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Association of AXL and PD-L1 Expression with Clinical Outcomes in Patients with Advanced Renal Cell Carcinoma Treated with PD-1 Blockade

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1 **Association of AXL and PD-L1 expression with**
2 **clinical outcomes in Patients with**
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5

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95 **Translational relance:**

96 This is the first study to demonstrate in a sizable cohort of cases that high tumor AXL
97 expression may affect outcomes of patients with advanced clear-cell renal cell
98 carcinoma treated with anti-PD1 therapy. This study also reveals AXL expression is
99 associated with tumor PD-L1 expression and that co-occurrence of PD-L1 positivity
100 and high AXL expression coincides with reduced overall survival in patients treated
101 with PD-1 blockade. This finding may aid in the selection of treatment for advanced
102 RCC patients.
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Abstract

A minority of patients currently respond to single agent immune checkpoint blockade (ICB) and strategies to increase response rates are urgently needed. AXL is receptor tyrosine kinase commonly associated with drug-resistance and poor prognosis in many cancer types including in clear-cell renal cell carcinoma (ccRCC). Recent experimental cues in breast, pancreatic and lung cancer models have linked AXL with immune suppression and resistance to antitumor immunity. However, its role in intrinsic and acquired resistance to ICB remains largely unexplored. In this study, tumoral expression of AXL was examined in ccRCC specimens from 316 metastatic patients receiving PD-1 inhibitor, nivolumab, in the GETUG AFU 26 NIVOREN trial after failure of anti-angiogenic therapy. We assessed associations between AXL and patient outcomes following PD-1 blockade, as well as the relationship with various markers including PD-L1, VEGFA, the immune markers CD3, CD8, CD163, CD20, and the mutational status of the tumor suppressor gene *VHL*. Our results show that high AXL expression levels in tumor cells is associated with lower response rates and a trend to shorter progression-free survival following anti-PD-1 treatment. AXL expression was strongly associated with tumor PD-L1 expression, especially in tumors with *VHL* inactivation. Moreover, patients with tumors displaying concomitant PD-L1 expression and high AXL expression had the worst overall survival. Our findings propose AXL as candidate factor of resistance to PD-1 blockade, and provide compelling support for screening both AXL and PD-L1 expression in the management of advanced ccRCC.

INTRODUCTION

135
136
137 Kidney cancer is responsible for about 175000 deaths each year [1]. The most
138 common type of kidney cancer is clear-cell renal cell carcinoma (ccRCC) accounting
139 for 70%-80% of cases. Treatment with immune checkpoint blockade (ICB) has
140 resulted in striking clinical benefits and changes in the management of patients with
141 advanced ccRCC since anti-PD1 (nivolumab) therapy has been approved as second-
142 line treatment for recurrent metastatic diseases, as well as the anti-PD-1 (nivolumab)
143 plus anti-CTLA-4 (ipilimumab) combination approved as first-line treatment for
144 patients with intermediate and high risk metastatic RCC followed by the combination
145 of PD-1 inhibitor with VEGFR TKIs in any risk group in front line [2–5]. Cell surface
146 receptor PD-1 is especially expressed by T lymphocytes, which can prevent T cells
147 from killing tumor target cells upon binding to its ligands PD-L1 and PD-L2,
148 dampening the immune response. Therefore, PD-1 blockade using specific
149 neutralizing antibodies is able to inhibit interaction of PD-1 with its ligands, which may
150 at least partially restore their activity and boost antitumor T-cell immunity.
151 Nevertheless, only a small proportion of patients benefits from these treatments as
152 shown by the low response rate (RR) on one hand, and high rates of disease
153 progression on the other hand, even in initial responders. This illustrates primary and
154 acquired resistances to PD-1 blockade. In an era of progress where anti-PD-1-based
155 combination approaches are expected to increase RR and prolong treatment efficacy
156 [6,7], it is urgent to better define the underlying biology of resistance and response to
157 PD-1 blockade. Thus, new biomarkers and targets that can be easily and routinely
158 inspected in pathological sections might facilitate treatment decisions and give new
159 therapeutic options for metastatic ccRCC (m-ccRCC).
160 Notably, ccRCC has specific features: 1) In contrast to most tumor types, ccRCC can

161 be highly infiltrated by CD8+ T cells, but this infiltration is often associated with poor
162 prognosis [8]; 2) RCC has relatively low mutational load and this parameter does not
163 appear to be a good predictor of response to PD-1 blockade [5]; 3) controversies also
164 exist regarding the utility of PD-L1 expression to predict response. Indeed, PD-L1
165 expression is associated with poor prognosis in RCC, but its value to predict
166 response to PD-1 blockade is still a matter of debate [5,9,10]. Furthermore, genetic
167 alterations in *PBRM1* and *BAP1* genes as well as focal loss in chromosome
168 10q23.31 have demonstrated interesting properties to estimate patients who are
169 likely to benefit from ICB [11,12]. The *von Hippel-Lindau (VHL)* tumor suppressor
170 gene is also altered in a majority of RCC cases. Defects in *VHL* gene include
171 germline or somatic mutations, loss of heterozygosity through chromosomal loss of
172 3p and promoter hypermethylation [13]. It is often considered that two “hits” must
173 occur to inactivate *VHL*. Thus, a combination of these alterations can cause bi-allelic
174 inactivation of *VHL* in 60-90% of RCC cases leading to a complete loss-of-function
175 (LOF) of *VHL* gene in some cases, whereas in other cases, such function likely
176 remains intact. *VHL* LOF in tumor cells results in chronic accumulation of hypoxia-
177 inducible factors (HIFs) in the cells entering a pseudohypoxic state likely contributing
178 to tumorigenesis, angiogenesis and changes in the tumor microenvironment (TME),
179 in particular *via* induction of the HIF-target gene, *VEGFA*. Recent studies reported
180 clinical benefits for combination of VEGFR TKIs with ICB [5]. However, the impact of
181 the VHL/HIF/VEGF pathway, beyond anti-angiogenic therapy, in regulating refractory
182 responses to ICB remains unclear.

183
184 AXL is member of the TAM (TYRO3-AXL-MER) family of receptor tyrosine kinases,
185 which can regulate tumor cell survival, proliferation, migration, angiogenesis, and
186 interactions with TME [14]. AXL has been associated with drug-resistance and poor

187 prognosis in many cancer types including in RCC [14]. Notably, AXL expression In
188 human RCC has been associated with high risk of relapse after surgery [15], worse
189 clinical outcome in metastatic patients treated with anti-angiogenic agents, as well as
190 epithelial-mesenchymal transition (EMT) features and fibrosis [16]. Recent evidence
191 in breast, pancreatic and lung cancer models have also linked AXL with immune
192 suppression and resistance to antitumor immunity [17–20]. Yet, the role of AXL in
193 resistance to anti-PD1 therapy remains unknown.

194 In this study, we aimed to elucidate the potential impact of AXL expression on clinical
195 outcomes of patients with advanced RCC following anti-PD1 treatment.

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Materials and Methods

200 Patients and samples

201 The renal cancer samples have been collected as part of the NIVOREN GETUG-AFU
202 26 phase II trial assessing the activity and safety of nivolumab (anti-PD1) in 729
203 patients with m-ccRCC who failed vascular endothelial growth factor–directed
204 therapies (ClinicalTrials.gov identifier: NCT03013335). This trial was conducted by
205 UNICANCER, approved by the human research ethics committee of UNICANCER
206 and conducted in accordance with guidelines from the International Conference on
207 Harmonization and the Declaration of Helsinki. Patients were enrolled across 27
208 institutions between February 12, 2016 and July 27, 2017. Safety activity data was
209 reported previously [21]. The follow-up was censored on January 2019.

210 As part of a biomarker cohort, 324 archived formalin-fixed paraffin-embedded (FFPE)
211 tumor tissue specimens were retrospectively analyzed by IHC for immune cell
212 profiling (CD3, CD8, PD-L1, CD20, CD163, VEGFA, and AXL). Specimens were
213 mostly obtained prior anti-angiogenic therapy as 1st or 2nd line regimens (comprising

214 Sunitinib (83.3%), Axitinib (27.5%), Pazopanib (23.8%), Sorafenib (6.5%),
215 Bevacizumab (3.4%), Cabozantinib (0.3%)), or mTOR inhibition (Everolimus 11%).
216 Cases with paraffin block showing less than 40% of tumor or more than 40% of
217 necrosis, or corresponding to another histological subtype (TFE3 translocation RCC,
218 chromophobe RCC or papillary RCC) were excluded.

219

220 **Alterations in *VHL* gene**

221 *VHL* inactivation status was determined by PCR, FISH and promoter methylation
222 analysis. Evaluation of *VHL* point mutations was evaluated by gene sequencing [22].
223 The three exons and exon–intron junctions of the *VHL* gene were sequenced on
224 DNA extracted from tumor samples, as described previously [23]. Search for deletion
225 of the *VHL* genomic region was performed by FISH using ZytoLight® SPEC
226 *VHL/CEN3* Dual Color Probe (Zytovision, Germany) reviewed by FD. Promoter
227 hypermethylation of *VHL* was assessed by methylation-specific multiplex ligation-
228 dependent probe amplification as described [23]. Presumably two hits are needed for
229 *VHL* inactivation to occur. Therefore, tumors were classified in two groups. When
230 biallelic alteration was evidenced, the case was classified in a group with *VHL*
231 inactivation, also referred to as *VHL*^{-/-}. Another group consisted of non-inactivated
232 cases, with no alteration or one allelic alteration, referred to as *VHL* ^{+/+}; ^{+/-}. A few
233 cases were excluded from this analysis when biallelic or monoallelic alterations could
234 not be ascertained because of non-interpretable results, or when the mutation
235 detected was simply defined as possibly damaging with no clear loss of *VHL* function.

236

237 **mRNA extraction and analysis of RNA-Seq datasets from RCC patients**

238 RNA-seq data were available for 83 FFPE cases of the Nivoren cohort [24]. Briefly,
239 archived FFPE samples were retrieved before anti-PD-1 treatment, and upon review
240 of H&E slides, the tissue blocks with the highest tumoral content were selected for
241 further processing. Blank slides were produced with 5 μm thickness and
242 macrodissected to only include tumoral tissue, using a total surface area of 50-1800
243 mm^2 . RNA was extracted using the Maxwell RSC RNA FFPE kit (Promega)
244 according to the manufacturer's instructions. Subsequently cDNA libraries were
245 prepared using the Forward QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina
246 (Lexogen) according to the manufacturer's instructions with 5 μL of RNA as input and
247 16 PCR cycles. cDNA concentrations and fragment length were measured with the
248 QubitTM dsDNA HS assay (Thermofisher) and Bioanalyzer HS DNA electrophoresis
249 (Agilent). Illumina cBOT was used for clonal cluster generation and RNAseq was
250 performed using the HiSeq 4000 kit (Illumina) according to the manufacturer's
251 instructions. For subsequent survival analysis, samples were classified as AXL high
252 (defined by *AXL* mRNA expression \geq highest 1/3) or AXL Low (defined by *AXL*
253 mRNA expression \leq lowest 2/3) in the dataset. Progression Free Survival (PFS) was
254 estimated using the Kaplan-Meier method to explore the impact of AXL the different
255 subsets. For association studies, *VHL* status was determined as above. AXL
256 immunostaining also served to classify tumors as AXL^{high} or AXL^{neg/low}, and in this
257 case, RNA-seq data was interrogated to evaluate changes in the expression of
258 known gene expression signatures [25–27].

259 CheckMate RNA-Seq data, clinical, and genomic information were interrogated from
260 the Braun et al. study [11] for 181 advanced ccRCC treated with anti-PD1 in the
261 CheckMate 009, 010 and CheckMate 025. The CheckMate cohorts consist mostly of
262 previously treated anti-angiogenic-refractory patients. Experimental details including

263 whole-exome and RNA sequencing analyses have been reported previously [11].
264 Among the samples with both RNA and genomic information, 93% exhibited loss of
265 chromosome 3p carrying *VHL* gene, and 64% were reported with a point mutation in
266 *VHL*. Cases with at least one WT copy of *VHL* were considered as non-inactivated
267 *VHL* cases, whereas cases with at least two alterations were considered as
268 inactivated. Samples were classified as above with *AXL*^{high} (defined by *AXL* mRNA
269 expression \geq highest 1/3) and *AXL*^{low} cases (defined by *AXL* mRNA expression \leq
270 lowest 2/3). PFS and OS estimates using the Kaplan-Meier method were generated
271 to explore the impact of *AXL* in all patients (n=181), *VHL*-inactivated (n=78), or *VHL*-
272 non-inactivated (n=44) subgroups.

273

274 **Immunohistochemistry**

275 All tumor specimens were centrally reviewed by a genitourinary pathologist (NRL).
276 For each case, one representative archived FFPE tissue block was used for IHC.
277 Primary antibodies to stain for PD-L1 (clone 22C3, Dako), CD8 (clone C8/144B,
278 Dako), VEGF (clone SP28, Spring Bioscience Corp), CD163 (clone 10D6 Diagnostic
279 BioSystem), CD20 (clone L26, Agilent), CD3 (polyclonal rabbit antibody, Agilent)
280 were used according to standard protocols. For PD-L1, *AXL*, CD8, and VEGF, slides
281 were independently evaluated by a genitourinary pathologist (JA, NRL) blinded to the
282 patients' treatment outcomes. For *AXL* IHC assessment, epitope retrieval was
283 performed in a citrate buffer pH 7.3 (Diapath) during 30 min in a water bath set to
284 98°C. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide
285 for 10 minutes. Blocking was performed with IHC/ISH Super Blocking (Leica
286 biosystems) for one hour at 37°C. The primary antibody (clone C89E7, Cell Signaling
287 Technology), was incubated overnight. Omission of the primary antibody was used

288 as negative control. A multiplicative H-score (0-300) was calculated as the product of
289 intensity scores and the percentage of stained tumor cells. Representative stainings
290 are shown in **Supplementary Fig. S1**. To note, AXL staining could also be detected
291 in various non-tumor cells, in particular immune cells with morphology evocative of
292 myeloid cells (i.e. monocytes/macrophages/DC) (**Supplementary Fig. S1**). A score
293 of 0-3 was given based on the abundance of AXL⁺ immune cells.

294 Patients were considered with Negative (H-score 0), Low (>0 <=50), or High (>50)
295 AXL tumor expression. H-score > 50 was used as cutoff to discriminate between
296 AXL^{high} and AXL^{neg/low} expression. For PD-L1, VEGF and CD8, stainings were
297 performed using a Discovery XT research instrument (Roche) using standardized
298 protocols. For PD-L1 (anti-PD-L1, clone 22C3, Dako). The % of positive tumor cells
299 was evaluated. % ≥ 1 was used as cutoff to discriminate between PD-L1 TC^{neg} and
300 PD-L1 TC⁺ expression. For assessment of PDL1 staining in TILs, a manual semi
301 quantitative 3-point scale was employed with score 0 (no PDL1⁺ TILs), score 1 (rare
302 and focal PDL1⁺ TILs), score 2 (several foci of PDL1⁺ TILs), score 3 (diffuse and
303 numerous PDL1⁺ TILs). For subsequent analysis, the cutoff ≥ 1 was used to
304 discriminate PD-L1^{neg} and PD-L1⁺ in TILs. CD8 positivity (anti-CD8, clone C8/144B,
305 Dako) in TILs was scored according to the proposal of the International
306 ImmunoOncology Biomarkers Working Group [28], using a manual semi quantitative
307 four point scale : score 0 (no CD8⁺ TILs or very rare CD8⁺ TILs), score 1 (rare
308 diffuse or focal CD8⁺ TILs), score 2 (diffuse numerous CD8⁺ TILs), and score 3
309 (diffuse and numerous CD8⁺ TILs with some aggregates of TILs). Anti-VEGF (clone
310 SP28, Spring Bioscience Corp) was used and the % of positive tumor cells assessed.
311 For CD3 (polyclonal rabbit antibody, Agilent), CD20 (clone L26, Agilent), and CD163
312 (clone 10D6 Diagnostic BioSystem) immunostaining, tissues were deparaffinized and

313 rehydrated by successive baths of Clearene (Leica Biosystems, 3803600E) and
314 ethanol gradient (100%, 90%, 70% and 50%). Antigen retrieval was performed with a
315 PT-link (Dako) at pH 6.1 (EnVision FLEX Target Retrieval Solution, Low pH 50x
316 concentrated Citrate buffer, Dako, K8005) by an incubation of 30 minutes at 97°C. 15
317 minutes of Endogenous Peroxidase block (H₂O₂ 3% Gifrer, 1060351) and 10
318 minutes of Protein Block (Dako, X0909) reagents were used for alkaline phosphatase
319 and FcR blocking. The primary and secondary antibodies were incubated for 30
320 minutes respectively. All these markers were processed on an automated
321 immunostainer (AutostainerPlusLink 48, Dako). Secondary antibodies were revealed
322 by Permanent HRP Green (Zytomed Systems, ZUC070-100) for CD3 (10 minutes),
323 High-Def red IHC chromogen (AP) (Enzo, ADI-950-140-0030) for CD20 (5 minutes)
324 and AEC Substrate Kit, Peroxidase HRP: 3-amino-9-ethylcarbazole (Vector
325 Laboratories, SK-4200) for CD163 (23 minutes). The nuclei were counterstained with
326 hematoxylin (Dako, S3301) during 5-7 minutes and slides were scanned with a
327 Nanozoomer (Hamamatsu) after mounting with Ecomount (Biocare, EM897L). For
328 CD20 and CD3, and glycergel Mounting Medium (Dako, C056330-2) for CD163. The
329 density of positive cells/mm² was quantified in the Tumor Core (TC) by two
330 independent analysts (AB, GL) using Halo10 software (Indica labs).

331

332 **Statistical analysis**

333 Qualitative data are reported by frequency and proportion. The Chi-square and
334 Fisher's exact tests were used to identify associations between biomarkers, status, or
335 groups as categorical variables. Median follow-up is calculated using the reverse
336 Kaplan-Meier method and presented associated with its 95% CI. PFS was defined as
337 the time between treatment initiation and first progression or death, and Overall

338 Survival (OS) was defined as the time between treatment initiation and death or date
339 of final contact for patients alive. OS and PFS were estimated using the Kaplan-Meier
340 method and were described in terms of median or specific time point estimation in
341 each subgroup, along with the associated two-sided 95% CI for the estimates and
342 were compared with the log-rank test. A multivariate cox regression model was used
343 to estimate the hazard ratio and 95% CI and adjusted for the following baseline
344 characteristics: International Metastatic Renal Cell Carcinoma Database Consortium
345 (IMDC) risk groups, age, sex and number of previous systemic therapies. Objective
346 Response Rate (ORR) as per central radiology review using Response Evaluation
347 Criteria in Solid Tumors (RECIST) version 1.1 and previously described [21].
348 Quantitative data are reported by means and range. Non-parametric Mann-Whitney
349 U or Kruskal-Wallis test were applied. A value of $p < 0.05$ was considered statistically
350 significant, and all p values were two-sided. Data analyses were carried out using
351 GraphPad (GraphPad Prism), Excel (Microsoft Corp) and SAS 9.4 (SAS Institute Inc,
352 Cary, NC).

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Results

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368 **AXL expression is associated with worse PFS and resistance to anti-PD-1**

369 **therapy**

370

371 AXL tumor cell staining was detectable in 54.4% of cases (172/316) and AXL
372 expression was considered with high expression (H-score >50) in 23.7% of cases
373 (75/316). Population characteristics of patients with AXL^{high} vs AXL^{neg/low} tumors were
374 comparable in terms of gender, age at diagnosis, metastatic features, radiotherapy,
375 IMDC (International Metastatic RCC Database Consortium), or Eastern Cooperative
376 Oncology Group (ECOG) Performance Status (**Table 1**). Median follow-up was 23.5
377 months (95% CI, 22.6 to 24.0) and median PFS was 4.5 months (95% CI, 2.9 to 5.2).
378 ORR was 25% for the evaluable cases (78/312). In the AXL^{high} group of patients (H-
379 score > 50), median PFS was shorter (2.8 months (95% CI, 2.5 to 4.8)) than in the
380 AXL^{neg/low} group (4.6 months (95% CI, 3.5 to 5.3); p=0.11). Hazard ratio (HR) for
381 disease progression was 1.26 (95%CI, 0.95 to 1.68), and 12-month PFS rates were
382 21.3% (12.9-31.2) for AXL^{high} compared to 28.6% (23.0-34.4) for the AXL^{neg/low} group
383 (**Fig. 1A**). On multivariate analysis adjusting for age, sex, IMDC and number of
384 previous therapy lines (2 vs >2), high AXL expression was associated with a trend
385 toward reduced PFS (HR = 1.28 (95%CI, 0.96 to 1.71), p=0.098 (**Supplementary**
386 **Table S1**). The ORR in patients with AXL^{high} tumors was 15.5% (11/71) vs 27.8%
387 (65/234) in patients with AXL^{neg/low} tumors, indicating an inverse association between

388 AXL expression and response to anti-PD-1 (**Fig. 1A**, $p=0.036$). For OS, univariate
389 and multivariate analyses indicated no statistical difference (**Fig. 1A** and
390 **Supplementary Table S2**). Additional analysis showed that patients whose tumors
391 exhibited AXL^{low} or AXL^{neg} profiles had similar clinical outcomes and characteristics
392 (**Supplementary Fig. S2A** and **Supplementary Table S3**). No association was
393 observed between AXL staining in immune cells and OS, PFS or ORR (not shown).
394 To further explore the link between AXL expression and clinical outcomes, we
395 exploited the RNA-seq data available for a subset of the cases ($n=79$). Despite the
396 small number of cases, we recapitulated our findings. A higher AXL expression in the
397 specimens tended to be associated with reduced 12-month PFS ($p=0.15$) and OS
398 rates ($p=0.63$) (**Fig. 1B**). We then interrogated expression data from the Braun et al.
399 study [11]. This cohort included patients with advanced RCC patients enrolled in
400 CheckMate (CM-009, CM-010 and CM-025 trials) who were treated with an mTOR
401 inhibitor (Everolimus), or nivolumab after progression on at least one prior anti-
402 angiogenic treatment [11]. The potential effect of high AXL expression on survival
403 and ORR was examined in the anti-PD-1 treated arm. High AXL expression was
404 associated with worsened OS under anti-PD-1 (HR for death, 1.46; 95% CI, 1.00 to
405 2.22, $P<0.05$) (**Fig. 1C**). Median OS was 19.8 months in patients with tumors
406 expressing high levels of AXL mRNA vs 35.1 months in cases displaying reduced
407 levels of AXL mRNA. A trend was observed towards shorter PFS under anti-PD-1
408 blockade and lower ORR in tumors with high AXL mRNA expression (19%, 11/58
409 cases) compared to tumors with low AXL expression (24.56%, 28/114), although the
410 differences could not reach statistical significance.

411 Together, these findings suggest that AXL, especially when highly expressed in
412 cancer cells, may be predictor of tumors with reduced response rate and worse
413 progression following PD-1 blockade.

414

415 **AXL expression is associated with increased tumor expression of PD-L1**

416

417 We then explored the TME composition of these tumors by investigating associations
418 between AXL and various markers as assessed by IHC. This included VEGFA, PD-
419 L1 and markers of the most common immune cell populations CD3, CD8 (T cells),
420 CD163 (macrophages) and CD20 (B-cells). PD-L1 tumor cell staining was detected in
421 23.9% (76/317) of evaluable ccRCC cases and PD-L1 on TILs in 58,6% (186/317) of
422 cases. We then stratified cases according to AXL expression and PD-L1 expression
423 status in tumor cells, and tested putative associations between the two markers (**Fig.**
424 **2A**). A significant enrichment of PD-L1 positivity within tumors with AXL^{high} profile
425 was observed (p=0.0031). Moreover, the mean % of PD-L1 positive tumor cells was
426 increased in the AXL^{high} group compared to tumors with AXL^{neg/low} profiles (p=0.0019)
427 (**Fig. 2A** and **Supplementary Fig. S2B**). In contrast, no significant associations were
428 observed between AXL and PD-L1 TIL expression (not shown, p > .99). AXL
429 expression was associated with a greater proportion of tumor cells positive for VEGF
430 (p=0.002) (**Fig. 2B**). We observed a trend toward a higher level of tumor-infiltrating
431 CD8+ T cells in AXL^{high} vs AXL^{neg/low} cases, and no association between AXL and
432 CD3, CD163, CD20 densities in the tumor core (**Figure 2B-C, Supplementary Fig.**
433 **S2B**). RNA-seq data revealed a trend toward greater inflammation (p=0.072),
434 immune suppression (p=0.105), and monocytic lineage (p=0.052) signatures in
435 AXL^{high} tumors (**Fig. 2D**).

436

437

438

439

440 **High AXL combined with PD-L1 expression is associated with poor survival in**
441 **patients treated with PD-1 blockade**

442

443 We set out to determine the extent to which combining AXL and PD-L1 markers
444 would impact on OS under anti-PD-1 treatment (**Fig. 3**). PD-L1 positive staining in
445 tumor cell alone (using cutoff 0;0.5 versus ≥ 1) associated with worse 12-month
446 survival rate (70% (58.6-78.8) versus 77.3 (71.5-82.1)) with HR for death 1.51; log-
447 rank $p = 0.02$ (**Fig. 3A**). No association was found with PFS (HR = 1.10, $p = 0.5$) or
448 the ORR (28.6% in PD-L1⁺ vs 23.9% in PD-L1^{neg} subsets) (**Supplementary Fig.**
449 **S3A**). PD-L1 staining in TILs had limited impact on OS (12-month survival rate 73.3%
450 (66.4-79) vs 78.7 (70.7-84.8)), HR to death 1.34; log-rank $p = 0.09$), no impact on
451 PFS ($p = 0.16$), nor on the ORR (25.1% in PD-L1⁺ vs 25% in PD-L1^{neg}) (**Fig. 3B** and
452 **Supplementary Fig. S3B**).

453 The combination of the two markers AXL and PD-L1 on tumor cells had significant
454 impact on OS since the group of patients whose tumors exhibited both high levels of
455 AXL and PD-L1 tumor cell positivity had the lowest survival rate at 12 months 60.7%
456 (40.4-76.0%), HR to death 1.98 (1.17-3.35), Logrank $p = 0.046$, compared to the
457 three other groups (AXL^{neg/low}/PD-L1⁺ ; AXL^{neg/low}/PD-L1^{neg} ; AXL^{high}/PD-L1^{neg}) (**Fig.**
458 **3A**). On multivariate analysis adjusting for age, sex, IMDC risk groups and number of
459 previous therapy lines (≤ 2 vs > 2), high AXL combined with PD-L1 expression was
460 associated with a trend to reduced OS (HR = 2.01 (95%CI, 1.18-3.44), $p=0.084$

461 **(Supplementary Table S5)**). We further interrogated the prognostic value of
462 combining AXL and PD-L1 TC markers specifically in patients with IMDC
463 intermediate-risk/poor-risk RCC. In this setting, a AXL^{high}/PD-L1⁺ TC profile was
464 associated with the lowest overall survival rate at 12 months 56.0% (34.8-72.7%) and
465 HR to death 2.05 (1.21-3.50), p=0.053 **(Supplementary Fig. S4)**.

466 There was no significant difference between ORR across the different groups
467 (p=0.123), and AXL/PD-L1 TC combination had little if no further influence on PFS
468 compared to AXL alone **(supplementary Fig. S3A)**. However, it is noteworthy that
469 the combination of high AXL expression with PD-L1 staining in TILs displayed
470 notable features, with patients in the AXL high/PD-L1⁺ TIL group showing the worst
471 clinical outcomes particularly in terms of ORR (12%, p=0.04) and PFS, with relatively
472 low PFS rate at 12 months (15.9% (7.0-28.1%), p=0.14) **(Supplementary Fig. S3B)**.

473 We examined the repartition of the different markers in each subgroup, namely, PD-
474 L1 tumor, PD-L1 TIL, VEGF, CD3, CD8, CD163 and CD20. Patients whose tumors
475 were positive for PD-L1 had increased immune cell infiltration. Hence, both AXL^{high}
476 and AXL^{neg/low} cases within the PD-L1⁺ subset demonstrated high immune cell
477 infiltration compared to PD-L1^{neg} groups **(Fig. 4A-B)**.

478 These data indicate that AXL and PD-L1 staining can identify a particular group of
479 patients with poor outcomes when receiving anti-PD-1 treatment.

480

481 **VHL status does not interfere with clinical outcomes or AXL expression in**
482 **advanced ccRCC treated with Nivolumab**

483

484 Since *VHL* is the most common genetic alteration in RCC, we characterized *VHL*
485 alterations in the ccRCC cases of the NIVOREN cohort. Point mutations were found

486 in 76.7% (231/301), LOH in 84.1% (254/302), and promoter hypermethylation in
487 19.1% (27/141) of cases. *VHL* status could be ascertained in 260 cases. 74% of
488 cases (193/260) were found to have a biallelic alteration of *VHL* which supposes LOF
489 of the gene. Among the remaining cases, 7.7% (20/260) presented with no *VHL*
490 alteration (+/+), and 18% (47/260) had one *VHL* alteration (+/-) implying one
491 remaining functional copy. No significant differences in ORR (chi-square $p=0.40$),
492 PFS (HR 1.00 (0.73-1.37), $p=0.98$) or OS (0.92 (0.61-1.38), $p=0.68$) were observed
493 between patients with biallelic *VHL* inactivation (*VHL*^{neg/neg}) and those presenting with
494 at least one unaltered *VHL* copy (*VHL*^{+/+} and *VHL*^{+/-})(**Supplementary Fig. S5A-B**).
495 AXL expression was homogeneously distributed across cases with or without biallelic
496 *VHL* alterations excluding the possibility that AXL expression is dependent on *VHL*
497 status (**Supplementary Fig. S5C**). When stratified based on *VHL* status, a trend was
498 found towards increased PD-L1 positivity in TILs within tumor specimens harboring a
499 *VHL*^{neg/neg} profile (**Supplementary Fig. S5C**), whereas *VHL*^{+/+}/*VHL*^{+/-} tumors
500 tended to be enriched with PD-L1⁺ tumor cells (**Supplementary Fig. S5C**) in line with
501 a previous study [22]. Analysis of the Nivoren RNAseq dataset [24] indicated that
502 *VHL*^{+/+}/*VHL*^{+/-} tumors expressed more PD-L1 ($p=0.01$) and *VHL* mRNAs ($p=0.03$)
503 (**Supplementary Fig. S5D**). As expected, *VHL*^{neg/neg} tumors exhibited increased
504 angiogenesis ($p=0.02$) and hypoxia signatures ($p=0.02$). *VHL*^{+/+}/*VHL*^{+/-} tumors
505 displayed a higher VEGF tumor expression, suggesting a *VHL*/*HIF*-independent
506 regulation of VEGF in this group, as previously proposed [29] (**Supplementary Fig.**
507 **S6A**). Together, this data support that despite similar clinical outcomes under PD-1
508 blockade, *VHL*^{neg/neg} and *VHL*^{+/+}/*VHL*^{+/-} tumors are biologically different and
509 associated with a distinct microenvironment.

510

511 **Association between AXL, PD-L1 and survival according to *VHL* status**

512 We then questioned whether AXL staining would yield comparable prognostic and
513 biologic insights in patients with complete inactivation of *VHL* compared to those with
514 a non-inactivated status. We categorized cases into four groups based on AXL
515 expression >50 and the *VHL* status. Patients whose tumors exhibited high AXL
516 expression and *VHL*^{neg/neg} appeared to have the worst median PFS and 12-month
517 PFS compared to cases with AXL^{neg/low} expression within *VHL*^{neg/neg} subset, though
518 the overall difference was not significant in the log-rank tests comparing the four
519 groups (**Fig. 5A**). In the *VHL*^{neg/neg} subset, a separation in the PFS curves was
520 evident between AXL^{high} vs AXL^{neg/low} groups after ~5 months and continued to show
521 differences in time as supported by the 12-month PFS rates of 19.6% versus 29.9%,
522 respectively (**Fig. 5A**). In contrast, in the *VHL*^{+/+}/*VHL*^{+/-} tumors, there were no signs
523 of such differences. Of note, when interrogating RNAseq data for a subset of
524 patients, a trend in the same direction was observed toward a negative impact for
525 AXL on PFS in the inactivated *VHL* subset but not in the non-inactivated *VHL* subset
526 (**Fig. 5B**). We then conducted a similar analysis using the Checkmate RNA dataset
527 and generated Kaplan–Meier curves for PFS and OS according to tumor *VHL* status.
528 AXL^{high} expression associated with worsened survival (PFS and OS) compared to
529 AXL^{neg/low} in the inactivated *VHL* group (n=78; P=0.05), whereas no difference was
530 observed in patients conserving at least one WT copy of *VHL* in their tumor (n=44;
531 p=0.49) (**Fig. 5C**). Therefore, the influence of AXL on clinical outcomes was mainly
532 apparent in a *VHL*^{neg/neg} tumors. The results also suggest that both OS and PFS
533 could be impacted by high AXL expression in this setting.

534 Finally, we sought to examine the putative interaction between AXL and PD-L1
535 according to tumoral *VHL* status. The enrichment for PD-L1 positivity in AXL^{high} cases

536 was restricted to *VHL*^{neg/neg} tumors (p=0.0003) and accompanied by a significant
537 increase in the % of PD-L1⁺ tumor cells in *AXL*^{high} *VHL*^{neg/neg} cases (**Figure 5D**). In
538 *VHL*^{+/+}/*VHL*^{+neg} cases, PD-L1 staining was detected, but the proportion of positive
539 cases was identical in both the *AXL*^{neg/low} and *AXL*^{high} groups (p> 0.99). In contrast,
540 the relationship between *AXL* with VEGF was perceptible in both *VHL*^{neg/neg} and
541 *VHL*^{+/+}/*VHL*^{+neg} subsets (**Supplementary Fig. S6B**). We observed no particular
542 effects of *AXL* on the immune infiltrate (CD8, CD3, CD163, CD20) in the *VHL*^{neg/neg}
543 and *VHL*^{+/+}/*VHL*^{+neg} specimens(**Supplementary Fig. S6B** and **S6C**).

544

545

546

Discussion

547 Immunotherapy approaches are increasingly used in clinical practice. Yet many
548 challenges remain to improve efficacy and RR. Most patients exhibit primary or
549 acquired resistance to immunotherapy for reasons that remain unclear. The
550 development of markers predictive for response or resistance is warranted. While
551 recent studies have already shown promising results of immunotherapies as first-line
552 therapy, it will be critical to provide the best appropriate combinatorial therapy for
553 each patient, and this implies the development of tools to capture the unique
554 microenvironment of their tumor.

555 In this study, patients with ccRCC tumors exhibiting high expression levels of *AXL*
556 had poorer clinical response (PFS and ORR) after Nivolumab treatment, but not
557 altered OS. The median follow-up in this study was 23.5 months. Outcome measures
558 derive from a still active multicenter phase II study. Extended follow-up should better
559 inform on the impact of *AXL* on OS. A final analysis at a four-year minimum follow-up

560 is planned. The findings obtained from the analysis of the CheckMate dataset are
561 concordant with a role for AXL on OS.

562 AXL expression was strongly associated with PD-L1 TC positivity on tumor cells, and
563 patients with tumors displaying concomitant PD-L1 TC expression and high AXL
564 expression had the worst OS. We found no significant differences for PFS. Such
565 discordance between PFS and OS should be interpreted with caution since prior
566 work indicates that PFS may not be an ideal predictor of OS in the setting of anti-PD-
567 1 monotherapy [9].

568 The absence of correlation between PD-L1 expression and ICB therapy response in
569 this cohort is interesting. It could be due to possible interference between factors
570 such as AXL and PD-L1 expressed by tumor cells or TILs, or discrepancies in PD-L1
571 expression in different tumor sites. Intriguingly, patients with tumors exhibiting high
572 AXL TC expression and PD-L1 positivity in TILs demonstrated a much lower
573 response rate, suggesting that AXL might play a substantial role in tumors infiltrated
574 with PD-L1 expressing TILs. The combination of AXL and PD-L1 biomarkers could
575 identify various subgroups of patients displaying high or reduced tumor-infiltrating
576 immune cells. The findings support the hypothesis that AXL may be one factor
577 interfering with the predictive value of PD-L1, though it's not clear yet whether such
578 effects could act directly on tumor cells, TILs or indirectly *via* the TME components, a
579 question that should be addressed in future investigations.

580 It will be important to explore the connections between AXL, microenvironment
581 components and therapeutic outcomes in the context of other treatment regimens,
582 namely the anti-PD-1/anti-CTLA4 combination emerging as a standard-of-care first-
583 line therapy option [10,30], anti-PDL1-based combinations used to treat patients with
584 advanced RCCs [5,31,32], as well as anti-PD-1 based combinations integrating

585 agents targeting AXL such as cabozantinib, expected to target VEGFR2, MET, RET
586 and AXL [5,33]. Recent data have demonstrated activity of cabozantinib after ICB in
587 m-ccRCC [34]. This treatment might enable to overcome the deleterious effects of
588 AXL.

589 Although AXL expression was not significantly associated with tumor infiltration by
590 macrophages, B or T cells, a broader assessment of the TME and immune
591 checkpoints is required. Our RNA-seq analysis suggested potential interactions
592 between AXL expression and increased inflammation, immune suppression and
593 monocytic lineage signatures. Such analysis likely suffered from the small number of
594 cases analyzed. Studies using sizeable cohorts of patients will be needed to validate
595 these findings. Multiplex IHC or IF approaches would be needed to fully characterize
596 tumor and immune cell infiltrates in this cohort, dissect intratumor heterogeneity, and
597 test potential associations with therapeutic outcomes.

598 Our analysis is retrospective and has other potential limitations. A number of cases
599 with FFPE block showing <40% of tumor or more than 40% of necrosis were
600 excluded. The archival tumor specimens were often obtained before anti-angiogenic
601 therapy or at an early-stage disease. This is in line with routine clinical practice for
602 treatment decision-making. However, a longitudinal survey with a multiple sampling
603 scheme may be necessary to fully appreciate the TME composition and monitor the
604 influence of previous treatments. Diseases with specific features (e.g, Sarcomatoid,
605 low grade, IMDC favorable-risk groups) also need further investigations, as the
606 current study was limited by the number of cases in these categories. As reported by
607 Boysen et al. [35], we noted a trend toward a greater sarcomatoid differentiation in
608 AXL positive cases (n=25, p=0.09) as well as an enrichment of high grade tumors
609 (Furhman III and IV) in cases with high AXL expression.

610 Interestingly, AXL association with PD-L1 and worse PFS was observed in tumors
611 harboring *VHL*^{neg/neg} genetic profile, but it has been unclear if this also occurs in those
612 with *VHL*^{+/+}/*VHL*^{+/neg} status where the number of cases was lower. It is postulated
613 that the TME from *VHL*^{neg/neg} tumors differs from those displaying *VHL*^{+/+} or *VHL*^{+/neg}.
614 It would be interesting to see if among the identified subgroups, some are enriched
615 for genomic alterations reported with values for predicting anti-PD-1 responsiveness
616 including *BAP1* and *PBRM1* [11]. This could explain, in part, some of the
617 demonstrated effects on PFS, OS or ORR. Previous studies have demonstrated that
618 loss of *PBRM1* functions can accentuate the transcriptional response of HIF in
619 *VHL*^{neg/neg} RCC [36]. We postulate that a molecular link exists between AXL and loss
620 of *PBRM1* in *VHL*^{neg/neg} RCC tumors.

621 Moreover, tumor cells may have intrinsic characteristics which likely affect the TME.
622 AXL expression has been associated with EMT, epithelial-mesenchymal plasticity,
623 and phenotypic changes of tumor cells in various cancer types [37]. Compelling
624 evidence also exists that differentiation of tumor cells to a more mesenchymal
625 phenotype can greatly influence the TME *via* increased secretion of
626 immunosuppressive substances such as TGF-beta, VEGF, PD-L1 or IL-8 [38–43]. In
627 a lung cancer study, Thompson and colleagues observed that NSCLC tumors
628 displaying a more mesenchymal phenotype had reduced clinical responses to PD-1
629 blockade. From this work, the investigators derived an EMT/inflammation-based gene
630 expression signature for predicting clinical response of metastatic NSCLC patients
631 [44]. In a cohort of patients with metastatic urothelial cancer treated with nivolumab,
632 Wang et al. found that particularly in patients with T-cell infiltrated tumors, higher
633 EMT/stromal-related gene expression was associated with lower response rates and
634 poor outcomes [45]. Moreover, Chakravarthy et al. showed that an extracellular

635 matrix (ECM) transcriptional program involving TGF- β signalling and EMT-related
636 genes is associated with worse prognosis in melanoma and bladder cancer patients
637 treated with anti-PD-1 [46]. In melanoma, Hugo and colleagues identified a gene-
638 expression signature associated with innate PD-1 resistance (IPRES) of metastatic
639 melanoma which involves the upregulation of EMT-related factors, including AXL,
640 immunosuppressive cytokines, hypoxia and pro-angiogenic factors [47].
641 In our survey, AXL staining in immune cells had no prognostic value. However,
642 because the panel of markers analyzed was limited, we cannot exclude the possibility
643 that a particular immune cell subpopulation expressing AXL contributes to anti-PD-1
644 responsiveness. Others have reported evidence of associations between AXL
645 expressing immune cell populations, and the establishment of an immunosuppressed
646 TME [48–50]. Future studies are warranted to elucidate the interplay between
647 pseudo-hypoxia status, epithelial-mesenchymal plasticity, AXL, microenvironmental
648 changes and their impact on anti-PD-1 responsiveness.

649
650 In summary, we identified associations between expression of AXL, PD-L1
651 expression and *VHL* status in advanced ccRCC which could contribute to disease
652 progression under ICB therapy. Furthermore, we demonstrated that the combined
653 assessment of AXL and PD-L1 expression may be useful to predict outcomes and
654 discriminate patients who are more likely to be resistant or to benefit from anti-PD-1
655 immunotherapy.

656

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670

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Table 1: Patient characteristics

Variables	AXL Tumor cell score		All Patients n=316	Tests
	[0;50] n=241	>50 n=75		
Age (years)				
N	241	75	316	T-test
Mean (Std)	62.6 (10.9)	61.9 (10.4)	62.4 (10.7)	0.608
Median (min; max)	64.0 (22.0; 87.0)	62.0 (41.0; 86.0)	64.0 (22.0; 87.0)	
Gender				
Male	194 (80.5%)	63 (84.0%)	257 (81.3%)	Chi-2
Female	47 (19.5%)	12 (16.0%)	59 (18.7%)	P = 0.497
IMDC group				
Favorable	42 (17.4%)	12 (16.0%)	54 (17.1%)	Chi-2
Intermediate	142 (58.9%)	49 (65.3%)	191 (60.4%)	P = 0.580
Poor	57 (23.7%)	14 (18.7%)	71 (22.5%)	
ECOG				
Missing	8	3	11	
0 or 1	199 (85.4%)	63 (87.5%)	262 (85.9%)	Chi-2
2 or 3	34 (14.6%)	9 (12.5%)	43 (14.1%)	P = 0.656
M Stage				
Missing	14	5	19	
0	83 (36.6%)	28 (40.0%)	111 (37.4%)	Chi-2
1	59 (26.0%)	20 (28.6%)	79 (26.6%)	P = 0.657
X	85 (37.4%)	22 (31.4%)	107 (36.0%)	
Furhman				
Missing data	7	2	9	
I, II	69 (29.5%)	13 (17.8%)	82 (26.7%)	Chi-2
III, IV	165 (70.5%)	60 (82.2%)	225 (73.3%)	P = 0.049
Brain Metastasis				
Missing	16	3	19	
NO	197 (87.6%)	65 (90.3%)	262 (88.2%)	Chi-2
YES	28 (12.4%)	7 (9.7%)	35 (11.8%)	P = 0.533
Surgery				
	241 (100%)	75 (100%)	316 (100%)	
Nephrectomy				
NO	9 (3.7%)	3 (4.0%)	12 (3.8%)	Chi-2
YES	232 (96.3%)	72 (96.0%)	304 (96.2%)	P = 0.916
Radiotherapy				
NO	148 (61.4%)	47 (62.7%)	195 (61.7%)	Chi-2
YES	93 (38.6%)	28 (37.3%)	121 (38.3%)	P = 0.845

829
830

Figure legends

831 **Figure 1 High AXL expression is associated with a trend to shorter PFS and**
832 **lower ORR in patients with advanced ccRCC treated with Nivolumab. A,**
833 Kaplan–Meier survival curves for PFS and OS of RCC patients when stratified
834 according to AXL H-score into two groups, negative/Low [0;50] vs High >50.
835 Corresponding Objective Response Rates, hazard ratios, median PFS (mPFS), and
836 survival estimates at 12 months post-treatment are shown. **B,** Kaplan–Meier PFS and
837 OS curves, and outcome measures deriving from RNA-seq data available for a
838 subset of cases. Nivolumab-treated RCC patients divided into two groups based on a
839 cutoff \geq highest 1/3 (High) vs \leq lowest 2/3 (Low) AXL mRNA expression to assess the
840 impact of AXL on survival outcomes. **C,** Kaplan–Meier PFS and OS curves derived
841 from CheckMate RNA-seq data available from the study of Braun [11]. Nivolumab-
842 treated RCC patients were considered and divided into AXL^{high} or AXL^{low} expression
843 groups as in **B**.

844
845 **Figure 2 AXL is associated with PD-L1 expression. A,** Distribution of PD-L1 (% of
846 cases and % of positive cells) in tumors with AXL^{neg/low} vs AXL^{high} profiles. **B**
847 Distribution of VEGF, CD8, CD3, CD163, CD20 expression in AXL^{Neg/Low} versus
848 AXL^{high} cases. **C,** A heatmap is shown to illustrate the biomarker composition across
849 the tumor specimens according to AXL groups. **D,** RNA-Seq-derived expression of
850 various gene signatures in AXL^{neg/low} vs ^{high} cases. Violin plots are shown with box
851 representing the 25th to 75th percentiles and central line representing the median
852 (Mann-Whitney test).

853
854 **Figure 3 High AXL expression plus PD-L1 TC positivity is associated with**
855 **worse OS in patients treated with Nivolumab. A,** Kaplan-Meier estimates for OS
856 according to PD-L1 TC status (pos/neg), or AXL expression and PD-L1 TC status
857 (four biomarker subgroups). **B,** Kaplan-Meier estimates for OS according to PD-L1
858 TIL status (pos/neg), or AXL expression and PD-L1 TIL status (four biomarker
859 subgroups).

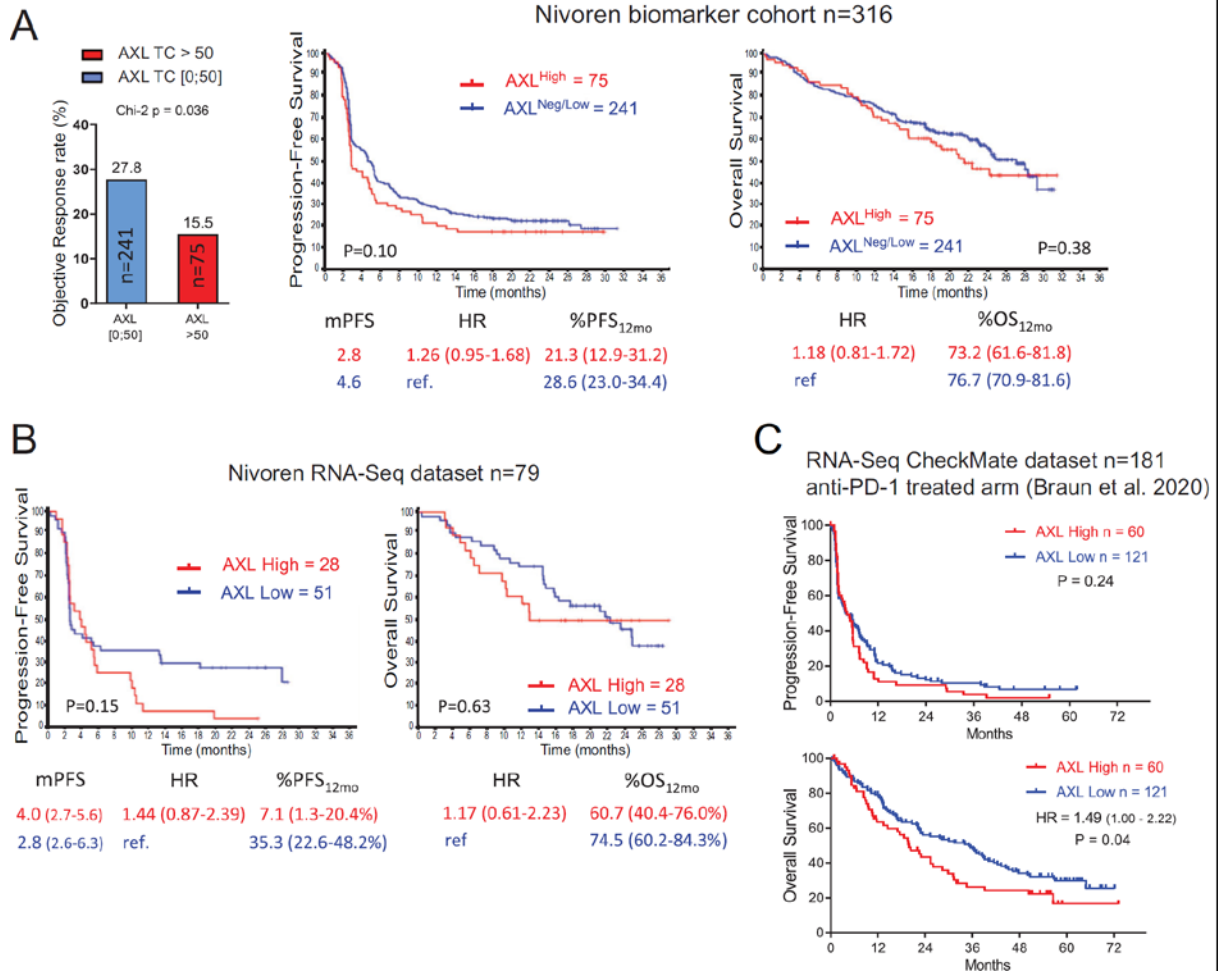
860
861 **Figure 4 biomarker distribution across groups stratified based on PD-L1 TC**
862 **and AXL expression**

863 **A,** Bar graphs comparing the biomarker composition in the corresponding subgroups.
864 Data are presented as means. Errors bars are s.e.m. Mann-Whitney test. **B,** A
865 heatmap showing the biomarker distribution across the tumor specimens in the
866 indicated subgroups.

867
868 **Figure 5 AXL association with PD-L1 and outcomes in *VHL*^{neg/neg} and *VHL*^{+/+}**
869 **/*VHL*^{+/-} tumors A,** Kaplan–Meier estimates for PFS in patients divided into four
870 groups based on AXL expression and *VHL* status. In the *VHL*^{neg/neg} subset, patients
871 with tumors expressing high levels of AXL (red line) trended to worse PFS compared
872 to those with AXL^{Null/Low} expression (grey line). **B,** Kaplan–Meier PFS curves
873 generated after categorization using RNA-seq data on a subset of cases in *VHL*-
874 inactivated (*VHL*^{neg/neg}) and *VHL* non-inactivated (*VHL*^{neg/+} / *VHL*^{+/+}) cases. **C,**
875 Kaplan–Meier estimates for PFS and OS as in B, but using the CheckMate dataset
876 [11]. P values from Log rank tests are shown. **D,** AXL expression associated with PD-
877 L1 TC expression especially in the *VHL*^{neg/neg} tumors. Distribution of PD-L1 (% of

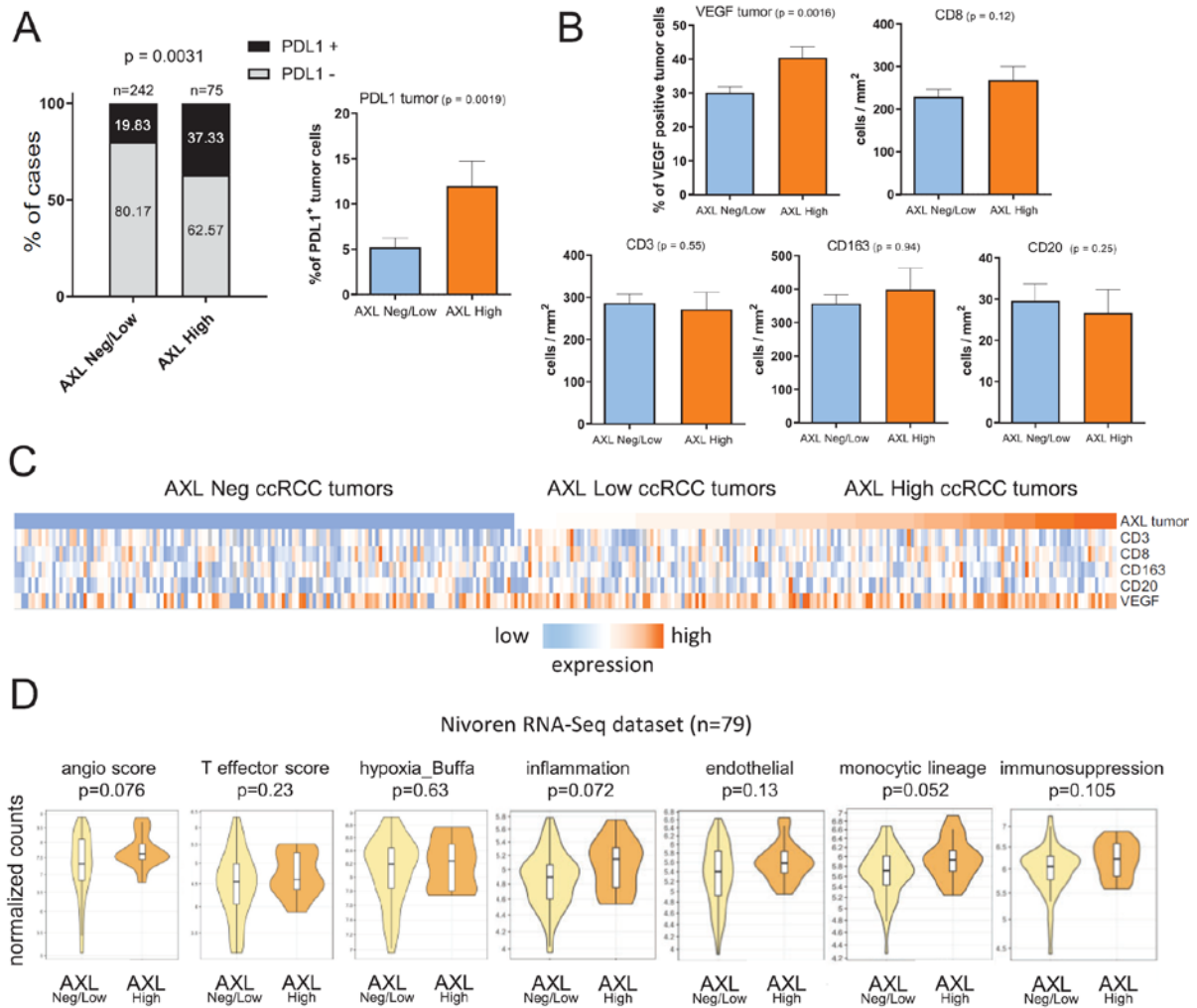
878 cases and % of positive cells) across the tumor specimens according to the indicated
 879 subgroups. Data are presented as means. Errors bars are s.e.m. Chi-square and
 880 Mann-Whitney tests were applied.
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 882

FIGURE 1



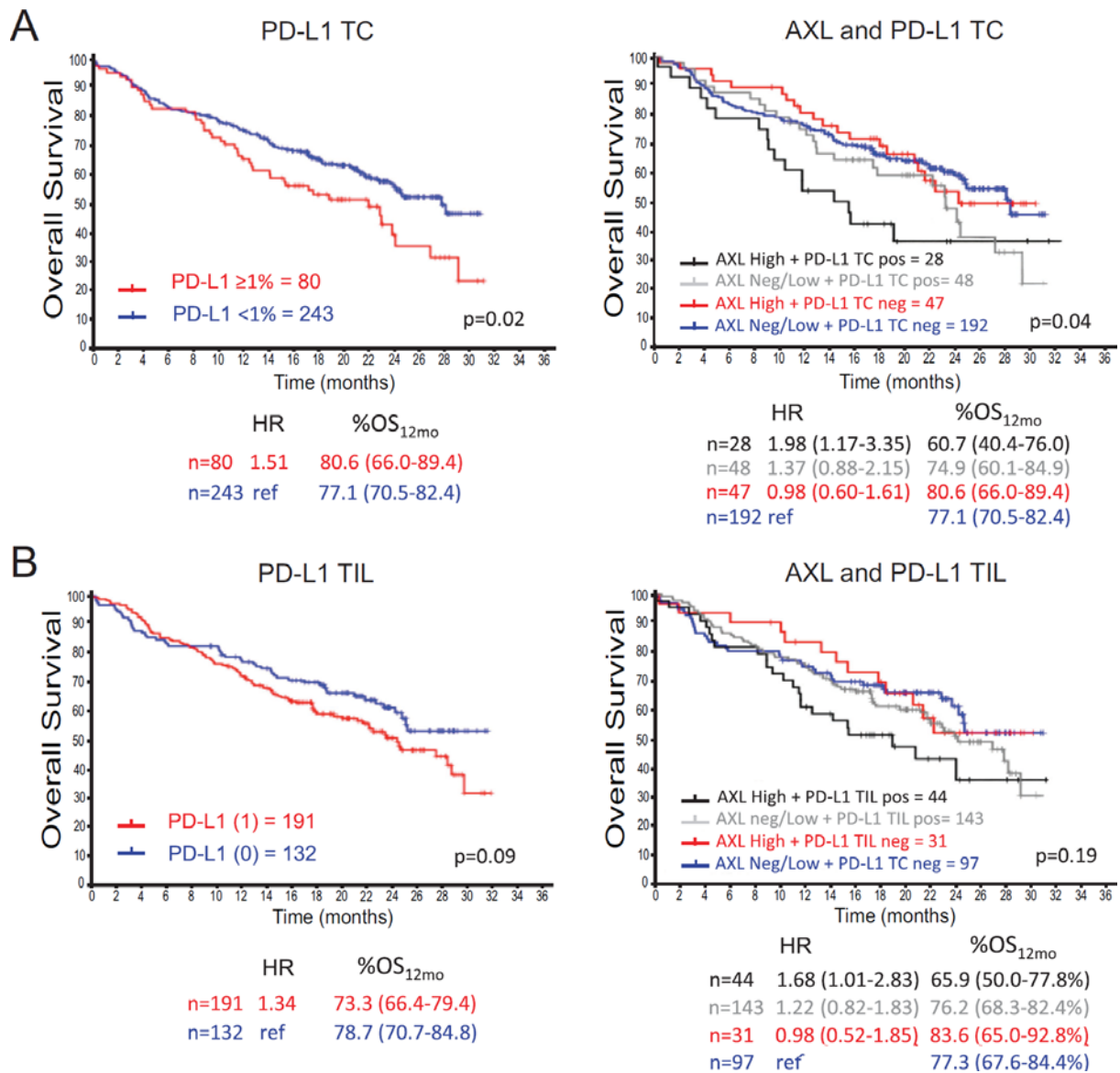
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FIGURE 2



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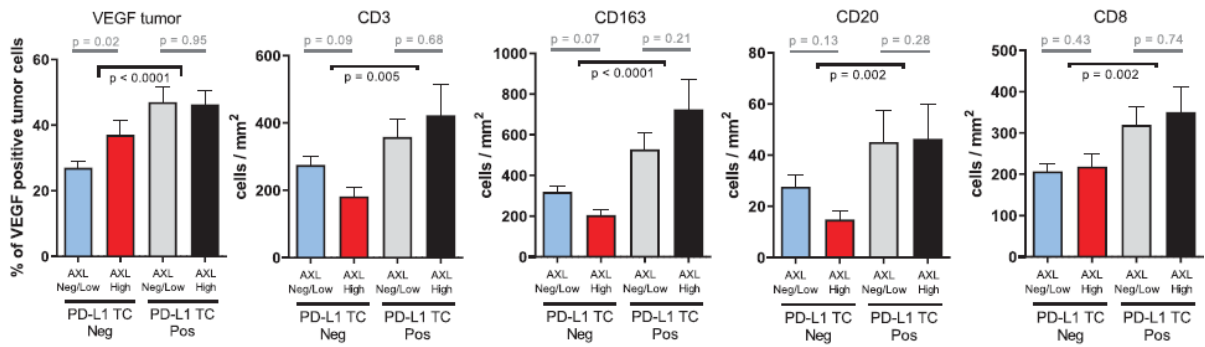
FIGURE 3



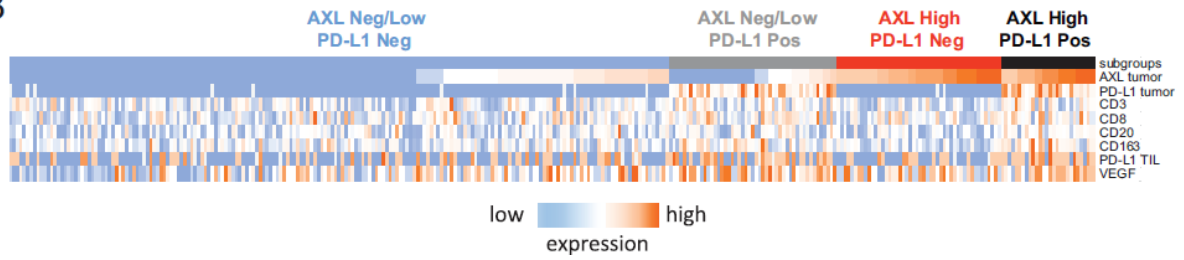
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FIGURE 4

A

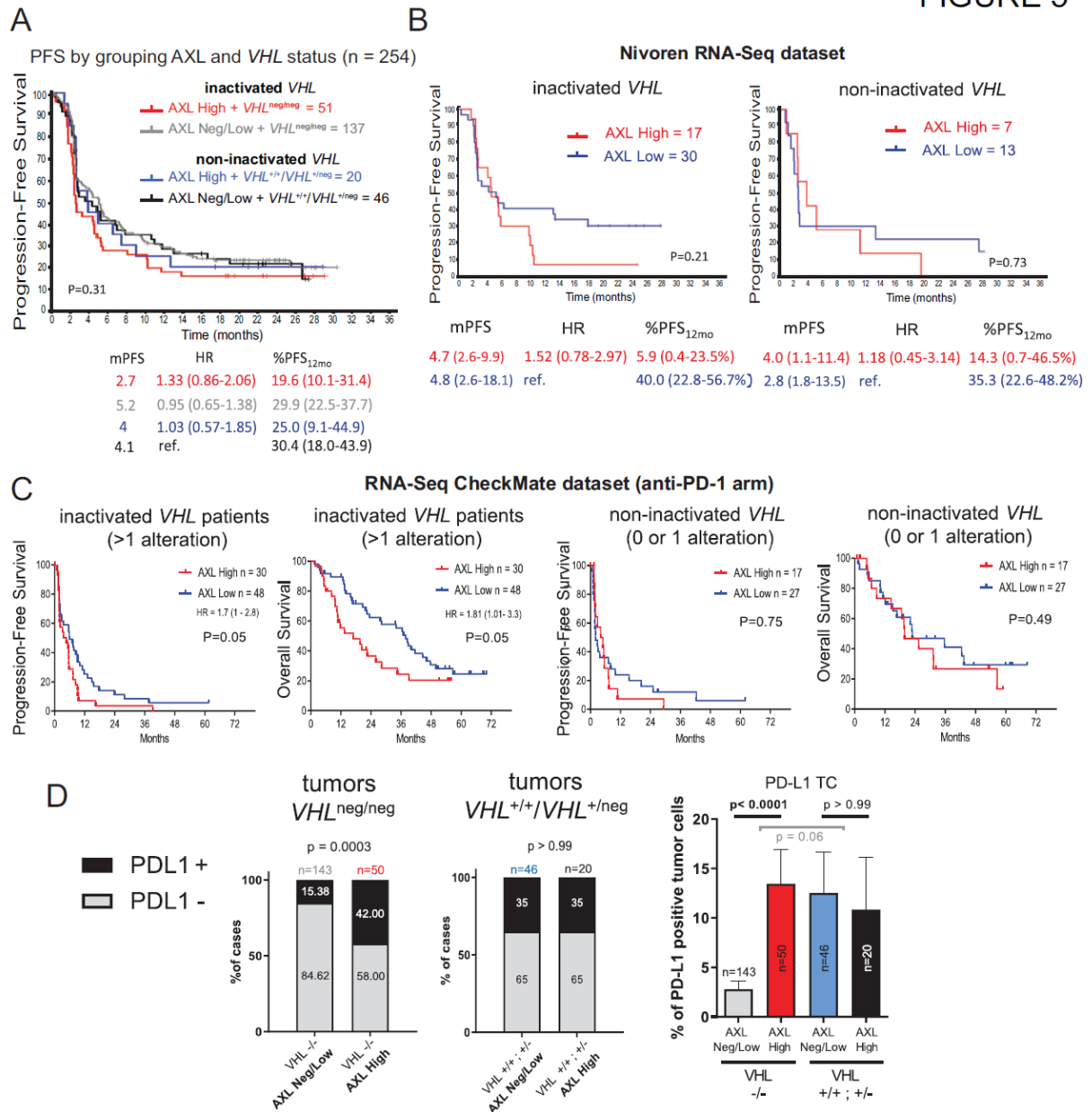


B



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FIGURE 5



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