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ORIGINAL RESEARCH ARTICLE

Targeted Therapy in Salivary Gland Cancer: Prevalence of a Selected Panel of Actionable Molecular Alterations in a German Tertiary Referral Center Patient Cohort

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Abstract

Objective Salivary gland carcinomas (SGC) are a heterogeneous group of malignancies, with 24 subtypes defined by the World Health Organization (WHO). The standard of therapy is surgical resection, with adjuvant radiotherapy in most cases. However, disease recurrence (R) or metastasis (M) is common and no active systemic therapies are currently available for RM-SGC resulting in a 5-year survival rate of only 20%.

Patients and Methods Overall, 55 SGC patients with seven different histological tumor subtypes were included in this study. formalin-fixed paraffin-embedded (FFPE) tissue samples were used for immunohistochemical (IHC) staining targeting HER2/neu, androgen receptor (AR), PD-L1, EGFR, panTRK, and TROP2. Fluorescence in situ hybridization (FISH) was performed for detecting HER2/neu amplifications and NTRK1/2/3 translocations in selected cases with relevant HER2/neu and panTRK protein expression, respectively. IHC and FISH results were correlated with patients' clinical and histopathological data.

Results The overall prevalence of druggable molecular alterations, defined as an immunoreactive score ≥ 9 in at least one of the analyzed targets, was 54.4% with the highest percentage in oncocytic carcinomas (100%) and lowest percentage in acinic cell carcinomas (10%). EGFR overexpression proved to be the most common alteration (32.7% of cases) followed by overexpression of TROP2 (27.3%), AR (10.9%), HER2/neu (7.3%), PD-L1 (1.8%), and panTRK (1.8%). HER2/neu amplifications were found in 50% and NTRK translocations were found in 100% of all cases with elevated Her2/neu and panTRK protein expression, respectively.

Conclusions Our data indicate that targeted therapy using e.g., trastuzumab deruxtecan, bicalutamide, pembrolizumab, cetuximab, entrectinib or sacituzumab govitecan might be a promising option especially for a relevant subset of patients with RM-SGC not suitable for salvage surgery. However, evidence from clinical studies regarding response rates to these therapies remains sparse, which underlines the need of multicenter clinical trials.

Key Points

Salivary gland cancers (SGC) show potentially druggable molecular alterations in 54.4% of cases.

The prevalence and patterns of molecular alterations differ significantly depending on histological subtype.

EGFR, TROP2, and AR were the most common detectable targets in our SGC cohort.

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1 Introduction

Salivary gland cancer (SGC) is a group of very rare tumors that develop from the large (parotid gland, submandibular gland, and sublingual gland) and more seldom from the small accessory salivary glands (SSG) with an incidence of 1:100.000 per year [1]. Histologically, SGCs comprise of very heterogeneous entities with 24 subtypes currently defined by the World Health Organization (WHO) on the basis of histopathological as well as genetic features [2, 3]. The standard of therapy is surgical resection, with adjuvant radiotherapy in most cases [4]. However, disease recurrence (R) or distant metastasis (M) is common, affecting more than half of all patients with SGC and there is a relevant lack of active systemic therapies that are available for RM-SGC, which results in a 5-year survival rate of only 20% [1, 5, 6]. Owing to the rarity of this disease, the only specific targeted therapies that are currently approved for SGC treatment in Europe are NTRK- and RET-inhibitors [7, 8]. FDA pan-cancer approvals in solid cancer cases with HER2 overexpression, BRAF p.V600E mutation, MMRd, and TMBhigh findings offer additional therapeutic options in the USA [9].

Over the past years, single case studies and small clinical phase I/II trials were reporting targeted therapy approaches in patients with RM-SGC including e.g., HER2/neu, androgen receptor (AR), NTRK, PD-L1, EGFR, tyrosine kinases, VEGFR2, and RET as potential therapeutic targets [6, 7, 10–24]. However, those studies were investigating target-specific treatment of only one selected molecular alteration, respectively. Data on the prevalence and therapeutic relevance of multiple molecular alterations in this cancer entity, e.g., umbrella studies remain sparse [25]. Additionally, most studies included only patients with one histological SGC subtype and reported highly heterogeneous response rates ranging from 4 to 100% [5, 14]. Evidence from large scale phase III trials as well as a head-to-head comparisons of targeted therapy approaches with clinical gold standard i.e., cytostatic chemotherapy is still missing [14]. Comparably, there exist only sparse data on the overall prevalence of multiple druggable molecular alterations in well characterized and large-scale patient cohorts, which would be of great importance to answer the questions of (i) how many patients with SGC are suitable for targeted therapy approaches and (ii) what panel of predictive molecular biomarkers should be tested.

Against this background, our study aimed to analyze the prevalence of six druggable molecular alterations in a cohort of 55 patients with SGC to elucidate the potential of targeted therapy approaches in this highly malignant and aggressive human cancer entity. Our analyses included immunohistochemical analyses of HER2/neu, panTRK, TROP2, EGFR, PD-L1, and AR expression as well as fluorescence in situ hybridization (FISH) analyses for potential HER2/neu gene amplifications and NTRK1/2/3 gene fusions. Those molecular targets were carefully selected on the basis of current literature evidence on clinical activity in SGC patients as well as the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) [26]. Immunohistochemical (IHC) and FISH results were correlated with the patients clinical and histopathological data as well as patient outcome.

2 Materials and Methods

2.1 Patients and Tissue Samples

In total, 55 patients with salivary gland cancer were retrospectively included in our study with seven different histological subtypes as shown in detail in Table 1. All patients were treated at the department of otorhinolaryngology, head and neck surgery at Saarland University Medical Center (Homburg, Germany) between 2012 and 2022. Tumor node metastasis (TNM) and American Joint Committee on Cancer (AJCC) stages were defined according to the 8th version of the AJCC/Union for International Cancer Control (UICC) head and neck cancer staging system [27]. Cases that presented before 2018 were reclassified to improve comparability between all patients of our cohort. Tumor tissue of the patients was obtained during surgical tumor resection.

The Saarland Medical Association ethics review committee approved the scientific use of the patients' tissue and clinical data (index number 218-10). All experiments were performed according to the relevant guidelines and regulations. Written informed consent was obtained from all patients.

2.2 Immunohistochemistry

Immunohistochemical staining was used to analyze expression levels of six different proteins that represent potential targets of therapeutic strategies. Figure 1 gives an overview of those targets and currently available, clinically tested antibodies, antibody–drug conjugates (ADC) and small molecules that can be used to target those proteins. The selection of targets was on the basis of current evidence for a potential therapeutic benefit in patients with SGC as shown in preclinical data and early clinical trials (see discussion for details). All target proteins met the ESCAT (ESMO Scale for Clinical Actionability of molecular Targets) criteria for defining targets for cancer precision medicine according to the ESMO Translational Research and Precision Medicine Working Group [26]. For immunohistochemical staining, the

Table 1 Clinical and histopathological data of the included patients.

		Number of patients
Total		55
Sex	Male	31
	Female	24
primary tumor	PG	43
	SMG	10
	SSG	2
T-Stage	T1	17
	T2	20
	T3	12
	T4	6
N-Stage	N0	36
	N1	5
	N2	10
	N3	4
M-Stage	M0	42
	M1	13
Tumor histology	ACC	15
	AciCC	11
	ADC	8
	MEC	7
	SDC	7
	MUC	5
	OCC	2
Therapy	Surgery	18
	Surgery + RT	26
	Surgery + PBT	1
	Surgery + CRT	9
	Primary CRT	1
AJCC Stage	1	15
	2	13
	3	5
	4	22

PG, parotid gland; SMG, submandibular gland; SSG, small salivary glands; ACC, adenoid cystic carcinoma; AciCC, acinic cell carcinoma; ADC, adenocarcinoma; MEC, myoepithelial carcinoma; SDC, salivary duct carcinoma; MUC, mucoepidermoid carcinoma; OCC, oncocytic carcinoma; RT, radiotherapy; CRT, chemoradiotherapy; PBT, proton beam therapy

tumor tissue was formalin fixed, paraffin embedded and cut into 3 µm slices with a Leica RM 2235 rotation microtome (Leica Microsystems, Wetzlar, Germany). The slices were then transferred onto Superfrost Ultra PLUS microscope slides (Menzel-Gläser, Braunschweig, Germany) and dried in an incubator at 37°C overnight. Hematoxylin and eosin (HE) staining was performed for all tissue samples according to a standard protocol for morphological control. For immunohistochemical detection of the target proteins TROP2, HER2/neu, panTRK, EGFR, PD-L1 and AR, heat-induced epitope unmasking was performed upon deparaffinization in a rice cooker using 10 mM sodium citrate buffer, pH 6.0. In a next step, unspecific protein binding was blocked by incubating the slides in PBS (pH 7.2) with 3% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, USA) for 30 min at room temperature (RT). The slides were then incubated with the primary antibody at 4 °C overnight. The final concentrations of the primary antibodies were 1:2000 for TROP2 (ab227691, Abcam, Cambridge, UK), 1:500 for HER2/neu (ab214275, Abcam, Cambridge, UK), 1:1500 for panTRK (ab76291, Abcam, Cambridge, UK), 1:1000 for EGFR (ab52894, Abcam, Cambridge, UK), 1:400 for PD-L1 (ab210931, Abcam, Cambridge, UK), and 1:1500 for AR (ab133273, Abcam, Cambridge, UK) in PBS/1% BSA v/v, each. Immunohistochemical staining was visualized with streptavidin-labeled alkaline phosphatase and chromogen red using the Dako REAL Detection System Alkaline Phosphatase/RED (K5005, Dako Agilent, Santa Clara, CA, USA) following the manufacturer's instructions. In a final step, the slides were counterstained with hematoxylin (Sigma Aldrich) and mounted with Entellan (Merck, Darmstadt, Germany). Every staining series included negative controls by omitting the primary antibody as well as appropriate positive controls (head and neck squamous cell carcinoma for TROP2, EGFR, and PD-L1; invasive ductal breast cancer for HER2/neu; mouse brain tissue for panTRK; prostate cancer for AR). The stained slides were semiquantitatively analyzed using an immune reactive score (IRS) according to Remmele and Stegner [28]. First, the relative number of stained tumor cells was valued from 1 to 4 (0—no positive tumor cells, 1—< 10% positive tumor cells;+, 2—11–50% positive tumor cells, 3—51–80% positive tumor cells, and 4—> 80% positive tumor cells). Second, the staining intensity of positive cells was valued from 0 to 3 (0—no staining, 1—low staining intensity, 2—medium staining intensity, 3—strong staining intensity). Both values were then multiplied, resulting in an IRS ranging from 1 to 12. For PD-L1, tumor proportion score (TPS; percentage of viable tumor cells showing partial or complete membrane staining for PD-L1 at any intensity) and combined positive score (CPS; number of PD-L1 positive cells including tumor cells and leucocytes relative to all viable tumor cells) were additionally estimated due to their clinical relevance. Three examiners including one board-certified pathologist independently analyzed every IHC staining. All examiners were blinded for the clinical diagnosis and the other examiners' scoring.

2.3 Fluorescence in Situ Hybridization (FISH)

FISH was carried out on 3-µm-thick sections cut from each formalin-fixed paraffin-embedded (FFPE) sample using the ZytoLight FISH-Tissue Implementation Kit (Z-2028,

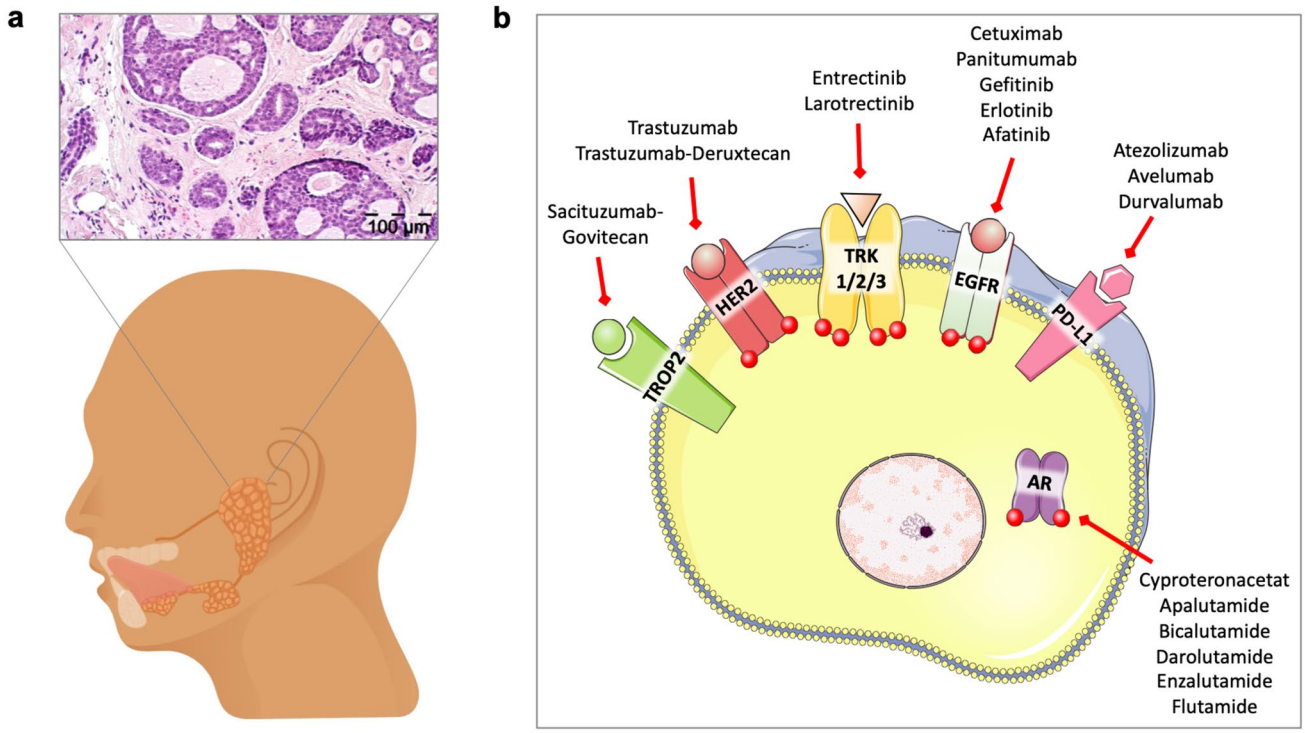

Fig. 1 **a** Location of the major salivary glands and histological image of an adenoid cystic carcinoma representing the second most common SGC subtype. **b** Potential therapeutic targets and respective drugs for a targeted therapy of patients with SGC

ZytoVision, Bremerhaven, Germany) according to the manufacturer's instructions.

HER2 amplification was assessed by FISH using ZytoLight SPEC ERBB2/D17S122 Dual Color Probe (Z-2190, ZytoVision), and defined as HER2 copy number per centromere of chromosome 17 signal ratio is greater than 2. For all tumor samples, FISH signals were counted by two independent observers in a total of 50 randomly selected and non-overlapping nuclei. As internal control, an ERBB2 Control Slide Set (E-4007-2, ZytoVision) was used.

NTRK1, NTRK2, and NTRK3 fusion detection was performed on all pan-TRK positive cases ($n = 6$) by three separate assays using specific break-apart probes for each gene (ZytoLight SPEC NTRK1 Dual Color Break Apart Probe (Z-2167); ZytoLight SPEC NTRK2 Dual Color Break Apart Probe (Z-2205); ZytoLight SPEC NTRK3 Dual Color Break Apart Probe (Z-2206); ZytoVision). FISH signals were counted by two independent observers in up to 100 randomly selected and non-overlapping nuclei. FISH was considered positive for an NTRK fusion if ≥ 15% tumor cells showed a separation of red and green signals with a minimum of two signal diameters. As internal control, an NTRK positive breast cancer tumor sample was hybridized in parallel.

Slides were counterstained with DAPI in an antifade solution and the hybridized slides were examined using a BX61 fluorescence microscope (Olympus, Hamburg, Germany). Images were captured using a Hamamatsu C11440-36U camera (Hamamatsu City, Japan) and the CellSens imaging system Software version 4.1.1 (Olympus, Hamburg, Germany).

2.4 Statistical Analysis

For statistical analyses and figure design, Prism v10.2.3 (GraphPad Software Inc., Boston, MA, USA) was used. D'Agostino & Pearson omnibus normality test, Anderson-Darling test, Shapiro–Wilk test, and Kolmogorov–Smirnov test were used to determine if datasets follow a Gaussian distribution in each comparison. Gaussian distribution was only assigned if the data sample passed ≥ 2 of the aforementioned normality tests. If the data showed a normal distribution, parametric tests were performed (two-tailed unpaired *t*-tests). If the data showed no normal distribution, non-parametric tests were applied (Mann–Whitney *U* test). For survival analyses, a log rank test was used. p Values < 0.05 were considered statistically significant ($\alpha = 0.05$). In the figures, statistically significant results are labeled with asterisks: $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. AVATAR graphics for illustration of alteration data were designed using publicly available avatar software (https://github.com/sysbio-bioinf/avatar) [29].

3 Results

3.1 Prevalence of Actionable Molecular Alterations

In total, 55 patients with SGC were included in our study comprising 56% male and 44% female patients with a mean age of 60.7 years. Median patient follow-up was 61.1 months. Most patients showed tumors of the parotid gland (n = 43, 78%) followed by tumors of the submandibular gland (n = 10, 18%) and tumors of the small salivary glands (n = 2, 4%). Details on TNM and AJCC stages as well as clinical data including therapeutic regimens are shown in Table 1.

First, FFPE tissue samples of all included patients were used for IHC staining targeting HER2, panTRK, TROP2, EGFR , PD-L1, and AR (Fig. 2a–f). The staining results were quantified using an immunoreactive score ranging from 0 (no staining) to 12 (strong staining). A relevant expression of the abovementioned proteins sufficient for a targeted therapy approach was defined as IRS ≥ 9. Additionally, copy number alterations of the HER2/neu gene as

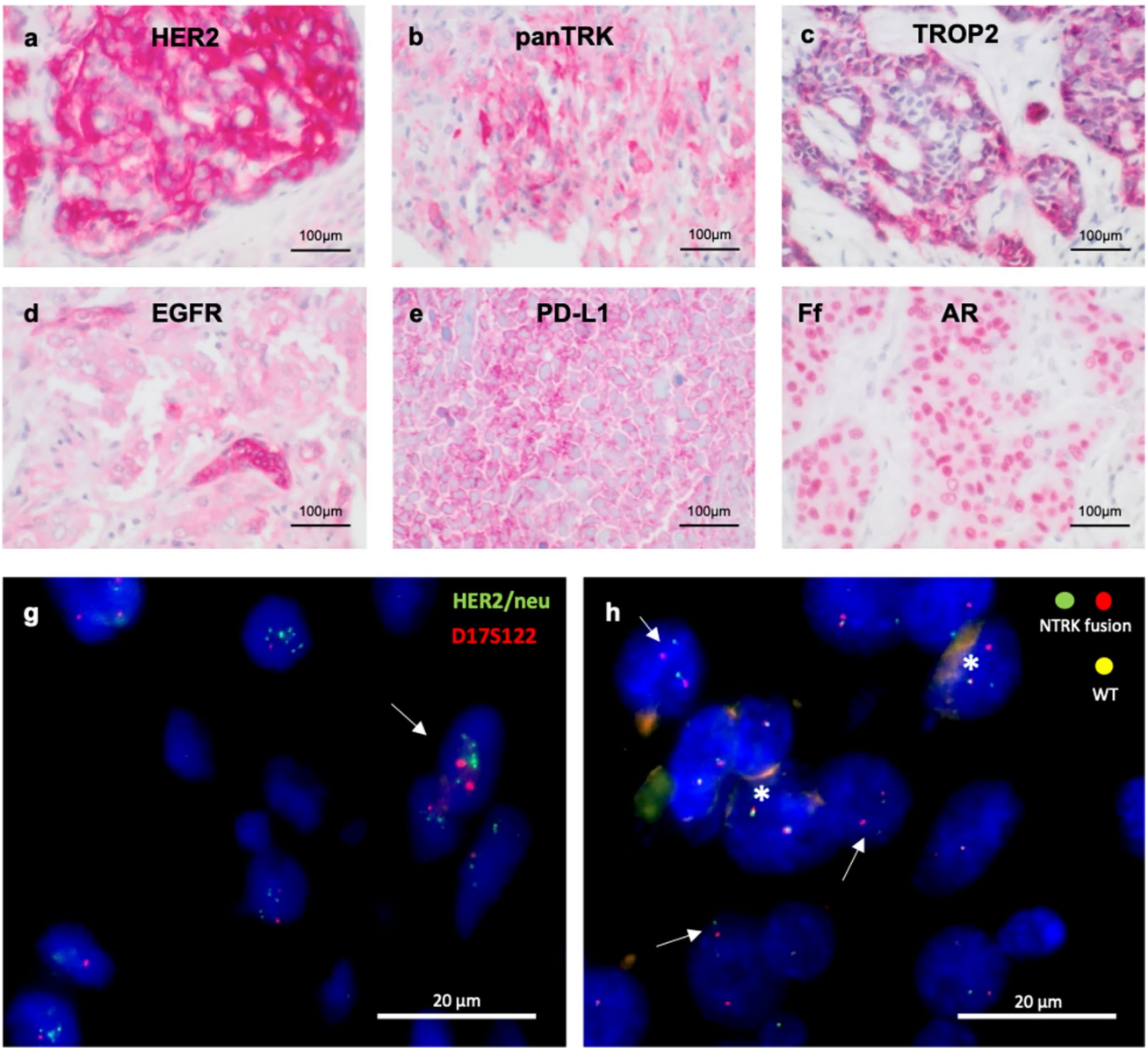

Fig. 2 Exemplary images for positive immunohistochemical staining of SGC cases targeting **a** HER2/neu, **b** panTRK, **c** TROP2, **d** EGFR, **e** PD-L1, and **f** AR. In **a–f** positive IHC signals are shown in red. **g** HER2/neu FISH of an ACC case showing amplifications of the HER2/neu gene (white arrow). **h** NTRK3 FISH of an AciCC case using break-apart probes and showing physiological signals (yellow signals, labeled with white stars) and gene translocations indicated by separated green and red signals (labeled with white arrows)

well as translocations of the NTRK1, NTRK2, and NTRK3 gene were analyzed using FISH with dual color probes and break-apart probes, respectively (Fig. 2g–h). In total, 54.4% (30/55) of tumors showed a strong expression (IRS ≥ 9) of at least one actionable target with 34.5% (19/55) showing overexpression of one target, 18.1% (10/55) showing overexpression of two targets and 1.8% (1/55) showing overexpression of three targets. Overexpression of the EGFR gene was the most frequent molecular alteration with an IRS ≥ 9 in 32.7% (18/55) of all cases, followed by overexpression of TROP2 (27.3%; 15/55), AR (10.9%; 6/55), HER2/neu (7.3%; 5/55), PD-L1 (1.8%; 1/55), and panTRK (1.8%; 1/55; Fig. 3). For better understanding, those alteration data have also been visualized as AVATAR graphic (Supplementary Fig. 1). Additionally, all SGC cases with a moderate to strong HER2/neu expression [IRS ≥ 6; $n = 14$: 4 ADC, 3 adenoid cystic carcinoma (ACC), 2 AciCC, 2 OCC, 2 SDC, and 1 MUC] were analyzed for copy number alterations of the HER2/neu gene using FISH. Copy number gains and/or amplifications were observed in 7/14 cases (2 ADC, 2 SDC, 1ACC, 1 AciCC, and 1 OCC). Moreover, all SGC cases with a moderate to strong panTRK expression (IRS ≥ 6, $n = 6$) were screened for translocations of the NTRK1, NTRK2, and NTRK3 gene. All investigated cases showed at least translocations for NTRK1, two cases also showed translocations for NTRK2, and three cases for NTRK3. For PD-L1, CPS and TPS were calculated in addition to the immunoreactive score. Here, $n = 30$ cases (54.5%) showed a CPS ≥ 1, $n = 4$ cases (7.3%) showed a CPS ≥ 20 and $n = 1$ case (1.8%) showed a TPS ≥ 50% (detailed results for PD-L1 IRS, CPS, and TPS are shown in supplementary Table 1).

3.2 Correlation of Molecular Alterations with Clinical and Pathological Features

Next, we correlated the expression levels of EGFR, TROP2, AR, HER2/neu, PD-L1, and panTRK with histological and clinical features including TNM stage, AJCC stage, sex, and primary tumor site. Here, patients with distant metastases showed significantly higher PD-L1 expression levels ($p = 0.0034$) and significantly lower panTRK expression levels ($p = 0.034$) compared with patients without distant metastases. No further correlations were observed.

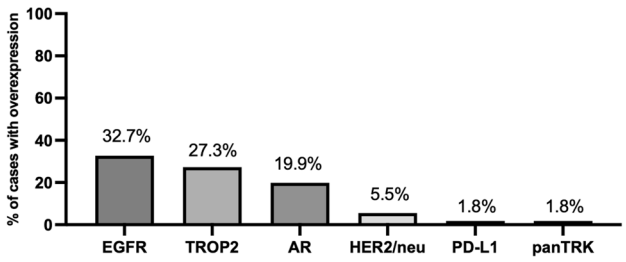

Fig. 3 Percentage of SGC cases with overexpression (IRS ≥ 9) of the respective molecular target

3.3 Prognostic Relevance of Clinical and Histopathological Features

To identify clinical and histopathological factors that significantly influence patient overall survival, Kaplan–Meier plots were used for a univariate analysis of prognostically relevant factors. Median overall survival of the included patients was 81 months (6.8 years) with a 5-year overall survival rate of 71%. Advanced AJCC stages, advanced T-stages, the presence of lymph node metastasis as well as distant metastasis significantly correlated with a poor outcome (Fig. 4b–e). Furthermore, tumor histology had a significant impact on overall survival with the best prognosis for ADC followed by AciCC, ACC, MEC, SDC, and MUC (Fig. 4a). Regarding the potential therapeutic targets that were investigated in this study, only PD-L1 expression in tumor cells showed a significant influence on overall survival with high expression levels being correlated with worse outcome (Fig. 4f). Patient age, sex, treatment strategy, and tumor localization showed no significant correlation with patient outcome (data not shown).

3.4 Expression Patterns Depending on Histological Subtype

Next, the frequency of actionable molecular alterations was analyzed separately for the 7 histological subtypes that were included in our study. Thereby, OCCs showed the highest percentage of cases with overexpression of at least one actionable target (2/2, 100%), followed by ADC (7/8, 87.5%), MUC (4/5, 80%), MEC (5/7, 71%), SDC (4/7, 57%), ACC (6/15, 40%), and AciCC (2/10, 20%; Fig. 5a). The most frequent molecular alterations were EGFR overexpression in ACCs, AciCCs, MECs, and MUCs, TROP2 overexpression in ADCs and AR overexpression in SDCs. Notably, 46% of all TROP2 overexpressions were found in ADCs, and 50% of all AR overexpressions were found in SDCs.

4 Discussion

Salivary gland carcinomas (SGC) are a rare and highly heterogeneous malignancy of the large and small salivary glands that are associated with a poor prognosis especially in recurrent and metastatic cases. Currently there is a relevant lack of active systemic therapies that are available for RM-SGC and there exist only sparse data on the overall prevalence of multiple druggable molecular alterations in well characterized and large-scale patient cohorts.

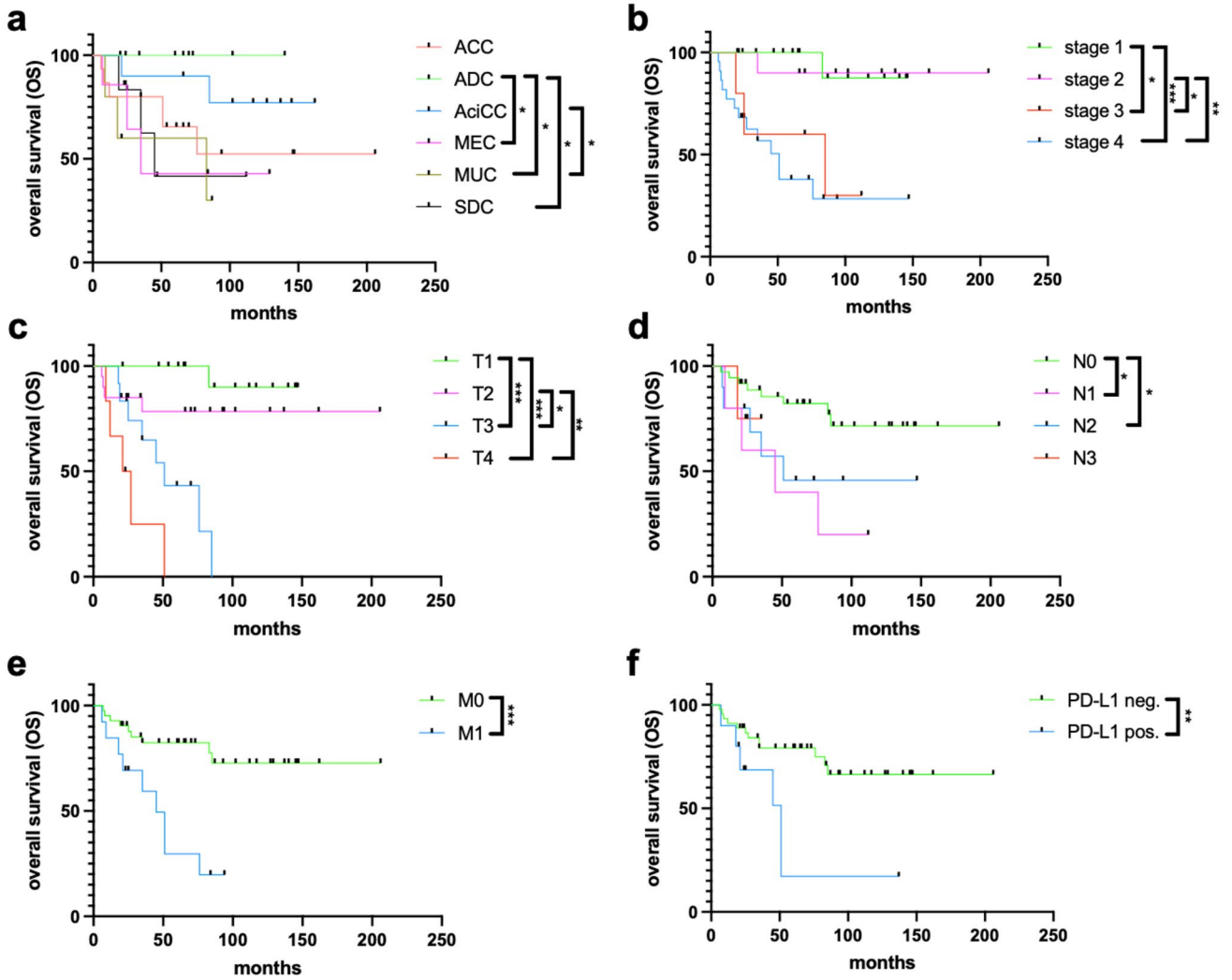

Fig. 4 Clinical, histopathological and molecular factors with a significant impact on patients' overall survival. **a** Comparison of OS between histological subtypes (only histological subtypes with > 3 cases were included), **b** OS depending on AJCC stage, **c** OS depending on T stages, **d** OS depending on N stages, **e** OS depending on the presence of distant metastasis, and **f** OS depending on PD-L1 expression in tumor cells (positive staining defined as IRS≥2). OS, overall survival

Our study investigated the prevalence of six potentially druggable molecular alterations in a cohort of 55 patients with SGC with seven different histological subtypes using IHC and FISH. Here, the overall prevalence of druggable molecular alterations was 54.4% with EGFR overexpression being the most common alteration followed by overexpression of TROP2, AR, HER2/neu, PD-L1, and panTRK. Supplementary HER2/neu amplifications and NTRK translocations were found in 50% and 100% of cases preselected by IHC, respectively.

Several groups have investigated expression of in most cases only one of the aforementioned molecular targets in different patient cohorts. A panTRK overexpression as a consequence of NTRK gene fusions in patients with SGC was reported in only a few studies with a prevalence ranging from 0.5 to 5% [30]. Remarkably, NTRK fusion positive SGC showed in clinical studies a response rate of 90.9% to larotrectinib [10] and 85.7% to entrectinib [7] with a median duration of response of 35.2 and 10 months, respectively. For clinical testing, European and US clinical guidelines recommend either a screening by panTRK-IHC followed by NTRK-fusion specific FISH in IHC-positive cases or next generation sequencing based approaches [31, 32]. Our study showed a comparably high prevalence of NTRK fusion genes of 10.9% that could be detected not only in SGC cases with strong panTRK expression (i.e., IRS ≥ 9) but also in cases with moderate panTRK expression (i.e., IRS 6–8). Hence, we would recommend to test any SGC case with immunohistochemically detectable panTRK expression for the presence of NTRK fusion genes given the high predictive

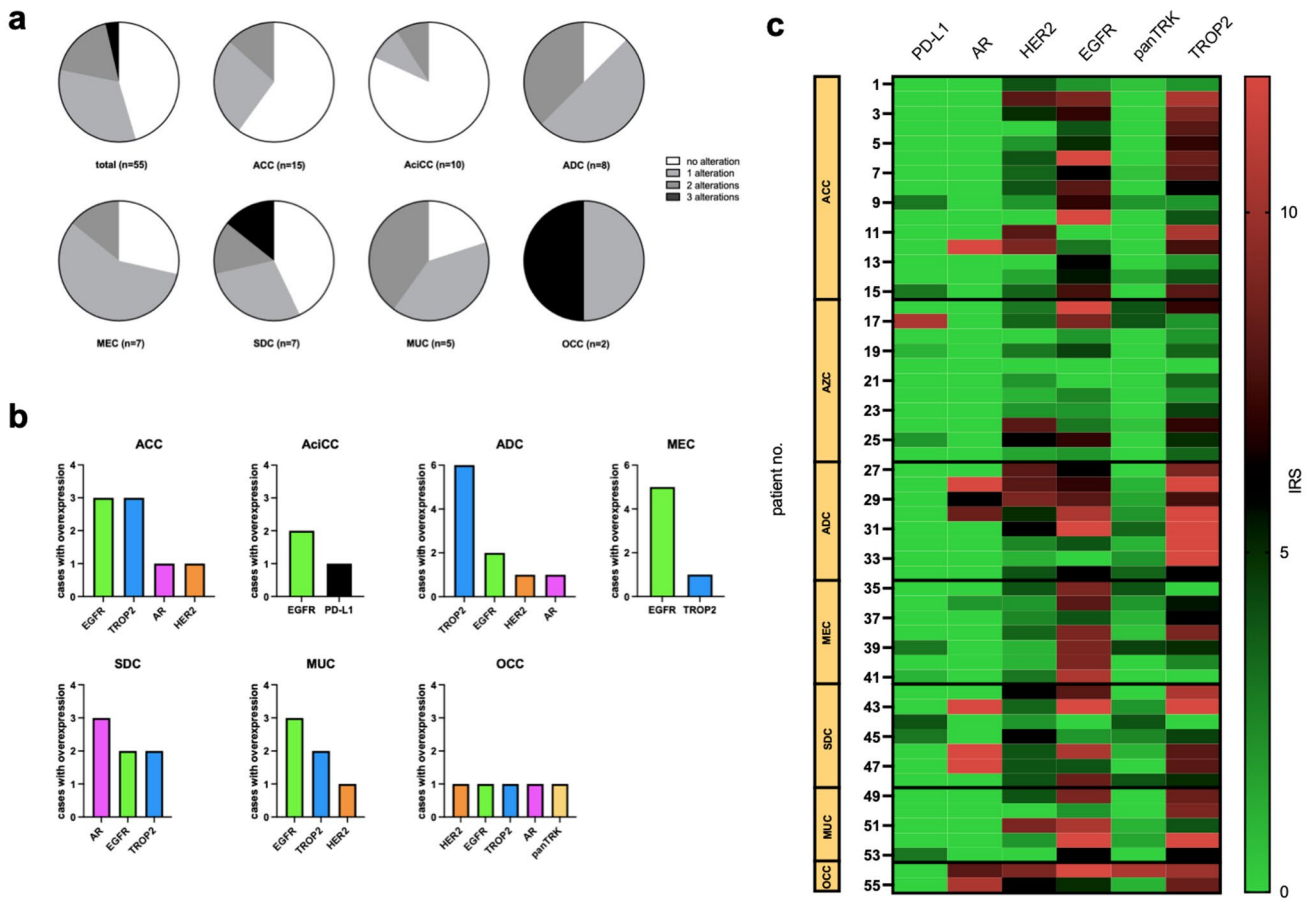

Fig. 5 Distribution of actionable molecular alterations for the seven different histological SGC subtypes. **a** Number of cases with three (black), two (dark grey), one (light grey), or no (white) molecular alteration delineated for the seven different histological subtypes. **b** Frequency of overexpression of potential therapeutic targets depending on histological subtypes. **c** Heat map illustrating IRS values for all five targets delineated for all included cases.

value for clinically relevant response to NTRK inhibitors once a fusion gene is detected [7, 10]. Nonetheless, it must be noted that we did not test cases with low panTRK expression (i.e., IRS 2–5) for the presence of NTRK fusions using FISH in our study.

For PD-L1, studies of the past years showed a heterogeneous prevalence of PD-L1 positive SGC patients ranging from 30 to 60% [33], which is much higher than in our study with only 1.8% positive cases. This discrepancy can mainly be attributed to the threshold of an IRS ≥ 9 that we predefined for assigning a tumor as positive. Any PD-L1 expression independent of IRS was found in 18% of patients in our cohort, 54.4% of cases showed a CPS ≥ 1 and only one case (1.8%) showed a TPS $\geq 50\%$. In terms of a potential therapeutic approach, several prospective phase II trials investigated Anti-PD-1 treatment of SGC patients: in the KEYNOTE-028 basket trial PD-L1 positive SGC patients ($n = 28$) were treated with pembrolizumab and showed an objective response rate of 12% [11]. The NISCAHN trial reported a 6-month non-progression rate of 23.2% in patients with SGC treated with nivolumab and the KEYNOTE-158 trial found an objective response rate of 10.9% in $n = 109$ patients with SGC (MSI-high or DNA mismatch repair deficiency) treated with pembrolizumab [12]. Currently ongoing and future studies will investigate combinations of Anti-PD-1 checkpoint inhibition with cytotoxic chemotherapy as well as other immunotherapy approaches including tumor vaccination to increase response and clinical benefit rates [14]. Which one of the afore mentioned biomarkers (PD-L1 IRS versus TPS versus CPS) correlates best with therapy response to Anti PD-1 inhibition needs to be addressed in future studies.

EGFR overexpression in SGCs was reported in literature in 74–91% of cases [34]. A potential clinical use of EGFR directed therapy in patients with SGC was tested for cetuximab, lapatinib, and gefitinib—all in comparably small and early-phase clinical trials without pre-therapeutic EGFR testing. Together, those studies showed poor response rates with no long-lasting remissions [21, 22, 24]. However, all of those studies did not consider EGFR

expression level on tumor cells for patient selection, which was shown to predict response to Anti-EGFR therapy in other cancer entities e.g., non-small cell lung cancer independent of EGFR mutation status [35, 36]. In this context, a phase-I/II trial was recently initiated that investigates clinical efficacy of EGFR inhibitor therapy in pretested EGFR patients with overexpressing recurrent or metastatic SGC (NCT02069730).

TROP2 represents a glycoprotein of the plasma membrane that is involved in several cellular processes [37]. TROP2 is overexpressed in various human tumor entities and associated with poor patient outcome [38]. Hence, several TROP2-based antibody–drug conjugates (ADC) have been developed over the past years [39]. In 2020, sacituzumab govitecan being the first TROP2-ADC to be approved by the FDA for treatment of non-resectable or metastatic triple-negative breast cancer. So far, only one study investigated TROP2 expression in SGC patients observing a high expression in 44% and a moderate expression in 38% of $n = 114$ patients with SGC [40]. However, to date no clinical trial data are available for TROP2-targeted therapy in salivary gland cancer.

Androgen receptor expression was found in 67–98% of tumor cells in salivary duct cancer (SDC) while AR expression in other SGC subtypes is extremely rare [15]. Anti-androgenic therapy was investigated only in few phase-II trials with small patient cohorts showing response rates to anti-androgenic therapy in patients with AR-positive SGC between 4 and 65% with a duration of response between 5.6 and 11 months [13–15].

Of all potentially actionable molecular alterations investigated in our study, the strongest evidence exists for HER2/neu amplification and/or overexpression in SGC. More than 50 studies have analyzed HER2 expression in SGC over the past years reporting positivity rates from 3 to 84% depending on histological subtype [41]. In this context, first phase-II clinical trials investigated a potential use of Anti-HER2 therapy in a palliative setting of patients with RM-SGC with overall response rates between 60 and 100% with a duration of response up to 18 months in HER2/neu-positive SGC patients [14, 16, 25]. With respect to the detection method, we used both HER2/neu IHC and FISH for those cases with relevant protein expression. For clinical use of trastuzumab in breast cancer either HER2/neu protein overexpression or gene amplification are sufficient for in-label use according to the European Medicines Agency (EMA) and Food and Drug Administration (FDA). However, IHC and FISH showed different power for predicting response to HER2-targeted therapy in this cancer entity [42]. For SGC, clinical evidence regarding the predictive value of HER2/neu IHC versus FISH in trastuzumab treated patients remains sparse, so that no clear recommendation on the preferable detection method can be made.

Altogether, those data on the expression level and potential therapeutic use of the molecular targets that were investigated in our study confirmed the overall low prevalence of their expression across different SGC subtypes. In conclusion, a whole panel of actionable targets, rather than a single biomarker must be tested to identify at least one targeted therapeutic approach in a relevant subset of patients as recently also described for advanced cases of other cancer entities, e.g., head and neck squamous cell carcinomas [43]. In our study, the analysis of six potentially actionable molecular alterations led to the identification of ≥ 1 targeted therapeutic approach in 54.4% of patients. There are undoubtedly several other targets that could have been addressed in our biomarker panel e.g., c-kit, RET, BRAF, and VEGFR with evidence from preclinical and clinical studies for a potential therapeutic relevance in SGC [6]. Nevertheless, we focused on targets for which therapeutic strategies are available, i.e., drugs that are yet approved in patients with non-SGC tumor and that meet the ESCAT criteria for targets in cancer precision medicine [26]. Comparable studies that investigated a panel of different actionable molecular alterations in SGC remain sparse and in most cases focused only on one single SGC subtype, especially SDC. Hence, reliable conclusions on the overall prevalence of druggable alterations in SGC are not possible, which was the basic motivation for our study. Comparably, there are no phase-III clinical trials that compare the efficacy of the abovementioned targeted therapy approaches in patients with SGC with standard cytotoxic chemotherapy, which underlines the urgent need for large-scale multi-center clinical trials. Owing to the rarity and heterogeneity of this disease patient recruitment will remain a major challenge for generating evidence from clinical trials so that new study designs, e.g., umbrella or basket studies should be considered to overcome those barriers [25].

There are several important caveats to this analysis. First, our study investigated a small cohort of 55 patients with SGC. These are rare cancer types, but nevertheless, the six-target-panel should be investigated in a much larger patient cohort in order to confirm the prevalence numbers we have observed in our center. A second limitation is the heterogeneity across different malignant salivary gland histologies: we selected seven SGC subtypes, and do not have data for even more rare histologies. Regarding the potential therapeutic relevance of the molecular alterations tested in our study, we have to note that, owing to the retrospective nature of our study, only two of the included patients with SGC actually received a targeted therapy approach. One patient with SDC with liver and bone metastases and strong AR expression in a biopsied liver nodule treated with bicalutamide showed a partial response for 23 months. Another patient with ACC metastasized to the lung and histologically proven EGFR-expression in the tumor cells treated with Gefitinib showed stable disease for 17 months. For all other patients,

one can only speculate if biomarker expression would have indicated response to a targeted therapy approach. Given the evidence of the available phase-II clinical trials, one would not expect every patient with an actionable molecular alteration to experience tumor response after targeted therapy. Nonetheless, complete and ongoing responses were reported in single cases for all of the therapeutic approaches discussed above so that we strongly recommend molecular testing especially in RM-SGC cases with no available therapeutic alternatives. In addition to the clinically feasible and cost-sparing IHC testing as used in our study combined techniques with next generation sequencing (NGS) approaches as recently proposed for head and neck squamous cell carcinoma should also be considered [44] and can be used in combination with IHC/FISH techniques. Though NGS is a comparably cost-intensive technique that is not available at the majority of medical centers in the world especially outside of industrialized countries, it should nowadays be considered the gold-standard of screening for therapeutically relevant molecular alterations in human cancer. In our study, we aimed to present a clinically feasible and cost-sparing method to identify promising therapeutic targets in patients with SGC that can be used as primary screening approach in countries where NGS is not available or as adjunctive technique in combination with NGS techniques.

Another point that needs to be discussed is the IRS≥9 cut-off that we used to define a case as positive for the respective molecular alteration. In fact, none of the potential therapeutic strategies (apart from entrectinib and larotrectinib) that we tested are currently FDA- or EMA-approved for SGC treatment, so that there exist no pre-defined diagnostic thresholds that need to be met for an in-label use with respect to the biomarkers that we tested. Basically, it was the aim of our study to screen patients with SGC for clinically relevant molecular alterations that represent potential targets for a potent therapeutic strategy as based on clinical data from other human cancer entities. When taking a deeper look into literature, one can find that for targeted therapy approaches against all of the molecular alterations tested in our study, expression level at least in certain therapeutic contexts predicts response to therapy (HER2 [45, 46], PD-L1 [47], NTRK [31], AR [48], TROP 2 [49, 50], EGFR [35, 36]) with a strong expression indicating the highest probability to observe clinical response. Hence, we decided to only define those cases as "druggable" that show a strong biomarker expression according to the definition of Remmele and Stegner [28], i.e., an IRS ≥ 9. For sure, this does not mean that patients that still show expression of the respective protein but with an IRS < 9 cannot respond to the corresponding targeted therapy approach but with a much lower probability.

Taken together, our study has shown that therapeutically relevant molecular alterations including overexpression of HER2/neu, AR, PD-L1, EGFR, NTRK, and TROP2 can be detected in a relevant subset of patients with SGC. Future clinical trials will have to show if the detection of those alterations can also predict response to targeted therapy approaches using e.g., trastuzumab deruxtecan, bicalutamide, pembrolizumab, cetuximab, entrectinib, or sacituzumab govitecan.

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Declarations

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Competing Interests The authors ML, SW, FLB, SK, LAB, MK, GGK, MW, LGTM, and JPK have no relevant financial or non-financial interests to disclose.

Ethics Approval This study was approved by the Saarland Medical Association ethics review committee (index number 218-10). All experiments were performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent to Participate All patients included in this study gave their informed consent for the scientific use of their tissue and clinical data.

Data Availability Statement The data that support the findings of this study are available on request from the corresponding author.

Code Availability Not applicable.

Author Contributions M.L., L.G.T.M., and J.P.K. conceptualized the study; S.W., F.L.B., S.K., L.A.B., M.K., G.G.K., and M.W. carried out the formal analysis and investigation; M.L., S.W., L.G.T.M., and J.P.K. carried out writing—original draft preparation; All authors contributed to writing—review and editing; M.L. carried out funding acquisition; S.W. and S.K. acquired the resources; M.L., L.G.T.M., M.W., and J.P.K. supervised the study.

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