them, viral sequences were found integrated at the 8q24.21 locus, targeting the long noncoding RNA (lncRNA) homosapiens cancer susceptibility 21 (*CASC21*) sequences. This lncRNA, also identified in colon carcinogenesis (41) and recently recognized as an HPV integration site (42), acts as a competing endogenous RNA to sponge miR-7–5p, a micro RNA able to impair DNA damage repair through PARP1 and BRCA1 inhibition. The consecutive cell apoptosis accounts for the tumor suppressive properties observed for miR-7–5p (41), also recognized as a mediator of chemo-resistance in small cell carcinomas of the lung (43). Our recurrent observation of HPV18 integrants that target *CASC21* in small cell carcinomas of the cervix might help decipher the oncogenesis of this poorly documented tumor (44).

Whether HPV integration acts in carcinogenesis via the alteration of cellular genes remains a subject of debate (45, 46) but the role of viral mutational insertion in oncogenesis has been well documented in other models. For instance, it was recently reported that the HTLV-1/BLV proviruses insertion acts early in leukemogenesis via the cis-perturbation of driver genes (47). Three of the driver genes targeted by HTLV1 integration, *CDX1, KLF12, KMT2C*, were also the target of HPV insertions in our case panel and corresponding hybrids were identified in the blood of patients. The extent of the NGS-based tumor-associated HPV DNA analyses to the characterization of ctHPV DNA should be of value not only for diagnostic purposes, but also to facilitate the identification of specific tumor markers that may point to deregulated pathways (23, 48) or that allows optimal characterization of the viral sequences that constitute potential therapeutic targets (49).

We report that the detection of ct-HPV DNA and the characterization of the viral pattern in HPV-associated invasive carcinomas can be achieved via the analysis of a standard blood sample with a high level of sensitivity and specificity, whatever the tumor localization and the viral genotype. The clinical validation of the "liquid biopsy approach" in HPV-associated tumors should be helpful in certain circumstances to avoid more invasive diagnostic procedures. We show in addition that the complete characterization of the viral sequences provides the identification of specific tumor markers, and, potentially, of pathway disorders. Finally, our CaptHPV approach could be extended, using the same basic procedure, to capture and characterize other viral DNA/RNA sequences implicated in oncogenesis, such as MCPyV (50), EBV (51), or HTLV1 (52), opening up a systematic approach that provides screening of various viral-associated malignancies.

Authors' Disclosures

A. Harlé reports grants from Institut National du Cancer during the conduct of the study. No disclosures were reported by the other authors.

Authors' Contributions

X. Sastre-Garau: Conceptualization, formal analysis, supervision, validation, writing–original draft, writing–review and editing. **M. Diop:** Investigation. **F. Martin:** Investigation, writing–review and editing. **G. Dolivet:** Investigation. **F. Marchal:** Investigation. **C. Charra-Brunaud:** Investigation. **D. Peiffert:** Investigation. **L. Leufflen:** Investigation. **B. Dembélé:** Investigation. **J. Demange:** Formal analysis, investigation. **P. Tosti:** Project administration. **J. Thomas:** Investigation. **A. Leroux:** Investigation. **J.L. Merlin:** Investigation. **H. Diop-Ndiaye:** Investigation. **J.M. Costa:** Investigation. **J. Salleron:** Data curation, methodology, writing–review and editing. **A. Harlé:** Conceptualization, data curation, formal analysis, supervision, validation, writing–review and editing.

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