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**Prognostic factors of metastatic neuroendocrine carcinoma under first-line treatment with platinum etoposide with a focus on NEC score and Rb expression: Results from the multicentre RBNEC study of the Groupe d'Etude des Tumeurs Endocrines (GTE) and the ENDOCAN-RENATEN network**

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1 **Prognostic factors of metastatic neuroendocrine carcinoma under first line treatment**  
2 **with platinum etoposide with a focus on NEC score and Rb expression: results from**  
3 **the multicenter RBNEC study of the Groupe d'Etude des Tumeurs Endocrines (GTE)**  
4 **and the ENDOCAN-RENATEN network.**

5

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64 **Abstract** (245 words)

65

## 66 **Introduction & aim**

67 Neuroendocrine carcinomas (NEC) are aggressive malignant diseases. Platinum-etoposide  
68 (PE) combination is the standard first line treatment, whatever the primary location. NEC  
69 score but also Rb status have been suggested to be predictive/prognostic factors in NEC.

70 The primary objective of our multicentric retrospective study was to evaluate the prognostic  
71 relevance of NEC score and Rb status, assessed by immunohistochemistry in PE-treated  
72 metastatic NEC patients.

## 73 **Methods**

74 Seven centers participated. Inclusion criteria were: NEC whatever the primary site,  
75 metastatic stage, first line treatment with PE and tissue samples available. Rb status was  
76 determined centrally.

## 77 **Results**

78 We report multicentric data from 185 metastatic patients (37% female, median age 63).  
79 There were 108 small cell NEC (SCNEC, 58.4%), 50 large cell NEC (LCNEC, 27%) and 27  
80 not otherwise specified NEC (nosNEC, 14.6%). Primary sites were: thorax (37%), gastro-  
81 enteropancreatic (38%), unknown (15%) and other (9%). Mean Ki-67 index was 76% (range  
82 20-100). Rb status was interpretable in 122 cases. Rb expression was lost in 74% of the  
83 cases: 84% of SCNEC vs 60% and 63% of LCNEC and nosNEC, respectively ( $p=0.016$ ).  
84 Objective response (OR) was seen in 70% of SCNEC, 45% of LCNEC and 48 % of nosNEC  
85 ( $p<0.001$ ) and in 62% of Rb-negative tumors vs 46% of Rb-positive tumors ( $p=0.3$ ). There  
86 was no difference in median PFS or OS under according to Rb status. Age, NEC score and  
87 response to chemotherapy were the main factors associated with OS in our cohort.

## 88 **Conclusion**

89 In our series, Rb status had no prognostic impact in PE-treated metastatic NEC patients  
90 whereas age, NEC score and response to chemotherapy were the main factors associated  
91 with OS

## 92 INTRODUCTION

93 Neuroendocrine carcinomas are aggressive neuroendocrine neoplasms, which may  
94 arise in all organs and are defined by a poorly differentiated morphology usually associated  
95 with high proliferative capacities [1]. Two histological subtypes are recognized in all sites: the  
96 small cell (SCNEC) and the large cell (LCNEC) neuroendocrine carcinomas. In both types,  
97 the diagnosis is often done at the metastatic stage and the prognosis is poor. The standard  
98 recommended first-line treatment is the platinum-etoposide chemotherapy regimen [2,3],  
99 mostly based on retrospective results, whatever the primary site [4–7]. With this regimen,  
100 response rate (RR) is 40 to 70% but the median progression-free survival is between 4 and 9  
101 months [7,8]. Disease progression almost always occurs during or just after treatment.  
102 Median overall survival (OS) is only about 12-15 months for gastro-entero-pancreatic (GEP)  
103 NEC and of 8 to 10 months for pulmonary NEC [9,10]. Similar results have been reported for  
104 either cisplatin or carboplatin [7,8] or either intravenous or oral etoposide [11,12].

105 The main clinico-pathologic prognostic factors in metastatic NEC reported so far are  
106 performance status, number of metastatic sites, presence of liver metastasis, Ki-67 index  
107 and pathological subtype [7,8,13–15]. Recently, new potential prognostic and/or predictive  
108 factors have been proposed. One example is the GI-NEC score which gathers clinical,  
109 biological and pathological features to predict prognosis in GEP NEC [16]. However, its  
110 potential relevance in other sites has not been evaluated. Another example is Rb  
111 (retinoblastoma protein) status. Rb is encoded by the *RB1* gene and is involved in cell cycle  
112 regulation. *RB1* inactivation, usually associated with the loss of expression of Rb protein, is  
113 frequent in NEC. It is almost constant in SCNEC, frequently associated with *TP53* alteration,  
114 while it is less frequent in LCNEC, in line with the molecular heterogeneity recently  
115 evidenced in this tumor type, with either “SCNEC-like” or “adenocarcinoma-like” molecular  
116 signatures in the lung, the colon, the pancreas and other sites [17–20].

117 It has long been known that the objective response rate (ORR) to platinum-etoposide  
118 seems to differ between SCNEC and LCNEC. This difference has been reported for  
119 pulmonary NEC with an ORR of around 30% in LCNEC [10] as compared to 50-60 % in

120 SCNEC [9,21,22] and for pancreatic NEC with an ORR of 68 and 44% for SCNEC and  
121 LCNEC respectively [23]. Recently, concurrent reports have suggested that Rb status might  
122 be predictive of the response to chemotherapy in NECs and have hypothesized that the  
123 molecular heterogeneity of these tumors have to be taken into account for deciding the most  
124 adapted therapeutic strategy [19,24]. The heterogeneity in Rb expression has also been  
125 reported in NEC of the digestive tract: Rb loss of expression, indicative of *RB1* inactivation, is  
126 found in 100% of SCNEC versus 31% of LCNEC of the colon [20]; Rb loss is also found in  
127 89% of SCNEC versus 60% of LCNEC of the pancreas [17].

128         A retrospective analysis from the Dutch registry of pulmonary NEC has shown that  
129 LCNEC without Rb expression loss had a significantly longer median overall survival (OS) of  
130 9.6 months (95% CI, 7.4–11.8) when treated with a “NSCLC-like” chemotherapy regimen  
131 (with either gemcitabine or paclitaxel) as compared to “SCLC-like” chemotherapy (platinum-  
132 etoposide) with a 4.8 months (95% CI, 3.9–5.7) median overall survival [14]. In another  
133 retrospective series of pancreatic G3 NEC, the response rate to first-line platinum-based  
134 chemotherapy was 68% in the 31 patients with SCNEC versus 44% in the 18 patients with  
135 LCNEC and 80% vs 38.4% in, Rb-negative and Rb-positive tumors, respectively, [23].  
136 Recently, Lacombe et al reported higher response rate in NEN G3 with Rb H-score below  
137 150 [25].

138         We were therefore prompted to evaluate and compare the potential relevance of the  
139 recently proposed prognostic factors NEC score and Rb status in a large series of NEC from  
140 different primary sites. The Groupe d’Etude des Tumeurs Endocrines (GTE) and the French  
141 expert network for the management of neuroendocrine neoplasms ENDOCAN-RENATEN.  
142 designed a multicentric retrospective study including 185 cases of metastatic neuroendocrine  
143 carcinoma, whatever the primary location, treated in first-line with platinum and etoposide.

144 **METHODS**

145 ***Patients***

146 This was a multicenter retrospective study of 7 tertiary centers of the French expert  
147 network for the management of neuroendocrine neoplasms ENDOCAN-RENATEN  
148 (approved by the French National Cancer Institute, INCa). Patients were diagnosed between  
149 July 1999 and January 2020. Inclusion criteria were 1) locally confirmed diagnosis of  
150 neuroendocrine carcinoma, based on WHO recommendations including [26]: suggestive  
151 morphology, immunohistochemical demonstration of neuroendocrine differentiation,  
152 determination of histological grade according to site-specific WHO criteria; 2) metastatic NEC  
153 with platinum and etoposide combination as first-line systemic treatment chemotherapy and  
154 at least one cycle administered, 3) available formalin-fixed paraffin-embedded (FFPE) tumor  
155 tissue material for determination of the Rb status, 4) measurable disease according to  
156 RECIST 1.1. Well differentiated neuroendocrine tumors with high proliferative capacities  
157 were excluded.

158

159 **Data collection**

160 The following parameters were collected:

161 - baseline characteristics: age, gender, performance status, primary location, stage,  
162 number of metastatic sites, metastatic sites locations, lactate dehydrogenase (LDH),  
163 alkaline phosphatase (ALP). When data were available, the GI-NEC score was calculated,  
164 according to Lamarca et al [16], for all patients, whatever the primary, and referred to NEC  
165 score in the manuscript. The score is as follows: i) presence of liver metastases (0=No,  
166 1=Yes), ii) ALP (0 if  $\leq 82$  IU/l, 1 if 83-289 IU/l and 2 if  $\geq 290$  IU/l), iii) LDH (0 if  $\leq 827$  IU/l, 1  
167 if  $\geq 828$  IU/l, iv) ECOG PS (0= 0 or 1, 1 if  $\geq 2$ ) and v) Ki67 (0 if  $\leq 80\%$ , 1 if  $> 80\%$ ); NEC  
168 score is A (good prognosis) if total of points = 0-2, NEC score is B (poor prognosis) if total  
169 of points =3-6 [16].



- 170 - treatment characteristics: type of platinum used (carboplatin or cisplatin), number of  
171 cycles, reason for treatment stop and associated treatment modalities (radiation therapy,  
172 surgery, etc). Best response to treatment according to RECIST 1.1 [27] was collected.
- 173 - date and events: date of diagnosis, of day 1 cycle 1 of treatment, last date of treatment  
174 administration and date of disease progression or death during or after treatment, date of  
175 death.

176 This retrospective study complies with the French MR004 methodology regarding  
177 general data protection regulation (GDPR) for non-interventional retrospective health  
178 research (Délibération n° 2018-155 du 3 mai 2018) and received approval by internal ethics  
179 committee and review board (CSET N°2020-58).

180

### 181 ***Chemotherapy with platinum-etoposide***

182 Platinum-etoposide chemotherapy was administered according to French  
183 neuroendocrine neoplasms guidelines [29]. Patients received either cisplatin 100 mg/m<sup>2</sup> or  
184 carboplatin AUC5 on day 1, combined to etoposide 100 mg/m<sup>2</sup> on day 1, 2 and 3 as one  
185 cycle of treatment. Each cycle was repeated every 21-28 days until disease progression or  
186 for a maximum of 6-8 cycles. Choice between cisplatin and carboplatin, treatment duration  
187 and dose adaptations were made according to local practice in the different participating  
188 centers.

189

### 190 ***Pathological review and determination of Rb status***

191 The pathological diagnosis and histological subtyping, as either small cell (SCNEC) or large  
192 cell (LCNEC) NEC, were confirmed by a central review in accordance with WHO criteria for  
193 the corresponding body site. A few cases could not be confidently subtyped, usually because  
194 of the lack of suitable tissue material for an accurate morphological analysis (such as the  
195 presence of severe cellular artifacts like crushing); they will be thereafter referred to as not  
196 otherwise specified NEC (nosNEC). Ki-67 index was determined in each participating center  
197 according to WHO recommendations [26].

198 Rb status was determined by immunohistochemistry applied to FFPE tumor tissue samples.  
199 The technique was performed centrally by the Laboratory for Experimental and Translational  
200 Pathology (PETRA, CNRS UMS3655/INSERM US23), Gustave Roussy Cancer Center,  
201 Villejuif.

202 Briefly, 4  $\mu\text{m}$ -thick tissue sections were prepared and deparaffinized. Only freshly prepared  
203 sections were used. The clone 13A10 (Leica Biosystems, Buffalo Grove, IL), directed to Rb  
204 protein, was used as the primary antibody. A streptavidin-biotin technique was performed on  
205 an automated stainer (Ventana Benchmark Ultra, Tucson, AZ). Results were considered  
206 interpretable only when: (a) more than 1,000 tumor cells were evaluable, (b) internal positive  
207 controls (including stromal cells and/or peritumoral normal cells) were reactive and presented  
208 a distinct nuclear labeling. External controls (samples of lymphoid tissue) were included in  
209 each run to allow the comparison between the various runs necessary to complete the study.

210 Rb expression was considered retained when any proportion of tumor cells presented a  
211 distinct nuclear labeling, of any intensity. Rb expression was considered lost when no  
212 evidence of nuclear labeling was detected in any tumor cell. Rb status was defined as  
213 positive if Rb expression was retained and negative if Rb expression was lost.  
214 Representative examples are given in Figure 1.

215 All cases were assessed independently by two neuroendocrine pathologists (one junior, CK  
216 and one senior, JYS); in case of discrepancy, the sections would have to be reviewed  
217 simultaneously by the two pathologists at a multi-head microscope and a consensus decision  
218 would have to be achieved. No discrepancy occurred for Rb status determination.

219

## 220 **Statistics**

221 The progression-free survival (PFS) under treatment was defined as the time from the  
222 date of the first chemotherapy course to the date of the first event (cancer progression or  
223 death from any cause) or to the last follow-up. The overall survival (OS) was defined as the  
224 duration between the date of the first chemotherapy course to death from any cause or of the  
225 last follow-up.

226 Quantitative variables are presented in the form of mean +/- standard deviation or  
227 median (interquartile space) and compared using Student's t test or Wilcoxon test (2 groups)  
228 and ANOVA or Kruskal-Wallis test (more than 2 groups) depending on the application  
229 conditions. The qualitative variables are presented in count (percentage) form and compared  
230 using the Chi<sup>2</sup> test or Fisher's exact test depending on the application conditions. The  
231 survival curves are calculated according to the Kaplan-Meier method and compared by the  
232 Log-rank test.

233 For the univariable prognostic analysis, a Cox model was used. The threshold for  
234 entering the multivariate analysis was based on a p-value less than 0.05. The multivariate  
235 analysis used a top-down selection procedure based on the Akaike Information Criterion  
236 (AIC). All the tests presented are bilateral. A value of  $p < 0.05$  was chosen to indicate  
237 statistical significance. The analyses were carried out in the R 4.0 software (The R Core  
238 Team, 2020, Vienna, Austria).

239 **RESULTS**

240 ***Clinical characteristics of the patients***

241 Two hundred and nineteen patients with an initial diagnosis of neuroendocrine  
242 carcinoma treated by platinum-etoposide with at least one archival tumor sample available  
243 were identified. Pathological review in the expert centers excluded 12 cases, reclassified as  
244 well differentiated grade 3 neuroendocrine tumors. Among the remaining 207 patients, 22  
245 were treated for localized or advanced, non-metastatic disease and were excluded to gather  
246 a more homogeneous population of patients. Finally, 185 patients with metastatic NEC were  
247 included and eligible for Rb status determination and correlation with clinical data. The flow  
248 chart of the study is reported in Figure 2.

249 Characteristics of included patients, overall and according to the primary location, are  
250 summarized in Table 1. There was 117 males and 68 females. The median age was 62  
251 (range 21-88), 73% of patients had a good ECOG performance status (0-1). Primary was of  
252 gastro-entero-pancreatic (GEP), thoracic or unknown origin in 38, 37 and 15% of the cases,  
253 respectively. The 71 GEP primaries were located in the pancreas (n=24, 34%), colon (n=18,  
254 25%), rectum (n=12, 17%), esophagus (n=8, 11%), stomach and biliary tract (n=4, 6% each)  
255 and duodenum (n=1, 1%). There were 10 patients with gynecological primary (6 cervix, 3  
256 uterus and 1 ovary), 4 with head & neck and 3 with prostate primary.

257 Two thirds of patients had 1 or 2 distant metastatic sites, 54% of the patients with  
258 NEC of unknown primary had 3 or more metastatic sites which was significantly superior to  
259 patients with other primary locations (p=0.011). Most frequent metastatic sites were in the  
260 liver, found in 58% of the cases, more often in patients with gastro-entero-pancreatic  
261 primaries (p=0.024); in the lymph nodes (48%), more often in patients with GEP and  
262 unknown primaries (p=0.038); in the bone (31%) and in the lungs (23%) which were more  
263 often found in primaries of lung and unknown primaries (p<0.001 & p=0.01, respectively).  
264 Brain metastases were reported in 24 patients, 75% of whom had lung NEC. Of the 114  
265 patients with available data, 65% had a NEC score A.

266

267 ***Pathological characteristics***

268 Of the 185 metastatic patients with NEC, there were 108 small cell NEC (SCNEC,  
269 58.4%), 50 large cell NEC (LCNEC, 27%) and 27 not otherwise specified NEC (nosNEC,  
270 14.6%). Pathological characteristics according to the histological subtype are summarized in  
271 Table 2. SCNEC was more frequent in lung (77%), gynecological (90%), head & neck and  
272 prostate primary (100%) while LCNEC and nosNEC were more frequent in GEP (65%)  
273 primary site ( $p<0.01$ ). Ki-67 index was significantly different between SCNEC, LCNEC or  
274 nosNEC (ANOVA,  $p<0.001$ ): mean Ki-67 index was 70.7 % (SD 18.37) in LCNEC, 62.5% in  
275 nosNEC, significantly lower than in SCNEC (mean 80%, SD 16.59,  $p<0.001$ ).

276

277 ***Results of first line chemotherapy with platinum and etoposide***

278 All 185 metastatic patients received either carboplatin (60%) or cisplatin (40%)  
279 combined with etoposide for a median number of 6 cycles (range 1-26). Radiation therapy  
280 ( $n=15$ ) and surgery ( $n=5$ ) were done with a palliative intent, together with chemotherapy, in  
281 20 patients as part of a multimodal therapeutic strategy. Altogether, first-line chemotherapy  
282 led to a 60% objective response rate (ORR: complete response (CR) + partial response  
283 (PR)) and 23% of disease stabilization (SD); the main causes of treatment discontinuation  
284 were progressive disease (44%) or scheduled pause (39%). Seventeen percent of the  
285 patient had a primary refractory disease (Supplementary Table 1). Median PFS under first-  
286 line platinum-etoposide therapy was 5.9 months [95% CI 5.4-6.4] (Figure 3A). Median OS of  
287 metastatic patients was 12.0 months (95%CI 10-14) (Figure 3B). One year, 2-year and 5-  
288 year OS rates were 52%, 19% and 5%, respectively. Patients who progressed under first line  
289 chemotherapy had a worse overall survival (median 7.3 months [95% CI 5.5-10],  $p=0.001$ ) as  
290 compared to patients experiencing response or stable disease. Of note, patients with  
291 objective response (CR+PR) had a similar median survival (14 months [95% CI 12-17]) to  
292 patients who experienced only disease stabilization (median 12 months [95% CI 9.9-16]).

293

294

295 ***Clinico-pathological parameters, Rb status and correlations with clinical features,***  
296 ***platinum etoposide responses and outcomes***

297 There were no differences in ORR according to Ki-67 index and the previously  
298 published 55% cut-off [8]: ORR was 55% in patients with Ki-67 index > 55% and 52% in  
299 patients with Ki-67 index < 55% (p=0.7). Objective responses were significantly more  
300 frequent (65%) in SCNEC as compared to LCNEC (44%) and nosNEC (44%) (p<0.001).  
301 Histological subtype was the only prognostic factor for PFS. Patients with SCNEC had a  
302 significantly longer median PFS of 6.4 months [95% CI 5.4-6.4] (p<0.01) as compared to  
303 patients with LCNEC (median 4 months [95% CI 3.1-5.7], HR = 2.16 [95% CI 1.51-3.07]) or  
304 nosNEC (median 3.4 months [95% CI 2.5-6.5], HR = 1,86 [95% CI 1.17-2.83] (Figure 3C).  
305 There was no difference in terms of PFS according to Ki-67 index as a continuous variable or  
306 dichotomized according to the previously published 55% cut-off and no other prognostic  
307 factor on PFS was found (Supplementary table 2). Median OS were 13 months (95%CI 12-  
308 16) in SCNEC, 9.8 months (95%CI 7.7-13) and 10 months (95%CI 8.8-29), p=0.27) (Figure  
309 3D). There was no difference in overall survival according to primary, Ki67, number of  
310 metastatic sites (Supplementary Figure 1). NEC score (HR 1.9 [95% CI 1.11-3.27] for Score  
311 B, p=0.023) was the only independent prognostic factor on overall survival in the multivariate  
312 analysis (Supplementary table 3).

313 From the 185 metastatic patients, 156 pathological samples were of sufficient quality  
314 for Rb immunohistochemistry. Rb expression was lost in 90 samples (58%), conserved in 32  
315 samples (20%) and not interpretable in 34 cases (22%). Rb loss was more frequent in  
316 SCNEC (84% of the samples) as compared to LCNEC (60%) and nosNEC (63%) (p= 0.016)  
317 (Table 3 and Figure 3B). There were no statistically significant differences in Rb status and  
318 mean Ki-67 index according to primary site: Rb was lost in 76% of GEP, 81% of lung and  
319 65% of unknown origin NEC (p=0.46). Clinical characteristics of patients with available Rb  
320 status are reported in supplementary table 4. Objective responses were seen in 62% of  
321 patients with Rb negative NEC and in 46% of patients with Rb-positive NEC (p=0.23).

322 In metastatic patients with available tumoral samples and Rb interpretable results,  
323 median progression-free survival was 5.3 months (95% CI 4.4-6.4) in patients with Rb-  
324 negative NEC and 4.7 months (95% CI 3.8-6.5) in patients with Rb-positive NEC,  $p= 0.18$   
325 (Figure 3E). Median overall survival in patients with Rb-lost NEC was not statistically different  
326 (10 months (95% CI 9.2-13)) from patients with Rb-conserved NEC (12 months (95% CI 9.9-  
327 24)),  $p= 0.23$  (Figure 3F). However, the worst prognosis was observed for patients with Rb-  
328 positive LCNEC with a median OS of 7.5 months (95% CI 6.2-not evaluable) while Rb-  
329 positive SCNEC ( $n=11$ ) and nosNEC ( $n=7$ ) had median OS of 20 and 18 months,  
330 respectively but with few patients in both subgroups. Other subgroups did not differ in terms  
331 of prognosis (Figure 3G&H).

332

333 **DISCUSSION**

334 Here, we report the prognostic factors in patients with metastatic neuroendocrine  
335 carcinoma, either small cell or large cell, under first-line treatment with platinum-etoposide  
336 chemotherapy combination, whatever the primary with a focus on NEC score and Rb status.  
337 We showed that median progression-free survival was better in patients with SCNEC (6.4  
338 months) as compared to either LCNEC (4 months) or nosNEC (3.4 months) but did not  
339 translate into better overall survival contrary to what was reported in the pancreatic NEC G3  
340 studies [15,23]. Although 60% of our patients received second-line treatment, this is unlikely  
341 to explain this discrepancy since we did not observe any difference in terms of PFS under  
342 second-line treatment according to pathology subtype (SCNEC vs LCNEC).

343 In our study, NEC score [16] was the only independent prognostic factor on overall  
344 survival together with response to chemotherapy. When removing NEC score from our  
345 multivariate model, none of the factors composing this score (Ki67 index, ECOG  
346 performance status, presence of liver metastasis and serum levels of ALP and LDH [16]),  
347 taken alone, had an independent prognostic impact. These data suggest that this composite  
348 score reflect multiple aspects of the disease: metastatic burden and/or clinical status and/or  
349 aggressiveness of the disease with greater prognostic impact. Like in the CEPD FFCD-GTE  
350 cohort [7], we do not confirm in our independent study, the prognostic impact of the 55% Ki-  
351 67 index cut-off which was initially reported by the NORDIC NEC study [8] but was not  
352 confirmed in a recent pathological study [13], based, in part, on the same cohort of patients.  
353 This suggests that some biases in the composition of the population under study might  
354 influence the prognostic relevance of this parameter.

355 As suggested by “omics” data [18,19,24,30], as well as basic science results [31,32],  
356 *RB1* inactivation and the consequent loss of expression of Rb protein appears to be one of  
357 the core molecular features of bona fide neuroendocrine carcinoma. Therefore, we aimed at  
358 exploring the clinical relevance of Rb status, whatever the primary, since numerous reports  
359 have shown a high frequency of Rb inactivation in NEC of diverse origins [19,20,23,33]. We  
360 analyzed Rb status by immunohistochemistry as it could be easily performed in routine



361 practice, thus enabling a rapid diagnosis. It has been shown that the loss of Rb expression  
362 detected by immunohistochemistry is strongly correlated with *RB1* gene inactivation or bi-  
363 allelic loss [14,33]. However, Rb loss of expression (by immunohistochemistry) can also be  
364 found in the absence of detection of a *RB1* gene alterations in the tumor [14] or in circulating  
365 tumor DNA (CtDNA) [34]. In this study, as in most previous ones, we strictly defined the loss  
366 of Rb expression as the complete absence of detection of Rb protein in tumor cells, in the  
367 presence of positive internal controls. Others have used H-score, based on the evaluation of  
368 the percentages of tumor cells expressing Rb at variable intensities (from 0 to 3 on a semi-  
369 quantitative scale) [14,35,36]. However, it is known that Rb levels are variable from one cell  
370 to another according to the position in the cell cycle; h-scores might therefore be more  
371 related to proliferative capacities than to *RB1* gene inactivation. Interestingly, Lacombe et al,  
372 recently reported a prognostic effect of a Rb H-score <150 on progression-free survival while  
373 a complete loss of Rb expression (corresponding to the definition of Rb-negative cases in the  
374 present study) had a prognostic effect on overall survival [26]. This questions the  
375 methodology to choose and how to interpret the clinical correlations. It must be underlined  
376 that, the reproducibility and accuracy of H-scores are questionable, especially when samples  
377 with different pre-analytical processing are included, as in our multicenter study. The  
378 importance of pre-analytical processing is well exemplified by our data: immunohistochemical  
379 results could not interpreted in several cases because of the lack of positive internal controls,  
380 likely as a result of pre-analytical conditioning and/or storage conditions.

381 Consistent with previous reports, we found a better ORR of 65% in patients with  
382 SCNEC as compared to 44% in patients with LCNEC [23]. ORR was higher in patients with  
383 Rb loss in their tumor (62%) as compared to 46% when Rb was conserved but there was no  
384 difference of disease control rate (CR+PR+SD) between Rb-positive and Rb-negative NEC.  
385 These results differ from the data of the Japanese pancreatic G3 NEC cohorts where the  
386 ORR in patients with tumors showing Rb loss and *KRAS* mutations was 70% as compared to  
387 33% in those without Rb loss or *KRAS* mutation [15]. This discrepancy could be related to a  
388 more heterogeneous population of patients in the present study since we gathered patients

389 with gastro-entero-pancreatic, thoracic, other and unknown primaries. However, interestingly,  
390 in our cohort of 185 metastatic patients, we did not find any prognostic difference on median  
391 overall survival nor progression-free survival according to the primary.

392 We did not find any prognostic role for Rb expression on progression-free or overall  
393 survival in the whole cohort. In the Japanese pancreatic NEC study, there was a trend  
394 toward shorter overall survival for patients with Rb loss and/or KRAS mutation (median 239  
395 vs 473 days, hazard ratio 2.11; 95% CI 0.92-4.86, P=0.077) but this was not significant  
396 [15]. Our results on overall survival are also in accordance with the retrospective results of the  
397 Dutch registry on patients with pulmonary LCNEC where Rb expression was assessed by  
398 immunohistochemistry using an H-score [14]. In this study, there was no difference in overall  
399 survival according to RB1 IHC H-score in 108 patients LCNEC patients (6.9 months for H-  
400 score<50 vs 6.3 months for H-score≥50); there was also no difference in overall survival  
401 according to the *RB* mutational status in a subgroup of 78 patients. However, in a subset  
402 analysis, the authors showed a better overall survival in 14 patients with Rb-positive (H-  
403 score≥50 in their report) LCNEC of the lung treated with an “adenocarcinoma-like”  
404 chemotherapy combination of platin with either gemcitabine or paclitaxel as compared to 9  
405 patients treated with platinum etoposide. However, another “adenocarcinoma-like”  
406 chemotherapy combination of platin and pemetrexed did not confer any survival advantage  
407 over the platinum-etoposide combination in Rb-positive patients in the same report [14]. A  
408 recent study by Zhuo et al, on 63 patients with LCNEC found opposite results with longer  
409 PFS and OS in “NSCLC-like” LCNEC under platinum etoposide as compared to platinum  
410 combined to either gemcitabine or paclitaxel. A recent pilot study by Gerard et al. found that  
411 GEP-NEC patients with an “adenocarcinoma-like” profile on CtDNA (i.e. *KRAS* and *BRAF*  
412 mutations) had shorter median PFS under platinum-etoposide, while the two patients with  
413 *RB1* mutations had shorter PFS under FOLFIRI-based (adenocarcinoma-like) chemotherapy  
414 [34]. In our study, we cannot confirm the hypothesis of an overall survival advantage in  
415 treating Rb-positive LCNEC patients with an “adenocarcinoma-like” chemotherapy regimen  
416 since all our patients were treated with the “SCLC” standard chemotherapy platin and

417 etoposide. However, we found a decreased median overall survival of only 7.5 months in Rb-  
418 positive LCNEC patients which suggests that Rb prognostic stratification could apply to  
419 LCNEC subgroup only and that a platinum-etoposide-based chemotherapy may not be ideal  
420 in this subgroup of patients.

421 Our study has some limitations. First, by gathering primaries from different origin, we  
422 have gained power but also heterogeneity in the population on study. However, it is not clear,  
423 from data in the literature, whether NEC of different origins have really different biological  
424 mechanisms and behavior. Of note, a recent report suggested that cervical G3 NEC may  
425 have different molecular alterations than G3 NEC from other origins [37]. Only 10 patients in  
426 our cohort had a NEC of gynecologic origin. We do not find a primary effect on prognosis in  
427 our study. Second, the retrospective nature of the present work, although adding evidence to  
428 previous works, do not allow to draw definitive conclusion on the relevance of Rb status and  
429 the best way to personalize the treatment of patients with G3 NEC. Our results suggest that  
430 the histological subtype may have the main prognostic/predictive impact as compared to Rb  
431 expression status but this remains to be demonstrated in a prospective clinical study. This is  
432 one of the objectives of the phase II FOLFIRINEC – PRODIGE 69 trial (NCT04325425) that  
433 will randomize metastatic G3 NEC patients between platinum-etoposide and FOLFIRINOX,  
434 with a stratification according to histological subtype, coupled to a molecular profile including  
435 Rb immunohistochemistry. This trial will compare an “adenocarcinoma-like” to a “NEC-like  
436 chemotherapy”. This strategy may be worth exploring, especially in the Rb-positive LCNEC  
437 population of patients who might have a decreased overall survival, as was demonstrated in  
438 the present study.

439 To conclude, Rb expression loss, defined by the complete absence of detectable  
440 protein in tumor cells in the presence of positive internal controls is associated with greater  
441 objective response rate but has no prognostic impact on either median PFS under first-line  
442 PFS or OS in patients with metastatic NEC, whatever the primary. NEC score and response  
443 to chemotherapy were the main factors associated with OS. NEC score and response to

444 chemotherapy were the main factors associated with OS. Further prospective evaluation is  
445 needed and will come from randomized studies.

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458 None declared

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## **Figure 1: representative examples of Rb-negative and Rb-positive tumors.**

### **Figure 1 legend**

a: a Rb-negative tumor, in which no tumor cell shows any detectable expression of Rb protein while internal controls, ie stromal cells, are strongly positive.

b and c, two examples of Rb-positive tumors, one (b) with a strong homogeneous labeling of all tumor cells and the other (c) showing a heterogeneous labeling with an admixture of positive and negative tumor cells.

Immunoperoxidase; original magnifications: a x280, b x180, c x240.

## **Figure 2: Study flow chart**

### **Figure 2 legend**

NEC: Neuroendocrine Carcinoma, MiNEN: Mixed neuroendocrine neoplasm, NET: Neuroendocrine tumor, IHC: immunohistochemistry.

## **Figure 3: Overall and progression-free survival of patients with metastatic NEC (whole cohort and subgroups)**

### **Figure 3 Legend:**

Kaplan Meier survival curves (in months) with Log-Rank test p values between subgroups when applicable.

A: Progression-free survival under platinum-etoposide.

B: Overall survival.

C: Progression-free survival under platinum-etoposide according to NEC subtype.

D: Overall survival according to NEC subtype.

E: Progression-free survival under platinum-etoposide according to Rb status.

F: Overall survival according to Rb status.

G: Overall survival according to NEC subtype in patients with Rb-negative NEC.

H: Overall survival according to NEC subtype in patients with Rb-positive NEC.

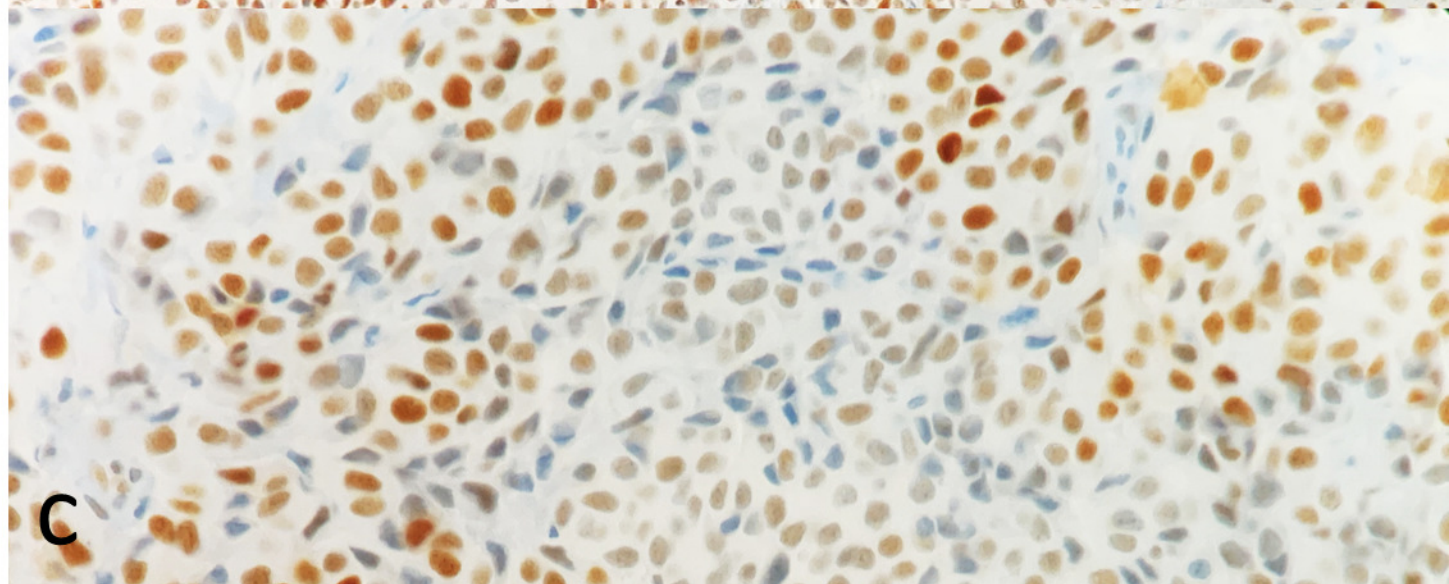
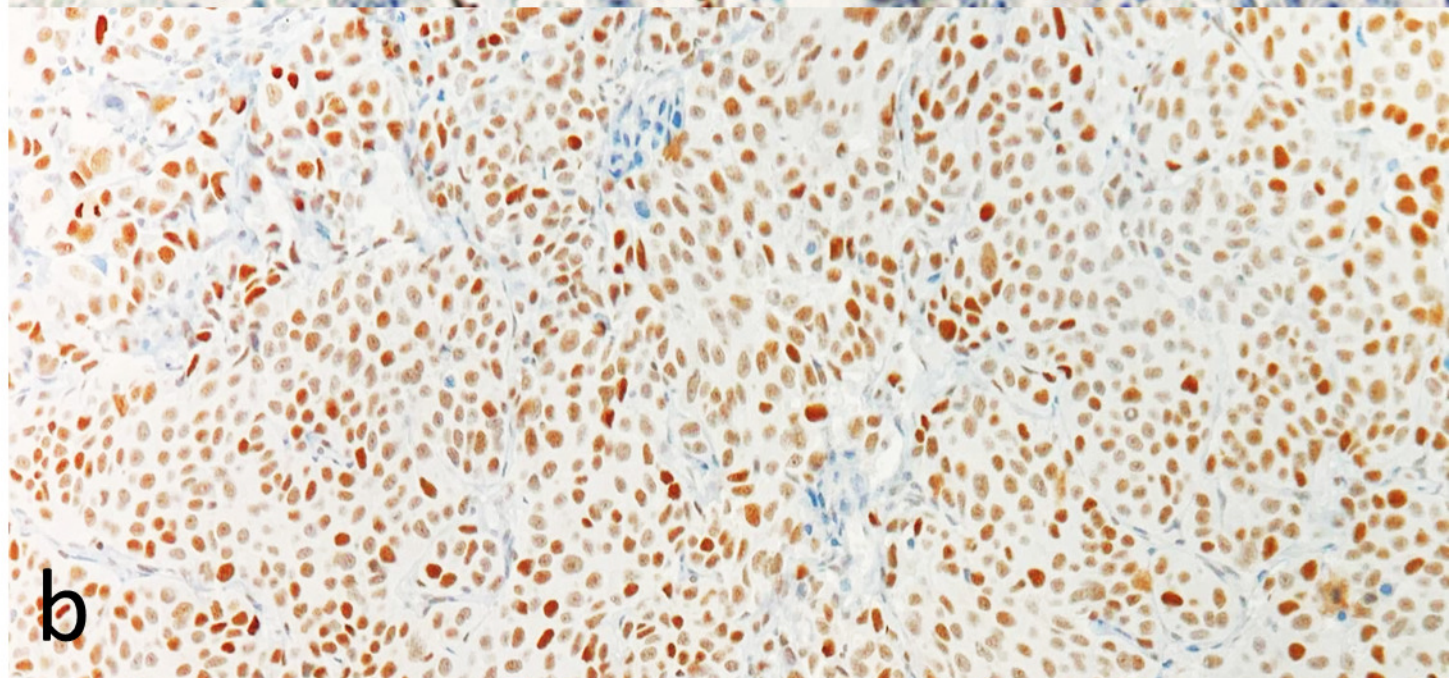
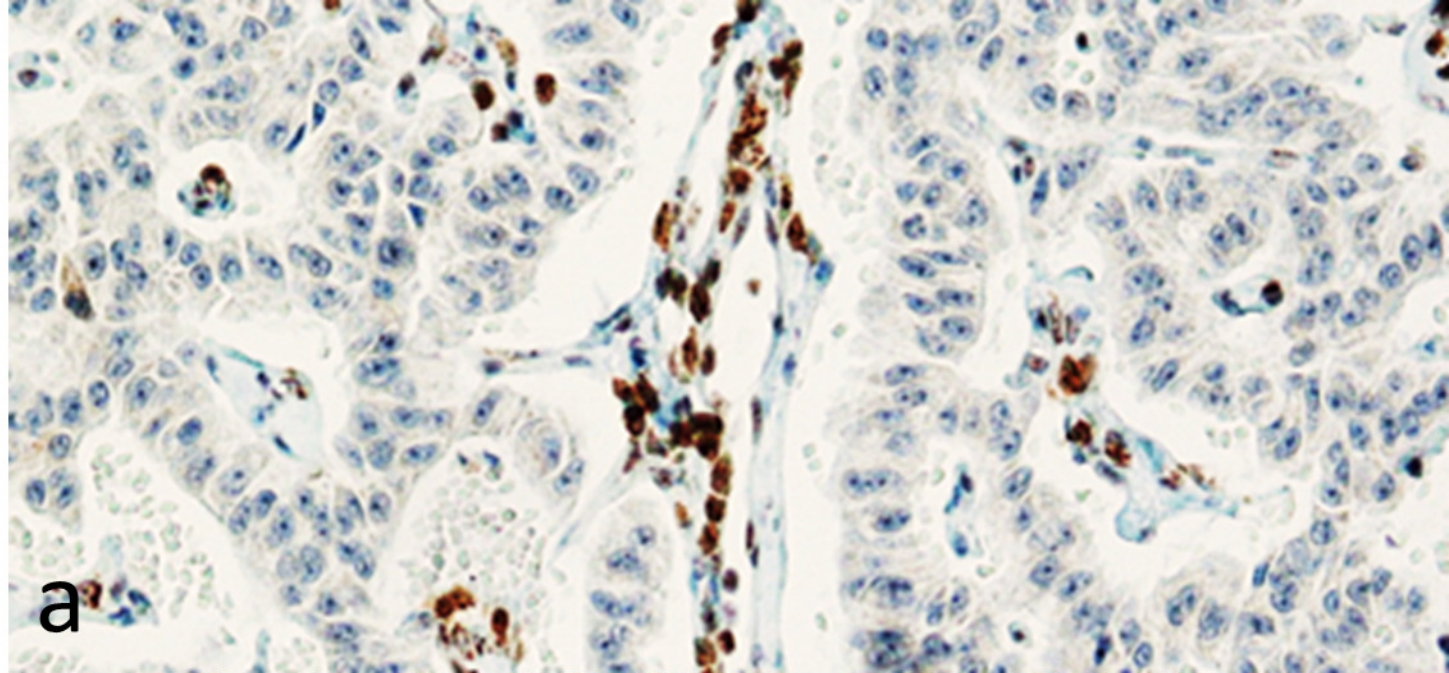


Figure 2: Study flow chart

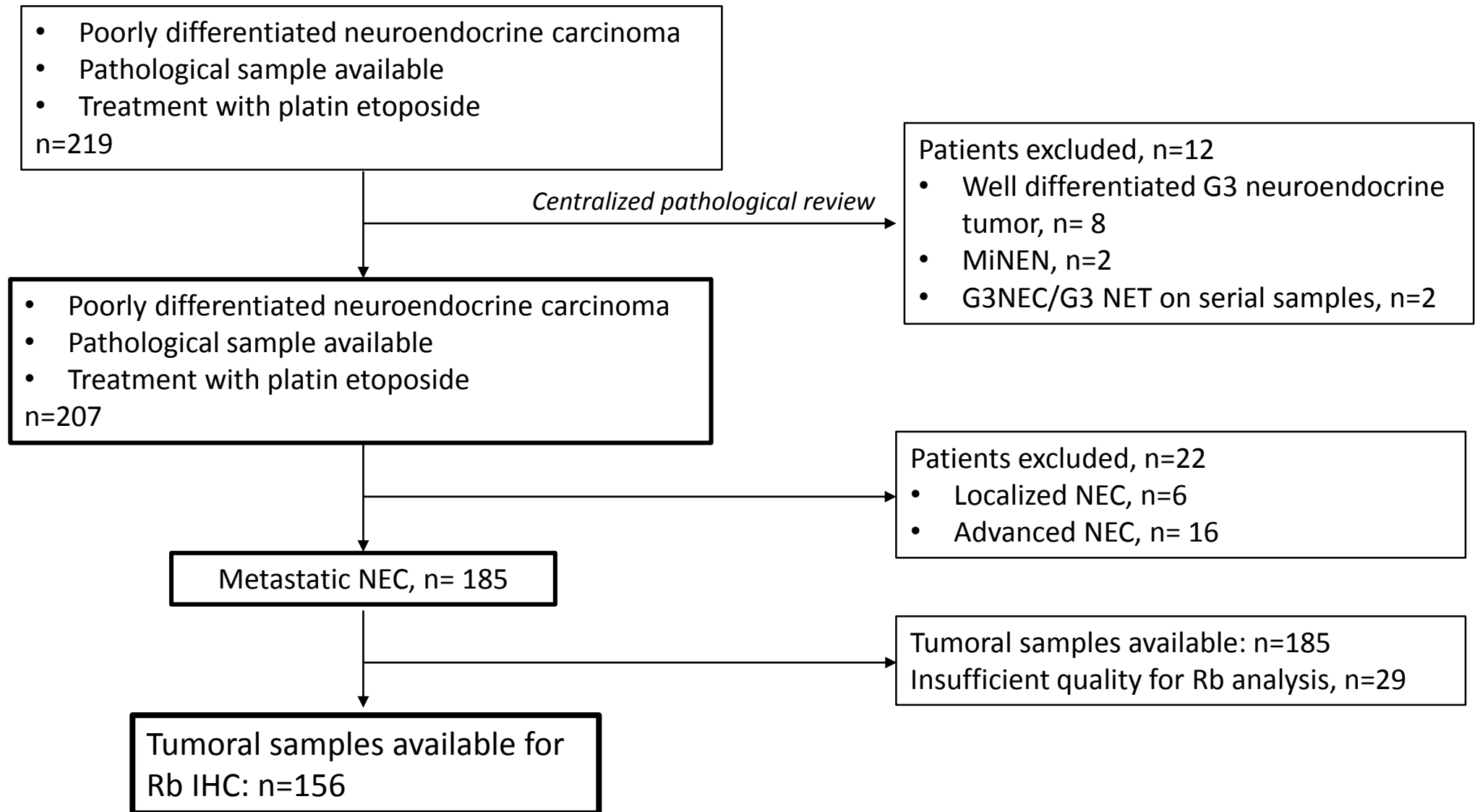


Figure 3A: Kaplan meier curve of progression-free survival (months) under platinum-etoposide

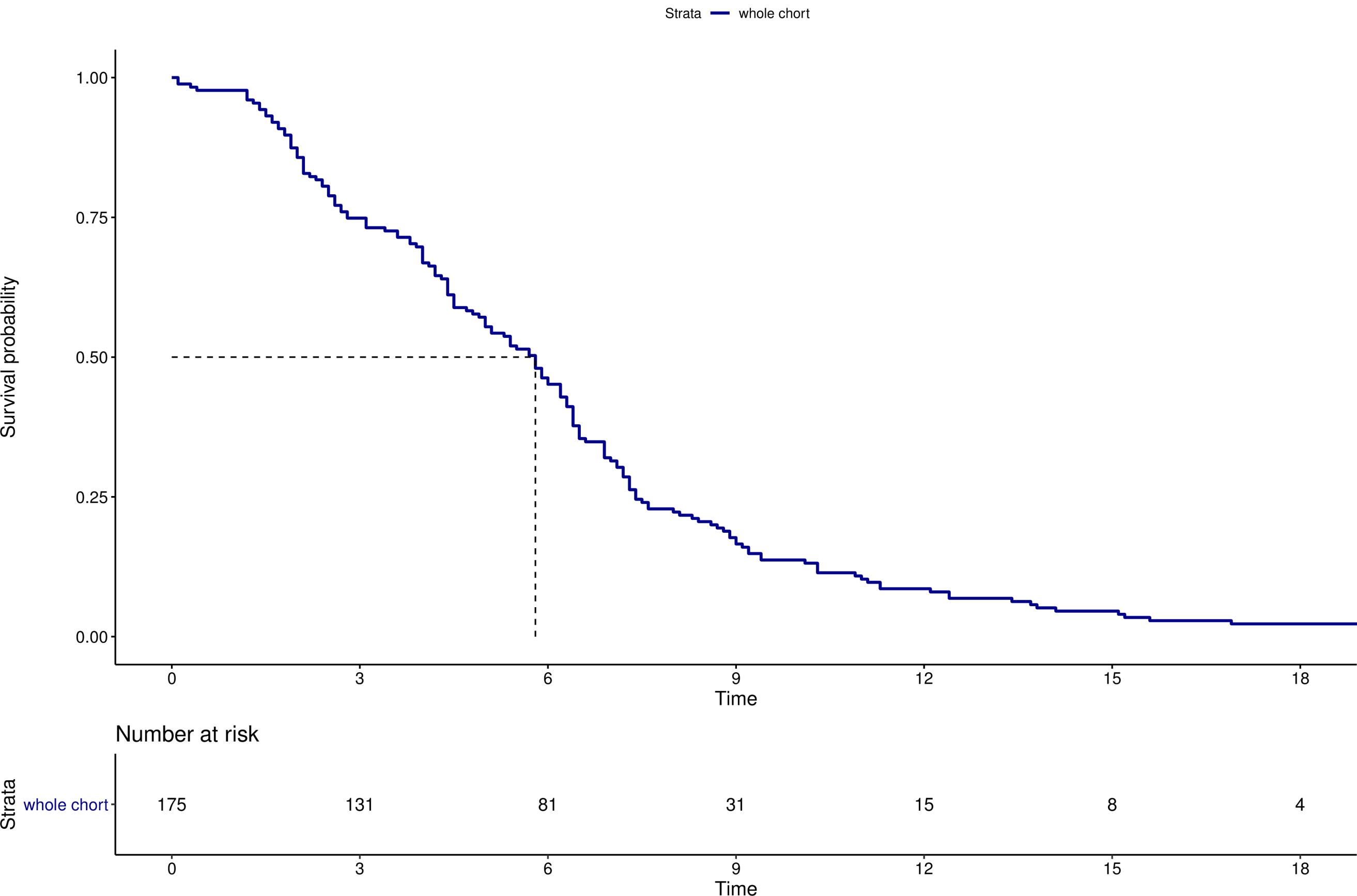


Figure 3B: Kaplan meier curve of overall survival (months)

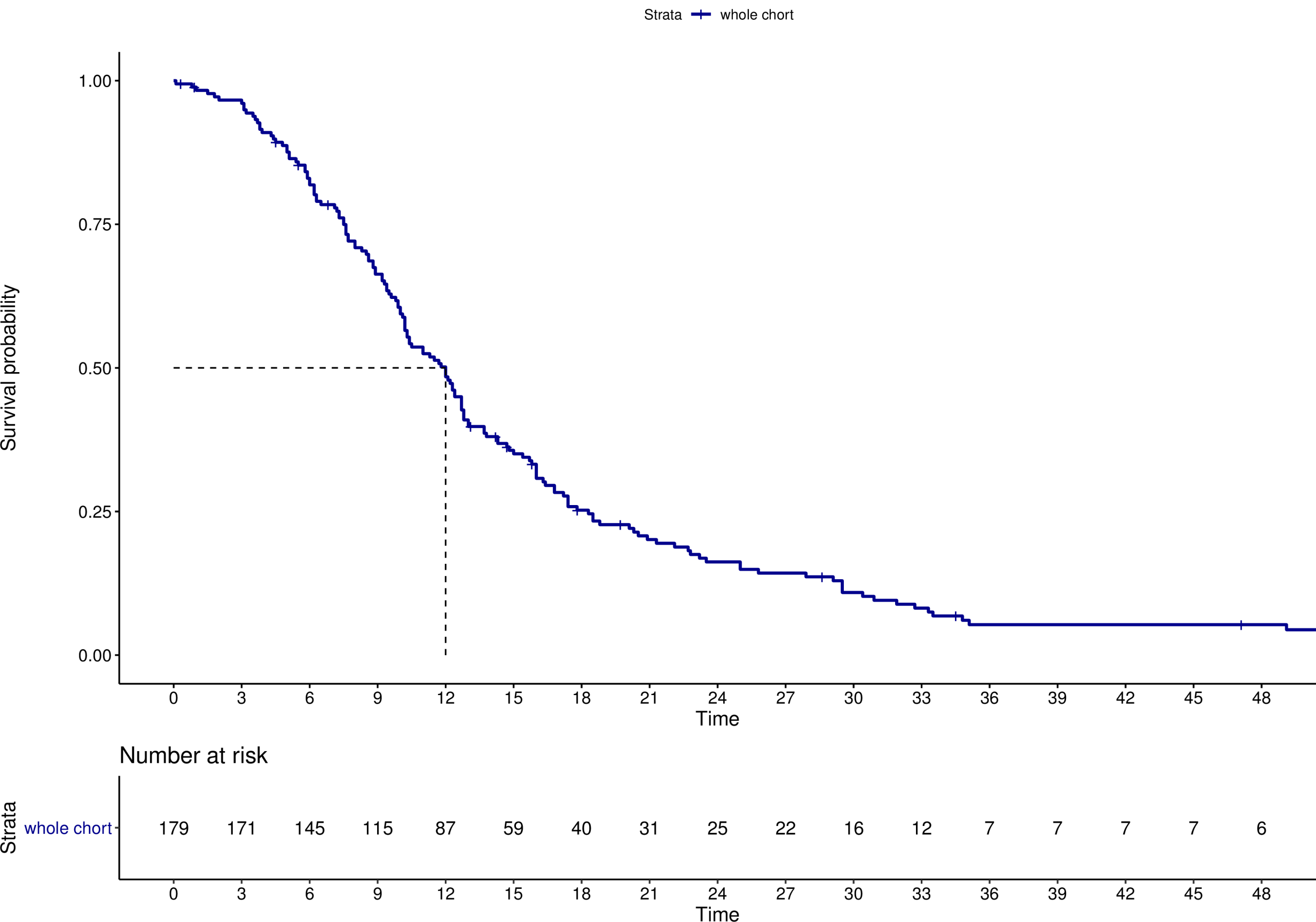
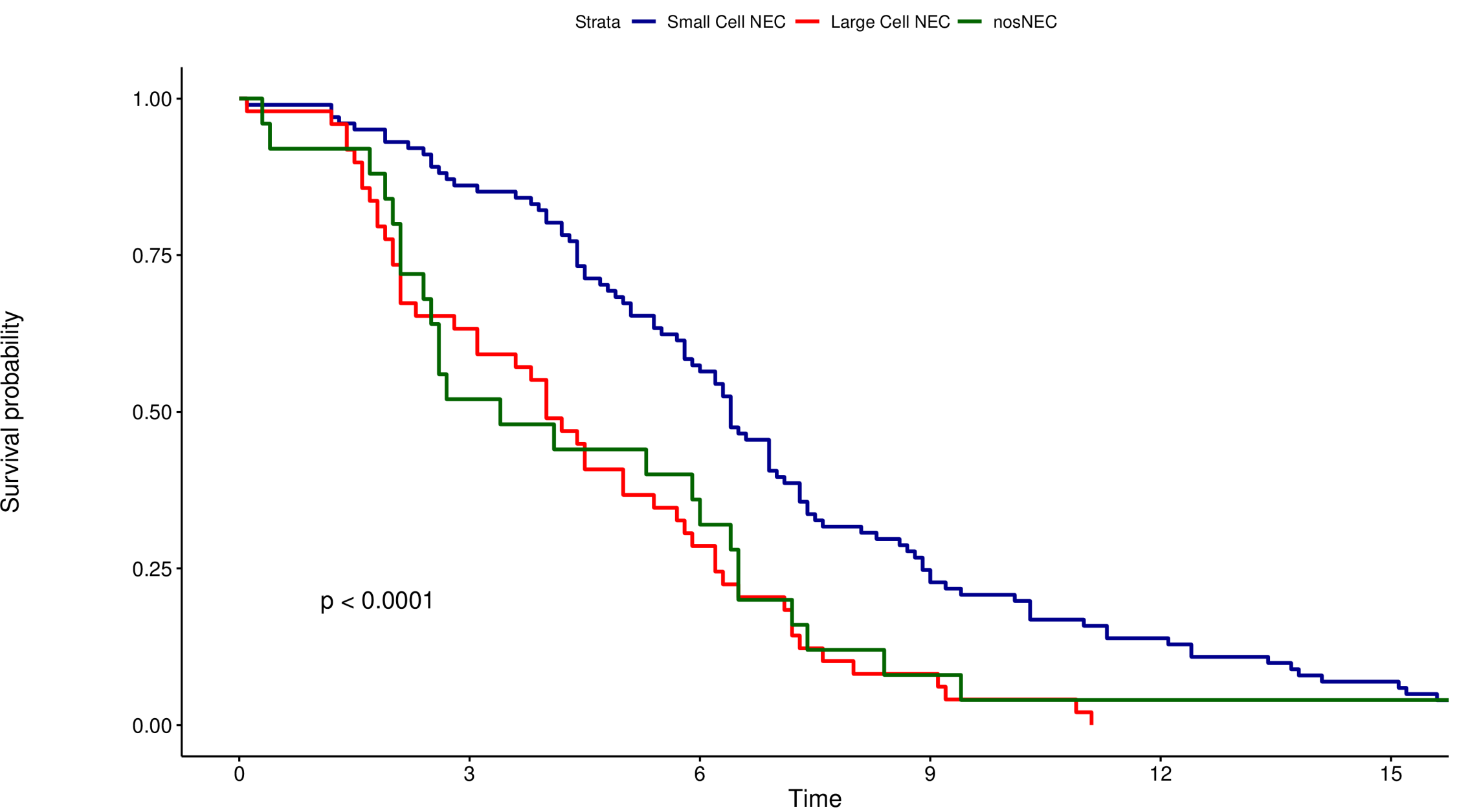


Figure 3C: Kaplan–meier curve of progression–free survival (months) under platinum–etoposide according

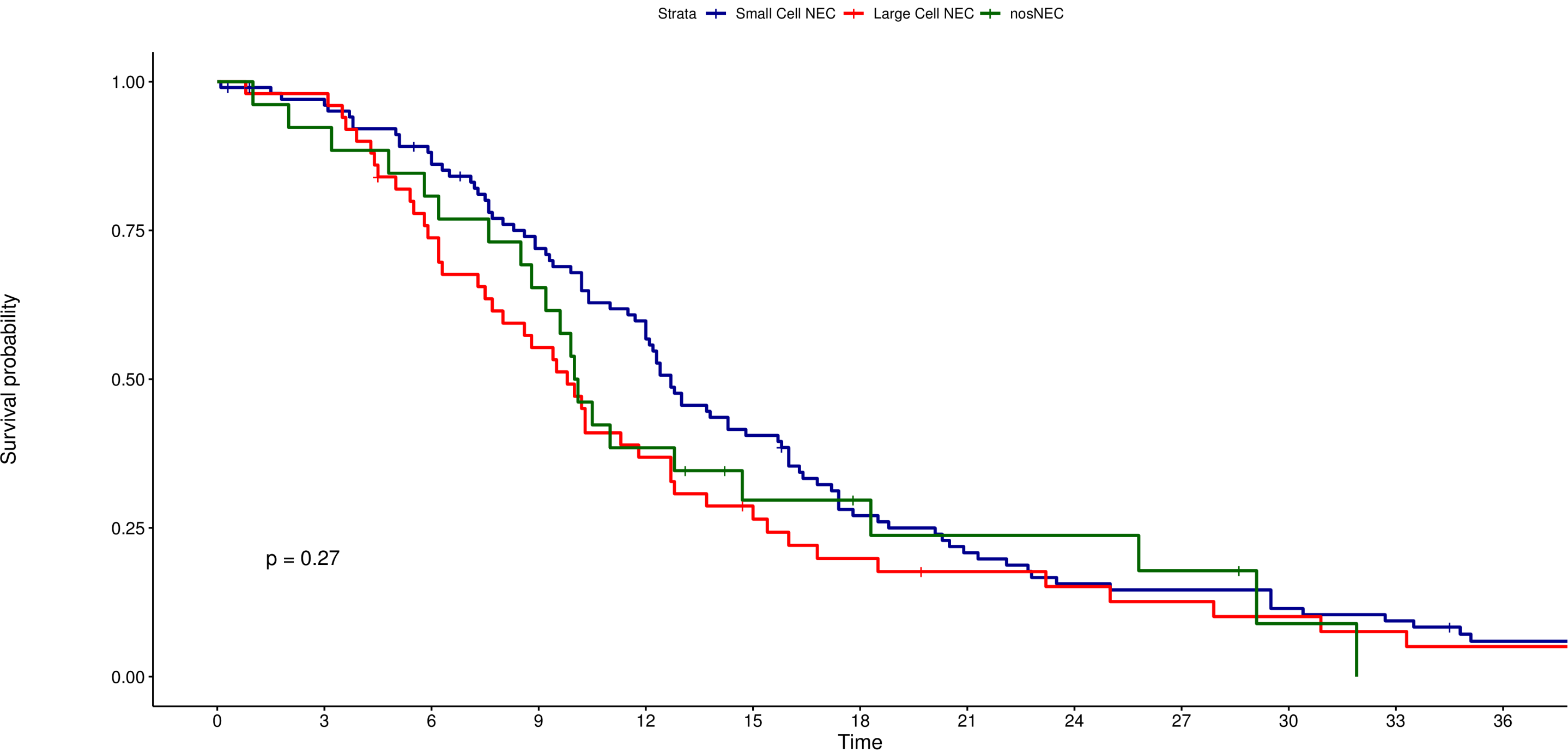


Number at risk

Strata	0	3	6	9	12	15
Small Cell NEC	101	87	58	25	14	7
Large Cell NEC	49	31	14	4	0	0
nosNEC	25	13	9	2	1	1

Time

Figure 3D: Kaplan–meier curve of overall survival (months) according to NEC subtype



Number at risk

Strata	0	3	6	9	12	15	18	21	24	27	30	33	36
Small Cell NEC	103	98	88	71	59	40	26	20	15	14	11	9	5
Large Cell NEC	50	49	36	27	18	13	9	7	6	5	4	3	2
nosNEC	26	24	21	17	10	6	5	4	4	3	1	0	0

Time



Figure 3E: Kaplan–meier curve of progression–free survival (months) under platinum–etoposide according to Rb sta

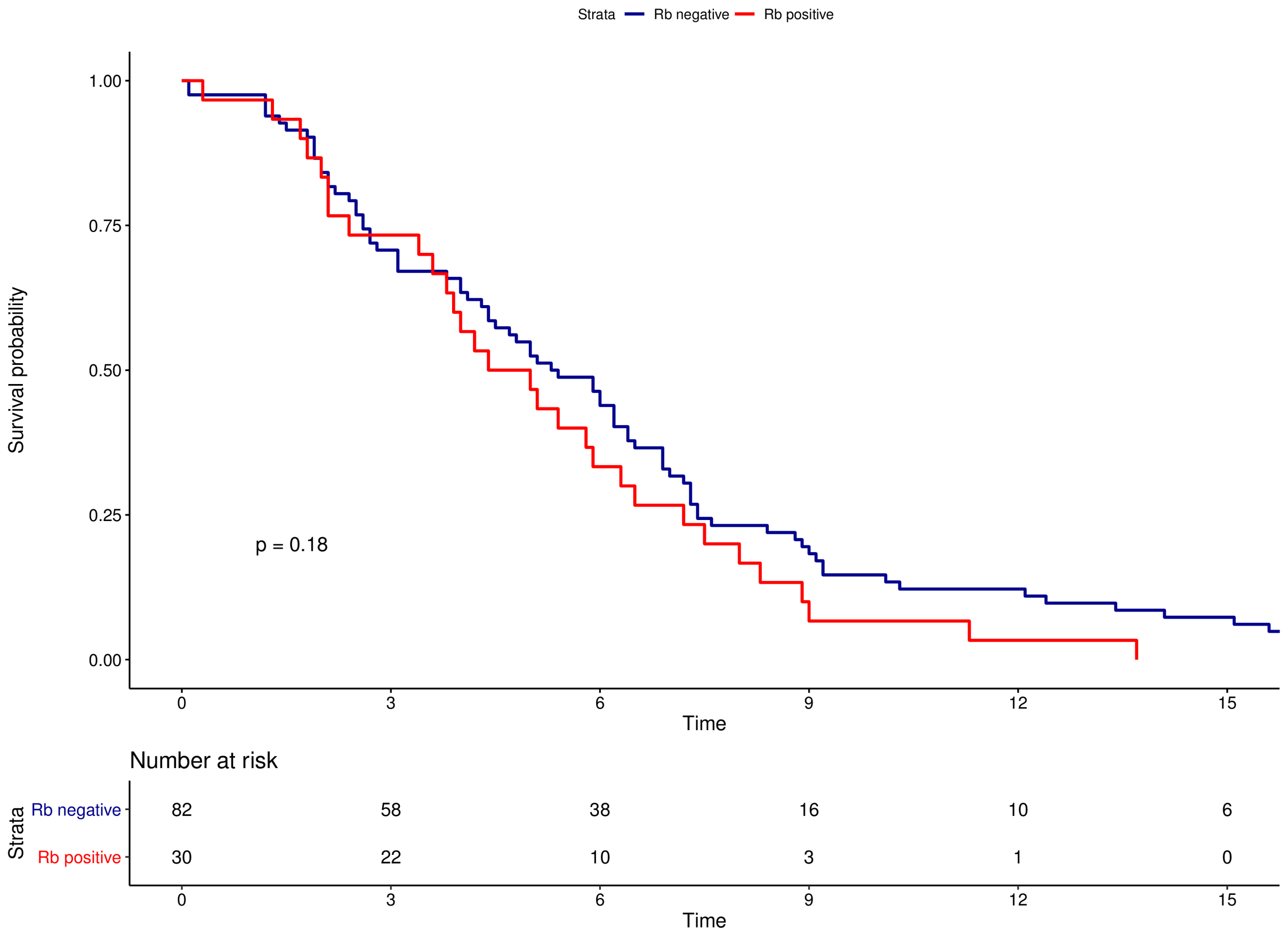


Figure 3F: Kaplan–meier curve of overall survival (months) according to Rb status

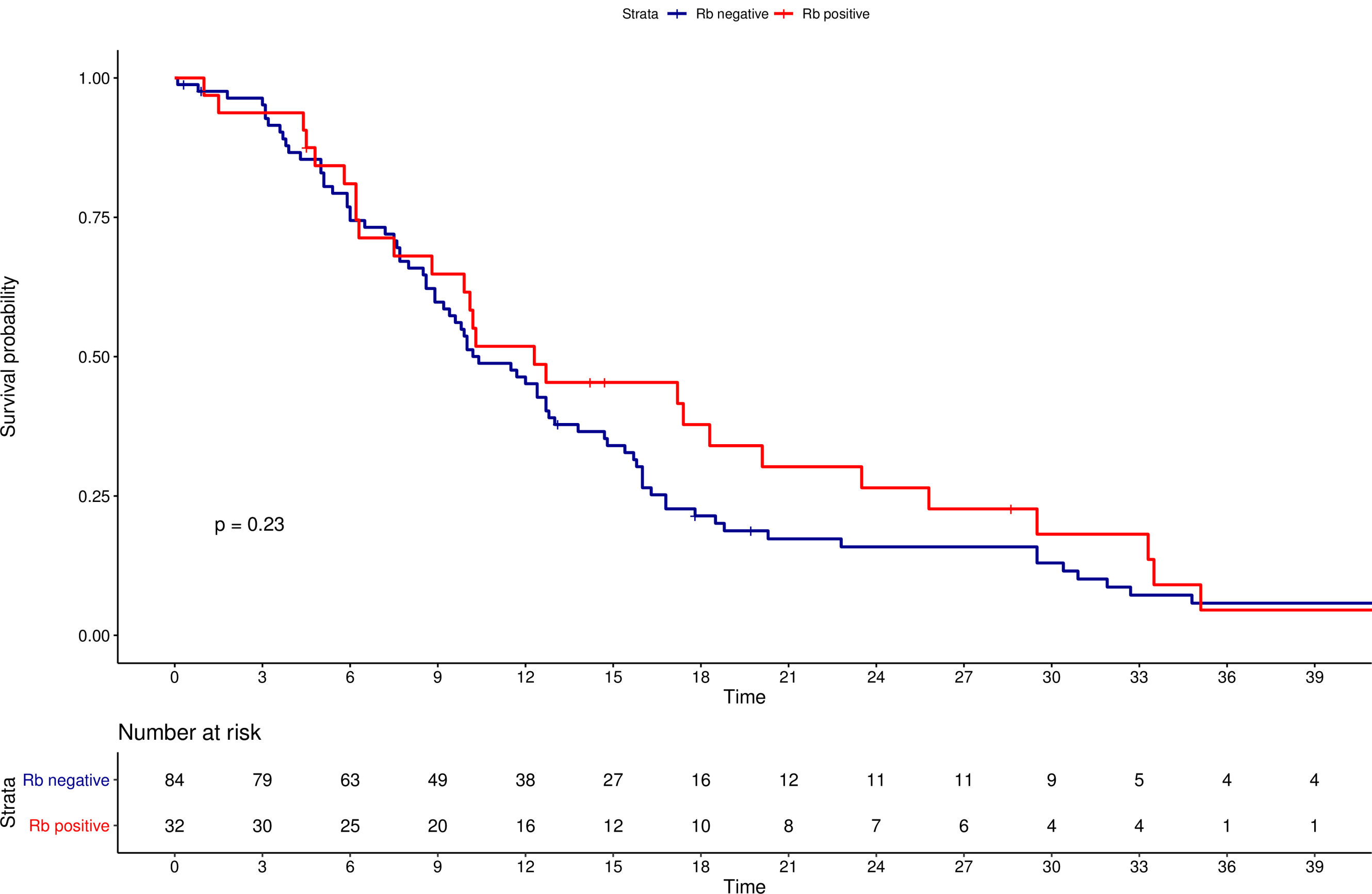
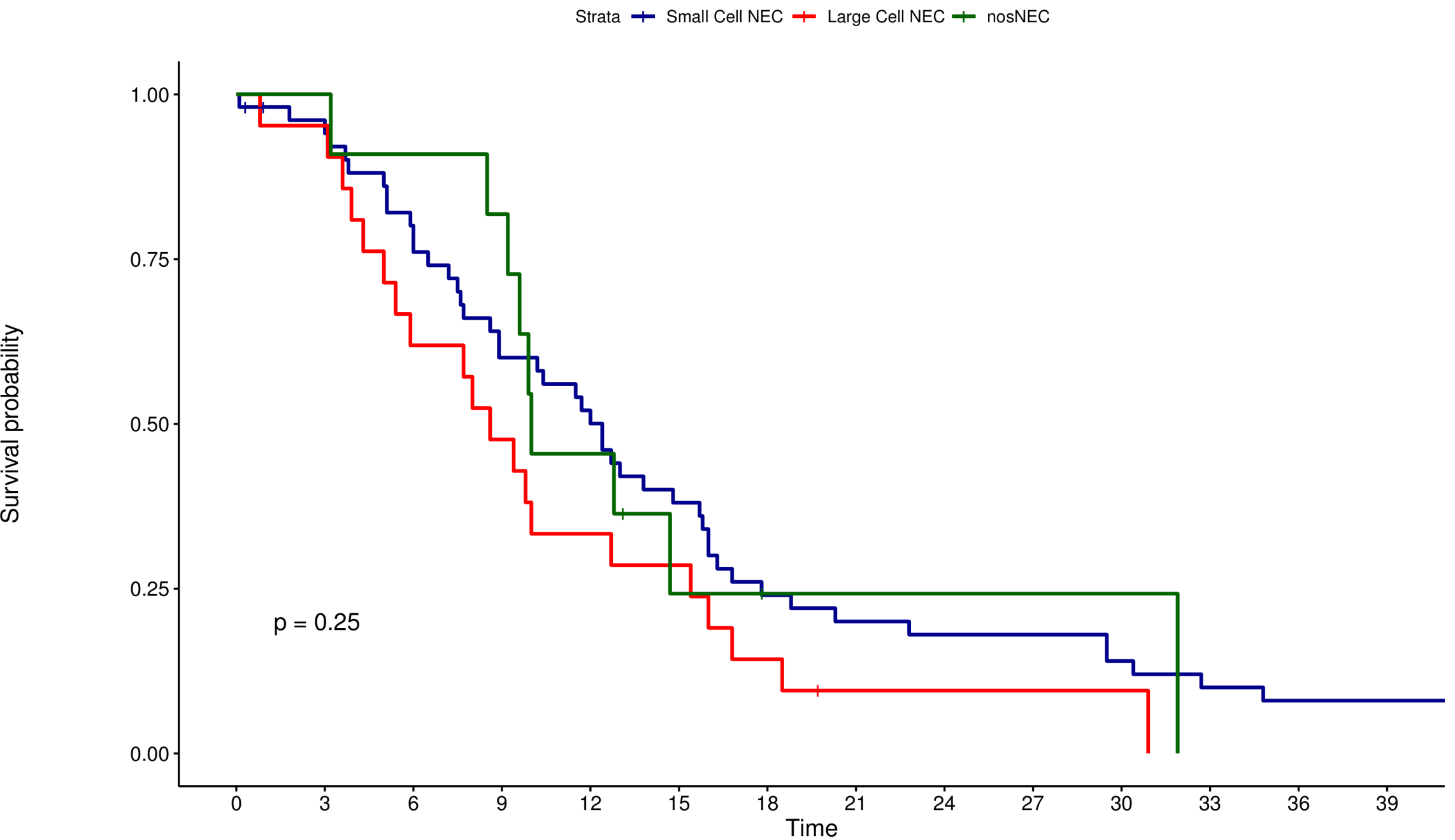


Figure 3G: Kaplan–meier curve of overall survival (months) according to pathological subtype in patients w

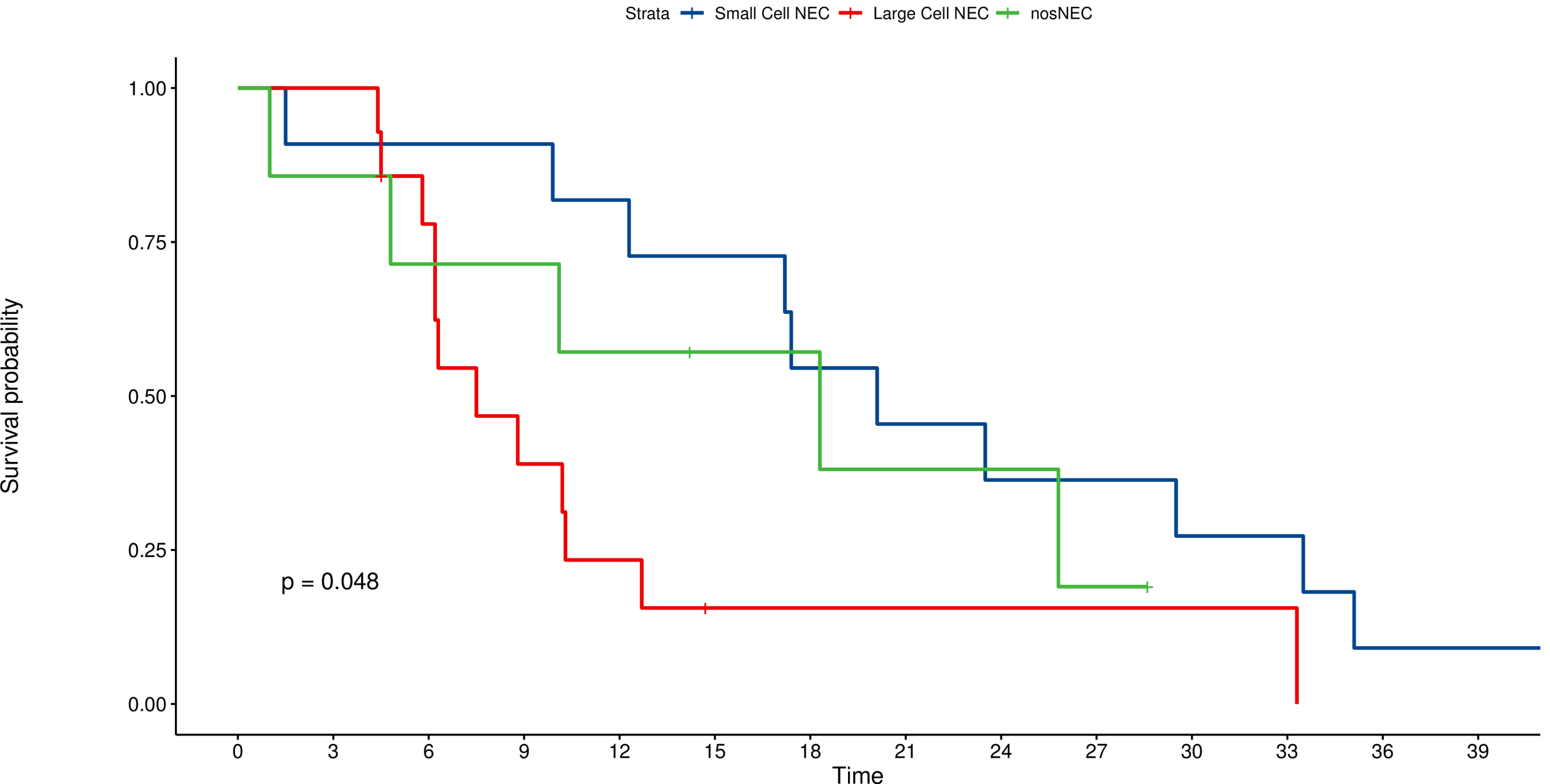


Number at risk

Strata	0	3	6	9	12	15	18	21	24	27	30	33	36	39
Small Cell NEC	52	48	40	30	26	19	12	10	9	9	7	5	4	4
Large Cell NEC	21	20	13	10	7	6	3	1	1	1	1	0	0	0
nosNEC	11	11	10	9	5	2	1	1	1	1	1	0	0	0

Time

Figure 3H: Kaplan-meier curve of overall survival (months) according to pathological subtype in patients with Rb-po



Number at risk

Strata	0	3	6	9	12	15	18	21	24	27	30	33	36	39
Small Cell NEC	11	10	10	10	9	8	6	5	4	4	3	3	1	1
Large Cell NEC	14	14	10	5	3	1	1	1	1	1	1	1	0	0
nosNEC	7	6	5	5	4	3	3	2	2	1	0	0	0	0

Time

**Table 1: Clinical characteristics**

Variables	N	Overall N = 185	Primary				p-value <sup>2</sup>
			GEP N = 71	Thorax N = 69	Unknown N = 28	Others N = 17 <sup>1</sup>	
<b>sexe</b>	185						0.025
F		<b>68 (37%)</b>	23 (32%)	23 (33%)	10 (36%)	12 (71%)	
M		<b>117 (63%)</b>	48 (68%)	46 (67%)	18 (64%)	5 (29%)	
<b>Age</b>	185						0.4
Mean (SD)		<b>61.8 (12.2)</b>	62.03 (11.96)	63.08 (11.29)	61.67 (12.90)	55.94 (15.00)	
Median (Range)		<b>63.0 (21.3, 87.9)</b>	63.00 (31.60, 87.90)	63.60 (24.20, 83.30)	64.45 (31.20, 80.80)	53.80 (21.30, 77.80)	
<b>ECOG</b>	115						0.2
0		<b>34 (30%)</b>	14 (27%)	8 (27%)	6 (29%)	6 (46%)	
1		<b>49 (43%)</b>	27 (53%)	9 (30%)	8 (38%)	5 (38%)	
2-3		<b>32 (28%)</b>	10 (20%)	13 (43%)	7 (33%)	2 (15%)	
<b>Number of metastatic sites</b>	185						0.011
1		<b>66 (36%)</b>	31 (44%)	24 (35%)	8 (29%)	3 (18%)	
2		<b>67 (36%)</b>	27 (38%)	25 (36%)	5 (18%)	10 (59%)	
3 and more		<b>52 (28%)</b>	13 (18%)	20 (29%)	15 (54%)	4 (24%)	
<b>Metastatic sites</b>	185						
Liver		<b>107 (58%)</b>	50 (70%)	33 (48%)	17 (61%)	7 (41%)	0.024
Bone		<b>57 (31%)</b>	10 (14%)	30 (43%)	12 (43%)	5 (29%)	<0.001
Lymph node		<b>89 (48%)</b>	39 (55%)	24 (35%)	15 (54%)	11 (65%)	0.038
Lung		<b>43 (23%)</b>	8 (11%)	19 (28%)	10 (36%)	6 (35%)	0.010
Peritoneum		<b>22 (12%)</b>	10 (14%)	3 (4.3%)	5 (18%)	4 (24%)	0.036
Brain		<b>24 (13%)</b>	2 (2.8%)	18 (26%)	3 (11%)	1 (5.9%)	<0.001
Skin		<b>6 (3.2%)</b>	1 (1.4%)	2 (2.9%)	2 (7.1%)	1 (5.9%)	0.3
Adrenal		<b>13 (7.0%)</b>	1 (1.4%)	10 (14%)	2 (7.1%)	0 (0%)	0.014
Other		<b>14 (7.6%)</b>	4 (5.6%)	1 (1.4%)	6 (21%)	3 (18%)	0.002
<b>NEC score</b>	71						0.7
A		<b>46 (65%)</b>	21 (66%)	14 (64%)	6 (55%)	5 (83%)	
B		<b>25 (35%)</b>	11 (34%)	8 (36%)	5 (45%)	1 (17%)	

<sup>1</sup> include 10 gynecologic, 4 head & neck and 3 prostate NEC

<sup>2</sup> Statistical tests performed: chi-square test of independence; Kruskal-Wallis test; Fisher's exact test

**Table 1 legend:**

ECOG PS = ECOG performance status, SD = standard deviation, GEP = gastroenteropancreatic

**Table 2: Pathological characteristics of the whole cohort, whatever the stage**

Variable	N evaluable	Overall N = 185	NEC subtype			p-value <sup>2</sup>
			SCNEC N = 108	LCNEC N = 50	nosNEC N = 27 <sup>1</sup>	
<b>Primary</b>	185					
GEP		71 (38%)	25 (23%)	25 (50%)	21 (78%)	p<0,001
Lung		69 (37%)	53 (49%)	13 (26%)	3 (11%)	
Unknown		28 (15%)	14 (13%)	11 (22%)	3 (11%)	
Gynecologic		10 (5.4%)	9 (8.3%)	1 (2.0%)	0 (0%)	
Head&Neck		4 (2.2%)	4 (3.7%)	0 (0%)	0 (0%)	
Prostate		3 (1.6%)	3 (2.8%)	0 (0%)	0 (0%)	
<b>Ki67</b>	148					
Mean (SD)		74.92 (18.37)	79.99 (16.59)	70.70 (18.37)	62.45 (18.18)	
<b>Ki67 cut-off</b>	148					0.021
Ki67 > 55%		124 (84%)	77 (91%)	33 (77%)	14 (70%)	
Ki67 < 55%		24 (16%)	8 (9.4%)	10 (23%)	6 (30%)	

<sup>1</sup>Not typeable

<sup>2</sup>Statistical tests performed: Kruskal-Wallis test; Fisher's exact test

**Table 2 legend:**

GEP = gastroenteropancreatic, SCNEC = small cell neuroendocrine carcinoma (NEC), LCNEC = Large cell NEC, nosNEC = not otherwise specified NEC, SD = Standard deviation, IHC= immunohistochemistry, Rb = retinoblastoma protein.

**Table 3: Rb status and correlations with clinical status**

Variables	N evaluable	Total	Rb status		p- value <sup>1</sup>
			Negative N = 90	Positive N = 32	
<b>Clinical Characteristics</b>					
<b>NEC PRIMARY SITE</b>	122				0.42
<b>GEP</b>		54	41 (76%)	13 (24%)	
Pancreas		20	15 (75%)	5 (25%)	
Colon		13	10 (77%)	3 (23%)	
Rectum		8	7 (88%)	1 (12%)	
Esophagus		7	6 (86%)	1 (14%)	
Stomach		3	1 (33%)	2 (67%)	
Biliary tract		2	2 (100%)	0 (0%)	
Duodenum		1	0 (0%)	1 (100%)	
<b>Lung</b>		31	25 (81%)	6 (19%)	
<b>Unknown</b>		26	17 (65%)	9 (35%)	
<b>Gynecologic</b>		5	3 (60%)	2 (40%)	
Cervix		3	1 (33%)	2 (67%)	
Uterus		2	2 (100%)	0 (0%)	
<b>Head &amp; Neck</b>		4	3 (75%)	1 (25%)	
<b>Prostate</b>		2	1 (50%)	1 (50%)	
<b>Pathological characteristics</b>					
<b>PATHOLOGY SUBTYPE</b>	122				0.016
Small Cell NEC	68	57 (84%)	11 (16%)		
Large Cell NEC	35	21 (60%)	14 (40%)		
nosNEC	19	12 (63%)	7 (37%)		
<b>Ki67 (%)</b>	110				0.7
Mean (SD)		75.5 (17.8)	75.5 (18.7)	75.67 (15.8)	
<b>Ki67 CUT-OFF</b>	110				>0.9
Ki67 > 55%		94	68 (72%)	26 (28%)	
Ki67 < 55%		16	12 (75%)	4 (25%)	
<b>First-line treatment with platinum etoposide results</b>					
<b>RECIST 1.1 reponse</b>	99				0.23
CR+PR		57 (58%)	44 (62%)	13 (46%)	

	SD		25 (25%)	15 (21%)	10 (36%)	
	PD		17 (17%)	12 (17%)	5 (18%)	
<b>MEDIAN PFS (months, 95% CI)</b>		112				0.18 <sup>2</sup>
	SCNEC	61	6.4 (5, 7.5)	6.3 (4.8-7.4)	7.5 (4.4-NE)	
	LCNEC	34	4.1 (3.1, 5.8)	4.4 (3.1- 7.2)	3.8 (2.1-NE)	
	nosNEC	17	4.1 (2.6, 6.5)	4.1 (2.6-NE)	4.7 (2.4 -NE)	
<b>MEDIAN OS (months, 95% CI)</b>		116				0.23
	SCNEC	63	13 (12-16)	12 (8.9-16)	20 (17-NE)	
	LCNEC	35	9.8 (7.7-13)	8.6 (5.4-16)	7.5 (6.2-NE)	
	nosNEC	18	10 (8.8-29)	10 (9.6-NE)	18 (4.8-NE)	

<sup>1</sup>Statistical tests performed: Fisher's exact test; Wilcoxon rank-sum test

<sup>2</sup> Log-rank test

**Table 3 legend:**

Rb = retinoblastoma protein, GEP = gastroenteropancreatic, SCNEC = small cell neuroendocrine carcinoma (NEC), LCNEC = Large cell NEC, nosNEC = not otherwise specified NEC, SD = Standard deviation, PFS = progression-free survival, CI= confidence interval, OS = overall survival, CR= Complete Response, PR = Partial response, SD = Stable Disease, PD = Progressive Disease