# Personalized biomarker-based treatment strategy in patients with recurrent/metastatic squamous cell carcinoma of the head and neck

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| | | | |
|---|---|---|---|
| 5-FU | 5-fluorouracil | dMMR | deficient mismatch repair |
| AACR | American Association for Cancer Research | dPCR | Digital PCR |
| GENIE | Genomics Evidence Neoplasia Information Exchange | ECCO | European CanCer Organisation |
| ACE | Areca nut extract | ECOG | Eastern Cooperative Oncology Group |
| aCGH | Comparative genomic hybridization | EDNRB | Endothelin receptor type B |
| AE | Adverse event | EGFR | Epdermal growth factor receptor |
| ATR | ataxia telangiectasia and Rad3-related protein | EMA | European Medical Agency |
| BEAMing | Bead-emulsion-amplification-magnetics | EORTC | European organization of research and treatment of cancer |
| BER | Base excision repair | ESMO | European Society of Medical Oncology |
| BRCA1/2 | Breast Cancer 1/2 | EVS | extracellular vesicles |
| CAPP-seq | Personalized profiling by deep sequencing | FADD | Fas-associated protein with death domain |
| CCND1 | Cyclin D1 | FDA | Food and drug administration |
| CCNDE1-2 | Cyclin E1-2 | FDG | fluorodeoxyglucose |
| CDK | Cyclin-dependent kinase | FFPE | Formalin-fixed paraffin-embedded |
| CDKN2A | cyclin-dependent kinase Inhibitor 2A | FGFR | Fibroblast growth factor receptor |
| CDx | Companion diagnostic assay | FGFR | Fibroblast growth factor receptor |
| cfDNA | cell-free DNA | FHIT | Fragile Histidine Triad Diadenosine Triphosphatase |
| CI | Confidence interval | GSP | glutathione S-transferase |
| CNA | Copy number alterations | HER | Human epidermal growth factor receptor |
| COSMIC | Catalogue of Somatic Mutations in Cancer | HLA-E | Human leukocyte antigen |
| CPS | Combined positive score | HNC | Head and neck cancer |
| CRT | Chemoradiotherapy | HNCG | Head and neck cancer group |
| CTC | Circulating tumor cells | HPV | Human papillomavirus |
| ctDNA | circulating tumor DNA | HR | Homologous recombination |
| CYP2E1 | Cytochrome P450 2E1 | HRD | Homologous recombination deficiency |
| DCC | Deleted in colorectal carcinoma | ICI | Immune checkpoint inhibitors |
| ddPCR | Digital droplet PCR | iDES | Integrated digital error suppression |
| DDR2 | discoidin domain receptor tyrosine kinase 2 | IHC | immunochemistry |

| | | | |
|---|---|---|---|
| IMRT | Intensity-modulated radiotherapy | PFS | Progression-free survival |
| IO | Immunotherapy | PI3K | phosphoinositide-3-kinase |
| JAK | Janus kinase | PIK3CA | Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha |
| LA-SCCHN | loco-regionally advanced SCCHN | PTEN | phosphatase and tensin homolog tumour suppressor protein |
| LB | Liquid biopsy | QoL | Quality of life |
| LOH | Loss of heterozygosity | R/M | Recurrent/metastatic |
| LRC | locoregional control | Rb | Retinoblastoma |
| mAb | monoclonal antibody | RT | Radiotherapy |
| MAPK | Ras/Raf/mitogen-activated protein kinase | RTOG | Radiation Therapy Oncology Group |
| MRD | Minimal residual disease | Safe-SeqS | Safe-sequencing system |
| MSI-H | microsatellite instability-high | SCC | Squamous cell carcinoma |
| mTOR | mammalian target of rapamycin | SCCHN | Squamous cell carcinoma of the head and neck |
| NCI | National Cancer Institute | SD | Stable disease |
| NER | Nucleotide excision repair | SEER | Surveillance, Epidemiology, and End Results |
| NGS | Next-generation sequencing | SEPT9 | Septin 9 |
| NK | Natural killer | SHOX2 | Short stature homeobox 2 |
| NKG2A | Natural killer group 2A | STAT3 | signal transducer and activator of transcription 3 |
| NSCLC | non-small cell lung cancer | TCGA | The cancer genome atlas |
| NTRK | Neurotrophic tyrosine receptor kinase | TKI | Tyrosine kinase inhibitor |
| OPC | Oropharyngeal cancer | TLM | transoral laser microsurgery |
| OPSCC | oropharyngeal squamous cell carcinoma | TMB | Tumor mutational burden |
| ORR | Objective response rate | TORS | transoral robotic surgery |
| OS | Overall survival | TRK | tropomyosin-related-kinase |
| PARP | Poly ADP ribose polymerase | UDPGT | uridine diphosphate-glucuronosyl transferase |
| PCR | Polymerase chain reaction | UMI | unique molecular identifiers |
| PD | Progressive disease | VEGF | Vascular endothelial growth factor |
| PD-1 | Programmed cell death-1 | VMAT | Volumetric modulated arc therapy |
| PD-L1 | Programmed death-ligand 1 | | |
| PET | positron emission tomography | | |

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# 1. INTRODUCTION

## 1.1 Squamous cell carcinoma of the head and neck (SCCHN)

Head and neck cancer (HNC) is a broad term that covers 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] Globally, HNC is the fifth most common malignancy with 887 659 new cases reported worldwide in 2018, accounting for 4.9% of all cancers.[2]

While HNC has different histological subtypes, 90% are derived from squamous cells.[3] Squamous cell carcinomas arise from the epithelial cells that line the mucosal surfaces of different anatomical subsites in the head and neck region (Figure 1-1).

This thesis focuses on squamous cell carcinomas of the oral cavity, oropharynx, hypopharynx, and larynx (SCCHN).

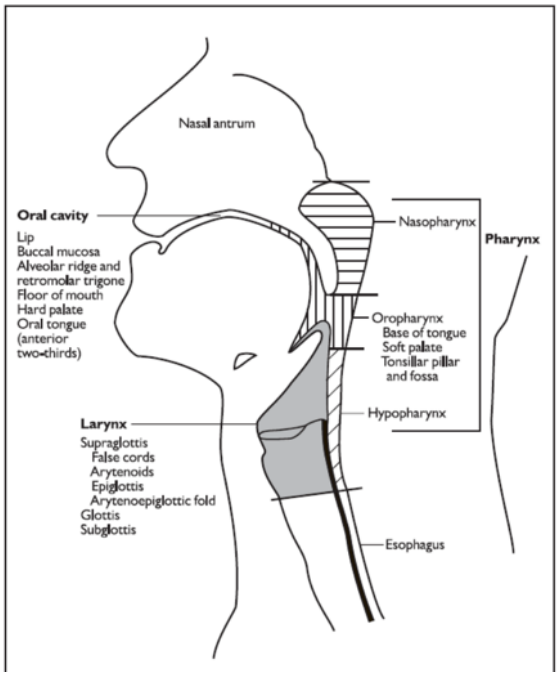


**Figure 1-1. Anatomical subsites of SCCHN**

Treatment for SCCHN depends on the location of the primary tumour, the stage of the disease, and the expected oncological and functional outcomes.[4] Early stage SCCHN is usually treated with one single treatment modality (surgery or radiotherapy (RT)); however, approximately 60% to 80% of SCCHN patients presents with 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 (PD-1) monoclonal antibodies (pembrolizumab or nivolumab), and/or cetuximab).

### 1.1.1 Epidemiology

In 2018, 705 781 cases of SCCHN were reported worldwide, ranking it as the seventh most common cancer.[2] SCCHN is responsible for 3.7 % of all cancers deaths.[2] The incidence of SCCHN varies according to geographical area. In the United States, more than 54 000 new cases were diagnosed in 2018, resulting in an annual incidence of 15 per 100 000, with 12 345 deaths attributed to the disease. In Europe, the incidence and mortality rates of SCCHN are higher, with approximately 145 000 new cases diagnosed in 2018, corresponding to an annual incidence of 43/100 000.[2] Oral cavity cancer is the most common subsite (40%), followed by laryngeal (20%).[2] SCCHN is more frequent overall in males than females, and the ratio ranges from 2:1 to 4:1 depending on the anatomical subsite. 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 (Figure 1-2), reflecting declining smoking habits. In 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 behaviours.

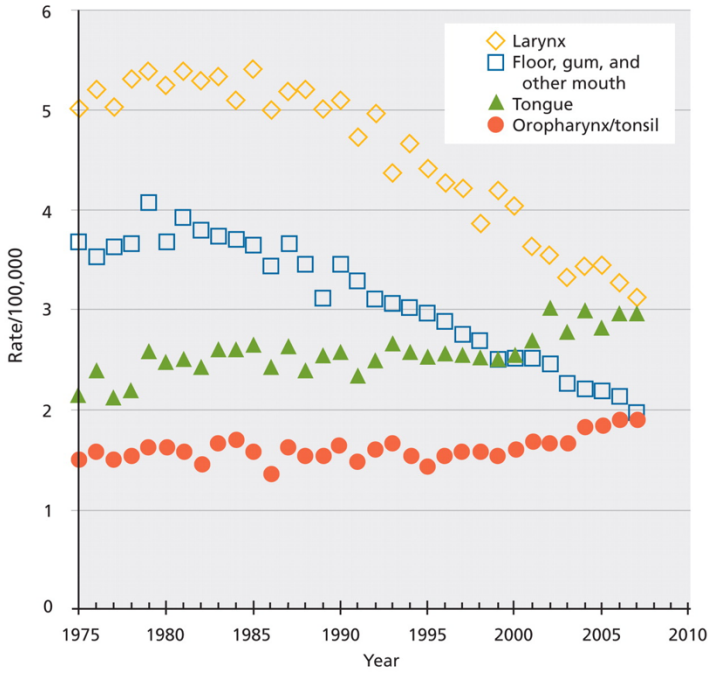


**Figure 1-2. Age-adjusted SEER incidence rates for laryngeal cancers, floor of mouth/gingival/other mouth cancers, tongue cancers, and oropharyngeal cancers in the United States.**

### 1.1.2 Risk factors

Cancer is generally caused by a progressive accumulation of genomic modifications leading to the inactivation of tumour suppressor genes or the activation of proto-oncogenes, the latter of which express proteins that are altered in quantity or quality.[5-9]

Tobacco use and alcohol consumption are the main risk factors associated with SCCHN, accounting for approximatively 70% of all cases.[10,11] The human papillomavirus (HPV) is another risk factor essentially for oropharyngeal cancer.[12] The prevalence of SCCHN attributable to HPV varies widely across the globe but is estimated to be around 30%.[12-14] HPV-positive oropharyngeal cancer patients have

a significantly better outcome with greater responses to chemotherapy and RT than patients diagnosed with HPV-negative disease.[15-18]

#### 1.1.2.1 Tobacco and alcohol

Acting synergistically, tobacco use and alcohol 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.[19] Benzopyrene, the component of tobacco smoke, is a well-known carcinogen that forms covalent DNA adducts after metabolic activation and induces mutations.[20] Acetaldehyde, the first metabolite of ethanol, is also mutagenic and it interferes with DNA repair machinery. However, not all smokers or drinkers will develop cancer. Genetic polymorphisms in xenobiotic-metabolizing-enzymes have been described and these determine an individual's susceptibility to carcinogens.[21] 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.[21,22] Similarly, detoxifying enzymes, such as glutathione S-transferase (GSP) or uridine diphosphate-glucuronosyl transferase (UDPGT), can deactivate some carcinogens but their protective activity can be decreased by polymorphisms.[23] 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.[21,24,25]

Compared to non-smokers, tobacco users have 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.[26,27] Alcohol also increases the risk of upper aerodigestive tract cancer.[28] Chronic alcohol exposure results in increased activation of carcinogens by cytochrome P450 2E1 (CYP2E1).[29] On the other hand, the accumulation of acetaldehyde and other alcohol metabolites could affect gene transcription or have mutagenic effects.[23]

#### 1.1.2.2 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. HPV-positive SCCHN arises mostly in deep tonsillar crypts and the base of the tongue (oropharynx).[30,31] HPV is a well-established independent risk factor, accounting for 20 - 60% of all oropharyngeal cancers (OPC). In North America, 56% of oropharyngeal SCCHN are HPV-positive, followed by 52% in Japan, 45% in Australia, 39% in northern and western Europe and 13% in the rest of the world.[13] The incidence of HPV-related OPC has increased over the last decades and this 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.[32] 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 with a smaller primary tumor associated with multiple cystic lymph nodes.[16,32,33] HPV-associated oropharyngeal cancer is associated with a favorable prognosis compared to HPV-negative tumors.[34] The signification of HPV infection in subsites other than oropharynx is unknown and controversial.

#### 1.1.2.3 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] Areca nut extract (ANE), the major component of betel quid, has been documented to induce negative oxygen species and to consequently cause genetic damage.[36] ANE usage is tightly linked to oral cancer. However, the mechanisms of carcinogenesis remain unclear.[37]

Other reported risk factors include: Plummer–Vinson syndrome,[38-40] long-term immunosuppression,[41] gastro-esophageal reflux,[42,43] poor oral hygiene,[44-47] and ill-fitting dentures.[48,49] 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.[50] Fanconi's

anemia,[51,52] Dyskeratosis congenital,[53,54] Bloom's syndrome,[55] ataxia telangiectasia,[56] Li-Fraumeni syndrome [57] and xeroderma pigmentosum[58] are inherited causes of head and neck cancer.

### 1.1.3 Prognostic factors

In early stage SCCHN (stage I/II), there is a high cure rate of 80-90% with surgery and/or RT;[59,60] however, more than 50% of patients present with locally-advanced disease (stage III/IV).[61] Despite multimodal treatment of locally-advanced SCCHN with combined surgery and/or (chemo)RT, less than 60% of patients will remain disease-free at three years.[62] Patients with recurrent/metastatic disease that are not amenable to RT or surgery have a median survival of 12-15 months. Prognosis depends on different factors including tumor-related factors (staging, location, HPV status, molecular markers), patient-related factors (age, toxicity habits, comorbidities) and treatment related factors (surgical margins, response to chemotherapy/RT). Risk stratification is required to determine the optimal treatment modalities and prognosis.

#### 1.1.3.1 Tumor-related factors

The most important prognostic factor is tumor staging based on TNM classification.[63] 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 primordial 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%).[61] Malignancy grade,[64] perineural invasion and vascular invasion have also been reported as being related to poor outcome.[65]

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. 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, greater or less than 10 pack-years of tobacco smoking, tumor stage, and nodal stage.[15] HPV-status has been implemented in the new 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 are not yet standard of care.[66-68] There is sufficient evidence to strongly recommend that future clinical trials, including those that evaluate 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.

Some molecular markers are also known to influence patient outcomes. *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 worst outcome.[69,70] 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.[71] Upregulation of Cyclin D1 (encoded by *CCND1*), a protein regulating the G1 phase of the cell cycle, is also linked to poor prognosis,[72,73] although some studies suggest that Cyclin D1 overexpression may increase a patient's sensitivity to definitive radiation.[74,75] Finally, epidermal growth factor receptor (EGFR) protein overexpression, or increased *EGFR* gene copy number, is associated with radioresistance and poor prognosis.[76-78]

#### 1.1.3.2 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. In contrast, advanced age, presence of comorbidities,[79] poor performance

status, active drinking and/or smoking[80,81] and malnutrition or weight loss [82] have been reported to be predictive of worse outcome.

#### 1.1.3.3 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.[83] In one study, the presence of positive margins was found to be an independent predictor of poor survival,[84] although this was not confirmed in other reports. Positive surgical margins are an indication for adjuvant RT combined with chemotherapy (CRT).[4] 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 linked to favorable prognosis.[85]

### 1.1.4 Carcinogenesis and molecular landscape of SCCHN

Carcinogenesis is the biological process by which a normal cell is transformed into a cancer cell. It is characterized by the 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 genetic alterations differ (Figure 1-3). Klussmann and colleagues described the genetic differences between HPV-related and -unrelated tumors of the oropharynx.[86] They found that chromosomal alterations and amplifications were significantly lower in HPV-positive, as compared with HPV-negative, OPC. The molecular landscape of 279 SCCHN was published by the The Cancer Genome Atlas (TCGA) consortium in 2015, and it included both HPV-negative and HPV-positive tumors.[87] 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 1-4 illustrates the most frequent alterations in the driver genes of

HPV-negative and HPV-positive SCCHN that will be discussed more in details in the following paragraphs.

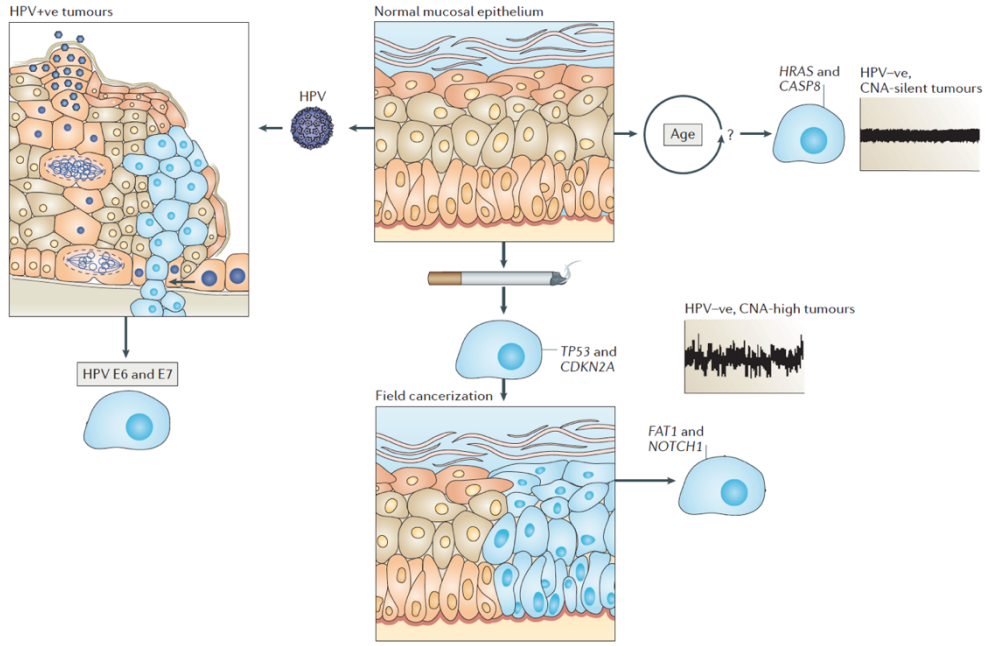


**Figure 1-3. Carcinogenesis models of SCCHN**

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 not of the of 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 seems to be ageing. These tumors harbor *HRAS* and *CASP8* mutations. More details are indicated in the main text. Figure adapted from Leemans and colleagues.[88]

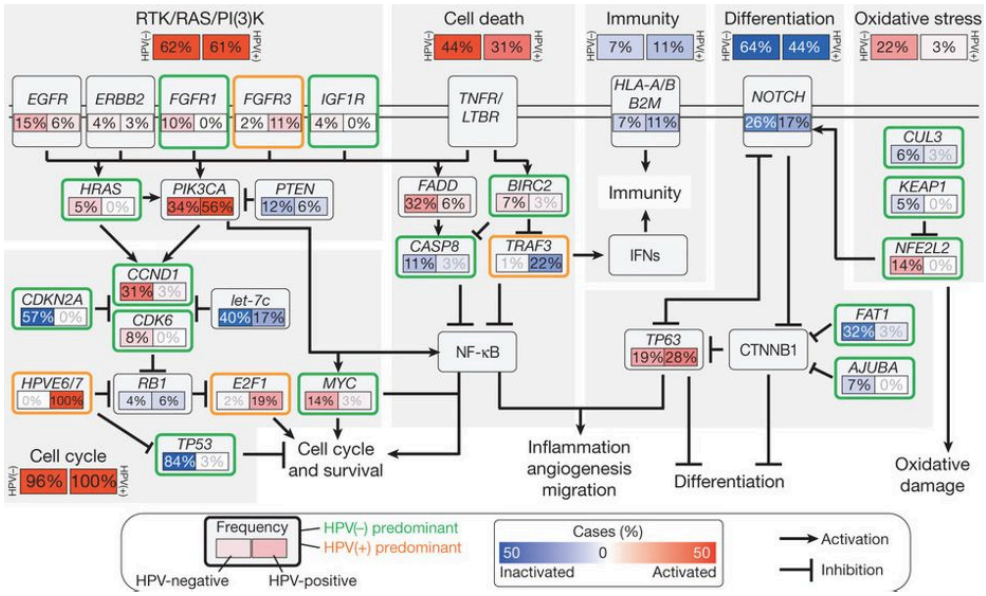


**Figure 1-4. The frequency (%) of alterations in the driver genes of HPV-negative and HPV-positive SCCHN**

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 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.[87]

#### 1.1.4.1 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.[89] The majority of patients will 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).[90] 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.[91] 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.[92] 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,[93-95] indicating that they are early events in HNC carcinogenesis. *FHIT* and *CDKN2A* are located at the 3p14.3 and 9p21 loci, respectively, and are tumour suppressor genes. These genomic alterations were also observed in the mucosal epithelial cells of surgical margins, which explains the high recurrence rate.[96,97] *TP53* is a tumour 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.[87]

Changes occurring later in carcinogenesis include the amplification of chromosomal bands 11q and 3q.[98] The frequently amplified 11q13 region includes the *CCND1*, *CTTN* and Fas-associated protein with death domain (*FADD*) genes, resulting in cell cycle deregulation and migration.[99] One of the genes located at 3q23 is the ataxia telangiectasia and Rad3-related protein (*ATR*).[100] This gene induces differentiation and aneuploidy and eliminates radiation-induced $G_1$ arrest.[100] Another important region for cancer growth is the 3q26.3-qter region. This region amplifies, among others, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA)*, a key molecular mediator implicated in many of the downstream signals from tyrosine kinase membrane receptors, including the EGFR.[101,102] The most frequently altered pathways are discussed hereunder.

A. Tyrosine kinase receptors

Gene alteration of several growth factor receptors and their downstream cascade-associated proteins has been reported in HPV-negative SCCHN.[87] EGFR is a member of the transmembrane tyrosine kinase human epidermal receptor (HER) family, which also includes HER2 (ErbB2), ErbB3 and ErbB4.[103] Binding of ligands to EGFR results in its homo- or heterodimerization with other HER receptors, subsequent autophosphorylation, and activation of downstream mitogenic signaling cascades

including the Ras/Raf/mitogen-activated protein kinase (MAPK), the phosphoinositide-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), and the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathways.[104,105] EGFR overexpression is an early marker of SCCHN carcinogenesis, supporting its role as a driver gene.[106] EGFR overexpression is observed in 90% of SCCHN and results mainly from increased mRNA synthesis and, to a minor extent, *EGFR* amplification (10 - 30%).[106] EGFR overexpression and a high number of *EGFR* gene copy numbers are associated with poor prognosis.[107]

MET is a receptor tyrosine kinase able to stimulate similar pathways as HER proteins.[108] MET overexpression is observed in 80% and increased *MET* copy numbers are seen in 13% of SCCHN.[87] MET expression is associated with reduced disease-free and overall survival in HPV-negative SCCHN and has been associated with resistance to radiation, cisplatin, and cetuximab.

Members of the fibroblast growth factor receptor (FGFR) family can also be altered. Mutation or amplification of *FGFR1* is observed in 5 - 10% of HPV-negative patients, while *FGFR3* mutations are more frequent in HPV-positive patients (1-12%). Genetic alterations of *FGFR2* are less frequent and are observed in 2 - 4% of all SCCHN patients.[87]

B. Signaling pathways

Activation of the MAPK pathway regulates cell growth, proliferation, differentiation, migration and apoptosis.[109] *HRAS* mutations are found in 4% – 5% of SCCHN cases.[88,110-112] PI3K/Akt/mTOR pathway activation, implicated in cell growth and survival, may occur via several mechanisms. Loss of the phosphatase and tensin homolog tumour suppressor (PTEN) protein is reported in 10% of SCCHN, and mutation or amplification of the *PIK3CA* gene, coding for the catalytic subunit of PI-3-kinase, is found in 39% of SCCHN.[88,113,114]

The recruitment of STAT3 by JAK to the activated EGFR leads to STAT3 translocation to the nucleus where STAT3 promotes cell proliferation, apoptosis suppression and angiogenesis.[115] STAT3 is constitutively activated and overexpressed in the majority of patients with SCCHN.[115] High levels of activated STAT3 correlate with advanced tumor stage and poor patient prognosis.[116]

C. Cell cycle alterations

Figure 1-5 illustrates cell cycle deregulation in SCCHN. The most frequent genetic alterations in HPV-negative SCCHN are losses of chromosome 9p, containing the tumor suppressor gene *CDKN2A* (coding for p16), and inactivation mutations of another tumour suppressor gene, *TP53*. Both are involved in cell cycle regulation.[87,88] In addition, 20 - 30% of these tumours have *CCND1* amplification, and cyclin D1 overexpression is observed in 30% - 58% of cases. Cyclin D1 plays a central role in regulating the cell cycle as it promotes cell cycle transition from the G1 to S phase. Briefly, cyclin D1 binds to cyclin-dependent kinas (CDK)4/6 to form an active kinase, which induces the phosphorylation of retinoblastoma (pRb). The phosphorylation of Rb leads to the release of E2F transcription factors and induces the transcription of genes responsible for cell cycle progression and cellular proliferation. The role of the p16 protein is to disrupt the cyclinD1-CDK4/6 complexes.[117] In SCCHN, the loss of *CDKN2A*, encoding p16, combined with the frequently amplified *CCND1,* may lead to uncontrolled DNA replication. In response to DNA damage, p53, encoded by *TP53*, arrests the cell cycle and activates repair or initiates apoptosis.[118] SCCHN appears to be the most common p53 mutation-carrying cancer, with TP53 mutation reported in 50 –80% of cases.[87] *TP53* mutation is associated with tobacco use and alcohol consumption in SCCHN and is predictive of resistance to treatment and poor prognosis.[119] Another alteration impacting cell cycle regulation is the amplification of the proto-oncogene *Myc* in 14% of patients.[87] c-Myc is a proto-oncoprotein that directly regulates cell cycle transition by inducing the transcription of genes involved in the cyclin-D-CDK4/6-RB pathway such as *CDK4*, *CCND2*, and *CCNE1-2.*[120]

D. DNA Repair

Multiple pathways are implicated in DNA repair mechanisms and each focus on restoring the structure of DNA to maintain the genome. A defect in one of the DNA repair mechanisms can lead to the accumulation of genomic damage, referred to as genomic scars. Homologous recombination (HR) is one DNA repair pathway for double-strand breaks. It uses the intact copy on the sister chromatid to repair DNA in an error-free manner.[121] Breast Cancer (*BRCA)1* and *BRCA2* are key genes implicated in the HR pathway. When mutated in breast or ovarian cancer, they can confer sensitivity to platinum-based therapies[122] or to PARP inhibitors.[123] Other

genes can be altered and lead to homologous recombination deficiency (HRD). A comprehensive analysis for HRD was performed on 5371 tumors representing 15 cancer types (The Cancer Genome Atlas).[124] The aim of the study was to investigate the distribution of previously characterized genomic scars associated with HRD. The authors ranked the heterozygosity (HRD-LOH) scores and found that HRD was associated with ovarian cancer, lung cancer, SCCHN and bladder cancer. Another study reported the frequency of mutation in HR genes and observed mutations in 6.8% of head and neck tumours.[125]

E. WNT signaling and NOTCH pathway

The Wnt signaling cascade plays an important role in cell orientation and cell fate and is implicated in tumorigenesis.[126] *FAT1,* localized on region 4q35 and coding for a cadherin-like protein, has been shown to potentially supress cancer cell growth *in vitro* and *in vivo* by normally binding ß-catenin (encoded by the *CTNNB1 gene*) and antagonizing its nuclear localization, thus inhibiting its function in nuclear transcription.[127] Losses at chromosome 4q, or inactivating mutations by the *FAT1* tumor suppressor gene, are observed in up to 30% of SCCHN.[88] *AJUBA* and *NOTCH1* are two other tumor supressor genes that are linked to the Wnt pathway by regulating ß-catenin degradation, and these are altered in 8% and 22% of tumors, respectively.[88] However, besides NOTCH 1 inactivating mutations supporting its tumor suppressor role, activating mutations are also described supporting an oncogenic role. Thus, NOTCH activity is contextual in SCCHN. [128]

F. Copy number alterations (CNA)-silent HPV negative tumors

A particular subgroup of HPV-negative tumors is characterized by very few copy number alterations (CNAs) and a *TP53* wild type genotype. These tumors retain chromosome 3p, which is commonly lost in *TP53* mutated SCCHN.[129] They typically harbor more frequently activating *HRAS* mutations and inactivating mutations in the apoptosis-related *CASP8* gene. This subgroup of patients seems to also have a better prognosis.[87]

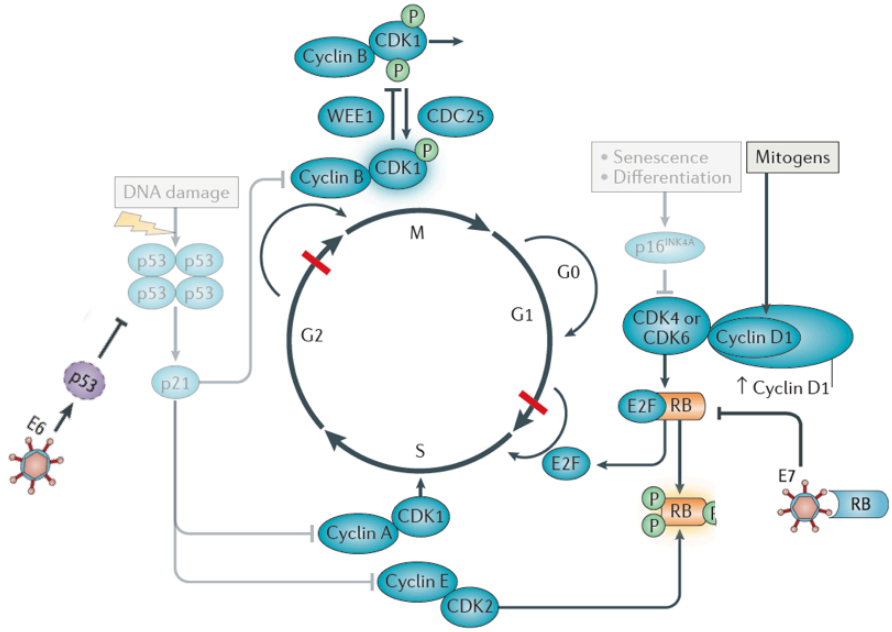


**Figure 1-5. Deregulation of the cell cycle in SCCHN.**

The cell cycle is regulated by several cyclin-dependent kinase (CDK) complexes. Cyclin-dependent kinases 4 and 6 (CDK4/6) form complexes with D-type cyclins and regulate cell cycle progression at the G1 restriction point (red bar), predominantly through the phosphorylation of Rb, a tumor suppressor protein. Hyper-phosphorylated Rb proteins release the transcriptional factor, E2F, that drives the transcription of genes involved in DNA replication and cell cycle progression. Negative regulation of the cyclinD1-CDK4/6 complex is controlled by $p16^{INK4A}$. The G2 restriction point (red bar) is predominantly regulated by p53, a tumor suppressor protein, by driving the gene transcription of p21, which can inactivate several CDK/cyclin complexes, leading to cell cycle arrest. WEE1 kinase can also regulate the cyclineB-CDK1 complex. The genes CDK inhibitor 2A (*CDKN2A*), encoding p16INK4A, and *TP53* are generally inactivated in HPV-negative SCCHN, indicated by faded colors, while Cyclin D1 is overexpressed. The HPV genome encodes two viral oncoproteins, E6 and E7, which inactivate the p53 and Rb proteins, respectively, with subsequent cell cycle progression. Figure adapted from Leemans and colleagues.[88]

#### 1.1.4.2 HPV-positive SCCHN

In HPV-positive tumours, malignant transformation begins with degradation of the p53 tumor suppressor protein mediated by the HPV oncoprotein E6 (Figure 1-5). There is a lower incidence of *TP53* mutations found in HPV-positive tumours compared with HPV-negative tumours.[130] E7, the second HPV oncoprotein, inactivates the retinoblastoma (Rb) tumour suppressor protein.[131] The *E2F1* gene is amplified in approximatively 20% of HPV-positive tumors, adding to cell cycle deregulation. In HPV-positive tumours, p16 (encoded by the *CDKN2A* gene) is overexpressed due to the loss of negative feedback induced by the inactivation of Rb by HPV E7.[132,133] 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).[134,135]

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.[87,136] 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 tumour-suppressor genes *TRAF3* and *ATM1*, respectively, have been reported. Finally, preclinical studies have shown that HPV-positive SCCHN have DNA double strand repair defects.[137]

#### 1.1.4.3 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 is limited.[138-140] 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%),[138] a higher frequency of alterations in the histone modifier *BAP1* gene and the DNA mismatch repair *MSH2* gene in persistent/recurrent laryngeal SCC,[140] 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%).[138] 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 circulating tumor DNA (ctDNA) of 112 baseline samples and found a genetic profile in line with previous reports with *TP53, FAT1, CDKN2A, NOTCH1* being the most frequently mutated genes. [141] Siu and colleagues reported the results of a comparison of three genomic databases:[142] (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).[142] 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.[139] 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.[143] Mutations not present in the primary tumors included *SMAD4* and *ALK* in two metastatic samples, and *FLT3, CDKN2A*, *KIT*, *TP53* and a *HRAS* mutations in recurrent samples. These limited data illustrate the potential intra-patient tumor heterogeneity through space and time.

### 1.1.5 Treatment strategies for SCCHN

Curative treatment of SCCHN depends on the tumour's localization and extension, patient age and performance status, and the anticipated functional outcome and long-term toxicity.[144] SCCHN should be staged according to the 8th edition TNM

system.[145] A multidisciplinary treatment schedule should be established in all cases.[144,146]

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.[59,60]

Approximately 60% to 80% of SCCHN patients presents with loco-regionally advanced disease (LA-SCCHN) at time of diagnosis.[60] One treatment option is surgery plus postoperative RT and, for high-risk patients (nodal extracapsular extension and/or R1 resection), post-operative chemoradiotherapy (CRT).[146] Radical CRT is another treatment option, even in resectable 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.[67,145]

#### 1.1.5.1 Locoregional treatment

A. Surgery

In early stage SCCHN, transoral surgery can be recommended as single modality treatment for oral cavity, oropharyngeal, and laryngeal lesions.[147] Surgical techniques depend on the location of the tumour and patient-related factors. Minimally invasive surgical treatments, including transoral laser microsurgery (TLM) and transoral robotic surgery (TORS), could offer the potential for organ preservation with less functional morbidity than open surgery and often less long-term toxicity than radiation therapy, providing they do not jeopardize the functional outcome (e.g. speech and swallowing) and do not require post-operative radiotherapy due to residual tumour.[148-150] 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 changing patient profile has strengthened interest in functional organ preservation surgery to improve functional outcomes and patient quality of life (QoL). Although

there is a lack of randomized trials, recent data suggest that the oncological outcomes of TORS for oropharyngeal cancer is comparable to open surgery and (C)RT.[151,152] 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.[153] Even though the head and neck is relatively accessible, access and manoeuvrability are sometimes limited by anatomic resections. TLM is currently considered a standard of care for early glottic cancer, but TORS has also been used to treat early-stage glottic carcinomas despite limited data.[154,155] When surgery is the primary treatment, neck dissection is recommended for most tumours except for early tumours 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%.[156] Neck dissection is also recommended in patients treated with primary chemoradiation when residual disease is suspected 12 weeks after the end of chemoradiation.[157-159]

B. 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 tumour 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 nonresectable 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 the absolute 5-year survival by 6.5%.[146,160] High-dose cisplatin (100 mg/m$^2$ day 1, 22 and 43) remains the standard radiosensitizer for the treatment of SCCHN. 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.[161,162] 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.[163]

Cetuximab is a chimeric mouse-human IgG1 monoclonal antibody (mAb) that binds to the EGFR extracellular domain and prevents its ligand-mediated activation.[164] 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.[165] 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/m$^2$) and RT (70 Gy), or weekly concomitant cetuximab (250 mg/m$^2$), 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.[166,167] How concomitant cetuximab-RT is comparable to concomitant cisplatin-RT in patients with locally advanced HPV-negative driven tumours is unknown, but the recommendation is to favour chemoradiation, and to only use cetuximab for patients not fit for platinum-based chemotherapy.

To minimize toxicity following RT for SCCHN, there is a shift away 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. [168] IMRT is therefore a standard of care in SCCHN. Today, all patients receiving RT should be treated with IMRT or its variant - volumetric modulated arc therapy (VMAT).

#### 1.1.5.2 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 HNSCC patients.[169,170]

A. 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 tumours express multiple immune checkpoint molecules that are implicated in decreased antitumour immunity.[171] Binding of the immune checkpoint programmed death-1 (PD-1) receptor, expressed on activated T-cells to its ligand PD-L1, which is expressed on tumour cells and tumour-infiltrating immune cells, led to a decrease of T-cell anti-tumour activity.[171] Pembrolizumab and nivolumab are two ICI that, by targeting PD-1, may reactivate the anti-tumour T-cell immune response.

The KEYNOTE-048 study showed that a combination of chemotherapy (cisplatin or carboplatin + 5-FU) plus pembrolizumab, a mAb targeting PD-1, significantly improves OS over the EXTREME regimen (cisplatin or carboplatin + 5-FU + cetuximab): 13 vs 10.7 months (p-value: 0.0034).[172] 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.[172] 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.

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 tumour shrinkage is needed. Of note, 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 PD-L1. In addition, the impact on survival of pembrolizumab for patients with a CPS of between 1 and 19 in SCCHN needs to be clarified.

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 tumours (CPS ≥ 1). The European Medical Agency (EMA) has approved pembrolizumab with or without chemotherapy only for PD-L1 expressing tumours (CPS $\underline{\geq}$ 1).

For patients who progress within six months of platinum therapy given either as palliative treatment or as part of multimodal curative treatment, nivolumab, a mAb targeting PD-1, 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).[173] 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.[174] In the population with a PD-L1 tumour 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 tumours express PD-L1 with a TPS ≥ 50%.

Furthermore, pembrolizumab and nivolumab offer a better toxicity profile and improvements in QoL compared to standard therapies.[175]

B. 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.[176] 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.[177] 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.[178] 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).

C. Other chemotherapies

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

D. 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 (Table 1-1). Besides Cetuximab, no other compounds targeting the HER pathway have shown meaningful clinical activity in unselected populations. Multiple efforts have been done 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 $\alpha/\beta/\delta/\gamma$ subunit) was added to Paclitaxel.[179] The biomarker analysis of this study did not find any correlation between PI3K pathway activation and drug activity.[141] Recently, Buparlisib monotherapy in patients with SCCHN harbouring *PIK3CA* mutation showed limited activity, even in this preselected population.[180] Encouraging results were found with the combination of Cetuximab and Palbociclib, a CDK4/6 inhibitor, in a single-arm phase II study[181]. 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.[182] Studies targeting the VEGF pathway have resulted in limited efficacy but substantial toxicity.[183] 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.[184] Recently, lenvatinib, a multikinase inhibitor, has shown encouraging results in combination with pembrolizumab.[185] Only a few biomarker-driven trials are dedicated to SCCHN. They are described in chapter 1.2.4.2 (Table 1-5).

| Drug | Target | Study design | Population | ORR | OS | Reference |
|---|---|---|---|---|---|---|
| **HER pathway** | | | | | | |
| **Gefitinib** | EGFR TKI | Phase III, RCT<br>Gefitinib 250mg/day versus Gefitinib 500mg/day versus MTX | R/M SCCHN<br>after radical radiation therapy, no restriction on prior platinum | Gefitinib 250mg:2.7%<br>Gefitinib 500mg:7.6%<br>MTX: 3.9% | Gefitinib 250mg: 5.6 mo<br>Gefitinib 500mg: 6.0 mo<br>MTX: 6.7 mo | Stewart et al, 2009[186] |
| **Afatinib** | Pan-HER TKI | Phase III, RCT,<br>Afatinib versus MTX | R/M SCCHN<br>after platinum | Afatinib: 10%<br>MTX: 6% | Afatinib: 6.8 mo<br>MTX: 6.0 mo | Machiels et al, 2015[170] |
| **Duligotuzumab** | Dual Ab against EGFR and HER3 | Phase II, RCT<br>Duligotuzumab versus Cetuximab | R/M SCCHN<br>after chemotherapy | Duligotuzumab: 12%<br>Cetuximab: 14.5% | Duligotuzumab: 7.2 mo<br>Cetuximab: 8.7 mo | Fayette et al, 2016[187] |
| **IGF-1 Receptor** | | | | | | |
| **Figitumumab** | IGF-1R | Phase II, single-arm | R/M SCCHN<br>after platinum | 0% | 63 days | Schmitz et al, 2012[188] |
| **MET** | | | | | | |
| **Tivantinib** | MET TKI | Phase II, RCT<br>Tivantinib (T) + Cetuximab (C) vs Cetuximab | R/M SCCHN<br>after platinum | T + C: 8.5%<br>C: 5.3% | T + C: 8 mo<br>C: 8 mo | Vokes et al, 2015[189] |
| **PI3K/AKT/mTOR pathway** | | | | | | |
| **Temsirolimus** | mTOR | Phase II, single-arm | R/M SCCHN<br>after platinum and cetuximab | 0% | 152 days | Grunwald et al, 2015[190] |
| **Temsirolimus + erlotinib** | mTOR + EGFR | Phase II, single-arm | R/M SCCHN<br>after platinum | 0% | 4 mo | Bauman et al, 2013[191] |

| | | | | | | |
|---|---|---|---|---|---|---|
| **PX-866 + cetuximab (C)** | Pan-isoform PI3K + EGFR | Phase II, RCT<br>PX-866 + C versus C | Pretreated R/M SCCHN | PX-866 + C: 10%<br>C: 7% | PX-866 + C: 8.5 mo<br>C: 8.5 mo | Jimeno et al, 2015[192] |
| **Buparlisib + Paclitaxel** | Inhibitor of PI3K p110 α/β/δ/γ subunit | Phase II, RCT<br>Buparlisib + Paclitaxel versus Paclitaxel + placebo | R/M SCCHN<br>after platinum | Buparlisib + Paclitaxel : 39%<br>Paclitaxel + placebo : 14% | Buparlisib + Paclitaxel : 10.4<br>Paclitaxel + placebo : 6.5 | Soulières et al, 2017 [179] |
| **Cell Cycle** | | | | | | |
| **Palbociclib + cetuximab (C)** | CDK4/6 + EGFR | Phase II, RCT<br>Palbociclib + C versus C + placebo | R/M SCCHN<br>after platinum | NA | Palbociclib + C : 9.7 mo<br>C + placebo : 7.8 mo | Adkins et al, 2019[182] |
| **Angiogenesis** | | | | | | |
| **Bevacizumab + Chemotherapy (CT)** | VEGF | Phase III, RCT<br>Bevacizumab + CT versus CT alone | R/M SCCHN, first-line | Bevacizumab + CT: 35.5%<br>CT alone: 24.5% | Bevacizumab + CT: 12.6 mo<br>CT alone: 11 mo | Argiris et al 2019[184] |
| **Sunitinib** | VEGFR1-3, FGFR 1-4, PDGFRα/β, FLT3, RET, CSF1R, KIT | Phase II, single arm | R/M SCCHN after platinum | 3% | 102 days | Machiels et al, 2010[183] |
| **Pembrolizumab + Lenvatinib** | VEGFR 1−3, FGFR 1-4, PDGFRα, RET, KIT | Ib/II | R/M SCCHN | 36.4% | NA | Taylor et al, 2018[185] |

**Table 1-1. Selected clinical trials evaluating targeted agents, other than cetuximab, in SCCHN**

Ab, antibody; mo, months; MTX, methotrexate; NA, not available , ORR, Objective response rate;; OS, overall survival; RCT, randomized controlled trial; R/M, recurrent/metastatic; SCCHN, squamous cell carcinoma of the head and neck; TKI tyrosine kinase inhibitor; CSF1R, receptor for macrophage colony-stimulating factor, EGFR, epidermal growth factor receptor; FLT3, FMS-like tyrosine kinase-3, FGFR, fibroblast growth factor receptor; HER, human epidermal growth factor receptor; IGF-1R,insulin growth factor-1 receptor; MET, Mesenchymal epithelial transition; PDGFR, platelet-derived growth factor receptor ; RET, glial cell-line derived neurotrophic factor receptor; VEGFR, vascular endothelial growth factor receptor; CDK, Cyclin-dependent kinase

## 1.2 Precision Medicine

This chapter is adapted from Galot et al. (Annals of Oncology, 2018).[193]

### 1.2.1 The concept of precision medicine

According to the National Cancer Institute (NCI),[194] "precision medicine," also called "personalized medicine," is a form of medicine that uses information from a person's genes, proteins, and environment to prevent, diagnose, and treat disease. Different forms of precision medicine are already used in routine oncology practice. Environmental factors, such as a family history of cancer, help to determine screening strategies and, from a molecular viewpoint, the presence of a germline *BRCA1/2* mutation can be used to prevent breast or ovarian cancer by way of surgical strategies. Molecular analysis is used with diagnostic intent, for example, the *EWS-FLI-1* fusion gene is pathognomonic for Ewing's sarcoma. However, in cancer, the term 'precision medicine' usually refers to therapeutic strategies that are expected to have greater benefit in patients whose tumors harbor specific molecular characteristics.[195] Typically, it refers to genomic medicine based on DNA aberrations. Importantly, precision medicine can use other "-omics" based strategies including aberrations in the proteome, transcriptome, metabolome or epigenome.[196] Treating *HER2* amplified breast cancer with trastuzumab,[197] or *EGFR* mutated NSCLC with EGFR-tyrosine kinase inhibitors (TKI),[198] are biomarker-based therapeutic strategies that have been implemented successfully and improve patient outcomes.

The concept of precision medicine has emerged in parallel with the advent of new technologies, such as next-generation sequencing (NGS), which have revolutionized the study of genomics and molecular biology. Our growing understanding of the molecular biology of cancer has led to the identification of potentially targetable cancer-specific molecular alterations and the development of a multitude of new drugs to target these. Trying to select patients upfront who are most likely to benefit from a specific treatment may be a way to improve outcomes. Next-generation clinical trial designs have emerged to address these challenges and to study these types of drugs more efficiently.

### 1.2.2 Precision medicine in clinical practice

For decades, treatment decisions in oncology were guided by the cell of origin, the cancer's histological features, anatomical subsite and TNM classification. Trastuzumab for HER2-positive breast cancer, determined by HER2 IHC, was the first drug-companion diagnostic assay (CDx) combination to be approved by the FDA, and it paved the way for a new paradigm of biomarker-based oncology. Since then, many other targeted drugs have shown great outcome benefits in selected populations enriched for specific biomarkers, resulting in the FDA approval of different targeted drug-CDx combinations. Table 1-2 provides an overview of biomarker-based treatment strategies already implemented in the clinic based on FDA approvals. Most of the implemented biomarker-driven strategies are histology specific. Recently, however, the FDA approved two drugs in a histology-agnostic approach: pembrolizumab for microsatellite instability-high or mismatch repair deficient cancers[199] and larotrectinib for cancers harbouring Neurotrophic tyrosine receptor kinase (NTRK) fusions.

The recent FDA approval of pembrolizumab as monotherapy in the first-line R/M setting according to the PDL-1 status (CPS >/= 1) will be the first biomarker-driven approach to guide the treatment of SCCHN.

| Cancer | Biomarker | Targeted drug |
|---|---|---|
| **Breast** | HER2 overexpression/*HER2* amplification | HER2 inhibitors (trastuzumab, pertuzumab, lapatinib, ado-trastuzumab emtansine) |
| | *PIK3CA* mutation | PIK3CA inhibitor (alpelisib) |
| | *BRCA1/BRCA2* germline mutations | PARP inhibitors (olaparib, talazoparib) |
| | PD-L1 expression in TNBC | Anti-PD-L1 (atezolizumab) |
| **NSCLC** | *EGFR* mutation | EGFR TKI/pan-HER inhibitors (erlotinib, gefitinib, afatinib, osimertinib, dacomitinib) |
| | *ROS* fusions | TKI (crizotinib) |
| | *ALK* rearrangement | TKI (crizotinib, ceritinib, alectinib, brigatinib lorlatinib) |
| | *BRAF* mutation | MEK-inhibitor + BRAF inhibitor (trametinib + dabrafenib) |
| | PD-L1 expression | Anti-PD-1 (pembrolizumab) |
| **SCCHN** | PD-L1 expression | Anti-PD-1 (pembrolizumab) |
| **Ovarian** | *BRCA1/BRCA2* germline or somatic mutations<br>HRD-positive tumours | PARP inhibitors (olaparib, rucaparib, niraparib) |
| **Cervical** | PD-L1 expression | Anti-PD-1 (pembrolizumab) |
| **Urothelial** | *FGFR3* mutation or *FGFR2/3* fusions | FGFR inhibitor (erdafitinib) |
| | PD-L1 expression | Anti-PD-1 (pembrolizumab), Anti-PD-L1 (atezolizumab) |
| **Gastric** | *HER2* amplification | HER2 inhibitor (trastuzumab) |
| | PD-L1 expression | Anti-PD-1 (pembrolizumab) |
| **ESCC** | PD-L1 expression | Anti-PD-1 (pembrolizumab) |
| **Colorectal** | *RAS* wild type | EGFR inhibitors (cetuximab, panitumumab) |
| **Melanoma** | *BRAF* mutation | MEK-inhibitor/BRAF inhibitor (vemurafenib, trametinib + dabrafenib, encorafenib + benimetinib) |
| **GIST** | *c-KIT/PDGFRA* mutations | TKI (imatinib) |
| **All cancers** | MSI-H or dMMR | Anti-PD-1 (pembrolizumab) |
| | *NTRK* fusions | TRK inhibitor (larotrectinib, entrectinib) |

**Table 1-2. Biomarker-based treatment strategies based on FDA approvals**

ALK, anaplastic lymphoma kinase; BRCA, BReast CAncer gene; dMMR, deficient mismatch repair; ESCC, esophageal squamous cell carcinoma; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; GIST, gastrointestinal stromal tumor; HER2, human epidermal growth factor receptor 2; MSI-H, microsatellite instability-high; NSCLC, non-small cell lung cancer; NTRK, neurotrophic receptor tyrosine kinase 1; PARP, poly-ADP ribose polymerase; PD1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PDGFRA, platelet derived growth factor receptor alpha; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ROS1, ROS proto-oncogene 1; SCCHN, squamous cell carcinoma of the head and neck; TKI, tyrosine kinase inhibitors; TNBC, triple-negative breast cancer; TRK, tropomyosin receptor kinase

### 1.2.3 Lessons learned from biomarker-driven studies

As discussed previously, the treatment landscape in oncology is moving very fast from a "one treatment fits all" strategy with cytotoxic chemotherapy to a "precision medicine" approach that is mainly based on biomarkers. New targeted drugs and their associated biomarkers still need proper testing in clinical trials to validate their efficacy and safety, and this work is ongoing. In the USA, for example, the presence/absence of a genomic alteration in trial designs increased over 5-fold between 2006 and 2013; and in 2018, over one-third of trials were using biomarkers to stratify patients. Meta-analyses have shown that trials with a biomarker-driven selection have better outcomes than trials without biomarkers.[200-202]

The standard approach is to investigate one or two interventions in a single disease enriched for one biomarker (Figure 1-6). With this strategy, investigating biomarkers with a high incidence, like *KRAS* status in colorectal cancer (40%), is feasible. However, this type of design becomes very challenging for low-incidence biomarkers as clinical trial recruitment will be slow and the number of patients to screen will be high, making it an expensive and time-consuming exercise.

'Next-generation clinical trial design' has emerged as a way to efficiently integrate several biomarkers into clinical trials. The main aim of this design is (i) to facilitate screening and patient accrual, (ii) to answer clinical questions more efficiently and in less time and (iii) to optimize operational efficiency.

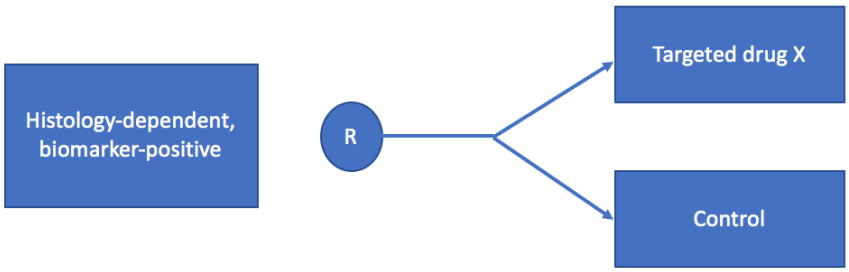


**Figure 1-6. Classical biomarker-driven trial design**

#### 1.2.3.1 Next-generation clinical trial designs

"Master protocol" terminology refers to a framework in which several (sub)studies that investigate multiple therapies are operated in parallel under one 'overarching' master protocol.[203] Master protocols include mainly two different study designs: basket and umbrella trials (fig 1-7). Table 1-3 summarizes the opportunities and drawbacks of these designs.

***Basket trials*** are biomarker-driven clinical trials that include patients based on pre-defined specific molecular tumour abnormalities, irrespective of tumour origin and histology.[204] One of the advantages of this histology agnostic approach is to investigate the activity of targeted drugs across different cancer types, even in rare cancers for which clinical trials do not exist. They also offer the possibility to target low incidence molecular alterations.

***Umbrella trials*** are biomarker-driven clinical trials that are histology specific, investigating different therapeutic interventions in a single cancer type.[205] A histology specific approach is interesting to avoid the heterogeneity due to different biology across various tumour types.

***Strategy trials*** investigate if selecting the treatment based on molecular alterations results in superior outcome compared to standard therapy, independently of the drug, the disease, and the studied biomarker(s).[206]

***Molecular screening programs*** have been implemented to facilitate the access to precision medicine trials. These screening initiatives can be histology-agnostic or histology-specific.[207]

Table 8-1 and 8-2 in the supplementary data gives a detailed overview of selected histology-agnostic and histology-specific biomarker-driven approaches respectively, that will be further discussed in this chapter.

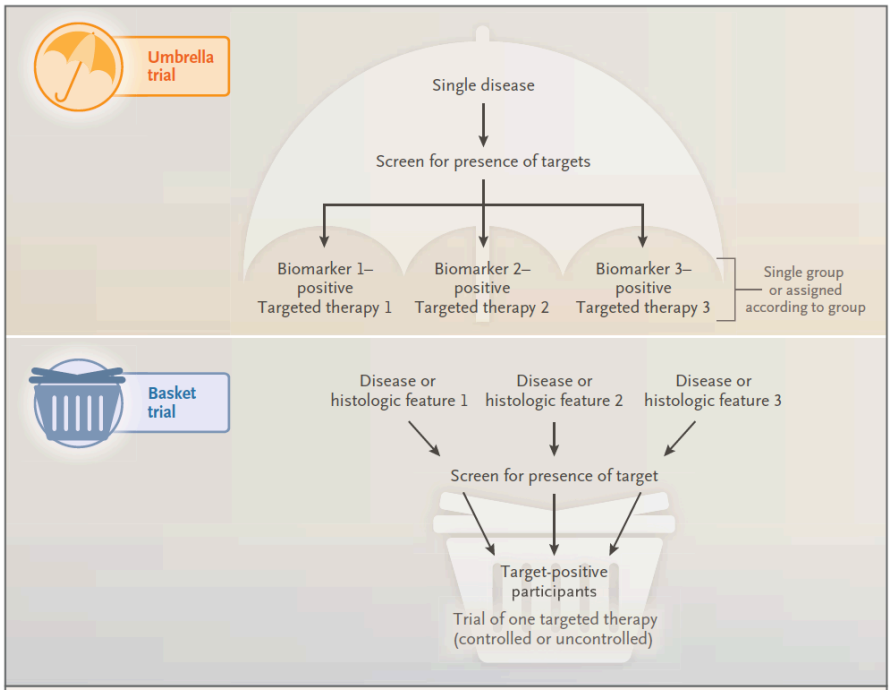


**Figure 1-7. Schematic representation of umbrella and basket trials.**

An umbrella trial (top) is a biomarker-driven clinical trial investigating different biomarker-targeted therapy pairs in a single cancer type. A basket trial (bottom) may involve different types of cancers and one or more biomarker-targeted therapy pairs will be tested in a tumor-agnostic approach. Figure adapted from Woodcock et al.[208]

| | ADVANTAGES | DISADVANTAGES |
|---|---|---|
| **MASTER PROTOCOLS** | | |
| **BASKET trials**<br>**Histology agnostic** | Can include rare cancer types<br><br>Can target low incidence actionable/targetable molecular alterations | Assumes that molecular biology can replace histology and that a specific genetic alteration has the same signification across different tumor types |
| **UMBRELLA trials**<br>**Histology specific** | Targets molecular alterations in one cancer type and avoid heterogeneity due to multiple cancer histologies<br><br>Enables to get more conclusive results for one tumor type. | Feasibility limited for rare cancers |
| **SCREENING PROGRAMS** | Have the potential to identify an actionable/targetable genetic alteration<br><br>Can facilitate the access to early development clinical trials | If an actionable/targetable alteration is present, the specific drug is not always available with the risk that a low number of patients finally benefits from this program |
| **STRATEGY TRIALS** | Have the potential to identify an actionable/targetable genetic alteration | Effect of the strategy can be diluted by less effective target-drug pairs |

**Table 1-3. Advantages and pitfalls of "biomarker-driven" clinical trial designs**

#### 1.2.3.2 Theranostic and molecular screening tools

Different diagnostic tests are routinely used to predict the activity or resistance of some targeted therapies. Most of them are evaluated on tumor biopsies, although liquid biopsies are entering into the clinic (e.g. *EGFR T790M* mutation in non-small cell lung cancers (NSCLC)). Biomarkers can be evaluated at the proteomic level such as the estrogen receptor status assessed by IHC but also at the genomic level such as *HER2* amplifications or *EGFR* activating mutations.

The tumor molecular profile has been obtained in 67% to 93% of screened patients in biomarker-driven clinical trials.[205-207,209-213] Most of them use DNA sequencing on tumor biopsies. Reproducibility and reliability of the molecular screening tools are important. Most of the trials use certified laboratories, but the analysis is not always centralized. In these cases, some trials performed an inter-laboratory analytical validation before starting the trial [214] or validated the assay.[215]

A fresh biopsy is probably more reliable than an archival one. Indeed, the cancer molecular profile can change during disease evolution.[139] IMPACT [207,211] used archival paraffin-embedded tissue (FFPE). In the LUNG-MAP trial [216] and LUNG-MATRIX trial [213], both archival or fresh-taken tissues are accepted. In the MOSCATO 01 [210], NCI-MPACT [204], NCI-MATCH [204], BATTLE [205], and SHIVA [206] trials, a fresh tumor biopsy has/had to be taken for the trial purpose.

Interestingly, the WINTHER trial, incorporated transcriptomics in patient selection. [217] A fresh biopsy was analyzed for every patient including a broad comprehensive approach on the DNA level (NGS, 263 genes), but also transcriptomics approach (dual biopsies of tumor and matched normal tissue to differentiate gene expression and microRNA).

Genomic profiling of tissue can be limited because of unaccessibility of the lesions to perform a new biopsy, the risk of clinical complications, but also due to the quality of the biopsies. An attractive alternative is liquid biopsies. TARGET was a molecular profiling screening program that used ctDNA to screen patients with advanced cancer to match them with early phase clinical trials.[218] They showed a good concordance rate between tumor and ctDNA for somatic mutation detection (70/94 patients, 74.5%).

#### 1.2.3.3 Actionable genomic alteration frequency and enrolment rate

According to European Society of Medical Oncology (ESMO) glossary,[195] *targetable genomic alteration* encodes an altered protein against which a drug exists or can be synthesized and an *actionable genomic alteration* includes both targetable alterations and genomic alterations that cannot be directly targeted but that lead to dysregulation of a pathway in which there are possible targets.

The percentage of patients that had an actionable genomic alteration identified through screening programs ranged from 46% to 63%.[207,210,211,219] However, the number of patients who were finally treated with a matched targeted therapy were low: 13%, 16%, and 19% in SAFIR01,[219] IMPACT (first published report [211]), and MOSCATO 01,[210] respectively. This number increased to 27% in the most recent IMPACT publication, [207] probably related to the extension of the screening panels. Different reasons may explain these low enrolment rate: tumor tissue issues, decline in the performance status or rapidly progressing disease, the absence of a targetable event, and the access to matched clinical trials or drugs. As IMPACT and the MOSCATO 01 were screening programs, patients were referred to enrolling clinical trials with obvious limitations in the treatment possibilities. The same enrollment rate was observed in TARGET,[218] allocating patients to early phase clinical trials based on ctDNA detection. Fourty-one out of the first 100 (41%) of patients had an alteration considered as actionable, of whom 11 (11%) were treated with a matched therapy. The main reason for being treated with non-matched therapy was non-availability of trial and clinical deterioration. Although the proportion of patients treated with a matched therapy was low, this trial showed the feasibility of using ctDNA to refer a subset of patients to early-phase clinical trials.

A way to partially solve these issues is to include the access to drugs into the clinical trial design. The NCI-MATCH basket trial pre-planned the access to some targeted compounds. However, only 12% of the patients were finally enrolled in the trial.[212] This low enrolling rate might be due to the low incidence of the targeted variants since only 18% of the screened tumors were found to have a genomic alteration that matched one of the 30 treatment arms. In contrast, in BATTLE and LUNG-MAP, two umbrella trials for NSCLC, 75% and 37% of the patients were included in one of the sub-studies, respectively.[205,216] The number of treated patients is higher in these

two last trials due to a pre-planned access to matched targeted therapies. In addition, for the Battle trial, the molecular profile strategy was disease-specific and adapted to NSCLC, explaining the high prevalence of some of the investigated biomarkers.

Not all the patients will harbor a targetable genomic alteration and when present, they might not be sufficient for efficacy prediction. Other techniques than classical DNA sequencing could be used for biomarker selection, like gene expression. The recently published WINTHER trial, [217] allocating patients to treatment based either on DNA alterations or based on RNA analysis that compared tumor to matched normal tissue, showed that transcriptomics enhanced the number of patients treated with a matched therapy from 23% to 35% of consented patients.

#### 1.2.3.4 Treatment efficacy in Master protocols

Different endpoints are used in biomarker-driven trials. In MOSCATO 01,[210] the primary endpoint was the progression-free survival (PFS) ratio calculated for each patient, that must be > 1.3 to define clinical benefit (PFS ratio = PFS on the molecular-profile selected therapy/PFS on prior therapy). The approach is judged efficient if it modifies the natural history of the disease and is associated with a longer PFS than the previous line of treatment. Thirty-three percent of patients treated with a targeted therapy had a PFS ratio > 1.3. However, the number of patients who benefited from the personalized approach represented only 7% of the screened patients.

In IMPACT, the clinical outcomes of patients with molecular aberrations treated with matched therapy were compared with those of consecutive patients who were not treated with a matched therapy. They reported a better objective response rate (ORR) (11% vs 5%), a longer failure-free survival (3.4 vs 2.9 months), and a longer OS (8.4 vs 7.3 months) in the matched group.[207] The clinical benefit rate in the matched group, defined as the proportion of patients with either a stable disease lasting more than 6 months or a partial response or complete response, was 29% (111/381) as compared to 24% (56/238) in the non-matched group. However, only 8% of the whole population finally experienced a clinical benefit. The use of non-optimal targeted drugs or sub-optimal dosages in phase 1 trials, and sometimes the

level of evidence concerning the investigated biomarker(s) may explain the limited treatment efficacy observed.

In MyPathway basket trial,[220] the ORR was 23% in 14 different tumor types, a clinically significant result for advanced refractory disease. In the SUMMIT trial, [221] a basket trial studying neratinib in patients with a tumor harboring either *HER2* or *HER 3* mutations, the primary endpoint was reached only for breast cancer, and not for lung, bladder, and colorectal cancers, underlining the importance of the histology and the tissue of cancer origin. In BATTLE,[205] the 8-week disease control rate and ORR were 46% and 4%, respectively. The first data of the ongoing Lung-MAP trial reported an ORR of 4-7% for the first 3 biomarker-driven cohorts.[222-224]

The SHIVA trial was the first randomized trial comparing a molecularly targeted therapy based on tumor molecular profiling versus conventional therapy for advanced cancer.[206] This study tested the overall strategy of a biomarker-driven treatment approach versus standard therapy. The trial did not meet its primary endpoint (PFS). Several reasons could explain this overall negative result. First, they used drugs that were marketed in France at that time and not necessarily the best in class to target the molecular alteration identified. Second, the experimental arm was also heterogeneous with multiple drugs and various tumor types. This could have blinded the benefit of some drugs in some specific cancer(s). The ongoing NCI-MPACT trial [204] is also a strategy trial. To avoid a negative trial linked with inadequate target modulation by the selected agents, all the targeted agents used in NCI-MPACT have been validated to engage their purported targets and have at least an established phase II dose. A recent update on the trial however, showed that 3 of the 4 targeted treatment cohorts were closed for futility. Due to the large number of patient withdrawal, the comparison between the targeted arm and the control arm was uninterpretable. The trial was redesigned to a non-randomized design to complete the assessment of the activity of the last targeted cohort.[225]

Until now, the off-label use of targeted therapies should be discouraged outside of clinical trials. Recently, 3 new basket trials have been designed, with the aim to identify signals of drug activity for off-label use of approved/commercially available targeted therapies matched to pre-specified genomic targets in patients with advanced cancer: TAPUR in the USA,[226] DRUG in the Netherlands,[227] and CAPTUR[228] in Canada. The 3 designs are similar and include different cohorts that are defined

by a study drug, a histologic tumor type, and a molecular profile. The efficacy is analyzed per cohort and primary endpoints are objective response rate and/or stable disease at 16 weeks, and grade ≥3 adverse events. An update on the "DRUG" trial was reported at ASCO 2019; 101 cohorts are opened testing 22 different treatments; out of 870 patients submitted for review, 365 patients (42 %) have started a treatment.

One of the factors that could limit the efficacy in precision medicine trials is the use of monotherapy that targets only one molecular alteration. The I-PREDICT study hypothesized that personalized combination therapy would improve outcome.[229] Based on genomic profiling (NGS on 236-405 genes, Foundation Medicine) combined, when possible, with immunotherapy predictive factors (PD-L1 IHC, tumor mutational burden (TMB), microsatellite instable (MSI) status) and ctDNA analysis, patients were treated with drug combinations to target the majority of genomic alterations. Eighty-three patients of the 149 that consented to the trial were treated, 73 of whom received a precision therapy consisting of $\geq 1$ molecularly matched treatment (median of 2 administered drugs and median of 2 genomic alterations targeted per patient). The matching rate of 49% (73 of 149 patients) is higher than in the other trials. Analysis was reported according to a "matching score" for each patient, it is the total number of molecular alterations matched to the drugs administered and divided by the total number of characterized genomic aberrations. They reported better outcome for patients classified as "matching score high" (>50%, n=28) vs "matching score low "(<50%, n=55), with a higher disease control rate (50% vs 22.4%, p=0.028), a longer PFS (6.5 mo vs 3.1 mo, p=0.01) and OS (not reached after median follow-up of 8.5 mo vs 10.2 mo). Although this study has some limitations, including the lack of control group and the frequent match of immunotherapy related to TMB status in "the matching score high subgroup", this suggest that targeting the majority of molecular alterations could improve outcome compared to monotherapy.

### 1.2.4 Biomarker-driven treatment strategies for SCCHN

The only targeted therapies and immunotherapies that are currently approved for SCCHN are Cetuximab, a monoclonal antibody against EGFR, and the PD-1 targeting agents, Pembrolizumab and Nivolumab. Until recently, none of these were

biomarker-driven. With the results of the Keynote-048 trial, Pembrolizumab as first-line treatment for R/M SCCHN based on PD-L1 status, is the first biomarker-driven approach for SCCHN. Although the genomic landscape of SCCHN harbors potential actionable events, targeted therapies have shown disappointing results, as discussed previously. [183,188,189] This is mainly due to the fact that they were tested in unselected populations. Recently, encouraging activity was observed with tipifarnib, a farnesyl transferase inhibitor, in patients with recurrent/metastatic *HRAS*-mutant SCCHN (n=15, ORR 53%). [230]

#### 1.2.4.1 Actionable events in SCCHN

NGS technologies have identified potentially actionable/targetable genomic alterations in SCCHN [87,112,113], as discussed previously in this manuscript. The majority of these data were obtained through sequencing of primary tumors. Targetable genomic alterations in HPV-negative SCCHN include events in genes related to kinase growth factor family receptors, like *EGFR* (15%), *FGFR 1-3* (14%), *HER2* (5%) or their downstream molecular pathways, including *PIK3CA* (34%), and *HRAS* (5%). HPV-negative SCCHN has also potentially actionable cell cycle genomic alterations: *TP53* mutation (70%), *CCND1* amplification (20-30%), and *CDKN2A* inactivation (80-90%). The NOTCH pathway could also be a potential target as *NOTCH1* mutations are seen in 10-19%, although it needs to be context specific. NOTCH-inhibitors could be of interest for SCCHN with *NOTCH* activating mutations.[128] Preclinical data suggest that SCCHN with *NOTCH1* loss of function mutation could be sensitive to PI3K/mTOR inhibitors. [231]

In HPV-positive OPC, where the oncoprotein E6 and E7 inactivate respectively p53 and Rb, *PIK3CA* amplifications/mutations are found in 56% whereas the other genomic alterations are rare.

Table 1-4 gives an overview of potentially targetable genomic alterations in SCCHN.

| Alteration | Drug |
|---|---|
| *EGFR amplification/mutation* | HER inhibitors |
| *HER2 amplification/mutation* | HER inhibitors |
| *FGFR amplification/mutation/fusion* | FGFR-inhibitors |
| *MYC amplification* | BET-inhibitors |
| *PIK3CA amplification/mutation* | PI3K inhibitors |
| *HRAS mutation* | Farnesyl transferase inhibitors |
| *TP53 mutation* | Small molecules targeting mutant p53 or targeted agents against DNA damage repair proteins (eg. WEE-1 inhibitors) |
| *CCND1 amplification* | CDK inhibitors |
| *CDKN2A mutation/deletion* | CDK inhibitors |
| *NOTCH1 mutation* | NOTCH inhibitors or PI3K/mTOR inhibitors |
| Mutations in DNA–repair related genes | PARP inhibitors |
| *NTRK fusions* | Pan-TRK inhibitor |

**Table 1-4. Potentially targetable genes in SCCHN**

BET, Bromodomain and extra-terminal motif; CCND1, Cyclin D1; CDK, Cyclin dependent kinase; CDKN2A, cyclin-dependent kinase Inhibitor 2A; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; HER, human epidermal growth factor receptors; mTOR, mammalian target of rapamycin ; NTRK, neurotrophic tyrosine receptor kinase ; TRK, tropomyosin-related-kinase ; PI3K, Phosphoinositide 3-kinase ; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-kinase catalytic subunit alpha; PARP, poly(ADP-ribose) polymérase

#### 1.2.4.2 Biomarker-driven clinical trials for SCCHN

Some groups reported on their first experience with precision medicine. Chau et al incorporated NGS assays into the routine care of SCCHN patients. They were able to enroll 8 out of these 213 SCCHN patients in clinical trials with a compound that matched the genomic alteration detected within their tumor. Four (50%) experienced a partial response.[232] Another group performed NGS on 151 patients with advanced head and neck cancer, including 53 with SCCHN.[233] The molecular analysis guided therapy in 13/53 (25%) SCCHN patients. Seven of these patients (13%) received targeted therapies: 4 patients with alterations in *PIK3CA* or *PTEN* treated with PI3K inhibitors, 1 patient with *HRAS* mutation treated with a farnesyl transferase inhibitor, 1 patient with *MAPK1* mutation treated with an ERK inhibitor and 1 patient with *SMARCB1* deletion treated with a histone methyl transferase Enhancer of Zeste Homolog 2 (EZH2) inhibitor. The other 6 patients were enrolled on immunotherapy trials.

Only a few biomarker-driven trials are dedicated to SCCHN (Table 1-5). Some phase II trials are selecting patients upfront based on a rare specific genomic alteration (*HRas* proto-oncogene (*HRAS)* mutations or *Fibroblast Growth Factor Receptor* (*FGFR)* mutations/amplifications/translocations). However, these trials offer only one potential therapeutic option for the very low percentage of patients harboring these rare genomic events. This results in a high rate of screening failure. There is another ongoing trial in Korea assessing personalized therapy for recurrent/metastatic SCCHN and oesophageal cancer (NCT03292250) where patients are allocated to different treatment arms after first line platinum-based therapy according to molecular characterization. Another option to evaluate the benefit of some targeted approaches in SCCHN, especially for low-incidence biomarker, is including the patients in BASKET trials, like NCI-MATCH[204] or TAPUR[226].

| Study title | ClinicalTrials.gov identifier and status |
|---|---|
| Pan FGFR kinase inhibitor BGJ398 in treating patients with *FGFR1-3* translocated, mutated, or amplified recurrent head and neck cancer | NCT02706691<br>Terminated (Study stopped due to poor enrollment) |
| Phase II study of tipifarnib in squamous head and neck cancer with *HRAS* mutations | NCT02383927<br>Recruiting |
| Safety and efficacy of tipifarnib in head and neck cancer with *HRAS* mutations and impact of *HRAS* on response to therapy (AIM-HN/SEQ-HN) | NCT03719690<br>Recruiting |
| Copanlisib in association with cetuximab in patients with recurrent and/or metastatic SCCHN harboring a *PI3K*CA mutation/amplification and/or a PTEN Loss | NCT02822482<br><br>Active, not recruiting |
| SF1126 in recurrent or progressive SCCHN and mutations in *PIK3CA* gene and/or PI-3 Kinase pathway genes | NCT02644122<br>Terminated (Slow enrollment) |
| Evaluation of ABEMACICLIB monotherapy in patients with locally advanced/metastatic head and neck cancer after failure of platinum and cetuximab or anti-EGFR-based therapy and harboring a homozygous deletion of *CDKN2A*, and/or an amplification of *CCND1* and/or of *CDK6* (ABORL) | NCT03356223<br>Recruiting |
| Korean Cancer Study Group: Translational biomarker driven UMbrella Project for head and neck (TRIUMPH), esophageal squamous cell carcinoma- Part 1 | NCT03292250<br><br>Recruiting |
| Single-arm study with Bimiralisib (orally available pan-PI3K/mTOR inhibitor) in patients with head and neck squamous cell carcinoma harboring *NOTCH1* loss of function mutations | NCT03740100<br>Recruiting |

**Table 1-5. Biomarker-driven studies for squamous cell carcinoma of the head and neck**

CCND1, cyclin D1; CDK6, cyclin-dependent kinase 6; CDKN2A, cyclin-dependent kinase inhibitor 2A EGGR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; HRAS, *HRas* proto-oncogene; PI3K, Phosphatidylinositol-3-Kinase PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-kinase catalytic subunit alpha, SCCHN, squamous cell carcinoma of the head and neck;

## 1.3 Current applications and challenges of circulating tumor DNA (ctDNA) in SCCHN

This chapter is adapted from Galot R and Machiels JP (Cancer Treatment Reviews, invited review, submitted).

Liquid biopsies (LB) are emerging in the oncology field with promising data as new diagnostic, prognostic and treatment-monitoring tools.[234-236] LB refers to the sampling and analysis of several biological fluids as opposed to solid biopsy which analyzes a tissue sample.[237] The most popular form of LB is the analysis of blood samples, but LB can also refer to samples of saliva, urine, ascites, cerebrospinal fluids and pleural effusions. Different composites are exploitable in a blood-based biopsy to characterize tumors, including circulating tumor cells (CTCs),[238] circulating tumor DNA (ctDNA), circulating cell-free RNA, circulating extracellular vesicles (EVs), proteins and metabolites. Besides genomics, including the mutational profile and copy number variations, LB can provide information regarding transcriptomics,[239] epigenomics,[240] proteomics[241] or metabolomics.[242] Currently, only assays analyzing CTCs and ctDNA have been approved by the FDA. CTC enumeration (CellSearch®, Menarini Silicon Biosystems, Huntingdon Valley, Pennsylvania USA) is FDA-approved and has a prognostic value in metastatic breast,[243,244] colorectal,[245] and prostate cancers,[246] but its clinical utility is still to be demonstrated. The FDA also approved the cobas® EGFR Mutation Test v2 (Roche Molecular Diagnostics, Pleasanton, California, USA) to detect *EGFR* mutation in the cell-free DNA (cfDNA) of lung cancer patients;[247,248] and Epi proColon® (Epigenomics, Berlin, Germany) to assess the methylation status of the *SEPT9* promoter in cfDNA to screen patients for colorectal cancer.[249-251]

The gold standard for cancer diagnosis remains tissue biopsy as, in contrast to liquid biopsy, it provides histological features of the tumor in addition to molecular characterization. However, obtaining a tissue biopsy can sometimes be challenging and technically difficult due to disease localization. LB is easily accessible, less invasive and more comfortable for the patient. At the molecular level, LB should theoretically be able to capture intra-patient tumor molecular heterogeneity as it allows ctDNA released from multiple tumor regions to be analyzed. Therefore, it

should be able to reflect the different genomic alterations between the primary tumor and metastatic lesions.[252] In addition, LB may also address tumor heterogeneity over time[253] by enabling multiple samples to be taken over the course of the disease.

### 1.3.1 Potential clinical applications of ctDNA

ctDNA is cell-free DNA from tumoral origin that is essentially derived from apoptotic or necrotic tumour cells,[254,255] although active release is also implicated.[256] The analysis of ctDNA can provide information on tumor-specific genomic alterations including somatic mutations, copy number alterations, fusions or epigenetic alterations. Below, we briefly describe the potential clinical applications of ctDNA.

#### 1.3.1.1 Cancer diagnosis and early detection

The first potential application of ctDNA is cancer screening and early diagnosis in the hope that earlier treatment will lead to improved outcome. Some studies have focused on quantitative analyses of cfDNA as an indicator of tumor burden. Indeed, the level of cfDNA has been shown to be higher in cancer patients compared to healthy controls.[257,258] Moreover, cfDNA levels seem to correlate with tumor stage. However, knowing only the quantity of cfDNA is not particularly informative as other parameters, including inflammation, exercise, auto-immunity, smoking, pregnancy and heart dysfunction, can influence cfDNA levels.[259] In contrast, ctDNA is more attractive as it detects genomic alterations that are tumor-specific, and this has been explored in various studies. Bettegowda et al reported ctDNA detection across various cancer types in 82% of patients with stage IV and in 47% of patients with early stage disease.[260] Using the targeted error correction sequencing (TEC-Seq) approach, Phallen et al detected somatic mutations in the plasma of 71%, 59%, 59%, and 68 % of patients with early-stage (I/II stage) colorectal, breast, lung or ovarian cancers, respectively.[234] Another blood-based test, CancerSEEK, which combines mutations in cfDNA and circulating proteins, was positive in 70% of 1005 patients with non-metastatic clinically detected cancers (43%, 73%, and 78% in stage I, II and III, respectively).

Changes in methylation patterns are early events in carcinogenesis and may also be useful in cancer detection. Aberrant circulating DNA methylation patterns have been shown to be a potential tool for the early diagnosis of disseminated breast cancer[261] or ovarian cancer.[262] Copy number alterations detected by whole genome sequencing have led to the detection of early ovarian cancer.[263] These studies outline the potential application of early cancer detection through ctDNA analysis. However, important challenges remain before this evolving diagnostic field can be confidentially implemented. Clonal hematopoiesis, or the presence of somatic mutations within nonmalignant hematopoietic cells, is described in up to 10% of individuals aged over 65 years and this could lead to false positive results.[264,265] Furthermore, some studies have described the presence of somatic mutations in normal cells from apparently healthy individuals.[266,267] Recently, results of a substudy from the Circulating cell-free Genome Atlas (CCGA) study on 2301 participants (1422 cancer, 879 non-cancer), showed that targeted methylation technology had a sensitivity ranging from 59 to 86% (across cancer types and stages) with a 99% specificity.[268] This low false-positive rate is critical before population-based screening test can be implemented. Using a novel multivariate cancer risk score (NCRS) model that combines shallow whole genome sequencing of cfDNA and protein markers, Mao et al reported 71.8% sensitivity and 97.7% specificity for detection of multiple cancer types.[269]

#### 1.3.1.2 Prognosis and detection of minimal residual disease

ctDNA can be used to detect minimal residual disease (MRD), defined as the persistence of microscopic cancer after treatment that cannot be detected with current medical imaging modalities.[270] MRD is correlated with an increased risk of disease recurrence and poorer outcome. Several studies have shown the feasibility of ctDNA to detect MRD after curative treatment in various cancer types including breast[271] and colon cancers.[272] MRD detection often requires the identification of a somatic mutation in the solid tumor biopsy at baseline that will be followed longitudinally by digital polymerase chain reaction (PCR)[271] or next generation sequencing (NGS)-based assay.[272] However, Chaudhuri et al have shown that ctDNA may be useful to detect MRD even without prior knowledge of a tumor's

mutational status.[235] In a post-treatment sample, ctDNA detection was reported in 94% of lung cancer patients with disease recurrence. In these studies, ctDNA detection often preceded clinical recurrence.

#### 1.3.1.3 Characterization of molecular landscape, precision medicine and treatment selection

Several studies have shown that ctDNA has the potential to reflect the mutational landscape of a tumor. High concordance rates between liquid and solid biopsies have been reported for driver oncogenes, including *KRAS* and *EGFR*.[273,274] This can be of particular interest to identify actionable mutation(s). For example, many efforts have been made to detect *EGFR* mutations in the plasma of patients with lung cancer as obtaining biopsies can be challenging in lung cancer and *EGFR* mutations are predictive of response to EGFR tyrosine kinase inhibitors (TKI). Although the sensitivity of *EGFR* mutation detection in plasma was reported to be only 65.7%,[273] these tests can avoid invasive tumor biopsies to a significant number of patients.

#### 1.3.1.4 Monitoring of treatment and emergence of resistant clones

The short half-life of ctDNA and the ease of sampling make LB an interesting tool for monitoring treatment response longitudinally. ctDNA dynamics have been shown to correlate with tumor response in many cancers.[236,275,276] In breast cancer, ctDNA outperformed cancer antigen 15-3 in predicting response to chemotherapy and relapse.[236] A study in non-small cell lung cancer found that ctDNA could distinguish between residual disease and treatment-related imaging modifications.[276] This illustrates the complementary role that ctDNA can provide to imaging for disease monitoring. Somatic mutation analysis can also provide information regarding potential resistance mechanism(s) such as the emergence of *RAS* mutations in colorectal cancer patients[277] treated with EGFR-targeted therapies, or the appearance of the *EGFR* T790M mutation in lung cancer patients that confers resistance to gefitinib and erlotinib.[278]

### 1.3.2 Technical aspects of ctDNA somatic mutation analysis

The optimal sequencing technique should consider the end goal(s) of ctDNA analysis. The main challenge with ctDNA analysis is that the proportion of ctDNA can sometimes be extremely low (<1%).[275] Therefore, the detection of MRD, for example, will require a highly-sensitive technique that is able to detect ctDNA at very low proportions.

Table 1-6 and Figure 1-8 provide an overview of ctDNA sequencing approaches. There are multiple techniques to analyse ctDNA and these vary in the number of genomic alterations covered by the sequencing technique and in the limit of detection. The limit of detection is the lowest mutant allele fraction or the lowest allele concentration that will confidently discriminate the relevant mutations from the background noise.[279] Below this threshold, the analysis will be hampered by technical artefacts such as PCR or sequencing errors. The mutant allele fraction (expressed in percentage) is the ratio of the number of mutant reads divided by the total number of reads at a specific genomic position number, and the mutant allele concentration (expressed in copies per mL) is the number of mutant DNA fragments per unit volume.

The extent of ctDNA analysis can vary from a single mutation or a limited multiplex panel through to whole genome.

The cobas® *EGFR* mutation test v2 is an example of an allele-specific real-time PCR-based test which focuses on *EGFR* mutation detection with a limit of detection of 25 copies/mL. In digital-PCR based technologies, like digital droplet PCR (ddPCR)[280,281] or bead-emulsion-amplification-magnetics (BEAMing)[282], the partitioning of the DNA sample into thousands of individual fractions before PCR-amplification enables absolute quantification without standard curve and a lower limit of detection compared to real-time PCR. These techniques, however, only have the potential to detect a limited number of mutations of interest. They can be used for hotspot mutation detection[278] or detection of MRD.[271] In the latter, knowledge of the presence of specific mutations in the tumor tissue biopsy of each

individual patient is required. Advantages of these techniques include the absence of bio-informatic analysis and a rapid turn-around time.

Targeted NGS techniques, using PCR amplicons or hybrid-capture, enable broader gene panels to be targeted. For standard NGS techniques, detection is limited to variants with a mutant allele fraction of 1% because of the background error rate.[283-285] These errors can be reduced by implementing barcode based approaches or specific bioinformatic tools. For instance, the Safe-sequencing system (Safe-SeqS)[285] uses unique molecular identifiers (UMI) or molecular barcodes that will tag sequences from the same DNA fragment to identify possible errors. Personalized profiling by deep sequencing (CAPP-seq)[276] is a capture-based enrichment strategy that includes a specialized bioinformatic workflow. It can be further improved by the coupling of integrated digital error suppression (iDES) to eliminate artefacts observed in cfDNA sequencing.[286] These techniques do not need patient-specific tumor analysis and have the advantage over standard NGS approaches[283,287] to offer a lower limit of detection.

Finally, whole exome or whole genome sequencing offers the most comprehensive approach but at the cost of a lower limit of detection estimated at 5% because of the lower sequencing depth.[288] Shallow whole genome sequencing with a 0.1 to 0.2 x low sequencing coverage is used for copy number alteration detections.[289,290] The drawback of NGS-based techniques is the bioinformatic expertise needed to analyze the data and the higher cost compared to digital PCR-based techniques.

| Extend of analysis | Examples of technology | Assay limit of detection | Potential clinical applications |
|---|---|---|---|
| Single mutation or limited multiplex panel | BEAMING[291]<br>ddPCR[280,281] | 0.01%<br>0.001% | Detection of hot-spot mutations[278]<br><br>Disease monitoring and detection of minimal residual disease[271] |
| Targeted panel gene (10kb to 50Mb) | Standard NGS[283,292]<br>Safe-SeqS[285]<br>SiMSen-Seq[293]<br>CAPP-seq [276]<br>iDES | 1%<br>0.1%<br>0.1%<br>0.02%<br>< 0.01% | Mutational landscape[294]<br><br>Detection of actionable mutations[283,295]<br><br>Disease monitoring and detection of minimal residual disease[235,272,296]<br><br>Detection of *de novo* mutations implicated in resistance mechanisms |
| Whole Exome<br><br>Whole Genome | Hybrid-capture exome sequencing[288]<br><br>Plasma-seq[289] | >5%<br><br>5-10% | Detection of resistance mechanism [288]<br><br>Genomic landscape[289] |

**Table 1-6. Overview of sequencing technologies for cfDNA analysis (non-exhaustive)**

BEAMING, bead-emusion-amplification-magnetics; CAPP-seq, personalized profiling by deep sequencing; ddPCR, digital droplet PCR; iDES, integrated digital error suppression kb, kilobases Mb, megabases; NGS, next generation sequencing; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing

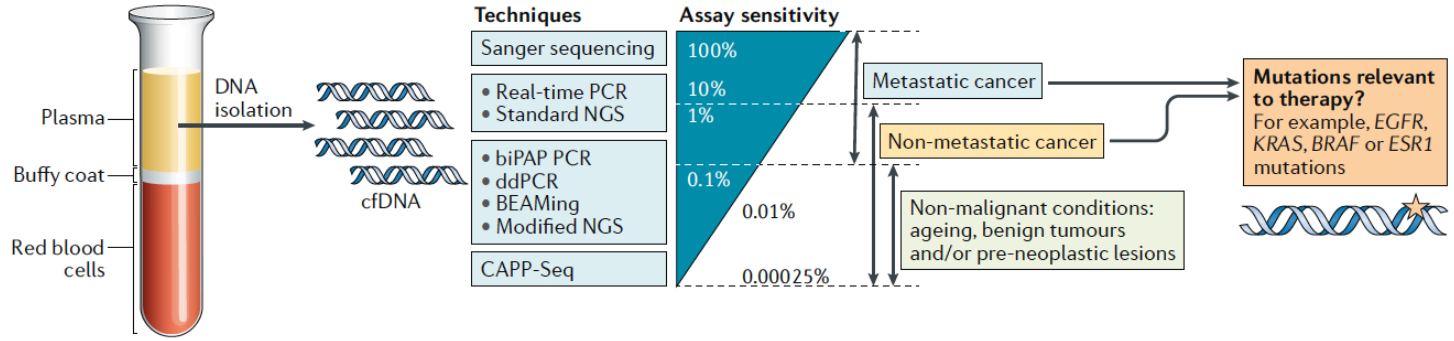


**Figure 1-8. Sequencing technologies for cfDNA analysis**

Cell-free DNA (cfDNA) can be extracted from plasma samples and sequenced to detect tumor-specific genomic alterations. The schema represents the possible sequencing techniques that can be used with their respective assay sensitivity. It also shows the range of circulating tumor DNA that have been reported at different stages of cancer disease (metastatic versus non-metastatic), and highlights that cfDNA with tumor-associated mutations can also be found in healthy individuals (benign or pre-malignant lesions, clonal haematopoiesis).

BEAMING, bead-emusion-amplification-magnetics; CAPP-seq, personalized profiling by deep sequencing; ddPCR, digital droplet PCR; NGS, next generation sequencing; PCR, polymerase chain reaction. Figure adapted from Pantel et al.[270]

### 1.3.3 cfDNA in SCCHN

Some studies have explored LB as a diagnostic, prognostic or monitoring tool for SCCHN, but data are limited compared to other cancers and there is currently no clinical application. LB could, however, be of interest at different stages of SCCHN disease. For instance, 40 - 50% of patients treated with curative intent will recur. The suspicion of local relapse is currently based on physical examination, including flexible head and neck fiberoptic endoscopy and imaging. The differential diagnosis with treatment-induced fibrosis or radionecrosis can be difficult, leading to delayed diagnosis and treatment. Additionally, in some cases of locoregional recurrence without distant metastases, salvage surgery or (chemo)radiation can still be curative. Therefore, early diagnosis of locoregional recurrence is primordial. ctDNA could be useful to distinguish the difference between local relapse and treatment-related complications, and to detect MRD, with the ultimate goal of identifying patients who need additional treatment.

Another interesting field for LB is precision medicine. The characterization of the SCCHN molecular landscape has identified potential therapeutic targets with new treatment opportunities.[193,297] SCCHN is a heterogenous disease, and multiple molecular assessments during the disease course may be required to adapt the treatment strategy. Identifying potentially actionable alterations through LB could be an attractive alternative when obtaining a biopsy is challenging or not feasible.

Blood-based DNA analysis for SCCHN includes not only the detection of tumor-specific mutations but also the detection of the human papillomavirus (HPV) DNA for HPV-positive oropharyngeal cancer.[298-300] Furthermore, different studies have investigated ctDNA microsatellite alterations,[301,302] methylation levels,[303,304] and copy number aberrations.[305]

#### 1.3.3.1 ctDNA detection trough somatic mutation analysis

Somatic mutations in ctDNA can be analyzed using two different approaches: a *targeted approach* and a *tumor-agnostic approach*. The targeted approach identifies genomic alteration(s) in tumor tissue that can be used as marker(s) for ctDNA presence through different high-sensitive sequencing techniques. Another approach is the *tumor-agnostic approach* or *non-targeted approach*, whereby ctDNA is analyzed without prior knowledge of the mutational status of the tumor.[235] This could also be of high interest when tumor biopsies are not feasible or difficult.

A. Targeted approach

Different studies in SCCHN have used a targeted approach to detect ctDNA in plasma based on somatic mutation(s) previously identified in the tumor (Table 1-7). The main clinical applications for this approach are baseline ctDNA detection and disease monitoring or minimal residual disease detection.

The largest study was reported by Wang et al.[306] The group explored the detection of tumor-specific DNA in the saliva and/or plasma of 93 SCCHN patients including 20 patients (22%) with early stage (I or II) disease. At least one genomic alteration was detected in the tumor of each patient including the detection of HPV16 DNA in 30 patients (32%), a somatic mutation in genes commonly altered in SCCHN in 58 patients (62%), or another driver mutation/translocation in the last five patients. Subsequently, digital PCR or Safe-SeqS for the detection of HPV DNA and point mutations/translocation, respectively, was applied to the saliva (n=93) and plasma (n=47). The investigators detected ctDNA in 87% of the 47 patients whose plasma was tested. This detection rate increased to 96% when both plasma and saliva were combined. Importantly, it was demonstrated that a limited panel of genes, containing HPV DNA 16 sequences and the most common altered genes in SCCHN (*TP53*, *PIK3CA, CDKN2A, NOTCH1*), led to the detection of ctDNA in 95% of SCCHN. Therefore, ctDNA detection seems feasible in SCCHN when highly sensitive techniques are used.

In the same patient cohort, 14 patients with sequenced plasma had locoregional recurrent disease. ctDNA was detected in all of them, highlighting the potential of ctDNA to help diagnose recurrent disease. Although it was not the main purpose of their work, Wang and colleagues also examined the follow-up samples of nine patients who had detectable ctDNA before surgery. In three of these patients, tumor DNA was detected in the saliva or plasma after surgery and before clinical relapse, but none of the five patients without recurrence had detectable tumor DNA. The same investigators also showed on this limited number of patients that those with undetectable tumor DNA in their saliva/plasma after surgery had a better disease-free survival, suggesting a prognostic value.

In contrast, Perdomo[307] et al detected ctDNA in only 42% of patients using a targeted approach. As cfDNA samples used for this analysis were up to 10 years old, sample storage and handling may explain these modest results.

Van Ginkel et al[308] focused on the *TP53* gene. His group performed *TP53* testing in surgically resected primary tumor samples of six SCCHN patients, and designed mutation-specific ddPCR assays to detect the mutation in plasma. The mutation was detected in all six plasma samples with an allele frequency down to 0.01%, demonstrating that a highly sensitive PCR-based technique could be used as a post-treatment monitoring tool in SCCHN.

The first study focusing on detection of minimal residual disease was recently published.[309] Seven patients, with identifiable mutations in the primary tumor, were tested for the presence of these same patient-specific mutations in their plasma. Plasma-sequencing assays (SiMSen-Seq) were designed based on the mutations identified in the tumors (2-7 mutations per patient). In six patients (86%), ctDNA mutations were detected at baseline and these were followed for up to 18 months after therapy. Out of the four patients diagnosed with clinical recurrence, three had at least one mutation detected in their longitudinal plasma samples.

All these studies demonstrate that targeted approaches based on high-sensitive techniques are feasible with promising potential clinical applications.

| Study | N | Sample | Analysis | Technique(s) | Outcome |
|---|---|---|---|---|---|
| **Wang et al, 2015**[306] | 93 SCCHN<br>Stage I-II (n=20)<br>Stage III-IV (n=73)<br>Oral cavity (n=46)<br>Oropharynx (n=34)<br>Larynx (n=10)<br>Hypopharynx (n=3) | Tumor, plasma and/or saliva | Somatic mutations or HPV gene | Identification of at least 1 genetic alteration in each tumor<br>(HPV16 DNA detection (n=30), somatic mutation of one of the following genes *TP53, PIK3CA, CDKN2A, FBXW7, HRAS, and NRAS*) (n=58) or other driver mutation/translocation (n=5))<br><br>DNA detection in saliva/plasma:<br>- Digital PCR for HPV16 DNA (*E7* gene)<br>- Safe-SeqS for translocations and point mutations | ctDNA detection:<br>- plasma alone: 87% (n = 47)<br>- saliva alone: 76% (n = 93) (100% for oral cavity)<br>- plasma and saliva: 96% (n = 47)<br><br>Detection of tumor DNA in saliva or plasma post-surgically in 3 patients before clinical recurrence, but in none of the 5 patients without recurrence. |
| **Van Ginkel et al, 2017**[308] | 6 SCCHN (stage II-IV) | Tumor Plasma | Patient-specific *TP53* mutation detected in each tumor | *TP53* determined in surgically resected primary tumor samples<br><br>Mutation-specific ddPCR assays for pre-treatment ctDNA detection | *TP53* mutations detected in all plasma samples with fractional abundance down to 0.01% |
| **Egyud et al, 2019**[309] | 8 SCCHN at diagnosis | Tumor Plasma | Somatic mutations | Tumor sequencing to identify specific mutations<br><br>ctDNA analysis: SiMSen-Seq<br>(range 2-8 mutations per patient) | Baseline ctDNA positive in 6 patients.<br><br>ctDNA detection in 3/4 patients with recurrence. |
| **Perdomo et al, 2017**[307] | 36 HPV-negative SCCHN with known tumor mutation | Tumor and Plasma | Targeted approach to detect mutations in plasma, previously detected in the tumor | Plasma: Targeted sequencing (amplicon-based, ion torrent™ proton sequencer) in 5 genes<br>(*TP53, NOTCH1, CDKN2A, CASP8, PTEN*) | ctDNA detection in 15/36 (42% of patients)<br><br>Across 36 SCCHN patients, 65 mutations in 5 genes detected in the tissue samples, only 28% detected in plasma |

**Table 1-7. Selected studies focusing on ctDNA in SCCHN using a targeted approach**

AF, allele frequency; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; HPV, human papillomavirus; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; PCR, polymerase chain reaction; SCCHN, squamous cell carcinoma of the head and neck

B. Tumor-agnostic approach

Non-targeted or tumor-agnostic approaches sequence cfDNA without knowing the genomic alterations of the corresponding solid tumor. The main clinical applications are characterization of the molecular landscape, identification of actionable events and investigation of treatment resistance mechanisms.

Table 1-8 provides an overview of tumor-agnostic studies performed in SCCHN. Mazurek et al[258] studied the cfDNA of 200 patients with the primary aim of correlating cfDNA quantification with clinical characteristics. Higher cfDNA levels were found in oropharyngeal cancer as well as in clinical N2-N3 and in stage IV disease. The investigators also looked for plasmatic HPV16/18 DNA, *KRAS* G12C and *EGFR* mutations. HPV DNA was detected in 14% of patients but no *KRAS* or *EGFR* mutations were detected; these two latter alterations are, however, not frequent in SCCHN. Perdomo et al[307] also explored the non-targeted approach by sequencing the *TP53* gene independently in the tumor, saliva and plasma of 37 patients with late-stage SCCHN. As expected, *TP53* mutations were detected in 28/37 of solid tumor biopsies (76%), but surprisingly, these mutations were only observed in three plasma samples. Only in one patient was the same mutation detected in tumor, plasma and oral rinses. Again, the low plasmatic detection rate observed by Mazurek and colleagues might be explained by the long storage of plasma samples. An important finding of the study was the identification of *TP53* mutations in the oral rinses of a few non-cancer individuals with an allele frequency ranging from 0.001 - 0.004%. The detection of *TP53* mutations has been previously reported in the plasma, peritoneal fluid and skin of healthy controls.[266,267,310,311] Clonal hematopoiesis has also been reported as potential origin of false-positives.[264] These findings need to be taken into account when considering ctDNA for early cancer detection.

A targeted NGS-approach has been used to screen for the emergence of specific *EGFR* ectodomain or activating *RAS* mutations in patients treated with cetuximab-based therapy.[312] The primary tumor at baseline before cetuximab treatment was analyzed for the same specific mutations. The appearance of an activating *RAS* mutation was found in the cfDNA of 6/13 (46%) patients that had progressive disease during cetuximab-based treatment, while all the tumor baseline samples of these patients were *RAS* wild type. This underlines the possibility to detect hotspot mutations using ultra-deep NGS testing as well as *de novo* mutations possibly

implicated in treatment resistance. The detection of a hotspot *PIK3CA* mutation was also reported in 9/29 (31%) patients with treatment-naïve SCCHN using an allele specific amplification technique.[313] This could be of clinical interest as *PIK3CA* p.E545K is a potentially targetable event, and the technique that is used has a short turn-around time. Recently, another group detected *HRAS* mutations in three patients with locally-advanced SCCHN that were not present in the baseline tumor samples.[295] Furthermore, the investigators described the appearance of *HRAS* mutations in three other patients who progressed on nivolumab. Such data could potentially inform a targeted treatment strategy as SCCHN patients harboring a *HRAS* mutation could potentially benefit from tipifarnib, a farnesyltransferase inhibitor.[297]

| Study | N | Sample | Analysis | Technique(s) | Outcome |
|---|---|---|---|---|---|
| **Mazurek et al, 2016**[258] | 200 SCCHN | Plasma | cfDNA quantification<br><br>HPV16/18 DNA detection<br><br>Mutations in *EGFR/KRAS* | Plasma DNA concentration by Taqman- based TERT amplification<br><br>Taqman-based E6/E7 HPV16 and HPV18 DNA<br><br>TaqMan-MGB assay for *KRAS* G12C and *p.E746—A750del* mutation of *EGFR* | Higher cfDNA levels in OPSCC, in clinical N2-N3, and in stage IV<br><br>14% of HPV-positive based on plasma analysis<br><br>No detection of the pre-defined *EGFR/KRAS* mutations |
| **Braig et al, 2016**[312] | 20 SCCHN treated with cetuximab, 13 with progressive disease under cetuximab | Serum (post-cetuximab)<br><br>Tumor (baseline, before cetuximab) | *RAS* mutation and epitope-modifying *EGFR* mutations | NGS to screen *EGFR* exon 12, *KRAS/NRAS* exon 2,3,4 and *HRAS* exon 2/3 (mean number of > 20,000 reads per exon) | 6/13 patients with progressive disease during cetuximab-based therapy acquired activating *RAS* mutations detectable in cfDNA<br><br>No *EGFR* ectodomain mutations were found |
| **Perdomo et al, 2017**[307] | 37 stage III-IV SCCHN | Tumor, plasma, and oral rinses | Independent *TP53* mutation detection in each sample type: tumor, plasma and oral rinses | Sequencing of entire coding region of *TP53* gene | *TP53* mutations in 28/37 of solid tumor cases (76%), but only in 3 cfDNA (8.1%)<br><br>*TP53* mutations in oral rinses higher for oral cavity and oropharynx<br><br>Low concordance between tumor, oral rinses, plasma |
| **Porter et al, 2017**[314] | 60 HNC<br>OPSCC (n=21)<br><br>Salivary gland and thyroid cancer | Blood<br><br>ctDNA data compared to tumor NGS when available (n=29) | 70 gene panel | Guardant360, a 70-gene ctDNA NGS platform | Most frequent mutations: *TP53* (98%), *PIK3CA* (43%), *NOTCH1* (38%), and *ARID1A* (36%).<br><br>73% (n = 22) of patients had alterations identified in ctDNA, not present in tumor specimens<br><br>Actionable mutations in 66% of SCCHN and in 50% of salivary gland |
| **Soulières et al, 2018**[141] | 112 recurrent/metastatic SCCHN, after platinum | Plasma<br><br>archival tumor | 542 gene panel<br><br>44 gene panel | Targeted NGS with a panel of 542 genes | Molecular landscape: most frequently mutated genes: *TP53, FAT1, TET2, KMT2D, PIK3CA, NOTCH1, NFE2L2, NOCTH2, CCND1, CDKN2A*<br>Concordance archival tissue - ctDNA:<br>89% for HPV, 63% for *TP53* status |

| | | | | | |
|---|---|---|---|---|---|
| **Schmidt et al, 2018**[313] | 29 treatment naïve SCCHN patients (stage III-IV) | Plasma | *PIK3CA* p.E545K mutation | PlexPrime™ and PlexZyme™ technology in real-time PCR, allele-specific amplification technology | *PIK3CA* E545K detection in 9/29 patients (31%) |
| **Ping Wu et al, 2018**[315] | 27 SCCHN | Plasma and saliva pre- and post-surgery<br><br>Tumor | 1021 gene panel | Targeted NGS with a panel of 1021 genes | Tumor-specific mutations observed in 70% in plasma and 52% in saliva<br><br>ctDNA more likely detected in saliva from oral cancer (83%) versus 46% and 40% in pharynx and hypopharynx<br><br>Disease-free-survival was lower in patients with detectable postoperative ctDNA compared to undetectable |
| **Fostira et al, 2019**[295] | Cohort 1: 54 locally-advanced SCCHN<br><br>Cohort 2: 15 metastatic SCCHN | Cohort 1: Plasma at baseline, end of treatment and PD<br><br>Tissue at baseline<br><br>Cohort 2: Plasma at baseline and post 2nd cycle | *TP53, CDKN2A, HRAS* and *PIK3CA* | Plasma and tissue: Sequencing with SafeSEQ (*TP53, CDKN2A, HRAS* and *PIK3CA*) | Cohort 1: ctDNA detection in 56% of patients whose tumor harbored mutation at baseline<br>In 3 patients, detection of *HRAS* mutations in plasma, not detected in tissue at baseline<br>At progression, new mutations were detected in 40%<br><br>Cohort 2: ctDNA detection in 93% of metastatic patients prior to and during treatment with nivolumab<br>3 patients had emergence of *HRAS* mutations during treatment |

**Table 1-8. Selected studies focusing on ctDNA in SCCHN using a non-targeted approach**

cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; HPV, human papillomavirus; HNC, head and neck cancer; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; SCCHN, squamous cell carcinoma of the head and neck; TERT, human telomerase reverse transcriptas

#### 1.3.3.2 Analysis of cfDNA for methylation patterns, microsatellite alterations or copy number alterations

Other cfDNA analyses can be performed, including the investigation of methylation patterns (Table 1-9). Mydlarz[304] et al investigated the ability of three methylation probes (endothelin receptor type B (*EDNRB*), deleted in colorectal carcinoma (*DCC*), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) hypermethylation probes) to detect altered methylation in serum samples from 100 patients with untreated SCCHN and 50 healthy controls. *EDNRB* hypermethylation was reported in the serum of 10% of patients whilst none was observed in the control samples, highlighting a high specificity for this test but limited sensitivity as a biomarker for SCCHN. In contrast, Schrock et al[303] reported that *Short stature homeobox 2* (*SHOX2*) and *Septin 9* (*SEPT9*) DNA methylation had a higher sensitivity (52%) and a specificity of 95%. They also found that methylation levels predicted overall survival (OS) and correlated with tumor and nodal category.

Different studies have investigated microsatellite alterations in ctDNA.[301,302] Nawroz et al detected microsatellite alterations in the serum of 47% patients, and these were identical to the ones seen in the corresponding tumors. Patients with microsatellite alterations detectable in their cfDNA had a higher rate of distant metastasis but there was no correlation with OS. Hamana[302] et al also looked at the microsatellite status of patients with SCCHN and analyzed pre- and post-operative samples. Allelic imbalances (AI) were observed in 38 out of 64 tumor tissues. They detected at least one AI in the serum of 33 of these 38 patients. All the serum AI patterns were matched with the corresponding tumor. Serum AI were detected preoperatively in 44% (28 of 64) and postoperatively in 20% (13 of 64) of patients. In the pre-operative positive patients, 20 (71%) had no detectable ctDNA postoperatively and none recurred. Two patients with detectable post-operative ctDNA died within 44 weeks of surgery.

Finally, copy number alterations (CNA) can also be analyzed through cfDNA. Schirmer et al analyzed ctDNA derived CNA in SCCHN patients with low-coverage NGS.[305] They calculated a copy number instability score (CNI) from the number of copy imbalances as a general measure of the genomic instability that occurs in the

tumor and in the concentration of ctDNA. They found sensitivity for tumor detection that ranged from 46% for pT1 tumors to 94% for pT4. Patients with a CNI above the median (>72) had worse survival with an OS survival rate at three years of 62% compared to 90% in patients with CNI < 72.

| Study | N | Sample | Analysis | Technique(s) | Outcome |
|---|---|---|---|---|---|
| **Mydlarz et al, 2016**[304] | 100 SCCHN<br>50 healthy controls | Serum | *EDNRB*, *CDKN2A* and *DCC* DNA methylation | Quantitative methylation specific PCR (qMSP) | *EDNRB* hypermethylation in 10% of SCCHN patients versus none in the control group |
| **Schrock et al, 2017**[303] | 649<br>Training study: 284 SCCHN/122 controls<br><br>Validation study: 141 SCCHN/102 controls | Plasma | *SHOX2* and *SEPT9* DNA methylation | Methylation was determined using SHOX2/SEPT9/ACTB (actin beta) triplex quantitative PCR | Training study:<br>Sensitivity: 59% Specificity: 96%<br><br>Higher risk of death in patients with positive SEPT9 and SHOX2 plasma levels compared to negative patients<br><br>Disease relapse detected in 47% of patients before clinical recurrence<br><br>Validation study:<br>Sensitivity: 52%, Specificity: 95%,<br><br>OS prediction: patients with positive SEPT9 and SHOX2 plasma levels were at higher risk of death compared to methylation-negative patients<br><br>Correlation with tumor and nodal category |
| **Hamana et al, 2005**[302] | 64 OSCC | Serum: preoperatively, postoperatively, and 4 weeks post-operatively<br><br>Tumor samples and normal controls | Microsatellite blood assay (9 microsatellite markers) | PCR | At least 1 allelic imbalance in cfDNA in 87% (33 of 38) of the patients who had an allelic imbalance detected in tumor DNA |
| **Nawroz-Danish et al, 2004**[301] | 152 SCCHN | Tumor<br>Serum | Microsatellite blood assay | PCR | 45 % (68/152)<br>had microsatellite alterations of ctDNA identical to the corresponding tumor DNA.<br><br>Patients with microsatellite |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | alterations detectable in their cfDNA had higher rate of distant metastasis (16.2% (11/68) versus 6% (5/84), RR=2.7)<br><br>OS did not differ between positive and negative serum tests |
| **Schirmer et al, 2018**[305] | 103 SCCHN without distant metastasis<br><br>Control group: 142 tumor-free individuals | Plasma | ctDNA-derived CNA | Low-coverage NGS | CNAs were combined in a genome-wide copy number instability score (CNI):<br>ctDNA analysis to detect tumors:<br>- specificity :95%<br>- sensitivity: 46% (pT1), 94% (pT4)<br><br>CNI above the median (>72), OS was worse |

**Table 1-9. Selected studies focusing on cfDNA methylation patterns, microsatellite alterations or copy number aberrations**

CDKN2A, cyclin-dependent kinase inhibitor 2A ;cfDNA, cell-free DNA; CNA, copy number alterations; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; DCC, deleted in colorectal carcinoma; *EDNRB*, endothelin receptor type B; HPV, human papillomavirus; Safe-SeqS, safe-sequencing system; SiMSen-Seq, simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing; SHOX2, *Short stature homeobox 2*; SEPT9, *Septin 9* ; SCCHN, squamous cell carcinoma of the head and neck; PCR, polymerase chain reaction; qMSP, quantitative methylation specific PCR; OS, overall survival; RR, relative risk

#### 1.3.3.3 Circulating HPV DNA

In HPV-related OPC, there is expression of viral-specific oncogenes (E6 and E7).[316] Some studies have focused on the detection of these viral oncogenes in LB to use HPV cfDNA as a biomarker for HPV-positive OPC (Table 1-10). Ahn et al reported a sensitivity and a specificity of 76% and 100%, respectively, when HPV DNA analysis of pretreatment saliva and plasma samples were combined.[298] The sensitivity of LB alone was 67.5%. More recently, Chera and colleagues reported a sensitivity of 89% with a more sensitive digital PCR technique.[317]

HPV cfDNA levels seem to correlate with tumor burden as patients with stage IV and high N category had a higher rate of detectable pretreatment serum HPV DNA as reported by Dahlstrom et al.[65] This was confirmed by Hanna et al[318] who tested HPV cfDNA in 17 patients with R/M disease. The median HPV cfDNA levels increased with disease spread: 4.6 copies/mL in locoregional disease, 163 copies/mL in pulmonary-only disease, and 452.8 copies/mL in extra-pulmonary disease.[318] In this study, the median plasma HPV cfDNA level negatively correlated with survival, suggesting a prognostic value. The opposite was however shown in another study where low pre-treatment HPV cfDNA was associated with worse outcome, possibly related to lower tumor HPV copy numbers and greater likelihood of HPV integration which are adverse tumor genomic features.[317]

Finally, evidence is emerging that cfDNA could be used for monitoring. One study reported that HPV DNA detected in post-treatment saliva was associated with a higher risk of recurrence;[298] in two other studies, changes in HPV cfDNA levels confirmed treatment response or progression.[318,319] Recently, Chera and colleagues showed in a prospective biomarker trial that a rapid clearance profile of HPV cfDNA, defined as having a high baseline copy number (> 200/mL) and > 95% clearance by day 28 of CRT, could predict disease control in patients with HPV-positive OPSCC treated with definite CRT.[317] However, patients with detectable HPV cfDNA at the end of week 6 of CRT did not have a higher incidence of residual disease or recurrent disease.

| Study | N | Sample | Analysis | Technique(s) | Outcome |
|---|---|---|---|---|---|
| **Cao et al, 2012**[319] | 40 HPV-positive OPSCC<br>24 HPV-negative SCCHN<br>10 non-cancer | Plasma<br>Tumor | HPV 16/18 DNA E6 and E7 | Plasma: real-time PCR<br><br>Tumor HPV status confirmed by p16 IHC, HPV 16/18 PCR or HPV ISH | HPV cfDNA detected in 65% of pretreatment plasma samples in HPV-positive OPC and in none of HPV negative SCCHN or non-cancer controls.<br><br>Serial measurement in 14 patients showed rapid decline in HPV DNA and undetectable levels at RT completion |
| **Ahn et al, 2014**[298] | 93 OPSCC or unknown primary SCC (81 HPV-positive, 12 HPV-negative) | Tumor<br>Salivary rinses<br>Plasma | HPV 16 DNA E6 and E7 | Real-time PCR | Combined saliva and plasma analysis on pretreatment samples (HPV-16 DNA) compared with HPV status of the tumor<br><br>Sensitivity: 76% (plasma alone: 67.3% and saliva alone: 52.8%)<br>Specificity: 100%<br>Negative predictive value: 42%, Positive predictive value: 100%<br><br>In a multivariable analysis, positive posttreatment saliva HPV status was associated with a higher risk of recurrence |
| **Dahlstrom et al, 2015**[300] | 262 newly diagnosed, previously untreated OPSCC | Tumor<br>Pretreatment serum | HPV 16 DNA E6 and E7 | Tumor HPV status determined by PCR<br><br>Serum HPV DNA detection by real-time PCR | Stage IV and high N category had a higher rate of detectable pretreatment serum HPV DNA<br><br>HPV-positive patients with negative pretreatment HPV DNA serum had a trend of better PFS |
| **Hanna et al, 2018**[318] | 17 recurrent/metastatic HPV-positive OPSCC | Plasma: baseline sample and up to 5 additional plasma samples at 14-21 days interval | HPV DNA 16/18/31/33/45 E7 | ddPCR | Total tumor burden correlated with HPV cfDNA levels<br><br>Changes in HPV cfDNA levels with a median of 16 days prior to restaging scans confirmed treatment response or progression |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | Median HPV cfDNA levels negatively correlates with survival |
| **Chera et al, 2019**[317] | 103 non-metastatic p16-positive OPSCC after definitive CRT<br><br>55 healthy volunteers and 60 with non-HPV associated malignancy | Plasma at baseline, weekly during CRT and at follow-up | HPV DNA 16/18/31/33/35 E7 | ddPCR | HPV cfDNA testing<br>Sensitivity: 89%<br>Specificity: 97%<br><br>HPV cfDNA Kinetics (n=67):<br>19/67 (28%) had favorable clearance profile: 100% of persistent/recurrent regional disease-free survival (RDFS) at 18 months |

**Table 1-10. Selected studies focusing on HPV cell-free DNA**

cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; CRT, chemoradiation ddPCR, digital droplet PCR; HPV, human papillomavirus; IHC, immunohistochemistry, ISH, in situ hybridization; OPSCC, oropharyngeal squamous cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; PCR, polymerase chain reaction; squamous cell carcinoma of the head and neck; RT, radiotherapy; PFS, progression-free survival.

#### 1.3.3.4 Saliva

Most of the studies that investigate LB in SCCHN are based on blood analyses, although another interesting "liquid" in this disease, as previously mentioned, is saliva. Saliva can contain molecular biomarkers through ultrafiltration from blood, passive diffusion, or active transport.[320] Moreover, DNA can be released immediately into the upper aerodigestive tract by the necrosis of SCCHN epithelial cells. In the study by Wang et al, tumor DNA was detected in 76% of saliva samples (n=93). Combined analysis of saliva and plasma increased the detection of ctDNA from 87% to 96% compared to plasma analysis alone. This same trend was observed for the detection of HPV cfDNA when plasma and saliva samples were combined.[298] The sensitivity of ctDNA detection in saliva seems to be site-dependent. Tumor DNA detection was achieved in 100% of oral cavity cancers but only in 47%, 70% and 67% of oropharynx, larynx, and hypopharynx cancers, respectively.[306] Perdomo et al[307] described a *TP53* mutation detection rate in oral rinses that was also higher in oral cavity (54.2%) and oropharynx (60%) cancers compared to larynx cancer (16.7%).

# 2. AIMS AND OBJECTIVES OF THE THESIS

## 2.1 To design a biomarker-driven clinical trial for recurrent/metastatic SCCHN

We hypothesized that having a common biomarker screening platform for recurrent/metastatic SCCHN could be an effective strategy to identify patients upfront who will receive the greatest benefit from the novel available targeted agents. Therefore, the first objective of this thesis was to design a biomarker-driven clinical trial for recurrent/metastatic SCCHN.

## 2.2 To study the utility of liquid biopsy for recurrent/metastatic SCCHN

Obtaining a new biopsy of the disease at recurrence or metastatic SCCHN can be challenging. Liquid biopsy is a non-invasive tool to detect circulating tumor DNA in various types of cancer, as discussed in the previous chapter. Therefore, the second objective of this thesis was to study the utility of liquid biopsy to characterize the mutational profile of recurrent/metastatic SCCHN and, when possible, to investigate the concordance between the variants identified by liquid and solid biopsies.

# 3. THE EORTC-1559-HNCG TRIAL: BACKGROUND AND STUDY DESIGN

Adapted from Galot et al (2018)[193]:

## "Personalized biomarker-based treatment strategy for patients with squamous cell carcinoma of the head and neck: EORTC position and approach."

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**Abstract**

The molecular landscape of squamous cell carcinoma of the head and the neck (SCCHN) has been characterized and actionable or targetable genomic alterations have been identified. However, targeted therapies have very limited activity in unselected SCCHN and the current treatment strategy is still based on tumor location and disease stage and not on tumor biology**.**

Trying to select upfront the patients who will benefit from a specific treatment might be a way to improve patients' outcome. With the objective of optimizing the activity of targeted therapies and immunotherapy, we have designed an umbrella biomarker-driven study dedicated to recurrent and/or metastatic SCCHN patients (EORTC-1559-HNCG).

In this chapter, we review the background of each treatment cohort and we describe the design of the EORTC-1559-HNCG protocol.

## 3.1 Introduction

The current treatment strategy of patients with SCCHN is still based on tumor location and disease stage and not on tumor biology.[87,112,113] Targeted therapies have shown disappointing results.[183,188,189] Trying to select upfront the patients who will benefit from a specific treatment might improve the outcome. Our main objective was to design a biomarker-driven study dedicated to SCCHN patients. Below, we describe the overall study design as well as the different treatment cohorts of the EORTC-1559-HNCG trial, the first international biomarker-driven umbrella trial in recurrent SCCHN.

## 3.2 EORTC-1559-HNCG design

The EORTC-1559-HNCG trial is a biomarker-driven umbrella trial that enrolls patients with recurrent/metastatic SCCHN, progressing after first-line platinum-based chemotherapy. Each patient must undergo a fresh tumor biopsy. NGS is performed to identify somatic mutations and copy number alterations with a custom panel that has been designed for the trial. This panel covers 13 oncogenes and tumor suppressor genes: *EGFR, HER2, TP53, PIK3CA, CCND1, NRAS, KRAS, HRAS, PTEN, FGFR1, FGFR2, FGFR3,* and *cMET*. The analysis also includes p16 (p16 positive = Histo-score ≥ 210) and PTEN (PTEN High = Histo-score > 150) determined by IHC.[321] All these analyses are performed centrally in an ISO 15189 certified laboratory (OncoDNA, Belgium). mRNA FGFR expression will be evaluated by RNA-scope in another centralized laboratory (Targos, Germany). The details of the methodology can be found in the supplementary data (supplementary data 8.2).

Based on the molecular alterations identified, each patient is allocated to one of the cohorts. If the patient is not eligible for one of the biomarker-driven cohorts, he/she is included in one of the immunotherapy cohorts. The global design of the trial as well as the molecular rules for treatment allocation and prioritization are depicted in figures 3-1 and 3-2.

The full protocol includes a core protocol and several addenda. The core protocol describes the overall study design, the objectives and endpoints, the inclusion/exclusion criteria, the study flow chart, the statistical hypotheses, the data analysis plan, and the biobanking processes. For each experimental treatment,

there is one separate addendum that contains the confidential information related to the drug. The national health regulatory authorities, the ethical committee, and the investigators have access to the core protocol and all the addenda. The pharmaceutical companies have access to the core protocol but they can view and comment only the addendum/addenda concerning the cohort(s) for which they are supporting.

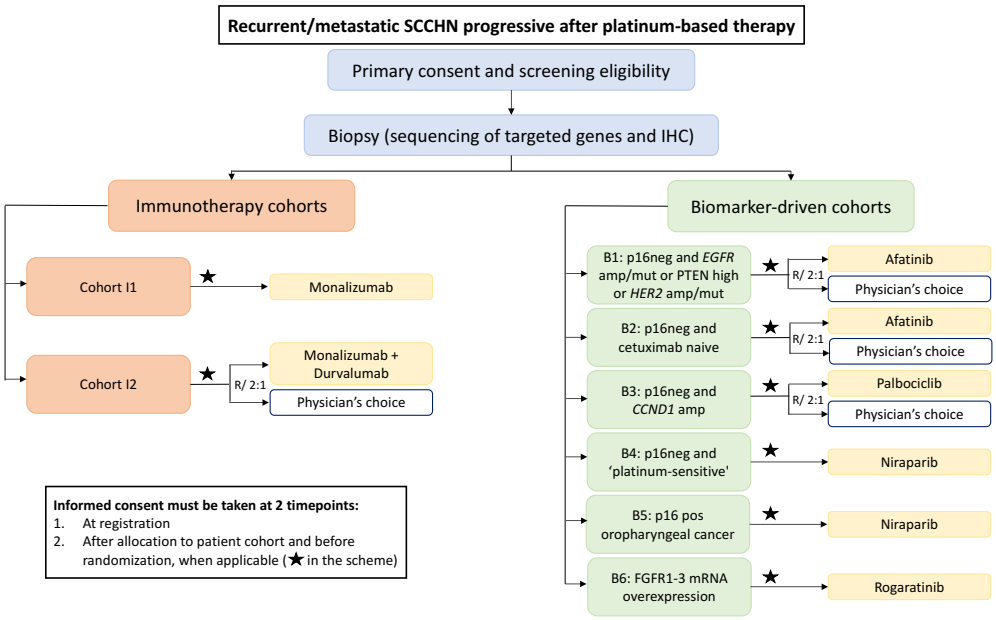


**Figure 3-1. General design of the EORTC-HNCG-1559 umbrella trial**

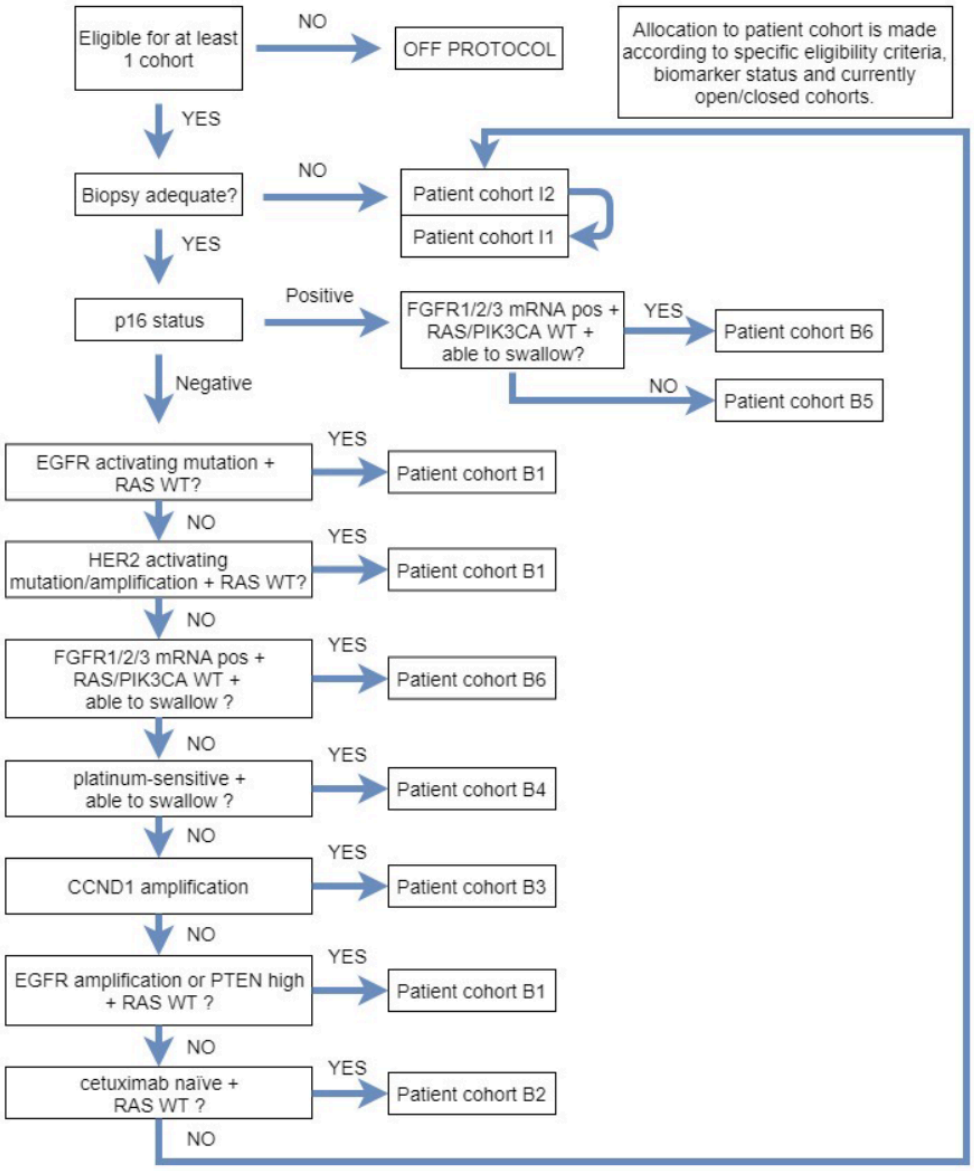


**Figure 3-2. Prioritization algorithm for the allocation to different patient cohorts**

## 3.3 EORTC-1559-HNCG biomarker-driven and immunotherapy cohorts

Each patient cohort is designed as a phase II study with its own statistical hypothesis (Table 3-1). The primary endpoint is either objective response rate (ORR) or PFS rate. Sample sizes vary from 20 to 57 patients across cohorts. The study can be amended to add other cohorts based on drug availabilities or other biomarker hypotheses.

### 3.3.1 Pan-human epidermal growth factor receptor (HER) inhibitor cohorts

*EGFR* mutations/amplifications are described in 15% of HPV-negative SCCHN and *HER2* is altered (mutation/amplification) in 5%.

The ORR with cetuximab monotherapy is 13%.[322] In contrast to colon cancer where *RAS* mutations are predictive markers of resistance, *RAS* alterations are found in only 4% of HPV-negative SCCHN. Although *RAS* mutations might also play a role in cetuximab resistance in SCCHN, [312] other mechanisms including activation of other HERs could be involved.[323,324]

Pan-HER inhibitors target all the dimers forms by HER family and have the potential to overcome anti-EGFR therapy resistance caused by cross-talk between EGFR and the other HERs. In unselected SCCHN patients who progress after platinum therapy, afatinib, an irreversible pan-HER inhibitor, improves PFS compared with methotrexate: median PFS 2.7 versus 1.6 months.[170] However, afatinib does not increase OS. Biomarkers analyses were performed within this trial.[321] Median PFS favored afatinib in patients with p16-negative, *EGFR*-amplified (defined as ≥ 50% of cells with ≥ 4 copies, or ≥1 cell with ≥ 8 copies), HER3-low (defined as H-score ≤ 50), and PTEN-high (defined as H-Score > 150) tumors. In the MCC15780 trial where 38 SCCHN patients were treated with cetuximab,[325] PFS was also significantly increased in PTEN-high tumors compared to PTEN-low tumors.[326] The fact that afatinib seemed to be more active in case of HER3-low and PTEN-high disease suggests that pan-HER inhibitors could be more active when the PI3K pathway is

not or less activated. Cetuximab-naïve patients with p16 negative tumor had also a significant benefit from afatinib (ORR: 27%).

We designed two biomarker-driven cohorts in the EORTC-1559 trial where the patients are randomized between afatinib or investigator's choice. The first cohort includes patients with p16 negative SCCHN harboring either an *EGFR* mutation/amplification or *HER2* mutation/amplification or PTEN high (H-score > 150). We did not include patients with HER3 low disease as this IHC is not always reproducible.[327] The second cohort includes cetuximab-naïve SCCHN patients with p16-negative tumor. SCCHN with any *RAS* mutations are excluded.[312]

### 3.3.2 Fibroblast growth factor receptor (FGFR) inhibitor cohorts

FGFRs can activate the RAS-MAPK, PI3K, STAT, and PLCγ pathways.[328] *FGFR1* mutation/amplification are found in 5-10% of HPV-negative SCCHN, while *FGFR 3* mutations are more frequent in HPV-induced OPC (1-12%). Genetic alterations of *FGFR2* are observed in only 2-4%.

In preclinical study, Goke et al demonstrated that FGFR1 expression was associated with BGJ398 sensitivity, a potent and selective FGFR inhibitor, in SCCHN cell lines and predicted FGFR inhibitor sensitivity in patient-derived tumor xenografts.[329] A phase 2 study of erdafitinib, a pan-FGFR (FGFR1-4) inhibitor, in patients with metastatic or unresectable urothelial carcinoma and *FGFR* alterations (including activating mutations and fusions) showed a 40% confirmed ORR (RECIST 1.1) (3% CR, 37% PR).[330] Based on this study, the FDA granted accelerated approval to erdafitinib for patients with locally advanced or metastatic urothelial carcinoma, with susceptible *FGFR3* or *FGFR2* genetic alterations, that has progressed during or following platinum-containing chemotherapy, including within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy. Twenty-four percent of patients with urothelial cancer overexpressing FGFR1-3 mRNA achieved ORR with Rogaratinib, another pan FGFR inhibitor. Interestingly, some responding patients had elevated tumor FGFR3 mRNA levels without corresponding genomic alterations. In the dose- expansion phase of this study, there was a separate cohort for SCCHN. The prevalence of FGFR1-3 mRNA positivity among 46 SCCHN patients was 56.5%. Out of the 7 patients with SCCHN and FGFR overexpression treated with rogaratinib, they observed 1 PR and 2 SD.[331] Currently, clinical data with FGFR

inhibitors in SCCHN are limited. The large number of patients who will be screened in this study offers the unique opportunity to test FGFR inhibitor in this disease. We will investigate Rogaratinib in cases of high FGFR mRNA levels assessed by RNA-scope.

### 3.3.3 Cell cycle inhibitor cohort

The vast majority of HPV-negative SCCHN harbors genetic alterations (*TP53* mutations, *CCND1* amplification, and p16 inactivation) that enable them to circumvent the mitotic checkpoints through aberrant cyclin-dependent kinase (CDK) activation. Since p16 inactivates CDK4/6 whereas cyclin D1 activates CDK4/6, there is a rationale to test CDK4/6 inhibitors in patients with p16 negative and *CCND1*-amplified SCCHN. Palbociclib in combination with cetuximab has been investigated in recurrent SCCHN with promising preliminary results (ORR: 35%).[332] Recently, the PALATINUS randomized phase II trial failed to demonstrate a statistically significant benefit of the palbociclib/cetuximab combination over cetuximab alone in unselected HPV-negative patients with SCCHN who progress after platinum-therapy.[182]

However, currently, there are no data with palbociclib monotherapy in SCCHN. We will investigate palbociclib in patients with p16 negative tumors harboring *CCND1* amplification.

### 3.3.4 Poly ADP ribose polymerase (PARP) inhibitor cohorts

DNA repair deficiency increases sensitivity to platinum-based chemotherapy and PARP inhibitors.[124] The PARP proteins are a family of proteins involved in DNA repair. They play key roles in single strand break repair through the base excision repair pathway. This pathway is particularly important in cells that are deficient in a second cellular mechanism for high-fidelity DNA repair of double strand breaks, homologous recombination (HR). Homologous recombination deficiency is generally induced by inactivation of genes such as *BRCA1, BRCA2*, ataxia telangiectasia mutated (*ATM*) and others. Therefore, tumors with a deficiency in the homologous recombination DNA repair pathway are particularly sensitive to

PARP inhibitors,[333] a situation known as synthetic lethality. In summary, PARP inhibition will inhibit the single strand break repair that will evolve to a double strand break. Tumors with homologous recombination deficiency, by *BRCA* mutations or other mechanisms, will not be able to repair that double strand DNA damage and this will lead to cell death. Niraparib, an oral potent, selective PARP-1 and PARP-2 inhibitor, has been shown to improve PFS in patients with platinum-sensitive recurrent ovarian cancer, regardless of the presence or absence of gBRCA mutations or homologous recombination deficiency (HRD) status. However, the benefit is higher in patients with gBRCA mutations or HRD.[334]
A comprehensive analysis for HRD was performed on 5371 tumors representing 15 cancer types (The Cancer Genome Atlas). The aim of this study was to investigate the distribution of previously characterized genomic scars associated with HR deficiency, a DNA repair deficiency that causes increased sensitivity to platinum-based genotoxic chemotherapy and PARP inhibitors.[124] They ranked the cancer types by average telomeric allelic imbalance (NtAI), large scale transition (LST), and loss of heterozygosity (HRD-LOH) scores. They found that HR deficiency was associated with ovarian cancer, lung cancer, SCCHN, and bladder cancer. Moreover, preclinical studies have shown that HPV positive SCCHN have DNA double strand repair defects and they showed increased sensitivity to another PARP-inhibitor, veliparib.[137] These data support the investigation of niraparib in p16 positive OPC as well as platinum-sensitive p16 negative SCCHN. Implementing HRD testing in our screening biopsy would have considerably increased the screening period. Therefore, we took platinum-sensitivity as marker for this cohort by analogy with ovarian cancer. Platinum-sensitivity is defined:

- If platinum-based regimen was given as palliative treatment: patients must not have progressed during treatment
- If platinum-based regimen was a part of a multimodal curative treatment (chemoradiation): patients must not have recurred within 6 months after the end of treatment

### 3.3.5 Immunotherapy cohorts

PD-1/PD-L1 blockers have activity in SCCHN but the 2-year's OS rate is still low: 16.9%.[335] Therefore, other immunotherapy approaches have to be investigated.

Human leukocyte antigen-E (HLA-E) is a non-classical major histocompatibility complex molecule that constitutes a way for cancer cells to escape immune surveillance. HLA-E is highly expressed in 70% of SCCHN.[336] HLA-E binds to the Natural killer group 2A (NKG2A) receptor on (Natural killer) NK cells and T-lymphocytes to inhibit the cytotoxic functions of CD8+ T lymphocytes and NK cells. Monalizumab is a human IgG4 antibody targeting the NKG2A receptor. In the first immunotherapy cohort, patients will receive monalizumab monotherapy. In the second immunotherapy cohort, patients will be randomized to receive the combination of durvalumab and monalizumab versus physician's choice.

| Patient Cohort | Biomarker(s) | Targeted drug/IO | Design | Sample size (max) | Statistical hypothesis |
|---|---|---|---|---|---|
| **Biomarker-driven patient cohorts** | | | | | |
| **B1*** | p16 negative and *EGFR* amplification/mutation or PTEN high or *HER2* amplification/mutation | Afatinib | Phase II, randomized, open-label, multi-center study<br><br>Simon 2 Stage design | 55 | H0: PFSR at 16 weeks = 20%<br>H1: PFSR at 16 weeks = 40% |
| **B2*** | p16 negative and cetuximab naïve | Afatinib | Phase II, randomized, open-label, multi-center study<br><br>Simon 2 Stage design | 55 | H0: PFSR at 16 weeks = 20%<br>H1: PFSR at 16 weeks = 40% |
| **B3** | p16 negative and *CCND1* amplification | Palbociclib | Phase II, randomized, open-label, multi-center study<br><br>Simon 2 Stage design | 55 | H0: PFSR at 16 weeks = 20%<br>H1: PFSR at 16 weeks = 40% |
| **B4** | p16 negative and 'platinum-sensitive' | Niraparib | Phase II, single arm, proof-of-concept, multi-center study<br><br>Simon 2 Stage design | 32 | H0: ORR over first 16 weeks = 5%<br>H1: ORR over first 16 weeks = 20% |
| **B5** | p16 positive oropharyngeal cancer | Niraparib | Phase II, single arm, proof-of-concept, multi-center study<br><br>Simon 2 Stage design | 32 | H0: ORR over first 16 weeks = 5%<br>H1: ORR over first 16 weeks = 20% |
| **B6**** | FGFR1/2/3 mRNA overexpression | Rogaratinib | Phase II, single arm, proof-of-concept, multi-center study<br><br>Simon 2 Stage design | 20 | H0: ORR over first 16 weeks = 5%<br>H1: ORR over first 16 weeks = 25% |
| **Immunotherapy cohorts** | | | | | |
| **I1** | NA | Monalizumab | Phase II, single arm, proof-of-concept, multi- center study<br><br>Simon 2 Stage design | 34 | H0: ORR over first 16 weeks = 3%<br>H1: ORR over first 16 weeks = 15% |
| **I2** | NA | Monalizumab + Durvalumab | Phase II, randomized, open-label, multi-center study<br><br>Simon 2 Stage design | 57 | H0: ORR over first 16 weeks = 3%<br>H1: ORR over first 16 weeks = 15% |

**Table 3-1. Different patient cohorts of EORTC HNCG 1559 trial**

*Patients included in the afatinib arms should not have activating mutation in *RAS* ** Patients included in the rogaratininb arm should not have activating mutation in *RAS* or *PIK3CA;* IO, immunotherapy; ORR, objective response rate; PFSR, progression-free survival rate

# 4. THE EORTC-1559-HNCG TRIAL: FEASIBILITY

## 4.1 History and timelines

The idea of designing an umbrella trial for R/M SCCHN was born in early 2015 during my clinical fellowship in the department of Medical Oncology at Cliniques universitaires Saint-Luc (Brussels). I had the chance to participate in the ECCO-AACR-EORTC-ESMO workshop on Methods in Clinical Cancer Research (Flims, Switzerland) in June 2015 to further advance the project. The initial design was meant to be a randomized phase II Belgian study with only one statistical hypothesis to test the strategy of personalized treatment over standard care. At Flims, faculty members Dr. J Bogaerts, Dr. K Edward, Dr. I Wistuba and Dr. C Thomas challenged me to design a more ambitious and larger trial.

When I started my PhD in October 2015, we initiated discussion with the EORTC. The EORTC head and neck group decided to endorse the concept and to help me to develop the trial. Finally, the trial became the first international umbrella trial dedicated to R/M SCCHN. The UPSTREAM acronym was chosen for Personalized Strategy for REcurrent And Metastatic squamous cell carcinoma of the head and neck.

Figure 4-1 details the UPSTREAM trial timeline showing major events and related activities. It took us two years, between October 2015 and November 2017, to get the trial activated and open for inclusion in the first country. Version 1.0 of the protocol was released in April 2017. This version contained initially three biomarker-driven cohorts and two immunotherapy (IO) cohorts to test monalizumab monotherapy: one in PD(-L)1 inhibitor naïve patients and one in PD(-L)1 pretreated patients. With the approval of nivolumab and pembrolizumab for patients who progress during or after platinum therapy, recruitment to the PD(-L)1 naïve cohort was considered challenging and those two cohorts were merged into the one ‘I1’ cohort (first amendment). This v2.0 of the protocol, containing one IO cohort (I1) and three biomarker-driven cohorts (B1/2 with afatinib and B3 with palbociclib) was submitted to the competent authorities and activated in November 2017 in Belgium. Specific versions of the protocols (v2.1 for France and United Kingdom) needed to be created due to country- specific regulatory requests. The first sites in France, Italy and the United Kingdom were activated in December

2017, May 2018, and February 2019, respectively. Global recruitment was rapidly successful and the I1 cohort closed for interim analysis in July 2018. This cohort was definitively closed after the interim analysis. The results of this cohort will be discussed in detail in section 5 of this manuscript. In view of the results of the I1 cohort, the second IO cohort (I2), that was initially a randomized substudy with three arms (monalizumab + durvalumab vs monalizumab vs investigator's best choice) was then modified to a two arm substudy with the decision to not open the monalizumab arm. In the meantime, discussions were ongoing with other companies to create new cohorts. We designed one cohort that would test entrectinib in SCCHN harboring *NTRK* fusions, but unfortunately, after initial interest, the company decided to withdraw its support. In February 2019, we activated version 4.0 of the protocol with new cohorts, including a second IO cohort (I2) and two biomarker-driven cohorts, to study the efficacy of niraparib in two different subgroups. In May 2019, the B1 cohort evaluating afatinib passed the success criteria at the interim analysis and therefore accrual continued. The last version of the protocol, v5.0, added a new biomarker-driven cohort (B6) that plans to investigate rogaratinib. At the time of this writing, this new amendment is under review by the competent authorities.

The global design of UPSTREAM offers the opportunity for new patient cohorts to be added as amendments, and this has been successfully achieved. The running platform makes the set-up of new cohorts easier and faster rather than considering each new separate cohort a new trial as the logistics are already in place. However, the fact that UPSTREAM is one overarching protocol means that a new amendment for the whole trial is required for each modification to a specific cohort. As such, there have already been multiple amendments to UPSTREAM (Table 4-1) for cohort-specific requirements. For example, a design modification (change in statistical design - suppression of monalizumab arm for the I2 cohort), a change in dosage for one of the drugs, or an update to the investigator's brochure(s). By trying to group some of the cohort-specific requirements into one amendment to decrease the administrative load, we experienced delays in cohort activation as the information needed for each amendment was sometimes not available in time.

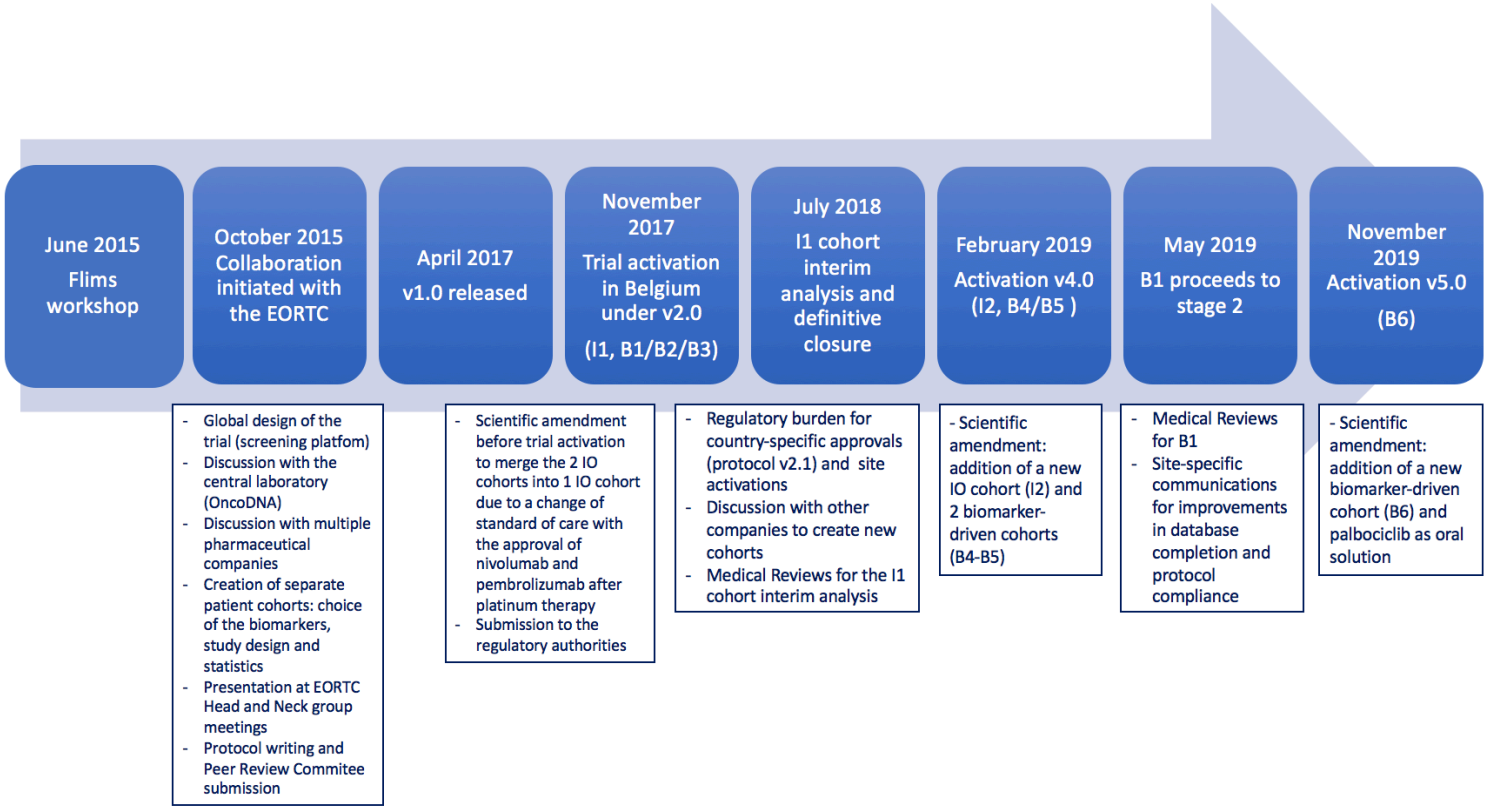


**Figure 4-1. Timeline of UPSTREAM noting major events and related activities**

| Protocol version (v) | Date of PRC approval | Amendment reference | Content | Protocol design | Protocol activated |
|---|---|---|---|---|---|
| V1.0 | April 27, 2017 | / | | Initial protocol<br>- Two IO cohorts: patient cohort 1 and 2 (monalizumab)<br>- Three biomarker-driven cohorts: patient cohort 3, 4 (afatinib versus control) and patient cohort 5 (palbociclib versus control) | Never activated |
| V2.0 | June 12, 2017 | 1 (scientific) | - Merging of the two IO cohorts and adaptation of the statistical design for the merged IO cohort (I1)<br>- Change in the names of all cohorts<br>- Change in the reference documents for afatinib and palbociclib from investigator's brochure (IB) to summary of product characteristics (SmPC) | First activated protocol<br>- One IO cohort I1 (monalizumab)<br>- Three biomarker-driven cohorts: B1, B2 (afatinib versus control) and B3 (palbociclib versus control) | - Belgium: November 16, 2017<br>- Italy: May 14, 2018 |
| V2.0 | August 01, 2017 | 2 (scientific) | - Change in the safety language from the updated IB of monalizumab. | Not applicable (NA) | NA |
| V2.1 | November 09, 2017 | 3 (Administrative) | Country- specific changes for France and the United Kingdom (UK) | NA | France: December 18, 2017 (Never opened in UK) |
| V3.0 | January 30, 2018 | 4 (scientific) | - Addition of one IO cohort: I2 (3 arms, monalizumab + durvalumab vs monalizumab vs best investigator's choice)<br>- Addition of two biomarker-driven cohorts: B4 and B5 (niraparib, single arm)<br>- Addition of one biomarker-driven cohort: B6 (entrectinib, single arm)<br>- Amendment to cohort I1: change of monalizumab dose (750 mg, flat dose)<br>- Amendment to general and cohort specific inclusion/exclusion criteria<br>- Screening period before cohort allocation has been extended from two weeks to three weeks<br>- Change in the prioritization algorithm of the cohorts | - Two IO cohorts: I1 and I2 (monalizumab + durvalumab versus monalizumab versus investigator's best choice)<br>- Six biomarker-driven cohorts: B1, B2, B3, B4, B5 (niraparib), and B6 (entrectinib) | Never activated (B6 with entrectinib never activated) |

| | | | | | |
|---|---|---|---|---|---|
| | | | - Data collection for patients who have biomarker assessments but who did not receive the allocated treatment due to screening failure / ineligibility | | |
| V4.0 | May 15, 2018 | 5 (scientific) | - Removal of cohort B6 due to withdrawal of support from Roche<br>- Small update addendum cohort I2 due to comments from Astra Zeneca (wording in drug reconstitution guidelines and small changes in toxicity management guidelines)<br>- Update Patient Information Sheet and Informed Consent form (PISIC) for cohorts B4 and B5 due to an update to the niraparib IB (dated April 2018)<br>- Update screening PISIC due to removal of cohort B6<br>- Follow up of all patients who have undergone molecular screening for survival data. | - 2 IO cohorts: I1 and I2<br>- 5 biomarker-driven cohort: B1, B2, B3, B4, B5 | - Belgium: February 22, 2019<br>- France: February 25, 2019<br>- UK: February 26, 2019 |
| V5.0 | July 17, 2018 | 6 (scientific) | - New design of I1: Change from A'Hern to Simon two-stage design to allow interim analysis on the first 25 patients | NA | |
| V5.0 | April 17, 2019 | 7 (scientific) | -Based on the interim analysis results, IO cohort I1 (monalizumab single agent) was permanently closed for recruitment<br>- Redesign cohort I2 as a 2-arm randomized cohort<br>- Addition of a new biomarker cohort B6 (rogaratinib)<br>- Various updates in protocol, PISICs and addenda.<br>- New addendum to screening PISIC for enrolled patients to archival tissues (translational research) | - Two IO cohorts: I1 (permanently closed) and I2<br>- Six biomarker-driven cohorts: B1, B2 and B3, B4, B5, B6 (rogaratinib) | Ongoing |
| V6.0 | October 18, 2019 | 8 (scientific) | Urgent safety amendment for palbociclib (B3): At the request of the MHRA, urgent safety measures have to be taken regarding the risk of occurrence of non-infectious interstitial lung disease/pneumonitis<br>- Protocol and PISIC update for new patients, and an addendum to PISIC created for patients already included in the trial. | NA | Ongoing |

**Table 4-1. Overview of protocol versions and amendments**

Bx refers to "biomarker-driven cohort x", Ix refers to "immunotherapy cohort x"; IB, investigator's brochure; IO, immunotherapy; PISIC, patient information sheet and informed consent form; PRC, peer review committee; MHRA, Medicines and Healthcare products Regulatory Agency; NA, not applicable; SmPC, summary of product characterists.

## 4.2 Inclusion status of UPSTREAM trial

The trial opened for inclusion at the end of November 2017 and the first patient entered the study in December 2017. As of October 2019, 23 sites are active across four countries (Belgium, France, Italy and the United Kingdom). At the data cut-off on September 16 2019, 178 patients were registered and 99 were already allocated to a treatment cohort (Figure 4-2). From the 99 patients that were allocated to a cohort, 60 (61%) were allocated to a biomarker-driven cohort and 39 (39%) to an immunotherapy cohort. Figure 4-3 and Table 4-2 show the accrual curve and the inclusion status per cohort, respectively.

Forty patients were screen failures, and for 39 patients, allocation to a cohort is ongoing. Of note is that the latter group can include patients who are waiting for biopsy results, but also those who are (from a practical point of view) “screen failures” and not yet classified into this group as their data is still awaiting completion in the electronic case report forms (eCRF). Database cleaning and analysis is ongoing to determine the exact number of and reasons for screening failures. Potential reasons could be: inadequate biopsy but ineligible for IO cohort, inadequate biopsy but no IO cohort available (between July 2018 and February 2019 where no IO cohort was open), clinical deterioration, non-eligibility for another exclusion criterion or death.

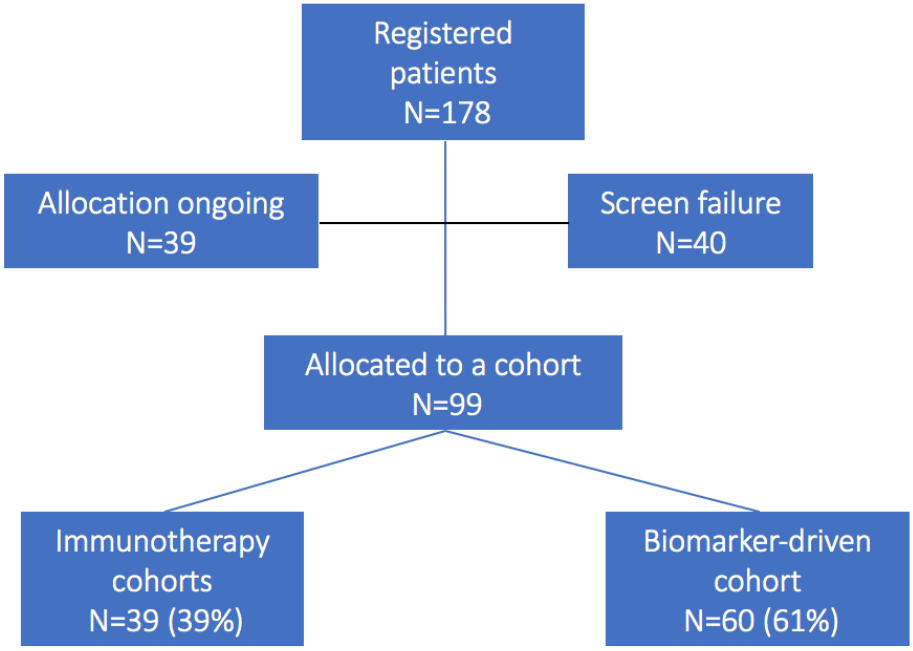


**Figure 4-2. Flow-chart of the UPSTREAM trial (Data cut-off September 16th, 2019)**

At the data cut-off on the 16th of September 2019, 178 patients were registered for the trial, and of these, 99 were allocated to a cohort. 40 patients were screen failures and for 39 patients, allocation to a cohort is ongoing. From the 99 patients who were allocated to a cohort, 61% were allocated to a biomarker-driven cohort and 39% to an immunotherapy cohort.

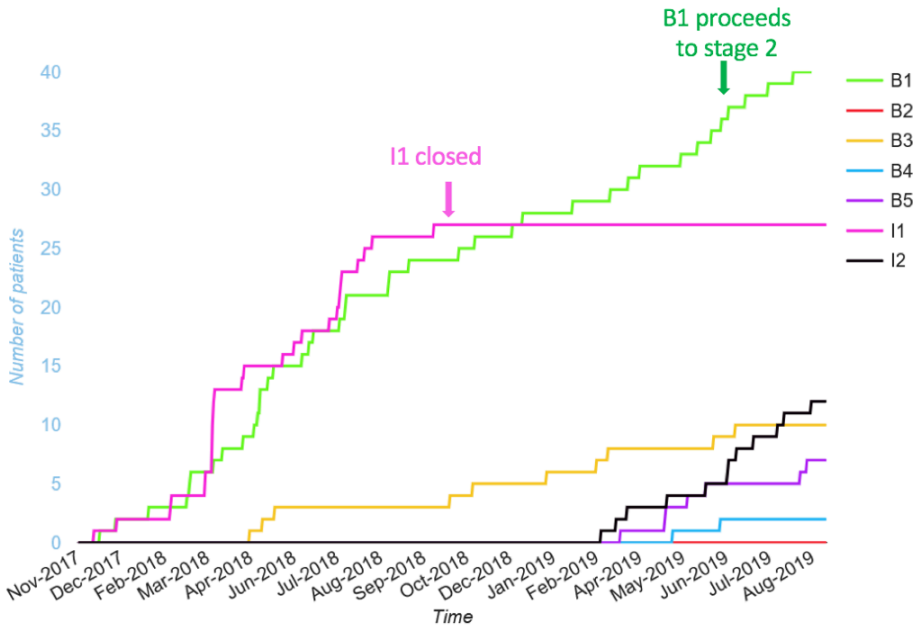


**Figure 4-3. Accrual graph of UPSTREAM (Data cut-off September 16th, 2019)**

The accrual graphs represent the accrual curve for each patient cohort. Green: cohort B1 (afatinib vs investigator best choice), red: cohort B2 (afatinib vs investigator best choice), yellow: cohort B3 (palbociclib vs investigator best choice), blue: cohort B4 (niraparib), purple: cohort B5 (niraparib), pink: cohort I1 (monalizumab), black: cohort I2 (monalizumab + durvalumab vs investigator best choice).

| Patient Cohort | Current N of patients | Planned N of patients (stage 1+2) |
|---|---|---|
| **B1 (Randomized 2:1, two-stage)** | 41 | 55 |
| Afatinib | 27 | 37 |
| Control | 14 | 18 |
| **B2 (Randomized 2:1, two-stage)** | 0 | 55 |
| Afatinib | 0 | 37 |
| Control | 0 | 18 |
| **B3 (Randomized 2:1, two-stage)** | 10 | 55 |
| Palbociclib | 6 | 37 |
| Control | 4 | 18 |
| **B4 (Single-arm, two-stage)** | | |
| Niraparib | 2 | 32 |
| **B5 (Single-arm, two-stage)** | | |
| Niraparib | 7 | 32 |
| **I1 (Single-arm, two-stage)** | | |
| Monalizumab | 27 (closed) | 34 |
| **I2 (Randomized 2:1, two-stage)** | 12 | 57 |
| Monalizumab + durvalumab | 7 | 38 |
| Control | 5 | 19 |

**Table 4-2. Accrual status per patient cohort, and per arm in case of randomized design (Data cut-off September 16th, 2019)**

Bx, biomarker-driven cohort; Ix, Immunotherapy cohort; N, number

## 4.3 Biopsies and frequency of molecular alterations

One main challenge of UPSTREAM is the requirement of new tumor biopsy for trial entry. The formalin-fixed paraffin-embedded (FFPE) sample is mandatory for biomarker-analysis and cohort allocation. In addition, the trial also requests two fresh frozen samples for future translational research. The FFPE sample is sent to the centralized laboratory (OncoDNA, Gosselies, Belgium).

Obtaining a new biopsy at the time of recurrent/metastatic disease can be challenging. The quality of the biopsy may be suboptimal (meaning not adequate for molecular testing), and time is needed for the results of the biopsy to come back before cohort allocation and treatment initiation. Since these parameters are crucial to the development of precision medicine for SCCHN, in the next paragraph, we present our preliminary results on these different parameters.

### 4.3.1 Quality of the biopsies

The centralized laboratory performs a quality check (QC) on the FFPE sample before molecular analysis (NGS and IHC). The tumor sample must fulfill the four following criteria to pass the quality check:

- > 10% tumor cells in the whole biopsy
- In the selected area used for NGS:
    - < 20% lymphocyte invasion
    - no necrosis
    - at least 4mm² of tumor cells

A detailed analysis of the quality of the biopsies is ongoing. Preliminary data are outlined below:

The first analysis was performed in March 2019 (cut-off date on 4$^{th}$ march 2019) and showed that 33/107 (31%) samples failed the QC. The main reason for failure was less than 4 mm² of tumor cells in the area selected for NGS (26/33, 79%).

Longitudinally follow-up of the biopsies showed an increased quality of the biopsies with at the cut-off date of the 7th of November 2019, 43 of the 178 analyzed biopsies (24%) with a non-optimal quality check. (Figure 4-4)

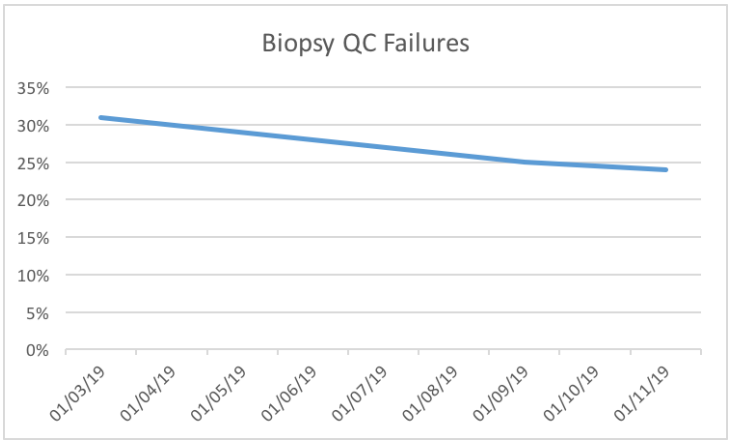


**Figure 4-4. Longitudinally follow-up of biopsy quality check (QC) failure**

Table 4-3 outlines the reason for quality failure. Of note, one sample can fail more than one criteria. The main criterion for QC failure is still the same, it is less than 4mm² of tumor cells in the area selected for NGS (84%).

| Reason of failure | Total (N=43) |
|---|---|
| Less than 10 % tumor cells in the whole biopsy | 21 (49%) |
| More than 20 % lymphocyte invasion in the area selected for next generation sequencing (NGS) | 10 (23%) |
| Necrosis in the area selected for NGS analysis | 7 (16%) |
| Less than 4 mm² of tumor cells in the area selected for NGS analysis | 36 (84%) |

**Table 4-3. Reasons for quality check failure (Data cut-off November 7th 2019)**

We are currently analyzing these data more in depth. Overall, 44% of the FFPE samples were biopsies from distant metastatic lesions and 46% percent of samples with QC failures were obtained by sampling a distant metastasis. Table 4-4 shows

the sampling technique that was used in samples that passed and failed the QC. Notably, 28% of samples that failed quality control were surgical specimens.

| Sampling technique | All samples (n=178) | Failed (n=43) |
|---|---|---|
| 5mm needle biopsy | 57 (32%) | 15 (35%) |
| 1mm needle biopsy | 11 (6%) | 3 (7%) |
| Needle aspiration | 3 (2%) | 2 (5%) |
| Surgical specimen | 40 (22%) | 12 (28%) |
| Unknown | 67 (38%) | 11 (25%) |

**Table 4-4. Sampling techniques used in samples that passed and failed quality control (Data cut-off November 7th 2019)**

### 4.3.2 Time intervals between sampling and results

An important factor in our trial is the interval of time between sampling and biomarker results. In clinical practice, the time it takes to organize consultations and plan treatment will also potentially delay the start of treatment even if the biomarker results are available. Therefore, it is important to analyze the time interval between sampling and the start of treatment. Table 4-5 outlines the different time intervals based on current observations in our database.

The median number of days between tissue sampling and biomarker results, and between biomarker results and treatment start, was 16 and 6 days, respectively, leading to a median number of 22 days between tissue sampling and treatment start.

| | **Median (days)** | **Range (days)** |
|---|---|---|
| **Number of days between (first) tissue sampling and final biomarker results (n = 109)** | 16 | 10 - 42 |
| **Number of days between (first) sample received at OncoDNA and final biomarker results (n=117)** | 10 | 6 - 34 |
| **Number of days between biomarker results and treatment initiation (n = 66)** | 6 | 0 - 23 |
| **Number of days between tissue sampling and treatment initiation (n = 80)** | 22 | 9 - 146 |

**Table 4-5. Time intervals (Data cut-off September 25 2019)**

### 4.3.3 Landscape of genomic alterations

The main advantage of mandating a biopsy in the trial is to enable the molecular landscape of recurrent/metastatic SCCHN to be characterized as data are relatively poor in the current literature. For the purpose of the trial, NGS on a limited panel of 13 genes is performed (*EGFR, ERBB2, TP53, PIK3CA, CCND1, NRAS, KRAS, HRAS, PTEN, FGFR1, FGFR2, FGFR3* and *cMET*) together with IHC for p16 and PTEN.

Table 4-6 outlines the frequency of each molecular alteration for 120 samples with available biomarker results (data cut-off 25 September 2019). In the first 120 analyzed patients, 29 (24%) had p16 positive SCCHN. *TP53* mutations were observed in 58 (48%) of all patients (p16+ and p16 -). Another cell cycle regulator, *CCND1*, was amplified in 18 patients (15%). Alterations in tyrosine kinase receptor genes were rather low: *EGFR* amplification or mutation was found in 7 (6%) and 2 (2%), respectively, and *ERBB2* amplification in 1 (1%). We detected one *FGFR1* amplification and one *FGFR3* mutation. Downstream signaling pathways were altered with *PIK3CA* mutations (n=17, 14%) being the most frequent. *HRAS* mutation was observed in three (2.5%) patients and *KRAS* or *NRAS* mutations were seen in one patient each.

The B1 cohort is evaluating afatinib in patients with p16 negative SCCHN harboring (i) an *EGFR* mutation/amplification or (ii) *ERBB2* mutation/amplification or (iii) PTEN H-score > 150 (high). These biomarkers were defined based on the results of the biomarker analysis of the phase III LUX Head & Neck 1 trial. The B1 cohort passed the first stage interim analysis and continues to recruit in the second stage. Table 4-7 provides an overview of the molecular alterations for the 41 patients entered in the B1 cohort. All but one patient (n=40; 98%) had a 'high' PTEN status. This is higher than what was expected based on the LUX Head & Neck 1 trial data, despite the fact that the same technology and cut-offs were used. Six patients harbored an *EGFR* amplification and one patient a *HER2* amplification. Unfortunately, the two patients with EGFR mutations in the whole UPSTREAM patient cohort were not entered into the B1 cohort.

| Alteration | N (%), n total = 120 |
|---|---|
| **Immunohistochemistry** | |
| **p16** | |
| Negative | 91 (76%) |
| Positive (H-score ≥ 210) | 29 (24%) |
| **PTEN** | |
| High (H-score > 150) | 84 (70%) |
| **Next-Generation-Sequencing** | |
| ***TP53*** | |
| Mutation | 58 (48%) |
| ***EGFR*** | |
| Amplification | 7 (6%) |
| Mutation | 2 (2%) |
| ***ERBB2*** | |
| Amplification | 1 (1%) |
| Mutation | 0 (0%) |
| ***FGFR1*** | |
| Amplification | 1 (1%) |
| Mutation | 0 (0%) |
| ***FGFR2*** | |
| Amplification | 0 (0%) |
| Mutation | 0 (0%) |
| ***FGFR3*** | |
| Amplification | 0 (0%) |
| Mutation | 1 (1%) |
| ***cMET*** | |
| Amplification | 0 (0%) |
| ***PIK3CA*** | |
| Amplification | 3 (2.5%) |
| Mutation | 17 (14%) |
| ***PTEN*** | |
| Mutation | 2 (2%) |
| ***HRAS*** | |
| Mutation | 3 (2.5%) |
| ***KRAS*** | |
| Amplification | 2 (2%) |
| Mutation | 1 (1%) |
| ***NRAS*** | |
| Mutation | 1 (1%) |
| ***CCND1*** | |
| Amplification | 18 (15%) |
| Mutation | 1 (1%) |

**Table 4-6. Overview of molecular alterations (Data cut-off September 25, 2019)**

| B1 cohort (N = 41) | |
|---|---|
| **Alteration** | **N (%)** |
| p16 negative | 41 (100% |
| *EGFR* amplification | 6 (15%) |
| *EGFR* mutation | 0 (0%) |
| *HER2* amplification | 1 (2%) |
| *HER2* mutation | 0 (0%) |
| PTEN high | 40 (98%) |

**Table 4-7. Overview of molecular alterations of patients allocated in the B1 cohort (Data cut-off September 25th, 2019)**

# 5. RESULTS OF THE I1 COHORT OF THE EORTC-HNCG-1559 TRIAL

Presented as an oral communication at ESMO 2019[337]

# "A phase II study of monalizumab in patients with recurrent/metastatic squamous cell carcinoma of the head and neck: results of the I1 cohort of the EORTC-HNCG-1559 trial (UPSTREAM)"

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**Abstract**

***Background***

Patients with recurrent and/or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) treated with PD-(L)1 inhibitors after platinum failure have a median overall survival (OS) of 7 - 8 months. Monalizumab is a monoclonal antibody against the inhibitory receptor NKG2A localized on natural killer (NK) cells and T cells. Its ligand, HLA-E, is overexpressed in SCCHN. By targeting the HLA-E-NKG2A pathway, monalizumab may enhance NK cell and T cell activity.

***Methods***

The UPSTREAM trial is a biomarker-driven umbrella trial evaluating the efficacy of several targeted therapies and immunotherapies (IO) in R/M SCCHN. Patients eligible for UPSTREAM have incurable R/M SCCHN (after platinum; ECOG 0-1, measurable disease (RECIST v1.1)). Based on biomarker testing, patients are allocated to biomarker-driven or IO cohorts. Non-eligible patients for the biomarker-driven cohorts are included in the IO cohort. The immunotherapy 1 (I1) cohort was a phase II, single arm, proof-of-concept substudy evaluating the efficacy of monalizumab monotherapy. Monalizumab was given as an infusion (10 mg/kg) on day 1 of each 14-days cycle. Primary endpoint was objective response rate (RECISTv1.1) during the first 16 weeks. A two-stage Simon design was used (H1 15%, H0 3%, $\alpha$ 8%, power 90%) with planned interruption of accrual if no response was seen after 25 patients. Secondary endpoints were toxicity (CTCAEv4.03), progression-free survival (PFS), response duration and overall survival (OS).

***Results***

Twenty-six eligible patients were enrolled in the first stage. Seventeen (65%) patients had received two or more previous lines of systemic treatment in the R/M setting and 15 (58%) were pretreated with PD(L)1 inhibitors. No response was seen and stable disease was observed in six patients (23%) with a median duration of 3.8 months (range: 2.7-9.1). All patients progressed during follow-up and median PFS was 1.7 months (95% CI: 1.5-1.8 months). At data cut-off, 24 patients had died and the median OS in the overall population was 6.7 months (95% CI: 3.0-9.6 months). Monalizumab was safe as no grade $\geq$3 treatment-related adverse events (AE) were seen. The most frequent grade 1-2 treatment-related AE was fatigue in 15% of patients and diarrhea, anemia and myalgia each observed in 7%.

***Conclusions***

Monalizumab monotherapy has limited activity in R/M SCCHN. The I1 cohort did not meet its primary endpoint and was closed at the interim analysis. Monalizumab is under investigation with durvalumab within the UPSTREAM trial.

### 5.1.1 Introduction

Squamous cell carcinoma of the head and neck (SCCHN) affects approximatively 700.000 patients per year worldwide, ranking it as the seventh most common malignancy. [2] Most patients present with locally-advanced disease, but despite multimodal treatment with surgery and/or (chemo)radiation, more than 50% will relapse.[160] Incurable SCCHN is treated with palliative systemic treatment. Platinum-based therapy [176,338] and pembrolizumab [321] improve survival in first line for patients with recurrent/metastatic (R/M) SCCHN. The median overall survival (OS) with programmed cell death 1 (PD-1) or programmed death-ligand 1 (PD-L1) inhibitors in second line is between 7.1 and 8.4 months. [173,174,339] There is no standard of care for patients who progress after platinum and PD-1 inhibitors, and patients have a dismal prognosis.

Although pembrolizumab and nivolumab are approved in SCCHN, a significant number of patients do not respond to PD-1 inhibitors and, ultimately, the majority of patients will progress. As the molecular landscape of SCCHN harbors potential actionable targets that could open the door to new treatment opportunities,[87] there is clearly a rationale to investigate new treatment strategies, including targeted therapies and immunotherapy. The European Organisation for Research and Treatment of Cancer (EORTC) is currently conducting the UPSTREAM trial (EORTC-HNCG-1559, NCT03088059), an umbrella trial dedicated to R/M SCCHN.[193] The UPSTREAM trial enrolls patients with R/M SCCHN who have progressed after platinum-based therapy. Based on potential biomarkers and molecular alterations identified in a new tumor biopsy taken for the purpose of the trial, patients are allocated to different patient cohorts which evaluate the efficacy of various targeted therapies or immunotherapies.

Immunotherapy cohort 1 (I1) was the first immunotherapy cohort of the UPSTREAM trial, studying the efficacy of monalizumab as monotherapy. Monalizumab is a human IgG4 antibody targeting the natural killer group 2A (NKG2A) receptor, an inhibitory receptor expressed on natural killer (NK) cells and CD8 +T-lymphocytes. [340] Its ligand, the human leukocyte antigen E (HLA-E), is a non-classical major histocompatibility complex (MHC) molecule that creates a way for cancer cells to escape immune surveillance. HLA-E is upregulated by different cancer cells, including colorectal cancer,[341] gynecological cancers [342] and SCCHN (70%). [336,343] The binding of HLA-E to NK2GA inhibits the cytotoxic functions of CD8+

T lymphocytes and NK cells.[336] Targeting NK2GA releases the brake on both T-cells and NK cells. A phase II, non-randomized trial showed promising data for the combination of monalizumab and cetuximab in R/M SCCHN with an objective response rate (ORR) of 27.5%. [344] However, at the time of writing, there are no data for single agent monalizumab in SCCHN. In this paper, we present the results of the I1 cohort, a phase II, single-arm, proof-of-concept study evaluating monalizumab in R/M SCCHN.

## 5.1.2 Methods

### 5.1.2.1 Patient eligibility

The UPSTREAM study design, investigated biomarkers, different treatment cohorts and the algorithm for cohort allocation have been previously described.[193] UPSTREAM includes patients with histologically confirmed R/M SCCHN of the oral cavity, oropharynx, hypopharynx or larynx not amenable to curative treatment who progress after platinum-based therapy, given as palliative treatment, or as part of multimodal curative treatment with progression within one year from the end of treatment. Previous treatment with PD-(L)1 inhibitors is allowed. Other main eligibility criteria include age greater than or equal to 18 years, Eastern Cooperative Oncology Group performance status (ECOG PS) 0-1, measurable disease according to Response Evaluation Criteria in Solid Tumors v1.1 (RECIST), and adequate hematologic, hepatic, renal and cardiac function. The disease must be amenable to a core biopsy as a fresh biopsy of the disease is mandatory for trial entry. The study is approved by a central ethics committee, by the health authorities of each participating country, and conducted in accordance with the Declaration of Helsinki (October 2000). Written informed consent is obtained for each patient twice: first at the screening phase (fresh tumor biopsy) and then again at the time of treatment allocation.

Patients enrolled in UPSTREAM were allocated to the I1 cohort if they met all the general inclusion criteria but were not eligible for one of the biomarker-driven cohorts defined as (i) failure of the biopsy to pass molecular testing quality control in the central laboratory, (ii) lack of predefined biomarkers, and (iii) allocation to one biomarker-driven cohort but with an exclusion criteria for the same cohort, or

the specified cohort was closed for accrual at the time of inclusion. The main cohort-specific exclusion criteria for the I1 cohort included systemic treatment with steroids or other immunosuppressive agents and active auto-immune disease/inflammatory disorder.

#### 5.1.2.2 Study objectives and endpoints

The primary objective of the I1 cohort was to assess the efficacy of monalizumab monotherapy in non-curable R/M SCCHN. The primary end point was objective response rate (ORR) over the first 16 weeks of study treatment, defined as the proportion of patients who met the criteria for complete response (CR) or partial response (PR) according to RECIST v1.1. The secondary end points were response duration, progression free survival (PFS), overall survival (OS), ORR according to the use of modified Response Evaluation Criteria in Solid Tumours in cancer immunotherapy trials (iRECIST), and toxicity evaluated with the Common Terminology Criteria for Adverse events (CTCAE) v4.03.

#### 5.1.2.3 Study design

The I1 cohort was a single-arm sub-study. Monalizumab was administered as an intravenous infusion at a dose of 10mg per kilogram over 60 minutes on day 1 of each 14-day cycle. No dose reductions were allowed. Disease evaluation was performed at baseline by contrast enhanced computed tomography (CT) and/or magnetic resonance imaging (MRI) at eight weeks and 16 weeks after the beginning of treatment and every eight weeks thereafter. Treatment was continued until unacceptable toxicity or disease progression. In the event of disease progression according to RECIST v1.1 and following iRECIST guidelines,[345] continued treatment with monalizumab was permitted for patients who were clinically stable until the next imaging assessment, performed at least four weeks but no more than eight weeks after the date of the initial progression.

#### 5.1.2.4 Statistical methods

A two-stage Simon design was used with 90% power to detect an ORR of 15% from a null hypothesis of 3% with a one-sided type 1 error of 8%. The total sample size required was calculated at 34 eligible patients with a planned interruption of accrual at the interim analysis if no response was seen after 25 patients. The study would be declared successful if at least three patients out of the 34 recruited experienced an ORR according to RECISTv1.1 during the first 16 weeks of treatment.

According to the statistical analysis plan, only evaluable patients were included in the efficacy analysis, and all patients exposed to a least one dose of monalizumab were evaluated for treatment toxicity.

PFS and OS were analyzed using the Kaplan-Meier method. Patients without any event of interest (progression or death) were censored at the date of last-follow-up. An exploratory analysis of OS according to exposure to PD-(L)1 inhibitors was performed using the same technique.

### 5.1.3 Results

#### 5.1.3.1 Patient characteristics

Between December 2017 and July 2018, 27 patients were enrolled in the I1 cohort at 14 centers in three European countries (Belgium, France and Italy). Twenty-six patients (25 men, 1 woman) were eligible and their baseline characteristics are described in Table 5-1. Nineteen patients (73%) had an ECOG PS of 1. The most frequent subsite of primary disease was the oropharynx (38%). Most of the patients (17/26, 65%) had received two or more lines of systemic treatment in the R/M setting. In total, 16 patients (62%) had been exposed to PD-(L)1 inhibitors: 15 and 1 before and after monalizumab, respectively. The reason for allocation to the I1 cohort was the absence of any of the predefined biomarkers in 14 (52%) patients, failure of the biopsy to pass quality control in 11 (44%), and the presence of exclusion criteria for the assigned biomarker-driven cohort in 1 (4%).

| Baseline characteristics (n=26) | |
|---|---|
| Median age – years (range) | 62 (44-76) |
| Male, n (%) | 25 (96%) |
| ECOG PS, n (%) | |
| 0 | 7 (27%) |
| 1 | 19 (73%) |
| Primary site, n (%) | |
| Oral cavity | 7 (27%) |
| Oropharynx | 10 (38%) |
| p16 negative | 6 |
| p16 positive | 3 |
| unknown | 1 |
| Hypopharynx | 7 (27%) |
| Larynx | 2 (8%) |
| Type of recurrence | |
| Locoregional only | 8 (31%) |
| Distant metastasis | 18 (69%) |
| Prior lines of systemic treatment in the R/M setting | |
| Number of previous lines, median (range) | 2 (0-4) |
| ≤ 1 line | 9 |
| 2 lines | 6 |
| ≥ 3 lines | 11 |
| Prior platinum-based therapy, n (%) | 26 (100%) |
| Prior cetuximab, n (%) | 23 (89%) |
| PD-(L)1 inhibitor exposure | 16 (62%) |
| Pre-study PD-(L)1 inhibitors, n (%) | 15 (58%) |
| Post-study PD-(L)1 inhibitors, n (%) | 1 (4%) |
| Reasons for exclusion in biomarker-driven cohorts | |
| Absence of one of the pre-defined biomarkers | 14 (52%) |
| Biopsy that did not pass molecular testing quality control | 11 (44%) |
| Other (other criteria for non-eligibility) | 1 (4%) |

**Table 5-1. Patient characteristics**

ECOG, Eastern Cooperative Oncology Group; PD-1, programmed cell death 1; PD-L1, programmed death-ligand 1; R/M, recurrent/metastatic

#### 5.1.3.2 Efficacy

For the 26 eligible patients, no objective response was observed (0%, 95% CI: 0-13.2%) (Table 5-2). Stable disease (SD) and progressive disease (PD) were recorded in six (23%, 95%CI: 9.0%-43.7%) and 20 (77%, 95%CI: 56.4%-91.0%) patients, respectively. The median duration of SD was 3.8 months (range: 2.7–9.1 months). One patient with SD died of tumor bleeding 9.6 months after the start of monalizumab without disease progression.

Five patients were not evaluable for radiological evaluation: two patients presented an early death and three patients had clinical progression, but disease evaluation according to RECISTv1.1 was not carried out as measurements were unavailable. All these patients were considered to have PD. Figure 5-1 shows the waterfall plot of the best change from baseline in the sum of the largest diameters of the target lesions during the first 16 weeks of treatment according to investigators for 21 out of the 26 evaluable patients. Tumor shrinkage of the target lesions was observed in one patient. The analysis of response according to iRECIST is ongoing.

Median follow-up was 13.8 months. All patients progressed during follow-up and the median PFS was 1.7 months (95% CI: 1.5-1.8 months) (Fig 5-2). Twenty-four deaths (92%) were reported. Median OS was 6.7 months (95% CI: 3.0-9.6 months) (Figure 5-3), with an OS rate at six and 12 months of 53.8% (95% CI: 33.3-70.6%) and 19.2% (95% CI: 7.0-36.0%), respectively. Sixteen patients were exposed to PD-(L)1 inhibitors. An unplanned exploratory analysis of OS according to PD-(L)1 inhibitor exposure showed a median OS of 8.6 months (95% CI: 2.7-10.7 months) in patients who had received PD-(L)1 inhibitors (n=16). The 1-year OS rate was 25% (95% CI: 7.8-47.2%) (Figure 5-4). In patients who were PD-(L)1 inhibitor naïve (n=10), median OS was 4.1 months (95% CI: 1.1-9.2 months) and the 1-year OS rate was 10% (95% CI: 0.6-35.8%) (Figure 5-4).

| Response (RECIST v1.1) (n=26) | |
|---|---|
| Best response during first 16 weeks of treatment, n (%) | |
| Complete/Partial response | 0 (0%) (95%CI: 0-13.2%) |
| Stable disease | 6 (23%) (95%CI: 9.0-43.7% |
| Progressive disease | 20 (77%) (95%CI: 56.4-91.0%) |
| Duration of disease stabilization (months), median (range)* | 3.8 (2.7-9.1) |

**Table 5-2. Best response according to RECIST v1.1 over the first 16 weeks of treatment**

*Computed over five patients with stable disease; another patient with stable disease died of tumor bleeding 9.6 months after start of treatment without disease progression.

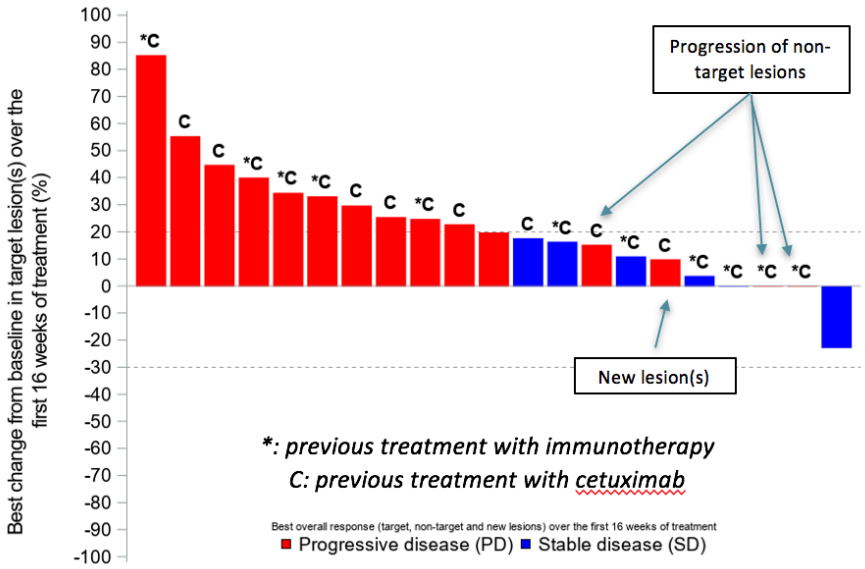


**Figure 5-1. Waterfall plot of tumor shrinkage according to investigator assessments**

The best change from baseline in the sum of the largest diameters of the target lesions according to RECISTv1.1 during the first 16 weeks of treatment is shown (%) for the 21 patients evaluable for this analysis. Patients with progressive disease are indicated in red and patients with stable disease (SD) in blue.

* = patients pretreated with PD-(L)1 inhibitors; C = patients pretreated with cetuximab.

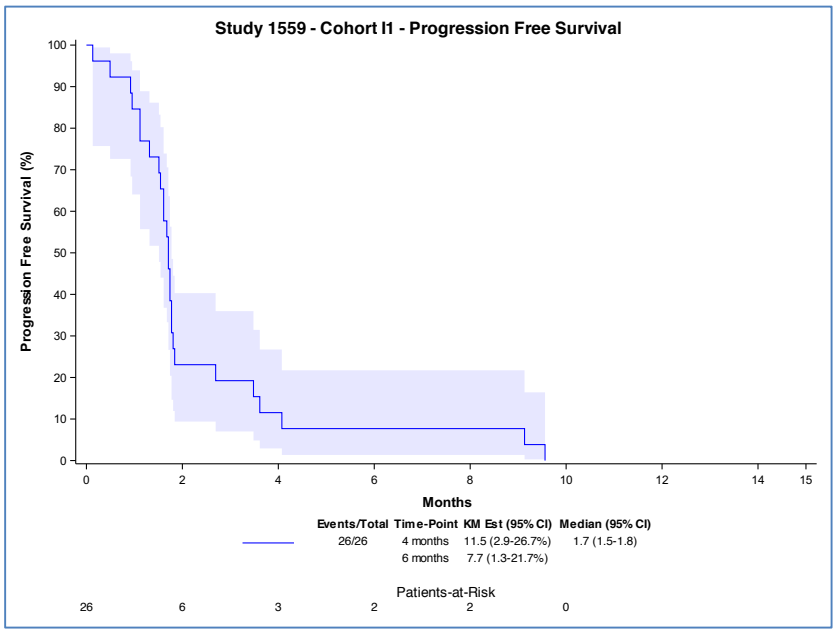


**Figure 5-2. Kaplan-Meier estimate of progression-free survival for all eligible patients (n=26)**

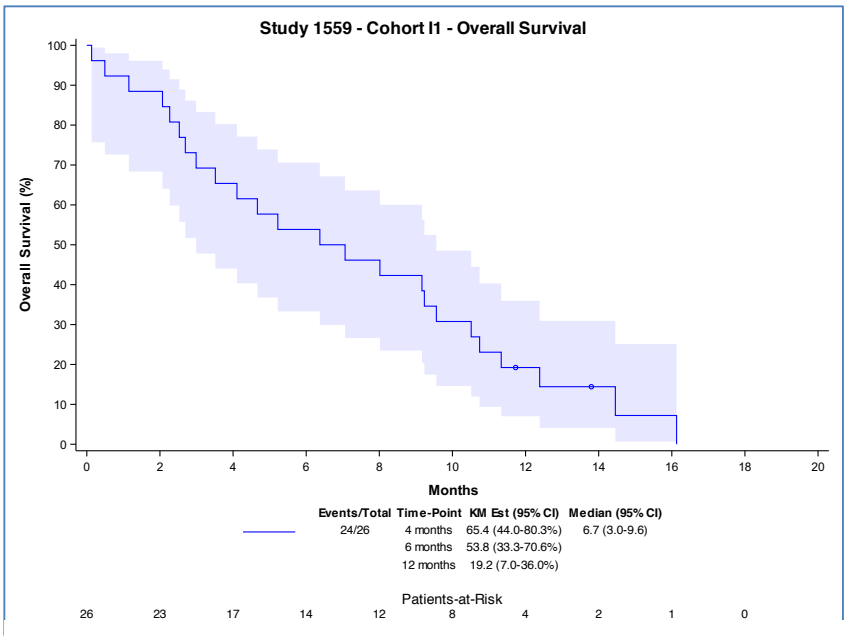


**Figure 5-3. Kaplan-Meier estimate of overall survival for all eligible patients (n=26)**

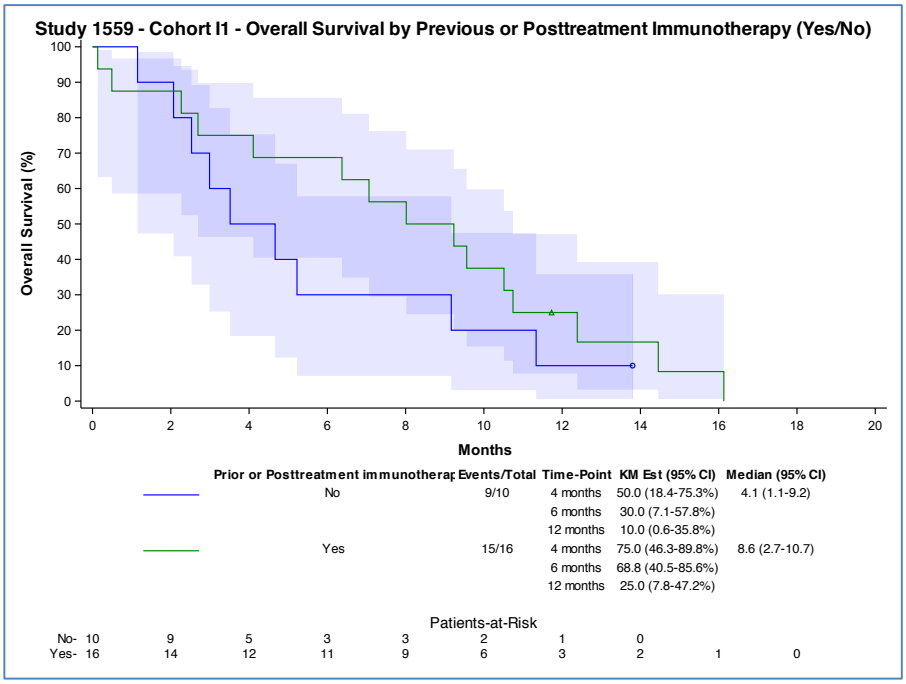


**Figure 5-4. Kaplan-Meier estimates of overall survival according to PD-(L)1 inhibitor exposure**

Green curve = patients exposed to PD-(L)1 inhibitors (n= 16). Blue curve = PD-(L)1 inhibitor naïve patients (n=10)

#### 5.1.3.3 Safety

In total, twenty of the 27 patients (74%) reported a grade 3 adverse event (AE) but none were related to treatment. Nine patients (33%) experienced a treatment-related grade 1/2 AE (Table 5-3). The most frequent grade 1/2 treatment-related AEs were fatigue in four patients (15%) and diarrhea, anemia and myalgia in two patients each (7%). No treatment discontinuation was related to toxicity.

| Adverse event | N=27<br>N (%) |
|---|---|
| Fatigue | 4 (15%) |
| Diarrhea | 2 (7%) |
| Anemia | 2 (7%) |
| Myalgia | 2 (7%) |
| Hypothyroidism | 1 (4%) |
| Allergic reaction | 1 (4%) |
| Stoma site infection | 1 (4%) |
| Pruritus | 1 (4%) |
| Maculo-papular rash | 1 (4%) |
| Oral mucositis | 1 (4%) |
| Oral dysesthesia | 1 (4%) |
| Alanine aminotransferase increased | 1 (4%) |
| Aspartate aminotransferase increased | 1 (4%) |

**Table 5-3. Grade 1-2 treatment-related adverse events according to CTCAE v4.03**

### 5.1.4 Discussion

The I1 cohort is the first immunotherapy cohort of the UPSTREAM trial. This sub-study failed to meet its primary endpoint as no response was seen. Disease stabilization was observed in six patients (23%). Median PFS was 1.7 months and median OS was 6.7 months in the overall population. Monalizumab monotherapy was safe as no grade 3/4 treatment-related adverse events were recorded.

The I1 cohort included a heavily pretreated population as 65% of the patients had received two or more previous lines of systemic treatment in the R/M setting. Although this may have impacted our results, the study design of UPSTREAM could have selected a more favorable patient group. Given that a fresh biopsy is

mandatory for study entry, the time frame (median 14 days, range: 10-22 days) needed for the biopsy results to come back could have selected patients in good condition with less rapidly progressing disease. Another potential confounding factor is that only those patients who were not eligible for one of the biomarker-driven cohorts were included in the I1 cohort. However, 48% of the I1 cohort's patients were allocated to I1 because of inadequate biopsy quality. These patients could thus potentially also harbor some of the predefined biomarkers, making them similar to the rest of the UPSTREAM population.

Monalizumab has been investigated in combination with cetuximab in patients with R/M SCCHN who progressed after platinum therapy and after two or less prior lines of therapy. [344] This single arm trial showed an ORR of 27%, a median PFS of 4.5 months, and a median OS of 8.3 months. These results compare favorably with cetuximab monotherapy after platinum therapy [322] (ORR: 13% and median OS: 178 days), suggesting that monalizumab could have some level of activity in combination with cetuximab. The hypothesis is that monalizumab may enhance cetuximab-induced antibody dependent cellular cytotoxicity (ADCC), as supported by in vitro data.[340]

We performed an exploratory OS analysis according to PD-(L)1 inhibitor exposure. Patients exposed to PD-(L)1 inhibitors had a median OS of 8.4 months (95% CI: 2.7-10.7 months), while for PD-(L)1 inhibitor-naïve patients, the median OS was 4.1 months (95% CI: 1.1-9.2 months). Interestingly, this finding was also reported in the trial that investigated the combination of monalizumab and cetuximab in R/M SCCHN where the OS of IO-pretreated patients was 12.8 months versus 7.8 months in IO-naïve patients.[344] Although patient numbers were low, confidence intervals were large and it was not a pre-planned analysis, the median survival of PD-(L)1 inhibitor-exposed patients in the two monalizumab SCCHN trials seems promising given that the median survival of patients treated with with PD-(L)1 inhibitors after platinum therapy is between 7.1 and 8.4 months.[173,174,339] Several hypotheses could explain these results: (i) the biased population which included a favorable group of patients, (ii) potential synergism between monalizumab and PD-(L)1 inhibitors, particularly if we consider that the biological activity of PD-1/PD-L1 inhibitors could still be present at the time we initiated monalizumab (15 patients had received

prior PD/PD-L1 inhibitors), (iii) or, in contrast, slower disease progression due to the activity of PD-(L)1 inhibitors alone, or better activity of subsequent treatment.

Preclinical data show that NKG2A is often co-expressed with PD-1 on CD8+ T cells.[340] [340] As PD-1 is a marker of exhausted T cells,[346] this might suggest that targeting NKG2A could reverse T-cell exhaustion. Moreover, it has been shown that HLA-E is more frequently expressed than PD-L1 in several cancer types and may potentially be responsible for the lack of response to PD-(L)1 inhibitors in some cancers.[340] Targeting the NKG2A-HLA-E pathway in combination with PD-1/PD-L1 blockade also improved tumor control in mice.[340] These data support the investigation of monalizumab in combination with PD-1/PD-L1 inhibitors.

In conclusion, single agent monalizumab has limited activity in R/M SCCHN, and the I1 cohort was closed at the interim analysis due to futility. Monalizumab continues to be investigated in combination with durvalumab within the UPSTREAM trial and with cetuximab in another phase II trial (NCT02643550). A randomized phase III study will compare cetuximab with or without monalizumab in patients with recurrent/metastatic SCCHN.

# 6. LIQUID BIOPSY FOR MUTATIONAL PROFILING OF LOCOREGIONAL RECURRENT AND/OR METASTATIC SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Adapted from Galot et al (2019), submitted

# "Liquid biopsy for mutational profiling of locoregional recurrent and/or metastatic squamous cell carcinoma of the head and neck"

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*Similar contribution

**Abstract**

***Background***

The molecular landscape of SCCHN harbors potentially actionable genomic alterations. We aimed to study the utility of liquid biopsy to (i) characterize the mutational landscape of recurrent/metastatic SCCHN using a comprehensive gene panel and (ii) estimate the concordance between DNA mutations identified from circulating tumor DNA (ctDNA) and matched tumor tissues.

***Methods***

Targeted next-generation sequencing (NGS) was performed on the cell-free DNA (cfDNA) of 39 patients with locoregional recurrent (n=19) and/or metastatic (n=20) SCCHN. Tumor biopsy (n=18) was sequenced using the same technique.

***Results***

ctDNA was detected in 51% of patients with a higher probability of detection in metastatic than locoregional recurrent disease (70% versus 30%, p=0.025). 81% and 58% of the tissue tumor variants were not detected in plasma when considering all patients and only metastatic patients with detectable ctDNA, respectively. In a multivariate analysis, the likelihood of detecting the tissue tumor variant in plasma was related to metastatic status (p=0.012), tumor variant allele frequency (p<0.001) and ctDNA quantity (p<0.001). 26% of the variants were detected only in liquid and not in the solid biopsy. Three patients without an available tumor sample had plasma containing three different potentially actionable *PIK3CA* mutations.

***Conclusions***

CtDNA detection and characterization using targeted NGS is feasible in metastatic SCCHN. Liquid biopsies do not reflect the complete mutation profile of the tumor but have the potential to identify actionable mutations when tumor biopsies are not available as well as other variants not found in matched tumor tissue.

## 6.1 Introduction

Cell-free DNA (cfDNA) refers to the presence of nucleic acids in circulation that are released by healthy and malignant cells. Circulating tumor DNA (ctDNA), originating from tumor cells, is characterized by the presence of tumor-specific somatic mutations. The analysis of cfDNA, commonly referred to as a liquid biopsy, is a non-invasive tool to detect somatic mutations in various types of cancer. [260,271,273,288,347] However, as the proportion of ctDNA can sometimes be extremely low (<1%), [275] detecting these mutations can prove challenging. Therefore, the investigation of the diagnostic value and clinical impact of ctDNA using different sequencing techniques across different disease stages is of crucial importance.

Treatment strategies for squamous cell carcinoma of the head and neck (SCCHN) are still based on disease localization and tumor staging and do not incorporate molecular characteristics. Despite multimodal treatment strategies that include surgery and/or (chemo)radiation, less than 60% of patients with locally advanced disease remain free of disease at three years. Patients with recurrent and/or metastatic (R/M) disease have a poor prognosis with a median survival of between 10-15 months. Recently, the characterization of the molecular landscape of SCCHN has identified potential therapeutic targets, opening the door to new treatment opportunities. [87] These are currently under investigation in clinical trials. [193]

SCCHN is, as other tumors, an evolutionary and heterogeneous disease.[88] The characterization of the active molecular landscape and the application of precision medicine in SCCHN thus requires to obtain fresh tumor biopsies multiple times over the course of the disease. In advanced SCCHN, obtaining sequential tumor biopsies might be challenging for multiple reasons: Local recurrence may sometimes require general anesthesia, and distant metastases may not be easily accessible. The risk of complications is also high when the tumor to be biopsied is close to blood vessels or localized in a previously irradiated area. Therefore, the ability to identify potential therapeutic targets using a liquid biopsy is an interesting alternative to tumor biopsy. Different studies have explored the feasibility of identifying somatic alterations using liquid biopsy in SCCHN. However, these were mainly single-mutation approaches and/or based on alterations previously identified in the

tumor biopsy. [258,306-308,312] Although the proof-of-concept of ctDNA detection for SCCHN has been shown, [258,306-308,312] the use of liquid biopsy to characterize the mutational landscape of SCCHN using a large comprehensive gene panel is underexplored. [141]

Using next-generation sequencing (NGS) on a custom panel of 604 genes, we investigated the feasibility of detecting ctDNA in a prospective cohort of patients with locoregional recurrent and/or metastatic SCCHN using a tissue-agnostic approach (without prior knowledge of somatic mutations in the solid tumor). As a second step, we assessed how ctDNA reflects the mutational landscape of the tumor.

## 6.2 Materials and methods

### 6.2.1 Study design and patient selection

The objective of the trial was to investigate the feasibility and utility of liquid biopsy in SCCHN, using a targeted cfDNA sequencing approach with a custom panel of 604 genes. Using a tumor tissue-agnostic approach (no prior knowledge of somatic mutations in the solid tumor), the two endpoints of our study were to determine: (i) the value of this technology in detecting ctDNA in two different groups of R/M SCCHN: patients with locoregional recurrent SCCHN without distant metastasis, and patients with metastatic disease with or without locoregional relapse; and (ii) the concordance of the mutational landscape between ctDNA and the matched tumor. To this end, we selected 20 patients with metastatic disease and 19 patients with locoregional recurrent disease.

The plasma samples used in this work were the baseline samples obtained at the first incurable relapse or at diagnosis of upfront incurable disease.

### 6.2.2 Sample collection

Patients with incurable locoregional recurrent and/or metastatic SCCHN treated with standard of care at our institution were prospectively enrolled in the UCL-ONCO-2013 trial, a non-interventional trial collecting whole blood (to isolate germline DNA), plasma samples (for cfDNA analysis) and, whenever possible, tumor tissue biopsies (NCT02139020). Patients with nasopharyngeal cancer, salivary gland tumors, and nasal or paranasal sinuses were excluded. The study was approved by our independent ethics committee and conducted in accordance with the Declaration of Helsinki (October 2000). Written informed consent was obtained for each patient.

All blood samples were collected in ethylenediamine tetraacetic acid (EDTA) blood collection tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). One EDTA tube for whole blood collection was immediately stored at −80 °C without further processing until germline DNA isolation. Twelve mL of blood were taken for plasma collection and two centrifugation steps were performed. The first centrifugation (10 minutes at 2000g at 4°C) was performed within 30 minutes of blood collection followed by a second (15 minutes at 2500g at 4°C). After this, plasma was aliquoted into 0.5 mL portions and stored at −80 °C until cfDNA isolation.

### 6.2.3 DNA extraction and quantification

Germline DNA was extracted from whole blood samples using the QiaAmp DNA Mini Kit (Qiagen, Q51304, Hilden, Germany). cfDNA was extracted from 4 mL of the baseline plasma sample using the Maxwell® RSC Circulating DNA Purification Large Volume Protocol Kit (AX1115) on the RCF Maxwell machine according to the manufacturer's instructions (both from Promega, Wisconsin, USA). DNA from formalin-fixed paraffin-embedded (FFPE) tumor tissues was extracted using the GeneRead DNA FFPE Kit (Qiagen, Q180134, Hilden, Germany). DNA concentrations were quantified by fluorometry using the Qubit™ dsDNA HS Assay Kit (high sensitivity assay kit (Q32854) on the Qubit® 2.0 Fluorometer - both from Thermo Fisher Scientific, Massachusetts, USA).

### 6.2.4 Targeted Next-Generation Sequencing

Targeted NGS of the germline, cell-free and tumor tissue biopsy-derived DNA at a median coverage depth of 1000x was performed using a custom panel of 604 genes, covering a list of frequently mutated tumor suppressor genes and oncogenes in SCCHN. Library preparation, sequence capture and sequencing were carried out by IntegraGen (Evry, France). Sequence capture, enrichment and elution were performed according to the manufacturer's instructions and protocols (SureSelectXT Custom kit 0,5-2,9Mb, Agilent Technologies, California, USA) without modification except for library preparation, which was performed with a NEBNext® Ultra™ kit (New England BioLabs, Massachusetts, USA). For library preparation, at least 2 ng of each genomic DNA were processed. Paired-end adaptor oligonucleotides from the NEB kit were ligated on repaired and dA-tailed fragments, then purified and enriched by 10 polymerase chain reaction (PCR) cycles. At least 800 ng of these purified libraries were then hybridized to the SureSelect oligo probe capture library for 72 hours (hr). After hybridization, washing and elution, the eluted fraction was PCR-amplified with 14 cycles, and then purified and quantified by quantitative polymerase chain reaction (qPCR) to obtain a sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina HiSeq®4000 as paired-end 75 bp reads. Image analysis and base calling was performed using Illumina Real Time Analysis (2.7.7) with default parameters.

Reads were aligned to the reference human genome sequence GRCh37 using Burrows-Wheeler Aligner 0.7.15 (Wellcome Trust Sanger Institute). Duplicate reads were marked and removed using Picard 1.107 (Broad Institute). Local realignment around indels and base quality score recalibration were performed using the Genome Analysis Toolkit 3.3 (Broad Institute). Germline single-nucleotide variants (SNV) and small indels were identified on the germline DNA samples using GATK Haplotype Caller 3.3, whereas somatic SNV and small indels were identified on the tumoral DNA and cfDNA using the MuTect2 algorithm based on GATK Haplotype Caller 3.7 (Broad Institute). Called variants were annotated, filtered and visualized using Highlander (http://sites.uclouvain.be/highlander/), an in-house bioinformatics framework. Eight different prediction software tools were used to estimate the pathogenicity of the variants: Sift, LRT, Mutation Assessor, FATHMM,

Mutation Taster, DEOGEN, Polyphen2 and CADD. Splicing effects were estimated by two ensemble learning methods [348]. A variant was considered if it was (i) supported by at least 10 reads, (ii) passed MuTect2 quality filters, and (iii) was present in less than 10 individuals in ascertained public sequence databases (ExAC, GoNL, 1000G). All retained variants were manually inspected for alignment.

### 6.2.5 Bio-informatics processing of the data and statistics

All analyses downstream of the variant calling were performed in R (version 3.5.1, http://www.R-project.com). Two statistical filters were applied to ascertain the somatic nature of the variant and distinguish it from background noise. These criteria were applied to both cfDNA and FFPE samples. We calculated a Z-score for each variant, with the alternative allelic frequency (alt AF) being the proportion of mutated reads in a sample:

$$Z\ score = \frac{alt\ AF\ in\ cfDNA/FFPE\ sample - \ mean\ alt\ AF\ in\ germline\ samples}{standard\ deviation\ of\ alt\ AF\ in\ germline\ samples}$$

A variant was retained if it had a Z-score $\geq 5$. Finally, if the variant was present in more than five reads in > 10% of samples of the same type (cfDNA or FFPE), it was discarded. Only the variants fulfilling these criteria were considered to be true somatic mutations.

Tumor tissue biopsy and cfDNA samples were paired to estimate the concordance of the findings. Results were correlated with clinic-pathological features. Mann-Whitney and Fisher's exact tests were used for inferential statistics depending on the type of data, with a two-sided level of significance of 0.05.

A logistic regression model was created to analyze the relationship between the detection of a tumor tissue variant in plasma (dependent binary variable) and five independent variables (metastatic status, the variant AF in the tumor tissue, ctDNA

quantity, classification of the variant as a driver or passenger based on the TCGA data, [87] and the time between tumor tissue biopsy and plasma sample collection).

### 6.2.6 Digital droplet PCR (ddPCR)

Mutation detection by ddPCR (Bio-rad Laboratories, QX200™ ddPCR system, California, USA) was used to confirm results obtained by NGS. For each mutation, wild type and mutant allele sequences were submitted for ddPCR assay design (Bio-rad Laboratories, California, USA). Reaction volume was prepared with 7 microliters (μL) of master mix containing 5μL of ddPCR Multiplex Supermix (no deoxyurudine triphosphate), 1μL of diluted restriction enzyme (HaeIII) and 1μL of the forward and reverse primers as well as the FAM-labeled mutant and HEX-labeled wild-type probe. Thirteen μL of DNA sample (cfDNA or FFPE-extracted DNA) was added to obtain a total reaction volume of 20 μL. The total amount of cfDNA tested ranged between 10 and 65 ng. The same amount was used for the FFPE samples and the positive and negative controls (wild type samples). Germline DNA from other cancer patients was used as a negative (wild-type) control and gBlocks® (sequence-verified, double-stranded DNA fragments, Integrated DNA technologies, Iowa, USA), diluted to various molar concentrations in wild-type germline DNA, were used as positive controls. The "No template controls" (NTC) wells contained 13 μL of purified water instead of DNA samples. The total 20 μL of reaction were used with 70 μL of generation oil for droplet generation. Droplets were then transferred to a 96-well PCR plate. The optimal annealing was determined for each assay independently before sample testing according to the manufacturer's instructions (Bio-rad Laboratories, California, USA). Thermal cycling conditions were set as follows: 95°C for 10 minutes (1 cycle), 94°C for 30 seconds, and previously determined optimal annealing temperature for 1 minute (40 cycles). This was followed by 10 minutes at 98°C and infinite hold at 4°C. For each sample tested, one NTC and seven negative controls were tested on the same run.

### 6.2.7 ddPCR data analysis

Data analysis was performed on QuantaSoft™ v1.7.4 software according to the manufacturer's instructions (Bio-rad Laboratories, California, USA). Thresholds were fixed manually based on the NTC and the negative controls. QuantaSoft™ software provided concentration in terms of copies of target per microliter (copies/μL). The false positive rate for each test was estimated by merging the results of the seven different negative control wells. The tested sample was considered positive if the error bars of the 95% CI of the concentration (copies/μl) between the negative controls and the test sample did not overlap. If the error bars did overlap, a t-test was used to determine whether the negative controls and test sample were significantly different ($p< 0.05$).

## 6.3 Results

### 6.3.1 ctDNA detection

Targeted sequencing was performed on the cfDNA of 39 SCCHN patients; 20 had metastatic disease and 19 were only locoregionally recurrent. A matched tumor biopsy was available for 18 patients (8 locoregional recurrent and 10 metastatic). Patient characteristics are listed in Table 6-1. In total, 285 variants were called in the cfDNA of 27 patients. Of these, 146 unique variants (51%) in 20 cfDNA samples passed our filtering criteria. ctDNA was thus detected in 20 patients out of 39 (51%). These patients carried a median of four variants (range 1-55 variants; Table 6-2). The plasma sample of patient HNSCC-62, who had p16-positive oropharyngeal cancer (p16+OPC), was the only outlier, with a high mutation rate (55 variants). This patient had a history of heavy smoking (> 10 pack-years) and a somatic mutation in a mismatch-repair gene, *MSH2*, that might explain the higher mutation rate [349]. The probability of ctDNA detection was higher for patients with metastatic disease compared to locoregional recurrent (70% vs 30%, $p=0.025$: Figure 6-1). The quantity of ctDNA, estimated as the median allele frequency (AF) of the somatic variants (passing filtering criteria) detected in the cfDNA, ranged from 1.16% to 20.25% (Table 6-2, Figure 6-1).

| Patients (n) | 39 |
|---|---|
| **Primary disease localization, n (%)** | |
| oral cavity | 8 (21%) |
| oropharynx | 22 (56%) |
| p16 negative | 17 |
| p16 positive | 5 |
| hypopharynx | 6 (15%) |
| larynx | 2 (5%) |
| unknown primary | 1 (2%) |
| **Locoregional recurrence, n (%)** | 19 (49%) |
| local | 12 |
| loco-regional | 7 |
| **Metastatic, n (%)** | 20 (51%) |
| non-visceral only (bone, mediastinal nodes) | 3 |
| visceral, lung-only | 10 |
| visceral, liver metastasis (with/without lung) | 7 |
| **Indication of systemic palliative treatment, n (%)** | |
| upfront incurable | 9 (23%) |
| recurrence after curative treatment | 30 (77%) |
| **Previous curative treatment, n (%)** | |
| radiotherapy | 3 |
| surgery | 2 |
| surgery + radiotherapy | 7 |
| surgery + adjuvant chemoradiation | 2 |
| chemoradiation | 14 |
| radiotherapy + concurrent cetuximab | 2 |

**Table 6-1. Patient characteristics**

| Patient ID | Disease status | estimated ctDNA(%) | Min AF(%) | Max AF(%) | Nb of variants |
|---|---|---|---|---|---|
| HNSCC-45 | M | 20.25 | 5.66 | 39.9 | 4 |
| HNSCC-27 | M | 14.25 | 1.33 | 60.18 | 8 |
| HNSCC-54 | M | 13.31 | 7.23 | 27.68 | 10 |
| HNSCC-59 | M | 12.36 | 10.82 | 15.81 | 4 |
| HNSCC-56 | LR | 5.97 | 2.03 | 11.82 | 6 |
| HNSCC-73 | LR | 5.05 | 5.05 | 5.05 | 1 |
| HNSCC-47 | M | 4.32 | 1.32 | 10.24 | 17 |
| HNSCC-62 | M | 3.43 | 1.86 | 27.08 | 55 |
| HNSCC-30 | LR | 2.87 | 2.87 | 2.87 | 1 |
| HNSCC-64 | M | 2.46 | 1.31 | 3.19 | 6 |
| HNSCC-31 | LR | 2.32 | 2.32 | 2.32 | 1 |
| HNSCC-34 | M | 2.22 | 0.98 | 4.74 | 7 |
| HNSCC-71 | LR | 2.21 | 1.67 | 2.75 | 2 |
| HNSCC-39 | M | 2.16 | 2.16 | 2.16 | 1 |
| HNSCC-68 | M | 1.99 | 1.98 | 4.75 | 3 |
| HNSCC-38 | M | 1.86 | 0.88 | 2.88 | 5 |
| HNSCC-44 | M | 1.86 | 1.15 | 3.1 | 4 |
| HNSCC-51 | M | 1.78 | 1.2 | 2.67 | 4 |
| HNSCC-60 | M | 1.55 | 1.49 | 1.6 | 2 |
| HNSCC-67 | LR | 1.16 | 0.81 | 1.32 | 5 |

**Table 6-2. cfDNA samples with detectable ctDNA**

AF, allele frequency; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; LR, locoregional; M, metastatic; Nb, number

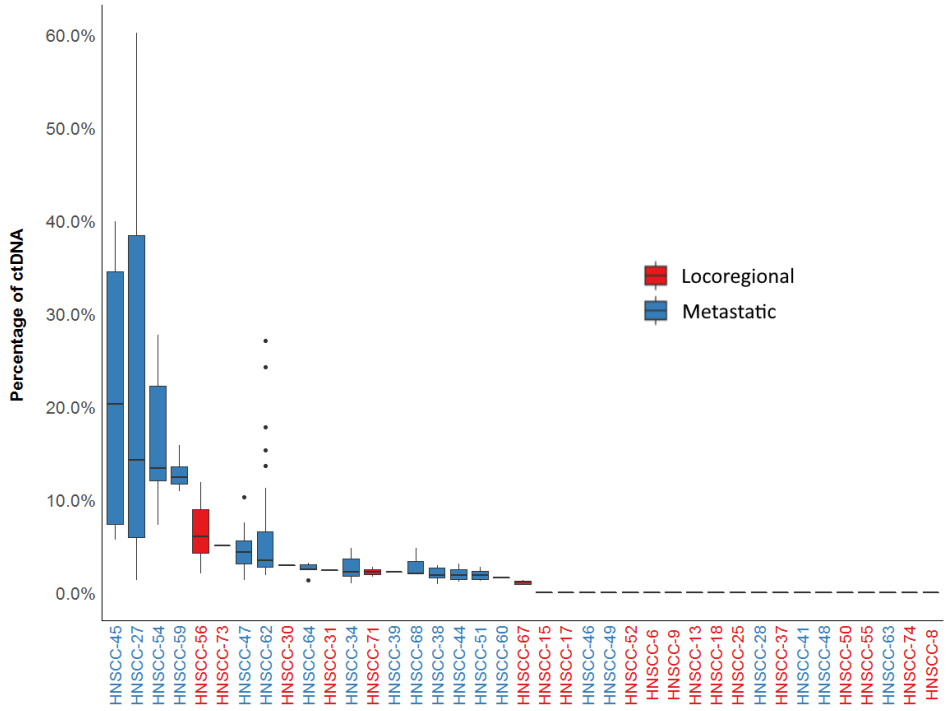


**Figure 6-1. ctDNA detection in 39 cfDNA samples from patients with SCCHN**

Box-plot showing the allele frequencies of all the somatic plasma variants (y-axis) for each cfDNA sample (x-axis). The quantity of ctDNA was estimated as the median of the allele frequencies of all the somatic variants retained in the plasma sample (indicated by the horizontal black bar in the box plot). Patients with metastatic disease and locoregional recurrence are indicated in blue and red, respectively.

### 6.3.2 Mutational landscape of ctDNA

Figure 6-2 represents the somatic mutational landscape of the 20 cfDNA samples in which ctDNA was detected. Only non-synonymous mutations are shown. As expected, the most frequently mutated gene in oral cavity, hypopharynx, larynx, and p16-negative oropharyngeal (p16-OPC) cancers was *TP53* (n=8/16; 50%). This was followed by mutations in genes implicated in the phosphoinositide 3-kinase (PI3K)-pathway, which were present in both groups (n= 3/20; 15%).

Other less frequent mutations were detected in multiple pathways that might be implicated in SCCHN oncogenesis. Mutations in different genes coding for tyrosine kinase receptors (TKR) were detected (*EGFR*, *ERBB3*, *AXL*, *CSF1R* and *RET*; each gene was mutated in one patient). Although we did not detect any *HRAS* mutation, the mitogen-activated protein kinase (MAPK) signaling pathway was altered by mutations in *MAPK1* or *NF1* (a negative regulator of RAS activation), in two other patients. Genes implicated in chromatin regulation are known to be altered in HPV-negative HNSCC [87] and in up to 33% of HPV-positive OPC. [350] In our cohort, we detected mutations in some of these genes (*KMT2C, KMT2B*, *NSD1, CREBBP, SMARCA2* and *SMARCA4*), in six patients. Mutations were detected in genes of the Notch, Hedgehog (HH) and Wnt pathways, as well as in the apoptosis-related gene CASP8, in six and one patient, respectively. Finally, four patients harbored mutations in genes implicated in DNA repair. Two mutations were seen in homologous recombination-related genes (*ARID1A, BRCA1*), and three mutations in mismatch repair genes (*MSH2, MLH1*).

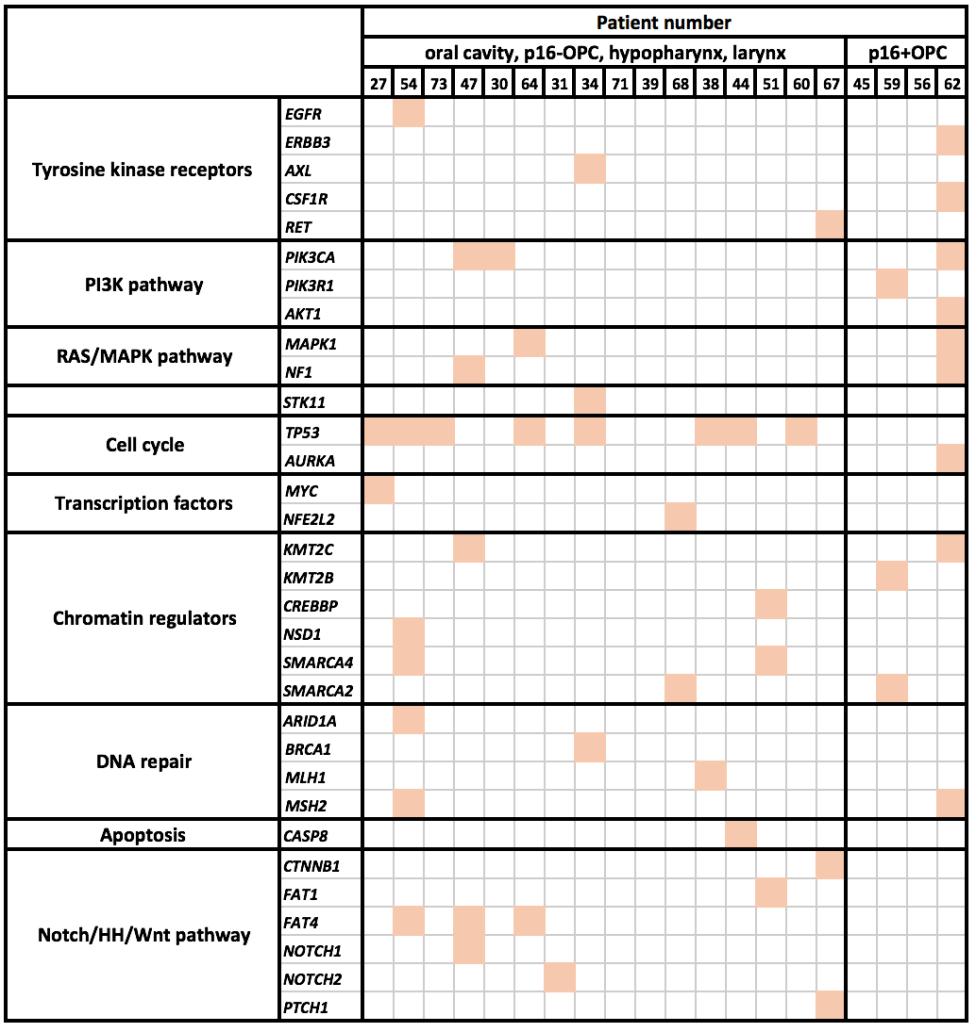


**Figure 6-2. Mutational landscape of the 20 cfDNA samples in wich ctDNA is detected**

The mutational landscape of the 20 ctDNA-positive cfDNA samples is presented. Genes with potential roles in SCCHN oncogenesis that have non-synonymous mutations in at least one sample are shown, sorted by pathway. OPC, oropharyngeal cancer

### 6.3.3 Concordance of variants between liquid and solid biopsy

For 18 patients, a corresponding tumor tissue biopsy was available. These biopsies were taken at the same time point as the sequenced plasma sample in all but two patients (HNSCC-34 and HNSCC-56, for which we used an archival biopsy).

In total, 209 variants were detected across these 18 tumor tissue biopsies (range: 4 - 21). In this group of 18 patients, ctDNA was detected in 12. The concordance rates were highly dependent on the ctDNA quantity harbored by the patient. Considering the 18 patients, 81% of the variants (169/209) identified in solid tumors were not detected in plasma. Conversely, 26% of the plasma variants (14/54) were not detected in the matched tumors, possibly reflecting tumor heterogeneity. Figure 6-3 shows the individual concordance for the 12 patients with detectable ctDNA and available paired tumor-biopsies. Considering only metastatic patients with detectable ctDNA, the rate of solid tumor variants also detected in plasma increased to 42%. For patients HNSCC-27, -54 and -59 harboring the highest ctDNA quantities (>10%), the amount of tumor variants detected in plasma was greater than 80%. However, for samples with a ctDNA quantity below 10%, the detection rate of tumor variants in plasma was lower (median: 14%, range: 0-55%).

In a logistic regression model, the likelihood of detecting tumor tissue variants in plasma was significantly impacted by the patient's metastatic status (odds ratio (OR) 4.50, $p=0.012$), the variant AF in the solid biopsy (OR=5.31, $p<0.001$), and the quantity of ctDNA (OR=4.13, $p<0.001$). Classification of the variant as a driver or passenger based on The Cancer Genome Atlas (TCGA) and the Catalogue of Somatic Mutations in Cancer (COSMIC) was, however, not relevant (OR=1.05, $p=0.948$), nor was the concordance of time between tissue biopsy and plasma sample collection (OR=1.39, $p=0.7$).

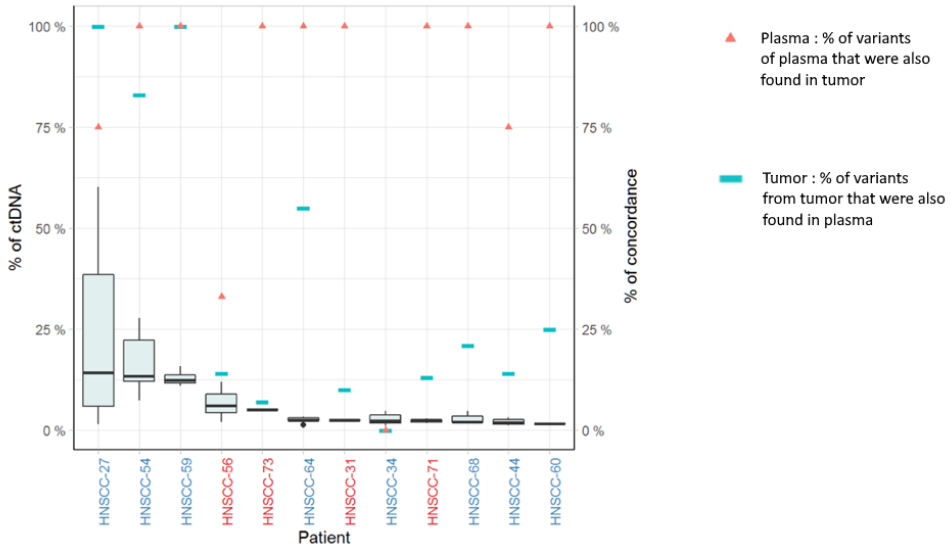


**Figure 6-3. Concordance between liquid and solid biopsy according to ctDNA quantity**

The concordance is shown for 12 patients with detectable ctDNA for which solid biopsy was sequenced. The box plot shows the allele frequencies of all the somatic plasma variants (right y-axis) for each cfDNA sample (x-axis). The quantity of ctDNA was estimated as the median of the allele frequencies of all the somatic plasma variants in that sample, indicated by the horizontal black bar in the box plot. For each cfDNA sample, the detection rate in the tumor of variants from plasma is indicated by the red triangle, whereas the detection rate in plasma of the variants from the tumor is indicated by the horizontal blue bar.

### 6.3.4 Concordance of driver events between liquid and solid biopsy

We performed a specific analysis focused on potential driver events. A driver event was defined as one of the following types of somatic alterations in a gene considered to be a SCCHN oncogenesis driver by TCGA [87]: (i) missense variants or in-frame indels predicted as pathogenic by at least four types of predictive software, (ii) frameshift or nonsense variant in genes for which loss of function is expected to be pathogenic, (iii) mutations predicted to affect splicing or (iv) known COSMIC mutations. In total, 23 driver events were detected in the cfDNA and/or FFPE samples of 17 patients (Table 6-3).

Figure 6-4 depicts the landscape of potential driver events in paired samples of both cfDNA and tumor tissue. As expected, *TP53* alterations were the most frequent driver events, both in the liquid and solid biopsies. We also detected other frequently reported driver alterations, including mutations in the PI3K pathway and one *EGFR* mutation. Forty-one percent (9/22) of solid tumor driver events were detected in plasma. After manual inspection, nine additional events that did not pass our background-noise threshold were found in plasma, underlining the limited sensitivity of the technique. Patient HNSCC-34 harbored one *TP53* mutation in the solid tumor but had a different *TP53* mutation in plasma.

| Patient | Disease status | ctDNA (%) | Gene | SNP effect | COSMIC ID | Detected in the solid tumor and cfDNA sample | AF in tumor (%) | AF in plasma (%) |
|---|---|---|---|---|---|---|---|---|
| HNSCC-27 | M | 14.25 | *TP53* c.919+1G>C | Splice-site | COSM13585 | Yes | 49% | 59% |
| HNSCC-27 | M | 14.25 | *MYC* c.1096G>A | Non-synonymous | NA | Yes | 21.8% | 9.2% |
| HNSCC-54 | M | 13.31 | *TP53* c.711G>A | Non-synonymous | COSM3378348 | Yes | 36% | 11.4% |
| HNSCC-54 | M | 13.31 | *EGFR* c.2584C>G | Non-synonymous | NA | Yes | 34.4% | 19.4% |
| HNSCC-59 | M | 12.36 | *PIK3R1* c.1699A>G | Non-synonymous | COSM1154507 | Yes | 26% | 11.9% |
| HNSCC-73 | LR | 5.05 | *TP53 c.731G>T* | Non-synonymous | COSM43652 | Yes | 60.6% | 5.1% |
| HNSCC-73 | LR | 5.05 | *CDKN2A* c.243delC | Frame-shift | NA | No | 62.5% | / |
| HNSCC-64 | M | 2.46 | *TP53 c.892G>T* | Stop-gain | COSM10710 | Yes | 58.6% | 2.4% |
| HNSCC-31 | LR | 2.32 | *TP53* c.559+1G>A | Splice-site | COSM131536 | No | 26.9% | / |
| HNSCC-34 | M | 2.22 | *TP53* c.949C>T | Stop-gain | COSM1709728 | No | / | 4% |
| HNSCC-34 | M | 2.22 | *TP53* c.919+1G>C | Splice-site | COSM13585 | No | 27.3% | / |
| HNSCC-68 | M | 1.99 | *TP53* c.520A>T | Non-synonymous | COSM3773316 | No | 37.2% | / |
| HNSCC-68 | M | 1.99 | *TP53* c.993G>T | Non-synonymous | COSM1158345 | No | 20.6% | / |
| HNSCC-68 | M | 1.99 | *MYC* c.629T>G | Non-synonymous | NA | No | 31.2% | / |
| HNSCC-44 | M | 1.86 | *TP53* c.818G>A | Non-synonymous | COSM1645335 | Yes | 19.7% | 2.3% |
| HNSCC-60 | M | 1.55 | *TP53 c.844C>T* | Non-synonymous | COSM1636702 | Yes | 71.7% | 1.6% |
| HNSCC-13 | LR | 0 | *TP53* c.892G>T | Stop-gain | COSM10710 | No | 77% | / |
| HNSCC-18 | LR | 0 | *TP53* c.581_585delTTATC | Frame-shift | NA | No | 5.6% | / |
| HNSCC-18 | LR | 0 | *PIK3CA* c.1034A>T | Non-synonymous | COSM94978 | No | 3.2% | / |
| HNSCC-41 | M | 0 | *TP53* c.517G>A | Non-synonymous | COSM2744864 | No | 26.6% | / |
| HNSCC-49 | M | 0 | *TP53* c.454C>T | Non-synonymous | COSM43582 | No | 31.4% | / |
| HNSCC-52 | LR | 0 | *TP53* c.637C>T | Stop-gain | COSM3378350 | No | 51% | / |
| HNSCC-55 | LR | 0 | *TP53* c.329G>T | Non-synonymous | COSM99929 | No | 4.5% | / |

**Table 6-3. Driver events in paired samples**

AF, allele frequency; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; LR, locoregional; M, metastatic; SNP, single-nucleotide polymorphism

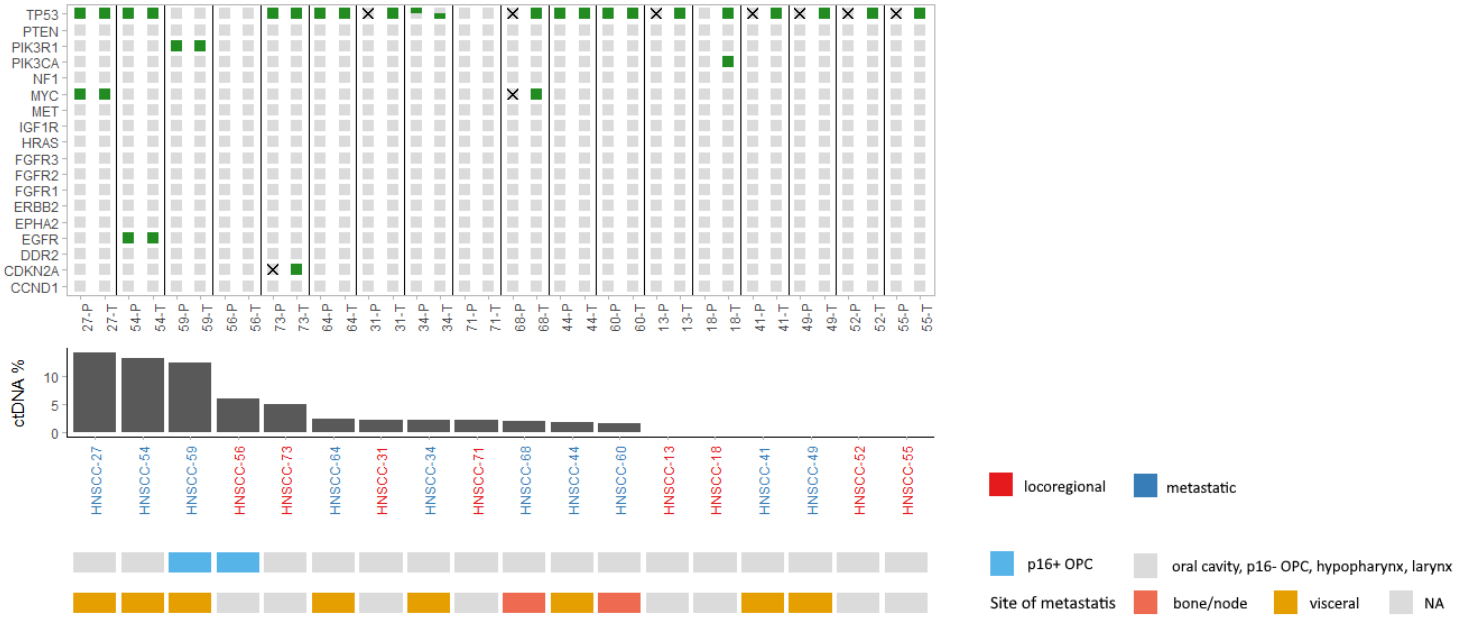


**Figure 6-4. Concordance of driver events between ctDNA and the matched tumor**

The concordance of driver events detected in ctDNA and matched tumor tissue of 18 patients is shown according to the ctDNA quantity, with clinical characteristics indicated at the bottom. Concordant driver events are shown in green. Black crosses indicate the driver events that were not retained by our classical filters but that were detected upon manual inspection of the sequencing data. HNSCC-34 harbors two different *TP53* mutations in the solid tumor versus the cfDNA sample.

### 6.3.5 Driver events in plasma samples without solid biopsy available

Of clinical interest, the plasma of three patients for whom no tumor was available carried three different potentially actionable *PIK3CA* mutations, including the p.E545K hotspot mutation.

### 6.3.6 Lowering thresholds only revealed more background-noise

As our retrospective manual analysis revealed the presence of variants that did not pass our background-noise threshold, we performed an analysis with less stringent criteria. We lowered the cut-off for variant detection in plasma to $\geq 5$ reads while retaining the downstream criteria. This resulted in the detection of four driver events that were not retained in our first analysis, enhancing the rate of detection of driver events to 60%. This less stringent analysis also revealed new and potentially interesting variants. We detected the somatic *ERBB22* S310Y variant (a hotspot activating mutation in breast cancer) in the cfDNA of three different patients. However, we considered them as false positive findings, as the ddPCR analysis of this variant in the three plasma samples was negative.

### 6.3.7 ddPCR to detect mutations with higher sensitivity

As shown in Figure 6-4 and as previously discussed, manual inspection of the data from plasma samples revealed nine driver events that were initially detected in the matched tumor sample but which failed to pass our background-noise threshold in plasma. We used ddPCR to screen these mutations in the tumor tissue biopsies and plasma samples of five patients for whom cfDNA was still available (Table 6-4). All five variants were detected in the matching plasma samples, one with an AF below 0.1%. ddPCR confirmed the variants in all five of the solid tumors tested and yielded a similar AF as that obtained by NGS. Slight variations in AF may be due to spatial tumor heterogeneity as new DNA extractions from the same FFPE block were required for ddPCR testing.

| Patient ID | Variant | Tumor AF (%) by NGS | Tumor AF (%) by ddPCR | Manual Plasma Detection (altered read/total reads) | Plasma AF ddPCR |
|---|---|---|---|---|---|
| 41 | *TP53* c.517G>A | 26% | 22.6% | 7/697 | 1.2% |
| 52 | *TP53* c.637C>T | 51% | 55.7% | 3/991 | 0.08% |
| 49 | *TP53* c.454C>T | 31.4% | 21.3% | 7/573 | 1.24% |
| 68 | *TP53* c.520A>T | 21% | 26.2% | 6/525 | 0.6% |
| 31 | *TP53* c.559+1G>A | 27% | 20.3% | 1/601 | 0.25% |

**Table 6-4. Mutation detection by digital droplet PCR**

AF, allele frequency; ddPCR, digital droplet PCR

## 6.4 Discussion

We explored the relevance of liquid biopsy to characterize the mutational landscape of R/M SCCHN using a panel of 604 cancer-related genes at deep coverage. We report ctDNA detection in 20/39 patients (51%) with a significantly higher probability for ctDNA detection in patients with metastatic disease compared to patients with only locoregional recurrence (70% vs 30%). This finding suggests a potential limitation of this technique given that around one third of recurrent SCCHN patients will have locoregional relapse without distant metastases. [169,170] The difficulty to detect ctDNA in localized disease using a similar sequencing approach has been also shown in other tumor types. [351] In SCCHN, Wang and colleagues reported a higher rate of ctDNA detection in early stage disease [306] using a PCR-based technique with detection of allele frequencies that are below our threshold of detection with targeted NGS. In addition, PCR-based approaches are restricted to a low number of genes.

Overall, the mutational landscape characterized by ctDNA in our study reflects the mutational spectrum that has been reported in the literature by tissue tumor

sequencing, with *TP53* mutations and genomic alterations in the PI3K pathway being among the most frequent events. We also detected a large amount of different mutations at low frequencies in pathways possibly implicated in SCCHN oncogenesis. These included the tyrosine kinase receptors, [87,352,353] MAPK, [87,138,352] chromatin regulation, [87,350] Notch/HH/Wnt, [87,112,113] and DNA repair pathways. [125] Surprisingly, we did not detect any *HRAS* or *CDKN2A* mutations in the cfDNA. This might be linked to the limited sensitivity of our technique and/or to low sample size. Moreover, p16-negative status is not only linked with *CDKN2A* mutations, but also with copy number variations (deletions) or epigenetic alterations, which were not analyzed in this work. Interestingly, three patients without available tumor harbored a potentially targetable *PIK3CA* mutation, supporting the diagnostic role of liquid biopsy when tumor biopsy is neither feasible nor available. Furthermore, 26% of the plasma variants were not detected in the matched tumors. This last finding suggests that liquid biopsies might also complement the tumor biopsy by identifying other variants that can be missed due to tumor heterogeneity, or in the absence of tumor biopsy.

Concordance between the variants, as determined by sequencing the tumor tissue and the cfDNA, was low in our cohort, with only 19% of tumor variants detected in the corresponding cfDNA sample. In line with our results, other studies have reported low concordance rates (8.6% - 22%) for broad gene-panel testing. [354-356] Series focusing on single gene analysis (using PCR-based platforms for specific mutation detection) have shown more encouraging results with a 93% agreement rate for *RAS* status in colon cancer, [274] or a 94.3% concordance rate with a sensitivity of 65.7% for the detection of *EGFR* mutation in lung cancer. [273] Testing for recurrent hotspot mutations in one specific gene with a highly sensitive amplification-based method performs better than broad panel testing but fails to capture the full molecular landscape and tumor heterogeneity. Nonetheless, higher concordance rates have also been described with more comprehensive gene panels. Using a hybrid capture-based panel of 62 genes in patients with non-small cell lung cancer, 78% of short variants from solid tumor were also detected in plasma (n=33). [294] This may be attributable to the high median unique exon coverage depth achieved (6873x), which was much deeper than with our technique. In a study dedicated to prostate cancer, 93.7% of the solid tumor genomic alterations were detected in plasma. However, different sequencing techniques were applied to the tumor

tissue and cfDNA, with a lower sequencing depth on the solid tumor, possibly resulting in underestimation of the number of mutations in the former as compared to the latter.[287] In contrast, a strength of our work is the application of the same sequencing technique and coverage depth to the germline samples, tumor tissues and the cfDNA. The high sequencing depth should capture a greater diversity of tumor subclones, not all of which are shed into circulation. Finally, two other studies have reported a 82 - 97% detection rate of tumor variants in cfDNA of advanced cancer patients using a panel of 50 and 46 genes. [283,292] Our larger panel of 604 genes may have led to the detection of more subclonal genomic alterations and passenger events, contributing also to a subsequent decrease in the concordance rate.

Beside the number of genes included in the panel and the sequencing technology that is used, a patient's clinical status can also influence the concordance rate between liquid and solid biopsies. All previous studies contained only patients with metastatic disease and, in many, concordance was reported only for patients in whom ctDNA was detected. [287,292,294] In our logistic multivariate regression model, the detection of a tumor tissue variant in plasma was significantly related to the patient's metastatic status and the quantity of ctDNA. For ctDNA-positive metastatic patients, our concordance for the detection of solid tumor variants in plasma increased to 42%.

For two patients, the liquid biopsy was not taken at the same time as the solid biopsy. This may be an important factor given that the samples from one of these patients (HNSCC-34) showed no molecular concordance. Two different *TP53* mutations were detected in this same patient's plasma and tumor tissue sample. As the FFPE sample was taken at the time of primary disease and the plasma sample at recurrence, molecular evolution of the disease could explain this result. Another confounding factor in HNSCC is that it is almost impossible at times to clinically distinguish between disease recurrence or the presence of a second primary tumor. This outlines the importance of developing non-invasive techniques to characterize the molecular landscape of HNSCC throughout the disease course.

One limitation of our study is the sensitivity of our targeted sequencing approach (median coverage 1000x), which was restricted to mutations with an allele frequency $\geq$ 1%. We have shown that lowering the thresholds for variant calling seemed to inflate false positives by retaining more background noise, illustrating the biases that can be introduced by the variant-filtering criteria applied. Similarly, a recent paper compared four different plasma NGS assays to matched tissue sequencing results. The investigators observed an increased proportion of false positive or negative results when the AF dropped below 1%, and these were mainly linked to technical variations (nonspecific variant calling and sequencing noise) rather than often-discussed biological factors, such as tumor heterogeneity.[357] Variants detected by NGS with an AF < 1% should be interpreted with caution and stringent criteria should be applied to avoid false positive results. ddPCR allowed us to confirm the presence of variants detected by manual inspection that did not pass our background-noise threshold, further highlighting the limited sensitivity of this NGS approach. Using ddPCR, we detected mutations with an AF < 0.1% and enhanced our concordance rate between solid and liquid biopsies for driver events up to 80% (18/22).

In conclusion, our findings show that ctDNA detection using a large targeted sequencing gene panel is technically feasible for patients with SCCHN, especially those with metastatic disease. Although the molecular landscape characterized by cfDNA is in line with the literature, it does not reflect the global picture of a particular tumor, preventing us from confidently using this sequencing technique for liquid biopsies in a tissue-agnostic approach. Importantly, actionable mutations were detected in three samples without an available tumor biopsy, supporting the complementary role that liquid biopsy can play in molecular diagnosis when a new tumor biopsy is not feasible.

# 7. CONCLUSIONS AND PERSPECTIVES

This PhD work focused on personalized biomarker-based treatment strategies for recurrent/metastatic SCCHN by (i) designing the EORTC-1559-HNCG trial and (ii) studying the utility of ctDNA sequencing to characterize the mutational profile of recurrent/metastatic SCCHN using a comprehensive panel.

The EORTC-1559-HNCG trial (UPSTREAM) is the first European multi-country umbrella trial to assess a personalized treatment strategy for patients with R/M SCCHN. We hypothesized that this approach could improve patient outcomes.

UPSTREAM has several strong points: one single protocol with pre-planned access to matched targeted therapies, one fresh tumor biopsy to deal with tumor evolution over time, an ISO-certified central laboratory, well-defined biomarker hypotheses, and the possibility of an ongoing protocol enabling new cohorts to be added. Finally, treatment allocation within the trial is not only based on DNA alterations but also on protein markers and clinical characteristics.

We demonstrated the feasibility of such a trial in Europe through a successful global accrual rate, the addition of new patient cohorts during the trial, and the release of results from the first immunotherapy cohort.

Below, we discuss the major challenges of this work and propose some perspectives for the future of precision medicine in SCCHN.

### *1. New tumor biopsies to guide treatment allocation*

One of the main challenges of UPSTREAM is that a new tumor biopsy is required for trial participation. This is based on the scientific rationale that the molecular landscape of cancers may change over time, likely due to the selection of molecular tumor subclones induced by previous treatments.[139,312] After the first 178 samples, we reported that 24% of the biopsies had failed the quality check for molecular NGS analysis. Although this failure rate may seem high, it compares favorably with other precision medicine trials.[206,217,358,359,360] The main reason for quality failure was low cellularity, as also reported in some of these trials. In the SHIVA trial, a precision medicine trial for patients with metastatic disease, a successful biopsy was not obtained in 28% of the patients.[206] A retrospective analysis of the quality of these biopsies showed that the success rate was higher in surgical or palpation-guided biopsy compared to CT-guided biopsy. Ongoing chemotherapy was also linked with a higher failure rate due to decreased tumor cell content. The absence of tumor, or

insufficient material, was the most frequent cause of a poor quality biopsy (46%). The remaining failures were linked to fibrosis and necrosis in 38% and 16% of cases, respectively.[361] Another group reported on the quality of percutaneous biopsies for NGS testing and showed that 30% of biopsies that were adequate for histological diagnosis were inadequate for NGS.[362] They also observed that patients who had never received systemic therapy before sampling had a greater probability to have a biopsy suitable for NGS. This might be linked to an increased proportion of fibrosis in lesions exposed to systemic therapy.[363] Taken together, these data and our results emphasize that samples might be sufficient for histopathological diagnosis but not meet the pre-analytical requirements for NGS testing.

From March 2019 to November 2019, the failure rate of biopsies to meet the NGS quality requirements decreased from 31% to 24% in UPSTREAM. Presentations at our bi-annual EORTC HNCG meetings and individual teleconferences with each site were carried out to emphasize the importance and challenges of obtaining the most suitable biopsy. We are currently investigating if the type of biopsy (surgical sample, percutaneous biopsy, image-guide biopsy, endoscopic biopsy), the site of biopsy (primary site, lymph node biopsy, metastatic lesion), previous treatments and their relationship with the reasons of quality failure (no tumor, necrosis, grade of fibrosis), could help us to better define the best way to perform biopsies for NGS testing in R/M SCCHN. Functional imaging to guide biopsies in areas with viable cells may also be attractive, although a retrospective analysis of the image-guided biopsies of the MOSCATO trial did not find a relationship between the presence of necrosis on imaging and tumor cellularity. Similarly, hypermetabolism detected on 2′-deoxy-2′-[18F] fluoro-D-glucose positron emission tomography (FDG-PET) did not correlate with tumor cellularity.[364]

This failure rate needs to be considered as it impacts the number of patients who could potentially benefit from a personalized approach. The time to obtain the biomarker results and to initiate the allocated treatment is another limiting factor. To date, the median time between tissue sampling and treatment initiation in UPSTREAM is 22 days with a median of 10 days to obtain the results of the molecular analysis from our central lab. These time-frames are shorter than reported in some other precision medicine trials. The median time from biopsy to

randomization was 65 days (IQR 44–124) in the SHIVA trial, and in MOSCATO 01 the median time from biopsy to molecular board (encompassing sequencing and comparative genomic hybridization (aCGH) analyses) was 21 days (IQR 14–27).

The mandatory fresh tumor biopsy and the time needed for biomarker results could introduce a potential bias in the population of USPTREAM, with the inclusion of patients with slow progressive disease and better prognosis. The results of the control arm in the randomized cohorts to analyze this confounding factor will be critically important. The current rate of screen failures in the trial is in the range of 22%-40% (ongoing analysis). Although we do not yet have the definitive analysis outlining the reasons for this screen failure, clinical deterioration and rapidly progressive disease seem to account for most cases. This illustrates the importance of trying to implement precision medicine strategies earlier in the course of the disease in the future. Furthermore, technological advances will no doubt increase the success rate of tumor biopsies suitable for molecular characterization. Less invasive liquid biopsy may also complement the molecular diagnosis when tumor biopsies are of poor quality or challenging (cfr. Infra).

**2. *Biomarkers***

Based on the analysis of the first 120 patients, the frequency of genomic alterations found in UPSTREAM are in line with what is reported in the literature. Although the frequency of *TP53* mutations seems relatively low (48%), it is comparable with the 41% reported in the AACR GENIE database[142] and the rate reported by Chau et al in their routine clinical testing.[232] The targeted sequencing approach of our panel focusing mainly on hotspot mutations, and the high-level cut-off to determine gene amplification, can impact the frequency of reported mutations and amplifications, respectively. The high rate of PTEN high (H-score > 150) tumors is surprising as it is remarkably higher than the 30% reported in LUX-H&N1[321], even though the same antibody and scoring technique is used. As almost all patients in the B1 cohort are PTEN high, this might be a confounding factor when we will analyze the results of this cohort.

UPSTREAM is missing some treatment arms that could target relevant genomic aberrations in SCCHN. Tipifarnib, a farnesyl transferase inhibitor, has shown encouraging activity in the 5% of SCCHN harboring *HRAS* mutations.[230] *PIK3CA* alterations are found in 16 to 34% of HPV-negative and in up to 56% of HPV-positive SCCHN, respectively. In our patient cohort, *PIK3CA* activating mutations were observed in 14%. Patient-derived SCCHN tumor xenografts with *PIK3CA* activating mutations are sensitive to mTOR/PI3K inhibitors.[365] In the BERIL-1 trial, buparlisib improved OS when added to paclitaxel.[179] However, biomarker analysis of this trial did not show an OS benefit for the patients with SCCHN in which the PI3K pathway is activated (*PIK3CA* mutation and/or PTEN expression).[141] Another trial showed only limited activity of buparlisib monotherapy in R/M SCCHN, independently of *PIK3CA* mutations.[180] Although the PI3K pathway seems an attractive treatment target, the optimal biomarker and best treatment combination for PI3K inhibitors still need to be identified. Strategies are emerging to target *TP53* mutated tumors, including small molecules or peptides targeting altered p53 proteins and restoring p53 transcriptional activity as well as targeted therapies against proteins critical for DNA damage response, like WEE1 inhibitors.[366]

Finally, in the current design, immunotherapy cohorts are not linked to biomarker(s). Among others, HPV-positivity, PD-L1 overexpression, interferon-gamma gene expression signature, and mutational burden are potential biomarkers that have been associated with a higher efficacy of immunotherapy in SCCHN.[173,367,368] Umbrella trials represent an ideal platform to further investigate the predictive value of immune biomarkers but, until now, we have failed to convince our pharmaceutical partners to move to biomarker-driven immunotherapy. We hope that, in the future, we will be able to include biomarker-driven cohorts targeting TIM-3, TIGIT, LAG3 or other immune checkpoints.

The level of evidence for the studied biomarkers is paramount to the success of precision medicine trials. The markers used in our trial are based on DNA alterations, some protein markers and clinical characteristics. The level of evidence for the use of these biomarkers is low and limited only to hypotheses. Although this could be challenged, and some of the cohorts will not meet their primary endpoint, the prospective collection of material for translational research will enable us to

characterize in depth the molecular characteristics of very good responders to help us to refine our biomarkers.

In contrast to other cancers, like non-small cell lung adenocarcinoma, the high-level drivers for SCCHN are probably not yet identified. For heterogeneous cancers with multiple potential oncogenic drivers, biomarkers assessed only at the DNA level may not reliably predict drug response.[369] As an example, the different treatment responses to BRAF inhibition for *BRAF* mutated melanoma or *BRAF* mutated colon cancer could be linked to differences in EGFR expression.[370,371] In *HER2* amplified breast cancer, different gene expression profiles can modulate treatment response.[372] Conversely, tumors can present similar expression patterns, without harboring specific oncogenic driver mutations, like BRCA-like tumors without *BRCA*-mutation.[373] All these examples highlight the importance of exploring the molecular landscape beyond genomics and might explain the low success rate of precision medicine trials based on genotype only. The WINTHER trial showed that including transcriptomics could enhance the number of patients treated with a matched therapy.[217] Therefore, for further developments, we will have to take into account others parameters, such as transcriptomics or proteomics, to obtain a more comprehensive view of the molecular landscape to optimize treatment outcome prediction. The use of mRNA expression levels for the new biomarker-driven cohort in UPSTREAM evaluating rogaratinib in FGFR-overexpressed tumors is a first step forward. In urothelial cancers, responses have been seen with rogaratinib in tumors that were mRNA high and without alterations on the DNA level. As we will have the sequencing data for *FGFR* genes (mutations, amplification), we will be able to correlate the DNA and mRNA picture. [331]

### *3. Single agent approach versus combination therapy*

Tumor heterogeneity can cause treatment resistance and it is not addressed when targeted compounds are used as monotherapy. The I-PREDICT study was a precision medicine trial in which tissue genomic profiling was performed using NGS (Foundation Medicine, 236-405 genes) and, whenever possible, PD-L1 IHC, tumor mutational burden (TMB), microsatellite instability (MSI) and NGS on ctDNA.[229] Based on these data, a molecular tumor board selected customized multidrug combinations to target the majority of genomic alterations in each patient's tumor.

As no two molecular profiles were identical, most treatment regimens were not exactly alike. The 73 patients treated with matched therapies received a median of two drugs (range 1-5) targeting a median of two genomic alterations per patient (range 1-6). For each patient, they calculated a matching score equal to the total number of molecular alterations matched to the administered drugs divided by the total number of characterized genomic aberrations. Patients with a high matching score (> 50%) had a better disease control rate, a longer PFS and OS. This study illustrates not only that combination therapies can perform better than single agent therapy, but also the importance of biomarkers beyond a single genomic alteration and the personalization of combination therapies to each individual tumor. Indeed, the complexity and heterogeneity of the tumor genome together with the numerously available targeted therapies will lead to a multitude of possible treatment combinations that will only match a small number of patients. This will require the implementation of complex algorithms that will need to consider the broad profiling of each tumor.

However, we believe, as a first step, that it is still of scientific importance to know the activity of a compound in monotherapy before investigating it in combination. The I1 cohort data that evaluated monalizumab monotherapy, for example, will be important to help us correctly interpret the data of the ongoing I2 cohort evaluating the combination of durvalumab and monalizumab.

In the Palatinus study, the combination of palbociclib and cetuximab did not improve OS compared to cetuximab monotherapy in unselected HPV negative SCCHN.[374] Currently, there are no data on CDK4/6 inhibitors as single agents in SCCHN. The B3 cohort will be of interest in this regard. While *CCND1* amplification, as the selected biomarker, is debatable based on biomarker results in other cancers, translational research will help us to better refine biomarkers and maybe select an optimal drug to use in combination with a CDK4/6 inhibitor in SCCHN patients.

### *4. Regulatory challenges*

Besides the inherent complexity of these trials, numerous logistic challenges were encountered when designing this protocol. Although the collaborating pharmaceutical companies accepted the inclusion of different compounds into the

one protocol, complex negotiations were crucial to successfully ensure that all stakeholders agreed to (i) standardize processes, (ii) accept the pre-defined protocol structure, (iii) use the central biomarker laboratory, (iv) match company interests with academic wishes, and (v) align all companies on the same protocol wording, particularly for the inclusion/exclusion criteria. The protocol was submitted to four different countries (Belgium, France, Italy, United Kingdom) and is intended for the competent authorities (CA) and applicable ethics committees (EC) in Germany and Spain. Overall, the study was well received by the CA and EC without major comments on the study design. The main question received from the EC concerned the criteria to allocate patients to the different cohorts. From a regulatory strategy perspective, having all cohorts in the one study simplifies the submission process as it requires only one initial clinical trial application to each CA and one initial request of opinion to each EC. Also, each amendment can be grouped so that modifications are able to be made to more than one cohort at the same time. If we had considered each cohort as a separate trial, different submissions would have been necessary, increasing the regulatory workload and consequently slowing down the time to activation. As separate trials, the current cohorts could have been opened/closed independently across countries without the need for a main protocol amendment. The multiple amendments to the main protocol grouping cohort-specific requirement led to delays in cohort activation that could have been avoided. Additionally, liaison with stakeholders is easier with a one trial approach, as the number of stakeholders per trial is significantly reduced.

Several challenges remain. The involvement of multiple countries means that country-specific documents and their adaptation(s) need to be optimally managed. Keeping up with the rapidly advancing pace of research in head and neck cancers is also paramount. Effective communication to stakeholders may be the key to ensuring that deadlines are met and that new cohorts are activated without delay.

The new European clinical trials regulation 536/2014, which is foreseen to enter into force 6 months after the European (EU) portal is fully functional, may also bring a novel perspective for studies with complex designs. The EU portal's main characteristic is the Member States' coordinated assessment of some sections of the dossier (protocol, labels, Investigational medicinal product dossier (IMPD), ...),

resulting in a consolidated feedback sent to the applicant. This may reduce the volume of correspondence and facilitate the management of any protocol modifications if they are required.

## 5. Liquid biopsy-driven clinical trials

The clinical difficulties of obtaining a new tumor biopsy of optimal quality for NGS push us to envisage alternatives to characterize the molecular R/M SCCHN landscape. Archival biopsies are not optimal given that the molecular landscape can change over time with the emergence of genomic alterations potentially implicated in treatment resistance as shown, for example, with the appearance of *HRAS* mutations in recurrent SCCHN.[295,312] Liquid biopsy offers an attractive alternative due to the ease of sampling and the potential to capture tumor heterogeneity. A biomarker-driven trial with the identification of molecular alterations through liquid biopsy instead of tumor biopsy could potentially (i) include more patients, particularly those for whom tissue biopsy is not possible or difficult, (ii) reduce the time from sampling to treatment since the turnaround time can be shorter and less logistical organization is required in the clinic, and finally (iii) capture spatial tumor heterogeneity.

The proof of concept of a liquid biopsy-driven clinical trial has been shown recently in the TARGET study,[218] a molecular profiling program to match patients with advanced cancers to early phase clinical trials based on ctDNA analysis. The investigators of this study demonstrated good concordance with the matched tumors and a similar rate of detection of actionable events and of patients treated with a matched therapy (11%). Tumor-specific liquid biopsy-based clinical trials are ongoing for some cancers in the advanced clinical setting. For example, the BFAST trial in lung cancer (A study to Evaluate Efficacy and Safety of Multiple Targeted Therapies as Treatments for Participants with Non-Small Cell Lung Cancer), assigns patients to targeted treatment based on ctDNA analysis. This trial has already showed promising results for ALK-positive patients based on ctDNA analysis treated with alectinib.[375]

Identification of *HRAS* mutation through liquid biopsy in SCCHN has been successfully performed.[2,295] However, today it is unlikely that an umbrella, multiple cohort, biomarker-driven trial based on ctDNA *only* is feasible in patients with SCCHN. In UPSTREAM, the very low frequency of actionable mutations obliged us to use other proteomic or clinical parameters to allocate patients to the different treatment cohorts. It would be of interest in our study to investigate if liquid biopsy is able to replace the NGS analysis part of our trial. This would partly solve the issue of biopsy quality. Notably, we have shown that the concordance between tumor and liquid biopsy using a standard NGS technique on a comprehensive panel is low, precluding the use of this particular technology to confidently use liquid biopsy to characterize the molecular landscape of R/M SCCHN.

Another setting where ctDNA would be of interest is in the detection of minimal residual disease (MRD). Prospective liquid-biopsy based studies for MRD are already ongoing in colon (DYNAMIC II, ACTRN12615000381583, and III trials; ACTRN12617001566325) and breast cancer (c-TRAK TN study, NCT03145961), for example.

Studies of ctDNA have shown that small gene panels may be able to detect ctDNA in > 90% of patients with SCCHN. The potential of MRD detection through ctDNA should be further investigated in SCCHN to identify patients at a high risk of relapse. Interventional studies based on the detection of ctDNA should be designed. For example, over the next two years, adjuvant anti-PD1 therapy could become standard of care in SCCHN after primary curative treatment. However, it is likely that this expensive treatment will be prescribed to all patients due to the lack of biomarker(s). Given this context, we must investigate how best to identify patients with MRD based on ctDNA detection and select only those who will potentially benefit from this approach. Our head and neck research group has just initiated a research program in this field.

Other uncertainties include the optimal duration of treatment. The recommended duration of a PD-1 inhibitor in the adjuvant setting will probably be one year, reflecting most trials. The optimal duration of immunotherapy or targeted

therapies in the metastatic setting is also unknown. ctDNA dynamics early in the treatment course have been shown to predict treatment response. Monitoring the dynamics of ctDNA, such as the time to ctDNA clearance, may give some clues about optimal treatment duration, and should also be investigated in prospective interventional trials as potential markers to help determine the best treatment duration. Besides the use of ctDNA as a marker of treatment response, ctDNA could also identify resistant clones early in the piece with clinical implications on the choice of treatment.

# 8. SUPPLEMENTARY DATA

# 8.1 Supplementary data of chapter 1

| Study | Tumor | Study design | Biomarker | Methodology | Endpoint | Target identification and nb of treated patients | Results and impact on outcome |
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| **SCREENING** | | | | | | | |
| **IMPACT** [207,211,376] | All, advanced cancer | Screening program | Archival (FFPE)<br><br>PCR-based sequencing for selected genes (*PIK3CA, BRAF, KRAS and NRAS, EGFR, KIT, GNAQ, TP53 and MET*), Sanger sequencing for *RET* analysis, IHC for *PTEN* loss of expression and FISH for *ALK* translocation<br><br>*Update 2017*: Sequencing by NGS at MD Anderson (11, 46 or 50 genes depending on the panel), Foundation Medicine (182 genes), Knight Diagnostics (48 genes) or other CLIA-certified laboratories<br><br>**! Gene panels of different sizes were used for MP!** | Screening route to phase I<br><br>Assignment to phase I clinical trial based on the identification of MA | Clinical outcome of patients with MA treated with matched therapy vs patients not treated with matched therapy | - 1144/1283 patients had adequate tissue for molecular analysis **(89.2%)**<br>- 460/1144 analyzed patients had 1 or more MA (**40.2%**)<br>- 211/460 (45.8%)treated with matched therapy = **16.4% of total population**<br><br>*Update 2017*<br>- 1179/1436 patients had 1 or more MA (**82%**)<br>- 914/1179 had 1 or more targetable alteration (77.5%) | Analysis on 379 with 1 MA:<br>175 patients treated with matched therapy vs 116 non-matched (88 excluded from clinical outcome analysis):<br>- ORR: 27% in matched therapy vs 5% (*p<0.0001*)<br>- SD ≥ 6mo: 23% vs 10%<br>- OS: 13.4 mo vs 9 mo (*p=0.017*)<br><br>*Update 2017:*<br>- ORR: 11% vs 5% (*p=0.0099*)<br>- SD ≥ 6mo + CR +PR : 29%vs 24%<br>- FFS: 3.4 vs 2.9 mo (*p=0.0015*)<br>- OS: 8.4 vs 7.3mo (*p=0.41*) |

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| | | | | | | - 390/637 (45.8%)patients with at least 1 MA received matched therapy = **27%** of total population<br><br>*Update 2018*: 711/3743 patients that had molecular testing received MT (19%) | *Update 2018*<br>*3-y OS: 15% vs 7%*<br>*10y OS: 6% vs 1%* |
| **MOSCATO 01** [210] | All, advanced cancer | Screening program | New biopsy (Fresh-frozen)<br><br>At the start of trial: targeted sequencing (first Ion Ampliseq Cancer Panel covering 40 genes, then the Ion Ampliseq Cancer Hotspot Panel v2.0 in 50 genes and finally an Ion AmpliSeq custom design covering 75 genes), aCGH analysis and IHC for phospho-MET, RNA sequencing and whole exome sequencing were added during the trial | Screening route to phase I/II<br><br>Assignment to phase I clinical trial based on the identification of MA | Evaluate the clinical benefit as measured by % of patients presenting PFS on matched therapy (PFS2) 1.3-fold longer than the PFS on prior therapy (PFS1) | - 948/1035 included patients underwent biopsy<br>- MP obtained in 843/948 patients (89%)<br>- 411/843 patients had a MA (**49%)**<br>**-** 199 patients were treated with a targeted therapy **= 19%** of total population | PFS2/PFS1 ratio > 1.3 in 63/199 patients treated with targeted therapy (33%) = **7% of successfully screened patients.** |

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| **TARGET** [218] | All, advanced cancer | Screening program | ctDNA assay (641 gene panel)<br><br>Tumor samples: initially 19-gene Mass array assay (Sequenom OncoCarta v1.0), then 24-gene GeneRead PCR Amplicon assy (Qiagen) | Screening route to early phase clinical trials<br><br>Assignment to early-phase clinical trial based on the identification of MA in ctDNA | Determine the feasibility of using ctDNA relative to tissue-based testing | 41/100 patients had actionable alteration (41%)<br>11/100 patients (11%) were treated with a matched therapy | Concordance between tumor and ctDNA:<br>70/94 patients<br>74.5%<br>PR in 4% treated with matched therapy |
| **STRATEGY** | | | | | | | |
| **SHIVA** [206] | All, advanced cancer | Strategy trial<br><br>Multicentre, open-label, proof-of-concept, randomized, phase II trial | New biopsy<br><br>Mutations by targeted NGS (AmpliSeq cancer panel)<br>CNA by Affymetix<br>IHC for oestrogen, progesterone and androgen receptors | Patients with MA in 1of 3 molecular pathways that could be matched with 11 targeted drugs were randomized between targeted therapy and control arm | PFS | - 716/741 screened patiens underwent tumor sample<br>- 293/741 screened patients had at least 1 MA matching one therapy (**40%**)<br>- 196/741 patients were randomized (26%) | Negative trial:<br>Median PFS was 2.3 mo in the experimental group vs 2.0 mo in the control group (*p=0.41*) |
| **NCI-MPACT** [204,225] | All, advanced solid tumor | Strategy trial<br>Double-blind, randomized trial | New biopsy<br>NGS of > 380 unique actionable variants in 20 genes | Patients with specific mutation are randomized (2:1 ratio) to targeted therapy ( arm A) vs control (arm B, not specifically targeting the detected mutation/pathway of interest) | ORR and 4-month PFS | 193 patients underwent biopsies<br><br>>90% completed DNA sequencing, 96<br>50% had actionable mutation and | 3 cohorts were closed for futility (AZD1775 & carboplatin, everolimus and Trametinib.)<br><br>Due to large number of patient withdrawal, the |

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| | | | | 3 genetic pathways: DNA repair, PI3K, RAS/RAF/MEK pathway | | were randomized | comparison between arm A and B was uninterpretable<br><br>Trial amended for non-randomized design |
| **BASKET** | | | | | | | |
| **CREATE** [377-380] | Advanced tumours with MET and/or ALK alterations | Multinational, multitumour, prospective phase II clinical trial | Tumour containing tissue block (FFPE) from the primary tumour and/ or metastatic site: sequencing (bidirectional Sanger sequencing method of only 1 gene) (MET), FISH for copy number status | Treatment with crizotinib in the different patient cohorts | ORR | No biomarker-positivity needed for entering the trial | Results published per histology |
| **NCI-MATCH** [204] | All, advanced solid tumors | Master protocol<br><br>Phase II, multicenter, open-label, non-randomized Basket trial | New biopsy or recent biopsy of < 6 months with no interim therapy<br><br>sequencing assay for more than 4,000 different variants in 143 genes<br><br>Beginning: genetic sequencing at one of four NCI-MATCH Network Laboratories<br><br>Expansion to a larger group of labs across the | Patients with MA are assigned in one of predefined treatment cohorts | ORR | - successful laboratory testing for 93% of patients<br>- 18% of screened tumors was found to have a genetic mutation that matched the patient to 1 of the 30 treatment arms.<br>- 998 patients have been assigned to treatment, of | 62.5% of less common cancers included<br><br>Promising signals in some reported cohorts (Nivolumab in non-colorectal cancer with mismatch repair deficiency, FGFR inhibitor in *FGFR* fusions, AKT inhibitor in *AKT1* mutations, TDM-1 in *HER*-amplified cancer) |

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| | | | country (around 30 by the beginning of 2019) | | | which 69% have enrolled (12% of screened population) | |
| **Mypathway** [220] | Advanced refractory solid tumor harboring MA in *HER2, EGFR, BRAF* or Hedgehog pathway | Master protocol<br><br>Phase IIa, multicenter, non-randomized, multiple basket study | MP was not conducted as part of this study. | Patients are assigned to specific treatment cohorts based on the presence of a relevant target MA | Investigator-assessed ORR within each tumor-pathway cohort | NA, patients were only included if testing already performed outside the clinical trial | Efficacy analysis population: 230 patients<br><br>ORR: 23% within 14 different tumor types |
| **SUMMIT** [221] | Solid tumors harbouring *HER2* and *HER3* mutations | Master protocol<br><br>Multi-cohort basket study<br><br>Treatment with Niraparib | MP was not conducted as part of the study, locally reported HER2/3 mutations were confirmed centrally | Patients with HER2-mutant cohorts were enrolled into disease-specific cohorts and HER3 mutants into one cohort | Investigator-assessed ORR | NA | Total: 125 HER2 mutant patients and 16 *HER3* mutant patients<br><br>For *HER2* mutant tumors, primary endpoint was met only for breast cancer (ORR 32%) and not for lung, colorectal or bladder.<br><br>No responses were observed in the *HER3* mutant cohort |
| **TAPUR** [226] | All, advanced cancer | Basket trial a phase II, prospective, non- | MP not conducted as part of the study. Genomic profiling from any Clinical Laboratory | Eligible patients are matched to any of the 16 FDA approved study drugs based | primary study endpoint within each cohort: ORR | > 1000 participants have thus far been registered | Five study cohorts have permanently closed to enrollment due to |

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| | | randomized, multi-basket | Improvement Amendments certified, College of American Pathologists accredited laboratory is acceptable | multiple parallel cohorts defined by tumor type, genomic alteration and drug. | or SD of at least 16 weeks duration. | and more than 800 treated with a TAPUR study drug. | lack of anti-tumor activity and 12 have expanded to the second stage of enrollment due to promising preliminary activity |
| **NAVIGATION TRIAL** | | | | | | | |
| **WINTHER** [217] | All, advanced cancer | Prospective "navigation trial" | New biopsy (Dual tumor and biopsy)<br><br>DNA sequencing: 236 gene panel<br><br>RNA sequencing: comparing tumor to normal | The clinical management committee (CMC) recommended therapies based on biopsy-derived DNA sequencing (arm A) or RNA expression (arm B)<br><br>Genomic matches were prioritized<br><br>Treatment access: clinical trial, on-label approved or off-label approved drugs | PFS ratio = PFS Winther/PFS previous therapy > 1.5 | 253/303 patients (83.4%) that consent had dual tumor and normal biopsy<br><br>62% of patients with dual biopsy had adequate quality for genomic and transcriptomic analysis | Expanded personalized treatment from 23% based only genomics to 35% with transcriptomics<br><br>Efficacy analysis on 107 (35% of consented patients) who received R/ consistent with CMC<br>- SD > 6 mo/CR/PR: 26.2% (arm A 23.2%, arm B 31.6%)<br>- PFS ratio >1.5: 22.4% (arm A 20.3%, arm B 26.3%)<br>-Fewer previous therapies, better performance status and higher matching score correlated with better PFS and OS |

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| **I-PREDICT**[229] | All, advanced cancer | Prospective "navigation trial" | Tissue genomic profiling: NGS (Foundation Medicine, 236-405 genes), PD-L1 IHC, TMB, MSI and ctDNA | Molecular tumor board (MTB) selected multidrug combinations to target a majority of the genomic alterations | Proportion of patients who received molecularly targeted matched treatment | 149 patients consented<br>83 patients (56%) were treated, 73 (49%) with personalized therapy<br><br>Median nb of genomic alterations: 5<br>Median nb of targeted alterations: 2<br>Median number of drugs: 2 | Higher matching scores correlated with improved disease control rate, longer PFS and OS rate |

**Table 8-1. Selected histology agnostic biomarker-driven approaches**

aCGH: comparative genomic hybridization array, CNA: copy number alteration, DCR: Disease control rate, FFPE: Formalin-fixed, paraffin-embedded, FFS: failure-free survival, FISH: fluorescence in situ hybridization, IHC: immunohistochemistry, MA: molecular alteration, mo: months, MP: molecular profile, NGS: Next generation sequencing, ORR: overall response rate, PFS: progression free survival, vs: versus

| Study | Tumor | Study design | Biomarker | Methodology | Endpoint | Identification of target and number of treated patients | Results and impact on outcome |
|---|---|---|---|---|---|---|---|
| **The BATTLE trial** [205] | Non-small cell lung cancer | Master protocol<br><br>Randomized phase II, single-center, open-label study | Fresh biopsy (FFPE)<br><br>Testing of 11 prespecified biomarkers: PCR-based sequencing for mutations *(EGFR, KRAS, and BRAF),* CNA by FISH *(EGFR, CCND1)*, and protein expression levels by IHC | Multiples arms:<br>5 biomarkers groups with different targeted therapies<br><br>equal random assignment for 97 first pts, and adaptive randomization for next 158 | DCR at 8 weeks | 341patients enrolled:<br>299 with adequate tissue for analysis (88%)<br>255 patients were randomized (75%) | Overall 8w DCR: 46%<br><br>Biomarker groups less predictive than individual biomarkers |
| **SAFIR01** [219] | Metastatic breast cancer | Screening program | Fresh biopsy<br><br>aCGH for preselected genes and Sanger sequencing for mutational hotspots on PIK3CA and AKT1 | Screening:<br>Based on the identified genomic alteration, patients were treated with targeted therapy if possible (within clinical trial or not) | Proportion of patients for whom a targeted therapy could be offered | 423 patients included, biopsy obtained for 407 pts<br>Targetable alteration in 195 (46%) | Therapy could be personalized in 55/423 patients (13%) |
| **LUNG-MAP master protocol** [216,222-224] | Advanced lung squamous cell carcinoma | Master protocol<br><br>Phase II-III umbrella trial | Archival FFPE or fresh tumor biopsies<br><br>FoundationOne NGS assay (Foundation Medicine) for mutations, amplifications, rearrangements (324 genes) and some IHC | Mutiple arms:<br>Based on the molecular profile, each pt is enrolled in a sub-study with matched targeted therapy or in non-match sub-study | ORR | 1392 patients registered to the screening component<br>523 pts registered to a sub-study (37%) | First results for 3 biomarker driven cohorts (S1400B, S1400C and S1400D): ORR 4-7%<br>Cohorts closed due to futility at interim analysis<br><br>S1400A (immunotherapy): 16% ORR |

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| | | | | | | | Other sub-studies ongoing |
| **The National Lung Matrix** [213,381] | Advanced NSCLC | Master protocol<br><br>Phase II umbrella trial | Pre-screening of tumor biopsies through the Stratified Medicine Programme 2 (take place in parallel with the patient receiving first line treatment): adaptable 28-gene NGS sequencing platform designed by Illumina covering the range of molecular abnormalities being targeted | Multiples arms (8 investigational medicinal products, within 22 distinct cohorts)<br>Patients are allocated to the appropriate targeted therapy according to the molecular genotype of their cancer<br><br>Bayesian adaptive design<br><br>"No actionable mutation arm" for patients without specific eligibility for one of the targeted genomic aberrations | For single-agent pre-specified clinically relevant outcomes are mPFS > 3 mo, ORR and/or durable clinical benefit rate at 24 weeks > 30% | As of july 2016:<br>- 1664 patients tested<br>- 1229 passed QC step (74%), 1098 patients with NGS results (66%)<br>- 731 pts with aberration for MATRIX (44%)<br>- 458 pts (28%) with MA and eligible (not registered) for MATRIX | First results presented:<br>As at march 2019, 286 patients recruited.<br><br>6 palbociclib cohorts (all proficient Rb)<br>-mPFS in KRAS mutation: 5.8 mo<br>- CDKN2A loss in non-squamouss passed interim<br>->75% predicitive probability of success (PPoS)for *CCND1* amplification and CDKN2A loss squamous<br><br>Crizotinib<br>>90% PPoS in *ROS1* fusion and *MET* exon14 skipping but less clear in *MET* amplification<br><br>Capivasertib in *PIK3CA* amplification<br>< 15% PPoS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **FOCUS4** [382] | Advanced colorectal cancer | Master protocol<br><br>Phase II-III umbrella trial | FFPE block taken prior to commencement of standard chemotherapy<br><br>Mutations of some preselected genes + some IHC, mRNA EREG | Multiple arms<br>After induction chemotherapy, patients are enrolled in different cohorts on the basis of MA in the tumor, to test different targeted agents versus placebo or in a no-biomarker cohort testing standard capecitabine vs placebo as maintenance | PFS | Accrual as of 29th August 2018<br>1149 registered patients, 292 randomized | First results for 1 patient cohort (FOCUSD):<br>Median PFS 3.48 mo with placebo and 2.96mo with AZD8931: closed for futility |

**Table 8-2. Selected histology specific biomarker-driven approaches**

aCGH: comparative genomic hybridization array, CNA: copy number alteration, DCR: Disease control rate, FFS: failure-free survival, FISH: fluorescence in situ hybridization, IHC: immunohistochemistry, MA: molecular alteration, mo: months, MP: molecular profile, NGS: Next generation sequencing, ORR: overall response rate, PFS: progression free survival, vs: versus, QC: quality check

## 8.2 Supplementary data of chapter 3

### 8.2.1 Criteria of evaluation for integrated translational research in the EORTC-1559-HNCG trial

In this study, patients are allocated in cohorts, depending on the results of a biomarker analysis. This analysis is done in a centralized laboratory in Belgium (OncoDNA). The tests are ISO 15189 accredited. The time to get the results of the biomarker analysis is evaluated at 7 working days after the validation of the quality checks (QCs) made from the Hematoxyline and Eosine (H&E) slide of the biopsy. A fresh tumor biopsy will be performed between informed consent and registration. The aim is to obtain three tumor samples from each patient. Of those tumor samples, one will be formalin-fixed and paraffin-embedded (FFPE), and the two remaining will be frozen for exploratory studies. The immunohistochemistry (IHC) analysis and the molecular analysis will be performed on FFPE histology sections.

#### 8.2.1.1 DNA extraction

Genomic DNA from the FFPE samples is extracted using a QIAamp DNA FFPE Tissue kit, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). DNA quantity is measured using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

#### 8.2.1.2 Next-generation sequencing

The somatic mutations are assessed using a custom AmpliSeqTM Panel which is designed to amplify 88 amplicons covering either hotspot mutations, whole exon or genomic regions for copy number variation (CNV) from 13 oncogenes and tumor suppressor genes (Life Technologies, Carlsbad, USA). The panel covers the following genes: *EGFR, ERBB2, TP53, PIK3CA, CCND1, NRAS, KRAS, HRAS, PTEN, FGFR1, FGFR2, FGFR3* and *cMET*.
Briefly, the targeted sequencing libraries are generated using the Ion AmpliSeq Library kit 2.0 according to the manufacturer's instructions (Life Technologies, Carlsbad, USA). The starting material consists in 20ng from FFPE sample. The

primers used for amplification are partially digested by Pfu enzyme. The product of digestion is then ligated with corresponding bar-coded adapters and purified using Ampure Beads afterwards. The product of purification is amplified for 5 more cycles and purified using Ampure Beads afterwards. The quality of the libraries (we will use two libraries to targeted regions) is evaluated using a QPCR. 10pm of each library is put on the IonChef system for the emulsion PCR and the loading of the chip. According to the throughput required for each sample, we will use the PGM system.

We target an average coverage of 1000x to be able to detect variants down to 5%. The data are analyzed using the Variant Caller software. We use the somatic high stringency parameters as well as the targeted and hotspot pipelines (we use as bed files the ones provided by LifeTechnologies associated with the panels). All the variants identified are confirmed by visualizing the data through IGV (Broad Institute).

Criteria used to consider a variant as true positive one are:

- Coverage min 1000X
- Min 5% of the mutated base
- A ratio of the read forward/reverse min 10%/90% and vice versa
- Associated with an amino acid change (including frame shift)
- Not associated with a technological bias (we have our own database made from > 2000 sequencing)

#### 8.2.1.3 Definition for the copy number variations

For the CNV in hotspots, we calculate the median per sample of the coverage of all the amplicons. Per sample and per amplicon, we divide the raw coverage by this median. Per amplicon and for the whole sample set, we calculate the median of all those medians normalized per sample. Per amplicon and for the whole sample set, we calculate the stdev of all those medians normalized per sample. This corresponds to our data set.

Then for a new tumor sample, we calculate the median of the coverage of all the amplicons covering the gene. Per sample and per amplicon, we divide the raw coverage by this median. If for the amplicon, the normalized value for the tumor sample is > (median + 2*stdev) of the data set and that the ratio of the medians is > 8 for 90% of the amplicons covering the same gene, we conclude in CNV.

#### 8.2.1.4 Immunohistochemistry

The different immunohistochemistries that will be done for the assignment in each subgroup: p16, PTEN
Other immunohistochemistries that may be done: P4EPB1, CDK4, PhosphoRB, Ki67, HLA-E

A. Details of the technique for p16

Antibody: Mouse monoclonal (Clone E6H4) from Ventana
Scoring criteria: H-Score formula: HScore = (%1+c + %1+n)/2 + (%2+c + %2+n) + 3*(%3+c + %3+n)/2 for H-score between 0 and 300
c : cytoplasmic
n : nuclear
Scoring criteria: p16 positive: Histo-score ≥ 210

B. Details of the technique for PTEN

Antibody: Rabbit monoclonal (Clone 138G§6) from Cell Signaling
Scoring criteria: PTEN High: Histo-score > 150

#### 8.2.1.5 FGFR RNA-scope

FGFR expression will be assessed using the RNAscope® Technology, an in situ hybridization (ISH) assay, in a centralized laboratory in Germany, Targos. In order to substantially improve signal-to-noise ratio of RNA ISH, RNAscope® employs a probe design strategy much akin to fluorescence resonance energy transfer (FRET), in which two independent probes (double Z probes) have to hybridize to the target sequence in tandem in order for signal amplification to occur. This design concept ensures selective amplification of target-specific signals. The assay will be performed using the Leica BONDRx platform.

Analysis of RNAscope slides will be performed by a pathologist using standard light microscopy, based on an proprietary algorithm developed by Targos. The complete

slides will be evaluated. The percentage of cells showing a pre-defined staining pattern/signal number will be determined per category, with categories defined as:
0 = No staining or <1 dot per 10 cells
1 = 1-3 dots per cell
2 = 4-9 dots per cell with no or very few signal clusters
3 = 10-15 dots per cell and, if applicable, less than 10% of signals in clusters
4 = >15 dots per cell and, if applicable, more than 10% of signals in clusters
A sample is described as FGFR positive in case of high panFGFR or FGFR1,2,3 mRNA expression levels (RNAscope predominant score of 3+ or 4+) in fresh tumor biopsy specimen.

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