

## Longitudinal assessment of PD-L1 expression and gene expression profiles in patients with head and neck cancer reveals temporal heterogeneity

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1 **Abstract (226 words)**

2 **Background:** Programmed death-ligand 1 (PD-L1) is the most validated predictive biomarker used for the  
3 treatment of head and neck squamous cell carcinoma (HNSCC) with immune checkpoint inhibitors (ICI).  
4 Several gene expression-based signatures surrogate of the activation of IFN-gamma pathway and of the  
5 presence of tertiary lymphoid structures (TLS) have also been proposed as potential biomarkers. While they  
6 may have a potential therapeutic implication, the longitudinal changes of either PD-L1 or gene expression  
7 profiles between the initial and recurrent HNSCC lesions is unknown.

8 **Methods:** PD-L1 immunohistochemistry (IHC) and targeted RNA-sequencing of 2,549 transcripts were  
9 analyzed on paired specimens from the initial diagnosis and recurrent HNSCC. PD-L1 status was defined  
10 using the combined positive score (CPS). PD-L1 mRNA levels were compared with protein expression  
11 levels by IHC. Enrichment scores of surrogate signatures for TLS and IFN-gamma (IFN- $\gamma$ ) pathway  
12 activation were computed using the single sample gene set enrichment analysis (ssGSEA).

13 **Results:** PD-L1 status was 64% (21/33) concordant between the initial and recurrent lesions using a CPS 1  
14 threshold and 67% (22/33) concordant using a CPS 20 threshold. CPS score was associated with PD-L1  
15 gene expression levels. There was a 43% (15/35) and 66% (23/35) concordance for the IFN- $\gamma$  and TLS  
16 signature scores, respectively.

17 **Conclusion:** Our study reveals temporal heterogeneity of PD-L1 status and TLS/IFN- $\gamma$  gene expression  
18 surrogates in HNSCC that need to be considered when interpreting biomarker studies.

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35 **Key words:** Programmed Death Ligand 1, Transcriptome, Squamous Cell Carcinoma of Head and Neck, Gene  
36 expression.

37 **Abbreviation List**

38 **APC:** antigen-presenting cells

39 **CPS:** combined positive score

40 **CTLA4:** cytotoxic T-lymphocyte antigen 4

41 **FFPE:** formalin-fixed, paraffin-embedded

42 **GEP:** gene expression profiles

43 **GSEA:** gene set enrichment analysis

44 **HNSCC:** head & neck squamous cell carcinoma

45 **HPV:** human-papillomavirus

46 **ICI:** immune checkpoint inhibitors

47 **IFN- $\gamma$ :** interferon-gamma

48 **KIR:** killer immunoglobulin-like receptor

49 **OS:** overall survival

50 **OSCC:** oral squamous cell carcinoma

51 **PD-L1:** programmed death-ligand 1

52 **PFS:** progression-free survival

53 **RECIST:** Response Evaluation Criteria in Solid Tumors

54 **R/M:** recurrent/metastatic

55 **ssGSEA:** single sample gene set enrichment analysis

56 **SS:** surgical specimens.

57 **TB:** tumor biopsy

58 **TLS:** tertiary lymphoid structures

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## 74 **1. Background**

75 Head and neck squamous cell carcinoma (HNSCC) is the sixth most incident cancer worldwide [1]. Despite  
76 aggressive multimodal therapeutic approaches including surgical resection (often with neck dissection),  
77 radiation therapy and adjuvant chemotherapy, more than half of the patients with locally advanced disease  
78 experience locoregional or distant relapse not amenable to curative treatments [2].

79 Since the dawn of immunotherapy with checkpoint blockade and its establishment in HNSCC as a  
80 therapeutic standard in the incurable recurrent and/or metastatic (R/M) setting [3-6], major efforts have been  
81 deployed towards the development of robust predictive biomarkers [7], considering the high cost of this  
82 treatment, its toxicity profile and reports of induced disease acceleration, or “hyperprogressive” disease  
83 (HPD) [9-10]. Programmed death-ligand 1 (PD-L1) protein expression by immunohistochemistry (IHC) has  
84 been shown to be partially correlated with response to immune checkpoint inhibitors (ICI) in HNSCC but  
85 complete responses have been observed in PD-L1-negative patients [3-6]. The FDA approved  
86 pembrolizumab for use in combination with platinum and fluorouracil for all patients and as a single agent  
87 for patients with a CPS [(Combined Positive Score), which is the number of PD-L1 staining cells (tumor  
88 cells, lymphocytes, macrophages) divided by the total viable tumor cells, multiplied by 100) ]  $\geq 1$ .  
89 Nivolumab is approved for patients progressing after a first-line platinum-based therapy, after showing a  
90 32% reduction in the risk of death in the Checkmate 141 trial [2,3].

91 The value of PD-L1 expression by IHC as a predictive biomarker is undermined for several reasons which  
92 might explain the discordant results seen across studies. Beyond assays and cut-off diversity [11], an  
93 important reason for observed discordant results may be the spatial and temporal heterogeneity of PD-L1  
94 expression. Indeed, its expression, often assessed from single tumor biopsies, varies within a given tumor  
95 [12]. A Danish team prospectively investigated intratumor heterogeneity of PD-L1 expression in HNSCC  
96 using six random core biopsies from each unique surgical specimen to assess the concordance between  
97 multiple biopsies [13]. Concordance was as low as 36 to 70%, depending on the positivity cut-offs used for  
98 evaluation.

99 PD-L1 expression may also change during the course of the disease from the initial diagnosis to the time of  
100 disease progression as previously reported in other tumor types [14,15]. Longitudinal PD-L1 expression  
101 changes in HNSCC is poorly described and may impact its value as a predictive biomarker.

102 Other predictive biomarkers of response to ICI have been proposed including tumor mutational burden [16],  
103 intratumoral immune cell infiltration [17] and gene expression profiles (GEP) [18]. However, they have not  
104 yet been validated in prospective clinical trials. In this search for biomarkers, efforts to identify a pancancer  
105 GEP resulted in the elaboration of a number of gene signatures, including in HNSCC [19].

106 The role of interferon-gamma (IFN- $\gamma$ ) signaling as an inducer of PD-L1 expression [20,21] has led to several  
107 IFN- $\gamma$ /T-cell-inflamed signatures that were shown to correlate with response to ICI [22,23], including in  
108 HNSCC [24,25].

109 Tertiary lymphoid structures (TLS) are cellular aggregates that can be present in tumors and be the site of

110 antitumoral defense, with a structural organization resembling that of secondary lymphoid organs [26]. They  
111 contain activated and proliferating effector T-cells and memory B-cells, along with antigen-presenting cells  
112 (APC) [26]. For this reason, recent data has emerged regarding their value as a predictive biomarker of  
113 response to ICI [27,28]. As such, various gene expression-based signatures reflecting the presence of TLS  
114 have been proposed, related to the presence of chemokines [29,30] or specific cell populations known to  
115 play a major role in the formation of TLS [31,32].

116 Very little is known on the longitudinal changes of both TLS and IFN- $\gamma$ /T-cell-inflamed gene expression-  
117 based signatures in sequential biopsies of patients with HNSCC.

118 In this work, we assessed PD-L1 protein and gene expression as well as GEP surrogates of IFN- $\gamma$  and TLS  
119 in patients with HNSCC using paired tumor biospecimens from the same patients to get more insight into  
120 their longitudinal changes.

## 122 **2. Material and methods**

### 123 **Patient selection and data collection**

124 The study was approved by the Institutional review board and done in accordance with the Helsinki  
125 Declaration. All patients with a histologically confirmed R/M HNSCC treated at Centre Léon Bérard (Lyon,  
126 France) and Institut Curie (Paris, France) in a clinical trial evaluating an anti-PD-1/PD-L1 antibody alone or  
127 in combination with an anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) or an anti-killer immunoglobulin-  
128 like receptor (KIR) between March 2014 and November 2018 were examined. Those with at least two  
129 available formalin-fixed, paraffin-embedded (FFPE) tumor samples were included in this study. For a given  
130 patient, each specimen was sampled in a different disease stage (initial diagnosis and recurrence stage). In  
131 case of neoadjuvant chemotherapy, analysis of the initial diagnosis setting was performed on a sample  
132 provided before any systemic treatment. Collection of data and analysis was in accordance with guidelines  
133 of each of these trials. The following data were collected and recorded: gender, age, primary tumor location,  
134 smoking history, HPV status (p16 immunostaining and/or DNA in situ hybridization) and previous  
135 multimodal therapy at the initial stage.

136 The data collection cutoff date was September 15, 2020.

### 138 **Immunohistochemistry**

139 4- $\mu$ m thick FFPE tissue sections were prepared according to conventional procedures. For each sample,  
140 hematoxylin and eosin staining was performed to determine the percentage of tumor cells. IHC was  
141 performed on an automated immunostainer (Ventana Benchmark ultra, Roche, Meylan, France) using Ultra  
142 View DAB Kit according to the manufacturer's instructions. Sections were incubated with a rabbit  
143 monoclonal human anti-PD-L1 antibody (diluted at 1:50, Quartett, Berlin, Germany) clone QR1. The  
144 Ventana amplification kit was used and an anti-rabbit-HRP was applied on sections. Staining was visualized  
145 with DAB solution with 3,3'-diaminobenzidine as a chromogenic substrate. Finally, the sections were  
146 counterstained with Gill's hematoxylin. All samples were examined by two qualified anatomopathologists

147 (C.C and N.B) for determination of the CPS, defined as the number of PD-L1-positive cells (tumor cells,  
148 macrophages and lymphocytes) divided by the number of viable tumor cells  $\times 100$  (a minimum of 100  
149 viable tumor cells must have been present for the specimen to be considered evaluable) (Supplementary  
150 Figure S1 and S2). In case of inter-observer disagreement over the CPS, a supplementary review of the  
151 slides was performed simultaneously by the two pathologists for a final scoring.

152 In a previous publication, the authors have compared the correlation and concordance of the QR1 and the  
153 standard 22C3 antibodies by testing on 110 lung adenocarcinomas. They have found a strong correlation  
154 ( $r=0.82$ ) and concordance for the different thresholds between the two antibodies [33]. The QR1 antibody  
155 has received the CE-IVD (Conformité Européenne marking for In Vitro Diagnostic) designation in Europe  
156 [34]. For internal validation, we performed a blinded analysis of the QR1 and 22C3 antibodies on 17  
157 samples, showing a high concordance (14/17, 82%) (Table S1).

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### 159 **Gene expression profiling**

160 GEP were generated by targeted-RNA sequencing in FFPE samples using the HTG EdgeSeq technology  
161 [35] and the Oncology Biomarker Panel (OBP) (2,559 transcripts). For a given patient, GEP were generated  
162 on the initial diagnostic tissue sample (surgically resected specimen or per-endoscopic biopsy) and on a new  
163 biopsy sampled at the time of disease recurrence. Probe and assay design, sample preparation, nuclease  
164 protection assay, PCR tagging and library cleanup are detailed in supplementary material and methods.

165 Sequencing was performed on an Illumina NextSeq instrument. Sequencing libraries were loaded with a 5%  
166 PhiX spike-in. Standard Illumina protocols were used for library denaturation. 50 cycles of sequencing were  
167 performed with two 6-base barcode reads. The sequencer performed demultiplexing and fastq files were  
168 returned. The HTG EdgeSeq parser was used to align the probe sequences to the results; this program is a  
169 front end for bowtie2 software, using a 25-base match with one mismatch allowed to the first 25 bases of  
170 sequencing information. The final data table is a compilation of all such counts per probe per sample.

171 We tested a 25-gene IFN- $\gamma$  signature surrogate of the IFN-  $\gamma$  pathway activation proposed by *Sharma et al*  
172 [21]. We also tested a 12-gene chemokine-based TLS surrogate signature proposed by *Prabhakaran et al*  
173 [29]. For each surrogate signature and sample included in the analysis, enrichment scores were computed  
174 using the single sample gene set enrichment analysis (ssGSEA) tool [36] from GenePattern [37] in order to  
175 compute enrichment scores for each pairing of a sample and a gene set independent of status sample. Using  
176 this tool, gene expression values for a given sample were rank-normalized and an enrichment score was  
177 produced using the Empirical Cumulative Distribution Functions of the genes in the gene set and the  
178 remaining genes. A gene set's enrichment score represents the activity level of the biological process in  
179 which the gene set's members are coordinately up- or down-regulated.

180 To validate the stability of the GEP in the initial diagnosis setting, the IFN- $\gamma$  and TLS surrogate signatures  
181 were computed in an independent cohort of patients suffering from oral squamous cell carcinoma (OSCC)  
182 and treated at the Groupe Hospitalier Pitié-Salpêtrière (GHPS, Paris, France) between November 2017 and  
183 August 2018. For each patient, the HTG EdgeSeq technology was used to generate targeted gene expression

184 profiles in per-endoscopic tumor biopsy (TB) and paired surgical specimens (SS).

## 186 **Statistical analysis**

187 Descriptive statistics were used to summarize the baseline characteristics of the patients. Statistical analyses  
188 and plots were performed on R 4.0.0 using networkD3 v0.4 and ggplot2 v3.3.1 packages. Chi-squared or  
189 Fisher's exact test was used for statistical comparisons of categorical data. A Mann-Whitney-Wilcoxon non-  
190 parametric test was used to compare PD-L1 expression levels between two CPS groups. All statistical tests  
191 were two-sided and P values  $\leq 0.05$  were considered to be statistically significant. For multiple testing, a  
192 false-discovery rate (FDR) was used to adjust P value.

## 194 **3. Results**

### 195 **Patient characteristics and management**

196 Thirty-five patients with a histologically confirmed recurrent HNSCC were included. Baseline  
197 characteristics are summarized in Table 1. Median follow-up was 41 months (range 20-67) (Figure 1).  
198 Median age was 64 years (range 42–87), 71% of patients were males and 74% had a smoking history. At the  
199 time of diagnosis, the majority of tumors were locally advanced (cT3-T4 stages according to AJCC 8th  
200 edition). Twenty-two patients (44%) presented with clinical/radiological (cN+) and/or pathological (pN+)  
201 cervical lymph node involvement. Curative treatment modalities are summarized in Table 2. Ten (28%)  
202 patients had neoadjuvant chemotherapy, followed by surgery (tumor resection and/or neck dissection) for  
203 five (14%) of them. Nearly all patients (9/10, 90%) had adjuvant radiation therapy. The twenty-five patients  
204 who did not receive neoadjuvant chemotherapy were all managed with surgery followed by post-operative  
205 radiotherapy for 22/25 (88%) of them. In 55% of the patients, radiation was potentiated with chemotherapy  
206 or cetuximab. At relapse, all patients were considered as having an incurable locoregional recurrent disease.  
207 Detailed sampling information are available in supplementary Table S2.

### 209 **Temporal heterogeneity of PD-L1 expression**

210 A majority of samples with a positive CPS using a threshold of 1 at diagnosis showed a positive CPS at  
211 relapse (19/25, 76%) while the majority of negative CPS samples at diagnosis became positive at relapse  
212 (6/8, 75%). PD-L1 status was 64% (21/33) concordant between samples at diagnosis and at relapse (Table 3  
213 and Figure 2A).

214 Using a CPS threshold of 20, we found that the majority of negative CPS samples at diagnosis remained  
215 negative at relapse (17/24, 71%) and the majority of positive samples remained positive at relapse (5/9,  
216 56%). PD-L1 status was 67% (22/33) concordant between samples at diagnosis and at relapse (Table 3 and  
217 Figure 2B).

### 219 **Correlation between PD-L1 gene expression and IHC score**

220 A high CPS was associated with a higher PD-L1 gene level ( $p < 0.05$ ) in initial diagnostic samples using a

221 CPS 1 threshold. Similarly, a high PD-L1 CPS was significantly associated with a higher PD-L1 gene level  
222 ( $p < 10e-3$ ) in relapse samples regardless of the CPS cut-off (Figure 3).

### 224 **Temporal heterogeneity of the transcriptome**

225 For each sample, we used the single sample gene set enrichment analysis (ssGSEA) tool that calculates a  
226 gene set's enrichment score representing the activity level of the biological process in which the gene set's  
227 members are coordinately up- or down-regulated. Samples with a positive enrichment score were considered  
228 as 'high' and those with a negative score as 'low'. Based on the value of the enrichment score, half of the  
229 samples with a high IFN- $\gamma$  signature score at diagnosis showed low scores at relapse (13/26, 50%) while the  
230 majority of samples with high scores at relapse already had high scores at diagnosis (13/15, 87%). IFN- $\gamma$   
231 signature score was 43% (15/35) concordant (Table 4 and Figure 4A).

232 A majority of samples with a high TLS signature score at diagnosis showed low TLS scores at relapse  
233 (11/16, 69%). Interestingly, the majority of samples with low a TLS signature score at diagnosis remained  
234 low at relapse (17/19, 90%). TLS signature score was 66% (23/35) concordant (Table 4 and Figure 4B).

### 236 **Stability of the transcriptome in the initial diagnosis setting**

237 In order to validate the stability of GEP in the same initial diagnosis setting, we computed the IFN- $\gamma$  and  
238 TLS signature scores in the GHPS cohort of 28 per-endoscopic tumor biopsy (TB) and paired surgical  
239 resection (SR) specimens. No treatment was administered between the time of TB and SR. A total of 56  
240 samples (paired TB and SS) from 28 patients, were profiled for gene expression using the HTG EdgeSeq  
241 technology (Table S3). One sample did not pass HTG quality control, representing an overall failure rate of  
242 1.8% (1/56). Subsequent analysis was thus performed in 54 samples corresponding to 27 patients with  
243 paired biospecimens. There was a 78% (21/27) concordance for the IFN- $\gamma$  surrogate signature and a 81%  
244 (22/27) concordance for the TLS surrogate signature between the TB and SS (Figure S3 and S4). We  
245 hypothesize that there was a high concordance rate observed in these paired specimens (TB and SS) as they  
246 were both sampled in the initial diagnosis setting, contrary to our cohort (initial diagnosis and recurrent  
247 disease), and so in line with our observations.

## 249 **4. Discussion**

250 In our study, PD-L1 expression demonstrated a significant temporal heterogeneity, between 33 and 36%  
251 depending on the threshold used for the CPS. In a similar manner, we observed that two GEP-based  
252 signatures (that are surrogates of the presence of TLS and IFN- $\gamma$  pathway activation) are discordant in 34 to  
253 57% of the cases between initial and recurrent lesions. In line with our observations, the GEP were stable in  
254 paired endoscopic and surgically resected specimens from the same initial diagnosis setting.

255 The literature is scarce on the temporal heterogeneity of the PD-L1 protein expression. Detection of PD-L1  
256 in a 2017 study differed by 20% in primary malignant mesothelioma lesions sampled at multiple time points  
257 [38].



258 As previously mentioned, PD-L1 was spatially heterogeneous within a given tumor in HNSCC [12]. This  
259 seems to also be the case in other malignancies such as breast cancer [39] and non-small cell lung cancer  
260 [40]. To our knowledge, there is no data about the dynamics of PD-L1 expression between initial HNSCC  
261 and recurrent lesions.

262 We did not evaluate the prognostic impact of the expression of PD-L1 and GEP in initial or recurrent lesions  
263 as our cohort size was not sizeable enough to make valid interpretations.

264 Importantly, there was an association between high CPS and higher PD-L1 gene expression. This confirmed  
265 data of previous studies such as a large pancancer analysis in which PD-L1 gene (*CD274*) amplification was  
266 correlated with PD-L1 IHC positivity [41].

267 Our study has some limitations. First, around one-third of the samples analyzed were from biopsies and  
268 therefore sampling bias cannot be excluded in some specimens given the spatial heterogeneity of PD-L1.  
269 Second, concomitant treatment with irradiation at the initial stage of treatment was either chemotherapy or  
270 cetuximab, which may each harbor different effects on the expression of PD-L1 [42-44] and the gene  
271 expression profiles. For example, induction chemotherapy with docetaxel, platinum and fluorouracil (TPF)  
272 resulted in a significant increase of PD-L1 expression in locally advanced HNSCC patients [44]. The effect  
273 of cetuximab on the expression of PD-L1 in HNSCC is less known. Jie et al. found that the increased  
274 frequency of PD-1+ and TIM+ infiltrating lymphocytes (TILs) during cetuximab treatment inversely  
275 correlated with objective response [45].

276 In conclusion, PD-L1 expression and GEP in HNSCC show a marked temporal discordance, which should  
277 be taken into consideration as they are used and developed as predictive biomarkers for ICI. Since our work  
278 is retrospective in nature with a relatively small number of patients, larger prospective studies are needed to  
279 confirm our conclusions about this temporal heterogeneity and to better assess the impact of  
280 chemotherapy/cetuximab on the expression of PD-L1 and the dynamics of the transcriptome.

295 **Author contributions**

296 Conceptualization: AK, JF and JB; Data curation: AK, JB, LM, NB, CC, VA and JAP; Formal analysis: AK,  
297 JB, LM, JAP and PS; Funding acquisition: PS, PZ and JF; Investigation: AK, JB, LM, CC, NB, JAP, PS and  
298 NG ; Methodology: AK, NB, MK, VA, JF and PS; Project administration: PS, CLT, MK and JF; Resources:  
299 NB, PZ, JF, CLT, MK and PS; Software: LM, VA and JB; Supervision: PS, JF and CLT; Validation: PS, JF  
300 and CLT; Visualization: MK, PZ, MK and LM; Roles/Writing - original draft: AK and JB; Writing - review  
301 & editing: AK, JB and PS.

302  
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306 in this study.

332 **FIGURE CAPTIONS**

333 **Figure 1. Cohort follow-up (n=35) from the first surgery/biopsy to the last follow-up date.**

334

335 **Figure 2A. Comparison of PD-L1 expression between initial and recurrent lesions.**

336 PD-L1 was evaluated with immunohistochemistry (IHC) using the Combined Positive Score (CPS) with a positivity  
337 threshold of 1. PD-L1 status was 64% concordant (21/33) between initial and recurrent lesions.

338

339 **Figure 2B. Comparison of PD-L1 expression between initial and recurrent lesions.**

340 PD-L1 was evaluated with immunohistochemistry (IHC) using the Combined Positive Score (CPS) with a positivity  
341 threshold of 20. PD-L1 status was 67% concordant (22/33) between initial and recurrent lesions.

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343 **Figure 3. Correlation between PD-L1 gene expression and Immunohistochemistry (IHC) score.**

344 A high PD-L1 gene expression level correlated significantly with a higher CPS score (p-value ranging from 0.05-0.1)  
345 in initial diagnostic samples using a  $\geq 1$  CPS cut-off.

346 A high PD-L1 gene expression level correlated significantly with a higher CPS score ( $p < 10e-3$ ) in relapse samples  
347 regardless of the CPS cut-off.

348 "\*\*\*\*" for p-value  $< 10e-3$ ;

349 "•" for p-value ranging from 0.05-0.1

350 "NS" for p-value  $> 0.1$

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352 **Figure 4A. Evolution of the transcriptomic Interferon-gamma signature (*Sharma et al.2017*).**

353 IFN- $\gamma$  signature was 43% (15/35) concordant between initial and recurrent lesions.

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355 **Figure 4B. Evolution of the Tertiary Lymphoid Structure (TLS) signature (*Prabhakaran et al.2017*).**

356 TLS signature was 66% (23/35) concordant between initial and recurrent lesions.

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**Table 1. Baseline characteristics (n = 35)**

| Variables                     | Overall population |       |
|-------------------------------|--------------------|-------|
| Age (Median, range)           | 64                 | 42-87 |
| <b>Gender (N, %)</b>          |                    |       |
| Male                          | 25                 | 71%   |
| Female                        | 10                 | 29%   |
| <b>Smoking history (N, %)</b> |                    |       |
| Current/Former                | 26                 | 74%   |
| No                            | 9                  | 26%   |
| <b>Sample type (N, %)</b>     |                    |       |
| Biopsy                        | 11                 | 31%   |
| Surgical                      | 24                 | 69%   |
| <b>Disease site (N, %)</b>    |                    |       |
| Oral cavity                   | 15                 | 43%   |
| Oropharynx                    | 13                 | 37%   |
| Hypopharynx                   | 4                  | 11%   |
| Larynx                        | 2                  | 6%    |
| Maxillary Sinus               | 1                  | 3%    |
| <b>HPV status (N, %)</b>      |                    |       |
| Negative                      | 16                 | 46%   |
| Positive                      | 7                  | 20%   |
| Unknown                       | 12                 | 34%   |

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**T stage according to AJCC 8<sup>th</sup> edition (N, %)**

|               |    |     |
|---------------|----|-----|
| T1            | 2  | 6%  |
| T2            | 12 | 34% |
| T3            | 7  | 20% |
| T4            | 12 | 34% |
| Non evaluable | 2  | 6%  |

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**N stage according to AJCC 8<sup>th</sup> edition (N, %)**

|              |    |     |
|--------------|----|-----|
| N0           | 11 | 31% |
| N1           | 4  | 11% |
| N2           | 17 | 49% |
| N3           | 1  | 3%  |
| Missing data | 2  | 6%  |

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**Table 2. Curative treatment modality (n=35)**

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**Neoadjuvant chemotherapy (N, %)**

|              |    |     |
|--------------|----|-----|
| No           | 23 | 66% |
| Yes          | 10 | 29% |
| Missing data | 2  | 6%  |

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**Tumor resection (N, %)**

|              |    |     |
|--------------|----|-----|
| No           | 8  | 23% |
| Yes          | 25 | 71% |
| Missing data | 2  | 6%  |

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**Neck dissection (N, %)**

|              |    |     |
|--------------|----|-----|
| No           | 8  | 23% |
| Yes          | 25 | 71% |
| Missing data | 2  | 6%  |

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**Radiation therapy (N, %)**

|     |    |     |
|-----|----|-----|
| No  | 4  | 11% |
| Yes | 31 | 89% |

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**Concomitant systemic therapy (N, %)**

|              |    |     |
|--------------|----|-----|
| Chemotherapy | 12 | 39% |
|--------------|----|-----|

389 Cetuximab 5 16%

390 No 14 45%

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395 **Table 3. PD-L1 Combined Positive Score (CPS) in all samples (n = 33)**

|                    | Status at diagnosis | Status at relapse | Number (N, %) |
|--------------------|---------------------|-------------------|---------------|
| PDL1 CPS $\geq$ 1  | Positive            | Positive          | 19 (58)       |
|                    | Negative            | Positive          | 6 (18)        |
|                    | Positive            | Negative          | 6 (18)        |
|                    | Negative            | Negative          | 2 (6)         |
| PDL1 CPS $\geq$ 20 | Positive            | Positive          | 5 (15)        |
|                    | Negative            | Positive          | 7 (21)        |
|                    | Positive            | Negative          | 4 (12)        |
|                    | Negative            | Negative          | 17 (52)       |

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398 **Table 4. Interferon-gamma (IFN- $\gamma$ ) and tertiary lymphoid structure (TLS) signature enrichment scores in all**  
399 **samples (n = 35)**

|                            | Enrichment score at diagnosis | Enrichment score at relapse | Number (N, %) |
|----------------------------|-------------------------------|-----------------------------|---------------|
| IFN- $\gamma$<br>signature | High                          | High                        | 13 (37)       |
|                            | Low                           | High                        | 2 (6)         |
|                            | High                          | Low                         | 13 (37)       |
|                            | Low                           | Low                         | 7 (20)        |
| TLS signature              | High                          | High                        | 5 (14)        |
|                            | Low                           | High                        | 2 (6)         |
|                            | High                          | Low                         | 11 (31)       |
|                            | Low                           | Low                         | 17 (49)       |

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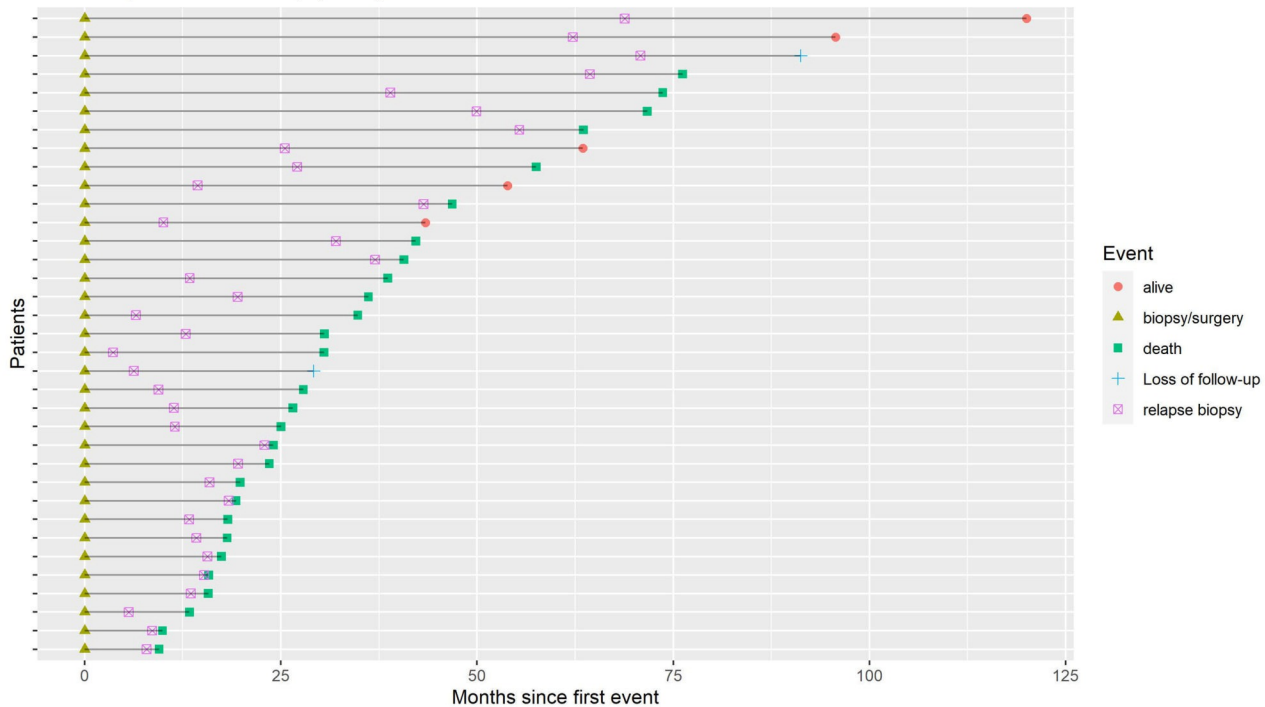
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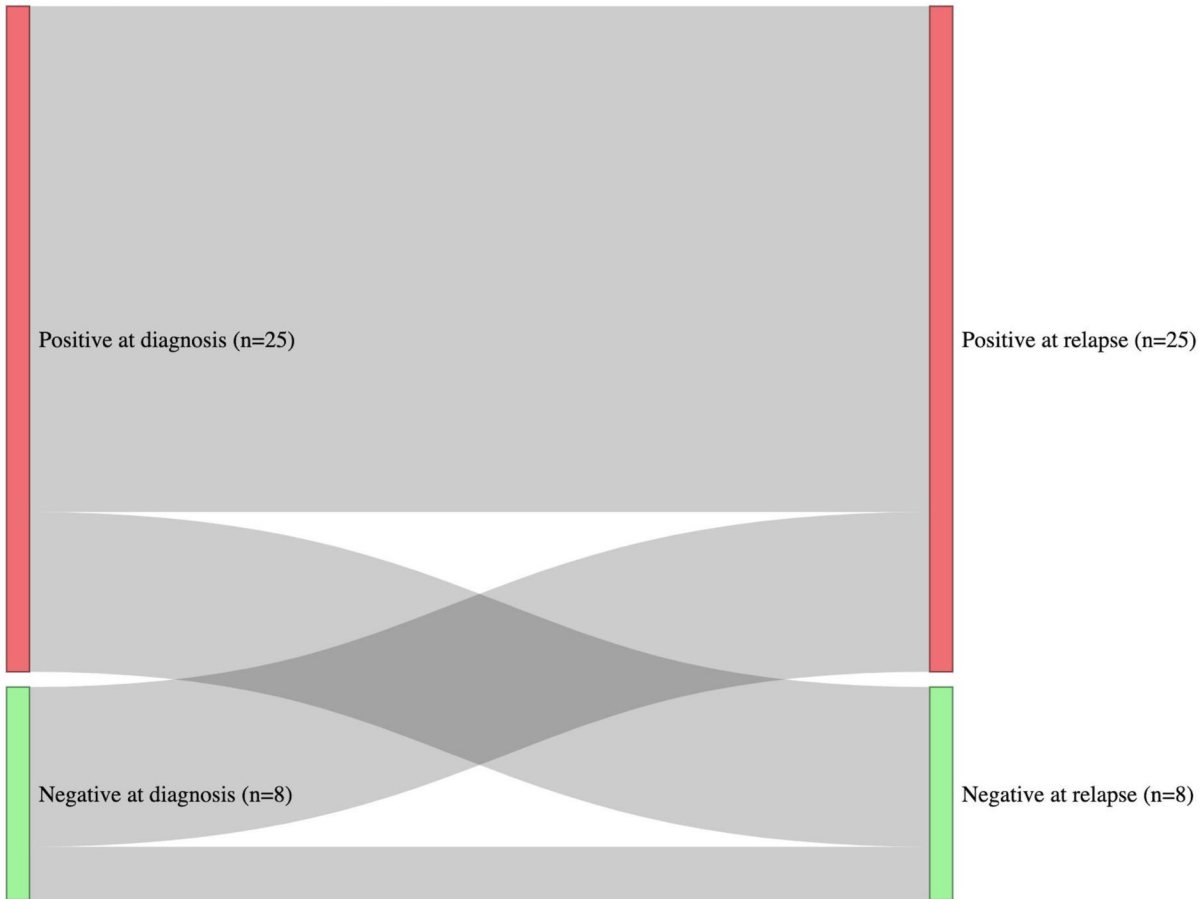
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#### 580 **Declaration of interest**

581 PS is a member of HTG Diagnostics Scientific Advisory Board and receives research grants from HTG  
582 Diagnostics, Inivata, ArcherDx, Bristol-Myers Squibb, Roche Molecular Diagnostics, Roche, AstraZeneca,  
583 Novartis, Bristol-Myers Squibb Foundation and Illumina. JF reports grants, personal fees and non-financial  
584 support from Bristol-Myers Squibb, personal fees and non-financial support from MSD, personal fees from  
585 Merck, personal fees and non-financial support from Astrazeneca, personal fees from Rakuten, personal fees  
586 from Biogen and personal fees from Innate Pharma. CLT participated in advisory boards from BMS, MSD,  
587 Merck Serono, Roche, Nanobiotix, Rakuten, Seattle Genetics, Rakuten, Astra Zeneca, GSK, Celgene.

HNSCC patients follow-up (n=35)



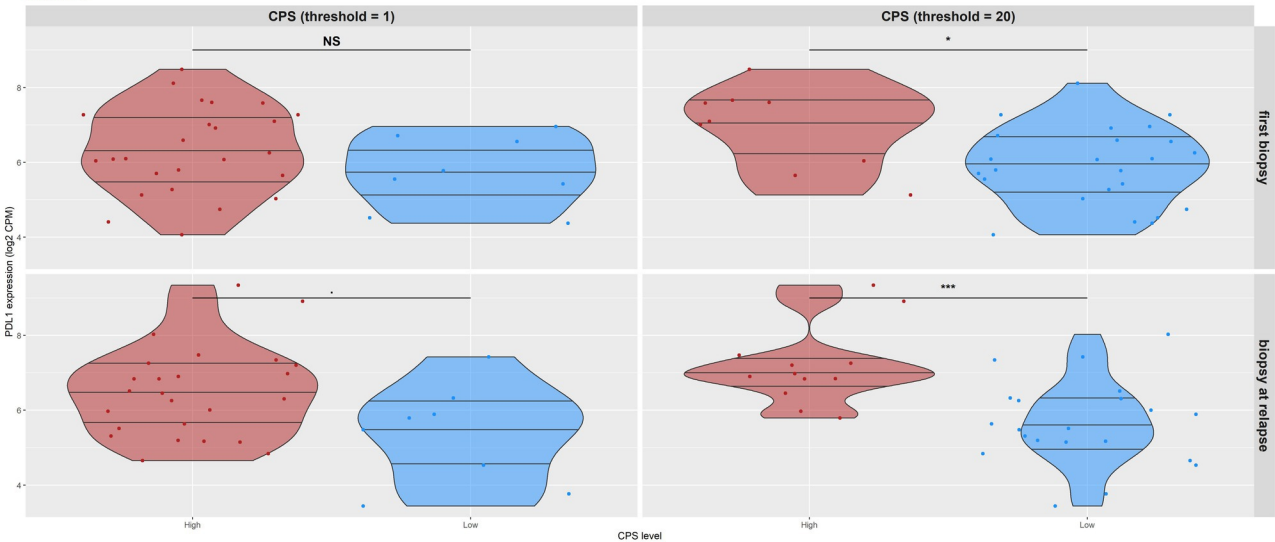


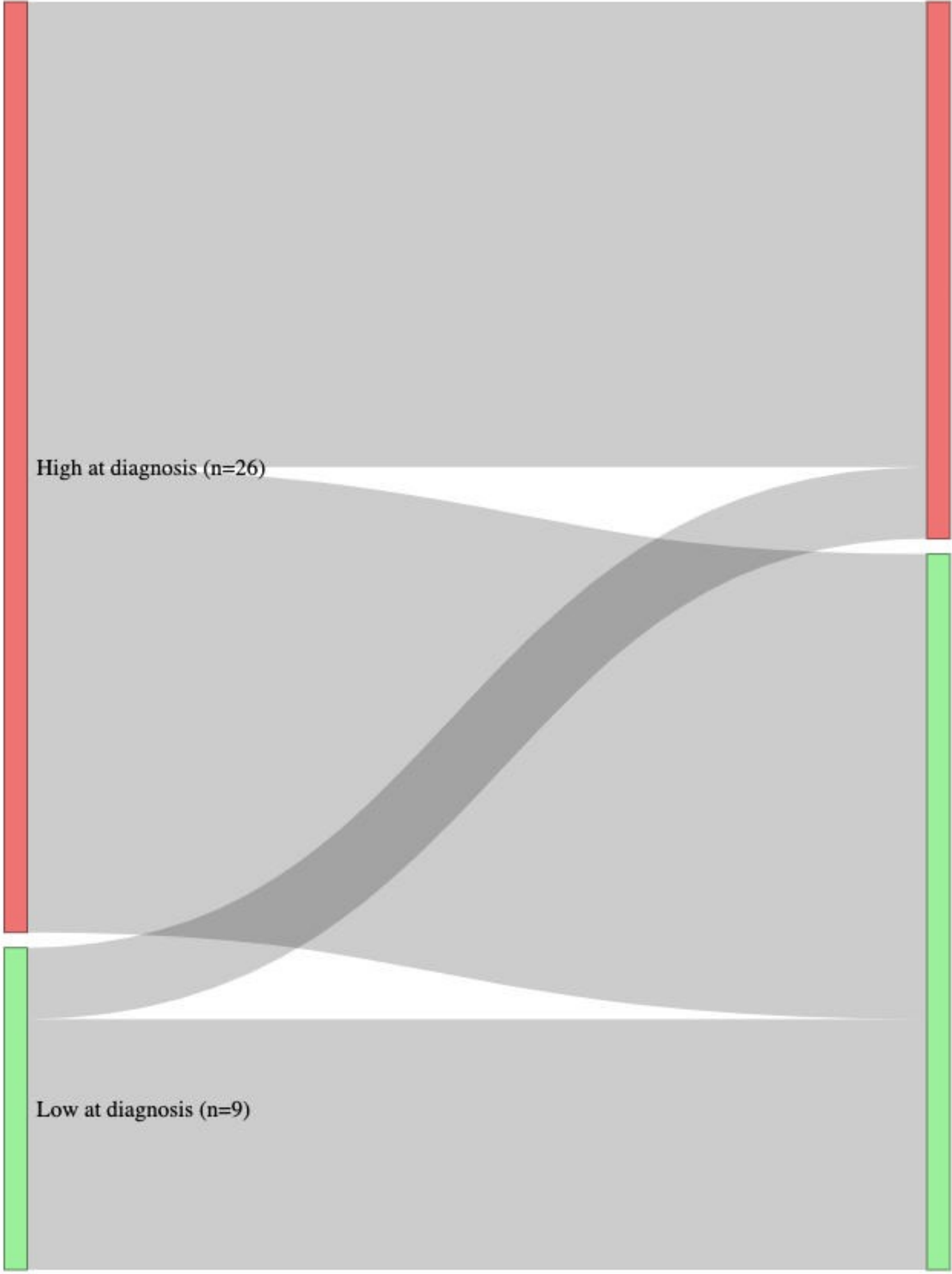




Correlation between PDL1 gene expression and CPS score (Immunohistochemistry)

Wilcoxon test



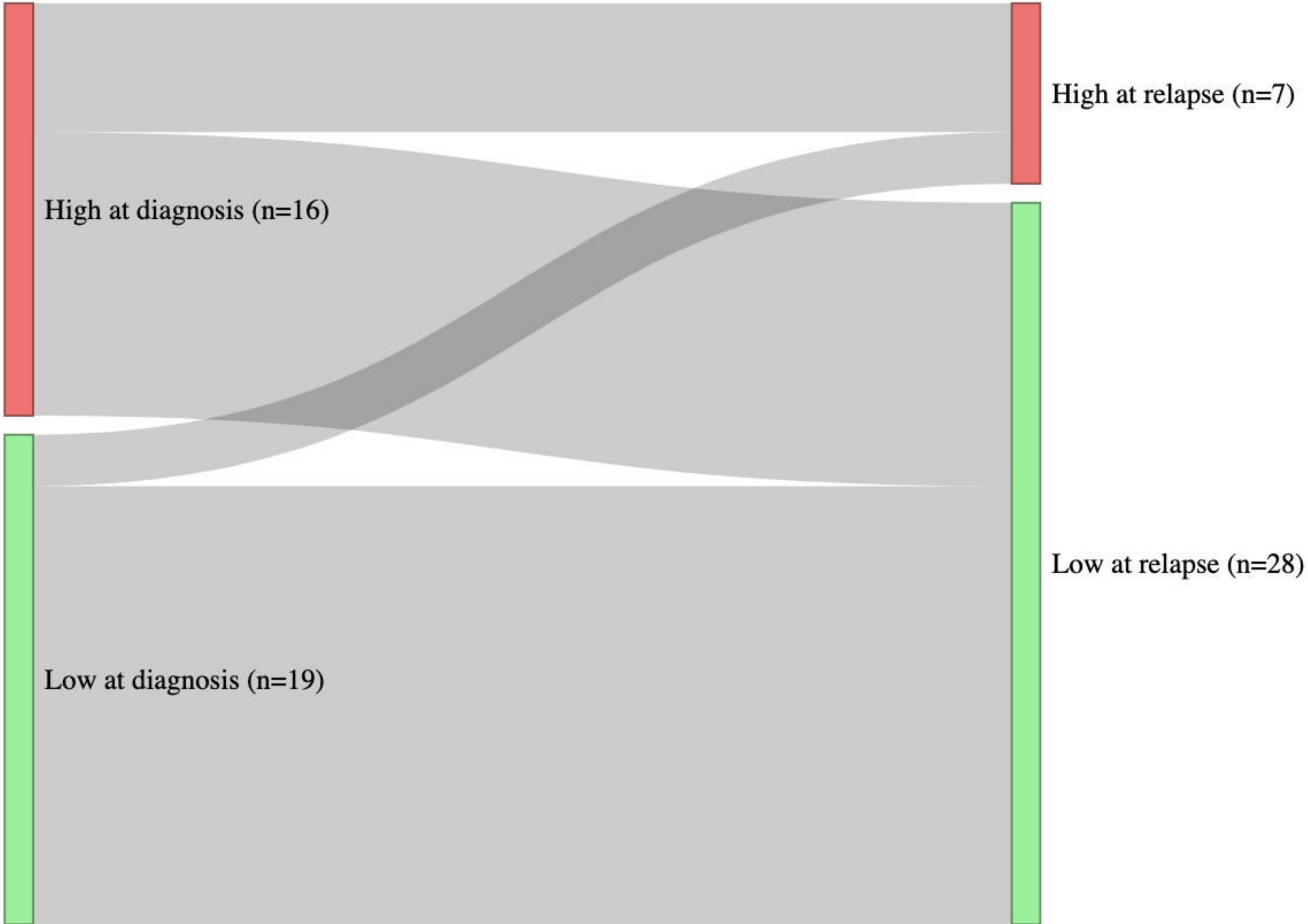


High at diagnosis (n=26)

Low at diagnosis (n=9)

High at relapse (n=15)

Low at relapse (n=20)



High at diagnosis (n=16)

High at relapse (n=7)

Low at diagnosis (n=19)

Low at relapse (n=28)