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EXTENSIVE MOLECULAR PROFILING OF SQUAMOUS CELL ANAL CARCINOMA IN A PHASE 2 TRIAL POPULATION: TRANSLATIONAL ANALYSES OF THE "CARACAS" STUDY.

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Abstract

Introduction

Advanced squamous cell anal cancer (aSCAC) is a rare and aggressive disease. No targeted therapies are currently approved and no standard therapies after first line are currently available. Molecular characteristics of SCAC are poorly explored. Immune checkpoint inhibitors (ICI) showed signs of activity in previous phase I/II trials, but predictive and prognostic biomarkers are lacking. High TMB and PD-L1 were associated to better response to ICI in other malignancies, but few data are available for SCAC.

Anti-EGFR have been tested given the well-known rarity of KRAS mutations in SCAC, with encouraging results. Earlier preclinical evidence suggests possible synergism between cetuximab (cet) and PD-L1 blockade

Materials and methods

In the phase II randomized trial CARACAS (NCT03944252), we tested avelumab (ave) alone (Arm A) or with cet (Arm B) in pretreated aSCAC, being overall response rate (ORR) the primary endpoint. With one-sided alpha error set at 0.05 and power of 80%, at least 4 responses out of 27 patients per arm had to be observed to declare the study positive. On pre-treatment tumor tissue samples, we assessed HPV status, PD-L1 expression, microsatellite status, tumor mutational burden (TMB) and next generation sequencing (NGS). Tumor-infiltrating lymphocytes (TILs) were characterized on HE-stained samples. Translational analyses were conducted on the 100% of patients enrolled in the clinical trial since all of them received ICI. Primary objective of the TranslaCARACAS study was to describe the clinical outcomes of ICI in the CARACAS trial population according to molecular analyses. Secondary objectives

were to assess progression-free survival (PFS) and overall survival (OS) according to molecular characteristics to individuate new prognostic biomarkers in SCAC.

Results

In the clinical trial, the Arm B reached the primary endpoint (ORR 17%). High TMB (3 10 mutations per megabase) was related to better OS (HR=0.09; 95%CI 0.01-0.68; p=0.019), showing the same trend in PFS (HR=0.44; 95%CI=0.15-1.27; p=0.129). High expression of PD-L1 (>40 measured with combined positive score, CPS) conferred significantly longer OS (HR=2.19; 95%CI=0.92-5.19; p=0.075) and PFS (HR=2.35; 95%CI=1.09-5.1; p=0.03). High TILs (>1.2) did not affect OS (HR=0.77; 95%CI=0.42-1.4; p=0.39) nor PFS (HR=1.19; 95%CI=0.57-2.48; p=0.645). Combined together and with high TILs, high TMB and PD-L1identified pts with significantly better prognosis in OS (HR=0.43; 95%CI=0.21-0.87; p=0.019) and PFS (HR=0.48; 95%CI=0.23-1.00; p=0.051). Remarkable responses were also observed in patients with high PD-L1 expression and TMB

Conclusions

To our knowledge, TranslaCARACAS was the first study to document prognostic role of TMB and PD-L1 in mSCAC treated with ICI. Further investigation in larger cohorts is warranted to confirm our findings

Introduction

Squamous cell anal carcinoma (SCAC) is a rare but aggressive malignancy, with a current prevalence of around 2% of all gastrointestinal tumours yet steadily rising annually, and characterised by elevated mortality in advanced stages, with 5-years survival of 34% in case of metastatic disease[1]; the frequent association with human immunodeficiency virus (HIV) infection contributes to deteriorate clinical conditions, already undermined by the high morbidity of this malignancy.

In addition to HIV infection, risk factors include anal intercourse, cigarette smoking and human papilloma virus (HPV) infection; in particular, more than 80% of patients diagnosed with SCAC are HPV-positive[2]. HPV is also an independent prognostic factor, being HPV DNA and p16 expression related to better survival (HR=0.07, 95% CI 0.01 to 0.61; p=0.016) [3]. On the contrary, male sex, cigarette smoking, tumor > 5 cm in diameter and nodal involvement are related to worse prognosis. HIV infection does not affect the overall prognosis *per se*: recently, similar toxicity and response to standard treatments, as well as survival, have been observed in patients treated with antiretroviral therapy and CD4 count ≥ 200/mmc compared to HIV-negative population with SCAC[4, 5].

Apart from the well-known rarity of RAS mutations[6], no validated biomarkers predictive of response have been individuated in SCAC; as well, few chemotherapeutical treatments and no standardised target therapies are currently established. Nevertheless, growing interest has been raised in the last decade, in order to better characterize this malignancy and to search for new treatment strategies.

Chung et al described frequent alterations in the PI3K/AKT/mTOR pathway in a cohort of 70 patients with SCAC at different stages[7]. Similar results in the same type of

patients were found by Smaglo et al, together with high percentages of mutations in the onco-suppressors TP53 and FBXW7[8].

More recently, Van Morris et al performed a dedicated, extensive genome profiling in a cohort made of 41 metastatic SCAC (mSCAC) cases, finding high rates of mutations in PIK3CA, MLL2 and MLL3 and low tumour mutation burden (TMB)[9].

TMB is gaining growing interest in recent years given its role in immunocheckpoint blockade (ICB). As a matter of facts, high TMB proved positive predictive value for ICB in several solid tumors: melanoma[10, 11]; non-small cell lung cancer[12, 13], bladder[14], and MSI-H tumors in general[15].

On the other hand, the role of TMB in HPV-related malignancies is still debated.

The presence of low TMB in SCAC is in line with other HPV-related squamous tumours. In head and neck squamous cell cancer (HNSCC), significant association was described between objective response to pembrolizumab and high TMB in case of HPV and EBV negative tumours; on the contrary, this association was not confirmed in HPV+ or EBV+ tumours[16].

These observations support the theory that in virus-related squamous neoplasms, viral neoepitopes might account for the process of immune-escape even more than somatic antigens.

In particular, the expression of highly antigenic viral proteins and the upregulation of programmed cell death protein 1 (PD)-1/ programmed cell death protein ligand 1 (PD-L1) axis, which is responsible of immune-escape, might lead to chronic HPV infection and cancer initiation[17].

In recent years, ICB demonstrated promising activity in terms of response and survival in gastrointestinal malignancies in several clinical trials[18, 19, 20]. These studies showed

that gastrointestinal tumours with high microsatellite instability (MSI-H) are more responsive to ICB[15]. Unfortunately, no data exist regarding MSI in SCAC; however, benefit from ICB therapy is not always observed in MSI-H patients, suggesting that other factors could play a role in determining sensitivity to these treatments.

MSI-H tumors are characterized by elevated neoantigen load, tightly related to TMB. As well, MSI-H tumors often show broad PD-L1 expression and presence of tumor-infiltrating lymphocytes (TILs).

Association between high TILs number and better survival outcomes from ICB has been recently reported in MSI-H mCRC[21]; furthermore, TILs have been related to better prognosis in a number of different malignancies[22, 23]. In SCAC, high TILs levels were related to improved outcomes and lower risk of relapse after chemoradiotherapy for limited stages[24, 25, 26].

In advanced SCAC (mSCAC), ICB has been tested with encouraging results: promising activity expressed as objective response and disease control rate was reported with pembrolizumab in patients with PD-L1 positive tumors[27]. As well, remarkable response rate was obtained with Nivolumab in a mSCAC population unselected for PD-L1 or other biomarkers. In this study, significantly higher expression of CD8, Granzyme B, LAG-3, TIM-3 CD45 and PD-1 on T cells and PD-L1 on tumor cells were found in responders compared to non-responders, supporting the possible predictive role of TILs and PD-L1 for ICB therapy in mSCAC[28]. As a prognostic marker in SCAC, however, PD-L1 proved benefit in survival in patients with SCAC at initial stages treated with standard chemoradioterapy[26]; on the contrary, no data are available regarding its prognostic role in mSCAC.

In SCAC PD-L1 is frequently expressed: in most cohorts, PD-L1 positive tumors represent more than 50%[29, 30].

PD-L1 upregulation is at the basis of the adaptive immune resistance: this process is known to be promoted by IFNγ secreted by natural killer (NK) cells and CD8+ T cells into the tumor microenvironment, and brings also to the upregulation of IDO and to the recruitment of Treg cells[31]. The anti-EGFR cetuximab exerts antibody-dependent cytotoxicity (ADCC) via NK cells[32]. In vitro studies demonstrated that secretion of IFNγ by activated NK cells may induce overexpression of PD-L1 on tumor cells, supporting the hypothesis of synergism between ICB and anti-EGFR therapy[33].

Moving from such background, we conducted an open-label, prospective, multicenter randomized phase 2 trial to evaluate safety and activity of the anti PD-L1 avelumab alone or in combination with the anti-EGFR cetuximab in pretreated mSCAC: the CARACAS study (NCT03944252)[34].

In parallel with this study, we designed an ancillary, prospective translational study to identify new biomarkers predictive of efficacy for ICB in mSCAC. The TranslaCARACAS study has the ambitious aim of simultaneously providing a comprehensive molecular characterisation of mSCAC, and to prospectively describe correlations between molecular profile and overall survival, progression free survival and response pattern to immunotherapy alone or combined with anti-EGFR drugs.

Materials and methods

Study design, participants and treatments – clinical trial

The CARACAS trial (NCT03944252) was a multicentre, open-label, "pick-the-winner", randomised phase 2 trial promoted by the Gruppo Oncologico Nord Ovest (GONO) Foundation. The study involved 17 centres throughout Italy and was coordinated by Veneto Institute of Oncology IRCCS in Padua.

Patients were deemed eligible if they had already received at least one previous line of treatment for metastatic disease or if experiencing progression of disease within six months after the completion of chemoradiotherapy for non-metastatic disease. Importantly, HIV-positive patients were also eligible.

Randomisation was conducted in a 1:1 ratio between two treatment arms: avelumab monotherapy 10 mg/kg intravenously on day 1 every two weeks (arm A) or cetuximab 500 mg/m2 plus avelumab 10 mg/kg both intravenously on day 1 every two weeks (arm B).

Translational analyses

The collection of archival or freshly-obtained tissue specimens was mandatory for study entry; a formalin-fixed paraffin embedded tumour block from primary tumour and/or metastasis was required during the screening phase. Histological diagnosis was centrally confirmed for all patients; HPV status was centrally assessed by means of HPV test performed with real time polymerase chain reaction (RT-PCR) in case of patients with missing information. Additional biopsy was recommended, but not mandatory, at the time of disease progression.

On both pre-treatment and post-treatment tissue, NGS was performed with FoundationOne CDx panel®, which encompassed also microsatellite status and TMB quantification. TMB was considered "high" if ≥ 10.

To validate the results of NGS, mutational status of genes of particular interest was assessed with MassArray® System by Agena®: *PIK3CA, KRAS, NRAS, BRAF*; microsatellite status was also obtained with immunohistochemistry (IHC).

Furthermore, in order to better understand immunogenicity of SCAC, PD-L1 expression on tumor cells was assessed with IHC; TILs were also quantified on both pre-treatment and post-treatment tissue in haematoxylin-eosin. PD-L1 was considered "high" if > 40 as assessed with combined positive score (CPS).

A dedicated database was created, in which the results of all the analyses previously described were tabulated together with PFS, OS and best response obtained during the experimental treatment.

Clinical endpoints

The primary endpoint of the clinical study was overall response rate (ORR), defined as the percentage of patients achieving a complete (CR) or partial (PR) response, according to RECIST 1.1 criteria[35] among the total of patients randomised in each arm. With one-sided α error set at 0.05 and power of 80%, at least 4 responses out of 27 patients per arm had to be observed to declare the study positive.

Secondary endpoints were Progression-Free Survival (PFS), Overall Survival (OS), and safety profile description.

Translational objectives

Primary objective of the TranslaCARACAS study was to describe the clinical outcomes of ICI in the CARACAS trial population according to molecular analyses. In particular, we searched for possible correlations between molecular and histological characteristics of the tumor specimens analysed and the response to experimental treatments received during the study, in order to find predictive markers of response to immunotherapy.

Secondary objectives were to assess progression-free survival (PFS) and overall survival (OS) according to molecular characteristics to individuate new prognostic biomarkers in SCAC.

PFS was defined as the time from randomisation to the first documentation of objective disease progression or death due to any cause, whichever occurred first. For alive patients free from progression at data cutoff, PFS was censored at the time of the last evaluable tumour assessment documenting absence of progressive disease.

OS was defined as the time from study enrolment to the date of death due to any cause. Patients still alive at the time of analysis were censored for OS on the last date they were known to be alive.

Clinical results

Clinical characteristics were well balanced between the two arms

From 18 September 2018 to 2 July 2019, 60 eligible patients in total were randomised, 30 in each arm, six more than initially planned in order to counterbalance possible dropouts. Remarkably, target accrual was completed 7 months before the planned date of 28 February 2020. Baseline characteristics of patients are illustrated in Table 1 and were well balanced between the two arms, with the only exception of female patients. HPV status was assessable for near 100% of patients; HPV+ was found in the large majority of patients. Of note, patients with higher tumor burden at the time of the enrolment were numerically higher in arm B.

	Arm A **Avelumab** **N=30**	**Arm B** **Avelumab + Cetuximab** **N=30**
Median age (range), years	65 (35–84)	63 (39–77)
Race, n (%) **Caucasian** **Asian**	 29 (97) 1 (3)	 30 (100) 0 (0)
Sex, n (%) **Female** **Male**	 24 (80) 6 (20)	 17 (57) 13 (43)
ECOG performance status, n (%) **0** **1** **2**	 16 (53) 12 (40) 2 (7)	 18 (60) 11 (37) 1 (3)
HIV status **Positive** **Negative**	 3 (10) 27 (90)	 1 (3) 29 (97)
HPV status **Positive** **Negative** ***Not evaluable***	 25 (89) 3 (11) *2*	 27 (93) 2 (7) *1*
Disease stage at diagnosis, n (%) **I–III** **IV**	 18 (60) 12 (40)	 18 (60) 12 (40)

Metastatic sites at diagnosis, n (%)		
1	5 (17)	4 (13)
2	2 (7)	2 (7)
≥ 3	5 (17)	6 (20)
Burden of disease at the enrolment, n (%)		
Local relapse/persistent disease only	8 (27)	3 (10)
Distant metastases	22 (73)	27 (90)
Previous lines of systemic treatment, n (%)		
0	9 (30)	6 (20)
1	14 (47)	14 (47)
≥ 2	7 (23)	10 (33)

Table 1: Patients and disease baseline characteristics according to the randomly allocated treatment arm.

Clinical primary endpoint was reached in arm B

With 5 partial responses (PR) observed in 30 patients, clinical primary endpoint was reached in combination arm. ORR was 10% (95% CI 2·1-26·5) and 17% (95% CI 5·6-34·7) in arm A and B, respectively. However, durable disease stabilization was observed in both arms (Figure 1)

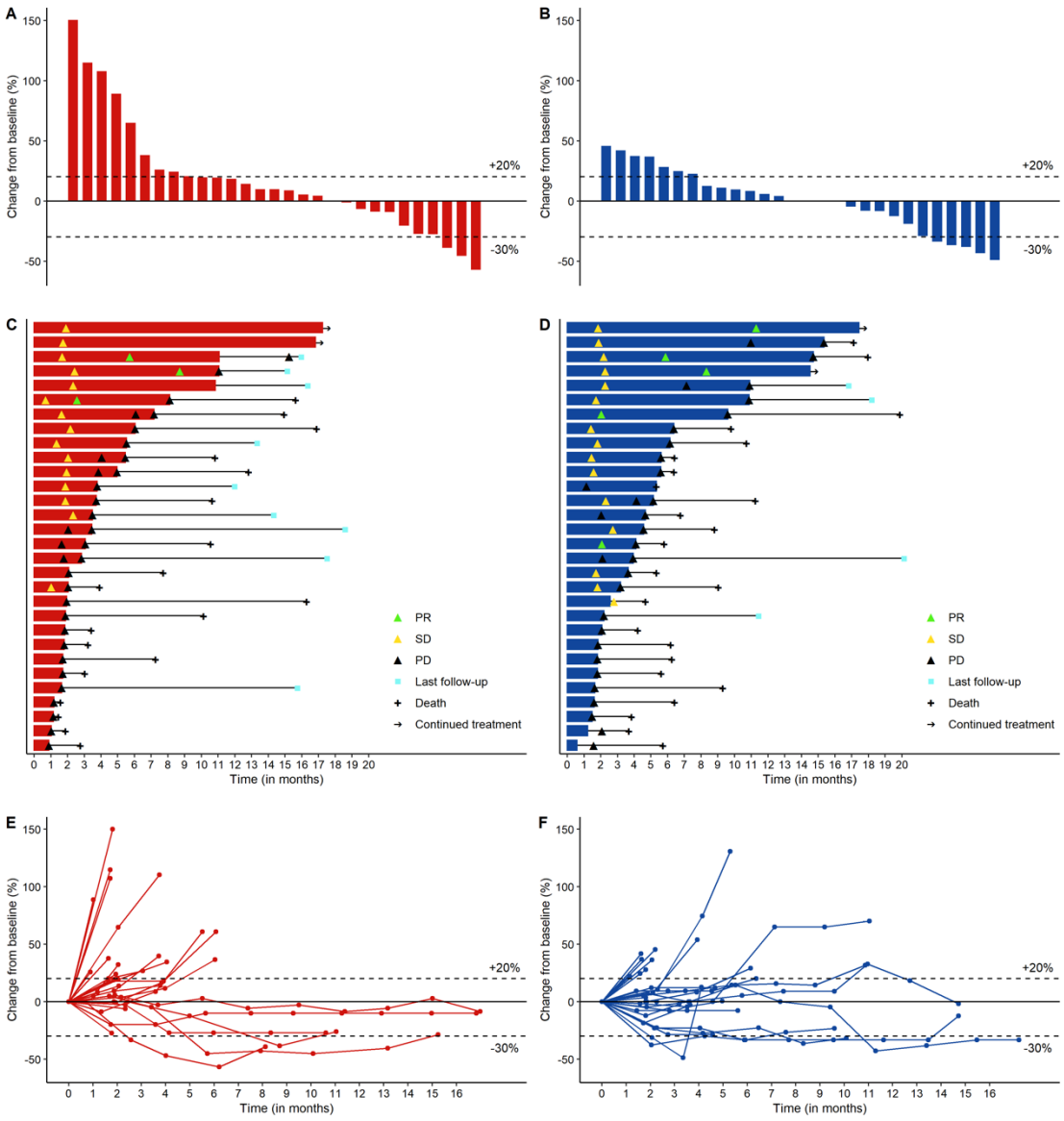

Figure 1: Plots describing response and duration of therapy in arm A (red colour) and B (blue colour). In sequence, waterfall plot (A, B) depicting RECIST v1.1 responses and their depth in 57 out of 60 patients evaluated for response; swimmer plot (C, D) depicting time-on-treatment and tumour dynamics in 60 evaluable patients; spider plot (E, F) depicting the longitudinal assessment of RECIST v1.1 response during treatment in 60 evaluable patients. PR: partial response; SD: stable disease; PD: progressive disease.

OS and PFS curves

Updated OS and PFS were obtained at cutoff date 15 July 2021: after a median follow up of 26.7 months (IQR 26.5-26.9), median PFS was 2.0 months (two-sided 95% CI 1.8-4.0) in Arm A and 3.9 months (two-sided 95% CI 2.1-5.6) in Arm B (Figure 3A-3B); median OS was 13·9 months (two-sided 95% CI 7·7-19.4) in arm A and 7.8 months (two-sided 95% CI 6·2-11.2) in arm B (Figure 3C-3D). A total of 56 progression and 52 death events occurred. (Figure 2)

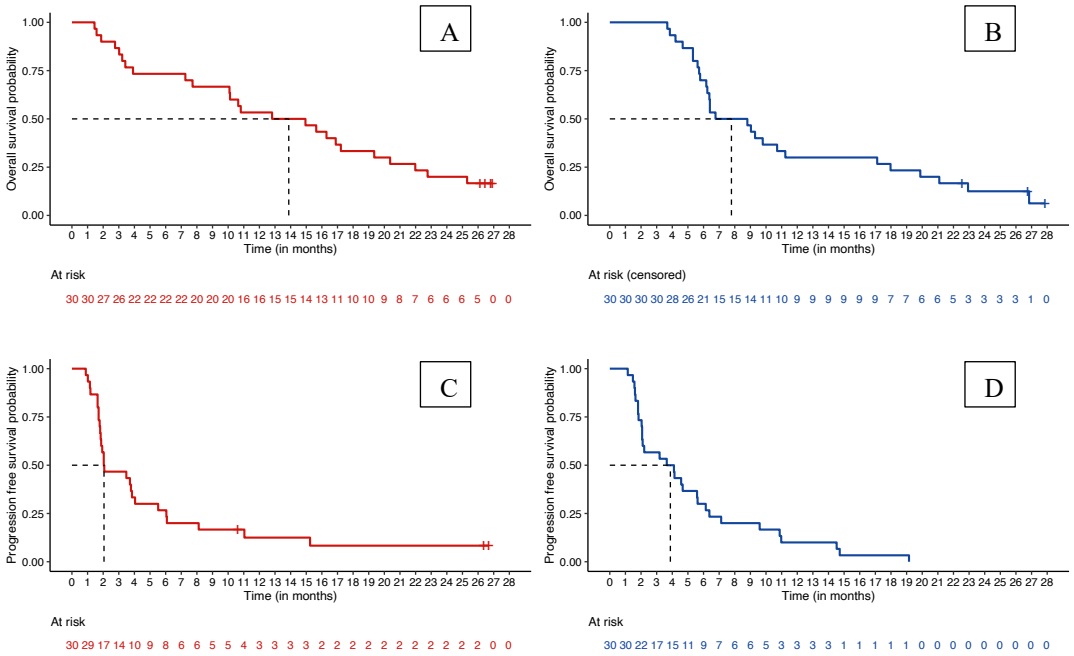

Figure 2: Kaplan-Meier curves depicting PFS (A-B) and OS (C-D) in arm A (red colour) and arm B (blue colour).

Translational results

Major molecular and histological drivers were well balanced between the two arms

No statistically relevant differences were observed between the two arms in the distribution of the main molecular and histological variables analyzed, as shown in Table 2. In line with data existing in literature, low rates of KRAS mutations and relatively high rates of PIK3CA mutations were reported. Of note, prevalence of low TMB tumors was confirmed. Extremely low rate of MSI-H tumors (2% of the overall population) was also observed. High PD-L1 was found in around half of the patients (measured with CPS, cutoff ≥10) (Table 2).

	Arm A **Tot =30** **n (%)**	**Arm B** **Tot =30** **n (%)**	**Overall population** **Tot = 60** **n (%)**
Histology **Keratinizing** **Non-keratinizing** ***Not evaluable***	11 (37) 19 (63) *0*	9 (30) 21 (70) *0*	20 (33) 40 (67) *0*
TILs **Present (≥1)** **Absent (<1)** ***Not evaluable***	16 (67) 8 (33) *6*	15 (56) 12 (44) *3*	31 (61) 20 (39) *9*
PD-L1 – TPS **High (≥10)** **Low (<10)** ***Not evaluable***	6 (22) 21 (78) *3*	4 (15) 23 (85) *3*	10 (19) 44 (81) *6*
PD-L1 – CPS **High (≥10)** **Low (<10)** ***Not evaluable***	11 (44) 14 (56) *5*	14 (52) 13 (48) *3*	25 (48) 27 (52) *8*
KRAS **Mutated** **Wild type** ***Not evaluable***	0 (0) 29 (100) *1*	2 (7) 27 (93) *1*	2 (3) 56 (97) *2*
BRAF **Mutated** **Wild type** ***Not evaluable***	0 (0) 29 (100) *1*	0 (0) 29 (100) *1*	0 (0) 58 (100) *2*
MSI **MSI-H** **MSS** ***Not evaluable***	0 (0) 29 (100) *1*	1 (4) 26 (96) *3*	1 (2) 55 (98) *4*
TMB **High (≥10 mut per megabase)** **Low (<10 mut per megabase)** ***Not evaluable***	3 (14) 18 (86) *9*	2 (11) 17 (89) *11*	5 (12) 35 (88) *20*
PIK3CA **Mutated** **Amplified** **Wild type** ***Not evaluable***	12 (41) 2 (7) 15 (52) *1*	5 (17) 1 (4) 23 (79) *1*	17 (29) 3 (5) 38 (66) *2*

Table 2: Patients and molecular and histological characteristics according to the randomly allocated treatment arm.

HPV positive tumors represented the large majority of our cohort

As expected, the large majority of our patients was diagnosed with HPV infection (91%). HPV+ cases were equally distributed between the two arms. In arm A and B respectively, HPV assessment was not possible for 2 and 1 patients. In both arms, the most common HPV genotype found was 16. (Table 3).

HPV Positive	**Overall population n=60**	**Arm A n=30**	**Arm B n=30**
Yes	52 (91%)	25 (89%)	27 (93%)
No	5 (9%)	3 (11%)	2 (7%)
Not evaluable*	*3*	*2*	*1*
HPV type on HPV-positive patients			
HPV Type	**Overall HPV-positive population n=52**	**Arm A HPV-positive patients n=25**	**Arm B HPV-positive patients n=27**
16	35 (95%)	13 (93%)	22 (96%)
Others†	2 (5%)	1 (7%)	1 (4%)
Not evaluable*	*15*	*11*	*4*

Table 3 HPV status among our patients

No statistically significant differences in distribution of major molecular, viral and histological characteristics between patients achieving disease control and patients with progressive disease (PD) as best response.

None of the major molecular and histological characteristics analyzed had statistically different distribution between patients reaching disease control vs patients with PD as best response. HPV positive and negative patients were also equally represented between patients with disease control and patients with PD as best response. The analyses were performed in the overall population without distinction per arm, since 100% of patients received immunotherapy. (Table 4).

	Best response SD or PR **Tot =32** **n (%)**	**Best response PD** **Tot =28** **n (%)**	**p Value**
Keratinizing	11 (34)	9 (32)	*p* = 1.0000
Non-keratinizing	21 (66)	19 (68)	
Not evaluable	*0*	*0*	-
TILs < 1	12 (44)	8 (33)	*p* = 0.5667
TILS ≥ 1	15 (56)	16 (67)	
Not evaluable	*5*	*4*	-
PD-L1 (CPS) < 10	12 (46)	15 (58)	*p* = 0.5793
PD-L1 (CPS) ≥ 10	14 (54)	11 (42)	
Not evaluable	*6*	*2*	-
TMB < 10	14 (78)	21 (95)	*p* = 0.1554
TMB ≥ 10	4 (22)	1 (5)	
Not evaluable	*14*	*6*	-
PIK3CA altered	7 (50)	7 (47)	*p* = 1.0000
PIK3CA WT	7 (50)	8 (53)	
Not evaluable	*1*	*0*	-
HPV+	27 (93)	25 (89)	*p* = 0.6701
HPV-	2 (7)	3 (11)	
Not evaluable	*3*	*0*	-

Table 4**:** Molecular and histological characteristics according to the best response.

No statistically significant differences in distribution of minor molecular characteristics between patients with PR/SD and patients with PD as best response

None of the minor molecular characteristics analyzed had statistically different distribution between patients reaching disease control vs patients with PD as best response. (Table 5)

	BEST RESPONSE SD or PR Tot =32 n (%)	BEST RESPONSE PD Tot =28 n (%)	p Value
NOTCH1 altered	3 (16)	1 (5)	$p = 0.3210$
NOTCH1 wild type	16 (84)	21 (95)	
Not evaluable	*13*	*6*	-
PTEN altered	2 (11)	3 (14)	$p = 1.0000$
PTEN wild type	17 (89)	19 (86)	
Not evaluable	*13*	*6*	-
CDKN2A altered	3 (16)	1 (5)	$p = 0.3210$
CDKN2A wild type	16 (84)	21 (95)	
Not evaluable	*13*	*6*	-
FBXW7 altered	2 (11)	3 (14)	$p = 1.0000$
FBXW7 wild type	17 (89)	19 (86)	
Not evaluable	*13*	*6*	-
MLL2 altered	5 (26)	4 (18)	$p = 0.7087$
MLL2 wild type	14 (74)	18 (82)	
Not evaluable	*13*	*6*	-
EP300 altered	2 (11)	2 (9)	$p = 1.0000$
EP300 wild type	17 (89)	20 (91)	
Not evaluable	*13*	*6*	-
MAPK1 altered	1 (5)	1 (5)	$p = 1.0000$
MAPK1 wild type	18 (95)	21 (95)	
Not evaluable	*13*	*6*	-
AKT altered	1 (5)	2 (9)	$p = 1.0000$
AKT wild type	18 (95)	20 (91)	
Not evaluable	*13*	*6*	-

Table 5: Molecular and histological characteristics according to the best response.

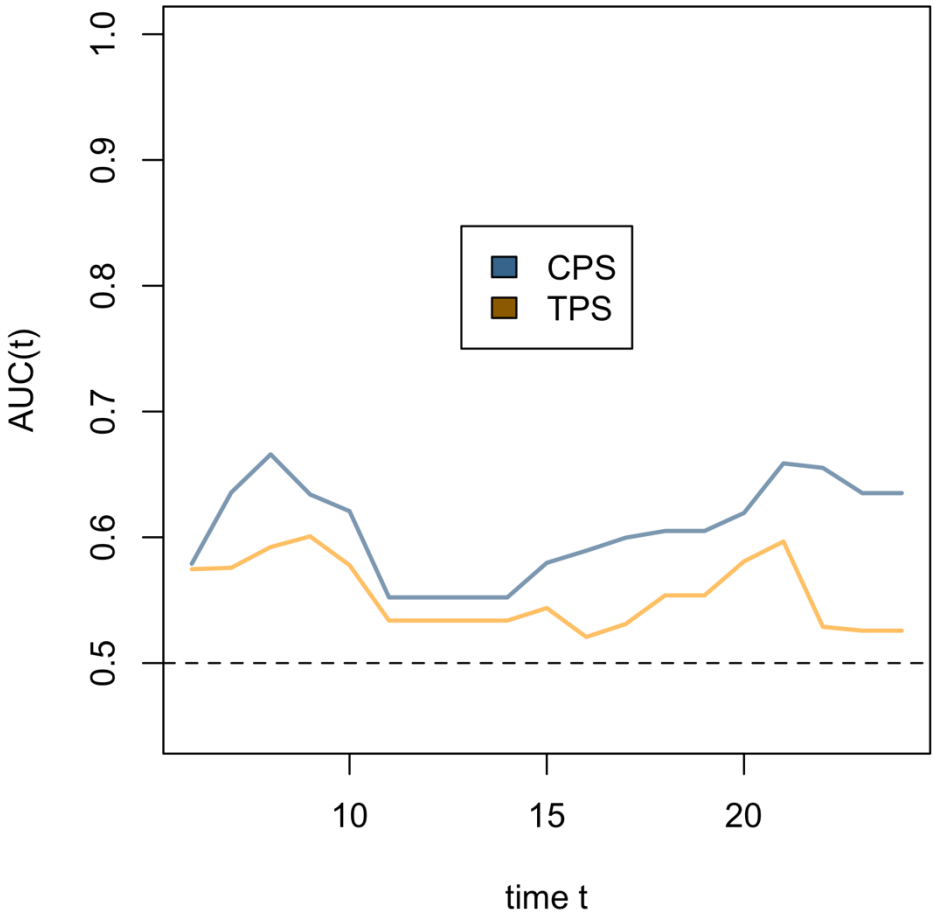

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Figure 3 ROC curves at 24 months for CPS and TPS

Investigating single biomarkers as prognostic: TMB and OS

When using cutoff ≥10 mutations per megabase, patients with high TMB had significantly higher OS compared to patients with low TMB, with HR=0.09; 95%CI 0.01-0.68; *p*=0.019. (Figure 4).

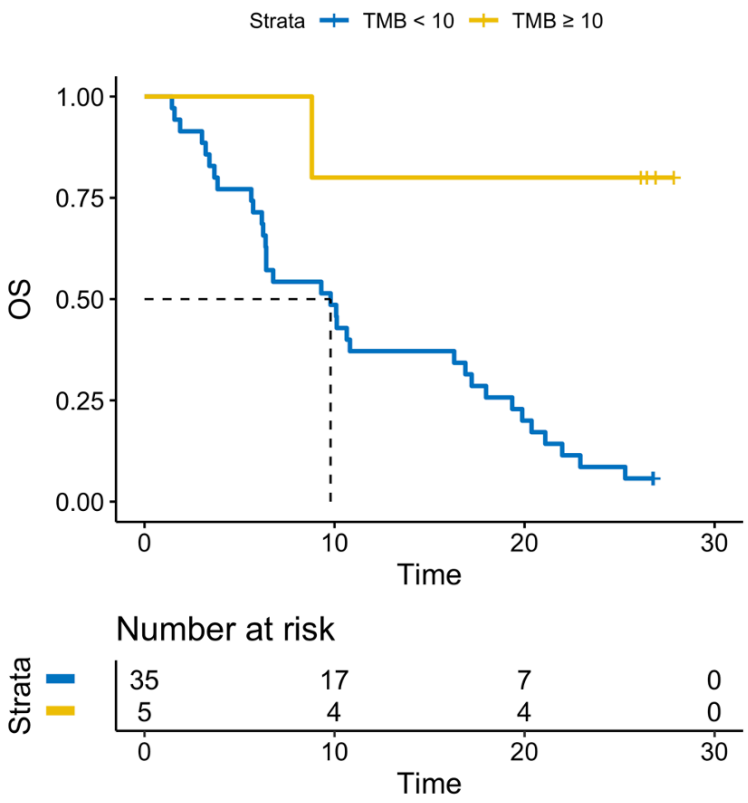

Figure 4 OS according to TMB

Investigating single biomarkers as prognostic: PD-L1 (CPS) and OS

When using CPS > 40 as cutoff, CPS was related to better OS, although statistical significance was not reached. HR=2.19; 95%CI=0.92-5.19; *p*=0.075 (Figure 5).

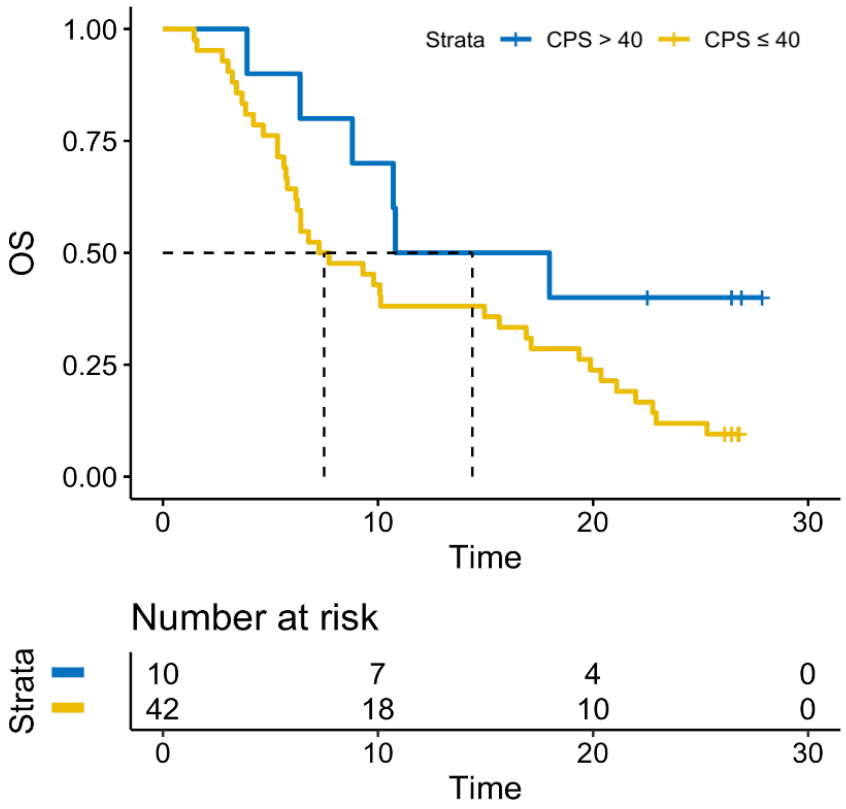

Figure 5 OS according to CPS

Investigating single biomarkers as prognostic: TILs and OS

TILs have an unclear prognostic value: when using the optimal cutoff, the cohort is split in a paradoxical way, having patients with less TILs better prognosis compared to patients with more TILs, with HR=0.77; 95%CI=0.42-1.4; *p*=0.39 (Figure 6).

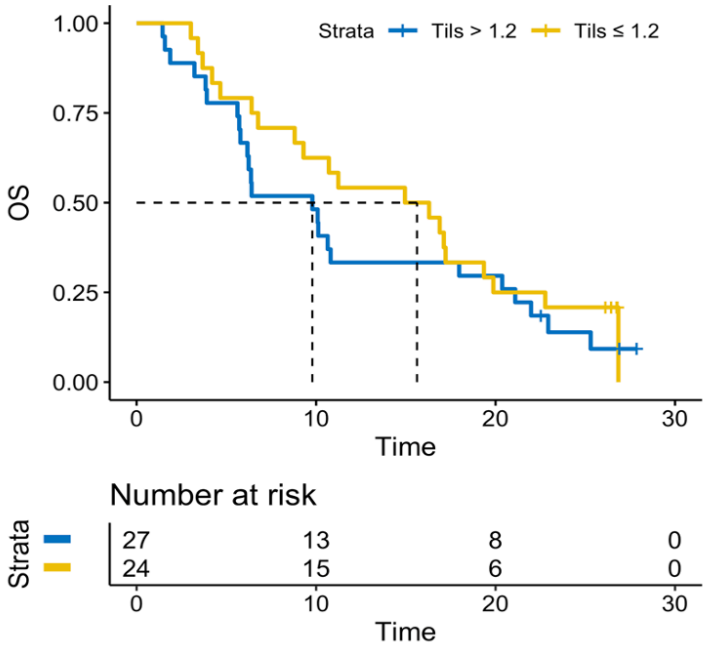

Figure 6 OS according to TILs

Combining TMB and CPS for OS

Presence of either high TMB or high CPS identifies a group of patients with longer OS (Figure 7)

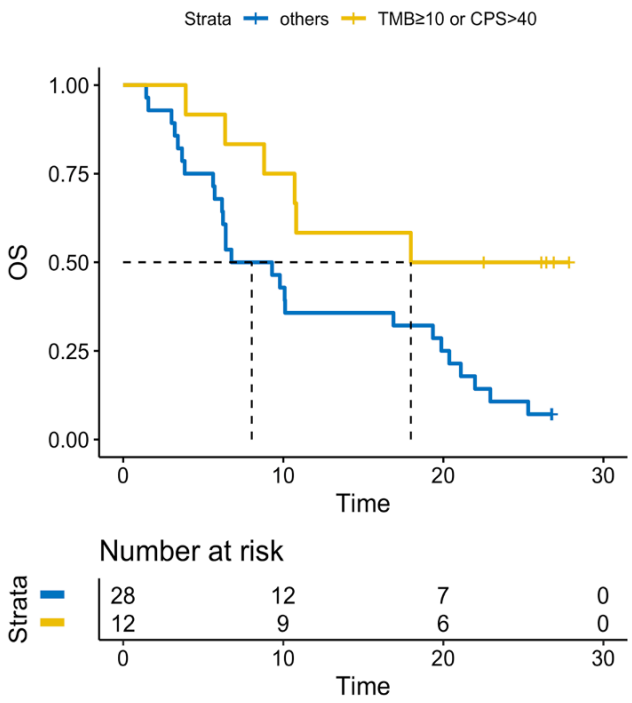

Figure 7 Better OS is related to TMB ≥ 10 or CPS ≥ 40

Combining TMB and CPS and TILs for OS

Adding also patients with Tils ≥ 4 enlarges the group with good prognosis, despite this finding being empiric and potentially influenced by cherry picking. HR=0.43; 95%CI=0.21-0.87; *p*=0.019 (Figure 8).

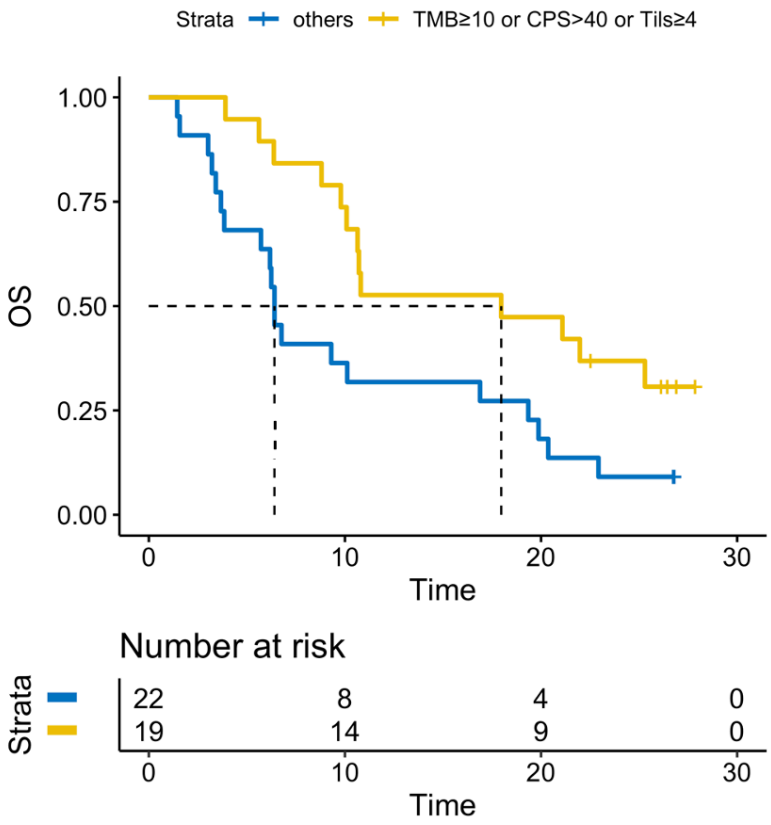

Figure 8 Combining TMB, CPS and TILS for OS

Prognostic impact of mutations on OS

Heatmap showing molecular profile of the cohort. No gene has a clear prognostic impact (Figure 9)

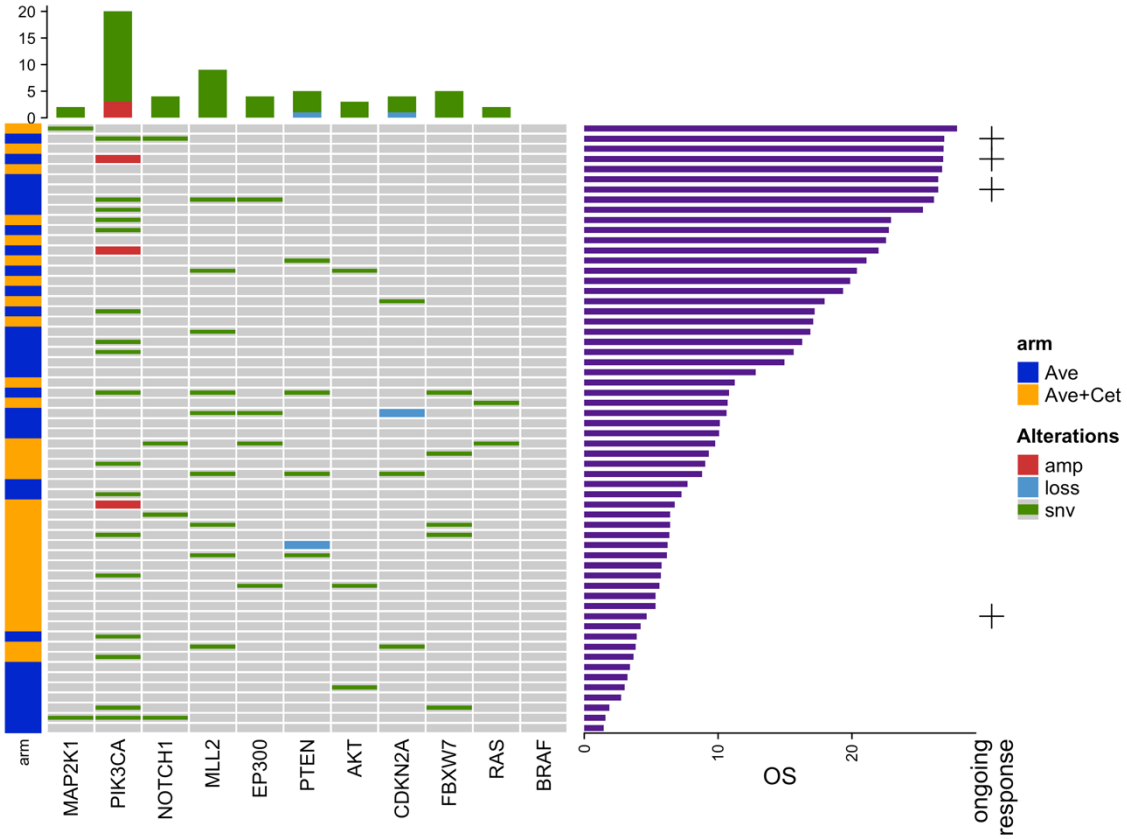

Figure 9 Impact of single genes on OS

Investigating single biomarkers as prognostic: TMB and PFS

Similar trend to OS is observed, although not statistically significant, for patients with TMB ≥ 10: they have better PFS compared to patients with TMB < 10 (HR=0.44; 95%CI=0.15-1.27; *p*=0.129) (Figure 10).

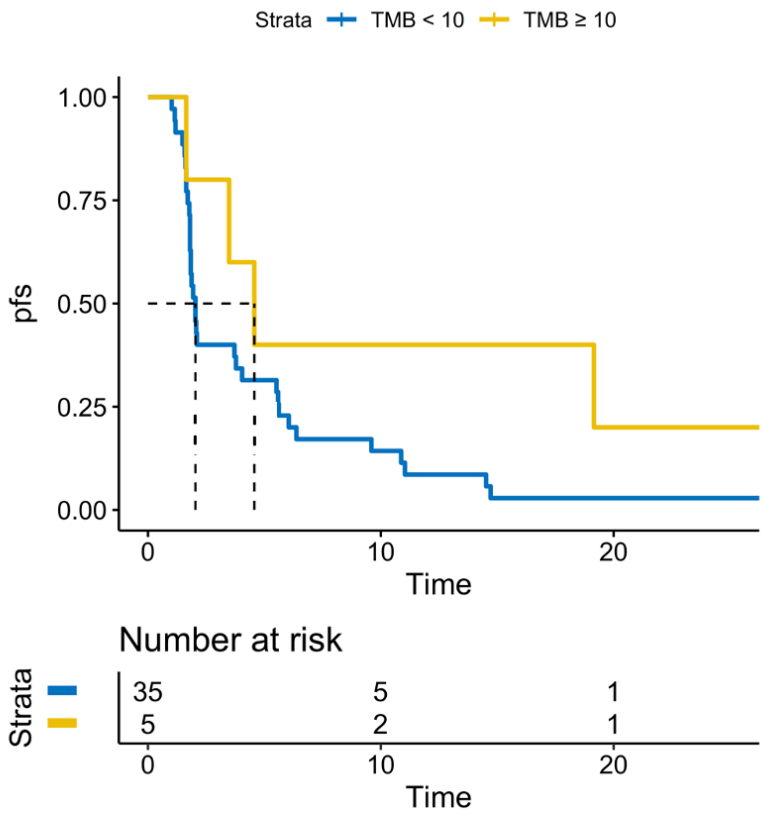

Figure 10 PFS according to TMB

Investigating single biomarkers as prognostic: CPS and PFS

As observed in OS, patients with CPS > 40 show better PFS compared to patients with CPS ≤ 40. However, in contrast with what observed for OS, in PFS the difference is statistically significant, with HR=2.35; 95%CI=1.09-5.1; *p*=0.03 (Figure 11).

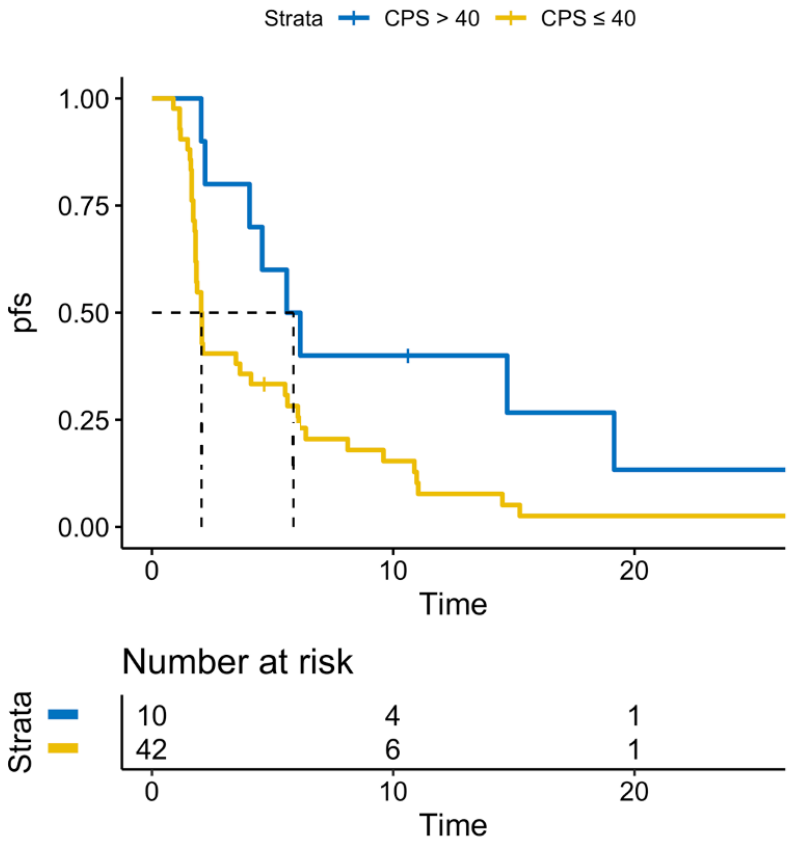

Figure 11 PFS according to CPS

Investigating single biomarkers as prognostic: TILs and PFS

As in OS, TILs do not have a clear impact on PFS, HR=1.19; 95%CI=0.57-2.48; *p*=0.645 (Figure 12).

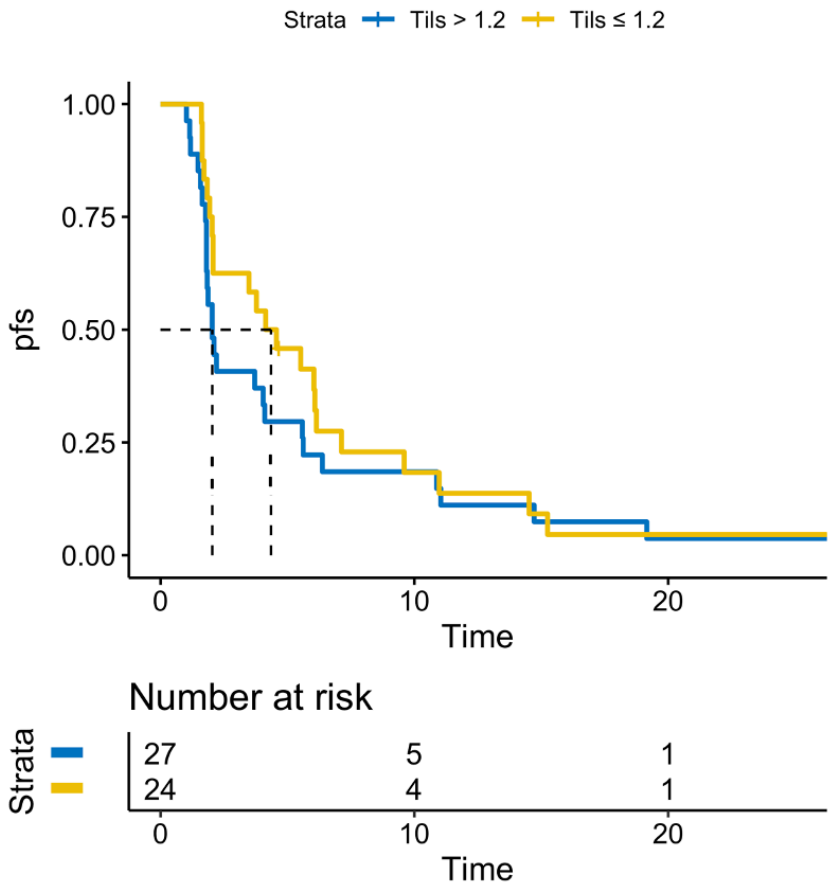

Figure 12 PFS according to TILs

Combining TMB and CPS and Tils in PFS

Presence of either high TMB or high CPS identify a group of patients with longer PFS, HR=0.48; 95%CI=0.23-1.00; *p*=0.051 (Figure 13).

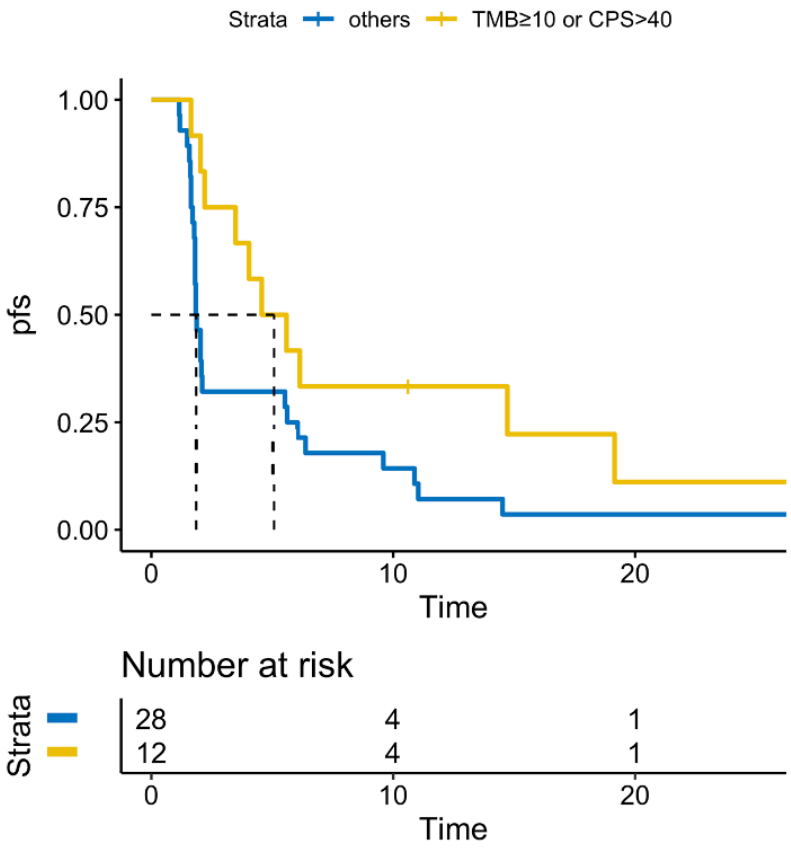

Figure 13 Combining TMB, CPS and TILS for PFS

Working on response

Patients with high TMB or high CPS show a better objective response. patients with both high TMB and high CPS are particularly good responders (Figure 14)

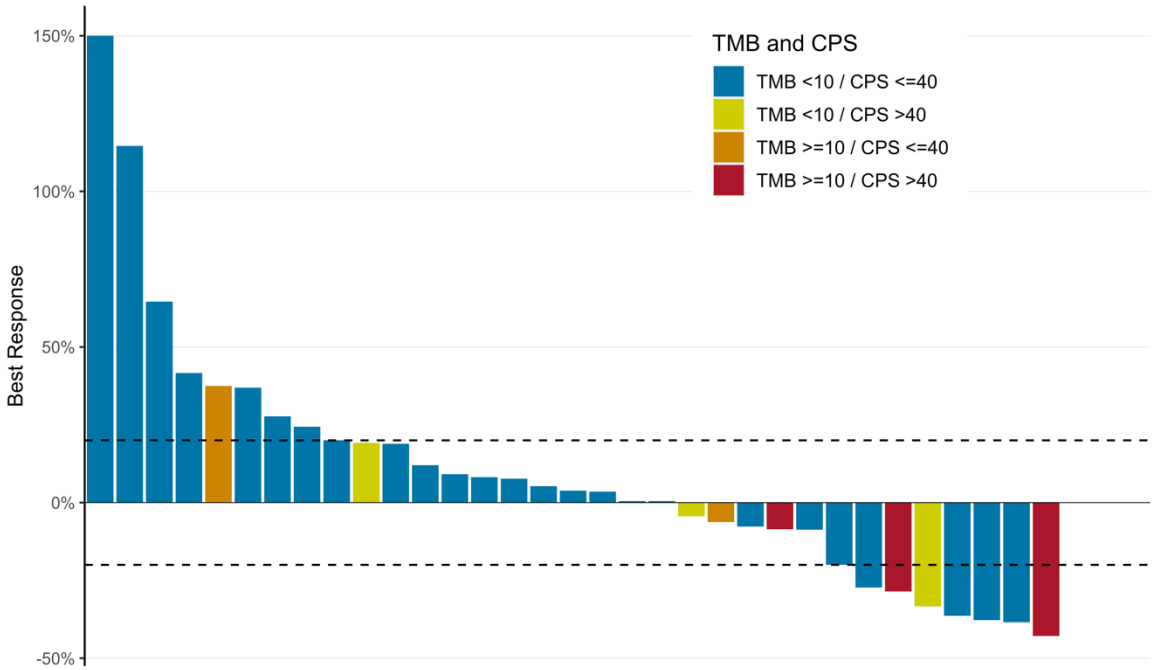

Figure 14 Relation between response, TMB and CPS

Discussion

In the TranslaCARACAS study, a comprehensive molecular characterisation of mSCAC treated with ICB with or without cetuximab was provided.

The most remarkable results of our work regard the predictive and prognostic role of TMB and PD-L1, associated with benefits in survival and with response to ICB with or without cetuximab in our cohort.

Consistently with other cohorts[36, 37], high TMB (≥ 10 mutations per megabase) was found in a small subgroup of patients (12%). In our work, tendence to positive predictive value of high TMB in therapy with ICI was also observed, especially when combined with high PD-L1 expression measured with CPS. Combination therapy of cetuximab and anti-PD-L1 was tested for the first time in SCAC in our study: due to the small number of patients with high TMB, we cannot draw any conclusion about the predictive role of TMB and PD-L1 in each of the two therapeutic strategies separately; nonetheless, the possibility that high TMB might predict response to the combination therapy more than to the ICI monotherapy is intriguing and cannot be excluded. Interestingly, in a recent study testing pembrolizumab in SCAC, a mean higher value of TMB was reported among responders compared to non-responders, even if significant correlation was not found[36].

In our cohort, positive correlation between OS and high TMB was also found, being the same trend observed in PFS; our was, to our knowledge, the first trial documenting TMB prognostic value in a cohort of patients with SCAC treated with ICI. In scientific literature, few data are available regarding prognostic value of TMB in SCAC. As far as we can see from these experiences, OS does not seem to

be influenced by TMB; however, these data concern patients treated with standard treatments, not ICI[37]. As a matter of facts, our finding opens new and interesting scenarios in the research for new prognostic biomarkers for SCAC treated with ICI and is worth of further investigation in larger cohorts.

Regarding PD-L1, our results confirm its frequent expression in SCAC. The prognostic role of PD-L1 expression has been already explored in cohorts of SCAC treated with standard chemoradiotherapy, showing benefit in survival and relapse-free rate[26, 29]. At the same time, Morris et al found positive correlation between PD-L1 expression and response to nivolumab in mSCAC[28]. However, no previous trials investigated on PD-L1 prognostic role in patients with SCAC treated with ICI. In our cohort of patients with SCAC treated with ICI with or without cetuximab, PD-L1 was related to significantly higher PFS; the same trend – although not reaching statistical significance – was observed in OS. Nevertheless, it must be underlined that in PD-L1 some issues are still open and need to be addressed: for example, there is no validated cutoff in SCAC; moreover, both TPS and CPS are widely used. In our study, CPS was chosen because it showed a better AUC for OS prediction at all timepoints; cutoff of > 40% was also considered optimal, while in other studies ≥ 1 was used. These discrepancies might account for differences in outcomes across the studies.

Differently from TMB and PD-L1, TILs did not show a clear prognostic and predictive value: when investigating on their role as prognostic factors, high TILs infiltrate seemed paradoxically related with worse survival. In scientific literature, TILs have been widely studied mainly in localised SCAC, and were found to be predictive of response to standard chemoradiotherapy and prognostic of better

survival outcomes[24, 38]. In other malignancies, like CRC, benefit in survival was confirmed in patients with high TILs infiltration[21]. In our cohort, several factors might account for this controverse result: first of all, TILs were assessed on haematoxylin-eosin without further analysis in IHC due to scarcity of available tissue, so a distinction between cytotoxic and regulator TILs could not be done. Secondly, TILs were quantified on specimens obtained from pre-treatment biopsies, being both primary tumor or metastases accepted: this might account for differences of TILs concentrations. Furthermore, it is known that, in keratinizing neoplasms, TILs are more concentrated in peripheric areas: being most of tissue specimens coming from bioptic material and not from surgery, information about the provenience of the specimen was not available. All these factors could explain the difficulty in drawing conclusions about prognostic and predictive value of TILs in our study.

Another factor deserving special consideration is HPV infection. In SCAC, HPV represents a fundamental driver and one of the most important prognostic factors; its predictive role in immune checkpoint blockade therapy, however, has not been fully explored. In HNSCC, durvalumab was tested in platinum resistant disease in the HAWK study: in this trial, response rate and survival were numerically higher in HPV+ tumors compared to HPV- ones[39].

The biologic rationale of a higher activity of anti-PD-L1 in HPV+ malignancies might be related to the observed upregulation of PD-L1 and PD-L2 on fibroblasts in these neoplasms[40]. In SCAC, HPV+ combined with evidence of high TILs in tumour microenvironment showed improved relapse-free survival (RFS) after

chemoradiotherapy; linear correlation between HPV infection and TILs infiltration was also described[24].

In our trial, however, HPV was not related to better survival and was not predictive of response. On the other hand, in our cohort the large majority of patients were HPV+: given the small sample size, our study might be underpowered to find differences in survival outcomes and response between HPV+ and HPV- patients.

Future directions

Experimenting new strategies is crucial to improve efficacy of PD-L1 blockade in SCAC. In other squamous malignancies, anti-PD-L1 agents have been successfully tested in association with chemotherapy. In SqNSCLC, in the KEYNOTE-407 trial, adding the anti-PD-1 pembrolizumab to standard chemotherapy doublets significantly improved PFS and OS, primary endpoints of the study, irrespectively of PD-L1 expression[41]; as well, the anti-PD-L1 atezolizumab showed benefit in PFS when in association with chemotherapy in the IMpower 131 trial[42]. Both studies were conducted in chemotherapy-naïve patients.

In the first-line setting in SCAC, modified DCF chemotherapy with or without atezolizumab is under investigation by a randomised, non-comparative phase 2 trial[43]. Also, carboplatin-paclitaxel with the anti-PD-1 monoclonal antibody retifanlimab or placebo is tested by the phase 3, placebo-controlled, double-blind InterAACT2 trial (NCT04472429). Regarding the combination with biological agents, a recent study did not show signals of synergistic activity when adding bevacizumab to atezolizumab in patients with previously treated SCAC[44].

Conclusions

To our knowledge, TranslaCARACAS was the first study to document prognostic role of TMB and PD-L1 in mSCAC treated with ICI. Undoubtedly, further investigation in larger cohorts is warranted to confirm our findings, and given the rarity of mSCAC on one hand and the lack of standard target therapies on the other hand, every effort could be precious for these patients. Considering the overall limited activity of ICI monotherapy in mSCAC, however, new biomarkers predictive of response are needed to select those patients who might benefit most from ICI. Moreover, testing new therapeutic associations or strategies comprising ICI could enhance their activity, in order to give new therapeutical options in this rare and aggressive disease.

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