Circulating Tumor DNA as a Biomarker in Squamous cell Carcinoma of the Head and Neck

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List of Abbreviations

5-FU	5-fluorouracil
AACR	American Association for Cancer Research
ANE	Areca nut extract
AP	apoptosis proteins
BER	base excision repair
bp	base pair
CA15.3	cancer antigen 15.3
CAPP-Seq	CAncer Personalized Profiling by deep Sequencing
CEA	carcinoembryonic antigen
cfDNA	circulating free DNA
CHIP	Clonal hematopoiesis of indeterminate potential
cHPV	circulating HPV
CNA	copy number alterations
COSMIC	Catalogue of Somatic Mutations in Cancer
CPS	combined positive score
CR	complete response;
CRC	colo-rectal cancer
CRT	chemoradiation, chemoradiotherapy
CSF	cerebrospinal fluid
ctDNA	circulating tumor DNA
CYP2E1	cytochrome P450
ddPCR	droplet digital PCR
DFS	disease-free survival
DNA	Deoxyribomucleic acid
DNMT	DNA methyltransferase
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EMA	European Medical Agency
ENE	extra nodal extension
FDA	Food and Drug Administration
FDG	fluorodeoxyglucose
gDNA	germline DNA
GSP	glutathione S-transferase
Gy	Gray

HNC	Head and Neck cancer
HPV	Human Papillomavirus
HR	hazard-ratio
IAP	apoptosis proteins inhibitors
IARC	International Agency for Research the on Cancer
ICI	Immune Checkpoint inhibitors
iDES	integrated Digital Error Suppression
IHC	immunochemistry
IMRT	intensity-modulated radiotherapy
LA	Locally-advanced
lncRNA	long non-coding RNA
LOH	Loss of heterozygosity
LRC	locoregional control
mAb	monoclonal antibody
mtDNA	mitochondrial DNA
MRD	minimal residual disease
NAC	neoadjuvant chemotherapy
NAT	neoadjuvant treatment
NE	Not estimable
NER	nucleotide excision repair
NGS	Next Generation Sequencing
NSCLC	non-small cell lung cancers
OPC	oropharyngeal carcinoma
ORR	objective response rate
OS	Overall survival
OSCC	oral squamous cell carcinoma
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed-cell death-1
PD-L1	programmed-cell death-liguand-1
PET	positron emission tomography
PFS	progression-free survival
PM	particulate matter
QoL	quality of life
qPCR	quantitative PCR
R/M	Recurrent/metastatic

RECIST	Response Evaluation Criteria in Solid Tumors
RFS	relapse-free survival
RNA	ribonucleic acids
RT	Radiotherapy
RTOG	Radiation Theray Oncology Group
SCC	squamous cell carcinoma
SCCHN	Squamous cell carcinoma of the head
SEER	Surveillande Epidemiology and End results
Sn	sensitivity
SSNV	somatic single-nucleotide variants
TCGA	The Cancer Genome Atlas
TLM	transoral laser microsurgery
TPS	tumor proportion score
TTMV	tumor tissue modified viral
UDPGT	uridine diphosphate-glucuronosyl transferase
UMI	Unique Molecular Identifier
VAF	variant allele frequency
VEGF	vascular endothelial growth factor
VMAT	volumetric modulated arc therapy
WES	Whole Exome Sequencing
WGS	whole genome sequencing
WHO	Wold Health Organization

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I. INTRODUCTION

I.1 Squamous cell carcinoma of the head and neck

Head and neck cancer (HNC) is an entity that regroups a diverse group of rare diseases originating from different anatomic subsites, including the lip and oral cavity, the pharynx (oropharynx, nasopharynx, hypopharynx), the larynx, the nasal cavities and the salivary glands. Each entity, taken separately, has an age-adjusted standardized world incidence rate of less than 6 per 100 000, which is on the threshold of rare cancers according to the definition of RARECARE.[1] While HNC has different histological subtypes, 90% are derived from squamous cells. Squamous cell carcinomas arise from the epithelial cells that line the mucosal surfaces of different anatomical subsites in the head and neck region (Figure I-1). This thesis focuses on squamous cell carcinomas of the oral cavity, oropharynx, hypopharynx, and larynx (SCCHN). Globally, SCCHN is the seventh most common malignancy with approximately 504,000 men and 157,000 women developing cancer originating from oral, oropharyngeal or laryngeal sites, accounting for 3.7% of all cancers.[2]

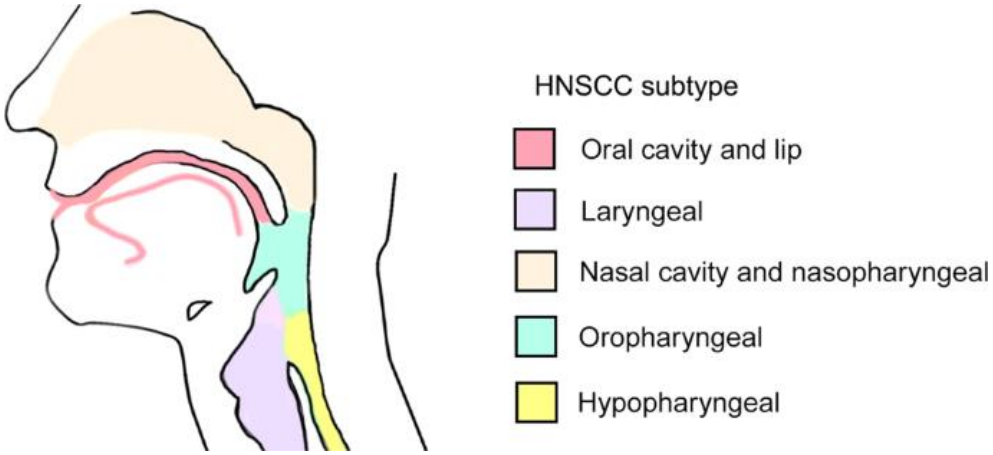

Figure I-1: SCCHN localizations.
Oral cavity cancer is found in the buccal mucosa, oral tongue, alveolar ridge, floor of the mouth, hard palate, and retromolar trigone. Laryngeal cancer is found in the supraglottis, vocal cords, glottis, and subglottis. Oropharyngeal cancer is found in the soft palate, base of the tongue, tonsillar pillars, and tonsillar fossa.[3] (Adapted from Shin, E. Exp Mol med 52, 1264–1274)

Treatment of SCCHN depends on the location of the primary tumor, the stage of the disease, and the expected oncological and functional outcomes. Early

disease stages are usually treated with one single treatment modality (surgery or radiotherapy (RT)); however, approximately 60% to 80% of SCCHN patients present loco-regionally advanced disease at diagnosis. The treatment of locally advanced disease is multimodal and includes surgery and/or RT and/or chemotherapy. Patients with incurable SCCHN are treated with palliative systemic treatment (platinum-based chemotherapy, anti-programmed cell death-1 (PD1) monoclonal antibodies (pembrolizumab or nivolumab), and/or cetuximab).[4,5]

I.1.1 Epidemiology

In 2020, more than 661 000 cases of SCCHN were reported worldwide, ranking it as the seventh most common cancer.[6] SCCHN is responsible for 3.7 % of all cancer related deaths.[6] The incidence of SCCHN varies according to geographical area. In North America, more than 57,502 new cases were diagnosed in 2020, resulting in an annual incidence of 15 per 100 000, with 13,521 deaths attributed to the disease. In Europe, the incidence rates of SCCHN are higher, with approximately 153,377 new cases diagnosed in 2020, corresponding to an annual incidence of 43 per 100 000. Oral cavity cancer is the most common subsite (40%), followed by laryngeal (20%). Overall, SCCHN is more frequent in males than females, except in Human Papillomavirus (HPV) induced cancers[7], and the ratio ranges from 2:1 to 4:1 depending on the anatomical subsite.[6] The median age at diagnosis is in the sixth decade of life. In Europe, the survival rate for adults with HNC is 72% at one year and 42% at five years. SCCHN incidence is influenced by risk factors. The Surveillance, Epidemiology, and End Results (SEER) data show that the incidence of tobacco and/or alcohol-induced malignancies has been decreasing since the early 1980s[8], reflecting declining smoking habits. By contrast, the incidence of Human Papillomavirus (HPV)-related malignancies of the tonsil, oropharynx and base of tongue is increasing, most likely due to changes in sexual behaviors.[9]

I.1.2 Risk Factors

Cancer is generally caused by a progressive accumulation of genomic modifications, leading to the inactivation of tumor suppressor genes or the activation of proto-oncogenes, the latter expressing proteins that are altered in quantity or quality. Tobacco use and alcohol consumption are the main risk factors associated with SCCHN, accounting for approximatively 70% of all cases.[8] The Human Papillomavirus (HPV) is another risk factor mainly for oropharyngeal cancer. The prevalence of SCCHN attributable to HPV varies widely across the globe but is estimated to be around 30%. HPV-positive oropharyngeal cancer patients have a significantly better outcome with greater responses to chemotherapy and RT than patients diagnosed with HPV-negative disease.[10]

I.1.2.1 Tobacco *and alcohol*

Acting synergistically, tobacco use and alcohol consumption are the two most important risk factors for the development of SCCHN. In a pooled analysis, the International Head and Neck Cancer Epidemiology Consortium reported that 72% of SCCHN were related to tobacco and/or alcohol, of which 4% was due to alcohol alone, 33% to tobacco alone, and 35% to tobacco and alcohol.[11] Benzopyrene, the component of tobacco smoke, is a well-known carcinogen that forms covalent deoxyribonucleotide (DNA) adducts after metabolic activation and induces mutations.[12] Acetaldehyde, the first metabolite of ethanol, is also mutagenic and interferes with DNA repair machinery. However, not all smokers or drinkers will develop cancer. Genetic polymorphisms in xenobiotic-metabolizing-enzymes have been described and determine an individual's susceptibility to carcinogens.[13] Most carcinogens need to be metabolized by specific enzymes before activation, and some studies suggest that polymorphisms on the cytochrome P450 group, which render the enzyme more active, could explain why some people have a higher risk of developing SCCHN when exposed to tobacco.[13,14] Similarly, while detoxifying enzymes, such as glutathione S-transferase (GSP) or uridine diphosphate-glucuronosyl transferase (UDPGT), can deactivate some carcinogens, their protective activity can be decreased by polymorphisms.[15] The induced damage may be repaired through the

nucleotide excision repair (NER) or the base excision repair (BER) systems; the latter is a correction system enabling the removal of a single base pair when a cytotoxic mutation is detected. Sequence variations in *NER/BER* genes may also explain inter-individual variations to tobacco toxins.[12,16] Compared to non-smokers, tobacco users have a 4 to 5-fold increased risk of developing cancer in the oral cavity, oropharynx, and hypopharynx, and a 10-fold increased risk of developing laryngeal cancer.[17,18] Alcohol also increases the risk of upper aerodigestive tract cancer.[19] Chronic alcohol exposure results in increased activation of carcinogens by cytochrome P450 2E1 (CYP2E1).[20] On the other hand, the accumulation of acetaldehyde and other alcohol metabolites could affect gene transcription or have mutagenic effects.[15]

E-cigarettes may also present danger regarding head and neck cancer: although less harmful than conventional tobacco use, e-cigarettes contain components, such as benzoanthracen, benzopyren, formaldehyde, arsenic, etc. known to cause HNC, through DNA damage and oxidative stress.[21,22] The role of marijuana smoking is still unclear[23], even though some studies have reported a dose correlation.[24] As e-cigarettes and marijuana use is rising, especially in the younger population, more epidemiology studies are necessary to better understand those possible risk factors.

I.1.2.2 Human *Papillomavirus*

The human papillomavirus is a small circular DNA virus that has the ability to infect the basal cells of the squamous epithelium and it can be transmitted through sexual contact.[25,26] HPV-positive SCCHN arises mostly in deep tonsillar crypts and the base of the tongue (oropharynx). HPV is a well-established independent risk factor, linked from 0 to 85% of all oropharyngeal cancers (OPC).[25] However, we observe a large range of incidence from one geographic area to another: 0% in South India, 85% in Lebanon,[7] 72% in North America,[27] 31% in northern and western Europe, and 13% in the rest of the world.[28,29]

The incidence of HPV-related OPC has increased over the last decades, which has been linked to an increase in oral sexual practices. High-risk HPVs include types 16, 18, 31 and 33, with HPV type 16 accounting for over 90% of HPV-positive oropharyngeal cancer.[30] HPV-positive SCCHN differs

demographically, molecularly and clinically from HPV-negative tumors. Clinically, HPV-positive oropharyngeal cancer patients are younger, have a better performance status, consume less alcohol and tobacco, have more sexual partners, and frequently present a smaller primary tumor associated with multiple cystic lymph nodes.[30-32] HPV associated oropharyngeal cancer is associated with a favorable prognosis compared to HPV-negative tumors.[33] The meaning of HPV infection in subsites other than oropharynx is unknown and controversial. Although the incidence of HPV-induced OPC has been rising over the last decades, the recent worldwide HPV-vaccine campaigns seem to have had a positive effect on the relative prevention percentage and has been evaluated to 84% for oropharyngeal cancers.[34]

I.1.2.3 Other risk factors

Betel quid chewing is a popular habit in India and Southeast Asia where the incidence of squamous cell carcinoma (SCC) of the oral cavity is the highest in the world.[35,36] Areca nut extract (ANE), the main component of betel quid, has been documented to induce negative oxygen species and to, consequently, cause genetic damage.[37] ANE usage is tightly linked to oral cancer. However, the mechanisms of carcinogenesis remain unclear.[38]

Similarly to lung cancers[39], air pollution has been shown to participate in head and neck cancers[21] and the World Health Organization (WHO)'s specialized cancer agency, the International Agency for Research on Cancer (IARC) has recently classified outdoor air pollution and particulate matter (PM) as IARC Group 1 carcinogen. The exact role in head and neck carcinogenesis is, however, still unclear.

Other reported risk factors include: Plummer–Vinson syndrome[40,41], long-term immunosuppression[42], gastro-esophageal reflux[43,44], poor oral hygiene[45,46], and ill-fitting dentures.[47] Rosenquist and colleagues reported that poor oral hygiene, inadequate dental status and malfunctioning complete dentures were independent risk factors for oral and oropharyngeal squamous cell carcinomas.[48] Fanconi's anemia[49,50], Dyskeratosis congenital[51], Bloom's syndrome[52], ataxia telangiectasia[53], Li-Fraumeni syndrome and xeroderma pigmentosum are inherited causes of head and neck cancer.[54]

I.1.3 Prognosis factors

In early stage SCCHN (stage I/II), there is a high cure rate of 80-90% with surgery and/or RT[55]; however, more than 50% of patients are diagnosed with a locally-advanced (LA) disease (stage III/IV). Despite multimodal treatment of locally-advanced SCCHN with combined surgery and/or (chemo)RT, less than 60% of patients will remain disease-free after three years.[56] Patients with recurrent/metastatic (R/M) disease that are not amenable to RT or surgery have a median survival of 12-15 months. The prognosis depends on different factors including tumor-related factors (staging, location, HPV status, PD-L1 expression, molecular markers)[57], patient-related factors (age, toxicity habits, comorbidities) and treatment related factors (surgical margins, response to chemotherapy/RT).[58] Risk stratification is required to determine the optimal treatment modalities and prognosis.

I.1.3.1 Tumor-related factors

The most important prognostic factor is tumor staging based on TNM classification.[59] Physical examination, imaging and pathological analysis enables the size and extent of the primary tumor (T), cervical lymph node status (N) and distant metastasis (M) to be determined. An accurate TNM staging for each patient is of utmost importance for adequate treatment selection. The anatomical subsite is also an important predictor of outcome with the hypopharynx having the worst prognosis (all stages, 5-year overall survival (OS) of 25%), whilst the larynx provides the best outcome (all stages, 5-year OS of 59%).[60] Malignancy grade[61], perineural invasion and vascular invasion have also been reported as being related to poor outcome.[62] It is well established that HPV is an independent prognostic factor. Ang and colleagues reported on the outcome of patients with OPC according to HPV status treated in the Radiation Therapy Oncology Group (RTOG) 0129 study.[63] In this trial, patients with advanced SCCHN were randomized to cisplatin with standard fractionated RT or accelerated fractionated RT. Patients with HPV-positive OPC had a better outcome than patients with HPV-negative OPC with a 3-year overall survival (OS) of 82.4% vs 57% ($p < 0.001$) and a 58% reduction in the risk of death (hazard ratio: 0.42; 95% CI: 0.27 to 0.66). Patients were classified as having a low, intermediate, or high-

risk of death on the basis of four factors: HPV status, more or less than 10 pack-years of tobacco smoking, tumor stage, and nodal stage. HPV status has been implemented in the 8th edition TNM staging system to take into consideration the characteristics and prognosis of this relatively new disease. Due to their better prognosis, de-escalating strategies are under investigation in HPV-positive OPC but still fail to prove clinical benefit.[64,65] There is sufficient evidence to strongly recommend that future clinical trials, including the ones evaluating new targeted therapies, investigate their effects in the two distinct subsets of HPV-negative and -positive disease. Randomized studies should also select, or at least stratify, patients according to their HPV status.[66]

An important prognosis[67] and predictive factor for anti-PD(L1) therapy efficacy in R/M SCCHN is the expression of PDL-1 on cell surface, evaluated by immunochemistry. For R/M SCCHN, the combined positive score (CPS) is calculated as follows:

$$CPS = \frac{Number\ of\ cells\ expressing\ PDL-1\ on\ their\ surface\ (all\ tumor\ and\ immune\ cells)}{Total\ number\ of\ viable\ tumor\ cells}$$

This score takes into account the PD-L1 expression on tumor and immune cells. In R/M SCCHN, pembrolizumab (anti-PD1) has been shown to be more active in patients with PD-L1 expressing SCCHN.[68,69]

I.1.3.2 *Patient-related factors*

Patient-related factors also need to be considered for outcome prediction. A good Eastern Cooperative Oncology Group (ECOG) performance status is linked to better survival.[70] By contrast, advanced age, presence of comorbidities, poor performance status, active drinking and/or smoking and malnutrition or weight loss have been reported to be predictive of worse outcome.[71-73]

I.1.3.3 Treatment-related factors

Margin status at the time of surgical resection is a key prognostic factor as residual carcinoma at the margins is a risk factor for local recurrence.[74,75] Positive surgical margins are an indication for adjuvant RT combined with

chemotherapy (CRT).[76] The presence of nodal extracapsular extension is also an indication for adjuvant CRT.

Another treatment-related prognostic factor is the response to chemotherapy and RT. A negative post-treatment fluorodeoxyglucose (FDG)-positron emission tomography (PET) scan is also linked to favorable prognosis.[77]

I.1.3.4 Molecular-related factors

Some molecular markers are also known to influence patient outcomes.[78-80] *TP53* is a tumor suppressor gene that negatively regulates the cell cycle and is implicated in DNA repair and apoptosis. *TP53* is called "the guardian of the genome" as it protects the cell from propagating mutations. It has been shown that *TP53* mutations are associated clinically with resistance to RT and chemotherapy and to worse outcome.[81] Other molecular markers have been linked to prognosis. Among others, vascular endothelial growth factor (VEGF) expression, a marker of angiogenesis, has been shown to correlate with locoregional recurrence, metastasis and poor survival.[82] Upregulation of Cyclin D1 (encoded by *CCND1*), a protein regulating the G1 phase of the cell cycle, is also linked to poor prognosis, although some studies suggest that Cyclin D1 overexpression may increase a patient's sensitivity to definitive radiation.[83-85] Finally, epidermal growth factor receptor (EGFR) protein overexpression, or increased *EGFR* gene copy number, is associated with radio resistance and poor prognosis.[86,87]

I.1.4 Carcinogenesis and molecular landscape of SCCHN

Carcinogenesis is the biological process by which a normal cell is transformed into a cancer cell, by accumulation of genetic, but also epigenetic, alterations in genes coding for proteins implicated in cell growth, survival, invasion and motility. As it becomes evident that HPV-negative SCCHN and HPV-positive OPC are two distinct entities, studies comprehensively analyzing the molecular landscape have shown that the carcinogenic process and genomic alterations differ (Figure I-2). Klussmann and colleagues described the genetic differences between HPV-related and HPV-unrelated tumors of the oropharynx.[88] They found that chromosomal alterations and amplifications were significantly lower in HPV-positive, as

compared with HPV-negative, OPC. The molecular landscape of SCCHN was published by The Cancer Genome Atlas (TCGA) consortium in 2015, and it included both HPV-negative and HPV-positive tumors.[89] These data highlighted the very common alteration of *TP53* and *CDKN2A* genes in HPV-negative SCCHN and the high frequency of *PIK3CA* alterations in HPV-positive SCCHN. Figure I-3 illustrates the most frequent alterations in the driver genes of HPV-negative and HPV-positive SCCHN that will be discussed in more details in the following paragraphs.

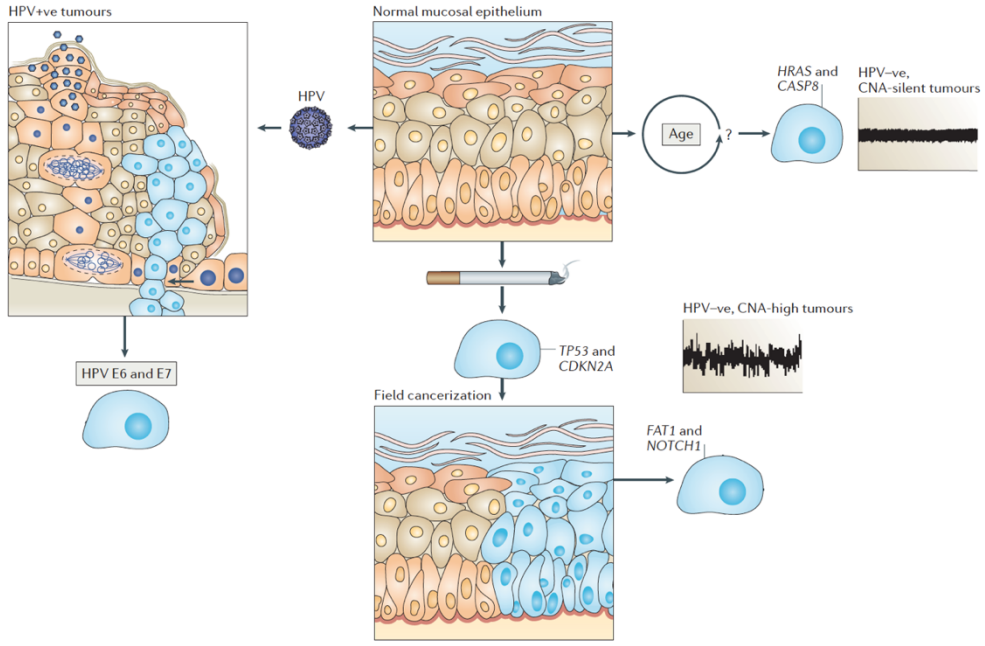

Figure I-2 : Different carcinogenic processes of SCCHN.A schematic overview of the different carcinogenic processes of SCCHN is shown. There are two main different processes and these are distinguished by the presence or absence of the human papillomavirus (HPV). In oral squamous epithelium, HPV infection will lead to productive infections. In specific oropharyngeal cripts, indicated in light blue, HPV infection might induce oncogenesis, mediated by the HPV oncoprotein E6 and E7. Tobacco induced HPV-negative SCCHN is characterized by the process of field cancerization, and early events include alterations in the TP53 and CDKN2A genes. Other frequently altered genes are FAT1 and NOTCH1. These tumors also have numerous copy number alterations (CNA). There is another group of HPV-negative SCCHN that are CNA-silent tumors for which the main risk factor appears to be ageing. These tumors harbor HRAS and CASP8 mutations. More details are indicated in the main text. Figure adapted from Leemans and colleagues.[10]

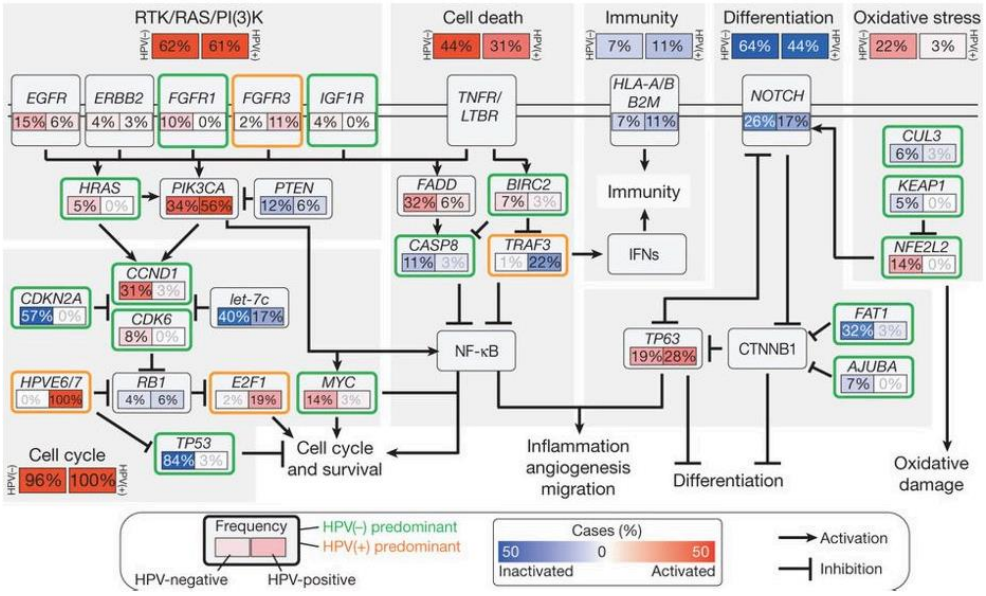

Figure I-3 : The frequency (%) of alterations in the driver genes of HPV-negative and HPV-positive
Pathways leading to cell survival, proliferation, migration or differentiation are frequently altered during carcinogenesis. The most frequent genetic alterations involved in the carcinogenesis of HPV-negative (left boxes) and HPV-positive (right boxes) SCCHN (n=279) are represented in the above figure. Genes may be activated (red) or inactivated (blue) by mutations, amplifications, heterozygous and homozygous losses, or epigenetic changes. Figure adapted from The Cancer Genome Atlas.[89]

I.1.4.1 HPV-negative SCCHN

HPV-negative SCCHN results from a multistep carcinogenesis process that enables the progressive transformation of the normal epithelium to hyperplasia, mild dysplasia, carcinoma *in situ* and finally invasive SCCHN.[90] The majority of patients present with *de novo* tumor, but some preneoplastic lesions are visible during clinical examination as white plaques (leukoplakia) or red areas in the mucosa (erythoplakia).[91] The anatomopathological analysis of these lesions allows the detection and grading of dysplasia. However, many pre-cancerous lesions are not visible during clinical examination and mucosal abnormalities are only diagnosed by pathological examination. These morphological alterations led

to the concept of “field cancerization” described in 1953 by Slaughter.[92] This phenomenon explains the high recurrence rate after treatment and the high prevalence of other secondary primary tumors in the mucosal linings. A comprehensive analysis of the genetic basis of this field cancerization was first described in the 1990’s, leading to the first genetic progression model of SCCHN.[93] The landscape of chromosomal loss increases with the progression of histopathological features. Hyperplasia is characterized by a loss of heterozygosity (LOH) on chromosomes 3p, 9p and 17p. Losses at these loci increase in frequency in dysplastic lesions and are found in more than 60% of patients with SCCHN, indicating that they are early events in HNC carcinogenesis.[94-96] *FHIT* and *CDKN2A* are located at the 3p14.3 and 9p21 loci, respectively, and are tumor suppressor genes. These genomic alterations were also observed in the mucosal epithelial cells of surgical margins, which explains the high recurrence rate.[97,98] *TP53* is a tumor suppressor gene mapped on chromosome 17p13.[94] Premalignant head and neck lesions harbor *TP53* mutations in 50% to 80% of tumors, suggesting that these mutations are also an early carcinogenic event in SCCHN.[89]

I.1.4.2 HPV-positive SCCHN

In HPV-positive tumors, malignant transformation begins with degradation of the p53 tumor suppressor protein mediated by the HPV oncoprotein E6 (Figure I-4). There is a lower incidence of *TP53* mutations found in HPV-positive tumors compared to HPV-negative.[99] E7, the second HPV oncoprotein, inactivates the retinoblastoma (Rb) tumor suppressor protein.[100] The *E2F1* gene is amplified in approximatively 20% of HPV-positive tumors, adding to cell cycle deregulation. In HPV-positive tumors, p16 (encoded by the *CDKN2A* gene) is overexpressed due to the loss of negative feedback induced by the inactivation of Rb by HPV E7.[101,102] The gold standard for diagnosing HPV-induced SCCHN is E6 and E7 mRNA detection by reverse transcription PCR, but it can be technically challenging. p16 overexpression is now considered to be a surrogate marker for HPV-induced OPSCC and is easily detected by immunochemistry (IHC).[103,104] The most frequent gene alteration in HPV-positive SCCHN is in the *PIK3CA* gene,which is altered in 56% of cases by mutation or amplification.[89,105] Other alterations in HPV-positive SCCHN are rare. *FGFR3* mutations are observed in 1-12% of HPV-positive patients. HPV-specific loss of chromosomes 14q32 and 11q,

containing the tumor-suppressor genes *TRAF3* and *ATM1*, respectively, have been reported. Finally, preclinical studies have shown that HPV-positive SCCHN have DNA double strand repair defects.[106]

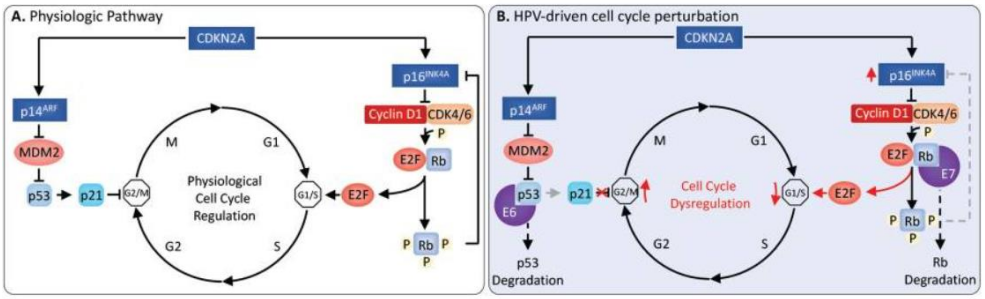

Figure I-4: Cell cycle perturbation by human papillomavirus
(A) Schematic representing physiologic cell cycle regulation. Tumor suppressor proteins are marked in blue, proto-oncoproteins are marked in red. The CDKN2A gene encodes $p14^{ARF}$ and $p16^{INK4A}$ proteins. $p14^{ARF}$ inhibits MDM2, thereby disinhibiting p53 to activate p21 and stop progression through the G2/M checkpoint into mitosis. $p16^{INK4A}$ inhibits the CyclinD1/CDK4 and CyclinD1/CDK6 complexes. These complexes catalyze phosphorylation of the retinoblastoma protein (Rb), inducing it to release E2F family transcription factors to enter the nucleus and activate transcription of S-phase promoting transcripts. Phosphorylation of Rb also results in feedback inhibition of $p16^{INK4A}$ expression. (B) HPV E6 oncoprotein binds and targets p53 for degradation, resulting in loss of G2/M checkpoint regulation. HPV E7 oncoprotein binds and targets Rb for degradation, resulting in the nuclear translocation of E2F and promotion of S-phase transition. In addition, downregulation of Rb results in the loss of feedback inhibition and overexpression of $p16^{INK4A}$. Figure adapted from Faraji et al.[107]

I.1.4.3 Recurrent/metastatic SCCHN

Most of the information on the genomic landscape of SCCHN has been obtained by exome studies of primary SCCHN, and data on the genomic profile of recurrent/metastatic (R/M) disease are limited.[108-110] Tumor evolution and subclonal proliferation, together with potential mutations induced by previous treatment (chemotherapy or radiation), suggest that the genetic landscape is different in the R/M setting. Two papers compared their sequencing data in R/M SCCHN to TCGA data. Their findings suggested a higher rate of *TERT* promoter mutations in R/M HPV-negative SCCHN (43%)[108], a higher frequency of alterations in the histone modifier *BAP1* gene

and the DNA mismatch repair *MSH2* gene in persistent/recurrent laryngeal SCC,[110] and the tendency for R/M HPV-positive tumors to present a "HPV negative-like" molecular profile with more *TP53* mutations (3/20, 15%), whole genome duplication (20%) and 3p deletions (55%).[108] These data were, however, limited by their small sample sizes. A study focusing on molecular alterations in samples from the BERIL 1 trial reported on the sequencing of tumor samples genetic profile in line with previous reports with *TP53 (38%), FAT1 (14%), CDKN2A (6%), NOTCH1 (8%)* being the most frequently mutated genes.[111] Siu and colleagues reported the results of a comparison of three genomic databases[112]: (i) data from TCGA on primary tumors; (ii) data from the American Association for Cancer Research Project Genomics Evidence Neoplasia Information Exchange (AACR project GENIE), an international data-sharing project of cancer genomic data that includes 700 samples of SCCHN, of which 40% had metastatic disease; and (iii) data from the Catalogue of Somatic Mutations in Cancer (COSMIC).[112] They found similar frequencies of the most common somatic mutations across the three databases. However, the ability to capture intratumor heterogeneity on an individual basis may also be important. A comparison of eight pairs of SCCHN primary tumors and metachronous recurrence revealed that 90.1% of the primary tumors' somatic single-nucleotide variants (SSNVs) were transmitted to the metachronous recurrent tumor. However, 40% of the SSNVs that were identified in the recurrent tumors were not present in the matched primary tumor, including mutations in the discoidin domain receptor tyrosine kinase 2 (*DDR2)* gene and *CCND1* amplifications.[109] Van Ginkel et al. analyzed 46 matched tumor pairs of primary tumor and regional/distant metastasis as well as 23 pairs of primary tumors and recurrences, revealing that 92% and 89% of the analyzed mutations, respectively, were concordantly present in both samples. Mutations not present in the primary tumors included *SMAD4* and *ALK* in two metastatic samples, and *FLT3, CDKN2A*, *KIT*, *TP53* and *HRAS* mutations in recurrent samples.[113] These limited data illustrate the potential intra-patient tumor heterogeneity through space and time.

I.1.5 Treatment strategies for SCCHN

Curative treatment of SCCHN depends on the tumor's localization and extension, patient age and performance status, and the anticipated functional outcome and long-term toxicity.[114] SCCHN should be staged according to the 8th edition TNM system.[4,115] A multidisciplinary treatment schedule should be established in all cases.[114,116] Limited or early-stage disease is the presenting stage in approximately 40% of patients and is usually treated with surgery or radiation alone; surgery and RT has shown similar locoregional control (LRC), based on non-randomized retrospective studies. Approximately 60% to 80% of SCCHN patients present with loco-regional advanced disease (LA-SCCHN) at time of diagnosis.[55] One treatment option is surgery plus postoperative RT and, for high-risk patients (nodal extracapsular extension and/or R1 resection), post-operative chemoradiotherapy (CRT). Radical CRT is another treatment option, even in resecable patients, when the anticipated functional outcome and/or the prognosis is so poor that mutilating surgery is not justified. The treatment of p16-positive oropharyngeal cancer is similar to p16-negative SCCHN. Due to its better prognosis, de-escalating strategies are under investigation in HPV-positive oropharyngeal cancer but are not yet standard of care.[64,117]

I.1.5.1 Local and locally advanced SCCHN

I.1.5.1.1 Surgery

In early stage SCCHN (stage I-II), a single modality treatment for oral cavity, oropharyngeal, and laryngeal lesions is recommended. The choice between surgery and radiotherapy depends on the localization, stage of the tumor, performance status and age of the patients and expected functional results. Minimally invasive surgical treatments, including transoral laser microsurgery (TLM) and transoral robotic surgery (TORS), could offer the benefit of organ preservation with less functional morbidity than open surgery and often less long-term toxicity than radiation therapy, provided that they do not impair the functional outcome (e.g., speech and swallowing) and do not require post-operative radiotherapy due to residual tumor.[118-120] For exophytic tumors limited to one vocal cord, TLM has shown promising results, without loss in survival and functional recovery compared to surgery or radiotherapy.[121]

This is especially relevant given the increasing incidence of HPV-positive disease, as these patients tend to be younger and have a better long-term prognosis than those with HPV-negative SCCHN. This change in patient profile has strengthened interest in functional organ preservation surgery to improve functional outcomes and patient quality of life (QoL). Similarly, TORS is associated with improved surgical outcomes in early-stage oropharyngeal cancer.[122,123] Despite the growing popularity of TORS in the treatment of oropharyngeal cancer, this approach has several potential drawbacks. Importantly, the use of TORS does not obviate the need for postoperative RT in some cases.[124] Even though the head and neck is relatively accessible, access and manoeuvrability are sometimes limited by anatomic resections. TLM is currently considered standard of care for early glottic cancer, but TORS has also been used to treat early stage glottic carcinomas despite limited data.[125,126]

When surgery is the primary treatment, neck dissection is recommended for most tumors except for early tumors of the vocal cord. The rationale for a selective neck dissection is based on known patterns of metastases from each site; the risk of occult lymph node metastases in patients with clinically N0 disease is around 30%.[127] Neck dissection is also recommended in patients treated with primary chemoradiation when residual disease is suspected 12 weeks after the end of chemoradiation.[128-130]

I.1.5.1.2 Radiotherapy and concomitant chemoradiation

In LA-SCCHN, RT is effective both as a primary modality concurrently with chemotherapy and as an adjuvant treatment following surgery.[4] Besides the treatment of the primary tumor itself, elective lymph node irradiation by external beam RT is necessary to eradicate subclinical disease. Concomitant platinum-based CRT is the standard of care in LA-SCCHN in both non-resectable and resectable patients when the anticipated functional outcome and/or prognosis is so poor that mutilating surgery is not justified.[4]

The updated meta-analysis of chemotherapy in combination with RT for SCCHN (MACH-NC) showed that the addition of chemotherapy concomitantly to RT improves absolute 5-year survival by 6.5%.[116,131] High-dose cisplatin (100 mg/m2 day 1, 22 and 43) remains the standard radiosensitizer for the treatment of SCCHN, although one underpowered trial

suggests that weekly cisplatin (40 mg/m2/weekly) is not inferior than high-dose cisplatin.[132] The role of induction chemotherapy with docetaxel, cisplatin and 5-fluorouracil (5-FU) is controversial as it failed to demonstrate a statistically significant difference in OS and progression-free survival (PFS) compared to CRT alone.[133,134] However, for larynx preservation, induction chemotherapy and concomitant CRT give similar long-term survivals even if concomitant CRT improves the rates of larynx preservation and local control at 5 and 10 years compared with induction therapy or RT alone.[135]

After surgery, peri-neural disease, vascular invasion or positive lymph nodes, and stage III/IV are indications of adjuvant radiotherapy. The addition of chemotherapy to radiation is indicated in case of positive surgical margins and nodal extracapsular extension.[136]

Cetuximab is a chimeric mouse-human IgG1 monoclonal antibody (mAb) that binds to the EGFR extracellular domain and prevents its ligand-mediated activation.[137] Cetuximab combined with RT improves the duration of locoregional recurrence free survival (24.4 vs 14months; p-value: 0.005) and OS (49 vs 29.3 months; p-value:0.03) compared to RT alone without increasing radiation-induced side effects.[138] The cetuximab-RT combination is therefore considered an alternative to CRT if patients cannot tolerate chemoradiation. Recently, two randomized trials have been reported in patients with p16-positive oropharyngeal SCCHN treated with either concomitant 3-weekly cisplatin (100 mg/m2) and RT (70 Gy), or weekly concomitant cetuximab (250 mg/m2), and the same RT regimen. Although these two trials enrolled slightly different patient populations (*i.e.* low risk patients in the UK De-Escalate study and all risk patients in the RTOG 1016 study), both trials demonstrated a lower OS in the cetuximab arm with no reduced acute and late morbidity rates.[139,140] Similar results were obtained in another phase 3 trial that compared conventional RT plus weekly cisplatin (40 mg/m2) versus RT (70 Gy) plus cetuximab.[141] Therefore, the recommendation is to favor cisplatin-based chemoradiation. Cetuximab plus RT can be a treatment alternative for some patients not fit for platinum-based chemotherapy.

To minimize toxicity following RT for SCCHN, there is a shift from the simpler RT techniques towards more advanced techniques. By varying the beam intensity across shaped radiation fields, intensity-modulated radiotherapy (IMRT) reduces the dose of radiation to organs at risk, such as the parotid glands, potentially reducing xerostomia. Randomized trials have shown that IMRT significantly reduces the incidence of xerostomia and improves QoL without jeopardizing LRC and OS.[142] IMRT is therefore standard of care in SCCHN. Today, all patients receiving RT should be treated with IMRT or its variant - Volumetric Modulated Arc Therapy (VMAT).

I.1.5.1.3 Immunotherapy for locally advanced SCCHN*

Pembrolizumab and nivolumab, two monoclonal antibodies (mAbs) targeting programmed cell death protein-1 (PD1), improve the overall survival of patients with inoperable recurrent and/or metastatic (R/M) SCCHN.[143]

For curable SCCHN, the role of immunotherapy is under investigation.[144] The potential role of anti-PD1/PD-ligand (L) 1 inhibitors in the curative treatment of SCCHN, either in combination with curative-intent (chemo)radiation or surgical treatment is described below.

I.1.5.1.3.1 Anti-PD1/PD-L1 mAbs in combination with curative-intent primary surgery.

Surgery remains a treatment of choice for head and neck cancers. However, in locally advanced (LA) disease, more than half the patients will recur even after curative surgery with pathological disease-free margins. There are currently many studies investigating the role of neo-adjuvant or adjuvant immunotherapy in the context of surgery to reduce the risk of disease recurrence.

Anti-PD1/PD(L)1 mAbs were investigated in several pre-operative window of opportunity studies. In a phase II study (NCT03021993), 12 patients with stage II-IVa oral squamous cell carcinoma (OSCC) were treated with three to four doses of nivolumab bi-weekly before curative surgery. The objective

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* *Section adapted from Honoré N., Beyaert S., Machiels JP. (2023). Immune Checkpoint Inhibitors in the Curative Setting: Pre-clinical and Clinical Data. In: Vermorken, J.B., Budach, V., Leemans, C.R., Machiels, JP., Nicolai, P., O'Sullivan, B. (eds) Critical Issues in Head and Neck Oncology. Springer, Cham.*

response rate was 33%, and 10 patients were still alive after a median follow-up of 2.23 years.[145] The phase I/II CheckMate 358 study investigated the safety and efficacy of two pre-operative doses of nivolumab in 26 patients with human papillomavirus (HPV)-positive SCCHN and in 26 patients with HPV-negative SCCHN. Nivolumab induced radiographic tumor shrinkage ≥30% according to RECIST criteria v1.1 in 12.0% and 8.3% of patients in the HPV-positive and HPV-negative cohorts, respectively. In addition, pathological regression was observed in 23.5% of patients with HPV-positive SCCHN and in 5.9% of patients with HPV-negative tumors.[146] A larger phase II study (NCT02641093) included 80 resectable p16-negative SCCHN patients with T3-T4 and/or two or more nodal metastases or clinical extra nodal extension (ENE). Patients were treated neoadjuvantly with pembrolizumab one to three weeks prior to surgery. After surgery, patients received pembrolizumab for a total of six doses with concurrent radiotherapy. Patients presenting a pathologic response had significantly improved disease-free survival (DFS) compared to patients without a pathologic response (93% vs 72%).[147]

Currently, although anti-PD1/PD-L1 therapy in combination with surgery with or without adjuvant (chemo)radiation gives promising signs of activity, the use of these drugs in combination with surgery for curative purposes is still not indicated in routine clinical practice.

Based on the above-mentioned results, several phase II/III studies are underway to better determine the role of anti-PD1/PD(L)1 mAbs in curable SCCHN treated with primary surgery.

I.1.5.1.3.2 Anti-PD1/PD-L1 mAbs in combination with primary curative-intent (chemo)radiation.

Anti-PD1/PD(L)-1 mAbs in combination with (chemo)radiation have been investigated in several indications: (i) as a de-escalation strategy in good prognosis patients (e.g., stage I/II p16-positive oropharyngeal cancer), (ii) to replace chemotherapy in cisplatin-unfit patients, and (iii) as treatment intensification in combination with chemoradiation in poor prognosis patients (e.g., p16-negative LA SCCHN and stage III p16-positive oropharyngeal cancer).[148-150]

As anti-PD1/PD(L)-1 mAbs are generally well tolerated and have limited toxicities compared to standard chemotherapy, immune checkpoint inhibitors (ICI) are being investigated as a potential de-escalation strategy to avoid chemotherapy in good prognosis tumors.[151] HN005 (NCT03952585) is a three-arm randomized trial for non-smoking patients with T1-2 N1 or T3 N0-N1 p16-positive tumors that compares standard chemoradiation to reduced dose radiotherapy (60 Gy + nivolumab) versus reduced dose chemoradiation (60 Gy). Another trial (NCT03799445) combines nivolumab and ipilimumab with a reduced dose of radiotherapy for T1 N2, T2 N1-N2 or T3 N0-N2 SCCHN. The CCTG HN.9/EORTC1740, a randomized phase II trial, is currently investigating durvalumab plus radiation (concomitant and adjuvant) versus standard chemoradiation in p16-positive intermediate risk oropharyngeal cancer. All these trials involve highly selected populations with mainly low-risk oropharyngeal p16-positive cancers.

ICI could also be used to replace chemotherapy in patients unfit for cisplatin. In PembroRad (GORTEC 2015-01)[152], patients with locally advanced SCCHN unfit to receive high-dose cisplatin were randomized between radiotherapy (RT) + cetuximab and RT + pembrolizumab. Pembrolizumab was given only during RT. Loco-regional control (LRC), progression free survival (PFS) and overall survival (OS) were similar between the two groups. However, acute toxicity was lower in the pembrolizumab-RT arm than in the cetuximab-RT arm: 74% versus 92% patients with at least grade ≥3 acute adverse events (p=0.006), mainly due to dermatitis in the radiation field, mucositis and cutaneous rash. In the REACH trial, patients unfit for cisplatin were randomized between avelumab + cetuximab + RT versus cetuximab + RT. In contrast to PembroRad, avelumab was not only administered concomitantly to RT but also for one year as adjuvant therapy. The primary endpoint was PFS. The avelumab-RT-based treatment did not significantly improve PFS compared to cetuximab-RT: the two-year PFS rates were 44% and 31%, respectively (p=0.15).[153]

ICI therapy has also been investigated in combination with cisplatin-based chemoradiation in patients with LA SCCHN. As previously stated, LA SCCHN patients have a high recurrence rate[144], and 40-50% will relapse within two years despite multimodal treatment. JAVELIN 100 compared high dose cisplatin chemoradiation combined with avelumab or placebo. Avelumab was

started one week before the initiation of chemoradiation, was continued every two weeks during chemoradiation, and then maintained as adjuvant treatment for one year. The trial did not meet its primary endpoint: median PFS was 16.9 months in the avelumab arm and not reached in the control arm (p=0.92). Subgroup analysis showed that PD-L1 expressing tumors might benefit from the addition of avelumab, although this analysis was impaired by the low number of patients. In the REACH study, cisplatin fit patients were randomized between high-dose cisplatin chemoradiation and RT + avelumab + cetuximab. The trial was, however, closed prematurely for futility.[153] The KEYNOTE-412 phase III trial investigated the safety and efficacy of pembrolizumab versus placebo concomitant and adjuvant to definitive CRT in LA SCCHN. The pembrolizumab/placebo was started 1 week before initiation of CRT and pursued in maintenance up to 1 year thereafter. This trial did not meet its primary endpoint with a median event-free survival that was not reached in the pembrolizumab group and 46.6 months in the placebo group, with a hazard-ratio (HR) of 0.83 (p-value= 0.0429). Other phase II and III studies are still ongoing in LA SCCHN. Among them, IMVoke010 is studying atezolizumab in the adjuvant setting only after primary treatment.

Immunotherapy has the potential to improve the efficacy of treatment in patients with LA SCCHN. Although we await the outcome of several studies, the first reported trials have been discouraging.

Innovative approaches are needed to investigate the best way(s) to integrate ICI with multimodal curative treatment. For example, strategies such as better patient selection (PD-L1 expressing tumors), ICI after chemoradiation (similar to the PACIFIC trial in lung – IMVoke 100), or ICI in the neoadjuvant setting before surgery (KEYNOTE 689) are worthy of exploration. Another hypothesis to explain the non-benefit of anti-PD/PD(L)-1 mAbs in combination with (chemo)radiation is the large field of irradiation to regional lymph nodes that might neutralise immune competent cells. To circumvent that possibility, the REWRITE trial (NCT03726775) is investigating the activity of durvalumab in combination with less extensive nodal radiation therapy (irradiation of adjacent lymph nodes only). The primary endpoint of this trial is the rate of relapse in non-irradiated regional lymph nodes in a highly selected population (T1-T4 with clinical status N0-N1 or N2a-N2b non-

palpable). Hopefully, the ongoing investigations and those of the future will guide how these agents can be best used in the curative setting.

I.1.5.1.4 Xevinapant

One of the hallmarks of carcinogenesis is apoptosis evasion. This event has been linked to overexpression of apoptosis proteins (APs). Inhibitors of APs (IAPs) are overexpressed in SCCHN and are associated with poor clinical outcome.[154,155] In light of these investigations, inhibitors of IAPs, such as xevinapant, have been developed. Xevinapant is a small, oral IAP inhibitor currently investigated in SCCHN. In a randomized phase II study, xevinapant vs placebo was evaluated concomitantly to standard RCT. Ninety-six LA SCCHN patients were randomized. The primary endpoint was the locoregional control at 18 months. A better locoregional control was observed in the xevinapant arm (decrease of locoregional failure by 54%) but did not reach statistical significance (p=0.089). The risk of progression or death was reduced in the xevinapant arm by 67% (p=0.0019). Overall survival (OS) was also prolonged in the xevinapant arm: median OS was not reached in the xevinapant group and was 36.1 months in the placebo group (p=0.01).[156] Seven hundred LA-SCCHN have been randomized between xevinapant plus standard RCT vs placebo plus standard RCT in the TrilynX (NCT04459715) phase 3 trial. The primary endpoint is the event-free survival and secondary endpoints are progression free survival, loco-regional control, overall survival and control.[157] Other studies are ongoing with xevinapant. The phase III Xray VISION (NCT05386550) trial is comparing xevinapant plus radiotherapy versus placebo plus radiotherapy as post-operative treatment in high-risk resected LA SCCHN patients that are ineligible for cisplatin. Xevinapant is also investigated in combination with radiotherapy (RAVINA, EORTC) in elderly and with cetuximab plus radiotherapy in unfit patients (NCT05930938).

I.1.5.2 Recurrent/metastatic SCCHN

I.1.5.2.1 Systemic therapy (recurrent and/or metastatic disease)

Patients with locoregional disease not amenable to surgery and/or radiation therapy, and those with metastatic disease, are eligible for a systemic treatment. Around 50% of patients with LA-SCCHN will relapse after primary treatment with distant metastases and/or local or regional disease. Loco-

regional relapse without any distance metastases occurs in around one third of recurrent SCCHN patients.[158,159]

I.1.5.2.2 Immune checkpoint inhibitors with or without platinum-based therapy

The standard of care for first-line R/M SCCHN has recently changed. Immune checkpoint inhibitors (ICI) are new validated treatments for R/M SCCHN. SCCHN tumors express multiple immune checkpoint molecules that are implicated in decreased antitumor immunity.[160] Binding of the immune checkpoint programmed death-1 (PD1) receptor, expressed on activated T-cells to its ligand PD-L1, which is expressed on tumor cells and tumor-infiltrating immune cells, led to a decrease of T-cell anti-tumor activity.[160] Pembrolizumab and nivolumab are two ICIs that, by targeting PD1, may reactivate the anti-tumor T-cell immune response. The KEYNOTE-048 study showed that a combination of chemotherapy (cisplatin or carboplatin + 5-FU) plus pembrolizumab, a mAb targeting PD1, significantly improves OS over the EXTREME regimen (cisplatin or carboplatin + 5-FU + cetuximab): 13 vs 10.7 months (p-value: 0.0034).[68] The objective response rate (ORR) and progression-free survival (PFS) were similar between the two groups as well as safety (ORR 35.6% and 36.3%; PFS: 4.9 and 5.1 months, grade 3-5 adverse events: 85.1% vs 83.3%, respectively). In the same trial, pembrolizumab monotherapy also improved median OS in patients with PD-L1 expressing SCCHN: 14.8 vs 10.7 months in the subgroup of patients with a combined positive score (CPS) ≥ 20, and 12.3 vs 10.3 months in those with a CPS ≥ 1.[68] As expected, pembrolizumab was better tolerated than EXTREME (grade 3-5 adverse events: 54.7% vs 83.3%). Nevertheless, the results in terms of PFS were not satisfactory for pembrolizumab monotherapy compared to EXTREME: PFS 3.4 vs 5.0 months in CPS ≥ 20 patients and 3.2 vs 5.0 months for those with a CPS ≥ 1. Similarly, ORR was 23.3% and 19.1% with pembrolizumab monotherapy in CPS $\underline{\geq}$ 20 and 1 compared with 36.1% and 34.9% in the EXTREME study, respectively. With a 4-year follow-up, first-line pembrolizumab and pembrolizumab-chemotherapy continued to demonstrate survival benefit versus cetuximab-chemotherapy in recurrent/metastatic head and neck squamous cell carcinoma. Around 20% of the patients are still alive at 5-year in the CPS ≥ 20 group.[4] Therefore, based on the results of KEYNOTE-048, two different

approaches are validated for patients with locoregional relapse and/or distant metastases. A “chemo-free” approach with pembrolizumab alone in patients with CPS ≥ 1 SCCHN should be considered, especially when rapid cancer shrinkage is not needed. A second option, independent of PD-L1 status, is the combination of pembrolizumab and chemotherapy (cisplatin or carboplatin + 5-FU) for all comers, particularly for symptomatic patients or when rapid tumor shrinkage is needed. It is worth noting that, with the data available today, we do not know if platinum/5-FU/pembrolizumab improves survival over platinum/5-FU/cetuximab for patients with SCCHN not expressing PDL1. The Food and Drug Administration (FDA) has recently approved pembrolizumab in combination with chemotherapy for first line treatment regardless of PD-L1 expression and pembrolizumab alone for patients with PD-L1 expressing tumors (CPS ≥ 1). The European Medical Agency (EMA) has approved pembrolizumab with or without chemotherapy only for PD-L1 expressing tumors (CPS ≥ 1).

For patients who progress within six months from platinum therapy given either as palliative treatment or as part of multimodal curative treatment, nivolumab, a mAb targeting PD1, has been shown to improve survival compared to single-agent systemic treatment (cetuximab or docetaxel or methotrexate): 7.5 vs 5.1 months (CheckMate 141).[143] In a very similar study design (Keynote 040), pembrolizumab prolonged median OS compared with standard of care: 8.4 months versus 6.9 months, although this difference was not statistically significant.[161] In the population with a PD-L1 tumor proportion score (TPS) of 50% or higher, median OS was 11.6 months with pembrolizumab and 6.6 months with standard of care. Nivolumab was FDA and EMA-approved in this setting. Pembrolizumab was approved with the same indication by the FDA, and the EMA approved if for patients whose tumors express PD-L1 with a TPS ≥ 50%. Furthermore, pembrolizumab and nivolumab offer a better toxicity profile and improvements in QoL compared to standard therapies.[162]

CheckMate 651 evaluated nivolumab + ipilimumab versus Extreme regimen (chemotherapy + cetuximab) as 1st R/M line treatment.[163] It showed clinical benefice only among the patients with CPS ≥20, but not in the overall cohort.

Other immune checkpoint inhibitors are currently being evaluated in clinical trials, in R/M setting, mostly as combination with other immune targets or targeted therapies. Eftilagimod-α (soluble LAG3) and pembrolizumab combination showed an objective response rate of ~30%, and up to 60% in patients with CPS ≥20.[164] Cabozantinib or Lenvatinib in combination to pembrolizumab gave promising results.[165,166] Interestingly, trials are ongoing for patients after platinum and immune checkpoint inhibitor: Interlink-1 (NCT04590963) is a phase III study evaluating cetuximab +/- monalizumab after anti-PD1 and platinum therapies. In LEAP-010 and -009 (NCT04428151), pembrolizumab is investigated in combination with lenvatinib in first-line R/M and after ICI.

I.1.5.2.3 Cetuximab-based therapy

Platinum/5FU/cetuximab (EXTREME) improves OS compared to platinum/5FU (10.1 vs 7.4 months) as first-line treatment in patients with R/M SCCHN.[167] A retrospective analysis from French sites showed an ORR of 30%, a median PFS of 3.6 months and OS of 7.8 months with the EXTREME regimen in patients who progressed on immune checkpoint inhibitors.[168] In first-line recurrent treatment, the EXTREME regimen is standard for patients with contraindications to anti-PD1 inhibitors or when the tumor does not express PD-L1. EXTREME could also be indicated in second-line after progression on immune checkpoint inhibitors for fit patients eligible for platinum-based chemotherapy. Cisplatin/docetaxel/cetuximab (TPEx) did not improve OS compared to EXTREME in a randomized phase III trial.[169] TPEx had less grade 4 toxicity than EXTREME (30 vs 44%, respectively) but was, nevertheless significantly toxic (grade 3 toxicity in 81%). TPEX could be considered as an alternative to the EXTREME regimen. After progression on platinum-based chemotherapy and anti-PD1 inhibitors, no standard of care exists. Cetuximab is approved by the FDA after platinum failure (despite the absence of randomized trials performed in this indication).

I.1.5.2.4 Other chemotherapies

Taxanes and/or methotrexate are frequently used after platinum failure although no randomized trials have demonstrated their benefit in this indication.

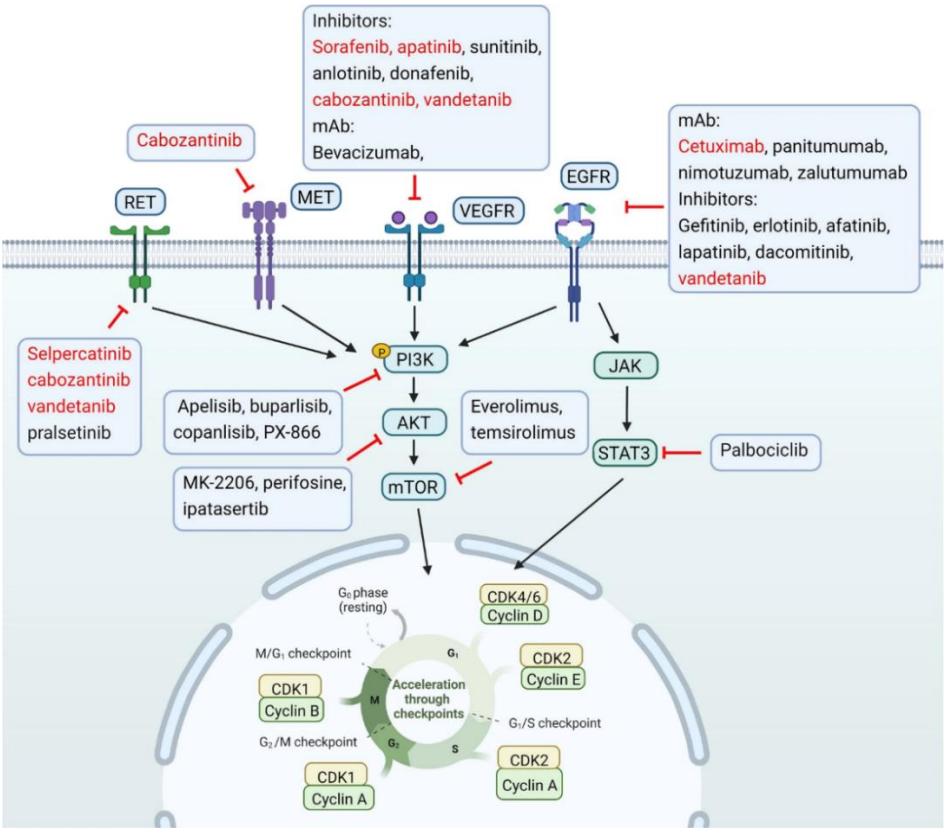

Figure I-5: Targeted therapies in clinical trials in HNC treatment.
EGFR epidermal growth factor receptor, MET mesenchymal–epithelial transition factor, AKT serine/threonine-specific protein kinase, mTOR mammalian target of rapamycin, CDK cyclin-dependent kinase, VEGF vascular endothelial growth factor, JAK jenus-activated kinase, STAT signal transducer and activator of transcription, mAb monoclonal antibody, RET rearranged during transfection, p phosphorylation. Figure adapted from Li et al.[170]

I.1.5.2.5 Targeted therapies

Cetuximab is the only targeted therapy approved for SCCHN. Other targeted therapies have also been investigated in unselected SCCHN populations with disappointing results. A visual summary of targeted therapies investigated in SCCHN is shown in Figure I-5. Besides Cetuximab, no other targeted therapies have demonstrated sufficient clinical benefit to be approved as standard of care in SCCHN. A non-exhaustive overview of

targeted therapies tested in clinical studies and their signaling pathways are listed in Table I-1.[170]

Type of molecule	Drugs
EGFR/HER inhibitors	
Monoclonal antibody	Cetuximab
	Panitumumab
	Nimotuzumab
	Zalutumumab
Selective EGFR inhibitors	Gefitinib
	Erlotinib
HER inhibitors	Afatinib
	Lapatinib
	Dacomitinib
	Poziotinib
VEGF inhibitors	
Multitarget kinase inhibitor of BRAF, VEGFR1-2	Sorafenib
Multitarget kinase inhibitor of VEGFR, PDGFR and c-kit	Sunitinib
	Apatinib
Monoclonal antibody	Bevacizumab
PI3K inhibitors	
	Buparlisib
AKT inhibitors	
	MK-2206
	Perifosine
	Ipatasertib
mTor inhibitors	
	Everolimus
	Temsirolimus

c-Met inhibitors	
	Tibantinib
	Foretinib
IDO1 inhibitor	
	Epacadostat
CDK 4/6 inhibitors	
	Palbociclib
ALK1 inhibitors	
	Dalantercept
HRAS inhibitor	
	Tipifarnib

Table I-1: A non-exhaustive overview of targeted therapies tested in clinical studies

Multiple efforts have been made to target the PI3K/AKT/mTOR pathway with limited success. The BERIL-1 trial was the only study that found a benefit in OS and PFS when Buparlisib (inhibitor of PI3K p110 a/b/d/g subunit) was added to Paclitaxel.[171] The biomarker analysis of this study did not find any correlation between PI3K pathway activation and drug activity.[111] Recently, Buparlisib monotherapy in patients with SCCHN harboring *PIK3CA* mutation showed limited activity, even in this preselected population.[172] Encouraging results were found with the combination of Cetuximab and Palbociclib, a CDK4/6 inhibitor, in a single-arm phase II study.[173] However, a randomized phase II study failed to demonstrate a survival benefit for this combination compared to cetuximab alone in cetuximab-naïve p-16 negative SCCHN patients that progressed during or after platinum therapy. Studies targeting the VEGF pathway have resulted in limited efficacy but substantial toxicity.[174] Bevacizumab did not improve OS when added to chemotherapy in a randomized phase III study but improved the response rate and progression-free survival with increased toxicities. A new emerging targeted therapy are RAS inhibitors. In R/M SCCHN, 4-8% of patients harbor *HRAS* mutations. Tipifarnib, a farnesyltransferase inhibitor, binds to *HRAS* and disrupts its function as shown in a phase I study with an objective response rate of 55%, median PFS of 5.6 months, and median OS of 14.5 months.[175] Clinical trials are currently ongoing to confirm in a larger cohort the potential benefit of this targeted therapy. Only a few biomarker-driven trials are

dedicated to SCCHN, there are, however, more and more molecular-driven basket trials such as the NCI-MATCH-trial in the U.S.A. or the PRECISION trial in Belgium.[176] The first umbrella trial dedicated to SCCHN, the UPSTREAM trial, was initiated in 2017 by Galot et al. (NCT03088059) but did not seem to improve the outcome of patients.[177,178]

I.2 Circulating tumor-DNA

I.2.1 Generalities

Liquid biopsy is defined as '*a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood, or for pieces of DNA from tumor cells that are in the blood*'.[179] It was first mentioned by Pantel and Alix-Panabières [180] to describe the use of a blood test to assess the presence and characteristics of a solid tumor. More generally, liquid biopsy refers to all biomarkers that can be detected in the blood: circulating deoxyribonucleic acid (DNA), proteins such as carcinoembryonic antigen (CEA) or cancer antigen 15.3 (CA15.3), tumor-associated cells, different types of ribonucleic acids (RNAs), and exosomes, among others. In this chapter, we focus on liquid biopsy performed specifically on blood samples but note that liquid biopsy also includes analyses of other bodily fluids such as cerebrospinal fluid (CSF), saliva, urine, etc.

Liquid biopsy has several advantages over tumor biopsy. First, it is usually less invasive, dangerous, and painful. Second, it may provide a more comprehensive molecular overview of tumor heterogeneity, possibly reflecting the characteristics of different cancer locations in a particular patient. This cannot be achieved by a single tumor biopsy.[181] Finally, it is easier to obtain repeated blood samples over time to understand the dynamics of response to a specific treatment.

We will focus on the detection of plasma circulating-tumor DNA (ctDNA), one of the most advanced and frequently investigated approach.[182]

ctDNA is characterized by tumor specific genomic alterations. The presence of ctDNA in the blood was first assessed in 1948[183] and is a proportion of circulating free DNA (cfDNA). Every cell is able to shed DNA into the

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Adapted from Honoré N, Galot R, van Marcke C, Limaye N, Machiels JP. Liquid Biopsy to Detect Minimal Residual Disease: Methodology and Impact. Cancers (Basel). 2021 Oct 26;13(21):5364. doi: 10.3390/cancers13215364. PMID: 34771526; PMCID: PMC8582541.

bloodstream through necrosis, apoptosis or active secretion.[184] Although cfDNA biology is not fully understood, the quantity of cfDNA depends on several parameters: it can be increased during pregnancy, in case of infection or cell death (i.e. crush syndrome or major physical exercise), or after organ transplantation.[185] In healthy individuals, most cfDNA is derived from hematopoietic cells[186], and is fragmented to a mean length of 166 bp.[187]

In the 1970's, Leon et al. showed that the quantity of cfDNA was greater in cancer patients.[188] Tumor cells have the same intrinsic capacity as normal cells to shed DNA, albeit with a higher probability due to higher cellular turnover. DNA with tumor-specific alterations can thus, be detected in blood.[189] The DNA, shed by tumor cells, is more fragmented than that of healthy cfDNA, with the mean length of ctDNA molecules being around 143bp.[187] The proportion of ctDNA is highly variable and can range from 0.1% to over 10% of the total cfDNA depending on the type of tumor, its size and localization, and tumor staging.[190] For example, ctDNA is detected in more patients with colon adenocarcinoma than in those with glioblastoma, and the amount of ctDNA is higher in case of metastatic spread compared to localized disease.[190]

I.2.2 Methodology and challenges

Several methods exist for ctDNA detection and characterization, based on the presence of tumor-specific genomic alterations. Some applications have already been implemented in daily clinical practice.[191] Next Generation Sequencing (NGS) refers to several different techniques of high-throughput nucleotide sequencing (sometimes referred to as massively parallel sequencing), based on the extreme miniaturization, parallelization, automation, and digitalization of polymerase chain reaction (PCR)-based "reading while copying" of DNA.[192,193] These methods allow scientists and clinicians to obtain increasingly large quantities of sequencing data within a few hours.[192] The main limiting factor of these sequencing tools is the error rate, which varies from 1% to 0.01% depending on the brand and type of sequencer.[194] High-confidence detection of variants, present at a fraction below 1%, requires sufficient depth of coverage (i.e., the number of sequences that "read" any given nucleotide position) in patient as well as

control samples, and the use of bioinformatic analyses and algorithms that allow for the application of adequate quality control criteria.

PCR-based techniques such as droplet digital PCR (ddPCR) allow for the detection of DNA variants up to 0.001%[180], although they are more suited to the assessment of limited numbers of pre-determined variants, rather than the discovery or determination of large numbers of different variants.

I.2.2.1 Personalized Methods

Tumor-personalized methodologies to detect ctDNA are methods based on prior knowledge of the genomic landscape of the tumor.

I.2.2.1.1 Tumor-customized based panels

One of the most validated methodologies to detect ctDNA is the specific interrogation of plasma-derived cfDNA for genomic alterations previously identified in the corresponding tumor. The tumor biopsy or surgical sample is sequenced (e.g., Whole Exome Sequencing (WES) or a large gene panel) to identify somatic mutations. A custom-made panel is designed accordingly, to sequence the altered positions in the plasma. This custom panel, specific to the patient's tumor, can employ PCR-amplification using specific primers, or hybridization-based capture using specific probes, in order to enrich the regions of interest for NGS[195,196] with a barcoding method typically incorporated to allow for sample multiplexing.

This design has been implemented in several clinical studies in the context of minimal residual disease (MRD) detection[196-199] and treatment monitoring, showing interesting results. The techniques used in these studies, and their main conclusions, are detailed in Table I-2. The main advantage of this methodology is the comprehensive view of the genomic landscape of the tumor that is achieved. This provides multiple targets for follow-up in the plasma, thereby optimizing the sensitivity and robustness of detection. By decreasing target size (*i.e.,* the number of nucleotides to be sequenced) as compared to WES or a large gene panel, the use of a patient-specific panel can exploit the considerable capacity of NGS sequencers to increase the depth of sequencing. This can allow the detection of variants present in very

low quantities (for example, variant allele frequency (VAF) <0.01%, based on a high pretest probability of detecting the variant), extremely useful in detecting MRD. Some technologies that combine deep sequencing with Unique Molecular Identifier (UMI) barcoding and bioinformatic pipelines such as Safe-Seq[199] or TARDIS[198], have been shown to detect tumor variant allele frequency as low as 0.002%. However, the choice of which tumor variants will make up the tumor-personalized panel may be subjective or arbitrary, often based on disease-pertinence bio-informatic algorithms and can omit driver mutations while selecting passenger mutations. The threshold for positivity of ctDNA is also a subjective decision with some authors setting the limit at one mutation per sample,[195] while others require the detection of at least two mutations per sample.[197]

This technique requires a tumor sample, which, in itself, creates several issues. First, obtaining a good quality biopsy can be challenging and sometimes carries risks (bleeding, pain) for the patient depending on the location (e.g., close to blood vessels). Second, patient-specific panels based on sequences from a particular tumor sample fail to capture genomic alterations in other parts of the same tumor, as well as those in distant metastases, due to tumor heterogeneity. Third, the genomic landscape of the primary tumor and its metastases can change over time as treatment and the natural course of the disease select specific tumor clones. The use of a tumor-guided panel therefore carries the risk of missing (potentially informative) alterations in the plasma.

Despite these drawbacks, this method has become the gold standard for detecting ctDNA due to its ability to detect tumor variants even when present in very low quantities.[200]

I.2.2.1.2 Custom-based PCR assay

Droplet Digital PCR (ddPCR) is a highly specific technique that allows for the detection of individual pre-defined genomic variants, down to a VAF of 0.01%, with promising results.[201] ddPCR has the benefit of giving quantitative results, unlike quantitative PCR, while also presenting a proportion of variant allele frequency within the same assay. It also offers a high-sensitivity compared to other techniques, as very low quantity of DNA (~1ng) can be used to detect a variant.[202]

ddPCR probes are designed to detect genomic alterations already identified in the tumor; this method shares many of the same advantages and disadvantages as the custom-based NGS panel technology described above. The use of ddPCR to detect MRD has been demonstrated in hematological neoplasia, when the carcinogenesis is due to a specific mutation (for example BRAF V600E in hairy cell leukemia[203]). Data, although still scarce in solid tumors, show promising results in tumors where hotspot mutations are well established as driver mutations.[204]

PCR has the very useful potential to reliably detect known single alterations such as the presence of an oncogenic virus. Chronic infections by viruses have been described as a cause of several hematologic and non-hematologic neoplasms.[205] The presence of the virus is usually determined by immunohistochemistry[206], immune-fluorometry assay[207], or PCR on the tumor biopsy.[206] It can also be detected either by circulating viral antigens specific assays or antibodies directed against the virus[208], or the presence of the viral genome in the bloodstream.[209] Studies have mainly focused on detecting specific viral oncogenes in the plasma using PCR-derived methods, for example, quantitative PCR (qPCR)[209], digital PCR (dPCR)[210,211], and droplet digital PCR (ddPCR).[212,213] Detection of the viral genome to assess MRD has shown interesting results, especially in Human Papilloma Virus (HPV)-derived and Epstein-Barr virus (EBV)-derived cancers. In HPV-related oropharyngeal cancers, Chera and colleagues[211] have demonstrated the prognostic value of detecting HPV-DNA in the plasma of patients after curative-intent treatment. The detection of EBV after curative-intent treatment has in turn also been shown to be predictive of disease relapse in EBV-related nasopharyngeal carcinoma.[209,214] NGS-based methods can also be used for viral detection[215,216], with the added benefit of assessing multiple subtypes of virus in a single assay.

I.2.2.2 Non-personalized methods

To address the potential of ctDNA as a biomarker, many have chosen a non-personalized or agnostic approach, i.e., one that is not guided by the specific genomic alterations present in a particular patient's tumor. Gene panels are typically the method of choice, due to their potential to detect any mutations in the neoplasm that are located in the targeted (i.e. sequenced) region of its genome, in one go. As we previously discussed, however, NGS is not the

most sensitive method to detect variants present at very low frequency[217,218], necessitating the use of supplementary methods to improve accuracy. For example, to separate real tumor variants from sequencing errors, the use of molecular barcodes (often termed UMIs, for Unique Molecular Identifiers) has been implemented, to lower the error-rate of variant-calling and increase the accuracy of VAF estimation.[219] Statistical and bio-informatic tools are used to further separate very low frequency variants from possible sequencing errors.[220]

To detect ctDNA, two main approaches have been studied: the use of (i) NGS gene panels, either comprehensive or specific to one tumor type, and (ii) PCR for mutations in very specific tumor subtypes.

A synthetic view of the main studies performed using gene panels can be found in Table I-2. As shown, it is feasible to use a large panel with over 1000 genes[221], but sophisticated bio-informatic tools and barcoding are essential to detect tumor variants with high confidence. One of the most widely-used strategies is CAncer Personalized Profiling by Deep Sequencing (CAPP- Seq).[222] It combines the deep sequencing of several genes (usually >300) implicated in carcinogenesis by amplification of the target regions, the use of molecular barcoding, and the integrated Digital Error Suppression (iDES) bio-informatic tool[223], specifically designed to detect ctDNA at a very low allele frequency. This technique has shown very promising results when it comes to detecting MRD in specific cancer types. Even without prior knowledge of the genomic mutations in the tumor, this method can detect ctDNA before treatment in >90% of localized non-small cell lung cancers (NSCLC).[222,224] It is however less effective in localized esophageal cancer, detecting ctDNA pre-treatment in only 60% of patients.[225] This may be explained by differences in the genomic landscape and the fact that esophageal cancer seems to shed less tumor DNA than NSCLC.[225] In the post-curative treatment setting, the success rate of the technique drops. Although it shows good positive predictive value, the negative predictive value still has room for improvement: when ctDNA is detected post-treatment, patients are significantly more likely to experience relapse in the following months.[196,226,227] However, due mainly to technical limitations, not detecting ctDNA after curative-intent treatment does not predict the tumor's definitive elimination (Table I-2). Despite these drawbacks, this technology

provides a much more comprehensive view of genomic heterogeneity than tumor-customized methods. It also enhances the understanding of the evolution of the disease as it can detect the emergence of certain clones. Based on these observations, several companies have developed either pan-cancer or cancer-specific panels (Roche Avenio ctDNA targeted kit, Foundation One® LiquidCDx, Guardant360®[228]) to implement this technology in clinical practice. These commercial panels were designed for diagnostic purposes but their use in the detection of MRD still needs to be proven. Other studies have used large panels, with or without UMIs, with interesting results[221,226,229,230]; however, the detection rate of ctDNA undeniably decreases as panel size increases (Table I-2).

Sequencing is not limited to human genome sequencing as several tools have been developed for viruses sequencing in virus-induced tumors.[231] In SCCHN, HPV-Seq provides a quantitative methodology to detect circulating HPV DNA.[232]

To increase assay sensitivity in particular populations of interest, several groups have designed smaller gene-panels, restricting target size in order to divert data-capacity towards maximizing sequencing depth, with promising results in disease detection by ctDNA. Reducing panel-size increases coverage depth, as the coverage depth depends on the capacity of the sequencing machine to generate a defined quantity of data per run, the number of samples per run and the target size *i.e.,* the number of bases to be sequenced. By reducing the panel-target size, coverage depth increases and tumor-variants may be detected at a much lower VAF. For example, Mes and colleagues detected ctDNA in 67% of patients with head and neck squamous cell carcinomas using a 12-gene panel.[233]

Opposite concepts are now emerging and breadth may supplant depth of sequencing to overcome the barrier of cfDNA abundance. Whole Genome Sequencing (WGS) could identify a tumor profiling capable of detecting the presence of ctDNA at very low VAF. This approach allows ultra-sensitive detection of ctDNA without retrieving information about the cancer genomic landscape.[234]

PCR techniques may also be used in a non-personalized setting, but their use is limited to specific known, recurrent genomic alterations and clinical situations. For example, the only FDA-approved PCR test, cobas® EGFR mutation test v2 (Roche), detects the presence of 42 *EGFR* mutations, including the hotspot T790M mutation, in patients with NSCLC previously treated with tyrosine kinase inhibitors.[235] This methodology can however only be used in a specific group of patients and is not a suitable option for most cancers.[236]

I.2.2.3 Other methods

Carcinogenesis can be driven by focal mutations (point mutations, small insertions/deletions) in the nucleotide sequence and/or by copy number changes in genes or groups of genes.[237] Tumor copy number alterations (CNA) can be detected by different types of sequencing: regular or shallow whole genome sequencing (WGS)[238,239], deep WES[240] or panel sequencing[241]. Detection of CNA in the bloodstream has been used for many years in the prenatal diagnosis of fetal abnormalities.[242] Although several bioinformatic tools have been designed to detect CNA in ctDNA[243-245], consensus on methodology is still lacking. Furthermore, the detection of tumor CNA perhaps, does not faithfully reflect persistence of tumor cells, having been found in treated breast cancer patients without evidence of relapse.[246] One hypothesis that has been tested in squamous cell carcinoma of the head and neck (SCCHN) is that the combined assessment of mutations and CNA[233] may improve ctDNA detection. CNA analysis is currently being explored in several settings and has shown promising results in cancer detection and treatment monitoring in multiple tumor types.[247-251]

Others methodologies to detect ctDNA presence are being developed, such as fragmentation analysis[244,252], where ctDNA is detected through the difference in bp between non-tumoral cell free DNA and ctDNA, or methylation analysis.[253,254] These methodologies are frequently combined to reach better ctDNA detection.

With the emergence of data about the implication of the microbiome in several diseases including cancer, genomic tools have been developed to assess the presence of bacteria or viruses through taxonomic sequence classifiers, such as Kraken or MMseq2.[255] Although PCR-based methods are

still widely used, taxonomic assignation pipelines are starting to emerge for microbiome profiling and for virus-induced cancers.[256,257] To the best of our knowledge, this methodology has never been used in cell-free DNA.

I.2.2.4 Challenges with ctDNA detection

Detecting ctDNA is still a complex issue as there are no standardized methods ready for implementation in daily practice.

The technical issues have been discussed above. While personalized methods use the most sensitive technologies and can accurately detect tumor variants, sequencing the tumor and then designing patient-specific NGS or PCR assays is costly and time consuming. Non-personalized methods would, therefore, theoretically be more practical in clinical practice, as one technique would serve multiple patients suffering from the same tumor type, and potentially even be used across different tumor types. Until recently, however, sequencing techniques have not been sensitive enough even with barcoding methodology. The risk of not correctly detecting ctDNA could negatively impact a patient's outcome. Moreover, large panels can yield huge numbers of variants, sorting through which to correctly identify driver variants can be challenging. To exclude non-pathogenic variants, sequenced reads would ideally be aligned not only to reference genomes, but also to the germline DNA of the patient using dedicated pipelines, in addition to excluding variants that are frequent in germline variant databases. This would require germline DNA sequencing for all patients.

Clonal Hematopoiesis of Indeterminate Potential (CHIP)[258] might also be a confounding factor - these variants arise from hematopoiesis and might incorrectly be called as tumor variants by bioinformatic tools. One way to separate CHIP variants from tumor variants would be to align the sequencing reads on germline DNA collected from circulating white blood cells, such as lymphocytes or peripheral blood mononuclear cells (PBMCs), which would also carry the former. Nevertheless, accurately differentiating non-tumor from tumor variants can remain difficult.[259-261] Finally, the treatment itself (e.g., radio(chemo)therapy) may induce variants, either in hematopoietic cells (CHIPs) or in cells of the exposed tissue. Separating these variants from

true tumor variants is difficult because treatment-induced variants will, like tumor variants, appear at low variant allele frequency and might also occur in genes implicated in carcinogenesis (*e.g., TP53*). Consortia on guidelines to discriminate tumor variants from other variants are emerging[262], and more databases are expanding their pipelines to sort variants, for *e.g.,* Varsome Clinical.[263] However, a systematic bioinformatic pipeline has yet to be proven useful in the clinical setting.

The timing of sampling is also important. ctDNA has a very short half-life (~30min)[184] and is dependent on active secretion by tumor cells and cell death. During the course of treatment, a decrease in ctDNA has been observed but a precise and universal wash-out time has not been defined.[196] Circulating DNA (cfDNA) can be released into the bloodstream after surgery, mainly due to tissue trauma, and can remain elevated up to four weeks later.[264] The best and optimal timing of ctDNA detection is still not known in all ctDNA potential applications. As discussed later, ctDNA detection might not be sensitive enough in tumor detection if harvested too early in carcinogenesis. The best timing after or during treatment to assess MRD or treatment monitoring is unknown. Adjuvant therapies usually start 6-8 weeks after primary curative treatment. Assessing for MRD within one from two weeks of curative treatment is, at least for now, not technically feasible, and on the other hand, delaying adjuvant therapy until results are available may lower patient prognoses. In treatment monitoring, there is no standard timing to evaluate treatment response: as the treatment is given by cycles, most of the studies evaluated ctDNA within the first cycles. Studies are warranted across different cancer types to determine the most effective moment of harnessing ctDNA as a biomarker. Indeed, a patient can be ctDNA-positive several months[265] or just a few weeks before relapse. Some studies have also shown that two positive ctDNA samples collected at two different time points can be more specific than a single positive sample obtained at a unique time point.[265]

One unresolved issue is the appropriate way of evaluating ctDNA quantity. For PCR-based techniques, quantity can be estimated knowing the quantity of probes and the number of amplification cycles used. In NGS-based techniques, the most convenient and easy way is to look at the VAF.

$$Variant\ allele\ frequency\ (VAF) = \frac{Number\ of\ reads\ with\ the\ alteration}{Total\ number\ of\ reads\ at\ the\ same\ position}$$

However, this proportion is influenced by many independent factors such as the sequencing depth at this location, the quantity of cell-free DNA, the sequencing error rate, etc. For example, if the normal proportion of non-tumoral cell-free DNA rises, due to infection or rhabdomyolysis, the proportion of ctDNA will decrease, although the real quantity of tumor-DNA in the bloodstream remains unchanged. Similarly, if the sequencing depth is not sufficient enough, the VAF might be abnormally increased or decreased. For example, if a variant is present at 0.1% in the plasma, a sequencing depth of at least 1000x is required for 1 read harboring the alteration. If sequencing is not sufficiently deep (500x *i.e.*), the variant allele frequency would be either 0% or 0.2% (if 0 or 1 read carries the variant). There are no studies that have managed to by-pass these issues and VAF remains the best way to evaluate ctDNA levels. This should be noted in analysing ctDNA VAF in studies.

Despite these drawbacks, ctDNA remains a very interesting tool, which can reflect tumor evolution, and has already been used in several applications.

I.2.3 Potential applications of ctDNA

I.2.3.1 Cancer diagnosis and early detection

The use of blood analysis as a diagnosis tool derives from pre-natal diagnosis. Similarly to pre-natal testing, sequencing technologies allow us to separate among cell-free DNA (cfDNA) DNA coming from normal cells (germline DNA, gDNA) from placental, fetal or tumor cells.[266,267] Screening tools are being developed, based on targeted sequencing[268], methylation patterns[269] or fragmentation.[244,270] Across these studies, it remains clear that cancer screening tools have a much higher sensitivity in locally-advanced stages than in early stages (stage I-II).[271,272] Further investigations are needed to improve detection rate in early stages, where chances of cure are higher. However, ctDNA technologies have a too high false positive rate, inducing in non-cancerous patients anxiety, and useless tests.[266] There are

ongoing trials using genome wide sequencing methods on cfDNA from healthy patients to improve cancer screening.[273-275]

In SCCHN, some studies have evaluated ctDNA as a screening tool, separating HPV-induced SCCHN from the others. In HPV-negative SCCHN, Perdomo and colleagues have studied the potential of targeted screening of ctDNA mutations and detection of *TP53* mutations in plasma and saliva. Although identifications of *TP53* mutations identified more SCCHN (76% vs 42%) than a 5 gene panel, it also showed more false positive rates, especially in saliva samples.[276] Cell-free hypermethylated DNA in plasma sample has recently shown good specificity for SCCHN detection (93%) but a too low sensitivity (33%) to be considered as a screening tool.[277] Methylation patterns as a screening tool in SCCHN remain promising and further investigations are warranted.[278] In HPV-driven SCCHN, HPV-DNA detection through ddPCR has shown good sensitivity and specificity (98% for both) as a non-invasive diagnostic approach for HPV-OPC.[279,280] New technologies have been developed to improve HPV detection in the bloodstream, like CHAMP-16 (ctDNA HPV16 Assessment using Multiple Probes), a new commercial assay, based on multiplex ddPCR that would be able to detect cHPV DNA with a low limit of detection.[281] The presence of HPV DNA in blood could precede actual SCCHN by several years,[282] which could induce too many screening tests and futile examinations with patient anxiety. There is also no way to distinguish circulating HPV (cHPV) origin: HPV-induced cancer can be found in the oropharynx, cervix and anus. Therefore, screening of cHPV DNA might not be the most specific tool for OPC.

I.2.3.2 Minimal Residual disease (MRD)

I.2.3.2.1 Definition

Minimal or Microscopic Residual Disease (MRD) is defined as "*a very small number of cancer cells that remain in the body during or after treatment*".[283] Tumor burden varies with disease and treatment. When there is a response to treatment, tumor burden decreases, and the tumor eventually becomes undetectable on imaging or clinical examination. However, if tumor cells remain in the patient's body after treatment, they may induce relapse, either locally or through distant metastases (Figure I-6). The importance of MRD

detection was first highlighted in hematological cancers and allowed clinicians to evaluate the success of therapy and to predict short and long-term relapse.[284] In solid tumors, the detection of MRD has enabled patients to be classified as having a high or low risk of relapse.[184] Hence, several research strategies are now being investigated to accurately detect MRD in solid tumors and integrate it into treatment strategies.

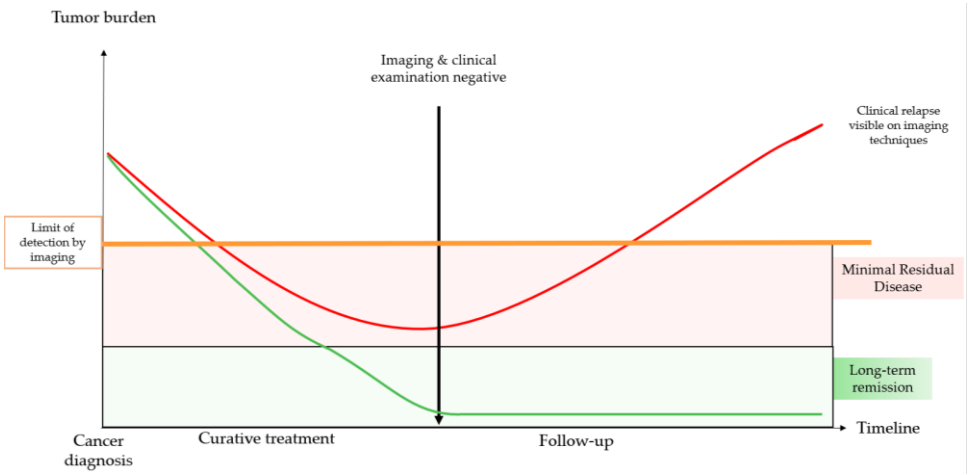

Figure I-6: Schematic view of Minimal Residual Disease (MRD). From Honoré et al. Cancers, 2020[285]

The ability to detect MRD may prove beneficial for patients and clinicians. After completion of standard therapy with curative intent, treatment could theoretically be adapted based on liquid biopsy results. Treatment intensification in MRD-positive patients has the potential to improve disease-free survival and overall survival if MRD is efficiently treated. On the opposite end, de-intensification strategies omitting adjuvant treatment (for example, systemic chemotherapy) in MRD-negative patients could reduce patient burden, decrease treatment related side effects, improve quality-of-life of the patient, and reduce financial costs for society without impairing survival rates.

New clinical trials are now being designed or initiated on diverse tumor types, in order to establish the role of MRD detection in the selection of the optimal treatment after curative local treatment.[182,286,287] This strategy is limited by the reliable detection of MRD, as it cannot be assessed by standard imagery

or clinical examination. Liquid biopsies have become a possible means to overcome this limitation. Although no consensus has yet been reached on the appropriate methodology to detect MRD through liquid biopsy, research has drastically increased, yielding technological and analytical improvements over recent years.[184,288,289]

I.2.3.2.2 Clinical impact of detecting MRD with ctDNA and perspectives

As discussed previously, clinicians wish to detect MRD for two main reasons: (i) to offer treatment escalation when MRD is detected after curative-intent therapy with the hope of improving the cancer outcome, and (ii) to de-escalate treatment when MRD is not detected with the goal of decreasing treatment toxicity. Therefore, accurate MRD assessment is likely to play an important role in cancer treatment personalization.

Multiple studies in various cancer types (Table I-2 and I-3) have demonstrated satisfactory positive predictive value of ctDNA detection, as the presence of MRD identified through ctDNA is associated with worse Disease-Free Survival (DFS). Nevertheless, as discussed previously, several hurdles maintain the negative predictive value of the technique at a low level: detection methods are not sensitive enough to detect ctDNA at very low proportions, new clones not captured by the selected technique can emerge, and the timing of post-treatment sampling can be inappropriate.

Reference	Number of patients included (n)	Tumor type and indication	Methodology	Conclusions
Early detection of metastatic relapse and monitoring of therapeutic efficacy by ultra-deep sequencing of plasma cell-free DNA in patients with urothelial bladder carcinoma [290]	68	Muscle invasive bladder cancer treated with neoadjuvant chemotherapy before cystectomy	Tumor sequencing: WES Plasma sequencing: 16 mutations/patient by multiplex PCR.	76% of ctDNA-positive patients post cystectomy had recurrence (median 96 days before). 0% of ctDNA-negative had recurrence.
Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer [195]	55	Early breast cancer patients receiving neoadjuvant chemotherapy	Tumor sequencing: NGS on panel with 14 known breast cancer driver genes (26). Plasma sequencing: 1 (or more) mutation(s) was (were) followed using ddPCR.	ctDNA was detected in the single post-operative blood test in 19% (7 of 37) of patients. ctDNA detection was predictive of early relapse (median 6.5 months).

Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer [198]	33	Stage I to stage III breast cancer	Tumor sequencing: WES Plasma sequencing: Using TARDIS (combinaison of NGS + PCR + UMIs): 6 to 115 mutations per patient.	Before treatment, ctDNA detected in 32 of 33 patients at tumor fractions of 0.002 to 1.06%. Plasma samples after completion of NAT were analyzed in 22 patients. ctDNA+ in 17 of 22 patients, including 12 of 13 patients with invasive or *in situ* residual disease and 5 of 9 patients with pathological CR. In patients who achieved pathological CR, median decrease in ctDNA was 96%, whereas in patients with residual disease observed at surgery, median decrease was 77%.
Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer[291]	94	Resectable colon cancers with plasma available	Tumor sequencing: NGS on custom targeted panel of 29 genes. Plasma sequencing: personalized ddPCR assays for each somatic mutation identified in the tissue.	ctDNA was detected in 63.8% at baseline. ctDNA was detected at 6-8 weeks post-surgery, before starting adjuvant chemotherapy, in 20.3% (14 of 69) patients with plasma available at this time. In ctDNA-positive post-op: 57.1% (8 of 14 patients) experienced recurrence. The presence of ctDNA immediately after surgery was associated with poorer DFS.
Circulating tumor DNA analyses as markers of recurrence risk and benefit	96	Stage III colon cancer	Tumor sequencing: NGS on 15 genes recurrently mutated in colorectal cancer.	A tumor-specific mutation was detected (ctDNA-positive finding) in the post-surgical plasma sample of 20 of 96 patients (21%).

of adjuvant therapy for stage III colon cancer [199]			Plasma sequencing: 1 mutation/patient with Safe-Seq (NGS + UMIs).	ctDNA was detectable in 15 of 88 (17%) post-chemotherapy samples. Post-surgical ctDNA was detectable in 10 of 24 patients (42%) with recurrence.
Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival [197]	84	High-risk early breast cancer patients with NAT (I-SPY2 Trial)	Tumor sequencing: WES Plasma sequencing: 16 mutations/patient by multiplex PCR	After NAC, all patients who achieved pCR were ctDNA-negative (n=17, 100%). For those who did not achieve pCR (n=43), ctDNA-positive patients (14%) had significantly increased risk of metastatic recurrence (HR 10.4; 95% CI, 2.3–46.6). Patients who did not achieve pCR but were ctDNA negative (86%) had a similar outcome than those who achieved pCR.
Circulating Tumor DNA predicts pathologic and clinical outcomes following neoadjuvant chemoradiation and surgery for patients with locally advanced rectal cancer[292]	29	Locally advanced rectal cancer	Tumor sequencing: WES Plasma sequencing: personalized ddPCR assays for each somatic mutation identified in the tissue.	Patients with detectable postoperative ctDNA experienced poorer RFS (hazard ratio, 11.56; P = 0.007). All patients (4 of 4) with detectable postoperative ctDNA recurred (positive predictive value = 100%), whereas only 2 of 15 patients with undetectable ctDNA recurred (negative predictive value = 87%).

Galaxy Study: Preoperative ctDNA levels are detectable in the majority of patients with resecable colorectal cancer[293]	808	Resecable CRC	Tumor sequencing: WES Plasma sequencing: personalized ddPCR assays for each somatic mutation identified in the tissue.	Longitudinal ctDNA positivity at postoperative week 4, 12 and 24 was significantly associated with inferior disease-free survival (DFS) with hazard ratio (HR) of 46.8. Sensitivity of relapse detection was 93.1%. Positivity at postoperative week 4 was significantly associated with inferior DFS with HR 19.5 overall, and HR 24.4 in pathologic stage I-III, indicating it is a suitable time point for ctDNA-based adjuvant study.

Table I-2:Non-exhaustive list of published trials with personalized methods to identify MRD through ctDNA detection.

WES: whole exome sequencing; PCR: polymerase chain reaction; ddPCR: droplet digital polymerase chain reaction; NGS: next generation sequencing, UMI: unique molecular identifier; NAT: neoadjuvant treatment; CR: complete response; NAC: neoadjuvant chemotherapy; pCR: pathological complete response; NSCLC: non-small cell lung cancer; PFS: progression-free survival; OS: overall survival; DFS: disease-free survival; RFS: relapse-free survival; HR: hazard ratio; RCT: radio-chemotherapy; PPV: positive predictive value; Sn: sensitivity; CAPP-Seq: cancer personalized profiling by deep sequencing; ctDNA: circulating tumor DNA.

Reference	Number of patients (n)	Tumor type and indication	Methodology	Conclusions
Early detection of molecular residual disease in localized lung cancer by circulating tumor DNA profiling [224]	40 54 healthy controls	Curative intent for stage I–III lung cancer	Plasma sequencing: CAPP-Seq 128 genes most frequently mutated in lung cancer.	94% of patients with MRD were ctDNA-positive in post-treatment plasma samples. Patients were ctDNA-positive before radiological relapse (72%) (5.2months). 53% of ctDNA-positive patients had actionable targets.
Circulating tumor DNA analysis for detection of minimal residual disease after chemoradiotherapy for localized esophageal cancer [225]	45	Stage IA to stage IIIB esophageal cancers (adeno-carcinoma or squamous cell carinoma)	Plasma sequencing: CAPP-Seq Esophageal specific panel	Baseline ctDNA-positive: 27/45 (60%). Post CRT ctDNA-positive: 5/31 (16%). Patients with detectable ctDNA post-CRT also had significantly increased risk of disease progression (HR 18.7, P < 0.0001), distant metastasis (HR 32.1, P < .0001), and disease specific death (HR 23.1, P < .0001).

Post-radiation circulating tumor DNA as a prognostic factor in locally advanced esophageal squamous cell carcinoma [226]	25	Resectable esophageal squamous cell carcinoma	Plasma sequencing: NGS on a custom designed 180 genes panel	At baseline, 100% ctDNA-positive. Post radiotherapy: 14/24 (58%) ctDNA-positive 10/24 (42%) ctDNA-negative In the 14 ctDNA-positive patients, 11 patients had a documented follow-up: 90.9% (10/11) had documented disease recurrence. In the 10 ctDNA-negative patients,8 patients had documented follow-up: 50% (4/8) had documented disease recurrence. Patients who were ctDNA-positive exhibited a marginally significant reduction in PFS (P=0.047) and a significantly decreased OS (P=0.005) compared to patients who were ctDNA-negative.
Minimal residual disease detection using a plasma-only circulating tumor DNA assay in colorectal cancer patients [227]	84	Resectable colorectal cancer	Plasma sequencing: Guardant Reveal™ test using NGS custom based panel for the detection of somatic and epigenitic abberations.	15 patients had detectable ctDNA and all 15 recurred. Of 49 patients without detectable ctDNA at the landmark timepoint, 12 (24.5%) recurred. Landmark recurrence sensitivity and specificity were 55.6% and 100%. Integrating epigenomic signatures increased sensitivity by 25-36% versus genomic alterations alone.

Prognostic implications of preoperative versus postoperative circulating tumor DNA in surgically resected lung cancer patients: a pilot study [294]	20	Stage IIA-IIIA lung cancer	Plasma sequencing: CAPP-Seq on a commercial 197 genes panel (Roche Diagnostics).	Eight patients (40%) were positive for preoperative ctDNA. Four patients (20%) were positive for postoperative ctDNA, and this was significantly correlated with histological grade (3 vs. 1 or 2, P=0.032). Postoperative positivity for ctDNA also predicted shorter recurrence-free survival (RFS).
Circulating tumor DNA as a prognostic biomarker in localized non-small cell lung cancer [229]	77	Resectable NSCLC	Plasma sequencing: NGS (cSMART assay) on a custom 127 gene panel	Postoperative ctDNA-positive patients also associated with a lower RFS (HR = 3.076, p = 0.0015) and OS (HR = 3.195, p = 0.0053). Disease recurrence occurred for 63.3% (19/30) of postoperative ctDNA-positive patients. Most of these patients (89.5% (17/19)) had detectable ctDNA within 2 weeks from surgery.
Circulating tumor DNA as a potential marker to detect minimal residual disease and predict recurrence in pancreatic cancer [221]	27	Operable pancreatic cancer	Plasma sequencing: NGS on a large (1.017) gene panel	ctDNA was detected in 18 of 27 preoperative plasma samples resulting in a detectable rate of 66.67%. Seven days after surgical resection, the status of ctDNA changed in 19 patients. Of these, one turned positive and 10 became completely negative. Patients who were ctDNA-positive postoperatively had a markedly reduced disease-free survival (DFS) compared to those who were ctDNA-negative. A positive postoperative ctDNA status was an independent prognostic factor for DFS

Deep sequencing of circulating tumor DNA detects molecular residual disease and predicts recurrence in gastric cancer [230]	46	Stage I-III gastric cancer	Plasma sequencing: NGS with Enrich Rare Mutation Sequencing (ER-Seq) assay on a custom driver mutation panel	ctDNA was detected in 45% of treatment-naïve plasma samples. All patients with detectable ctDNA in the immediate post-operative period eventually experienced recurrence. Post-operative samples (collected prior to any adjuvant chemotherapy; 9–48 days after surgery) showed that ctDNA was detected in 18% (7 of 38) of evaluable patients. ctDNA positivity after surgery was strongly associated with an increased risk of relapse (100% recurrence in the positive group vs 32% in the negative group), worse DFS ($p < 0.0001$) and worse OS ($p = 0.0007$).
Circulating tumor DNA analyses as a potential marker of recurrence and effectiveness of adjuvant chemotherapy for resected non-small cell lung cancer [295]	38	Resectable NSCLC	Plasma sequencing: NGS on a custom 425 genes panel	Preoperative plasma samples, ctDNA+ in 19 (50%) patients ctDNA was detected post-chemotherapy in 8 of 36 (22.2%) patients and was associated with an inferior RFS (HR, 8.76; $P < 0.001$).

Table I-3: Non-exhaustive list of published trials with non-personalized methods to identify MRD through ctDNA detection.
WES: Whole exome sequencing; PCR: Polymerase chain reaction; ddPCR: digital droplet polymerase chain reaction; NGS: Next generation sequencing, UMI: Unique molecular identifier; NAT: Neoadjuvant treatment; CR: complete response; NAC: Neoadjuvant chemotherapy; pCR: pathological complete response; NSCLC: non-small cell lung cancer; CRC: colo-rectal cancer; PFS: Progression-free survival; OS: Overall survival; DFS: Disease-free survival; RFS: Relapse-free survival; HR: Hazard ratio; RCT: Radiochemotherapy; VPP: Positive predictive value; Sn: Sensitivity; CAPP-Seq: Cancer personalized profiling by deep sequencing.

As we can observe, many studies have shown the added value of ctDNA as marker of MRD.

I.2.3.2.3 ctDNA to detect minimal residual disease in SCCHN

Several studies have investigated ctDNA as a biomarker of MRD in SCCHN, with separation between HPV-positive and -negative SCCHN.

In HPV-negative SCCHN, using tumor-personalized technologies, several studies have shown the utility of detecting MRD to identify the patients likely to relapse. In a small cohort of SCCHN patients (n=8), Eygud et al. diagnosed MRD in 50% of patients who recurred (2/4) and in none of the patients who did not relapse. Interestingly, this team detected ctDNA in 2/2 patients who had a local relapse, and in 0/2 who had a distant relapse.[296] Kogo et al. used a methodology based on a specific squamous cell carcinoma gene panel, and designed ddPCR probes to be tested in the plasma based on the mutations found in the tumor. Out of 26 patients, only 18 were tested. Patients with ctDNA detected after curative treatment had a worst prognosis than the ones who did not have ctDNA after treatment ($p<0.0001$).[297] In the LIONESS study, Flach and colleagues used the RaDaR™ technology. After tumor DNA sequencing, a personalized ctDNA assay was built combining multiplex PCR and targeted NGS, added to bioinformatic pipeline to obtain ctDNA detection as low as 0.006%. Using this technology, they recently identified in a small cohort of 17 SCCHN patients, ctDNA in 100% of the pre-surgical plasma samples. In the 5 patients who experienced recurrence, ctDNA was detected in post-surgical plasma samples in all patients (5/5, 100%), with a lead time ranging from 108 to 253 days.[298]

As viral HPV DNA is easier to diagnose and monitor than mutations in tumor suppressor genes, several studies have shown the relevance of detecting cHPV DNA in OPC in order to detect MRD. First, using PCR based methods, Akashi et al. showed cHPV DNA detection at the time of recurrence in 2/2 patients.[299] Boyle et al. also showed, with the use of ddPCR, that post-surgical cHPV DNA was a marker of residual disease and was present in plasma two months prior to recurrence.[300] Similarly, Routman et al. showed that the presence of cHPV DNA in post treatment plasma sample was correlated to worst recurrence-free survival (83%) compared with the patients with absence of cHPV DNA (100%).[301] In his study, Tanaka

compared predictive value of PET-CT and cHPV DNA by ddPCR. He showed that PET-CT and cHPV DNA had similar negative predictive value (84% and 89.7%), but cHPV DNA post-treatment had a much higher positive predictive value (100% vs 50%). This could indicate the possible added value of cHPV DNA to PET-CT to assess recurrence risk in HPV-OPC patients.[302] Chera et al. obtained a high positive predictive value (94%) to detect HPV DNA through digital PCR (dPCR) in the bloodstream to diagnose disease recurrence, with a median lead time of almost 4 months, in case of two positive samples.[211] Using Taq-man quantitative PCR (qPCR), Rutkowski and colleagues showed that the absence of cHPV DNA 12 weeks after curative intent radio(chemo)therapy was correlated with absence of recurrence, even in cases of incomplete radiological response at 12 weeks. However, cHPV DNA was detected in only 6 patients, with 3 of them presenting a recurrence.[303] Using the same q-PCR technique, Reder showed that cHPV DNA levels rose in 4/5 HPV-OPC prior to recurrence.[304] Berger and colleagues, using detection of tumor tissue modified viral (TTMV)-HPV DNA, a multiplex ddPCR based technique, showed an overall positive predictive value of HPV DNA in post-treatment HPV-OPC plasma samples of 95%, and a negative predictive value of 95%.[305]

Using NGS methodologies, such as HPV16-detect, an amplicon based next generation sequencing assay, Lee showed 100% sensitivity to predict recurrence in case of HPV16-detect positivity at the end of treatment. However, this concerns only one patient out of 37 patients.[306]

For HPV driven OPC, cHPV DNA detection showed good correlation with recurrence. However, because of the low recurrence rate in small cohort size, the sensitivity and specificity of the tests might not be as strong in larger cohorts.

Hilke et al. used a non-patient personalized methodology with a gene panel based on the sequencing of all patient's sequencing data. They tested this approach in HPV-negative SCCHN patients during radiochemotherapy (RCT). ctDNA detection after RCT did not show good correlation with disease recurrence, as ctDNA was detected in 2 out of 8 patients who relapsed (25%) and in none of the patients without relapse.[196]

Recently, Sanz Garcia and colleagues presented an interesting comparison between different methodologies to detect MRD in SCCHN. For HPV-positive tumors, they compared dPCR and HPV-Seq, a specific HPV sequencing assay. In their cohort, HPV-seq seemed to be more sensitive to detect MRD, as it detected cHPV DNA in post-treatment samples of 4/4 patients who relapse, while dPCR was positive in only 2/4 patients. In HPV-negative patients, they compared tumor-personalized assay RADAR™ to a non-personalized methodology, CAPP-Seq (based on sequencing of cfDNA with a specific squamous cell carcinoma panel). In their cohort, RADAR was able to detect MRD in 2/2 patients who relapsed within a year but not in the patients who relapsed more than 1 year after end of treatment. CAPP-seq was not correlated with relapse free survival (p=0.25).[307]

Although few attempts of ctDNA with non-personalized methods have been made in SCCHN[233,308], we can observe that there is room for improvement, especially in HPV-negative SCCHN.

I.2.3.3 ctDNA in treatment monitoring

I.2.3.3.1 Prognosis and Impact

As ctDNA reflects tumor burden, ctDNA dynamic has the potential to reflect disease evolution under a specific treatment and predict response. This propriety of ctDNA has been used in several studies, in different cancer types undergoing different treatment, for examples chemotherapy[309], targeted therapies[310,311], or other systemic treatment.[312]

Because immunotherapy is a relatively new treatment option in many cancer types and because factors playing a role in response to immunotherapy are not yet fully understood, many studies have tried to uncover prognosis or predictive markers of immunotherapy through ctDNA. ctDNA has been investigated in several immunotherapy settings: precision medicine[313], monitoring treatment efficacy[314], markers of response to immunotherapy [315-317], and potentially clonal evolution.[318] ctDNA dynamics can predict immunotherapy response in several cancer types, such as melanoma[319,320] and non-small cell lung cancers.[321]

I.2.3.3.2 Treatment monitoring in SCCHN

As shown in Table I-4, ctDNA has been investigated in SCCHN, with different aims. For assessing the potential use of ctDNA as a surrogate to tumor-biopsy sequencing for mutational profiling, Porter et al. showed that analysing ctDNA in R/M SCCHN could allow identification of actionable genomic alterations. Interestingly, 73% of these mutations were not present in the tumor,[322] supporting the utility of ctDNA profiling in pre-treatment evaluation. However, a meta-analysis of several trials highlighted the low number of trials dedicated to SCCHN and more trials are required.[323]

Treatment monitoring for SCCHN, through the use of ctDNA, has been evaluated in a few studies. In LA SCCHN, ctDNA could be used to assess response to radiation. In pre-clinical models, ctDNA was a good reflection of disease eradication during radiotherapy.[324] In LA HPV-positive OPC, Cao et al. showed ctDNA kinetics were predictive of CRT efficacy, and was comparable to imagery analysis to evaluate treatment response.[325] Similarly, in LA HPV-negative SCCHN receiving RCT, Hilke and colleagues showed that ctDNA kinetics were correlated to treatment response.[196]

In R/M SCCHN treated with buparlisib during the BERIL-1 trial, ctDNA analysis could help to identify worst prognosis biomarkers, such as low tumor burden, HPV-negative status and *TP53* alterations, to the drug.[111,171]

In R/M HPV-positive SCCHN patients treated with immunotherapy, Haring and colleagues showed that HPV ctDNA monitoring through ddPCR was correlated to treatment response and could predict progression with a mean lead time of 70 days.[326] In R/M HPV-negative SCCHN, Taylor et al. showed that early dynamics in ctDNA were predictive of overall survival and progression-free survival in patients undergoing immunotherapy, with or without concomitant chemotherapy.[327]

This non-exhaustive overview highlights the use of ctDNA in several settings in SCCHN. There is however no consensus in the methodology used among the studies.

Reference	SCCHN population	Number of SCCHN included	Methodology	Conclusions
Molecular profiling				
Next generation sequencing of cell free circulating tumor DNA in blood samples of recurrent and metastatic head and neck cancer patients[322]	R/M HNC	60 with 21 OPC	Guardant360, a 70-gene ctDNA NGS platform.	73% (n=22) of patients had alterations identified in their blood that were not present in tumor specimens. In patients with squamous cell carcinoma, 66% had an off-label option identified and 90% had a trial option identified, while 50% of patients with salivary primaries had off-label options identified and 75% had trial options identified.
The Prognostic and Therapeutic Value of the Mutational Profile of Blood and Tumor Tissue in Head and Neck Squamous Cell Carcinoma[328]	SCCHN	75	Guardant360 platforms (70 genes).	Concordance was 13.0%. 65.3% of patients had actionable ctDNA alterations. ctDNA alterations were significantly associated with decreased overall survival (OS)

Prevalence of DNA Repair Gene Mutations in Blood and Tumor Tissue and Impact on Prognosis and Treatment in HNSCC[329]	SCCHN	170 SCCHN patients	Guardant360 platform	The addition of ctDNA contributed to identification of a significantly increased incidence of patients with mutations in DDR. Patients with ctDNA mutations in all subsets of DDR genes analyzed had significantly worse overall survival in univariate and adjusted multivariate analysis.
Predictive marker				
Next-generation sequencing (NGS) profiling of matched tumor and circulating tumor DNA (ctDNA) in head and neck squamous cell carcinoma (HNSCC)[330]	SCCHN	62 stage I-IV SCCHN	Safe Sequencing System for four genes (TP53, CDKN2A, HRAS and PI3KCA)	Patients presenting with mutations in ctDNA had shorter OS

Tumor-Naïve Multimodal Profiling of Circulating Tumor DNA in Head and Neck Squamous Cell Carcinoma[331]	SCCHN	30 patients with stage I-IVA human papillomavirus-negative HNSCC	CAncer Personalized Profiling by deep Sequencing (CAPP-seq) and cell-free Methylated DNA (cfMeDIP-seq)	Patients with detectable pretreatment ctDNA by both methods demonstrated significantly worse overall survival (HR = 7.5; *P* = 0.025) independent of clinical stage, with lack of ctDNA clearance post-treatment strongly correlating to recurrence.
Plasma HPV cell-free DNA monitoring in advanced HPV-associated oropharyngeal cancer[332]	Advanced HPV+ OPC	22	ddPCR	Total tumor burden (TTB) strongly correlated to HPV cfDNA levels (R = 0.91, P = 2.3×10-6). All participants demonstrated a corresponding change in their HPV cfDNA levels at a median of 16 days (range 12-38) before restaging scans confirming treatment response or progression.
Monitoring response to treatment				

Circulating tumour DNA kinetics in recurrent/metastatic head and neck squamous cell cancer patients[327]	R/M HNSCC patients treated with systemic therapy, platinum-based chemotherapy (CT) or ICB	53	CAncer Personalized Profiling by deep Sequencing (CAPP-Seq)	Change in ctDNA VAF after one cycle of treatment (ΔVAF (T1-2)) was predictive of both PFS ($p< 0.01$) and OS ($p< 0.01$).
Personalized circulating tumor DNA analysis as a predictive biomarker in solid tumor patients treated with pembrolizumab[333]	Patients undergoing pembrolizumab treatment	94 patients, 16 SCCHN	Tumor-personalized assay	ctDNA kinetics during treatment was associated with PFS, OS clinical response and clinical benefit
Cell-free DNA and circulating tumor cell kinetics in a pre-clinical head and neck Cancer model undergoing radiation therapy[324]	Pre-clinical study for HPV-positive tumors	seven rabbits were inoculated with VX2 cells in the buccal mucosa	quantitative PCR for papillomavirus E6	Plasma ctDNA reflects the overall tumor burden within the animal model. ctDNA and lymph node volumes showed a positive correlation

Dynamics of cell-free tumour DNA correlate with treatment response of head and neck cancer patients receiving radiochemotherapy[196]	LA SCCHN undergoing RCT	20 patients with locally advanced HNSCC to identify driver mutations	Deep sequencing and UMI-based error suppression for the identification of driver mutations and HPV levels	ctDNA can be seen as a surrogate marker of disease burden, tightly correlatingto the gross tumour volume prior to the treatment start.
Early HPV ctDNA Kinetics and Imaging Biomarkers Predict Therapeutic Response in p16+ Oropharyngeal Squamous Cell Carcinoma[325]	LA HPV-positive OPC undergoing chemoradiation	34	HPV sequencing	Low pretreatment ctDNA and an early increase in ctDNA at week 2 compared with baseline were significantly associated with superior FFP (freedom from progression) ($P < 0.02$ and $P < 0.05$, respectively).
Cell-free human papillomavirus DNA kinetics after surgery for human papillomavirus-associated oropharyngeal cancer[300]	HPV+OPSCC undergoing surgery	33	ddPCR	ctHPVDNA levels are associated with the risk of residual disease in patients with HPV+OPSCC undergoing curative intent surgery

Circulating tumour HPV16 DNA quantification - A prognostic tool for progression-free survival in patients with HPV-related oropharyngeal carcinoma receiving curative chemoradiotherapy[334]	p16-positive OPSCC	136	qPCR	Disease burden was significantly correlated to baseline ctHPV16-DNA levels (R = 0.39, p=<0.001). Both lower baseline levels and AUC-ctHPV16DNA were associated with improved progression-free survival (p = 0.01 and p < 0.001), overall survival (p = 0.013 and p = 0.002). In multivariable analysis including tumour volume and treatment allocation (cisplatin vs cetuximab), AUC-ctHPV16DNA remained a significant prognostic marker of progression-free survival.
Human papilloma virus circulating tumor DNA assay predicts treatment response in recurrent/metastatic head and neck squamous cell carcinoma[326]	metastatic HPV+ OPSCC	12	ddPCR	Longitudinal changes HPV16 ctDNA correlate to treatment response and ctDNA responses are observed earlier than conventional imaging (average 70 days, range: 35-166).

Rapid Clearance Profile of Plasma Circulating Tumor HPV Type 16 DNA during Chemoradiotherapy Correlates with Disease Control in HPV-Associated Oropharyngeal Cancer[265]	oropharyngeal squamous cell carcinoma (OPSCC) undergoing CRT	103	dPCR	Nineteen of 67 evaluable patients had a ctHPV16DNA favorable clearance profile, and none had persistent or recurrent regional disease after CRT. In contrast, patients with adverse clinical risk factors (T4 or >10 pack years) and an unfavorable ctHPV16DNA clearance profile had a 35% actuarial rate of persistent or recurrent regional disease after CRT (*P* = 0.0049).
Molecular Alterations and Buparlisib Efficacy in Patients with Squamous Cell Carcinoma of the Head and Neck: Biomarker Analysis from BERIL-1	R/M SCCHN	158	NGS sequencing for TP53 mutations, WES for mutational load and PCR for HPV status	ctDNA analysis showed *TP53* alterations, HPV-negative status, low mutational load

Table I-4: Treatment monitoring in SCCHN with ctDNA R/M: recurrent/metastatic; SCCHN: squamous cell carcinoma of the head and neck; OPC: oropharyngeal cancer, HPV: human papillomavirus; ctDNA: circulating tumor DNA; OS: overall survival; DDR: DNA repair genes; NGS: next-generation sequencing; HR: hazard ratio; ddPCR: digital droplet PCR, TTB: total tumor burden; CT: chemotherapy; ICB: immune checkpoint bloquers; VAF: variant allele frequency, PFS: progression free survival; RCT/CRT: radiochemotherapy; UMI: unique molecular barcodes; FFP :freedom from progression; OPSCC: oropharyngeal squamous cell carcinoma; AUC: area under the curve; qPCR: quantitative PCR

II. Aims and objectives of the thesis

II.1 To design a tumor-agnostic ctDNA assay to detect minimal residual disease in LA SCCHN and predict relapse

We hypothesized that minimal residual disease could be detected in LA SCCHN with the help of ctDNA, and that we could accurately identify patients with high risk of relapse. Our objective was to develop an in-house non-personalized ctDNA assay, and to test its accuracy in a cohort of LA SCCHN patients. We compared these results with a commercial tumor-personalized assay (Signatera™).

II.2 To design a tumor-agnostic ctDNA assay to evaluate response to immune checkpoint inhibitors in R/M SCCHN

We hypothesized that ctDNA kinetics could be used to monitor the efficacy of anti-PD-1 therapy in R/M SCCHN patients. Our objectives were to predict treatment response and long-term outcome. We developed an in-house non-personalized ctDNA assay and evaluated its accuracy in a cohort of R/M SCCHN patients undergoing anti-PD1 treatment. We compared these results with a commercial tumor-personalized assay (Signatera™).

III. ctDNA as a biomarker of MRD in SCCHN

III.1 Plasma tumor-agnostic assay for circulating tumor DNA detects minimal residual disease and predicts the outcome in locally advanced squamous cell carcinoma of the head and neck

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Adapted from Honoré N, van Marcke C, Galot R et al. Tumor-agnostic plasma assay for circulating tumor DNA detects minimal residual disease and predicts outcome in locally advanced squamous cell carcinoma of the head and neck. Annals of Oncology [335]

ABSTRACT

Introduction

Forty to fifty percent of patients with locally advanced squamous cell carcinoma of the head and neck (LA SCCHN) relapse despite multimodal treatment. Circulating tumor DNA (ctDNA) enables the detection of minimal residual disease (MRD) after curative-intent therapy and to identify earlier which patients will progress. We developed a tumor-agnostic plasma ctDNA assay to detect MRD in unselected LA SCCHN with the aim of predicting progression-free survival and overall survival without the need for tumor sequencing.

Material & Methods

A 26-gene next-generation sequencing (NGS) panel was constructed that included the most frequently mutated genes in SCCHN and two HPV-16 genes. MRD was assessed in each patient through an in-house informatic workflow informed by somatic mutations identified in the corresponding pre-treatment plasma sample. The presence of MRD was defined as the detection of ctDNA in one plasma sample collected within 1-12 weeks from the end of curative treatment. The primary endpoint was the progression-free survival (PFS) rate at two years. At least 32 patients were planned for inclusion with the hypothesis that PFS at two years was <30% in MRD-positive patients and >80% in MRD-negative patients (α= 0.05, β= 0.9).

Results

We sequenced DNA from 116 plasma samples derived from 53 LA SCCHN patients who underwent curative-intent treatment. ctDNA was detected in 41/53 (77%) patients in the pre-treatment samples. Out of these 41 patients, 17 (41%) were MRD-positive after treatment. The 2-year PFS rate was 23.53% and 86.6% (73.4-100%) in MRD-positive and MRD-negative patients, respectively ($p<0.05$). Median survival was 28.37 [14.30-NE] months for MRD-positive patients and was not reached for the MRD-negative cohort ($p=0.011$).

Conclusions

Our ctDNA assay detects MRD in LA SCCHN and predicts disease progression and survival without the need for tumor sequencing, making this approach easily applicable in daily practice.

III.1.1 Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the seventh most common malignancy and affects around 600,000 patients per year worldwide.[4] Less than sixty percent of patients with locally advanced (LA) disease (Union for International Cancer Control stages III and IV) remain free of disease at three years despite aggressive multimodal local therapy with surgery and/or chemo-radiation.[4,335,336]

Recent randomized trials that investigated treatment intensification in LA SCCHN failed to meet their primary endpoints.[335-337] These results may partly be explained by the absence of markers able to identify minimal residual disease (MRD) after curative-intent therapy. Such markers could potentially improve patient selection. Studies conducted in different cancer types have demonstrated that circulating tumor DNA (ctDNA) has the potential to identify patients likely to recur with high specificity and sensitivity.[199,224,230] The early detection of patients at high or low risk of cancer relapse after curative-intent treatment could drastically modify patient surveillance and therapeutic strategies.

SCCHN is heterogenous with several disease locations. It has two main etiologies (tobacco/alcohol versus human papilloma virus (HPV)) and each one has a different prognosis. Genomic alterations are mainly found in tumor suppressor genes.[80,338] These factors make ctDNA detection challenging, and only limited data are available for SCCHN, particularly for HPV-negative SCCHN. Chera et al.,[211] using a multianalyte digital polymerase chain reaction (PCR) assay, showed that HPV-16 ctDNA detection in plasma samples during post-treatment surveillance has high positive-predictive value (PPV) and high negative-predictive value (NPV) for diagnosing disease recurrence, with a median lead time of 3.9 months in patients with HPV-driven oropharyngeal cancer (OPC). Flash et al.[298] used a deep-sequencing personalized assay (RaDaR™) to detect ctDNA in post-surgery samples in 17 HPV-negative SCCHN. In all patients who developed disease recurrence, ctDNA was detected prior to progression with lead times ranging from 3.6 to 8.4 months. To detect ctDNA, both approaches investigated personalized

methodologies based on prior knowledge of the patient-specific tumor genomic landscape.

To the best of our knowledge, no studies have shown that a tumor-agnostic MRD assay can detect ctDNA with clinically meaningful specificity and sensitivity in unselected LA SCCHN. Such agnostic technologies have the advantage of detecting ctDNA without the need for a tumor biopsy and not requiring a costly and personalized assay for each patient. However, their sensitivity may be decreased given that several genes generally need to be sequenced, and deep sequencing does not have the same low limit of detection as PCR-based technologies.[339]

Aiming to develop an agnostic assay able to predict disease progression, we investigated an in-house 26-gene panel to diagnose MRD within 12 weeks from curative-intent therapy in unselected LA SCCHN. Our results demonstrate that early MRD detection is associated with a shorter progression-free survival (PFS) and overall survival (OS).

III.1.2 Materials & Methods

III.1.2.1 Patient cohort, study design, and inclusion criteria

Upon obtaining written informed consent, all SCCHN patients in our institution treated with standard-of-care protocols are prospectively enrolled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional biomarker trial collecting whole blood, plasma samples, and sometimes tumor biopsies, before, during, and after therapy (NCT02139020). The clinical characteristics and disease outcome of each patient are also prospectively recorded in a secured database (REDCap[340]). The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria were: patients (i) enrolled in UCL-ONCO-2013 between 2019 and 2022 with LA- SCCHN (stages III/IVa/IVb p16-negative oropharyngeal, larynx, oral cavity, hypopharynx, and unknown primary cancers; and stage I (only N1)/II/III p16-positive oropharyngeal cancer (OPC) according to the 8th TNM classification), (ii) treated with curative-intent

therapy according to standard of care, (iii) for whom pre- and post-treatment plasma samples and whole blood were available, and (iv) with no clinical disease progression from the initiation of curative-intent treatment until collection of the post-treatment plasma sample. The post-treatment plasma sample had to be collected within 12 weeks from the end of the curative treatment. Nasopharyngeal, salivary, and sinonasal cancer were excluded. HPV-driven OPCs were defined as tumors that were positive for HPV by in-situ hybridization (ISH).

III.1.2.2 Study objective and endpoints

Our main objective was to develop a tumor-agnostic ctDNA assay to detect MRD within 12 weeks from the end of curative-intent treatment in unselected LA SCCHN. Our primary endpoint was the rate of progression-free survival (PFS) at two years in the MRD-positive and MRD–negative patients who had ctDNA detected in their pre-treatment plasma sample. Secondary endpoints were PFS and overall survival (OS) in MRD-positive and MRD-negative patients.

MRD was defined as ctDNA detection in the post-treatment sample collected within 12 weeks from the end of the curative treatment. PFS was defined as the time interval between the date of the first day of curative treatment and the date of SCCHN progression, the date of last follow-up or the date of death due to any cause. OS was defined as the time interval between the date of the first day of curative treatment until death due to any cause, or until the date of last follow-up.

III.1.2.3 Blood and plasma collections

Plasma samples were harvested from each patient before the start of curative-intent treatment and between one and 12 weeks after the end of curative-intent treatment. When possible, plasma samples were also collected during follow-up, every two to three months until relapse, or for up to three years following a patient's initial diagnosis. For each patient and timepoint, plasma was isolated from whole blood collected in two EDTA 7.5mL tubes after double centrifugation (2000g 10 minutes and 2500g 15 minutes) within 30 minutes at 4°C. Plasma was stored immediately at -80°C until further use.

III.1.2.4 Cell free DNA (cfDNA) and germline DNA extraction

cfDNA was extracted from 4mL to 6mL of plasma using the Promega® Maxwell® RSC ccfDNA LV plasma kit (RSC; Promega, Leiden, the Netherlands) and quantified with Qubit® (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). Matched germline DNA was extracted for each patient with the Promega® Wizard® Genomic DNA Purification kit (RSC; Promega, Leiden, the Netherlands) from a whole blood sample in EDTA 3.5mL tubes, frozen at -80°C and quantified with Nanodrop® technology (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). All sample characteristics and quantification can be found in Supplementary Table VI- 1.

III.1.2.5 cfDNA targeted next-generation sequencing (NGS) and variant calling

Plasma samples were analyzed following our in-house workflow, as described in Figure III-1. All pre-treatment plasma samples were first analyzed with Kraken2 (v.2.1.2, database “Standard”, March 2023),[341] a taxonomic sequence classifier that assigns labels to DNA sequences. A patient was considered HPV16 positive if at least one read was assigned to the HPV16 genome in the pre-treatment plasma sample, according to Kraken2. Otherwise, the patient was considered HPV16 negative.

Pre- and post-treatment plasma samples of HPV16 ctDNA-negative patients were analyzed using our gene panel. MRD was assessed in each patient through an in-house informatic workflow informed by somatic mutations identified in the corresponding pre-treatment plasma sample (details in Supplementary data, VI-1 material & methods 1). A variant was considered positive in the pre-treatment plasma sample if it was (i) supported by at least three reads, (ii) present in reads aligned in forward and reverse, (iii) passed modified Mutect2 quality filters, and (iv) present in less than one percent of healthy individuals in the public sequence databases (Gnomad). Read alignment for all variants was inspected manually. The list of all criteria can be found in Table 3 of the Supplementary data. Only variants fulfilling all criteria were considered true somatic pre-treatment variants. A pre-treatment sample was considered ctDNA-positive if at least one somatic variant was called. A variant was considered present in the post-treatment plasma sample if it was (i) previously identified in the pre-treatment sample, (ii) called in the BAM file and (iii) detected in three or more reads in the BAM files of

post-treatment plasma sample of the same patient. The ctDNA level was considered to be “high” if the higher allele frequency per sample was above the median of all variant allele frequencies per timepoint, and “low” if below the median.

Digital droplet PCR (ddPCR) and *HPV in-situ hybridization* are described in VI-1 Supplementary data (material & methods 2)

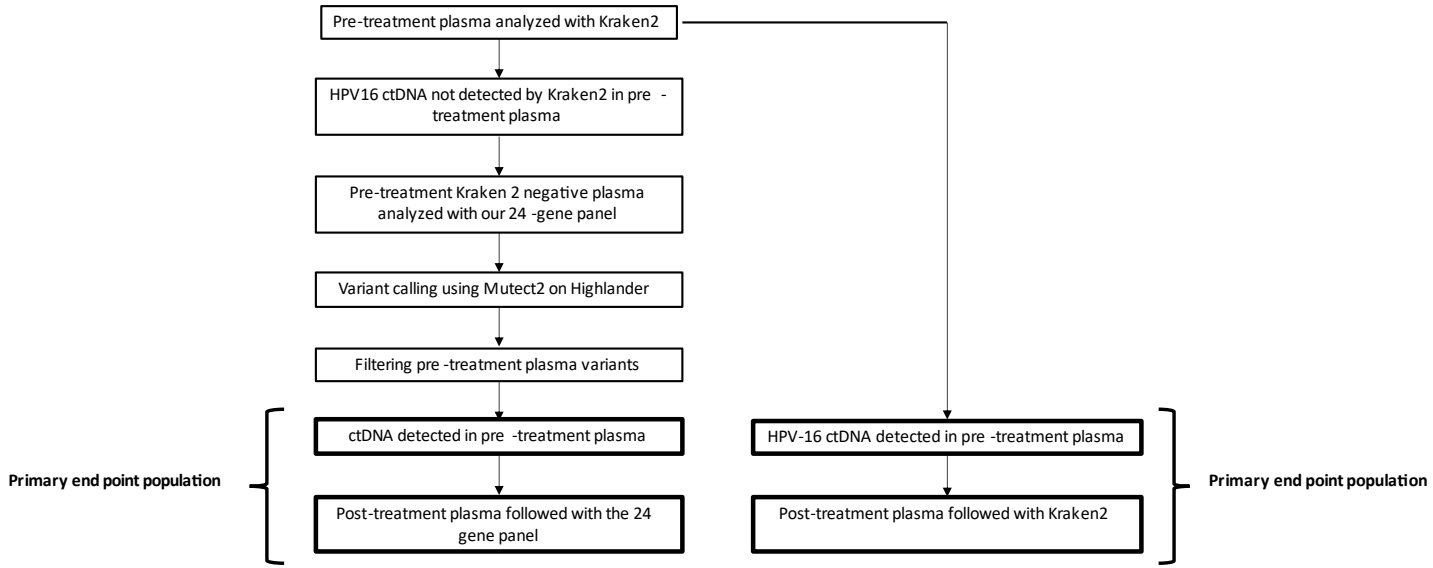

Figure III-1: In-house bioinformatic workflow.
ctDNA: circulating tumor DNA; HPV: human papillomavirus; Kraken2

III.1.2.6 Statistics

The primary endpoint was the PFS rate at two years in the population of patients who had ctDNA detected in the pre-treatment sample. The hypothesis was that the PFS rate at two years would be < 30% for MRD-positive patients and > 80% for MRD-negative patients. According to the 2-sided Z-test (unpooled variance), a minimum of 32 pre-treatment ctDNA-positive patients with at least two years of follow-up were needed (α= 0.05, power= 0.9).

PFS and OS were estimated using the Kaplan-Meier methods. Patients without any event (progression or death) were censored at the date of last-follow-up. The occurrence of another cancer was not considered to be an event. Treatment differences in PFS and OS were assessed using the log-rank test.

Cox proportional hazard regression models were used to assess the prognostic value of several parameters on OS and PFS. All analyses were done using SAS (v9.4) and R (v4.2.1).

III.1.3 Results

III.1.3.1 Patient characteristics

Between February 2019 and March 2022, 75 LA SCCHN patients were included in our prospective biomarker plasma collection trial. Fifty-three patients met our inclusion criteria (Figure III-2) and had pre-treatment and post-treatment plasma samples available. Patient characteristics are described in Table 3-1. The most frequent tumor site was the oropharynx (n=29, 54.7%). Seventeen (58.6%) patients had p16-positive oropharyngeal cancer and, of which, nine were HPV-positive by ISH. For one patient, ISH could not be performed due to insufficiency of tumor cells. Forty-nine (92.4 %) and four (7.6%) patients were treated with curative-intent (chemo)radiation and surgery as primary treatment, respectively. The estimated median follow-up was 31.0 months (range: 2.9-46.5). At two years, the PFS rate was 60.3% (95% CI: 48.0-75.8%) and OS was 78.0% (95% CI: 67.2-90.4%). The median PFS was 34.7 months (range: 1.7-44.0) and the median overall survival was not reached. Post-treatment plasma samples were collected between weeks 1 and 12 (median: 6 weeks) after the end of the curative-intent treatment.

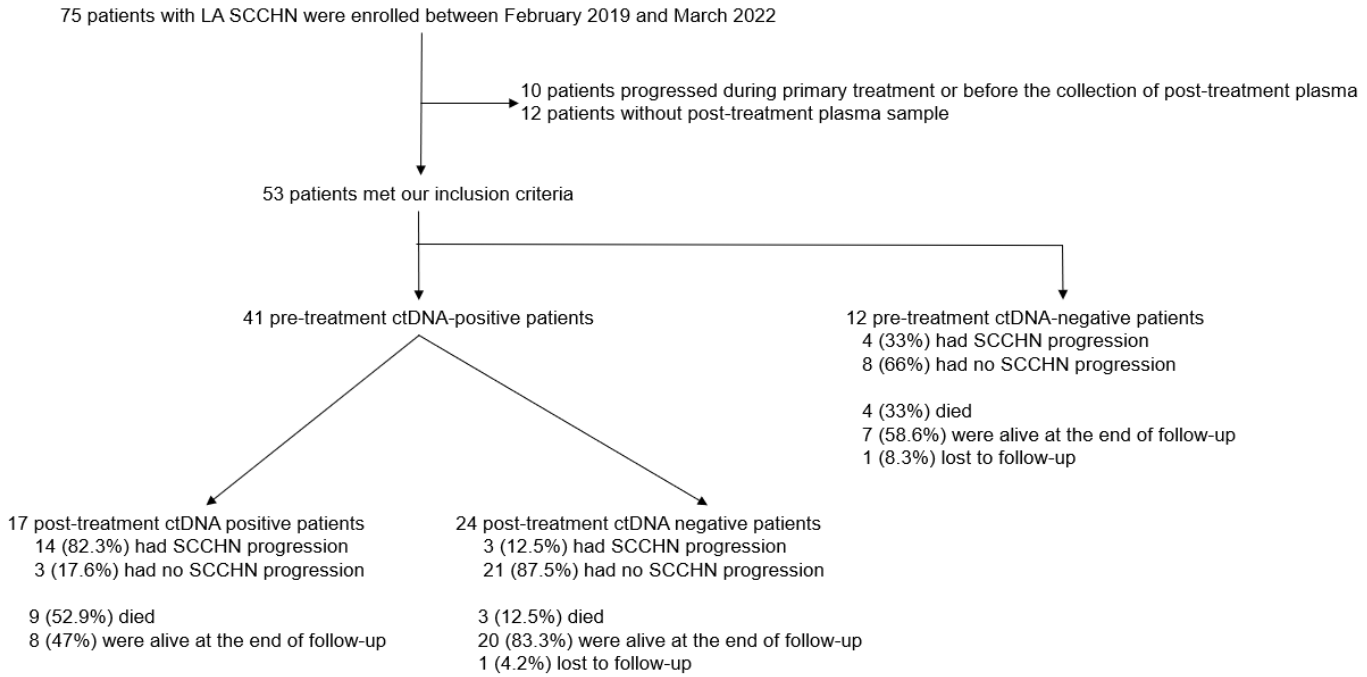

Figure III-2: Patient workflow
LA: Locally advanced; SCCHN: squamous cell carcinoma of the head and neck.

	Overall (N=53)
Sex	
Female	13 (24.5%)
Male	40 (75.5%)
Age (years)	
Median [Min, Max]	63.7 [41.1, 98.7]
Tobacco consumption (> 10 pack years)	
No	14 (26.4%)
Yes	39 (73.6%)
Alcohol consumption (> 2units/day)	
No	22 (41.5%)
Yes	31 (58.5%)
Primary localization of tumor	
Hypopharynx	8 (15.1%)
Larynx	9 (17.0%)
Oral cavity	5 (9.4%)
Oropharynx	29 (54.7%)
p16-negative	12 (41.3%)
p16-positive	17(58.6%)
HPV high risk ISH negative	7 (41.1%)
HPV high risk ISH positive	9 (53%)
HPV high risk ISH unknown	1 (5.8%)
Unknown primary	2 (3.8%)
Curative-intent treatment regimen	
Primary chemoradiation	39 (73.6%)
Primary radiation	10 (18.8%)
Primary surgery	4 (7.6%)
Staging according to 8th TNM edition	
p-16 positive oropharynx cancer	17(32.07%)
I (T1-T2, N1)	9(17.0%)
II	3 (5.7%)
III	5 (9.43%)
L, OC, H, UP cancers, p-16 negative OPC	36 (67.92%)
III	10 (18.86%)
IVa	17 (32.1%)
IVb	9 (17.0%)
Performance status (ECOG)	
0	35 (66%)
1	16 (30.2%)
2	2 (3.8%)
Disease progression	
No	32 (60.4%)
Yes	21 (39.6%)

Table III-1 : Patients' characteristics

ISH: in-situ hybridization; HPV: human papillomavirus; OPC: oropharyngeal cancer; L: larynx cancer; OC: oral cavity cancer; H: hypopharynx cancer; UP: unknown primary; ECOG PS: Eastern Cooperative Oncology Group performance status

III.1.3.2 Pre-treatment ctDNA detection rate

Overall, (HPV or somatic mutation(s)) ctDNA was detected in 41 (77%) out of the 53 pre-treatment plasma samples (Figure III-2).

The pre-treatment plasma samples of the 53 patients were first evaluated for the presence of HPV-16 ctDNA using Kraken2 (Figure III-1). The nine patients with HPV-related oropharyngeal cancer diagnosed by ISH and the patient for whom ISH could not be performed were found to have circulating HPV16 ctDNA in their pre-treatment samples. HPV ctDNA was not detected in the other patients.

Using the bioinformatic pipeline described in Figure III-1, 31 patients (72.1%) out of the 43 HPV16-negative patients by Kraken2 had at least one tumor variant in their pre-treatment plasma samples. Variants were found in the *TP53* (67.0%), *NOTCH1* (39.5%), *EGFR* (30.2%), *KMT2D* (25.5%), and *FAT1* (23.2%) genes. Variant allele frequency (VAF) ranged from 0.2% to 20.2% (median: 0.87%) of circulating cfDNA. Details of the variants found in the pre-treatment samples are included in the Supplementary Data (Table VI-5).

III.1.3.3 ctDNA detection within 12 weeks from completion of curative-intent treatment

Among the 41 pre-treatment ctDNA-positive patients, 17 (41.4%) had ctDNA detected in the paired post-treatment plasma sample collected within 12 weeks from the end of curative treatment. The VAFs ranged from 0.073% to 31.53% (median: 0.29%) in post-treatment plasma samples. Of these 17 HPV16-negative patients, 14 (82.3%) had disease progression (median time to progression after end of treatment: 10 months (range: 1-30 months)). Twenty-four patients were post-treatment ctDNA-negative, and 20 (83.3%) of these patients did not experience SCCHN relapse. The median time to disease progression for the three post-treatment ctDNA-negative patients who progressed was 14 months (4.8-27.4 months).

Overall, for the 41 pre-treatment ctDNA-positive patients, our MRD ctDNA assay had a sensitivity of 77.8%, specificity of 86.9%, positive-predictive

value of 82.3%, negative-predictive value of 83.3%, and accuracy of 79.1% for predicting disease progression.

Four patients were false negatives in that no ctDNA was detected by our assay after treatment despite confirmed disease progression. One of these patients had an HPV OPC, and HPV16 ctDNA was detected by ddPCR at 0.04 copies/mL in the Kraken2-negative post-treatment sample taken one week after treatment. One HPV-negative patient had no ctDNA detected in the post-treatment plasma sample taken at week one. In this patient, ctDNA was subsequently detected in the post-treatment plasma sample taken 13 weeks after the first. Unfortunately, the other two patients refused further plasma collection. Three patients were false positives in that ctDNA was detected after treatment without SCCHN progression. We analyzed the sequential plasma samples and the evolution of variant VAFs when available. One patient had a variant detected one week after curative-intent chemoradiation. This patient also refused sequential plasma sampling, making it impossible to determine if the variant disappeared thereafter. Interestingly, the other two patients developed non-SCCHN cancers during follow-up (Figure III-3). The first developed a lung adenocarcinoma and the second a pancreatic carcinoma at 20 and 12 months of follow-up, respectively. These second cancers were not detectable on 2'-deoxy-2'-[(18F)fluoro]-D-glucose positron emission tomography (18FDG-PET) nor on the computed tomography (CT) scans performed at the time of SCCHN diagnosis. For both patients, we sequenced the SCCHN biopsies obtained at diagnosis. The two ctDNA variants found in the pre-treatment sample of the lung adenocarcinoma patient were not found in the SCCHN biopsy and remained present in the plasma after SCCHN treatment (Figure III-3). They disappeared from the plasma following surgery of the lung adenocarcinoma, suggesting that this was where the variants originated from. In the patient with pancreatic adenocarcinoma, seven ctDNA variants were detected in the pre-treatment plasma sample. One of these variants was also found in the SCCHN biopsy but was cleared in the post-treatment plasma sample. However, the other six variants were not found in the SCCHN biopsy and were still present in the post-treatment plasma samples (Figure III-3), suggesting that they originated from the pancreatic cancer.

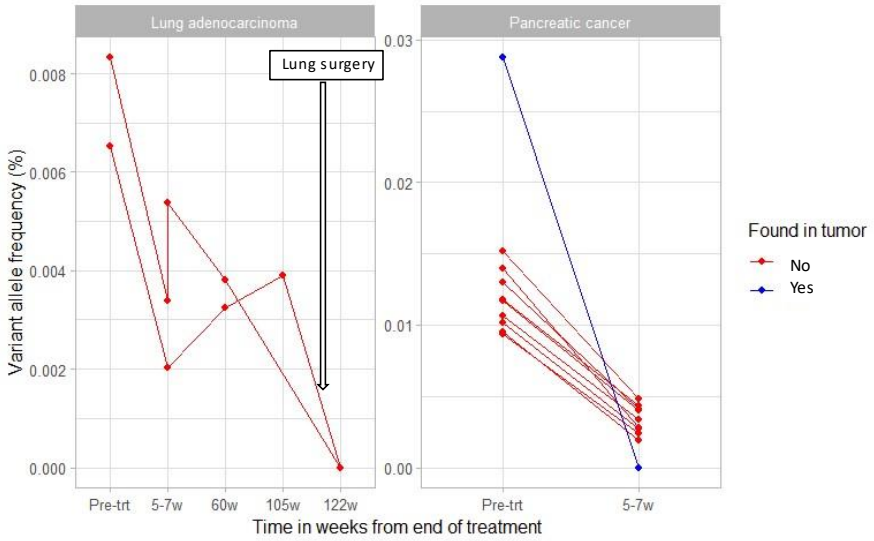

Figure III-3 : Kinetics of plasma variant detection in the two false-positive patients who developed another cancer
A Patient with lung adenocarcinoma. B Patient with pancreatic carcinoma. Variants found in the SCCHN are in blue, and variants not found in the SCCHN are in red.

III.1.3.4 The presence of MRD after curative treatment is associated with lower PFS and OS

For pre-treatment ctDNA-positive patients, the two-year PFS rate was 23.5% (IC 95%: 10-55%) and 86.6% (IC 95%: 73.4-100%) in the MRD-positive and MRD-negative patients, respectively ($p<0.05$). Median PFS was 11 months for the MRD-positive patients but was not reached for those who were MRD-negative ($p<0.0001$) (Figure 4a). Median survival was 28.4 months for the MRD-positive patients but was also not reached for the MRD-negative group ($p=0.011$) (Figure 4b).

We performed sensitivity analyses for PFS and OS in two subgroups: the pre-treatment ctDNA-positive HPV-negative population (n=31) and the eligible population enrolled in this study (n=53). For the latter analysis, pre-treatment ctDNA-negative patients were included in the MRD-negative

group. These additional analyses showed similar results to the ones observed in the primary endpoint population (Figure III-4).

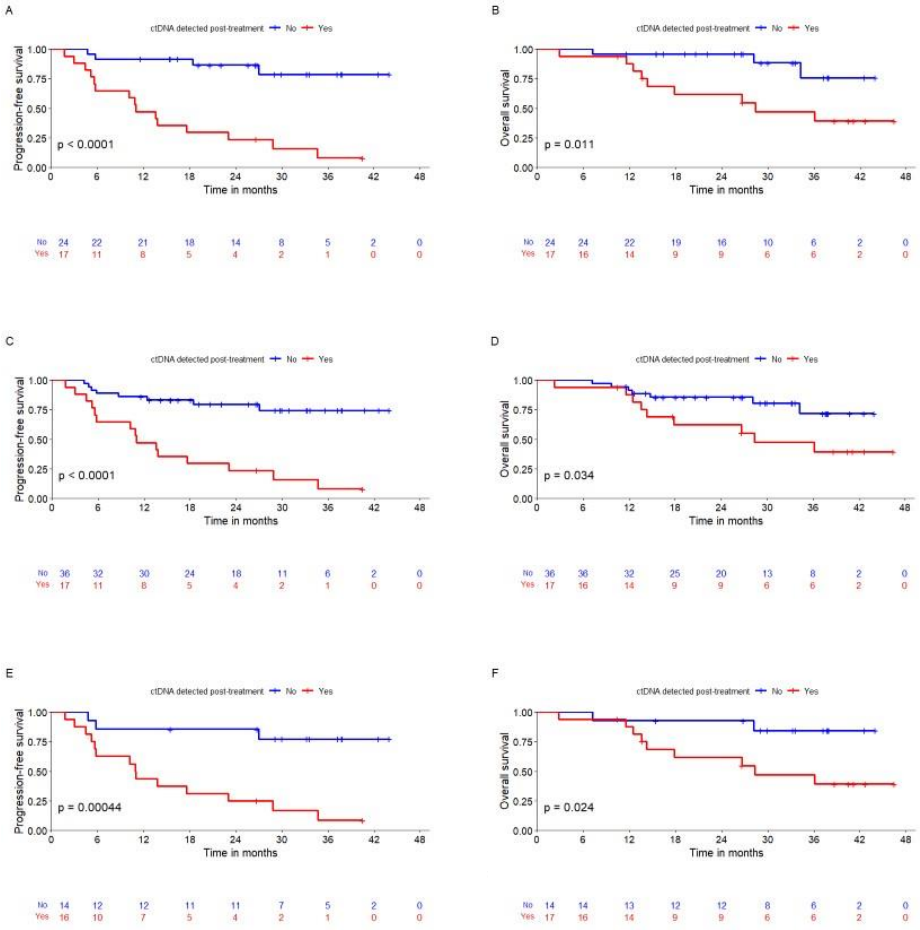

Figure III-4: Kaplan-Meier estimate of OS and PFS
Kaplan-Meier estimate of progression-free survival (A, C, E) and survival (Panels B, D, F) for MRD-positive and -negative patients in the pre-treatment ctDNA-positive population (n=41) (A, B), in the entire population (n=53) (C, D) and in the HPV-negative pre-treatment ctDNA-positive population (n=31) (E, F). Blue curves = MRD-negative patients; red curves = MRD-positive patients.

On the 53 patients who met our clinical inclusion criteria, we performed Cox univariate and multivariate analyses and included several prognostic factors: HPV status, disease stage, primary tumor location, Eastern Cooperative Oncology Group (ECOG) performance status, pre-treatment ctDNA level, and the presence of MRD) (Table III-2). In the univariate analysis, having a pre-treatment ctDNA level above the median value was also significantly correlated with worst PFS (HR=4.3 (1.82-10.39, p<0.001) but not OS (p=0.153). In the multivariate Cox model, MRD positivity remained an independent predictor of PFS (Table 2b) (HR=12.2; 95% CI [2.59-57-50]; p=0.002), and OS (HR=12.22; 95% CI [1.58-94.6]; p=0.017) (Table III-2).

A

Identification of potential prognostic factors for overall survival

		Univariate			**Multivariate**		
Factor	Value	p-value	Hazard-Ratio	HR 95%CI	p-value	Hazard-Ratio	HR 95%CI
ECOG	0-1	0.044	2.85	1.028-7.880			
	2	0.995	0.00	0.00-NE			
Pre-treatment ctDNA quantity	High	0.073	3.37	0.0894-12.73	0.039	4.60	1.078-19.60
Primary location	Hypopharynx	0.665	1.43	0.286-7.131			
	Oral Cavity	0.741	1.35	0.225-8.157			
	Oropharynx	0.692	0.76	0.188-3.027			
Staging TNM (8th version)	IV	0.592	1.37	0.435-4.299			
Presence of ctDNA in the pre-treatment sample	Yes	0.439	0.63	0.200-2.011			
HPV status	Positive	0.152	0.33	0.075-1.499	0.218	3.76	0.457-30.92
Presence of MRD	Yes	0.153	2.10	0.758-5.817	0.017	12.22	1.578-94.62

B **Identification of potential prognostic factors for progression-free survival**

		Univariate			Multivariate		
Factor	Value	p-value	Hazard-Ratio	HR 95%CI	p-value	Hazard-Ratio	HR 95%CI
ECOG	0-1	0.029	2.57	1.104-5.968			
	2	0.992	0.00	0.00-NE			
Pre-treatment ctDNA quantity	High	0.090	2.28	0.472-6.615	0.030	3.12	1.113-8.734
Primary location	Hypopharynx	0.399	1.77	0.472-6.615			
	Oral Cavity	0.352	2.04	0.454-9.201			
	Oropharynx	0.579	0.71	0.212-2.379			
Staging TNM (8th version)	IV	0.710	1.19	0.483-2.913			
Presence of ctDNA in the pre-treatment sample	Yes	0.653	1.28	0.434-3.791			
HPV status	Positive	0.019	0.18	0.041-0.752	0.671	1.48	0.242-9.060
Presence of MRD	yes	<0.001	4.34	1.817-10.39	0.002	12.21	2.593-57.50

Table III-2: Multivariate estimates for OS (A) and PFS (B)

III.1.4 Discussion

We explored an in-house tumor-agnostic ctDNA assay to detect MRD after curative-intent treatment in unselected LA SCCHN. Our study met its primary endpoint having demonstrated a two-year PFS of 23.5% (IC 95%: 10-55%) and 86.6% (IC 95%: 73.4-100%) in MRD-positive and MRD-negative patients, respectively ($p<0.05$). With our approach, we were able to identify, with a good degree of certainty, a subgroup of patients with poor prognosis who may benefit from additional treatment and/or surveillance.

ctDNA detection has shown promising results in other cancer types for treatment monitoring,[342] MRD detection,[343] and the identification of resistant clones.[344] However, most of these results were obtained with personalized tumor-informed assays, which require a tumor biopsy for each patient and the development of a time-consuming and expensive patient-specific assay.

Our methodology intends to loosen these constraints in the form of a single assay that can be applied to all LA HNSCC patients without the need for tumor sequencing.

Sanz Garcia et al. investigated MRD detection using another tumor agnostic assay, Cancer Personalized Profiling by deep sequencing (CAPP-seq), in 29 patients with LA SCCHN.[345] Preliminary results were recently presented, but MRD was not shown to be associated with relapse-free survival.

Since MRD was detected through an in-house bioinformatic workflow informed by the somatic mutations identified in the pre-treatment plasma samples, a possible downfall of our methodology is that pre-treatment ctDNA was only detected in 41 (77%) of the 53 patients included, with 100% ctDNA detection in HPV-driven OPC and 72% in HPV-negative SCCHN. However, personalized tumor assays are not without their own limitations. For example, they need a good quality tumor biopsy for each patient in order to identify the variants of interest to be tracked in plasma, and this is only achieved in 70%[297,330] to 100%[298,308] of all SCCHN cases. This might be due to the low quality or quantity of tumor-DNA extracted from the tumor biopsy or the inability to even obtain a tumor biopsy to start with.[339]

Several factors may likely influence the pre-treatment sensitivity observed in our MRD ctDNA assay. One is the quantity of cfDNA obtained since a lower quantity will negatively impact the detection limit.[346] ctDNA variants with a lower VAF could be missed as variants in genes not present in our panel. The minimal post-treatment VAF was 0.07%. This is comparable to other assays that are able to successfully detect MRD in other cancers.[225,292,347]

Two false-positive patients developed second new cancers that were not detected radiologically at the time of SCCHN diagnosis. Our data indicate that these second cancers are the likely reason for the presence and persistence of post-treatment ctDNA diagnosed by our assay. This highlights the potential use of ctDNA as a cancer screening tool as is currently being investigated in large cohort studies.[348,349]

Our study was designed to investigate the prognostic impact of post-treatment ctDNA within 12 weeks from the end of curative treatment. The

ability to detect patients with a high risk of relapse may help identify earlier those who could benefit from adjuvant therapy or those who need close follow-up with salvage treatment in mind. However, the best time to assess MRD is unknown and varies from one study to another.[350] In SCCHN, the situation is even more complex since the efficacy of primary chemoradiation is generally evaluated only 12 weeks later.[351] To the best of our knowledge, there are no studies that have evaluated the optimal timing of post-treatment ctDNA clearance after chemoradiation. Hilke et al.[196] detected MRD at six to 12 weeks after the end of chemoradiation in two out of eight patients that relapsed. Similarly, Burgener et al.[331] showed that the lack of ctDNA clearance three months after the end of curative therapy was strongly correlated with recurrence. In HPV-OPC, Chera et al.[265] observed, despite rapid clearance of HPV being a good prognostic factor, that patients with detectable HPV ctDNA at week six of chemoradiation did not have a poorer prognosis, and that some patients had even cleared their HPV ctDNA after six months. In our study, post-treatment plasma samples were collected between weeks one and 12 (median: six weeks) after the end of curative-intent treatment. Although we cannot exclude that the variability in timing may have influenced our results, we observed good correlation between the presence of MRD and disease progression. One false-positive patient had plasma collected one week post chemoradiation, and it cannot be excluded that this patient was subsequently able to clear his ctDNA. It would be interesting to evaluate the clearance of SCCHN ctDNA after chemoradiation on a weekly basis to determine optimal sampling timing.

To the best of our knowledge, Kraken2 has never been used in p16-positive SCCHN to detect HPV ctDNA. In clinical practice, HPV-driven tumors are detected through p16 immunochemistry with an estimated sensitivity of 96% and specificity of 92%.[352] In our cohort, 16 OPC had p16-positive staining and only 10 of these patients were HVP16 ctDNA-positive according to Kraken2. We observed 100% concordance with ISH to determine HPV status. This highlights the value of our assay in determining which tumors are HPV-driven without the need for ISH.

One limitation of our trial is the low proportion of patients with HPV-driven tumors (19%). HPV-positive patients have a better prognosis compared to those with HPV-negative disease.[4] We observed one patient who was not

diagnosed with MRD using Kraken2 at week one even though HPV ddPCR detected MRD. However, in this patient, HPV16 ddPCR detected only 0.04 copies/mL at this timepoint, which is at the limit of detection (Supplementary data, Table III-4). Since the number of HPV-positive patients in this trial was too low to assess the validity of our assay to detect MRD in HPV-positive tumors, larger cohorts are needed to fully assess the potential of this approach in this group of patients. It may also be the case that Kraken2 is not able to achieve the same sensitivity as HPV ddPCR.

III.1.5 Conclusions

Our study demonstrates the feasibility of detecting MRD in LA SCCHN using a tumor-agnostic approach. Although we investigated this approach on a limited number of patients, the results are striking, particularly for HPV-negative SCCHN. Since this technology does not require tumor sequencing and is not personalized, it has the potential to be used widely and at a lower cost per patient. Our assay could stratify patients according to their risk of relapse and identify candidates for future interventional trials investigating escalation and de-escalation strategies.

III.1.6 Addendum

In order to evaluate our methodology, we tested different variants with ddPCR (Table III-4). As recommended by the Bio-Rad® protocol, for each mutation, a temperature gradient was established to find the correct temperature of amplification, in order to get maximum positive droplets. Then for each variant, a gradient of 9 different concentrations (5%, 2%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02%, 0.005% and 0.002%) was established with DNA gblocks (IDT®). Ten nanograms of gblock DNA was used for each gradient well. To establish background detection, on the same plate, triplicates of NTC (no template control) and wild-type DNA wells were loaded to compare with the lowest limit of detection. The quantity of DNA used for each sample and the number of wells per sample are detailed in Table III-3. All ddPCR plates were analyzed according to protocol on Quanta Soft™ Analysis Pro software.

	Conc Qubit ng/µl	ng/well	Number of wells
133-290919-B	0.407	4	3
133-131119-FU	0.301	4	3
213-06-10-21-B	0.969	10	3
213-191121-FU	0.536	7	3
213-140122-FU2	0.308	4	3
102-150219-B	0.419	4	3
102-190419-FU	0.576	4	3
136-300919-B	0.246	3	3
136-191119-FU	0.372	4	3
150-021219-B	1.29	6	3
150-020120-FU	0.425	5	2
108-020419-B	0.524	5	3
108-180619-FU	1.03	5	3
208-010621-B	0.516	4	3
208-090921-FU2	0.342	4	3
208-090921-FU2	0.505	4	3
186-161120-B	0.086	1	3
186-110221-FU	0.133	1.7	3
186-260821-FU2	0.154	2	3
186-211021-FU3	0.637	4	3
186-301221-FU4	0.186	2.5	3
131-090919-B	0.312	4	2
131-301019-FU	2.78	6 or 30	3-6

Table III-3: Concentrations and number of wells used for ddPCR evaluation.
Conc: concentration; ng: nanograms; B: pre-treatment plasma sample; FU: plasma harvested at follow-up.

A major drawback for ddPCR, one of the most sensitive techniques to detect variants at a very low proportion, is the quantity of DNA loaded in the different wells. As described in the Bio-Rad® protocol, 10ng of DNA will allow a limit of detection of only 0.1%, the limit of detection often reached with our

gradient. With quantities of DNA mostly inferior to 10ng, variants were detected by ddPCR only if present in high VAF.

We did not find, amongst the variants tested, any variants found in the NGS results nor in ddPCR if the VAF was higher than the ddPCR limit of detection. There was one variant that did not pass our stringent filters and was found in the pre-treatment plasma sample by ddPCR, suggesting that, for some variants, our filters to select variants of interest may be too stringent.

Mutations		Probe (Bio-Rad)		Gblock				NGS		ddPCR					
		unique assay ID	Catalog #	gblock ref	gblock batch	Conc gblock ng/µl (Qubit)	Patient record ID	sample	VAF	Limite of detection gblocks	Detected in B sample	Detected in FU1 sample	Detected in FU2 sample	Detected in FU3 sample	Detected in FU4 sample
TP53 p.H179R	c.536A>G	dHsaMDV2010125	10049550	235294503	541635029	11.9	133	T	38.60%	0.10%	yes	yes			
								B	6.70%						
								FU1	26.90%						
TP53 p.H179R	c.536A>G	dHsaMDV2010125	10049550	235294503	541635029	11.9	213	T	51%	0.10%	no	no	no		
TP53 p.Y220C	c.659A>G	dHsaMDV2510536	10049550	235294504	541547283	10.3	102	T	23.90%	0.10%	yes	yes			
								B	11.60%						
								FU1	16.40%						

EGFR p.V769M	c.2305G>A	dHsaMDS203868197	10049047	235294505	541547293	10.9	133	T	85.00%	0.10%	yes	no			
								B	3.00%						
								FU1	26.90%						
TP53 p.R196*	c.586C>T	dHsaMDV2010121	10049550	235294506	541547262	8.49	108	T	65%	0.50%	no	no			
FAT1 p.V2582M	c.7744G>A	dHsaMDS157701253	10049047	235294507	541574936	9.19	136	B	0.30%	0.50%	no	no			
								FU1	0.20%						
TP53 p.I195T	c.584T>C	dHsaMDV2516898	10049550	235294508	541547295	8.3	186	B	0.20%	0.05%	yes	no	no	no	no
TP53 p.G245N	c.733_734GG>AA	dHsaMDS442514572	10049047	235294509	541657682	8.43	108	B	0.70%	0.10%	yes	yes			
								FU1	0.40%						
TP53 p.H193L	c.578A>T	dHsaMDV2516872	10049550	235294510	541574896	7.21	150	B	2.90%	0.10%	yes	no			

FAT1 p.R933C	c.2797C>T	dHsaMDS597745103	10049047	235294511	541547269	10.4	208	no did not pass quality filter	0.60 %	0.10 %	yes	no			

Table III-4: ddPCR results

Conc : concentration, ddPCR : digital droplet PCR ; VAF : variant allele frequency ; NGS : next generation sequencing; T: tumor; B: pre-treatment sample; FU: follow-up

III.2 ctDNA as a biomarker of minimal residual disease using a tumor-personalized approach

Abstract

Introduction

Despite multimodal treatment, locally advanced (LA) squamous cell carcinoma of the head and neck (SCCHN) have a recurrence rate of ~50%. Circulating tumor DNA (ctDNA) has emerged as a potential biomarker for minimal residual disease (MRD) detection.

Methodology

MRD was assessed using a bespoke multiplex-PCR tumor personalized assay (Signatera™) on plasma sample harvested within 12 weeks from the end of curative intent treatment. The primary endpoint was progression free-survival (PFS) of MRD-positive and negative patients.

Results

Out of a cohort of 50 LA SCCHN patients, this assay could be successfully designed for 43 patients. ctDNA was detected in 42/43 (97.7%) of the pre-treatment plasma samples, and in 4/43 of the post-treatment plasma samples. Out of the 4 MRD-positive patients, three had a recurrence. Ten out of the 39 MRD negative patients relapsed.

Conclusion

The bespoke multiplex-PCR tumor personalized assay (Signatera™) can detect ctDNA in untreated LA SCCHN but did not achieve enough sensitivity to detect MRD within 12 weeks from the end of the curative treatment.

III.2.1 Introduction

Squamous cell carcinoma of the head and neck (SCCHN) represent the 7th neoplasia in incidence worldwide. Despite multimodal treatment, locally advanced (LA) SCCHN have a recurrence rate of ~50%. Circulating tumor DNA (ctDNA) has emerged as a potential biomarker for minimal residual disease (MRD) detection.

In chapter III-1, we have shown the potential benefit of our in-house non-personalized assay to detect ctDNA as a biomarker of MRD. In this chapter, we evaluated a commercial tumor-personalized bespoke, multiplex-PCR, next-generation sequencing assay (Signatera™) to detect MRD in LA-SCCHN patients who underwent curative-intent treatment.

III.2.2 Materials & Methods

III.2.2.1 Patient cohort, study design, and inclusion criteria

Upon obtaining written informed consent, all SCCHN patients in our institution treated with standard-of-care protocols are prospectively enrolled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional biomarker trial (NCT02139020). The clinical characteristics and disease outcome of each patient are also prospectively recorded in a secured database (REDCap[340]). The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria were: patients (i) enrolled in UCL-ONCO-2013 between 2019 and 2022 with LA- SCCHN (stages III/IVa/IVb p16-negative oropharyngeal, larynx, oral cavity, hypopharynx, and unknown primary cancers; and stage I (only N1)/II/III p16-positive oropharyngeal cancer (OPC) according to the 8th TNM classification), (ii) treated with curative-intent therapy according to standard of care, (iii) for whom pre- and post-treatment

plasma samples and whole blood were available, and (iv) with no clinical disease progression from the initiation of curative-intent treatment until collection of the post-treatment plasma sample. The post-treatment plasma sample had to be collected within 12 weeks from the end of the curative treatment. Nasopharyngeal, salivary, and sinonasal cancer were excluded. HPV-driven OPCs were defined as tumors that were positive for HPV by in-situ hybridization (ISH).

III.2.2.2 Study objective and endpoints

Our main objective was to evaluate the ability of a bespoke tumor-personalized multiplex PCR assay (Signatera™) to detect MRD within 12 weeks from the end of curative-intent treatment in unselected LA SCCHN. Our primary endpoint was progression-free survival (PFS). Secondary endpoint was overall survival (OS).

MRD was defined as ctDNA detection in the post-treatment sample collected within 12 weeks from the end of the curative treatment. PFS was defined as the time interval between the date of the first day of curative treatment and the date of SCCHN progression, the date of last follow-up or the date of death due to any cause. OS was defined as the time interval between the date of the first day of curative treatment until death due to any cause, or until the date of last follow-up.

III.2.2.3 Blood and plasma collections

Plasma samples were harvested from each patient before the start of curative intent treatment (baseline or pre-treatment sample) and within 12 weeks from the initiation of treatment (post-treatment sample). For each patient and time-point, plasma was isolated from whole blood collected in two EDTA 7.5mL tubes after double centrifugation (2000g 10 minutes and 2500g 15 minutes) within 30 minutes at 4°C. Plasma was stored immediately at -80°C until further use. Two to 8 mL of plasma were used for DNA extraction, performed as previously described.[333]

III.2.2.4 Germline DNA extraction

Matched germline DNA (gDNA) was extracted for each patient with the Promega® Wizard® Genomic DNA Purification kit (RSC; Promega, Leiden, the Netherlands) from a whole blood sample in EDTA 3.5mL tubes, frozen at -80°C and quantified with Nanodrop® technology (Thermo Fisher Scientific,

Waltham, Massachusetts, U.S.A.). At least 40µL of gDNA was used for extraction, as previously described.[333]

III.2.2.5 Tumor preparation

For each patient, five to 10 unbaked slides (7µm) from needle core biopsy or surgical sample were used for tumor DNA extraction, accompanied by one fixed slide colored with Hematoxylin and Eosin (HE) for tumor-contouring purposes. Only samples containing more than 20% of tumor cells were deemed to have sufficient tumor DNA to be extracted. Tumor DNA (tDNA) was extracted as previously described.[333]

III.2.2.6 Personalized ctDNA assays

Design and application of personalized ctDNA (bespoke, multiplex-PCR, next-generation sequencing) assays was conducted with blinding to clinical data by Natera. Paired tumor and gDNA WES data were used to identify tumor-specific somatic mutations for each patient and clonality was estimated as described previously[353]. Multiplex-PCR primer pairs targeting 16 highly ranked tumor-specific variants were designed as previously described.[290,354,355] The primer sequences for each variant are from the Signatera™ assay and are proprietary of Natera®. Somatic small nucleotide variants present in the tumor but absent in the germline were used to select the 16 targets, with prioritization based on multiple factors, including the observed variant allele frequency in the tumor tissue and the associated background noise profile in the plasma. This prioritized list of variants was then used to design PCR amplicons based on optimized design parameters, ensuring uniqueness in the human genome, amplicon efficiency and primer interaction. Next, multiplexed targeted PCR was conducted followed by amplicon deep sequencing on an Illumina platform. A sample was considered ctDNA positive when ≥2 out of the 16 selected target mutations were present at above a predefined threshold. Details of the analytical validation of the assay were previously described.[353] Variant allele frequencies were determined for each of the 16 target mutations. The quantity of DNA in plasma sample was evaluated as “mutant molecules per mL” (MTM/mL), calculated as the quantity to cfDNA extracted per sample (converted in pg) multiplied by the haploid genome equivalent (3.3pg) multiplied by the variant allele fraction (VAF) on the volume of plasma extracted (mL). If no variant was detected in one sample, MTM/mL was established at 0 MTM/mL.

$$MTM/mL = cfDNA\ extracted\ (ng) \times 1000\ pg \times 3.3pg\ haploïd\ genome\ equivalent \times \frac{Variant\ Allele\ Fraction\ (VAF)}{Plasma\ volume\ extracted\ (mL)}$$

III.2.2.7 Statistics

PFS and OS were estimated using the Kaplan-Meier methods. Patients without any event (progression or death) were censored at the date of last-follow-up. The occurrence of another cancer was not considered to be an event. Treatment differences in PFS and OS were assessed using the log-rank test.

All analyses were done using R (v4.2.1).

III.2.3 Results

III.2.3.1 Patients' characteristics

Between February 2019 and January 2023, 100 LA SCCHN patients were included in our prospective biomarker plasma collection trial. Fifty patients met our inclusion criteria and were therefore included in this analysis. We hereby present the analysis on 50 patients, of whom 50 gDNA, 50 tDNA and 100 plasma samples have been analyzed. Clinical characteristics may be found in Table III-5. The majority of patients (30/50, 60.0%) had OPC. Eighteen were p16 positive. Thirty-nine (78.0%) had locally-advanced SCCHN (stage III/IV according to 8th TNM classification). The patients with stage I and II were p16 positive OPC, according to the 8th TNM classification.

	Overall
	(N=50)
Sex	
Female	12 (24.0%)
Male	38 (76.0%)
Age (years)	
Median [356]	63.7 [43.7, 76.1]

Tobacco consumption (> 10UAP)	
No	13 (26.0%)
Yes	36 (72.0%)
Missing	1 (2.0%)
Alcohol consumption (>2units/day)	
No	18 (36.0%)
Yes	32 (64.0%)
Primary localisation of tumor	
Oral cavity	6 (12.0%)
Oropharynx	30 (60.0%)
p16 status	
Negative	12 (40.0%)
Positive	18 (60.0%)
Hypopharynx	6 (12.0%)
Larynx	8 (16.0%)
Performance status (ECOG)	
0	31 (62.0%)
1	16 (32.0%)
2	3 (6.0%)
Staging according to 8th TNM	
I	8 (16.0%)
II	3 (6.0%)
III	17 (34.0%)
IVa	18 (36.0%)
IVb	4 (8.0%)

Table III-5: Patients' characteristics
YP: year packet; HPV: human papillomavirus; ECOG: eastern cooperative oncology group,*within a clinical trial

III.2.3.2 Samples characteristics

Out of the 50 patients, tDNA was successfully extracted and sequenced in 43 tumor samples. Six patients did not have their tDNA sequenced due to insufficient tumor content for processing and one did not achieve a mean sequencing coverage depth of 180x. For each patient, 16 tumor variants were selected and a 16-plex PCR was designed.

Out of the 86 plasma samples, median plasma volume was 6.95mL [range: 1.8-8.6mL] and median cell-free DNA (cfDNA) was 23 ng [range: 5-615 ng]. All samples passed Quality Control criteria except one post-treatment plasma sample (Figure III-5).

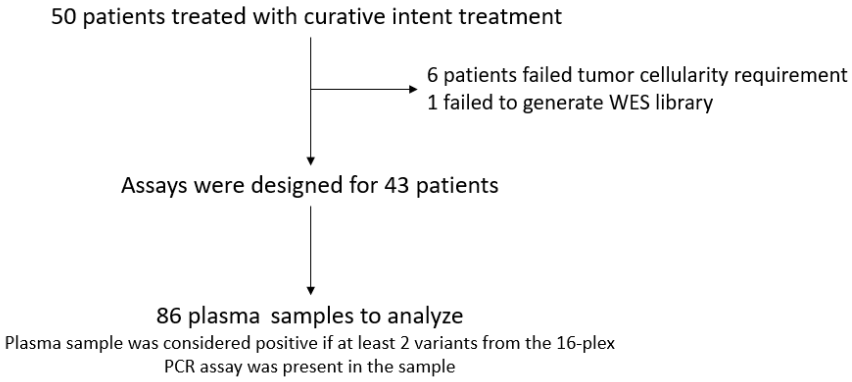

Figure III-5: Patients' workflow
LA: locally advanced; SCCHN: squamous cell carcinoma of the head and neck; tDNA: tumor DNA; ctDNA: circulating tumor DNA

III.2.3.3 Pre-treatment detection rate

At baseline, 42 out of 43 pre-treatment samples were considered positive for the presence of ctDNA, with a sensitivity of 97.7%. Median MTM was 5.8 MTM/mL. Median variant allele fraction was 0.386% across the 24 samples and a median of 15.5 variants was detected per assay.

III.2.3.4 Post-treatment detection rate

ctDNA was detected in 4 out of the 43 post-treatment plasma samples, with a median of 0.97 MTM/mL and a median of mean VAF of 0.07% variants

found in the 4 positive plasma samples. Three patients experienced relapse within 12 months after the end of treatment. The other one did not experience relapse. Among the 39 MRD-negative patients, 10 (25.64%) relapsed. Overall, this this MRD test could predict relapse with a sensitivity of 23%, a specificity of 96.7%, a positive-, negative- predictive value and an accuracy of ~74%.

III.2.3.5 ctDNA detection post-treatment was not predictive of PFS and OS

Median PFS was not reached in the MRD-negative patients and was 9.54 months [95%CI:1.32-NE] (p-value=0.0091) in MRD-positive group. Median OS was 14.74 months in the MRD-positive group and not reached in the negative group (p-value=0.041)(Figures III-6 & III-7). However, this comparison is not relevant due to low number of patients in the MRD-positive group.

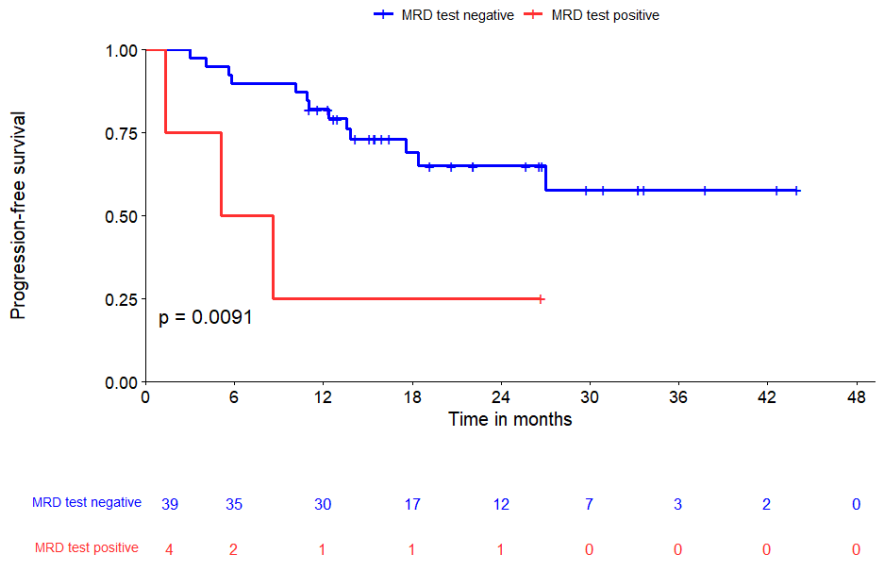

Figure III-6: Kaplan-Meier estimate for progression free survival
Kaplan-Meier estimate for progression free survival between the MRD positive group (red) and MRD negative group (blue)

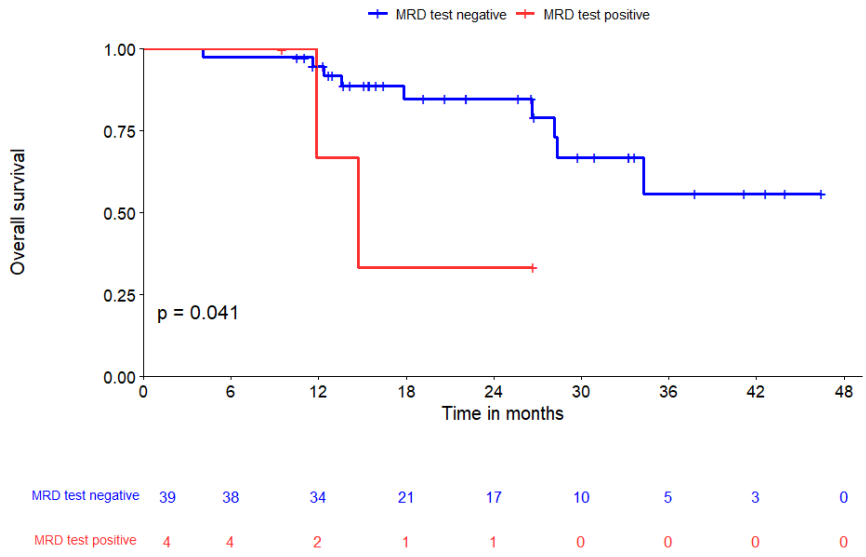

Figure III-7: Kaplan-Meier estimate for overall survival
Kaplan-Meier estimate for overall survival between the MRD positive group (red) and MRD negative group (blue)

III.2.4 Discussion

This analysis did meet its primary endpoint. However, results are to be analyzed with caution. Although one third (13/43) of the patients had a disease recurrence, this tumor personalized assay (Signatera™) detected MRD in only three (23%) of these patients. ctDNA was detected in another patient who did not experience recurrence even after more than 2 years of follow-up.

Circulating tumor DNA can be used in various settings such as monitoring of treatment response or detection of minimal residual disease. In those settings, in other tumor types, tumor-personalized multiplex-PCR assays have shown promising results with strong correlation with disease relapse-free survival in breast cancer[357] and bladder cancer.[290] To the best of our knowledge, this is the first study of minimal residual disease in SCCHN using this assay.

Although there is a good sensitivity at baseline (97.7%), this assay was not able to detect MRD in SCCHN with good sensitivity and accuracy. We could hypothesize that the threshold needed to detect MRD in SCCHN is very low. Indeed, ctDNA levels could depend on tumor type. SCCHN cells seem to shed less ctDNA than other cancers such as colon cancer and variant allele frequencies are usually lower in SCCHN than in other cancer types.[358]

The challenge of this study was to detect MRD within 12 weeks from the end of the curative treatment. Our results did not rule out the possibility that this assay would still be able to detect patients who will relapse but later in disease course (for example, more than 12 weeks after the end of curative treatment but before the radiological or clinical relapse).

One difficulty with tumor-personalized assays is the need for tumor sequencing. Tumor biopsy for SCCHN might be hazardous for anatomical reasons. Although tumor biopsy is required for diagnosis, tumor tissue sampling may sometimes be insufficient for WES, as we can observe in this cohort where only 86% (43/50) of the tumor biopsies had enough tumor cells (20%) for this ctDNA assay.

III.2.5 Conclusion

Even though the preliminary analysis did not show satisfactory results, this tumor-personalized assay showed very good sensitivity (97.7%) to detect ctDNA at baseline. That said, ctDNA as marker of MRD in SCCHN still needs refinement.

IV. ctDNA as a biomarker of response to ICI in R/M SCCHN

IV.1 Tumor-agnostic plasma assay for circulating tumor DNA predicts outcome in recurrent and/or metastatic squamous cell carcinoma of the head and neck treated with PD1 inhibitor

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Adapted from Tumour-agnostic plasma assay for circulating tumour DNA predicts outcome in recurrent and/or metastatic squamous cell carcinoma of the head and neck treated with a PD-1 inhibitor. Honoré, Natasha et al.European Journal of Cancer, Volume 195, 113372

Abstract

Background

Only 15-20% of recurrent and/or metastatic squamous cell carcinoma of the head and neck (R/M SCCHN) patients derive long-term benefits from nivolumab or pembrolizumab. We developed a ctDNA tumor-agnostic assay with the aim of predicting the efficacy of PD1 inhibitor monotherapy in R/M SCCHN at an early stage.

Patients and methods

Our tumor-agnostic assay included 37 genes frequently mutated in R/M SCCHN and two HPV16 genes. The primary endpoint was the concordance between ctDNA kinetics (ΔctDNA) and best overall response (BOR) according to Response Evaluation Criteria in Solid Tumors version1.1 (RECISTv1.1). ΔctDNA was defined as the difference in mean variant allele frequency (VAF) between the on-treatment sample harvested 6-10 weeks (FU1) after PD-1 inhibitor initiation and the pre-treatment plasma sample (ΔctDNA = mean FU1 VAF - mean pre-treatment VAF).

Results

ctDNA was detected in 35/44 (79%) in the pre-treatment plasma samples. The concordance between ΔctDNA and imaging response was observed in 74%. Median PFS was 8.6 months in the favorable ΔctDNA group and 2.5 months in the unfavorable ΔctDNA group (p=0.057). Median OS was 18.1 and 8.2 months in the favorable and unfavorable ΔctDNA groups, respectively (p=0.13). In patients with PD-L1 expressing SCCHN (Combined Positive Score ≥1), OS was significantly better in patients with favorable ΔctDNA compared with patients with unfavorable ΔctDNA: median OS was 8.4 and 41.5 months (p=0.033).

Conclusions

Tumor-agnostic ctDNA analysis for HPV-negative and HPV-positive R/M SCCHN is feasible. ctDNA kinetics show promising results in predicting the efficacy of PD1 inhibitor in R/M SCCHN.

IV.1.1 Introduction

Immune checkpoint inhibitors (ICI) have revolutionized cancer therapy, with low side effects and enhanced patient survival[359]. However, not all patients nor all tumor types respond to ICI.

Recurrent and/or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) patients who relapse after the primary curative treatment have a median survival between 10-17 months.[5,143,360,361] Pembrolizumab monotherapy, as a first-line treatment for R/M SCCHN, improves overall survival (OS) over a combination of platin, 5-fluorouracil, and cetuximab in patients whose tumors express programmed cell death ligand 1 (PD-L1) with a Combined Positive Score (CPS) ≥ 1.[5] Similarly, pembrolizumab in combination with chemotherapy increases OS in R/M first-line regardless of PD-L1 status.[5] Nivolumab and pembrolizumab also improve cancer outcomes in SCCHN patients who progress after platinum therapy.[143,362]

Although PD-1 inhibitors are now standard of care in R/M SCCHN, only around 15-20% of patients will derive long-term benefits from this therapy.[4] Many studies have tried to uncover predictive markers to enable those most likely to respond to be identified early. PDL-1 expression is the only marker currently used in daily clinical practice which selects patients who have a higher probability of response to a PD-1 inhibitor in R/M SCCHN.[69,363] Other predictive biomarkers, such as the abundance of tumor-infiltrating lymphocytes, tumor mutational burden and specific immune gene signatures, have been investigated;[364,365] however, they are far from perfect due to significant overlap between responders and non-responders.

ctDNA kinetics are currently being investigated as a biomarker to predict immunotherapy efficacy[366-370] with the hypothesis that changes in ctDNA quantity could allow early identification of patients likely to progress rapidly or derive long-term benefits. Bratman et al.[333] showed that early ctDNA

kinetics could predict pembrolizumab benefit in several tumor types using a tumor-informed assay but only 14 R/M SCCHN patients were included in the study. As such, this approach is clearly under investigated in SCCHN. In a small cohort of R/M human papillomavirus (HPV)-driven oropharyngeal cancer (n=12), Haring et al.[326] found that longitudinal changes in HPV16 ctDNA measured by droplet digital polymerase chain reaction (ddPCR) correlated to response to chemotherapy and/or immunotherapy. Using a tumor-agnostic assay (CAncer Personalized Profiling by deep Sequencing (CAPP-Seq)), Taylor et al.[327] observed that early dynamic changes in ctDNA level predicted both OS and progression-free survival (PFS) in R/M SCCHN treated with various systemic treatments that included platinum-chemotherapy, an anti-PD(L)1 inhibitor in monotherapy, or immunotherapy combination.

Here we show that early ctDNA kinetics, using an in-house tumor-agnostic assay, predict the efficacy of nivolumab or pembrolizumab monotherapy when administered as standard of care in R/M SCCHN.

IV.1.2 Materials & Methods

IV.1.2.1 Patient cohort, study design, and inclusion criteria

After obtaining written informed consent, all SCCHN patients were prospectively enrolled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional multicentric biomarker trial collecting whole blood, plasma samples every two months and, when feasible, tumor biopsies (NCT02139020). The clinical characteristics and disease outcomes of each patient were prospectively recorded in a secured database (REDCap[340]). The study was approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria for the work reported in this paper were: patients (i) enrolled in UCL-ONCO-2013 between 2017 and 2022 with R/M or incurable SCCHN (oral cavity, oropharynx, hypopharynx, larynx and unknown primary), (ii) treated with non-curative intent anti-PD-1 therapy without concomitant

chemotherapy according to standard of care, (iii) for whom whole blood as well as pre-treatment and first on-treatment plasma samples were available, and (IV) treated for at least six weeks with anti-PD-1 therapy to match the treatment resistance definition proposed by the Society for Immunotherapy of Cancer, which requested at least six weeks of immunotherapy exposition.[371] The first on-treatment plasma sample used to calculate ctDNA kinetics (ΔctDNA), defined as FU1, had to be collected within 6 to 10 weeks from the initiation of the PD-1 inhibitor. Nasopharyngeal, salivary and sinonasal cancers were excluded. HPV-driven oropharyngeal cancers (OPCs) were defined as tumors that were positive for HPV by in-situ hybridization (ISH).

According to the definition proposed by the Society for Immunotherapy of Cancer,[371] primary resistance was defined as disease progression within six months of anti-PD-1 therapy; secondary resistance was defined as progression after six months.

IV.1.2.2 Blood and plasma collections

For each patient, plasma samples were harvested before anti-PD-1 therapy and between six and 10 weeks after its initiation. When possible, plasma samples were also collected during immunotherapy treatment, every 2 to 3 months until progression or the end of therapy. For each patient and time-point, plasma was isolated from whole blood collected in two EDTA 7.5mL tubes after double centrifugation (2000g 10 minutes and 2500g 15 minutes) within 30 minutes at 4°C. Plasma was stored immediately at -80°C until further use.

IV.1.2.3 Cell free DNA (cfDNA) and germline DNA extraction

cfDNA was extracted from 4mL to 6mL of plasma using the Promega® Maxwell® RSC ccfDNA LV plasma kit (RSC; Promega, Leiden, the Netherlands) and quantified with Qubit® (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). Matched germline DNA was extracted for each patient with the Promega® Wizard® Genomic DNA Purification kit (RSC; Promega, Leiden, the Netherlands) from a whole blood sample in EDTA 3.5mL tubes, frozen at -80°C and quantified with Nanodrop® technology (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). All sample

characteristics and quantification can be found in Supplementary Table VI-7.

IV.1.2.4 cfDNA targeted next-generation sequencing (NGS) and variant calling

Libraries for germline DNA (gDNA) from whole blood and cfDNA from plasma were created according to the manufacturer's protocol using MF and EZ Twist Bioscience® (South San Francisco, California, U.S.A.) kits for cfDNA and gDNA, respectively. Targeted Next Generation Sequencing (NGS) was performed after capture using a custom-made in-house panel of 37 genes plus two HPV16 oncogenes (E6 and E7) designed by Twist Bioscience®, with an expected mean coverage of 2000x. The 37 genes were selected because they are frequently mutated in HPV-negative R/M SCCHN according to literature,[108,372] and our own in-house sequencing data (Supplementary Table VI-8). We added the following genes due to their possible implication in immune response: *BRCA2*, *JAK2,* and *B2M*. Sequencing ('paired-end' 2x100 bp reads, Illumina® NovaSeq600™) was carried out by IntegraGen® (Evry, France), using unique molecular identifier technology (UMIs).[219]

Raw data (.fasta files) were aligned to the reference human genome assembly (GRCh38) using BWA 0.7.15.[373] UMIs were removed using fgbiotools and Picard.[374] Aligned sequences (.bam files) were processed using GATK 4.2 "BQSR" (for base quality scores recalibration). Germline single-nucleotide variants and small indels were identified using GATK 4.2 "Haplotype Caller" whereas somatic single-nucleotide variants (SNV) and small indels were identified using Mutect2[375] (both following Broad Institute best practices). The generated variant calls (.vcf) files were annotated, imported and further analyzed on Highlander[376], an in-house bioinformatics framework within the Genomics and Bioinformatics platform of UCLouvain (PGEN:https://www.deduveinstitute.be/pgen-bioinformatics).[375,376]
Highlander provides extensive variant annotation, filtering and visualization.

Plasma samples were analyzed following our in-house workflow, as described in Figure IV-1. All pre-treatment plasma samples were first analyzed with Kraken2 (v. 2.1.2, database "Standard", March 2023),[341] a taxonomic sequence classifier that assigns taxonomic labels to DNA

sequences. A patient was considered HPV16 positive if at least one read was assigned to the HPV16 genome in the pre-treatment plasma sample, according to Kraken2. Otherwise, the patient was considered HPV16 negative.

Pre- and first on-treatment plasma samples of HPV16 ctDNA-negative patients were analyzed using our gene panel. A variant was considered positive if it was (i) supported by at least three reads, (ii) present in reads aligned in forward and reverse, (iii) passed modified Mutect2 quality filters and filtering criteria for selection of variants in Highlander, and (iv) present in less than one percent of healthy individuals in the public sequence databases (Gnomad[377]). The list of all criteria can be found in Table 3 of the Supplementary Data, and only variants fulfilling all criteria were considered true somatic variants. A pre-treatment sample was considered ctDNA-positive if at least one somatic variant was called. Variant allele frequency (VAF) was estimated by the number of reads carrying the nucleotide change divided by the total number of reads at the nucleotide change position in percentage.

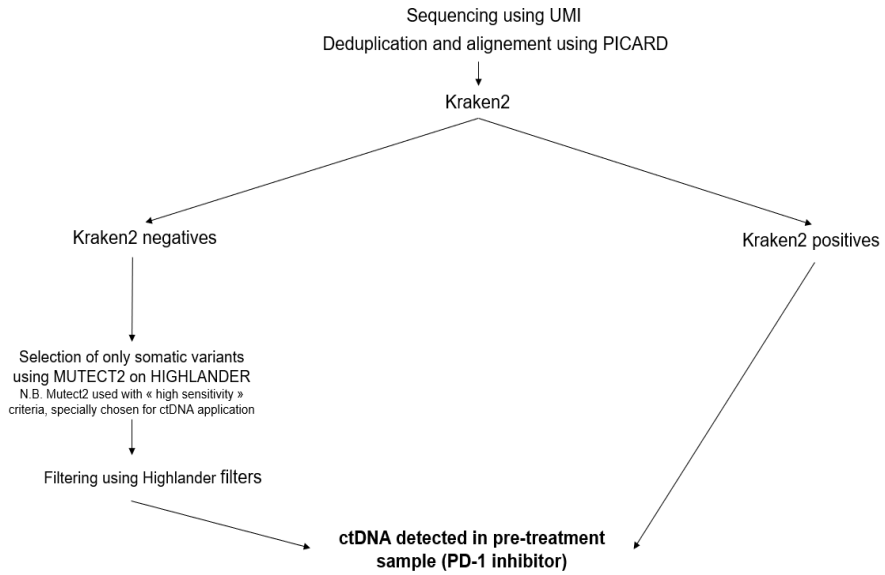

Figure IV-1: Bioinformatic workflow
In-house bioinformatic workflow. ctDNA: circulating tumor DNA; UMI: unique molecular identifier(s)

To study the association of treatment outcome with some specific mutations, we only considered variants that fulfilled all the above criteria and that were predicted to be “damaging” in at least nine out of twenty databases or algorithms, based on known prediction tools used in Highlander.[376] These mutations were related to cancer-related pathways using KEGG database.[378]

HPV in-situ hybridization is described in the Supplementary Data (VI-2 Material & Methods 1)

PD-L1 CPS was evaluated from the formalin-fixed paraffin-embedded (FFPE) biopsy slides stained with an anti-PD-L1 antibody and a 22C3 DAKO® (Agilent, Santa Clara, U.S.A.) antibody. PD-L1 was identified using the OptiView DAB IHC Detection Kit processed on the Ventana BenchMark Automated staining platform (Roche Diagnosis, Rotkreuz, Switzerland).

IV.1.2.5 Imagery evaluation

All imagery (computed tomography(CT)-scan and magnetic resonance imaging (MRI)) was evaluated according to RECISTv1.1[379] by a head and neck expert radiologist (AD) who was blinded to the ctDNA results and clinical evaluation.

IV.1.2.6 Study objective and endpoints

Our main objective was to develop a tumor-agnostic assay to evaluate ctDNA kinetics as a biomarker of response to therapy in HPV-negative and positive R/M SCCHN. Our primary endpoint was the concordance between best overall response (BOR) according to Response Evaluation Criteria in Solid Tumours version 1.1 (RECISTv1.1)[380] and ΔctDNA. Secondary endpoints were progression-free survival (PFS), overall survival (OS) and cancer-specific survival (CSS) with negative and positive ΔctDNA in patients who had ctDNA detected in their pre-treatment plasma sample. As an exploratory analysis, we investigated whether alterations in genes implicated in certain cancer-related pathways in the pre-treatment sample were associated with disease outcome.

ΔctDNA was defined for HPV16-negative patients as the difference in mean variant allele frequency (VAF) of all variants between the pre-treatment sample and the FU1 sample harvested 6-10 weeks after PD-1 inhibitor

initiation, as described previously (ΔctDNA = mean FU1 VAF - mean pre-treatment VAF).[327] For HPV16-positive patients, we defined ΔctDNA as the difference in number of reads assigned to the HPV16 genome by Kraken2[341] between the pre-treatment sample and the FU1-sample (ΔctDNA = FU1 HPV16 reads - pre-treatment HPV16 reads) . Therefore, positive ΔctDNA means that there was an increase in ctDNA in the FU1 sample compared with the pre-treatment sample and negative ΔctDNA means that there was a decrease in ctDNA in the FU1 compared with the pre-treatment sample. The pre-treatment ctDNA level quantity was defined as "high" in patients where the allele frequency was above the median allele frequencies of all patients, and "low" if it fell below the median. PFS was defined as the time interval between the date of the first day of anti-PD-1 treatment and the date of SCCHN progression according to RECISTv1.1, the date of last follow-up for patients still undergoing treatment, or the date of death due to any cause. OS was defined as the time interval between the date of the first day of anti-PD1 treatment and the date of death due to any cause, or until the date of last follow-up. CSS was defined as the time interval between the date of the first day of anti-PD1 treatment until the date of death related to the disease, or until the date of last follow-up. Best overall response (BOR) was defined as the best response according to RECISTv1.1 from the start of study treatment until the end of treatment.

IV.1.2.7 Statistics

Concordance between ΔctDNA and BOR in patients was evaluated using a Chi-square test. We hypothesized that patients with negative ΔctDNA will achieve as BOR either a CR, PR or SD whereas patients with positive ΔctDNA will experience PD as BOR. We compared the proportion of patients that fulfilled these criteria with the patients that did not fulfill them.

PFS, OS and CSS were estimated using Kaplan-Meier methods. Patients without an event (progression or death) were censored at the date of last-follow-up. Differences in PFS and OS between patients with negative and positive ΔctDNA were assessed using the log-rank test. A p-value < 0.05 was considered to be statistically significant.

All analyses were done using R (v4.2.1).

IV.1.3 Results

IV.1.3.1 Patient characteristics

Between June 2017 and July 2022, 97 R/M SCCHN patients treated with ICI were enrolled in the UCL-ONCO-2013 biomarker trial. Forty-four patients met the inclusion criteria (Figure IV-2). Patient clinical characteristics are described in Table IV-1 and in Supplementary Data Figure VI-1 A. The most frequent primary location was the oropharynx (17/44, 38%). Most patients had previously received treatment with curative intent (35/44, 79%). Twenty-four patients (54.5%) received nivolumab and 20 (45.5%) pembrolizumab as single agents. The median OS for the 44 patients was 14.4 [95% CI: 8.6-26.8] months and the median PFS was 2.9 [95% CI: 2.4-7.2] months. Patients with CPS ≥1 had a better OS (median OS 16.6 [95% CI: 12.5-41.5] months compared to patients with CPS < 1 (median OS 7.2 [95% CI: 6.6-not evaluable (NE)] months); p=0.027), but similar PFS (median PFS: 2.9 [95% CI: 2.4-11.9] months and 6.6 [95% CI: 1.7-7.2] months, respectively; p=0.38). No significant differences were observed between patients treated with pembrolizumab or nivolumab for OS (HR=0.92, [95% CI: 0.43-1.97]) or for PFS (HR=0.85, [95% CI: 0.44-1.68]). Similarly, there was no significant difference between patients treated with ICI monotherapy in first or second/third line of treatment (OS: HR=0.83 [95% CI: 0.38-1.79]; PFS: HR=0.74 [95% CI: 0.38-1.45]).

One hundred and forty-seven plasma samples were analyzed among these 44 patients (Supplementary Figure VI-2).

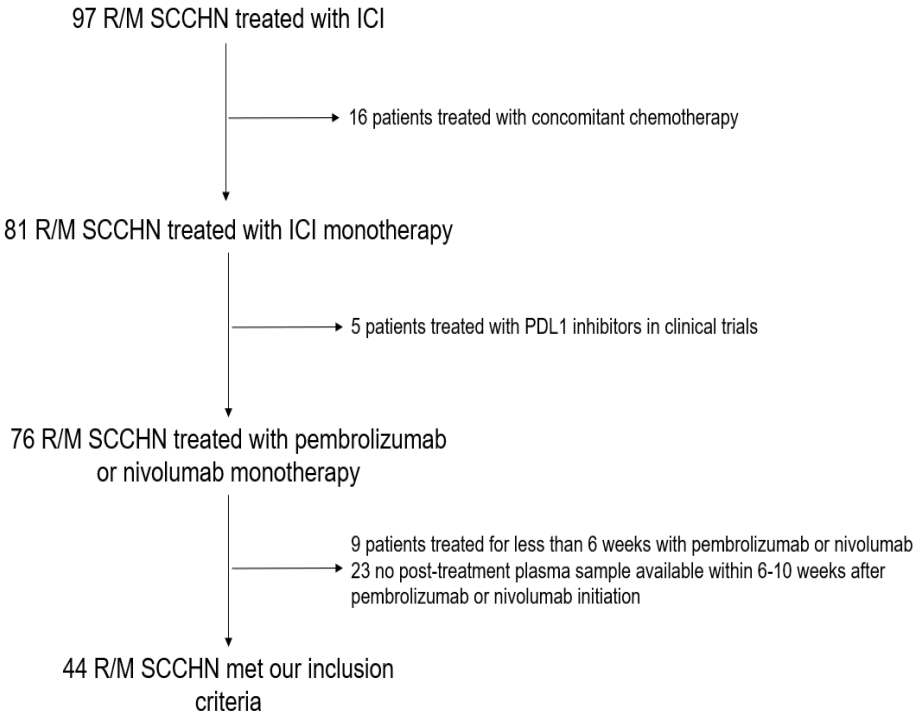

Figure IV-2: Patients' workflow
ICI: immune checkpoint inhibitor; R/M SCCHN: Recurrent and/or metastatic squamous cell carcinoma of the head and neck

IV.1.3.2 Tumor-agnostic panel detects pre-treatment ctDNA

Pre-treatment ctDNA was detected in 35 (80%) out of the 44 patients. HPV16 DNA was detected in three patients in the pre-treatment samples using Kraken2. The number of reads in the pre-treatment samples assigned to the HPV16 genome was 2459, 32 and 7 in each patient, respectively. Among the 41 HPV16-negative patients, ctDNA was detected in 32 patients (78%) in the pre-treatment samples. The most frequently mutated genes were *TP53* (32%), *SPEN* (18%), *EP300* (18%), *SMG1* (15.6%), and *KMT2C* (15.6%). Median VAF was 1.3% [range: 0.26% - 31.0%]. High and low ctDNA levels in the pre-treatment samples were not predictive of OS (median OS: 10.4 [95% CI: 7.1-NE] and 13.3 [95% CI: 8.0-26.8] months, HR=0.84 [95% CI:

0.4-1.9]; p=0.7) and PFS (median PFS: 2.8 [95% CI: 1.8-6.9] and 6.92 [95% CI: 2.5-13.0] months, HR=0.64 [95% CI: 0.31-1.32]; p=0.2).

	Overall
	(N=44)
Sex	
Male	33 (75.0%)
Female	11 (25.0%)
Age (years)	
Median [Min,Max]	68.5 [44.0, 79.0]
Primary location	
Oral cavity	13 (29.5%)
Oropharynx	17 (38.6%)
P16 status	
Negative	10 (22.7%)
Positive	7 (15.9%)
HPV-16 ISH	
Negative	4 (9.0%)
Positive	2 (4.5%)
Missing /not enough material	1 (2.3%)
Hypopharynx	4 (9.0%)
Larynx	3 (6.8%)
Head and Neck unknown primary	6 (13.6%)
Two locations (oropharynx and oral cavity)	1 (2.3%)
Previous curative-intent treatment regimen	
Primary chemoradiation	15 (34.1%)
Primary radiation	7 (15.9%)
Primary surgery +/- (chemo)radiation	13 (29.5%)
No previous curative treatment	9 (20.5%)
CPS Score	
<1	7 (15.9%)
1-19	16 (36.4%)
≥20	19 (43.2%)
Not enough material	2 (4.5%)

Tobacco consumption (>10PY)	
Yes	32 (72.7%)
No	12 (27.3%)
Alcohol consumption (>2U/day)	
Yes	30 (68.2%)
No	14 (31.8%)
PD1 Inhibitor	
Nivolumab	24 (54.5%)
Pembrolizumab	20 (45.5%)
R/M line in which immunotherapy was administered	
First-line	28 (63.6%)
Second-line	14 (31.8%)
Third-line	2 (4.5%)
Previously treated with platinum agents	
No	13 (29.5%)
Yes	31 (70.5%)
Disease location before the start of Immunotherapy	
Loco-regional	37 (84.1%)
Loco-regional & metastatic	7 (15.9%)
Metastatic only	0 (0.0%)

Table IV-1: Patients' characteristics
R/M: recurrent and/or metastatic, HPV16: Human Papilloma Virus 16, ISH: In-situ hybridization, CPS: combined positive score, PY: packet/year, U: units

IV.1.3.3 ΔctDNA is correlated with BOR

Among the 35 patients with ctDNA in their pre-treatment samples, 17 and 18 patients had a decrease (negative ΔctDNA) and an increase (positive ΔctDNA) in ctDNA mean VAF, respectively. ctDNA was no longer detectable in seven patients (6 HPV-negative and 1 HPV-positive SCCHN) in the first on-treatment plasma harvested within 6-10 weeks from treatment initiation (FU1).

In the overall cohort, the overall objective response rate (ORR) was 25.7% (9/35) (complete response (CR): 3/35 (8.6%); partial response (PR): 6/35

(17.1%)). Stable disease (SD) and progressive disease (PD) as BOR were achieved in 9 (25.7%) and 17 (48.6%) patients, respectively. Among these 35 patients, no patients had pseudo-progression, defined as initial PD followed by partial or complete response.

Among the 17 patients with negative ΔctDNA, 13 (76.4%) achieved either a CR, PR or SD and 4 (23.5%) had PD. Thirteen (72%) out of 18 with a positive ΔctDNA had PD as BOR (Table IV-2).

RECISTv1.1	**Negative ΔctDNA**	**Positive ΔctDNA**	**Total**
CR	2 (5.7%)	1 (2.9%)	3 (8.6%)
PR	5 (14.3%)	1 (2.9%)	6 (17.1%)
SD	6 (17.1%)	3 (8.6%)	9 (25.7%)
PD	4 (11.4%)	13 (37.1%)	17 (48.6%)
Total	17 (48.6%)	18 (51.4%)	35 (100%)

Table IV-2: Best overall response and ΔctDNA
CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, RECISTv1.1: Response Evaluation Criteria in Solid Tumors version 1.1

ΔctDNA correctly predicted the BOR according to RECISTv1.1 in 26 (74%) out of 35 patients, whereas 9 (26%) patients had imaging and ΔctDNA discordant results ($X^2 = 4.51$; p=0.033).

IV.1.3.4 ΔctDNA to predict PFS, OS, and CSS

Median PFS was 8.6 [95% CI: 2.73-18.1] months in the negative ΔctDNA group and 2.5 [95% CI: 2.0-3.0] months in the positive ΔctDNA group (p=0.057, Figure IV-3A). Median OS was 18.1 [95% CI 12.5-41.5] and 8.2 [95% CI 7.2-13.0] months in the negative and positive ΔctDNA groups, respectively (p=0.13) (Figure IV-3B). As six patients presumably died from causes other than their cancer (2 from pneumonia, 1 from hip fracture, 1 from sudden death, 1 from Covid infection, and 1 from cerebral vascular accident), we calculated the CSS. CSS was significantly better in the negative ΔctDNA group than in the positive ΔctDNA group: median CSS 41.5 [95% CI: 14.4-NE] and 8.35 [95% CI: 7.2-26.8] months, respectively (p=0.049) (Figure IV-3C).

IV.1.3.5 ΔctDNA predictive value in different subgroups

In patients with PD-L1 expressing SCCHN (CPS ≥1), there were significant differences between patients with positive and negative ΔctDNA for OS (median OS: 8.4 [95% CI: 7.5-13.0] and 41.5 [95% CI: 14.4-NE] months; p=0.033), and CSS (median OS: 8.6 [95% CI: 7.526.8] months and 41.5 [95% CI: 14.4-NE]; p=0.036), but not PFS (median PFS: 2.4 [95% CI: 1.9-3.03] and 11.9 [95% CI: 2.7-NE] months; p=0.054), (Figures IV-3D-F).

There was no difference in OS in patients who cleared their ctDNA in the first on-treatment plasma harvested within 6-10 weeks of treatment initiation (FU1), compared to those who did not (median: 14.7 [95% CI: 7.2-26.8] vs 12.5 [95% CI: 7.8-NE] months; p=0.9) and PFS (median: 6.64 [95% CI: 2.3-13.0] vs 2.9 [95% CI: 2.4-7.2] months; p=0.9).

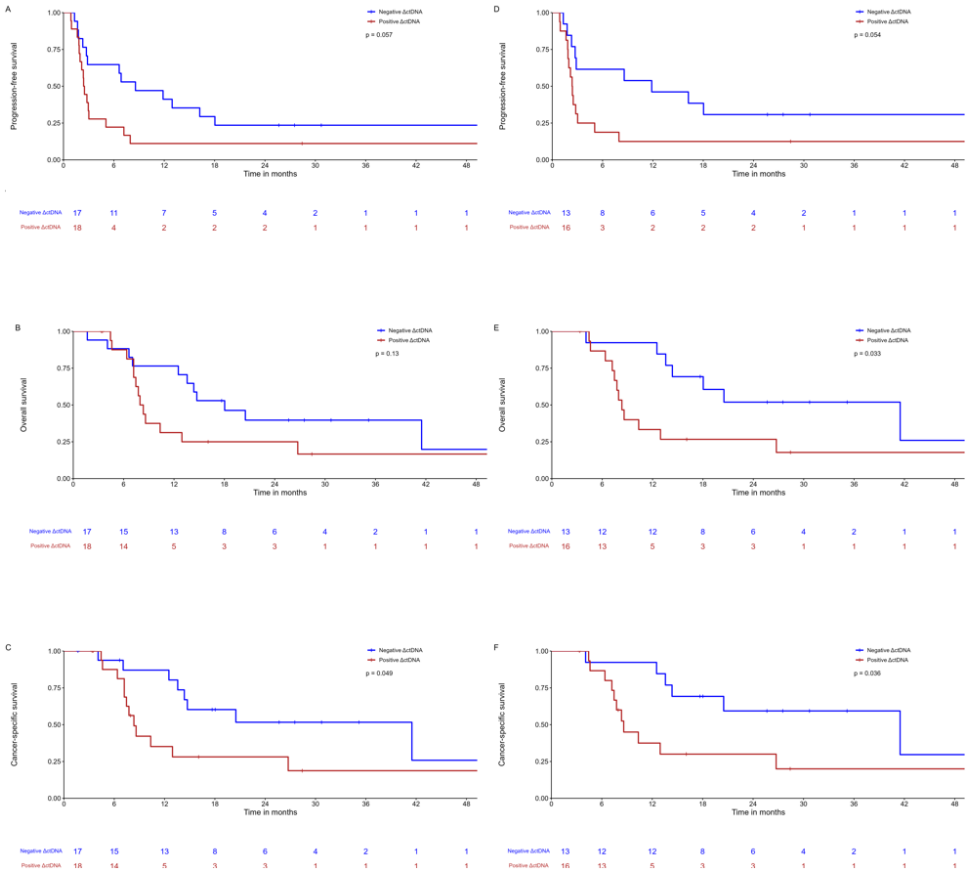

Figure IV-3: Kaplan-Meier estimations
Kaplan-Meier estimate of progression-free survival (Panels A), overall survival (Panels B), and cancer specific survival (Panels C) for positive and negative ΔctDNA patients in the whole population. Kaplan-Meier estimate of progression-free survival (Panels D), overall survival (Panels E), and cancer specific survival (Panels F) for positive and negative ΔctDNA patients in PD-L1 CPS ≥ 1 population. Blue curves = negative ΔctDNA patients; red curves = positive ΔctDNA patients.

Some patients had discordant results between ΔctDNA and imaging response. Four patients had a negative ΔctDNA with disease progression whereas five patients had a positive ΔctDNA with either a CR, PR or SD at first imaging evaluation. Interestingly, we observed that these discordant patients (n=9) had an intermediate prognosis. For the discordant group,

median OS was 13.6 [95% CI: 7.8-NE] months compared with 20.5 [95% CI: 15.5-NE] months for the patients with negative ΔctDNA and either a CR, PR or SD (p=0.5), and 8.4 [95% CI: 6.4-13] months for the patients with positive ΔctDNA and PD (p=0.2). Similarly, median PFS of the discordant group was 5.1 [95% CI: 2.3-8.0] months compared with 13.0 [95% CI: 6.9-NE] (p=0.2) and 2.2 [95% CI: 1.8-2.5] months (p<0.001), respectively (Figure IV- 4).

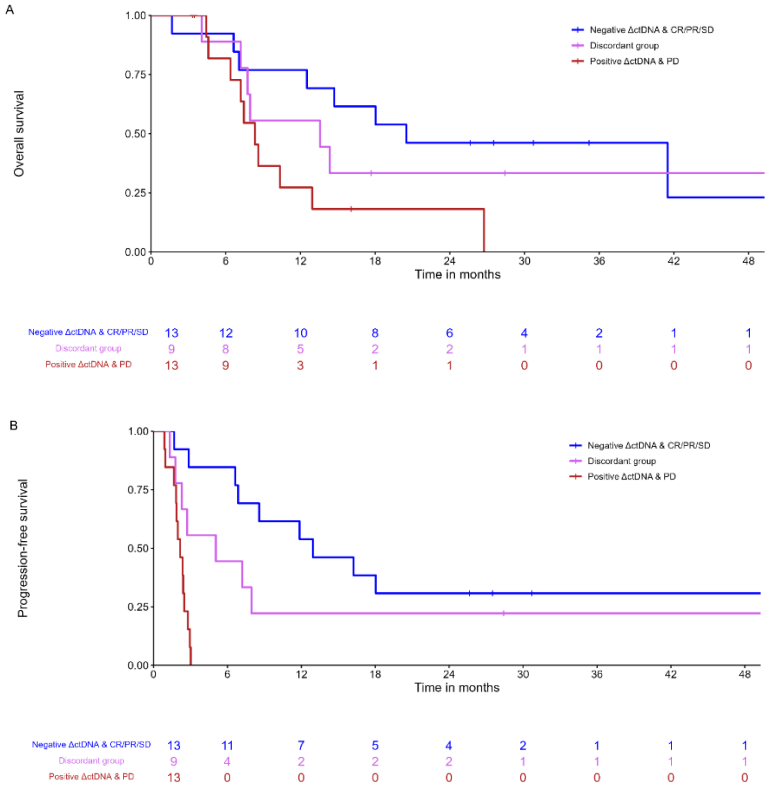

Figure IV-4: Kaplan-Meier estimation
Kaplan-Meier estimate of overall survival (A) and progression-free survival (B) for negative ΔctDNA & CR, PR and SD patients, positive ΔctDNA & PD patients, and discordant patients. Blue curves = negative ΔctDNA negative & CR, PR and SD; red curves = positive ΔctDNA patients & PD; pink curves = discordant patients.

IV.1.3.6 Prognostic value of pre- and on-treatment mutations

The mutations considered to be damaging and found in the pre-treatment plasma samples are depicted in Figure IV-5. Interestingly, we observed that inactivating mutations in genes implicated in the Notch/Hedgehog/Wnt pathways were only identified in patients with SD or PD as BOR and not in patients who had a CR or PR. There was a significant difference in PFS between patients with pre-treatment plasma harboring mutations in genes implicated in the Notch/Hedgehog/Wnt pathways compared to the others: median 2.07 [95%IC: 1.8-2.7] vs 6.8 [95%IC: 2.9-13.0] months (p=0.024). There was, however, no statistical difference in OS: 8.2 [95%IC: 6.6-13.6] vs 14.7 [95% IC: 8.6-41.5] months (p=0.083)(Supplementary Figures VI-2 & 3).

Another patient with primary resistance had a pre-treatment ctDNA *B2M* mutation. In contrast, in patients with long-term CR, we observed one patient with *CASP8 (*p.E123Q*)* mutation and another with *KMT2C* (p.C1150Y) and *LRP1B* (p.G683fs) mutations.

However, although ctDNA increased in most patients at disease progression, we could not link any damaging mutations with primary or secondary resistance (Supplementary Figure VI-4).

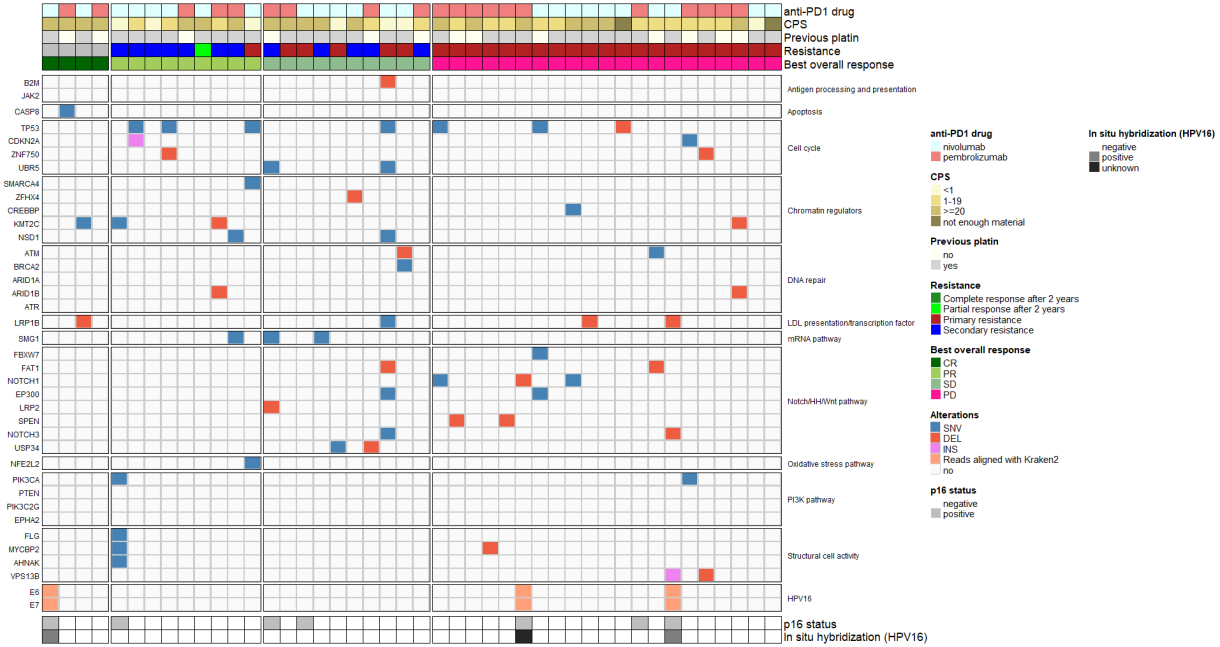

Figure IV-5: Mutations in pre-treatment plasma sample and patient characteristics. PD1: Programmed cell death 1, CPS: combined positive score, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, SNV: single nucleotide variation, DEL: deletion, INS: insertion

IV.1.4 Discussion

In our study, concordance between ctDNA kinetics and imaging response was observed in 26 (74%) out of 35 patients. ctDNA kinetics also identified a group of R/M SCCHN patients with better outcome when treated with nivolumab or pembrolizumab monotherapy. On the other hand, our analysis uncovered patients less likely to benefit from a PD1 inhibitor who might benefit from the rapid addition of chemotherapy or a change of therapy.

Several studies have already investigated the potential use of ctDNA kinetics to predict response to immunotherapy.[314,327,333,366] To the best of our knowledge, our series is the largest R/M SCCHN cohort treated with anti-PD1 monotherapy. We deliberately excluded patients who received a combination of chemotherapy and a PD1 inhibitor to avoid the confounding factor of chemotherapy in evaluating tumor response and outcome. This implies that our patient selection was also biased since, in first-line R/M, pembrolizumab monotherapy is more frequently given to patients with a lower disease burden who do not require immediate chemotherapy.[4]

Today, anti-PD1 therapy is mainly prescribed for patients with R/M SCCHN who express PD-L1. We therefore conducted a subgroup analysis in this setting and confirmed that ctDNA kinetics also predicted disease outcome in R/M SCCHN patients expressing PD-L1 (Figure 3D-F). Interestingly, in our trial, patients with a CPS $\geq$1 and negative ΔctDNA had a median survival of 41.5 months, suggesting that combining these molecular biomarkers should be further investigated in a larger group of patients. Surprisingly, some patients with a PD-L1 non-expressing tumor who were included in our study also seemed to derive benefits from the PD1 inhibitor (2 PR, 3 SD, 1 PD; data not shown), and this finding is higher than previously reported.[381] This highlights an important limitation of PD-L1 as the only biomarker to select patients. Although the small sample size and tumor heterogeneity may explain this observation, another possible reason is that the CPS analysis was performed retrospectively on archival tissues that were not harvested immediately prior to initiation of the PD1 inhibitor.

Some patients had discordant results between ΔctDNA and imaging response. Interestingly, we observed that these discordant patients had an intermediate prognosis compared with patients with non-discordant results. Similarly, Bratman et al.[333] also showed that ctDNA dynamics, when associated with imaging evaluation at two to three months, was also better at discriminating between poor and good prognosis patients in several cancer types.

We used a tumor-agnostic methodology that could be easily implemented in daily practice as it does not require a tumor biopsy. Although tumor-informed personalized methodologies have shown to have good ctDNA detection rate,[339] tumor sequencing might be problematic as lesions at progression might not be biopsied for safety reasons, and R/M SCCHN may have a different genomic landscape than primary tumors.[108] Additionally, quality issues of the tumor biopsy can also preclude confident variant analyses, particularly in the R/M setting.[382] However, our small in-house gene panel detected ctDNA in only 80% of patients prior to treatment initiation. This might be due to low amounts of ctDNA in some patients based on the inclusion of patients with low tumor burden who did not require chemotherapy, or because there were gene mutations not covered by our panel in this heterogenous population. According to results from Taylor et al.[327], a broader squamous cell specific panel using Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) may have a better ctDNA detection rate.

To the best of our knowledge, Kraken2 has never been used in R/M SCCHN HPV16 positive patients to evaluate the presence of circulating HPV16 DNA in plasma. Although only three patients in our cohort were HPV16 positive, we showed good correlation between the number of reads assigned to the HPV16 genome and clinical evolution. Unlike most of SCCHN ctDNA studies where HPV-positive and HPV-negative patients have to be analyzed separately, the use of Kraken2 allowed us to use the same sampling and sequencing techniques independently of HPV status.[326]

Although limited by the low number of genes in our panel and the small number of patients, we observed that some pre-treatment damaging ctDNA mutations seemed to be linked to disease outcome. The presence of

mutations in *CASP8*[383,384], *LRP1B,*[385,386] and *KMTC2*[387,388] has already been described as a possible biomarker of favorable outcome in patients undergoing immunotherapy in other studies.[383,389] Conversely, *B2M* is required for effective antigen presentation and this specific mutation has been previously described to predict anti-PD1 therapy resistance.[390] The Notch/Hedgehog/Wnt pathways may also be implicated in immunotherapy resistance or poor prognosis in SCCHN.[391,392] However, the on-treatment plasma samples did not reveal any specific pattern of ctDNA mutations associated with disease progression or response, highlighting the complexity of treatment resistance mechanisms.

IV.1.5 Conclusion

Tumor-agnostic ctDNA analysis for HPV-negative and HPV-positive R/M SCCHN is feasible. ctDNA dynamics show promising results in predicting response to immunotherapy in R/M SCCHN. Further investigations are necessary to integrate ctDNA kinetics with other prognostic and predictive biomarkers to better predict treatment and cancer outcomes.

IV.2 ctDNA tumor-personalized assay predicts treatment response for R/M SCCHN treated with PD1 inhibitors

Abstract

Introduction

For recurrent and metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN), pembrolizumab with or without platinum-based therapy as first-line treatment or nivolumab for the patients who progress after platinum improve overall survival. However, only 15-20% of R/M SCCHN will derive a long-term benefit from immunotherapy. We hypothesized that ctDNA kinetics could predict treatment efficacy of R/M SSCHN patients treated with PD1 inhibitors with or without chemotherapy.

Material & Methods

Using a bespoke multiplex-PCR tumor personalized assay (Signatera™), we evaluated ctDNA kinetics, defined as the difference in ctDNA levels between pre-treatment and on-treatment (obtained 6 to 10 weeks after treatment initiation) plasma samples. The primary endpoint was the concordance between ctDNA kinetics and best overall response, according to Response Evaluation Criteria in Solid Tumors version1.1 (RECISTv1.1). Secondary endpoints were overall survival (OS) and progression-free survival (PFS) according to ctDNA kinetics.

Results

Due to poor tumor biopsy quality, only twenty-seven out of 38 (71%) of our population could be evaluated with this assay. Among these patients, ctDNA was detected in 23 patients at treatment initiation. ctDNA kinetics correctly predicted best response in 81.2% of our patients. Median PFS was 8.1

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months [95%CI: 6.6-28.6] and 2.42 months [95%CI:2.3-3.9] in patients with favorable and unfavorable ctDNA kinetics (p<0.0001). Median OS was 15.6 months [95%CI: 7.96-NE] and 10.8 months [95%CI:4.4-15.1] in these two groups, respectively (p=0.21).

Conclusion

ctDNA kinetics, evaluated with a tumor-personalized assay, show promising results in predicting anti-PD1 therapy efficacy with or without chemotherapy.

IV.2.1 Introduction

Our in-house agnostic (non-personalized) ctDNA assay seems promising to predict response and disease outcome of R/M SCCHN patients treated with anti-PD1 therapy, as described and discussed in chapter IV-1.

Another possible approach is to use a tumor informed (personalized) ctDNA assay. We had the opportunity of testing a personalized commercial assay (Signatera™, Natera®) in a near identical population as the one investigated in chapter IV-1. This assay has already shown interesting results to monitor response to pembrolizumab in several tumor types. Bratman et al. evaluated the utility of this assay on a cohort of patients undergoing pembrolizumab in the INSPIRE phase II study. ctDNA kinetics ($\Delta ctDNA_{C3}$) were evaluated as the difference in ctDNA levels between initiation of treatment and at the time of the 3rd pembrolizumab cure in 73 patients with available plasma. $\Delta ctDNA_{C3}$ was predictive of benefit to pembrolizumab across all cancer types. Out of the 33 patients with favorable $\Delta ctDNA_{C3}$, 14 (42%) achieved an objective response, compared to only 1 (2%) of the 40 patients with unfavorable $\Delta ctDNA_{C3}$. Favorable $\Delta ctDNA_{C3}$ patients showed better OS (HR 0.36, 95%CI: 0.18-0.71) and PFS (HR 0.33, 95%CI: 0.19-0.58) across all cancer types. Interestingly, they showed that the combination of cycle 3 imagery tumor evaluation and $\Delta ctDNA_{C3}$ refined risk grouping: patients with favorable $\Delta ctDNA_{C3}$ had a better OS than the unfavorable $\Delta ctDNA_{C3}$ group, in patients presenting progressive disease (PD) (HR 0.69) and in patients presenting complete response (CR), partial response (PR) or stable disease (SD) (HR 0.62). However, only 14 R/M SCCHN were included in the Bratman's study.[333]

In this chapter, we evaluated a tumor-personalized bespoke, multiplex-PCR, next-generation sequencing assay (Signatera™) to predict anti-PD1 therapy efficacy in R/M SCCHN. Noteworthy, compared with our first cohort that evaluated our in-house agnostic assay (chapter III.1), in this study, we also included our patients treated with chemotherapy and pembrolizumab. We show that early ctDNA kinetics predict treatment efficacy.

IV.2.2 Materials & Methods

IV.2.2.1 Patient cohort, study design, and inclusion criteria

After obtaining written informed consent, all SCCHN patients in our institution treated with standard-of-care protocols are prospectively enrolled into UCL-ONCO-2013 (2013/12FEV/047), a non-interventional biomarker trial (NCT02139020). The clinical characteristics and disease outcome of each patient are also prospectively recorded in a secured database (REDCap[340]). The study is approved by our ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000).

Eligibility criteria for the work were: patients (i) enrolled in UCL-ONCO-2013 between 2017 and 2022 with R/M- or incurable SCCHN (oral cavity, oropharynx, hypopharynx, larynx and unknown primary), (ii) treated with non-curative intent anti-PD1 therapy with or without concomitant chemotherapy according to standard of care, (iii) for whom whole blood as well as pre-treatment and first on-treatment plasma samples are available, and (IV) treated with anti-PD1 therapy for at least six weeks. The first on-treatment plasma sample to calculate ctDNA kinetics, defined as FU1, had to be collected within 6 to 10 weeks after initiation of PD1 inhibitor. Nasopharyngeal, salivary, and sinonasal cancer were excluded. HPV-driven oropharyngeal cancers (OPCs) were defined as tumors that were positive for HPV by in-situ hybridization (ISH).

IV.2.2.2 Study objective and endpoints

Our main objective was to evaluate whether ctDNA kinetics, evaluated by a bespoke, multiplex-PCR assay (Signatera™) could predict best overall response (BOR) according to RECISTv1.1. Secondary endpoints were overall survival (OS) and progression free survival (PFS) according to ctDNA kinetics.

ctDNA kinetics were defined as the difference in ctDNA quantity, evaluated by mutant molecules per mL (MTM/mL), between pre-treatment and on-treatment sample (FU1). Best overall response was evaluated according to RECIST v1.1 by a radiologist, blinded to the ctDNA results. PFS was defined as the time interval between the date of the first day of treatment and the

date of SCCHN progression, the date of last follow-up or the date of death due to any cause. OS was defined as the time interval between the date of the first day of treatment and death due to any cause, or until the date of last follow-up.

IV.2.2.3 Blood and plasma collections

Plasma samples were harvested from each patient before the start of anti-PD1 treatment (baseline or pre-treatment sample) and between six and 10 weeks after the initiation of treatment (on-treatment sample). For each patient and time-point, plasma was isolated from whole blood collected in two EDTA 7.5mL tubes after double centrifugation (2000g 10 minutes and 2500g 15 minutes) within 30 minutes at 4°C. Plasma was stored immediately at -80°C until further use. Two to 8 mL of plasma were used for DNA extraction, performed as previously described.[333]

IV.2.2.4 Germline DNA extraction

Matched germline DNA (gDNA) was extracted for each patient with the Promega® Wizard® Genomic DNA Purification kit (RSC; Promega, Leiden, the Netherlands) from a whole blood sample in EDTA 3.5mL tubes, frozen at -80°C and quantified with Nanodrop® technology (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). At least 40µL of gDNA was used for extraction, as previously described.[333]

IV.2.2.5 Tumor preparation

For each patient, five to 10 unbaked slides (7µm) from needle core biopsy or surgical sample were used for tumor DNA extraction, accompanied by one fixed slide colored with Hematoxylin and Eosin (HE) for tumor-contouring purposes. Only samples containing more than 20% of tumor cells were deemed to have sufficient tumor DNA to be extracted. Tumor DNA (tDNA) was extracted as previously described.[333]

IV.2.2.6 Personalized ctDNA assays

Design and application of personalized ctDNA (bespoke, multiplex-PCR, next-generation sequencing) assays was conducted with blinding to clinical data by Natera®. Paired tumor and gDNA WES data were used to identify tumor-specific somatic mutations for each patient and clonality was estimated as described previously.[353] Multiplex-PCR primer pairs targeting

16 highly ranked tumor-specific variants were designed as previously described. The primer sequences for each variant are from the Signatera™ assay and are proprietary of Natera®. Somatic small nucleotide variants present in the tumor but absent in the germline were used to select the 16 targets, with prioritization based on multiple factors, including the observed variant allele frequency in the tumor tissue and the associated background noise profile in the plasma. This prioritized list of variants was then used to design PCR amplicons based on optimized design parameters, ensuring uniqueness in the human genome, amplicon efficiency and primer interaction. Next, multiplexed targeted PCR was conducted followed by amplicon deep sequencing on an Illumina platform. A sample was considered ctDNA positive when ≥2 out of the 16 selected target mutations were present above a predefined threshold. Details of the analytical validation of the assay were previously described.[353] Variant allele frequencies were determined for each of the 16 target mutations. Quantity of DNA in plasma sample was evaluated as "mutant molecules per mL" (MTM/mL), calculated as the quantity of cfDNA extracted per sample (converted in pg) multiplied by the haploid genome equivalent (3.3pg) multiplied by the variant allele fraction (VAF) on the volume of plasma extracted (mL). If no variant was detected in one sample, MTM/mL was established at 0 MTM/mL.

$$MTM/mL = cfDNA\ extracted\ (ng) \times 1000\ pg \times 3.3pg\ haploïd\ genome\ equivalent \times \frac{Variant\ Allele\ Fraction\ (VAF)}{Plasma\ volume\ extracted\ (mL)}$$

The favorable ctDNA kinetics group was defined as the patients for whom the on-treatment ctDNA MTM/mL decreased by ≥20% compared with the pre-treatment plasma sample. If not, patients were included in the unfavorable ctDNA kinetics group.

IV.2.2.7 Statistics

Concordance between ctDNA kinetics and BOR in patients was evaluated using a χ^2 test. We hypothesized that patients in the favorable ctDNA kinetics group will achieve as BOR either a CR, PR or SD whereas patients in the unfavorable ctDNA kinetics group will experience PD as BOR. We compared the proportion of patients who fulfilled these criteria with the patients who did not fulfill them.

Secondary endpoints were PFS and OS. PFS and OS were estimated using the Kaplan-Meier methods. Patients without any event (progression or death) were censored at the date of last-follow-up. Treatment differences in PFS and OS were assessed using the log-rank test.

All analyses were done using R (v4.2.1).

IV.2.3 Results

IV.2.3.1 Patients' characteristics

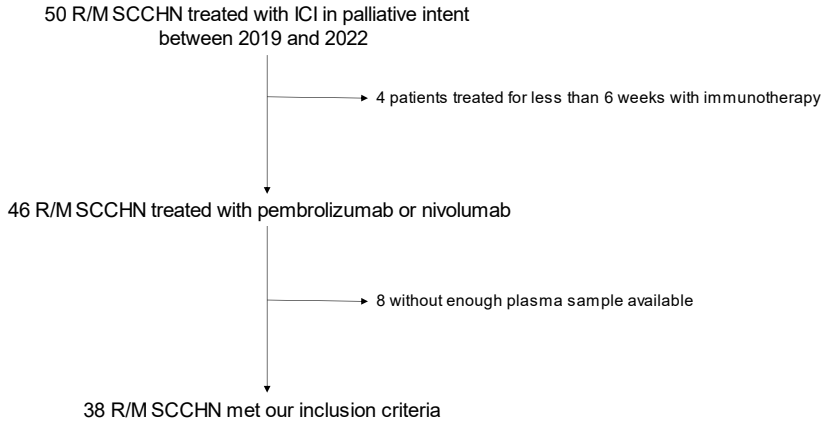


Figure IV-6: patients' workflow
Patient workflow. R/M: recurrent and metastatic; SCCHN: squamous cell carcinoma of the head and neck; ICI: immune checkpoint inhibitors

Between January 2019 and December 2021, 50 R/M SCCHN patients treated with ICI were enrolled in UCL-ONCO-2013 biomarker trial. Thirty-eight patients met our inclusion criteria (Fig IV-6). Patients' clinical characteristics are described in Table IV-3. The most frequent primary location was the oral cavity (12/38 (42.1%)). Most patients had previously received treatment with curative intent (25/38, 65.8%). Thirteen patients received nivolumab and 25 (35.8%) pembrolizumab, among whom 6 received concomitant treatment (one radiotherapy for antalgic purpose, and

five chemotherapy). The median OS for the 38 patients was 13.6 [95% CI: 9.2-16.9] months and the median PFS was 5.75 [95% CI: 3.3-8.1] months. Patients with CPS ≥1 had a better OS (median OS 13.8 [95% CI: 9.1-18.1] months compared to patients with CPS < 1 (median OS 7.5 [95% CI: 4.4-not evaluable (NE)] months); p=0.057), but similar PFS (median PFS: 5.94 [95% CI: 3.5-13.8] months and 4.2 [95% CI: 1.6-NE] months, respectively; p=0.13). No significant differences were observed between patients treated with pembrolizumab or nivolumab for OS (HR=0.78, [95% CI: 0.36-1.6]) or for PFS (HR=0.85, [95% CI: 0.4-1.2]). Similarly, there was no significant difference between patients treated with ICI in first or second/third line of treatment (OS: HR=0.92 [95% CI: 0.1-7.6]; PFS: HR=1.96 [95% CI: 0.2-16.0]).

	Overall
	(N=38)
Sex of the patient	
Male	27 (71.1%)
Female	11 (28.9%)
Age	
Median [Min, Max]	66.5 [41.5, 82.2]
Neoplasia primary location	
Oral cavity	16 (42.1%)
Oropharynx	13 (34.2%)
p16 status	
Negative	9 (69.2%)
Positive	4 (30.8%)
Hypopharynx	3 (7.9%)
Larynx	3 (7.9%)
Head and Neck unknown primary	2 (5.6%)
Two locations (oropharynx and oral cavity)	1 (2.6%)
Previous curative-intent treatment regimen	
Primary chemoradiation	11 (28.9%)
Primary radiation	2 (5.2%)
Primary surgery +/- (chemo)radiation	12 (31.6%)
No previous curative treatment	13 (34.2%)
CPS	
<1	4 (10.5%)
1-19	15 (39.5%)
>=20	17 (44.7%)
Not enough material	2 (5.3%)
Tobacco consumption (>10YP)	

No	12 (31.6%)
Yes	26 (68.4%)
Alcohol consumption (>2U/day)	
No	14 (38.8%)
Yes	24 (63.2%)
PD1 inhibitor	
Nivolumab	13 (34.2%)
Pembrolizumab	25 (65.8%)
Concomitant treatment	6 (25.0%)
Chemotherapy	5 (20.8%)
Radiotherapy	1 (4.1%)
R/M line in which immunotherapy was administered	
1	29 (76.3%)
2	7 (18.4%)
3	2 (5.3%)
Previously treated with platinum agents	
No	11 (28.9%)
Yes	27 (71.1%)
Disease location before the start of Immunotherapy	
Loco-Regional & Metastatic	26 (68.4%)
Loco-Regional	12 (31.6%)
Metastatic only	0 (0.00%)

Table IV-3: Patients' characteristics
R/M: recurrent and/or metastatic, CPS: combined positive score, PY: packet/year, U: units

Among the thirty-eight patients who met our inclusion criteria, twenty-seven (71%) patients were eligible for our primary endpoint. Ten patients failed tumor DNA WES, because 7 did not meet the adequate threshold of tumor cell proportion in the biopsy sample (proportion of tumor cells <20%), 1 failed DNA extraction, and 2 failed tumor DNA quality control. One patient failed assay design due to lack of designable PCR-probes. Details can be found in Figure IV-7.

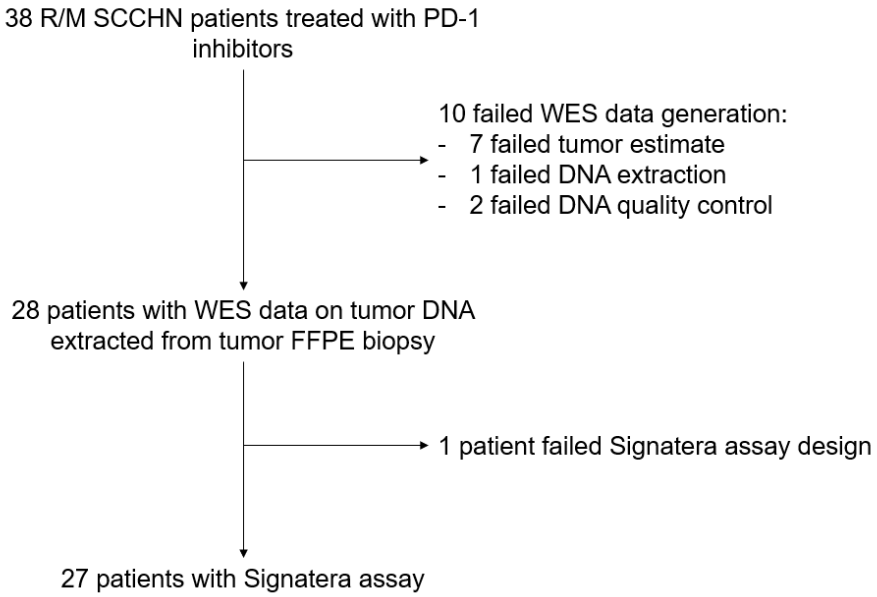


Figure IV-7: Assay design
R/M : recurrent and metastatic ; SCCHN : squamous cell carcinoma of the head and neck, WES: whole exome sequencing; FFPE: Formalin-fixed paraffin embedded

The mean coverage of the 27 tDNA succesfully sequenced was 180x. Fifty-four plasma samples were extracted, with a mean of 6.9mL [range: 1.6-7.7] per sample, and a median of 31.62ng [95%CI: 39.3-67.2] cell-free DNA (cfDNA) was extracted.

IV.2.3.2 Tumor-personalized assay detects ctDNA

In the pre-treatment plasma samples, ctDNA was detected in 23/27 (85.2%) plasma samples. Median MTM/mL was 8.7 [range: 0.0-986.0] and median variant allele frequency (VAF) 0.51%. A median of 15 variants were detected per assay. High and low ctDNA levels in pre-treatment plasma samples were not predictive of OS (median 12.0 months, 95% CI: 7.76-18.1 vs 13.6 months, 95% CI: 7.2-NE, in the high and low groups, respectively, (HR 0.81, 95%CI [0.3-2.1], p-val 0.7)) and PFS (median 4.8 months, 95%CI [25-19.7] vs 4.4 months, 95%CI [3.1-NE], (HR 1.15, 95%CI [0.5-2.8], p-val 0.8)], in the high and low groups, respectively.

In the on-treatment plasma samples, ctDNA was detected in 21/27 (77.8%) plasma samples. Median MTM/mL was 6.0 [range: 0.0-737.0], with a median VAF of 0.166%. A median number of 7 variants per sample were detected.

IV.2.3.3 ctDNA kinetics are correlated with BOR

In the overall cohort, objective response rate (ORR) was 29.6% (8/27) (complete response (CR) 3/27 (11.1%) and partial response (PR) 5/27 (18.5%). Six (22.2%) patients achieved a stable disease (SD) and 13 (48%) progressive disease (PD) as best response (Table IV-5).

Among the 27 patients, 15 (55.5%) had a decrease by more than 20% in ctDNA in the on-treatment samples harvested between week 6 and 10 after anti-PD1 therapy initiation (favorable ctDNA kinetics group). Eight (53.3%) achieved an objective response (3 complete response (CR) and 5 partial response (PR)). Four had a stable disease (SD) and 3 progressive disease (PD)). Twelve patients (44.4%) were in the unfavorable ctDNA kinetics group. None of them had an objective response to PD1 inhibitors: 10 had a PD (83.3%) and 2 a SD (8.3%).

Out of these 27 patients, 3 were treated with chemotherapy plus pembrolizumab. Two of them were in the favorable group and had a PR and the third one was in the unfavorable group and had a PD.

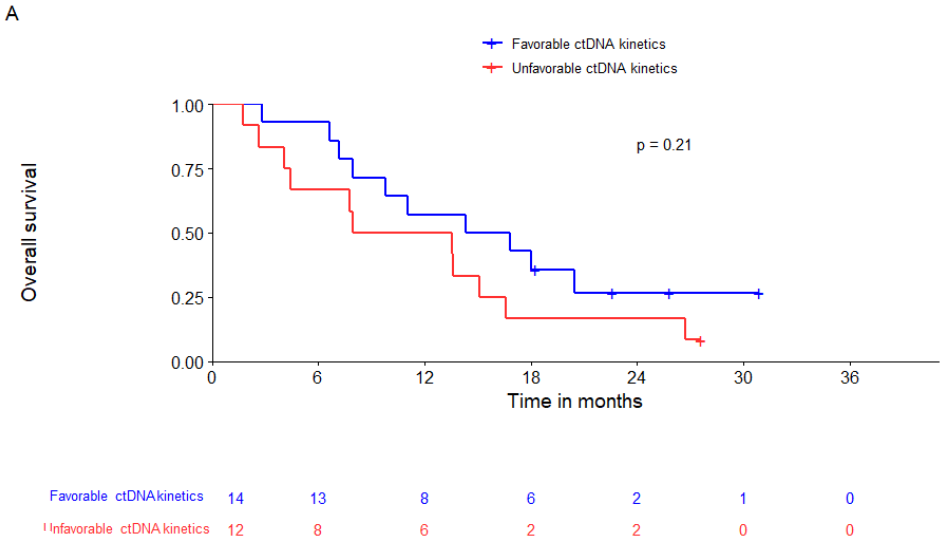
RECISTv1.1	**Favorable ctDNA kinetics group**	**Unfavorable ctDNA kinetics group**	**Total**
CR	3 (11.1%)	0 (0%)	3 (11.1%)
PR	5 (18.5%)	0 (0%)	5 (18.5%)
SD	4 (14.8%)	2 (7.4%)	6 (22.2%)
PD	3 (11.1%)	10 (37.0%)	13 (48.1%)
Total	15 (55.5%)	12 (44.4%)	27 (100%)

Table IV-4: Best overall response and ctDNA kinetics
CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, RECISTv1.1: Response Evaluation Criteria in Solid Tumours version 1.1

Therefore, ctDNA kinetics could predict best response in 81.4% (22/27 patients) according to RECISTv1.1, whereas 5 patients (18.5%) had discordant results between ctDNA kinetics and best response (chi-square = 8.32, p-val= 0.004)

IV.2.3.4 ctDNA kinetics are correlated with PFS but not OS

ctDNA kinetics were predictive of better PFS. In the favorable ctDNA kinetics group, median PFS was 8.1 months [95%CI: 6.6-28.6] compared to 2.42 [95%CI2.3-3.9] in the unfavorable ctDNA kinetics group (p<0.0001). Median OS was 15.6 months [95%CI: 7.96-NE] and 10.8 months [95%CI: 4.4-15.1] in these two groups, respectively (p=0.21).



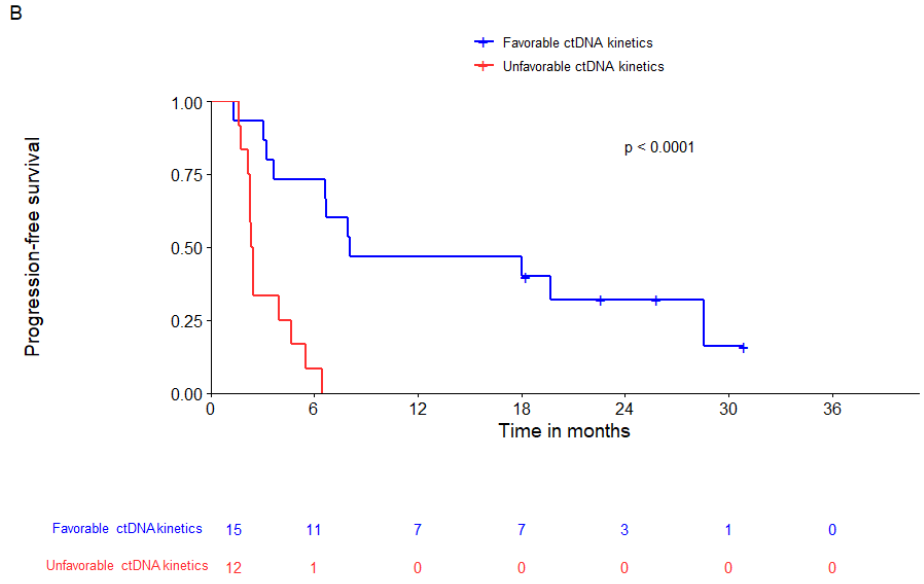


Figure IV-8: Kaplan Meier estimate for OS (A) and PFS (B)
Kaplan-Meier estimation of overall survival (A) and progression-free survival (B) for favorable and unfavorable ctDNA kinetics patients. Blue= favorable ctDNA kinetics group. Red: Unfavorable ctDNA kinetics group

IV.2.3.5 ctDNA kinetics are correlated with PFS but not OS in CPS ≥1

In the twenty-two CPS positive (≥1) patients, patients in the favorable ctDNA kinetics group had a significantly better PFS than patients in the unfavorable ctDNA kinetics group: median 13.1 months (95%CI: 3.7-28.6) vs 2.5 months (95%CI: 2.3-4.7) (p-value < 0.001). Median OS was 16.9 months (95%CI 9.8-NE), vs 8.0 months (95%CI: 4.1-15.1) in the favorable and unfavorable groups, respectively (p-value = 0.25).

IV.2.4 Discussion

ctDNA kinetics could predict best response in 81.4% of patients treated with anti-PD1 with or without chemotherapy. Patients with favorable ctDNA

kinetics during the first 6-10 weeks of treatment had a better PFS compared with patients with unfavorable ctDNA kinetics. We could not detect a significant survival difference between the unfavorable and favorable ctDNA kinetics groups. This might be explained by the low number of patients included. However, this approach could help the clinicians anticipate response to PD1 inhibitors and rapidly adapt treatment strategy.

Only 27 out of 38 (71%) of our population could be evaluated with this assay. 11/38 (29%) were not eligible for this methodology, either because of low tumor cell proportion in the biopsy or because of failure in designing the personal assay. This drawback may be improved by lowering the tumor cell proportion (20%) required for tumor DNA extraction or improving DNA extraction, although the impact on the assay performance will have to be evaluated under these conditions. In addition, ctDNA was not detected in 4 (11%) additional patients in the pre-treatment samples. This might be explained by the low abundance of ctDNA in some patients as well as the possible discordance between variants found in the tumor and the plasma, as previously described.[220] A fresh tumor biopsy is sometimes difficult to obtain in R/M SCCHN (for example, due to the close proximity of blood vessels or airway organs). Therefore, for some patients (n=9), we had to sequence archival tumor biopsies, sometimes taken several months before the initiation of PD1 inhibitors. As the tumor genomic landscape may change overtime, this may impact the accuracy of tumor-personalized approaches.

Personalized assays are costly. The bespoke multiplex-PCR tumor personalized assay investigated here requires 2 WES per patient (tDNA & gDNA) and the design of a 16-plex PCR assay. The assay design time has also needs to be accounted for, as the whole process can take several weeks. This delay could be challenging to implement this assay in the clinic.

In conclusion, this study shows the added value of ctDNA kinetics analysis to refine patients' outcome when treated with anti-PD1 inhibitors with or without chemotherapy. In the era of personalized medicine, ctDNA will play an important role in identifying early the patients who need treatment adaptation or modification.

IV.3 Evaluation of both approaches

We have evaluated two different assays (personalized and non-personalized) to measure ctDNA in R/M SCCHN patients treated with PD1-inhibitors. In both studies, ctDNA kinetics within 6-12 weeks from treatment initiation were correlated with tumor response and PFS.

However, there are differences in the results obtained with these two methodologies that requires further discussion (Table IV-5).

Thirty-three patients were included in the two cohorts described in chapters IV-1 and IV-2. Nine patients could not be analyzed by the Signatera™ assay, due to technical issues as discussed previously, and 3 were not analyzed using the non-personalized in-house approach. Therefore, twenty-two patients benefited from both analyses. There were 9 non-concordant results regarding ctDNA kinetics between the two assays (in red in Table IV-6). We did not identify any clinical parameters that could explain these discordances.

ctDNA kinetics are evaluated differently between the two assays. In literature, there are as many methodologies as assays to evaluate ctDNA kinetics. In our non-personalized approach, we evaluated ctDNA kinetics in a very simple way as previously described[327]: difference in mean VAF. Unlike the bespoke tumor-personalized multiplex-PCR assay, we did not take into account cfDNA quantity or concentration because there were no correlations between mean VAF and cfDNA quantity, concentration, mean read depth, or the plasma volume extracted (data not shown). For our in-house non-personalized assay, we tried to calculate ctDNA kinetics using other cut-off ($\geq$ 20% in ctDNA decrease) but this did not improve our results. It would be interesting to analyze the Signatera™ assay results using our approach to calculate ctDNA kinetics, to ascertain whether the results remain similar.

We conclude that ctDNA kinetics can predict response to PD1-inhibitor, regardless of the methodology used. No methodology has been proven to be superior to the other, each approach presenting advantages and drawbacks. Our results are, however, encouraging and pave the way to implementation of ctDNA evaluation in daily practice.

	Non-personalized approach (UCL in-house assay)		Personalized assay (Signatera)	
Total number of patients included	44		38	
Total number of evaluable patients	35		27	
Methodology to evaluate ctDNA kinetics	$\Delta ctDNA = MeanVAF\ (Baseline) - MeanVAF\ (FU1)$		Decrease in ctDNA in on-treatment samples = decrease of ≥20% in MTM/mL compared with pre-treatment samples. If not, considered as increase in ctDNA.	
Results	Decrease in ctDNA*	Increase in ctDNA*	Favorable ctDNA kinetics*	Unfavorable ctDNA kinetics*
	17 (48.6%)	18 (51.4%)	15 (55.5%)	12 (44.5%)
Best Overall Response				
CR	2 (5.7%)	1 (2.9%)	3 (11.1%)	0 (0%)
PR	5 (14.3%)	1 (2.9%)	5 (18.5%)	0 (0%)
SD	6 (17.1%)	3 (8.6%)	4 (14.8%)	2 (7.4%)
PD	4 (11.4%)	13 (37.1%)	3 (11.1%)	10 (37.0%)
Correct prediction of best response				
	74.5% (chi-square =4.51, p-value=0.03)		81.4% (chi-square = 8.32, p-value= 0.004)	
Survival analysis				
PFS Median in months	8.6 [95%CI:2.7-18.1]c	2.5 [95% CI: 2.0-3.0]	8.09 [95% CI: 6.6-28.6]	2.42 [95% CI: 2.3-4.0]
OS Median in months	18.1 [95%CI:12.5-41.5]	8.2 [95%CI:7.2-13.0]	15.6 [95% CI: 7.9-NE]	10.8 [95%CI: 4.4-15.1]

Table IV-5: Comparision of the two assays in the R/M cohort

*within 6-10 weeks from treatment initiation; ctDNA: circulating tumor DNA; VAF: variant allele frequency; B: Baseline, FU1: 1st on-treatment sample (6-10 weeks after treatment inititation); MTM/mL: mutant molecules per mL; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease

Record ID	Neoplasia primary location	p16 status	PD-L1 CPS score	PD1 inhibitor	Best Response (RECIST V1.1)	Number of days between initiation of treatment and death or last follow-up	Duration between initiation of treatment and progression or death or last follow-up	Signatera Assay	UCL-non personalized approach
89	Oral cavity	Negative	0	nivolumab	PD	506	66	Increase	
110	Oropharynx	Negative	<1	nivolumab	PR	513	204	Decrease	increase
115	Unknown	Negative	0	nivolumab	PD	159	90		increase
117	Hypopharynx	Negative	10	nivolumab	PR	549	549	Decrease	decrease
119	Oropharynx	Positive	5	pembrolizumab	PD	459	142	Increase	
122	Hypopharynx	Negative	5	nivolumab	PD	413	168	Increase	decrease
123	Oropharynx	Positive	<1	nivolumab	PD	813	120	Increase	increase
126	Hypopharynx	Negative	0	nivolumab	SD	202	202	Decrease	decrease
133	Oropharynx	Negative	20	pembrolizumab	PD	253	182		increase
134	Oral cavity	Negative	100	nivolumab	SD	381	261		decrease
148	Oral cavity	Negative	>50	pembrolizumab	PR	421	421		increase
150	Oral cavity	Negative	27	nivolumab	PR	1132	1132		increase
158	Oropharynx	Negative	4	pembrolizumab	PR	624	599	Decrease	decrease
168	Oral cavity	Negative	9	pembrolizumab	PD	135	50	Increase	increase
176	Oral cavity	Negative	80	pembrolizumab	SD	236	197	Increase	increase
181	Larynx	Negative	0	pembrolizumab	PD	437	99	Decrease	decrease

187	Oral cavity	Negative	100	pembrolizumab	CR	940	940	Decrease	decrease
188	Oral cavity	Negative	14	pembrolizumab	PD	394	106		increase
189	Oral cavity	Negative	1	pembrolizumab	PD	124	70	Increase	decrease
190	Oropharynx	Negative	50	nivolumab	CR		870	Decrease	increase
191	Oropharynx	Positive	30	pembrolizumab	SD	242	242	Decrease	increase
193	Unknown	Negative	33	pembrolizumab	PR	843	843		decrease
194	Oropharynx	Negative	60	pembrolizumab	PD	841	76	Increase	increase
196	Oropharynx	Negative	100	pembrolizumab	CR	688	688	Decrease	increase
198	Oropharynx	Positive	40	pembrolizumab	PD	219	93	Decrease	increase
199	Larynx	Negative	3	pembrolizumab	SD	786	786	Decrease	decrease
200	Oral cavity	Negative	10	pembrolizumab	PD	243	75	Increase	increase
207	Oropharynx	Negative	0	nivolumab	SD	53	53	Increase	decrease
217	Oral cavity	Negative	10	pembrolizumab	PD	618	74		
218	Oropharynx	Negative	0	nivolumab	PD	87	40	Decrease	decrease
222	Unknown	Negative	<1	nivolumab	SD	448	61		decrease
224	Oral cavity	Negative	0	pembrolizumab	PD	414	72	Increase	increase
225	Oral cavity	Negative	0	pembrolizumab	SD	556	556	Decrease	increase

Table IV-6: Patients who were tested by both assays CPS: combined positive score; RECIST: Response Evaluation Criteria in Solid Tumors version1.1 (RECISTv1.1), CR: complete response; PR: partial response, SD: Stable disease, PD: progressive disease.

v. Conclusions and perspectives

This PhD work is focusing on ctDNA as a biomarker in SCCHN. We first explored the utility of ctDNA as a biomarker for minimal residual disease (MRD) in locally-advanced disease and then as a biomarker for response to PD1 inhibitors in recurrent and/or metastatic patients.

Using a similar methodology, we developed two in-house *non-personalized* ctDNA assays with two different gene panels: one to detect MRD and the other one to monitor response to PD1 inhibitors. We demonstrated that non-personalized ctDNA assays have to potential to (i) detect MRD in LA SCCHN and predict disease progression and survival and (ii) to predict long-term efficacy of PD1 therapy. Our approach did not require tumor sequencing, making this approach easily applicable in daily practice.

We had the opportunity to investigate in similar populations the bespoke multiplex-PCR tumor *personalized* assay from Natera (Signatera™). This technology showed promising results to predict efficacy of PD1 inhibitors but was not able to detect MRD within 12 weeks after the end of curative treatment in LA-SCCHN.

Although the concordance between the cohorts was not of 100%, the majority of our patients were included in the evaluation of both the personalized and the non-personalized assays. This offers us the opportunity to discuss these two techniques.

We developed two SCCHN specific gene panels, based on the most frequent mutated genes in each clinical situation. In our two populations of interest (LA SCCHN and R/M SCCHN), we achieved a detection rate superior to 70% at baseline (74.5% in the MRD study and 79.5% in the PD1 inhibitor study). This is in line with previous reports of ctDNA detection rate in SCCHN, using a non-personalized approach (67% in Mes et al.[233], 92% in Wang et al.[308]). In these studies, the detection rate was improved by adding copy-number variations (CNV) analysis. We also tried to detect CNV but we did not succeed. CNV detection tools are not specific enough for MRD detection at the moment.

One major asset of our methodology is to combine in one assay HPV-negative and HPV-positive SCCHN patients with the use of taxonomic assignation to evaluate HPV presence. Taxinomic assignation tools[393] have been developed to evaluate the bacterial and viral landscape of tissues and, as the interest in studying the microbiome is increasing, bioinformatic pipelines to achieve this goal are becoming more accurate. Kraken2[341,393], the taxonomic assignment tool we used in this work, has a sensitivity of ~60% and a positive-predictive value of >95% to detect viral genome on training datasets, mainly constituted by shallow-WGS data. The use of such bioinformatic pipelines had never been used before in HPV-positive oropharyngeal cancers, as PCR-based technologies are efficient and standard of care. Furthermore, Kraken2 has never been used, to the best of our knowledge, on cfDNA sequencing data. The good correlation with Kraken2 between HPV16 ctDNA detection and tumor HPV16 in-situ hybridization was due, we hypothesized that, to the amplification step and the inclusion of HPV16 genes in our panel. To fully validate this tool, we should continue investigations on p16-positive oropharyngeal cancer patients to compare (i) in-situ hybridization, (ii) Kraken2 on cfDNA sequencing (as we have done in this thesis work), (iii) Kraken2 on cfDNA sWGS data and (iv) HPV 16 ddPCR as positive ctDNA control. If positive results emerge from this analysis, Kraken2, or any other taxonomic assignation tool, could be used on cfDNA sequencing data to improve comprehensive understanding of viral implication in SCCHN, HNC in general (including nasopharyngeal cancer), and any other types of cancer.

Below, we will discuss the major challenges of this work and develop future perspectives for ctDNA in SCCHN.

V.1 Challenges

First, one major challenge for ctDNA analysis is the limited amount of material available. In healthy patients, 1 to 10 ng/µL of cfDNA can be found in the bloodstream. In cancer patients, the amount of cfDNA can increase and in SCCHN can reach up to 100ng/µL. However, in our cohorts, we have shown that mean cfDNA quantity was 1.5ng/µL in the LA SCCHN and 2.27ng/µL in the R/M cohort. ctDNA being a proportion of cfDNA <1%, the absolute quantity of ctDNA in the bloodstream is very low. This is a limitation

to all techniques: NGS sequencing requires a minimal quantity of DNA (~10ng) and using UMIs become mandatory in order to detect reads present at very low frequency. PCR techniques are also restricted by DNA quantity. As shown in chapter III-3, the lowest limit of detection of a variant will depend on the quantity of DNA analyzed: for ddPCR, our limit of detection was ~0.1% with only 10ng of DNA engaged, which is consistent with Bio-Rad™ manufacturer protocol. Decreasing limit of detection would require a much higher quantity of DNA input, which means more blood harvested from our patients.

Another challenge already discussed in the introduction is the sampling timing. There are no standard defined time points for ctDNA detection neither for MRD nor during systemic treatment. A comprehensive approach would be to harvest blood sample every week from patients. However, this study would not only be very costly but also not convenient for the patients undergoing curative or palliative SCCHN treatment.

More and more data are highlighting the poor correlation between tumor DNA genomic landscape and ctDNA. In our analysis, the most frequent mutations occurred in *TP53* (67.0 and 32% in the LA- and the R/M- cohorts, respectively), *NOTCH1* (39.5%), *EGFR* (30.2%), *KMT2D* (25.5%), and *FAT1* (23.2%), which is consistent with SCCHN ctDNA analysis[233,308] and tumor analysis[10,89,109], even though the gene panels are different. Studies have already shown poor correlation between mutations found in the tumor and in the plasma.[220,297,330] In our MRD cohort, tumor DNA was sequenced using the same panel than the one use for cfDNA sequencing. Only 32/138 (23.2%) ctDNA mutations were concordant with the tumor and only 32/172 (18.6%) of tumor mutations were found in the plasma in the pre-treatment samples (Fig V-1).

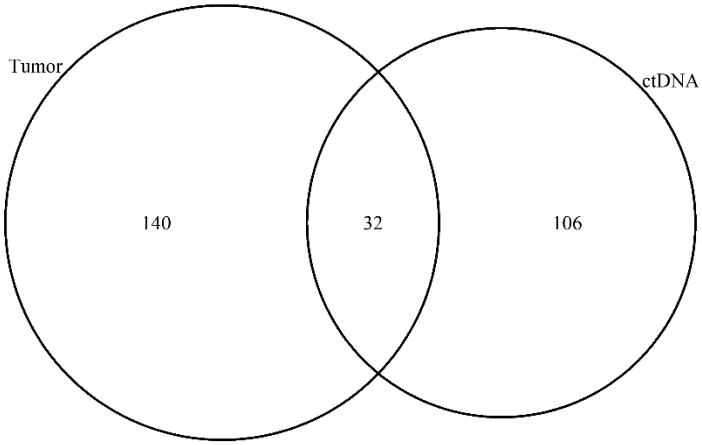


Figure V-1: Venn diagram between variants found in tumor biopsies and ctDNA at baseline
ctDNA: circulating tumor DNA

There might be several explanations for this low level of concordance: first, ctDNA shedding mechanisms by tumor cells are not fully understood[394]. Tumor proliferation, necrosis and apoptosis seem to show higher level of ctDNA shedding, and some tumors do not seem to shed significant amount of DNA in the bloodstream.[395] Tumor DNA analysis must also take into account the fact that there is time and spatial heterogeneity amongst the tumor, and DNA analysis of a biopsy may not reflect the genomic landscape of the whole tumor. For neoplasia that have already breached vessel barrier and invaded the lymph nodes, genomic landscape can be different[109], and may not be reflected in tumor-biopsy analysis. This non-exhaustive list of issues has to be taken into consideration in tumor-personalized methodologies but also in non-personalized approaches as ctDNA is so labile. This is also an issue to validate new ctDNA assays, as tumor DNA sequencing cannot be set as a gold standard.

Finally, a major challenge for bioinformaticians and oncologists is the selection of variants of interest. To separate sequencing errors from real tumor variants, we heavily rely on bioinformatic pipelines, like MUTECT2 in our work. Although many pipelines use MUTECT2, perfect accuracy to select

variants of interest has not been demonstrated. A few similar pipelines exist (VARSAN2[396], STRELKA[397], etc…) but none has shown superiority over the others. Furthermore, even the combination of two or more bio-informatic pipelines is not a guarantee of better variant selection.[398-400] Also, MUTECT2 was designed for somatic analysis of tumor DNA and not ctDNA analysis and detection of variants at a very low proportion. Built within the pipeline structure of MUTECT2 are variant filters that eliminate nucleotide changes present at a very low proportion. In our MRD study, to be able to detect variants present at frequency <0.01%, we had to get rid of these filters and apply a “high sensitivity” version of MUTECT2, without stringent filters. This means that MUTECT2 may allow more sequencing errors to be “called”, requiring an additional manual check, and quality filters for variant selection. Although this methodology cannot be used without guidance, it was very effective in retrieving variants in post-treatment MRD plasma samples of this work, when informed by the pre-treatment plasma genomic landscape. We verified the presence of some variants by ddPCR to confirm the accuracy of our findings.

Moreover, variant selection should avoid selecting Clonal Hematopoiesis of Indeterminate Potential (CHIPs).[401] CHIPs are variants present in non-tumor cells, more frequently white blood cells, that shed their DNA in the bloodstream. CHIPs are usually due to treatment, especially radiotherapy and chemotherapy, or age, and could led to confusion with a tumor variant. Several methodologies can be put into place to reduce the risk of confounding CHIPs and tumor variants: as CHIPs mainly come from white blood cells from the bone marrow to the bloodstream, germline analysis of PBMCs may eliminate CHIPs as it would be considered as germline DNA. However, to be eliminated as a germline variant, CHIPs should be present at a variant allele frequency higher than 45%, so in the large majority of the PBMCs. As CHIPs are induced in the bone marrow stem cells, white blood cells originating from different stem cells will not carry the same alterations and will be present in different proportions in the blood. Another way to eliminate CHIPs is to eliminate the genes in which CHIPs occur the most frequently.[402] However, one of the genes the most subjected to CHIPs is *TP53*, that is the major tumor suppressor gene mutated in SCCHN.[403-407] There is no consensus how to eliminate CHIPs at the moment.

When variants are selected, their implication and role in carcinogenesis are difficult to apprehend. Only a minority of tumor variants are implicated in carcinogenesis, mostly in oncogenes, like *KRAS* G12C or *BRAF* V600E and can be established as driver mutations. For mutations in tumor suppressor genes, apart from a few that have previously been described and evaluated in pre-clinical studies, we must rely on prediction scores and classification such as ACMG[408] or Belac[409]. These classifications are based on scores obtained if the variant is considered as damaging, depending on aminoacid change, location, etc… They also have a category of variants of unknown significance (VUS), grouping many variants with uncertain roles in carcinogenesis. This complicates the selection of mutations, and furthermore of “driver” mutations. Another challenge to tackle in ctDNA analysis is that we are never sure of the ctDNA quantity: in germline or tumor DNA analysis, a mutation would be considered as “driver” if it is present at a VAF corresponding to the tumor cells proportion estimation. For example, if there is 80% of tumor cells in the biopsy sequenced, and we find a variant, predicted as damaging and present with a VAF of ~40%, we may safely consider it as a driver mutation. However, in ctDNA analysis, except for analysis based on the length of cfDNA fragments, we cannot safely estimate the ctDNA proportion in the cfDNA. We rely only on prediction score and the highest VAF to estimate driver mutations. Nevertheless, in our studies, we bypassed this problem by not taking into account driver mutations. Any variants that passed our carefully selected filters were considered tumor variants, drivers or passengers, and were used to assess the presence of ctDNA with encouraging results in both cohorts.

Despite these challenges, ctDNA is becoming a cornerstone of SCCHN future.

V.2 Perspectives

We will discuss two points: liquid biopsies beyond ctDNA to improve detection rate and possible clinical trial designs.

V.2.1 Liquid biopsies beyond ctDNA

- Mitochondrial ctDNA

Mitochondrial circulating tumor DNA (mtDNA) is also shed by tumor cells and could potentially be a biomarker of cancer. Quantity of mtDNA may be elevated in some cancers, although not in all.[410] Until now, mtDNA mutations have not been extensively investigated in head and neck cancer, but a few studies have shown that levels of circulating mtDNA post operatively may be predictive of relapse.[411]

- Methylation patterns

Epigenetic modification could be divided into three main categories: methylation abnormalities, histone modification, and non-coding RNAs.

DNA methylation is a very powerful mechanism to regulate gene expression[412] : a methyl-group is added to the cytosine on CpG sites, by proteins belonging to the family of DNA methyltransferases (DNMT). DNMT3a and DNMT3b are mostly involved in *de novo* methylation, thus in embryonic differentiation. DNMT1 is responsible for maintenance of methylation during cell division. In normal cells, methylation occurs in a pattern specific to each cell type and does not occur in promotor regions, as they contain regulatory elements. In cancer cells, methylation patterns are disrupted, with hyper-methylation of promotor regions of tumor suppressor

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Adapted from Liquid Biopsy to Detect Minimal Residual Disease: Methodology and Impact; N. Honoré, R. Galot, C. van Marcke, N. Limaye and J. P. Machiels. Cancers (Basel) 2021 Vol. 13 Issue 21

genes and hypo-methylation of promotor regions of oncogenes.[412] This leads to a repression of the expression of tumor suppressor genes and an increased expression of oncogenes. Colon cancer is a seminal example of the role of methylation in tumorigenesis. The methylation of the *MLH1* gene promotor leads to the repression of MLH1 protein expression, resulting in inactivation of the mismatch repair machinery.[413] Several clinical trials are ongoing with therapies targeted at the mis-methylation process that occurs in cancer cells.[414]

Detection of cancer-associated methylation patterns in the blood was tested in SCCHN, as a risk stratification biomarker and for disease monitoring.[415] Although specificity was good (95%), methylation patterns should be further studied to improve sensitivity (52%). These finding have been confirmed in other studies in HNC.[277,416] Sensitivity could be increased by looking for more than one methylation pattern.[417,418] In these studies, a multiple-gene methylation pattern correlated with treatment response and was associated with worse progression-free survival. Met-ctDNA detection could thus be a powerful biomarker of MRD, but detection methods are not yet sensitive enough to be implemented in clinical practice.[419] Studies are currently ongoing to assess for methylation patterns in blood as a cancer detection tool (NCT04814407, NCT04511559), but also for monitoring disease and determining the presence of MRD (NCT03634826, NCT03737539). The detection of met-ctDNA might be a more powerful tool if combined with other biomarkers.

- Non-coding RNA

RNA is composed of a single strand of ribonucleic acids and can be divided into coding RNAs, also called messenger RNA (mRNA), and non-coding RNA (ncRNA). ncRNA is a family of RNA molecules defined by their function and length. ncRNA is subdivided into housekeeping RNA that includes transfer-RNA and ribosomal-RNA, and regulatory-ncRNA. Regulatory-ncRNA is also subdivided into multiple categories based on structure: long-ncRNA (> 200 nucleotides), circular-RNA, and functional ncRNA including micro-RNA, small-ncRNA, small nuclear-ncRNA and Piwi-interacting-RNA (piRNA) [420]. As these types of RNA are found in the nucleus and cytoplasm of cells, they can also be detected in the bloodstream or in other body fluids,

via the same mechanisms as ctDNA.[421] The most investigated are miRNA and lncRNA. miRNAs modulate the expression of genes by binding to mRNA and silencing translation [422]: cancer cells express specific miRNA signatures that can be detected in tumoral tissue and in the bloodstream.[421]

Data have shown that it is possible to detect cancer through the detection of cancer-specific miRNAs in head and neck cancer[356,423,424], and that lncRNA could be predictive of response to RCT.[425] Multiple studies are currently ongoing to assess the potential of miRNA as MRD biomarkers in several cancers, such as breast cancer (NCT04720508), pancreatic cancer (NCT04406831), prostate cancer (NCT04835454), head and neck cancer (NCT04305366), and many others (source: clinicaltrials.gov).

Long non-coding RNA (lncRNA) has also been investigated as a potential biomarker of tumor cells in many cancers.[426-430] LncRNA can be detected in the bloodstream and correlated with tumor stage and treatment response.[423,431-433] Despite showing good sensitivity (~70-80%), however, the specificity of lncRNA diagnostic tests is too low (~60%) to be applicable in routine practice.[431,434] Most ongoing clinical studies investigating lncRNA focus on its use as a cancer diagnostic tool (NCT03830619, NCT03469544, NCT04269746).

- Exosomes

Extracellular vesicles (EVs) have a phospholipid bilayer membrane containing bio-products derived from cells that secrete them via an active process. Exosomes are a subset of EVs released by living cells[435,436], ranging in size from about 40 to 160 nm (median 100nm).[437] Apart from cytosolic and transmembrane proteins, they contain single and double stranded DNA [438], RNA (mRNA, miRNA, lncRNA, cRNA etc. [437]), and cytosolic metabolites.[436] Exosomes are key players in inter-cellular communication. Their secretion allows cells to induce genetic, epigenetic and protein transformation of other cells. This method of communication has been investigated in conditions including pregnancy [439], auto-immune disease [440] and cancer. [437,441] Studies have shown that cancer cells may produce up to 20-fold more exosomes than non-cancerous cells.[435,442] Exosomes play a role in tumorigenesis by allowing cell-to-cell communication to induce, for example, Epithelial-to-

Mesenchymal Transition (EMT), invasion and migration of cancerous cells, and a tolerant immune environment.[437] Cancer-derived exosomes can be isolated using cancer-specific proteins expressed at their surface.[437] Cancer-specific DNA and RNA modifications are also found in exosomes.[437,443,444] Standardized isolation and quantification methods are lacking.[445,446] While clinical trials are ongoing to assess the diagnosis of tumors, also under investigation is the use of the modulating power of exosomes to deliver systemic therapies to specific cells. [447]

- Circulating Tumor Cells

Circulating Tumor Cells (CTCs) were first described in 1869 when Ashworth detected malignant cells in the bloodstream of a metastatic cancer patient. [448] It would be more than a century, however, before CTCs were defined and isolated. By definition, CTCs are tumor cells. They can be isolated from other circulating cells based on tumor-specific alterations (immuno-chemistry, size or density [448]). The tumor specific genomic and epigenetic alterations that they carry can also be harnessed to detect CTCs[449], with the advantage that their DNA, in contrast to ctDNA, is not fragmented.[450]

Although CTCs are indeed shed into the blood from primary tumors or secondary lesions [451], their presence does not necessary imply the presence, nor predict the development, of micro-metastases. For a cell to be able to metastasize in a specific organ, it needs to undergo MET (mesenchymal to epithelial transition) in order to breach the vascular endothelium and colonize tissue.[452] CTCs with metastatic potential would have to express specific proteins on their surface in order to be recognized by endothelial cells. The FDA-approved CellSearch®[453] isolation technique is based on the detection of one such cell-surface protein: the epithelial cell antigen EpCAM, on the surface of CTCs. This technique would however miss any CTCs without an epithelial phenotype.[454]

Notwithstanding their limitations, numerous clinical studies have investigated the prognostic role of CTCs. These studies are either quantitative, counting CTCs in the bloodstream, or qualitative, analyzing CTCs for their genome, transcriptome and/or proteome. The presence of CTCs after neoadjuvant chemotherapy was found to be associated with worse disease-free survival

(DFS) in early breast cancer [455,456], naso-pharyngeal carcinoma [457] and lung cancer [458], as well as a worse overall survival (OS) in melanoma.[459,460] There is to the best of our knowledge no interventional trial based on the detection of CTCs ongoing at the moment (source: clinicaltrials.gov). With the support of the European Liquid Biopsy Society, which is working on defining technical guidelines on the collection, processing and detection of CTCs, we expect more trials in this field to open in the coming years.[461]

- Circulating proteins

Today, the most validated MRD-biomarkers remain cancer-specific antigens such as CA125, CA15.3, carcinogen embryonic antigen (CEA), alpha-feto protein (αFP), and prostate-specific antigen (PSA).[462] However, there are no circulating proteins associated with HNC.

V.2.2 Prospective clinical trials

Ongoing clinical trials relying on ctDNA as a marker of MRD use four main designs as schematized in Figure V-2: Prognosis (A), Intensification (B), De-escalation (C) and Combined (D).

The first, (A), is a non-interventional design that evaluates the prognostic impact of ctDNA detection. Although ctDNA has already been shown to be a prognostic marker in some cancers, trials are warranted in tumors where ctDNA has been underexplored. Such trials are also necessary to evaluate the accuracy of the ctDNA detection assays under investigation. The optimal time to detect ctDNA following curative-intent therapy, and the impact of this timing on prognosis, can also be evaluated in these trials.

The second trial design (B) is an interventional trial that randomizes patients who are ctDNA-positive after receiving standard of care (SOC) treatment, to adjuvant intensification therapy or placebo. This design investigates whether adjuvant treatment can improve OS and/or PFS if ctDNA is detected. Although this trial design is elegant and can potentially improve survival rates, in the event of a negative outcome it can be difficult to evaluate whether it is the patient selection that is ineffective (ctDNA may not be a good

marker to select those in need of adjuvant treatment), or the adjuvant treatment.

The third trial design (C) is the de-escalation of SOC adjuvant treatment. In some tumor types, adjuvant treatment is routinely administered following curative-intent treatment (surgery), but adjuvant treatment may not be required for ctDNA-negative patients. If these trials show non-inferiority in terms of survival between cohorts, they might redefine SOC adjuvant treatment guidelines and spare a subset of patients the burden of additional treatment.

Finally, it is possible to combine these trial designs (D) so that the prognostic impact of ctDNA detection and the added value of adjuvant treatment in the event of post-treatment ctDNA positivity, can both be evaluated in a single study.

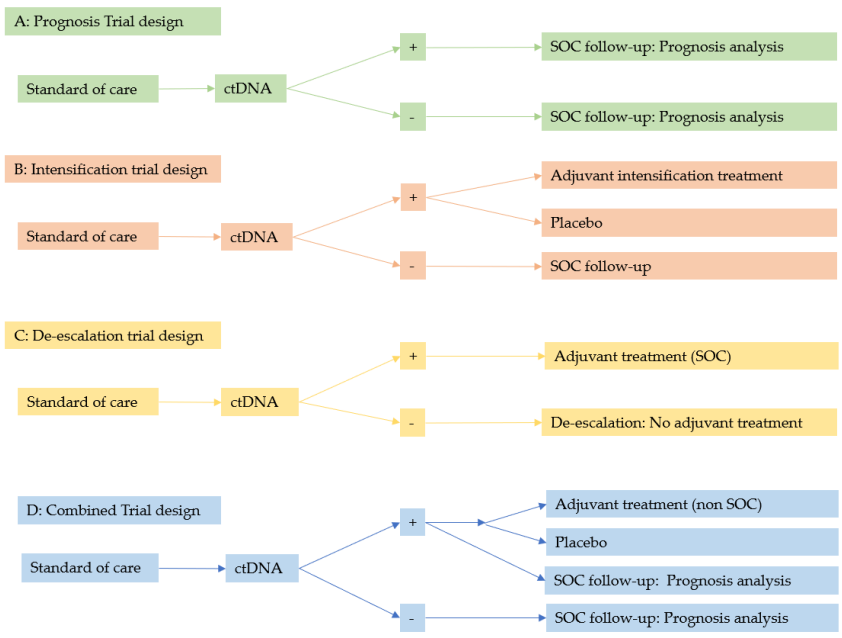

Figure V-2: Trial designs based on ctDNA

ctDNA: circulating tumor DNA; SOC: standard of care

In head and neck cancer, interventional studies are ongoing to assess the impact of ctDNA, especially in HPV positive SCCHN. We briefly review them in the following Table V-1:

Title	**NCT**	**Popu-lation**	**Intervention**
MRD			
A Study on Using Cell-Free Tumor DNA (ctDNA) Testing to Decide When to StartRoutine Treatment in People With Human Papilloma Virus (HPV)- Associated Oropharynx Cancer (OPC)	NCT05307939	HPV positive SCCHN, treated with surgery	Arm A: monitoring of ctDNA. If ctDNA -, no adj RT or delayed RT when ctDNA become +. Arm B: if ctDNA+, 3 weeks of CRT instead of 6-6.5 weeks of CRT
Molecular Residual Disease Interception in Locoregionally-Advanced High Risk HPV+ and HPV- SCCHN (MERIDIAN)	NCT05414032	LA HPV negative and positive SCCHN	Part A and Part B are investigational. Part D and E are follow-up ctDNA detection Part C is the randomized and interventional part of the study (n=60) for patients with MRD, detected with ctDNA. The patient will be randomized 3:1 to Arm A (treatment with AZD2936) or Arm B (observation).

Risk-adapted Therapy in HPV+ Oropharyngeal Cancer Using Circulating Tumor (ct)HPV DNA Profile - The ReACT Study	NCT04900623	HPV positive SCCHN	This research is being conducted to understand if treatment can be tailored for participants with HPV-related oropharynx cancers using both clinical features (stage of the tumor, smoking status) combined with an investigational HPV blood test.

The Sinai Robotic Surgery Trial in HPV-related Oropharyngeal Squamous Cell Carcinoma (SIRS 2.0 Trial)	NCT05419089	HPV positive SCCHN	In this trial, the study will be stratifying p16 positive patients with PCR detectable high-risk (HR) HPV DNA or RNA following TORS into risk groups based on final pathology to determine appropriate treatment intensity. Patients with low-risk pathologic disease and undetectable postoperative cfHPVDNA will receive no adjuvant therapy. Patients with high-risk pathologic disease and undetectable postoperative cfHPVDNA will receive de-intensified adjuvant radiation and chemotherapy.
Monitoring of immunotherapy response			
A Study of Pembrolizumab in Combination With Chemotherapy for Head and Neck Cancer	NCT05420948	R/M SCCHN	Immunotherapy with intermittent chemotherapy guided by measurements of tumor DNA in blood

Table V-1: current interventional trials in SCCHN using ctDNA

SCCHN: squamous cell carcinoma of the head and neck, OPC: oropharyngeal cancer; ctDNA: circulating tumor DNA; HPV: Human papillomavirus; cfHPVDNA: cell-free HPV DNA; RT: Radiotherapy; CRT: chemoradiation; MRD: minimal residual disease; TORS: trans-oral robotic surgery; R/M: recurrent and metastatic.

Based on our work, we could first try some strategies to improve the sensitivity and specificity of our test by adding, for example, methylation detection. To date, it is the epigenetic modifications that have shown promising results. This approach would not require additional cfDNA quantity, as the methylation detection is done on the same sequencing libraries as ctDNA detection. Increasing the number of genes in our panels may also results in a better sensitivity as suggest by Bratman et al.[333]

It would be interesting to evaluate our in-house MRD non-personalized assay in prospective observational clinical trials. Observational trials are needed to answer several questions raised by this work. We could imagine collecting plasma more frequently than in our work during the curative treatment and adjuvant treatment but also in R/M SCCHN to study the best timing of collection in regard of disease evolution and prognosis. This will be very helpful to design relevant interventional trials.

In LA-SCCHN, several interventional trial designs to investigate de-escalation and escalation strategies based on the presence or not of MRD are possible (Figure V-3). The choice of adjuvant treatment could be, for example anti-PD1 therapy in PD-L1 expressing SCCHN but other treatment modalities could also benefit from this strategy.[463]

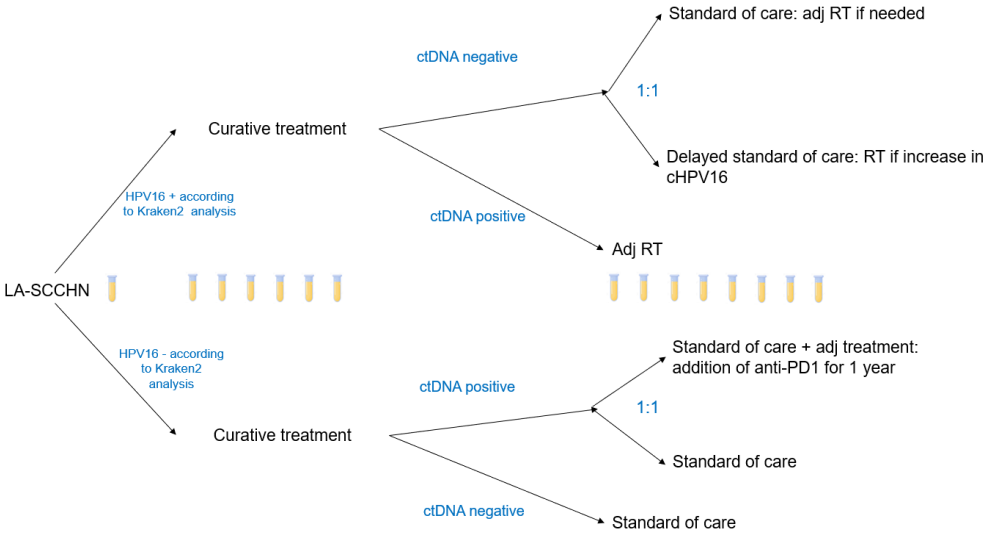

Figure V-3: trial design in locally advanced SCCHN
LA: locally advanced; SCCHN: squamous cell carcinoma of the head and neck; HPV: human papillomavirus; ctDNA: circulating tumor DNA; RT: radiotherapy; cHPV16: circulating HPV type 16; antiPD1: PD1 inhibitors; ajd: adjuvant; : blood sample for cfDNA extraction and analysis

In R/M SCCHN, it would be interesting to evaluate the potential of ctDNA in other settings such as chemotherapy, targeted therapy, etc…. In our work but also in other trials, ctDNA kinetics are investigated after several weeks of treatment. It is certainly of interest to study if earlier time points (2-3 weeks after treatment initiation) could give information about treatment efficacy to improve the rapidity of our clinical decisions. Finally, more interventional trials to investigate some strategies to adapt early the systemic treatment based on ctDNA kinetics are needed (Figure V-4).

Survival of patients treated with PD1 inhibitors is highly variable from one patient to another. A minority of the patients have a long-term benefit and some of them are cured. ctDNA could help us to identify the patients that do not require additional treatment and when to stop therapy, an open question today.

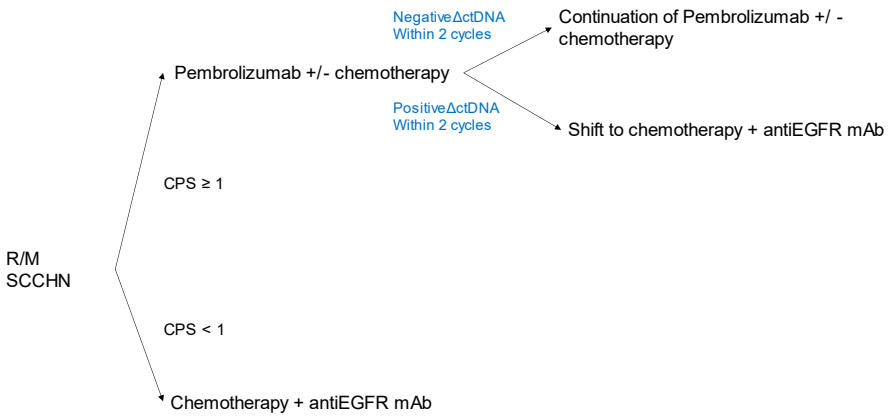

Figure V-4: trial design for R/M SCCHN
R/M: recurrent and/or metastatic; SCCHN: squamous cell carcinoma of the head and neck; ΔctDNA: difference in ctDNA levels according to assay; mAb: monoclonal antibody; CPS: combined positive score

I strongly believe that ctDNA will become a helpful biomarker in the management of SCCHN. Although some questions remain, this thesis work has shown the potential of ctDNA to help the clinicians to better stratify the patients and personalize therapy.

VI. Supplementary data

VI.1 Supplementary data for Chapter III

Supplementary material for

Material & Methods 1

Libraries for germline DNA (gDNA) from whole blood and cfDNA from plasma were conceived according to the manufacturer's protocol using MF and EZ Twist Bioscience® (South San Franscisco, California, U.S.A.) kits for cfDNA and gDNA, respectively. Targeted NGS was performed after capture using a custom-made in-house panel of 24 genes plus 2 HPV16 oncogenes (E6 and E7) designed by Twist Bioscience®, with an expected mean coverage of 2000x. The 24 genes were selected because they are frequently mutated in HPV-negative SCCHN[372] (Supplementary Table 2). Sequencing ('paired-end' 2x100 bp reads, Illumina® NovaSeq600™) was carried out by IntegraGen® (Evry, France), using unique molecular identifier technology (UMIs).[219]

Raw data (.fasta files) were aligned to the reference human genome assembly (GRCh38) using BWA 0.7.15.[373] UMIs were removed using fgbiotools and Picard.[374] Aligned sequences (.bam files) were processed using GATK 4.2 "BQSR" (for base quality scores recalibration). Germline single-nucleotide variants and small indels were identified using GATK 4.2 "Haplotype Caller" whereas somatic single-nucleotide variants (SNV) and small indels were identified using Mutect2[375] (both following Broad Institute best practices). The generated variant calls (.vcf) files were annotated, imported and further analyzed on Highlander[376], the in-house bioinformatics framework of the Genomics and Bioinformatics platform of UCLouvain (PGEN: https://www.deduveinstitute.be/pgen-bioinformatics)[375]. Highlander provides extensive variant-annotation, filtering and visualization.

Material & Methods 2

Digital droplet PCR (ddPCR)

In selected cases, mutation detection by ddPCR (Bio-Rad Laboratories, QX200™ ddPCR system, California, U.S.A.) was used to confirm the results obtained by NGS and the presence of the HPV16 genome (Supplementary Table VI-4). Data analysis was performed using Quantasoft™ v1.7.4 software according to the manufacturer's instructions (Bio-rad Laboratories, California, U.S.A.). The list of probes and ddPCR results can be found in Supplementary Data Table VI-4).

HPV In-situ Hydribization

HPV-driven tumors were evaluated using in-situ hydridization for high-risk human papilloma viruses (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66) (Ventana ISH iView Blue Plus Detection Kit). Staining was performed on a 3µm slide, using a Ventana-Roche GX autostainer (Roche Diagnosis, Basel, Switzerland). The presence of integrated HPV DNA in the nucleus of tumor cells was evaluated by an expert pathologist.

Tables

Id DNA Client	Quantity (ng)	Quantity of plasma extracted (mL)
112-20190527-B	53.5224	6
112-20190823-FU1	604.6036	6
129-20190902-B	89.146	6
129-20200106-FU1	44.4454	6
150-20191202-B	32.886	6
150-20200122-FU1	100.398	6
150-20200429-FU2	29.3886	6
170-20200609-B	295.9624	6
170-20210211-FU1	49.3812	6
170-20210416-FU2	45.5126	6
170-20220113-FU3	35.7802	6
175-20200714-B	83.8796	6
175-20200727-FU1	308.1134	6
175-20210114-FU2	56.8168	6
175-20210812-FU3	48.3024	6
175-20211007-FU4	72.4188	6
175-20220113-FU5	67.3844	6
182-20201012-B	32.422	6
182-20210125-FU1	20.7408	6
182-20210419-FU2	20.2536	6
182-20210517-FU3	17.4464	6
182-20211007-FU4	17.4	6
182-20211202-FU5	21.2686	6
182-20220121-FU6	21.7964	6
184-20201110-B	55.535	6
184-20210218-FU1	72.9234	6
184-20210812-FU2	18.8036	6
184-20211104-FU3	29.058	6
184-20220113-FU4	44.7702	6
185-20201116-B	42.4734	6
185-20210122-FU1	70.3714	6
185-20210826-FU2	21.373	6
185-20220224-FU3	31.1344	6

185-20220505-FU4	11.977	6
186-20201116-B	10.4458	6
186-20210211-FU1	11.5304	6
186-20210826-FU2	8.7	6
186-20211021-FU3	9.947	6
186-20211230-FU4	13.0384	6
186-20220505-FU5	8.9842	6
192-20210308-B	29.1218	6
192-20210714-FU1	37.4506	6
192-20211007-FU2	40.1824	6
192-20211216-FU3	31.8246	6
192-20220421-FU4	30.9488	6
197-20210419-B	100.4154	6
197-20210510-FU1	21.4252	6
197-20210621-FU2	31	6
197-20210709-FU3	24.1918	6
197-20211007-FU4	8.584	6
197-20211209-FU5	11.4144	6
197-20220202-FU6	11.4434	6
203-20210621-B	217.8016	6
203-20211104-FU1	74.7388	6
203-20220511-FU2	110.2928	6
206-20210705-B	62.4428	6
206-20211007-FU1	36.018	6
206-20211230-FU2	30.6066	6
206-20220421-FU3	43.4362	6
208-20210601-B	79.3788	6
208-20210708-FU1	52.461	6
208-20210909-FU2	17.3188	6
208-20211029-FU3	38.4656	6
208-20211230-FU4	35.8092	6
208-20220321-FU5	42.1428	6
209-20210920-B	108.4948	6
209-20211126-FU1	369.9356	6
209-20220210-FU2	99.238	6
210-20210927-B	88.8618	6
210-20211216-FU1	139.4494	6

210-20220127-FU2	83.4678	6
210-20220421-FU3	55.4944	6
211-20211011-B	37.3868	6
211-20220113-FU1	63.8232	6
211-20220224-FU2	38.7672	6
212-20211014-B	58.4002	6
212-20220127-FU1	73.2076	6
212-20220421-FU2	54.4678	6
213-20211005-B	100.3516	6
213-20211119-FU1	114.8574	6
213-20220114-FU2	48.5866	6
216-20211025-B	49.2304	6
216-20220210-FU1	163.6412	6
219-20211020-B	89.987	6
219-20211122-FU1	107.5146	6
219-20220224-FU2	101.906	6
219-20220421-FU3	80.0458	6
226-20220207-B	21.5934	6
226-20220421-FU1	17.0926	6
227-20220211-B	48.2328	6
227-20220602-FU1	22.7302	6
104-20190719-FU2	21.7036	6
106-20190828-FU2	41.093	6
106-20191120-FU3	28.188	6
106-20200212-FU4	43.4536	6
106-20200605-FU5	34.133	6
108-20220218-FU2	221.357	6
111-20200130-FU2	34.1504	6
111-20200409-FU3	20.4334	6
111-20200617-FU4	31.0126	6
111-20210305-FU5	33.6052	6
125-20191119-FU2	131.2366	6
132-20200525-FU2	20.8394	6
132-20200810-FU3	59.3456	6
132-20201103-FU4	32.9556	6
132-20211214-FU5	52.8264	6
160-20200720-FU2	78.9554	6

160-20210305-FU3	46.9336	6
160-20210813-FU4	57.2518	6
160-20211119-FU5	73.254	6
164-20210920-FU2	60.8768	6
164-20220128-FU3	38.135	6
164-20220429-FU4	25.8274	6
180-20211007-FU2	20.097	6
133-cfDNA-B	13.804	6
133-cfDNA-FU	13.92	6
136-cfDNA-B	28.768	6
136-cfDNA-FU	29.986	6
142-cfDNA-B	11.252	6
142-cfDNA-FU	84.1	6
143-cfDNA-B	25.52	6
143-cfDNA-FU	34.104	6
147-cfDNA-B	32.538	6
147-cfDNA-FU	183.86	6
151-cfDNA-B	40.6	4
151-cfDNA-FU	23.664	6
156-cfDNA-B	23.49	6
156-cfDNA-FU	28.884	6
160-cfDNA-B	33.176	6
160-cfDNA-FU	47.386	6
164-cfDNA-B	62.64	6
164-cfDNA-FU	47.386	6
167-cfDNA-B	36.946	6
167-cfDNA-FU	468.64	6
180-cfDNA-B	53.824	4
180-cfDNA-FU	64.96	6
181-cfDNA-B	71.34	6
181-cfDNA-FU	167.62	6
102-cfDNA-B	49.938	4
102-cfDNA-FU	78.3	4
103-cfDNA-B	24.65	6
103-cfDNA-FU	18.618	6
104-cfDNA-B	10.846	4
104-cfDNA-FU	15.312	4

105-cfDNA-B	78.3	5
105-cfDNA-FU	56.55	6
107-cfDNA-B	6.786	6
107-cfDNA-FU	23.606	6
108-cfDNA-B	52.896	4
108-cfDNA-FU	57.478	4
109-cfDNA-B	29.232	4
109-cfDNA-FU	14.5	6
110-cfDNA-B	113.1	4
110-cfDNA-FU	81.78	6
113-cfDNA-B	40.716	4
113-cfDNA-FU	52.954	6
114-cfDNA-B	28.884	3
114-cfDNA-FU	54.984	6
119-cfDNA-B	20.938	6
119-cfDNA-FU	20.59	6
120-cfDNA-B	34.104	4
120-cfDNA-FU	34.8	6
131-cfDNA-B	37.874	6
131-cfDNA-FU	217.5	6
132-cfDNA-B	39.672	4
132-cfDNA-FU	66.12	6
133-cfDNA-B	13.804	6
133-cfDNA-FU	13.92	6
136-cfDNA-B	28.768	6
136-cfDNA-FU	29.986	6

Table VI-1: Sample characteristics and quantification

AJUBA	
CASP8	
CDKN2A	
CTCF	
EGFR	
EPHA2	
FAT1	
FBXW7	
FLG	
FOSL2	
HIST1H1B	
HLA-A	
HRAS	
HRNR	
KMT2D	
KTR5	
NECAB1	
NOTCH1	
NSD1	
PIK3CA	
PSIP1	
PTEN	
TGFBR2	
TP53	
E6	HPV16 genome
E7	HPV16 genome

Table VI-2: Gene panel

Filter name	Explanation
exac_af	Allele frequency in total Exac samples ≤ 0.01
alternative_f1r2	Count of reads in F1R2 pair orientation supporting the alternate allele
alternative_f2r1	Count of reads in F2R1 pair orientation supporting the alternate allele
filters	GATK standard lowQual filters
	Should exclude minimum base quality, weak-evidence, mapping quality normal artifact
	List of all filters can be found on GATK and Mutect2 GitHub.
gnomad_wes_popmax_af ≤ 0.01	Maximum allele frequency accross populations
snpeff_effect	Planned effect according to SNEPFF
length	Length of reads
allelic_depth_alt	Number of reads supporting the alternate allele
read_depth	Number of reads in total
check-insilico	Manual evaluation of the variant

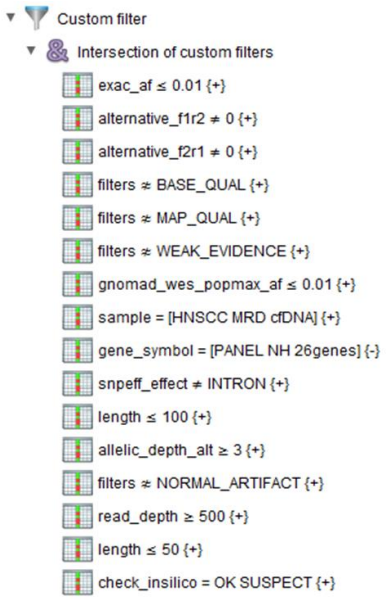

Table VI-3: Filters used for selection of baseline variants

Genome	Gene	Probe	Sample tested	Results
HPV16	E6	E6 2023 (FAM) Forward sequence, 5′-TCAGGACCCACAGGAGCG-3′; Reverse sequence, 5′-CCTCACGTCGCAGTAACTGTTG-3′; Probe (FAM-labeled) sequence, 5′-CAGAAAGTTACCACAGTTATGCACAGAGCT-3′	131-B; 131-FU	
HPV16	E7	E7 2023 (HEX) Forward sequence: CAGCTCAGAGGAGGAGGATG Reverse sequence: GTAATGGGCTCTGTCCGGT Probe sequence: AGATGGTCCAGCTGGACAAGCAGAACCGG Fluorophore: FAM	131-B; 131-FU	

Table VI-4: ddPCR probes for HPV

Multiplex results using 10 ng/well of cfDNA for sample 131-B (pre-treatment sample) and 30ng/well for sample 131-FU (post-treatment plasma sample). All results are for 3 wells merged (triplicates).

Well	Sample	Target	Allele frequency	Conc (copies/µL)	Supermix	DyeName(s)	TotalConfMax	TotalConfMin	PoissonConfMax	PoissonConfMin	Accepted Droplets	Positives	Negatives

M10	Patient 131 baseline	FAM	0.00284 %	0.03	ddPCR Supermix for Probes (no dUTP)	FAM	0.16	0.00	0.16	0.00	35210	1	35209
M10	Patient 131 baseline	HEX	0.01136 %	0.13	ddPCR Supermix for Probes (no dUTP)	HEX	0.32	0.00	0.32	0.04	35210	4	35206
M12	Patient 131 FU	FAM	0.00331 %	0.04	ddPCR Supermix for Probes (no dUTP)	FAM	0.19	0.00	0.19	0.00	30230	1	30229
M12	Patient 131 FU	HEX	0.00331 %	0.04	ddPCR Supermix for Probes (no dUTP)	HEX	0.19	0.00	0.19	0.00	30230	1	30229

Table VI-5: ddPCR results for HPV

Sample	chromosome	position	variant_type	hgvs_dna	hgvs_protein	cosmic_id	read_depth	allelic_depth_ref	allelic_depth_alt	allelic_depth_proportion_ref	allelic_depth_proportion_alt	gene_symbol
106-CF-BASELINE-TRT	1	152313880	SNV	c.1006C>G	p.Pro336Ala	COSV100911702	2117	2053	64	96.98%	3.02%	FLG
106-CF-BASELINE-TRT	1	152314336	SNV	c.550G>C	p.Glu184Gln		1864	1849	15	99.20%	0.80%	FLG
106-CF-BASELINE-TRT	2	28393807	SNV	c.87C>A	p.Gly29Gly		941	920	21	97.77%	2.23%	FOSL2
106-CF-BASELINE-TRT	9	21971037	SNV	c.322G>T	p.Asp108Tyr	COSV58682998	1977	1903	74	96.26%	3.74%	CDKN2A
106-CF-BASELINE-TRT	12	49033467	DEL	c.11235_11237delGCA	p.Gln3745del		1571	1553	18	98.85%	1.15%	KMT2D
106-CF-BASELINE-TRT	17	7673764	SNV	c.856G>A	p.Glu286Lys	COSV52664318	1821	1748	73	95.99%	4.01%	TP53
111-CF-BASELINE-TRT	1	152219720	SNV	c.1909G>T	p.Gly637Cys		1588	1577	11	99.31%	0.69%	HRNR

111-CF-BASELINE-TRT	2	28393782	SNV	c.62C>A	p.Ala21Glu		1679	1662	17	98.99%	1.01%	FOSL2
111-CF-BASELINE-TRT	3	179218303	SNV	c.1633G>A	p.Glu545Lys	COSV55873239	1925	1913	12	99.38%	0.62%	PIK3CA
111-CF-BASELINE-TRT	9	15465557	DEL	c.1554_1555delGA	p.Glu518fs		2324	2314	10	99.57%	0.43%	PSIP1
111-CF-BASELINE-TRT	9	21971121	SNV	c.238C>T	p.Arg80*	COSV58682746	2264	2242	22	99.03%	0.97%	CDKN2A
111-CF-BASELINE-TRT	9	136501780	SNV	c.5606C>A	p.Ala1869Asp		2469	2437	32	98.70%	1.30%	NOTCH1
111-CF-BASELINE-TRT	9	136501902	SNV	c.5484C>A	p.Pro1828Pro		2224	2204	20	99.10%	0.90%	NOTCH1
111-CF-BASELINE-TRT	12	49029061	SNV	c.14251G>T	p.Val4751Phe		1982	1973	9	99.55%	0.45%	KMT2D
111-CF-BASELINE-TRT	17	7674221	SNV	c.742C>T	p.Arg248Trp	COSV52662035	2500	2479	21	99.16%	0.84%	TP53

125-CF-BASELINE-TRT	2	28393807	SNV	c.87C>A	p.Gly29Gly		874	857	17	98.05%	1.95%	FOSL2
125-CF-BASELINE-TRT	5	177251853	SNV	c.4765G>C	p.Gly1589Arg		1462	1452	10	99.32%	0.68%	NSD1
125-CF-BASELINE-TRT	9	136501780	SNV	c.5606C>A	p.Ala1869Asp		1882	1867	15	99.20%	0.80%	NOTCH1
102-CF-B	14	22981278	DEL	c.988delG	p.Ala330fs		1733	1573	160	90.77%	9.23%	AJUBA
102-CF-B	17	7674872	SNV	c.659A>G	p.Tyr220Cys	COSV52661282	2311	2041	270	88.32%	11.68%	TP53
103-CF-B	9	21971006	SNV	c.353C>A	p.Ala118Asp	COSV100446147	1947	1931	16	99.18%	0.82%	CDKN2A
103-CF-B	9	136517811	SNV	c.1382G>T	p.Cys461Phe		1764	1735	29	98.36%	1.64%	NOTCH1
103-CF-B	12	49049883	INS	c.3704dupG	p.Gly1236fs		2021	1991	20	99.01%	0.99%	KMT2D

103-CF-B	12	49049890	SNV	c.3698A>T	p.Glu1233Val		2176	2155	21	99.03%	0.97%	KMT2D
103-CF-B	14	22976712	DEL	c.1109-15_1109-1delCTCTATTGTTTCTAG			1890	1878	12	99.37%	0.63%	AJUBA
103-CF-B	17	7675040	DEL	c.559+2_559+12delTGAGCAGCTGG			2045	2029	16	99.22%	0.78%	TP53
104-CF-B	4	186628552	SNV	c.4535G>A	p.Gly1512Asp	COSV71673337	1543	1537	6	99.61%	0.39%	FAT1
104-CF-B	4	186708985	SNV	c.843C>T	p.Cys281Cys		1801	1793	8	99.56%	0.44%	FAT1
104-CF-B	7	55157671	SNV	c.1216C>T	p.Leu406Leu		1569	1561	8	99.49%	0.51%	EGFR
107-CF-B	1	16135703	DEL	c.1379delC	p.Pro460fs		1834	1821	13	99.29%	0.71%	EPHA2
107-CF-B	1	152314434	DEL	c.451delA	p.Arg151fs		1781	1769	12	99.33%	0.67%	FLG

107-CF-B	4	186610010	SNV	c.9859G>A	p.Val3287Ile		1325	1320	5	99.62%	0.38%	FAT1
107-CF-B	7	55173911	DEL	c.2062-3delC			1588	1578	10	99.37%	0.63%	EGFR
107-CF-B	7	55205288	SNV	c.3304G>A	p.Ala1102Thr	COSV51804536	1866	1861	5	99.73%	0.27%	EGFR
107-CF-B	12	49028883	SNV	c.14327A>G	p.Lys4776Arg		1759	1751	8	99.55%	0.45%	KMT2D
108-CF-B	4	186628182	DEL	c.4781delA	p.Asn1594fs		1783	1770	13	99.27%	0.73%	FAT1
108-CF-B	4	186628235	SNV	c.4729G>A	p.Val1577Ile		2245	2230	15	99.33%	0.67%	FAT1
109-CF-B	7	55146623	SNV	c.442G>A	p.Val148Met	COSV51837186	1565	1554	11	99.30%	0.70%	EGFR
110-CF-B	9	21974696	SNV	c.132C>A	p.Tyr44*	COSV58689440	2042	2016	26	98.73%	1.27%	CDKN2A

110-CF-B	12	49044872	SNV	c.4835G>A	p.Arg1612His	COSV56484339	2034	2023	11	99.46%	0.54%	KMT2D
110-CF-B	17	7674229	SNV	c.734G>A	p.Gly245Asp	COSV52667838	1762	1739	23	98.69%	1.31%	TP53
113-CF-B	1	152312847	SNV	c.2039C>T	p.Ser680Leu		2086	2066	20	99.04%	0.96%	FLG
114-CF-B	4	186708192	SNV	c.1636C>T	p.Pro546Ser		1353	1347	6	99.56%	0.44%	FAT1
114-CF-B	5	177295440	SNV	c.8072C>T	p.Ala2691Val		1139	1133	6	99.47%	0.53%	NSD1
119-CF-B	1	152219442	SNV	c.2187C>T	p.His729His	COSV64256439	2362	2354	8	99.66%	0.34%	HRNR
119-CF-B	4	152326215	SNV	c.1435C>G	p.Arg479Gly	COSV55894999	1571	1561	10	99.36%	0.64%	FBXW7
119-CF-B	4	152337830	SNV	c.833G>A	p.Arg278Gln	COSV55928252	1319	1313	6	99.55%	0.45%	FBXW7

119-CF-B	9	136513393	SNV	c.2352C>T	p.Ser784Ser		1527	1521	6	99.61%	0.39%	NOTCH1
119-CF-B	12	49022063	SNV	c.16501C>T	p.Arg5501*	COSV56407191	1872	1866	6	99.68%	0.32%	KMT2D
120-CF-B	6	27866975	DEL	c.554delA	p.Lys185fs		1780	1767	13	99.27%	0.73%	H1-5
131-CF-B	10	87933074	SNV	c.315T>G	p.Cys105Trp	COSV64289216	1132	1114	18	98.41%	1.59%	PTEN
132-CF-B	5	177135095	SNV	c.-9C>T			1230	1225	5	99.59%	0.41%	NSD1
132-CF-B	5	177248246	SNV	c.4563C>T	p.His1521His		2141	2133	8	99.63%	0.37%	NSD1
132-CF-B	9	136505868	SNV	c.4028C>T	p.Ala1343Val	COSV53082167	1254	1211	43	96.57%	3.43%	NOTCH1
133-CF-B	7	55181314	SNV	c.2305G>A	p.Val769Met	COSV51802272	746	723	23	96.92%	3.08%	EGFR

133-CF-B	9	136499120	SNV	c.6074A>T	p.Asp2025Val		611	561	50	91.82%	8.18%	NOTCH1
133-CF-B	17	7675076	SNV	c.536A>G	p.His179Arg	COSV52661712	751	701	50	93.34%	6.66%	TP53
136-CF-B	1	16148923	SNV	c.278G>A	p.Arg93His	COSV64453099	1880	1874	6	99.68%	0.32%	EPHA2
136-CF-B	1	16155971	SNV	c.-39C>T			603	600	3	99.50%	0.50%	EPHA2
136-CF-B	1	152221053	SNV	c.576C>T	p.Arg192Arg		2055	2049	6	99.71%	0.29%	HRNR
136-CF-B	1	152313020	SNV	c.1866C>T	p.Ser622Ser		1738	1730	8	99.54%	0.46%	FLG
136-CF-B	2	201286626	DEL	c.*39delT			649	642	7	98.92%	1.08%	CASP8
136-CF-B	4	152326239	SNV	c.1419-8C>A			1155	1149	6	99.48%	0.52%	FBXW7

136-CF-B	4	186603840	SNV	c.10686C>T	p.Gly3562Gly	COSV101499365	1783	1777	6	99.66%	0.34%	FAT1
136-CF-B	4	186618718	SNV	c.7868C>T	p.Ala2623Val		1796	1792	4	99.78%	0.22%	FAT1
136-CF-B	4	186618842	SNV	c.7744G>A	p.Val2582Met	COSV71676110	1891	1886	5	99.74%	0.26%	FAT1
136-CF-B	5	177283902	SNV	c.6125T>C	p.Leu2042Pro		1699	1696	3	99.82%	0.18%	NSD1
136-CF-B	5	177295159	SNV	c.7791G>A	p.Gly2597Gly		1636	1627	9	99.45%	0.55%	NSD1
136-CF-B	8	90940783	SNV	c.748-3C>T			1560	1553	7	99.55%	0.45%	NECAB1
136-CF-B	10	87864242	SNV	c.-228G>A			861	857	4	99.54%	0.46%	PTEN
136-CF-B	12	49022714	SNV	c.16214G>A	p.Arg5405His	COSV56438012	1783	1776	7	99.61%	0.39%	KMT2D

142-CF-B	3	30671873	SNV	c.765G>A	p.Thr255Thr		1254	1217	37	97.05%	2.95%	TGFBR2
142-CF-B	12	49051394	SNV	c.2289G>A	p.Pro763Pro	COSV56483680	1381	1359	22	98.41%	1.59%	KMT2D
142-CF-B	17	7675234	SNV	c.378C>G	p.Tyr126*	COSV52662583	845	743	102	87.93%	12.07%	TP53
143-CF-B	9	136506571	SNV	c.3970G>A	p.Val1324Met	COSV53029836	2085	2075	10	99.52%	0.48%	NOTCH1
147-CF-B	3	179210480	SNV	c.1454A>G	p.Lys485Arg		1451	1387	64	95.59%	4.41%	PIK3CA
151-CF-B	17	7673790	SNV	c.830G>T	p.Cys277Phe	COSV52693726	1513	1401	112	92.60%	7.40%	TP53
156-CF-B	1	152220113	SNV	c.1516C>G	p.Arg506Gly		2120	2102	18	99.15%	0.85%	HRNR
156-CF-B	10	87933147	SNV	c.388C>T	p.Arg130*	COSV64288463	1812	1798	14	99.23%	0.77%	PTEN

160-CF-B	12	49032221	SNV	c.12484C>T	p.Arg4162Trp	COSV99040254	2025	2014	11	99.46%	0.54%	KMT2D
167-CF-B	1	152305091	SNV	c.9795G>A	p.Gly3265Gly		2400	2389	11	99.54%	0.46%	FLG
180-CF-B	6	27867023	DEL	c.506delA	p.Lys169fs		1123	1115	8	99.29%	0.71%	H1-5
181-CF-B	12	49042770	SNV	c.5753G>A	p.Arg1918His		1981	1971	10	99.50%	0.50%	KMT2D
21-CF-B	4	186618079	SNV	c.8507C>T	p.Ala2836Val		1583	1577	6	99.62%	0.38%	FAT1
4-CF-B	4	152346945	SNV	c.711G>A	p.Trp237*	COSV55907222	1322	1314	8	99.39%	0.61%	FBXW7
4-CF-B	4	186708247	SNV	c.1581C>T	p.Tyr527Tyr		1576	1568	8	99.49%	0.51%	FAT1
4-CF-B	12	49028883	SNV	c.14327A>G	p.Lys4776Arg		1403	1394	9	99.36%	0.64%	KMT2D

129-20190902-B	4	186603476	SNV	c.11050C>T	p.Gln3684*	COSV71675390	1472	1446	26	98.23%	1.77%	FAT1
129-20190902-B	17	7673544	INS	c.983dupT	p.Thr329fs		1678	1652	26	98.45%	1.55%	TP53
150-20191202-B	17	7674953	SNV	c.578A>T	p.His193Leu	COSV52663304	1105	1072	33	97.01%	2.99%	TP53
175-20200714-B	5	177260001	SNV	c.4979G>A	p.Arg1660His		1185	1181	4	99.66%	0.34%	NSD1
175-20200714-B	6	27867145	SNV	c.385G>A	p.Ala129Thr		1617	1609	8	99.51%	0.49%	H1-5
175-20200714-B	10	87864306	SNV	c.-164G>A			622	617	5	99.20%	0.80%	PTEN
175-20200714-B	12	49038090	DEL	c.9265delG	p.Val3089fs		1520	1508	12	99.21%	0.79%	KMT2D
175-20200714-B	14	22981291	SNV	c.976C>T	p.Arg326Trp		1108	1103	5	99.55%	0.45%	AJUBA

182-20201012-B	12	52515031	SNV	c.1684G>T	p.Gly562Trp		635	632	3	99.53%	0.47%	KRT5
184-20201110-B	3	179218303	SNV	c.1633G>A	p.Glu545Lys	COSV55873239	1364	1339	25	98.17%	1.83%	PIK3CA
184-20201110-B	7	55200350	SNV	c.2883C>T	p.Phe961Phe		1735	1720	15	99.14%	0.86%	EGFR
184-20201110-B	12	49043745	SNV	c.5357G>A	p.Arg1786His	COSV99980722	1228	1216	12	99.02%	0.98%	KMT2D
185-20201116-B	10	87925513	DEL	c.170delT	p.Leu57fs		1380	1371	9	99.35%	0.65%	PTEN
185-20201116-B	12	49049883	DEL	c.3704delG	p.Gly1235fs		1441	1429	12	99.17%	0.83%	KMT2D
186-20201116-B	1	152307653	SNV	c.7233A>C	p.Ser2411Ser		766	666	100	86.95%	13.05%	FLG
186-20201116-B	9	136517878	SNV	c.1315C>T	p.Gln439*	COSV53053276	528	490	38	92.80%	7.20%	NOTCH1

186-20201116-B	12	49032860	SNV	c.11845C>T	p.Gln3949*	COSV56425964	869	796	73	91.60%	8.40%	KMT2D
186-20201116-B	12	49044925	DEL	c.4780_4781delGA	p.Glu1594fs		722	673	49	93.21%	6.79%	KMT2D
186-20201116-B	12	49048740	SNV	c.4050G>A	p.Lys1350Lys		659	602	57	91.35%	8.65%	KMT2D
186-20201116-B	17	7674947	SNV	c.584T>C	p.Ile195Thr	COSV52664264	773	617	156	79.82%	20.18%	TP53
197-20210419-B	2	201286607	DEL	c.*32_*36delGTTTT			673	662	11	98.37%	1.63%	CASP8
203-20210621-B	17	7674872	SNV	c.659A>G	p.Tyr220Cys	COSV52661282	1692	1680	12	99.29%	0.71%	TP53
206-20210705-B	5	177269630	SNV	c.5332C>T	p.Arg1778*	COSV61785134	1610	1595	15	99.07%	0.93%	NSD1
206-20210705-B	10	87925513	DEL	c.170delT	p.Leu57fs		1460	1448	12	99.18%	0.82%	PTEN

208-20210601-B	4	186707031	SNV	c.2797C>T	p.Arg933Cys	COSV71675034	1507	1497	10	99.34%	0.66%	FAT1
208-20210601-B	12	49044253	SNV	c.5135A>G	p.Lys1712Arg		1053	1048	5	99.53%	0.47%	KMT2D
209-20210920-B	4	186636808	SNV	c.3749A>G	p.Tyr1250Cys		1765	1754	11	99.38%	0.62%	FAT1
209-20210920-B	12	49043672	SNV	c.5430A>G	p.Gly1810Gly		2130	2123	7	99.67%	0.33%	KMT2D
210-20210927-B	4	152329730	SNV	c.1178G>A	p.Arg393Gln	COSV55937382	1711	1704	7	99.59%	0.41%	FBXW7
211-20211011-B	3	179218303	SNV	c.1633G>A	p.Glu545Lys	COSV55873239	1169	1158	11	99.06%	0.94%	PIK3CA
211-20211011-B	12	49028883	SNV	c.14327A>G	p.Lys4776Arg		1309	1302	7	99.47%	0.53%	KMT2D
213-20211005-B	7	55146626	SNV	c.445C>T	p.Arg149Trp	COSV51841003	1439	1429	10	99.31%	0.69%	EGFR

216-20211025-B	17	76751055	INS	c.506dupT	p.Met169fs		1390	1348	42	96.98%	3.02%	TP53
219-20211020-B	17	76761552	DEL	c.216delC	p.Val73fs		2067	2042	25	98.79%	1.21%	TP53
226-20220207-B	1	161490 52	SNV	c.154-5C>A			1035	1023	12	98.84%	1.16%	EPHA2
226-20220207-B	1	152311563	SNV	c.3323G>T	p.Gly1108Val		1116	1105	11	99.01%	0.99%	FLG
226-20220207-B	2	201272682	SNV	c.633G>T	p.Leu211Leu		1167	1155	12	98.97%	1.03%	CASP8
226-20220207-B	2	201276909	SNV	c.920G>T	p.Arg307Leu	COSV51862120	1178	1166	12	98.98%	1.02%	CASP8
226-20220207-B	3	179219275	SNV	c.1744C>A	p.Gln582Lys		1000	990	10	99.00%	1.00%	PIK3CA
226-20220207-B	4	186596604	SNV	c.12936C>A	p.Pro4312Pro		1018	1007	11	98.92%	1.08%	FAT1

226-20220207-B	4	186618116	SNV	c.8470G>T	p.Gly2824Trp		1277	1266	11	99.14%	0.86%	FAT1
226-20220207-B	5	177135724	SNV	c.621G>T	p.Met207Ile		1072	1062	10	99.07%	0.93%	NSD1
226-20220207-B	5	177210538	SNV	c.2139G>T	p.Met713Ile	COSV100729794	1283	1270	13	98.99%	1.01%	NSD1
226-20220207-B	5	177210890	SNV	c.2491G>T	p.Gly831Trp		1279	1266	13	98.98%	1.02%	NSD1
226-20220207-B	5	177251744	SNV	c.4656G>T	p.Leu1552Phe		1053	1044	9	99.15%	0.85%	NSD1
226-20220207-B	5	177269672	SNV	c.5374G>T	p.Gly1792*		1409	1394	15	98.94%	1.06%	NSD1
226-20220207-B	5	177294014	SNV	c.6646G>T	p.Gly2216Trp		1188	1178	10	99.16%	0.84%	NSD1
226-20220207-B	6	29943346	SNV	c.422C>T	p.Ala141Val		765	743	22	97.12%	2.88%	HLA-A

226-20220207-B	7	55142353	SNV	c.156G>T	p.Gln52His		1299	1285	14	98.92%	1.08%	EGFR
226-20220207-B	9	21968148	SNV	c.*245C>A			808	797	11	98.64%	1.36%	CDKN2A
226-20220207-B	9	136500677	SNV	c.5809C>A	p.Arg1937Ser		1104	1091	13	98.82%	1.18%	NOTCH1
226-20220207-B	9	136506917	SNV	c.3700C>A	p.Arg1234Arg		1146	1130	16	98.60%	1.40%	NOTCH1
226-20220207-B	9	136517848	SNV	c.1345T>C	p.Cys449Arg		900	884	16	98.22%	1.78%	NOTCH1
226-20220207-B	11	534281	SNV	c.42G>T	p.Val14Val		1027	1012	15	98.54%	1.46%	HRAS
226-20220207-B	12	49024701	SNV	c.15929G>T	p.Gly5310Val		1078	1066	12	98.89%	1.11%	KMT2D
226-20220207-B	12	49030993	SNV	c.13571G>T	p.Arg4524Leu		1158	1147	11	99.05%	0.95%	KMT2D

226-20220207-B	12	49038383	SNV	c.8973C>A	p.Pro2991Pro		1332	1320	12	99.10%	0.90%	KMT2D
226-20220207-B	12	49038947	SNV	c.8409C>A	p.Pro2803Pro		1197	1183	14	98.83%	1.17%	KMT2D
226-20220207-B	12	49042802	SNV	c.5721G>T	p.Leu1907Leu		1249	1235	14	98.88%	1.12%	KMT2D
226-20220207-B	12	49043770	SNV	c.5332G>T	p.Gly1778Trp		923	909	14	98.48%	1.52%	KMT2D
226-20220207-B	12	49049693	SNV	c.3895C>A	p.Arg1299Ser	COSV99978343	921	911	10	98.91%	1.09%	KMT2D
226-20220207-B	12	52520212	SNV	c.85C>A	p.Arg29Ser	COSV99358360	1291	1275	16	98.76%	1.24%	KRT5
226-20220207-B	14	22981562	SNV	c.705G>T	p.Leu235Leu		1231	1215	16	98.70%	1.30%	AJUBA
226-20220207-B	16	67611112	SNV	c.280C>A	p.Gln94Lys		1072	1057	15	98.60%	1.40%	CTCF

226-20220207-B	17	7669573	SNV	c.*36C>A			733	723	10	98.64%	1.36%	TP53
227-20220211-B	1	152304752	SNV	c.10134A>G	p.Gly3378Gly		1576	1568	8	99.49%	0.51%	FLG
227-20220211-B	1	152313643	SNV	c.1243C>T	p.Arg415Trp	COSV100912235	1773	1765	8	99.55%	0.45%	FLG
227-20220211-B	4	186604404	SNV	c.10521G>A	p.Glu3507Glu		1206	1198	8	99.34%	0.66%	FAT1
227-20220211-B	12	49028883	SNV	c.14327A>G	p.Lys4776Arg		1593	1590	3	99.81%	0.19%	KMT2D
112-20190527-B	14	22981821	DEL	c.445delG	p.Ala149fs		1093	1052	41	96.25%	3.75%	AJUBA
112-20190527-B	17	7674229	SNV	c.734G>T	p.Gly245Val	COSV52666323	1462	1418	44	96.99%	3.01%	TP53

Table VI-6: List and characteristics of variants found at baseline

VI.2Supplementary data for chapter IV

Supplementary material from *Tumor-agnostic plasma assay for circulating tumor DNA predicts outcome in recurrent and/or metastatic squamous cell carcinoma of the head and neck treated with PD1 inhibitor.*

Material & Methods 1

HPV In-situ Hybridization

HPV-driven tumors were evaluated using in-situ hybridization for high-risk human papilloma viruses (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66) (Ventana ISH iView Blue Plus Detection Kit). Staining was performed on a 3µm slide, using a Ventana-Roche GX autostainer (Roche Diagnosis, Basel, Switzerland). The presence of integrated HPV DNA in the nucleus of tumor cells was evaluated by an expert pathologist.

Figures

Figure VI-1 A: Swimmer plot where each row = one patient, salmon line = duration of PD1 inhibitor treatment, blue dot: baseline sample, darkblue dot: first on-treatment plasma sample (FU1), black dot: other on-treatment plasma samples, green dot: last plasma sample without disease progression, red dot: plasma sample at progression.

Figure VI-2 B: Kaplan-Meier estimate of progression-free survival for patients harboring mutations in the Notch/Hedgehog/Wnt pathway or not. Blue curves = Wild-type for Notch/Hedgehog/Wnt; pink curves = positive for mutations in the Notch/Hedgehog/Wnt pathways.

Figure VI-3 C: Kaplan-Meier estimate of overall survival for patients harboring mutations in the Notch/Hedgehog/Wnt pathway or not. Blue curves = Wild-type for Notch/Hedgehog/Wnt; pink curves = positive for mutations in the Notch/Hedgehog/Wnt pathways.

Figure VI-4 D: Evolution of the variant allele frequency of mutations at different timepoints and according to response to treatment.

A

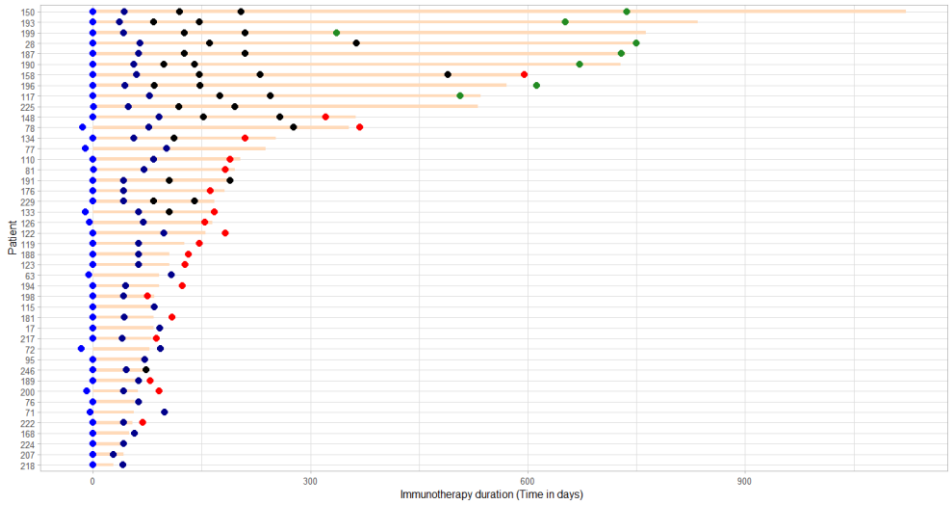

Figure VI-1: Swimmer plot for all samples

B

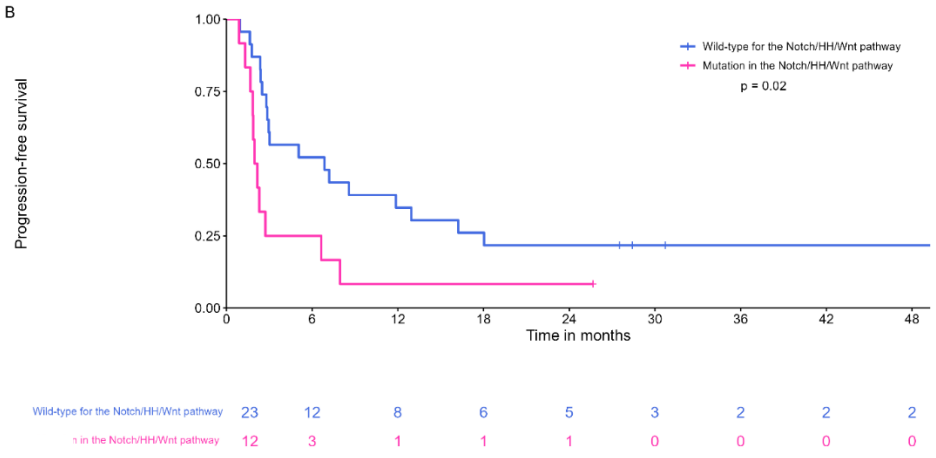

Figure VI-2: Kaplan-Meier estimate of progression-free survival for patients harboring mutations in the Notch/Hedgehog/Wnt pathway

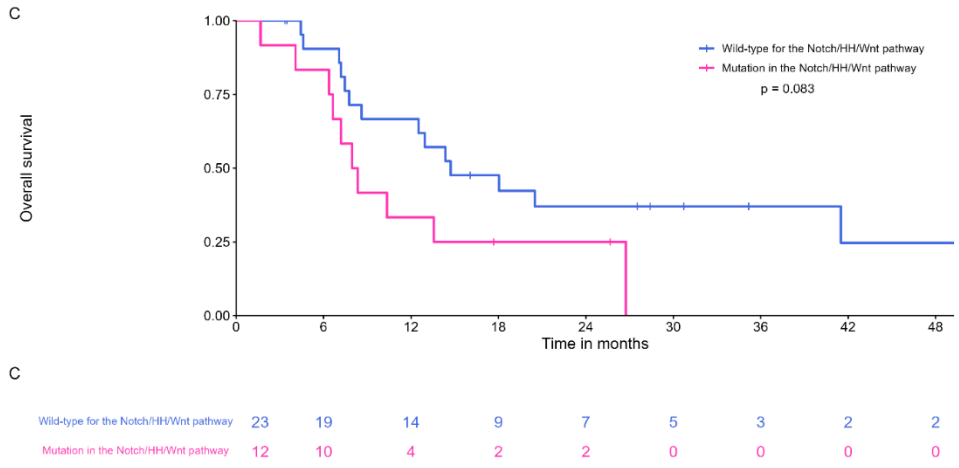

Figure VI-3: Kaplan-Meier estimate of overall survival for patients harboring mutations in the Notch/Hedgehog/Wnt pathway

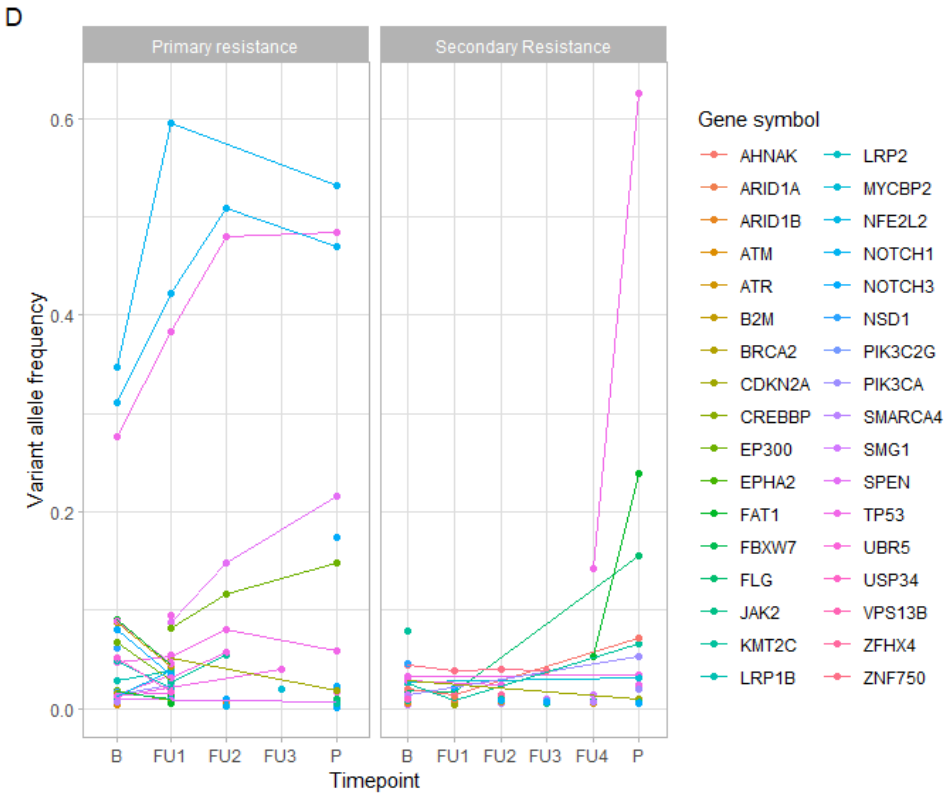

Figure VI-4: : Evolution of the variant allele frequency of mutations at different timepoints and according to response to treatment

Tables

Sample	Patient	Date	Timepoint	Concentration (ng/µL)	Quantity (ng)
110-1542020-B	110	15-04-20	B	1.5689	67.4627
110-21102020-P	110	21-10-20	P	2.7378	117.7254
110-872020-FU1	110	08-07-20	FU1	1.3143	56.5149
115-2122020-FU1	115	21-02-20	FU1	5.3584	230.4112
115-28112019-B	115	28-11-19	B	3.0039	129.1677
117-1622021-FU3	117	16-02-21	FU3	6.584	283.112
117-1662020-B	117	16-06-20	B	2.9376	126.3168
117-292020-FU1	117	02-09-20	FU1	0.7125	30.6375
117-5112021-END	117	05-11-21	END	0.8938	38.4334
117-8122020-FU2	117	08-12-20	FU2	0.6985	30.0355
119-172020-B	119	01-07-20	B	0.5289	22.7427
119-25112020-P	119	25-11-20	P	2.5915	111.4345
119-292020-FU1	119	02-09-20	FU1	0.8094	34.8042
122-3012020-P	122	30-01-20	P	10.0336	431.4448
122-310719-B	122	31-07-19	B	9.5969	412.6667
122-6112019-FU1	122	06-11-19	FU1	9.7351	418.6093
123-1662020-B	123	16-06-20	B	0.4632	19.9176
123-1882020-FU1	123	18-08-20	FU1	0.9917	42.6431
123-21102020-P	123	21-10-20	P	0.7408	31.8544
126-1262019-B	126	12-06-19	B	15.889	683.227

126-18112019-P	126	18-11-19	P	4.8946	210.4678
126-2682019-FU1	126	26-08-19	FU1	3.0065	129.2795
133-2552020-FU1	133	25-05-20	FU1	2.6835	115.3905
133-672020-FU2	133	06-07-20	FU2	1.1093	47.6999
133-792020-FU-P	133	07-09-20	P	2.9243	125.7449
134-1252020-B	134	12-05-20	B	1.2154	52.2622
134-192020-FU2	134	01-09-20	FU2	1.9137	82.2891
134-772020-FU1	134	07-07-20	FU1	1.2301	52.8943
134-8122020-P	134	08-12-20	P	1.6908	72.7044
148-1422020-B	148	14-02-20	B	1.1791	50.7013
148-1552020-FU1	148	15-05-20	FU1	0.7788	33.4884
148-1672020-FU2	148	16-07-20	FU2	1.7263	74.2309
148-291020-FU3	148	29-10-20	FU3	1.3327	57.3061
148-31122020-P	148	31-12-20	P	0.7429	31.9447
150-151220-FU3	150	15-12-20	FU3	0.8393	36.0899
150-162022-2Y	150	01-06-22	2Y	0.7543	32.4349
150-2292020-FU2	150	22-09-20	FU2	0.6563	28.2209
150-2552020-B	150	25-05-20	B	0.9586	41.2198
150-772020-FU1	150	07-07-20	FU1	0.8139	34.9977
158-1022020-B	158	10-02-20	B	0.3964	17.0452
158-1042020-FU1	158	10-04-20	FU1	0.3976	17.0968
158-1462021-FU4	158	14-06-21	FU4	1.881	80.883

158-2792021-P	158	27-09-21	P	1.5465	66.4995
158-2892020-FU3	158	28-09-20	FU3	0.391	16.813
158-672020-FU2	158	06-07-20	FU2	0.2705	11.6315
168-1252020-FU1	168	12-05-20	FU1	1.6838	72.4034
168-1632020-B	168	16-03-20	B	0.576	24.768
17-20171107-B-ICI	17	07-11-17	B	2.0796	120.6168
17-20180207-FU1-ICI	17	07-02-18	FU1	1.657	96.106
176-20200720-B-ICI	176	20-07-20	B	1.5747	91.3326
176-20200831-FU1-ICI	176	31-08-20	FU1	0.9749	56.5442
176-29122020-P	176	29-12-20	P	0.8423	36.2189
181-1242021-P	181	12-04-21	P	0.8341	35.8663
181-20201224-B-ICI	181	24-12-20	B	1.0156	58.9048
181-20210205-FU1-ICI	181	05-02-21	FU1	0.7212	41.8296
183-20201105-B-ICI	183	05-11-20	B	1.0091	58.5278
183-20201229-FU1-ICI	183	29-12-20	FU1	0.9704	56.2832
187-172021-FU3	187	01-07-21	FU3	0.5999	25.7957
187-2122022-END	187	02-12-22	END	0.6099	26.2257
187-3122020-B	187	03-12-20	B	1.1568	49.7424
187-422021-FU1	187	04-02-21	FU1	0.6818	29.3174
187-842021-FU2	187	08-04-21	FU2	0.5844	25.1292
188-22122020-B	188	22-12-20	B	0.9936	42.7248
188-2322021-FU1	188	23-02-21	FU1	3.635	156.305

188-352021-FU-P	188	03-05-21	P	0.7288	31.3384
189-1232021-P	189	12-03-21	P	2.9695	127.6885
189-20201223-B-ICI	189	23-12-20	B	2.3045	133.661
189-20210224-FU1-ICI	189	24-02-21	FU1	2.1377	123.9866
190-1122021-B	190	11-02-21	B	2.5085	107.8655
190-151222-END	190	15-12-22	END	1.2145	52.2235
190-172021-FU3	190	01-07-21	FU3	2.6947	115.8721
190-2052021-FU2	190	20-05-21	FU2	1.2585	54.1155
190-842021-FU1	190	08-04-21	FU1	1.0609	45.6187
191-1762021-FU2	191	17-06-21	FU2	3.3356	143.4308
191-20210304-B-ICI	191	04-03-21	B	5.2109	302.2322
191-20210415-FU1-ICI	191	15-04-21	FU1	2.833	164.314
191-992021-FU3	191	09-09-21	FU3	3.5573	152.9639
193-1032021-B	193	10-03-21	B	4.0587	174.5241
193-1642021-FU1	193	16-04-21	FU1	3.4082	146.5526
193-221222-END	193	22-12-22	END	2.5113	107.9859
193-262021-FU2	193	02-06-21	FU2	0.9089	39.0827
193-482021-FU3	193	04-08-21	FU3	1.2376	53.2168
194-1372021-P	194	13-07-21	P	1.1602	49.8886
194-20210312-B-ICI	194	12-03-21	B	0.498	28.884
194-20210426-FU1-ICI	194	26-04-21	FU1	0.5345	31.001
196-1242021-B	196	12-04-21	B	2.0118	86.5074

196-151222-END	196	15-12-22	END	1.0336	44.4448
196-2652021-FU1	196	26-05-21	FU1	1.5357	66.0351
196-672021-FU2	196	06-07-21	FU2	0.8508	36.5844
196-792021-FU3	196	07-09-21	FU3	1.1513	49.5059
198-20210503-B-ICI	198	03-05-21	B	1.7052	98.9016
198-20210614-FU1-ICI	198	14-06-21	FU1	3.9188	227.2904
198-2772021-P	198	17-07-21	P	2.8475	122.4425
199-1762021-FU1	199	17-06-21	FU1	0.6377	27.4211
199-2122021-FU3	199	02-12-21	FU3	0.4928	21.1904
199-652021-B	199	06-05-21	B	0.7649	32.8907
199-742022-END	199	07-04-22	END	1.6356	70.3308
199-992021-FU2	199	09-09-21	FU2	1.0172	43.7396
200-20210525-B-ICI	200	25-05-21	B	2.0422	118.4476
200-20210715-FU1-ICI	200	15-07-21	FU1	1.2831	74.4198
200-292021-FU-P	200	02-09-21	P	8.9965	386.8495
207-2792021-FU1	207	27-09-21	FU1	2.0287	87.2341
207-3082021-B	207	30-08-21	B	1.6291	70.0513
217-1712022-P	217	17-01-22	P	0.7572	32.5596
217-20211021-B-ICI	217	21-10-21	B	0.6091	35.3278
217-20211130-FU1-ICI	217	30-11-21	FU1	0.5556	32.2248
218-20220104-B-ICI	218	04-01-22	B	0.9556	55.4248
218-20220214-FU1-ICI	218	14-02-22	FU1	0.9156	53.1048

220-20211213-FU1-ICI	220	13-12-22	FU1	2.1724	125.9992
220-20220131-FU1-ICI	220	31-01-22	FU1	0.4155	24.099
222-15122021-B	222	15-12-21	B	1.0601	45.5843
222-2222022-P	222	22-02-22	P	11.5072	494.8096
222-2612022-FU1	222	26-01-22	FU1	2.5191	108.3213
224-20122021-B	224	20-12-21	B	0.1535	6.6005
224-3112022-FU1	224	31-01-22	FU1	0.3823	16.4389
225-2042022-FU2	225	20-04-22	FU2	1.0072	43.3096
225-23122021-B	225	23-12-21	B	2.6438	113.6834
225-672022-FU3	225	06-07-22	FU3	1.3021	55.9903
225-922022-FU1	225	09-02-22	FU1	0.4548	19.5564
229-121022-FU2	229	12-10-22	FU2	0.5581	23.9983
229-2072022-B	229	20-07-22	B	0.8447	36.3221
229-3182022-FU1	229	31-08-22	FU1	0.6241	26.8363
229-7122022-FU3	229	07-12-22	FU3	0.4	17.2
246-111222-FU2	246	11-12-22	FU2	1.0484	45.0812
246-141122-FU1	246	14-11-22	FU1	0.7439	31.9877
246-2992022-B	246	29-09-22	B	1.5026	64.6118
28-1672020-END	28	16-07-20	END	0.6163	26.5009
28-20180627-B-ICI	28	27-06-18	B	0.2788	16.1704
28-20180831-FU1-ICI	28	31-08-18	FU1	0.1427	8.2766
28-2662019-FU4	28	26-06-19	FU4	0.3996	17.1828

28-5122018-FU3	28	05-12-18	FU3	0.6302	27.0986
63-20170818-B-ICI	63	18-08-17	B	0.6351	36.8358
63-20171201-FU1-ICI	63	10-12-17	FU1	1.433	83.114
71-20180126-B-ICI	71	26-01-18	B	0.445	25.81
71-20180509-FU1-ICI	71	09-05-18	FU1	1.3144	76.2352
72-20180423-B-ICI	72	23-04-18	B	4.4086	255.6988
72-20180810-FU1-ICI	72	10-08-18	FU1	18.0679	1047.9382
76-20180426-B-ICI	76	26-04-18	B	0.4372	25.3576
76-20180628-FU1-ICI	76	28-06-18	FU1	0.7926	45.9708
77-20190222-B-ICI	77	22-02-19	B	0.7507	43.5406
77-20190614-FU1-ICI	77	14-06-19	FU1	0.8317	48.2386
78-1942019-FU4	78	19-04-19	FU4	0.4895	21.0485
78-1972019-FU-P	78	19-07-19	P	0.354	15.222
78-20180702-B-ICI	78	02-07-18	B	0.5133	29.7714
78-20181001-FU1-ICI	78	01-10-18	FU1	0.514	29.812
81-20180725-B-ICI	81	25-07-18	B	1.2863	74.6054
81-20181003-FU1-ICI	81	03-10-18	FU1	0.9969	57.8202
81-2312019-FU-P	81	23-01-19	P	0.7808	33.5744
95-2032019-B	95	20-03-19	B	19.1537	823.6091
95-3152019-FU1	95	31-05-19	FU1	4.6096	198.2128

Table VI-7: Sample characteristics

Gene Symbol	Genome
TP53	Human (GrH38)
PIK3CA	Human (GrH38)
LRP1B	Human (GrH38)
FAT1	Human (GrH38)
CDKN2A	Human (GrH38)
ZFHX4	Human (GrH38)
CREBBP	Human (GrH38)
KMT2C	Human (GrH38)
NOTCH1	Human (GrH38)
EP300	Human (GrH38)
MYCBP2	Human (GrH38)
AHNAK	Human (GrH38)
LRP2	Human (GrH38)
ZNF750	Human (GrH38)
VPS13B	Human (GrH38)
SMG1	Human (GrH38)
UBR5	Human (GrH38)
NSD1	Human (GrH38)
USP34	Human (GrH38)

ATM	Human (GrH38)
JAK2	Human (GrH38)
B2M	Human (GrH38)
EPHA2	Human (GrH38)
ARID1A	Human (GrH38)
PTEN	Human (GrH38)
SMARCA4	Human (GrH38)
BRCA2	Human (GrH38)
NFE2L2	Human (GrH38)
SPEN	Human (GrH38)
NOTCH3	Human (GrH38)
FBXW7	Human (GrH38)
CASP8	Human (GrH38)
PIK3C2G	Human (GrH38)
ARID1B	Human (GrH38)
ATR	Human (GrH38)
FLG	Human (GrH38)
TERT Promotor	Human (GrH38)
E6	Human Papillomavirus type 16
E7	Human Papillomavirus type 16

Table VI-8: Panel of selected genes

Elimination of common variants	
exac_af ≤ 0.01	Elimination of common variants from the Exac database
gnomad_wes_popmax_af ≤ 0.01	Elimination of common variants from de Gnomad database
Quality filters from Mutect2	
filters ≈ BASE_QUAL	Selection of quality filters based on MUTECT2[375]
filters ≈ MAP_QUAL	
filters ≈ WEAK_EVIDENCE	
filters ≈ CONTAMINATION	
filters ≈ NORMAL_ARTIFACT	
filters ≈ STRAND_BIAS	
Sequencing quality filters	
alternative_f1r2 ≠ 0	Selection of variants with at least 1 read forward and backward
alternative_f2r1 ≠ 0	
Quality of variant selected	
snpeff_effect ≠ INTRON	Selection of variants present in good sequencing locus, supported by at least 3 reads. Elimination of deletion larger than the sequencing (2x100bp) and variants in non-coding regions
length ≤ 100	
allelic_depth_alt ≥ 3	
read_depth ≥ 500	

Table VI-9: List of criteria for selecting variants

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