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Impact of Therapy Sequence with Alkylating Agents and MGMT Status in Patients with Advanced Neuroendocrine Tumors

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Abstract. *Background/Aim: Alkylating chemotherapeutics with either a streptozotocin-(STZ) or temozolomide-(TEM) backbone are routinely used in patients with progressive and unresectable pancreatic neuroendocrine tumors (PNET). In addition, dacarbazine (DTIC) was described as an alternative alkylating therapy option for PNETs. The optimal treatment sequence with alkylating compounds and a potential use of O6-methylguanine-DNA methyltransferase (MGMT) level as predictive biomarker have not yet been sufficiently elucidated. The aim of our study was the evaluation of therapy sequence with either STZ-based treatment followed by DTIC (group A) or the inverse schedule with upfront DTIC (group B) and to correlate MGMT status with clinicopathological characteristics and response to therapy. Patients and Methods: We retrospectively analyzed 28 patients with neuroendocrine tumors (NET) who were treated with STZ-based therapy and DTIC. Additionally, in a second group MGMT immunohistochemistry was performed from primary and metastatic tumor sites. For statistical evaluation Kaplan–Meier analysis, Cox regression methods and Fisher's exact test were used. Results: There was no difference of objective response and disease control between either STZ-based therapy followed by DTIC treatment (group A) after progression or the reverse sequence (group B). Median time to progression (TTP) was estimated to be 21 months in both arms. First-line STZ-based chemotherapy was not superior to first-line DTIC treatment (16 vs. 13 months; p=0.8). MGMT status did not correlate with clinicopathological characteristics or response to therapy with these alkylating agents. Conclusion: Upfront chemotherapy with either STZ-based treatment or DTIC monotherapy showed similar efficacy and median TTP rates. In this study, MGMT protein expression assessed by immunohistochemistry did not play an important role as a predictive marker for alkylating agents.*

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Pancreatic neuroendocrine tumors (PNET) represent a rare and heterogenous disease, accounting for approximately 5% of all pancreatic neoplasms (1, 2). While symptoms often occur late, the majority of PNET patients present with metastatic disease in up to 80% of cases (3, 4). Surgery remains the standard treatment for localized stages. In case of unresectable and metastatic disease medical treatment has been shown to improve the long-term outcome of patients. Recently, biotherapy with lanreotide demonstrated a benefit for PNET patients with Ki-67 values less than 10%. In this trial, however, most patients had stable disease prior to start of treatment (96%) and thus only reflect a subgroup of patients (5). For this reason the current guidelines of ENETS (European Neuroendocrine Tumor Society), NANETS (North American Neuroendocrine Tumor Society) and NCCN (National Comprehensive Cancer Network) recommend cytotoxic chemotherapy for patients with well-differentiated pancreatic neuroendocrine tumors and rapid tumor progress, symptomatic disease or high tumor load (6). In contrast to other neuroendocrine malignancies, PNETs are relatively chemosensitive. However, due to the limited number of randomized trials the value of chemotherapy is not well defined. Cytotoxic chemotherapy regimens commonly include alkylating agents such as streptozotocin (STZ), temozolomide (TEM) and dacarbazine (DTIC), with STZ and TEM commonly administered in combination with the anti-metabolites 5-FU and capecitabine (CAP), respectively (7, 8). Overall, first-line chemotherapy with STZ plus 5-FU can achieve response rates up to 40% and progression-free

survival times up to 20 months (9-13). Besides chemotherapy, a plethora of therapeutic options is available in advanced PNET patients. Loco-ablative and loco-regional approaches may affect a predominantly localized liver burden. Further systemic options include targeted therapies such as sunitinib and everolimus or the peptide receptor radionuclide therapy (PRRT). The latter has previously shown significant anti-tumor efficacy in midgut NET and was superior compared to somatostatin analogues SSA monotherapy (14). It is an ongoing debate how to select patients for the best treatment and sequence. To date, no comparative prospective trials are available which address the optimum therapy sequence. Currently, multiple alkylating agents in different therapeutic lines are frequently used, but evidence on their efficacy in sequential approaches is limited.

The mechanism of action by which alkylating agents affect tumor cells is based on diverse pathways. In this context the expression of the DNA repair enzyme O^6-methylguanine DNA methyltransferase (MGMT) has been suggested as main regulator of sensitivity to alkylating drugs. MGMT protein is essential for genomic stability and can prevent DNA replication or mismatch errors on position O^6 (15). Decreased MGMT activity might therefore be associated with enhanced effectiveness of alkylating compounds. MGMT detection can be achieved either with MGMT protein assessment *via* immunohistochemistry or by analysis of the MGMT promoter methylation status *via* PCR. There are no consistent data about correlation of protein and promoter methylation status in neuroendocrine neoplasms. However, recent studies have suggested an association of MGMT status and treatment with STZ or DTIC (16, 17).

The purpose of our study was to explore the efficacy of sequential treatment of STZ-based combination treatment followed by DTIC monotherapy or the reversed order in patients with advanced neuroendocrine tumors. Additionally, MGMT protein expression was assessed *via* immunohistochemistry to determine its prognostic or predictive impact in our cohort.

Patients and Methods

Patients. Patients (n=28) with histologically confirmed neuroendocrine tumors who received either STZ-based combination chemotherapy followed by DTIC (dacarbazine) monotherapy or the reversed sequence were retrospectively identified from a database at the comprehensive cancer center at the university hospital of Marburg. This study was conducted in accordance with the Declaration of Helsinki. Collection, storage, and evaluation of patient-related information in our neuroendocrine tumor (NET) database were performed after informed consent and with the approval of the local ethics committee at the university of Marburg.

Protocol treatment. Chemotherapy was applied either as combination therapy using STZ in combination with doxorubicine (Dox) or 5-fluorouracil (5-FU) or as dacarbazine (DTIC) monotherapy. The chemotherapeutic STZ/Dox regimen included STZ at a dose of 500 mg/m^2 on days 1-5 and Dox at a dose of 50 mg/m^2 on day 1 and 22. The regimen was repeated every 6 weeks. The STZ/5-FU regimen included short-term infusion of 5-FU at a dose of 400 mg/m^2 on days 1-5, in addition to STZ every 6 weeks. Dox was terminated after 5 cycles (before reaching the cumulative dose of 550 mg/m^2) and replaced by 5-FU. DTIC was given as short-term infusion at a dose of 650mg/ m2 every 4 weeks.

Follow up and evaluation of tumor response. Follow-up investigations were scheduled after three completed treatment courses and included history, physical examination, laboratory investigations and imaging (CT or MRI scan). Response to treatment was evaluated using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee (18).

Evaluation of MGMT status in paraffin-embedded tissues. A total of 24 tissue samples were available from an independent cohort of consecutive patients treated either with STZ combinations or DTIC, including 20 PNET patients and 4 non-PNET patients treated at the Marburg ENETS center (Table I). Paraffin sections (4 μm) were used for immunohistochemical analyses which were performed as described previously (16). The tissues were incubated with mouse monoclonal antibody to MGMT (dilution 1:25; Ab-1; clone MT 3.1; Thermo Scientific, Dreieich, Germany), a biotinylated secondary antibody (mouse IgG), and subsequent visualization with avidin-horseradish peroxidase (Vectastain Elite ABC Kit; Vector Laboratories, Eching, Germany) according to the manufacturer's instructions.

Immunohistochemical MGMT expression was estimated independently by three investigators without knowledge of the clinical data. Nuclear MGMT expression was measured with the Remmele-Stegner immunoreactivity score (IRS), which is defined as product of nuclear staining intensity and number of positive cells (19). The results of the MGMT staining were categorized into deficient (score 0) and intact (score >0). Non-neoplastic cells (lymphocytes and endothelial cells) served as an internal positive control in all tissue sections. The MGMT expression status was then correlated with the clinical outcome of the patients.

Statistical design and analysis. The comparisons between clinical response and tumor characteristics, disease stage and laboratory features were based on Fisher's exact tests. Time to progression (TTP) was measured from the beginning of treatment to progression, death, or last follow-up. TTP was measured by the method of Kaplan and Meier (20). The statistical differences in TTP between groups of patients were estimated by the log-rank test (21). All statistical calculations were performed using SPSS (IBM SPSS Statistics). Differences were considered statistically significant when the *p*-value was less than 0.05.

Results

Study population. Overall the study included 28 patients with advanced neuroendocrine tumors. Baseline characteristics are presented in Table II. Fifteen patients received STZ-based treatment followed by DTIC monotherapy (group A) and 13 patients received the reversed sequence (group B). Twenty-five patients suffered from a pancreatic NET, three patients (n=11%)

Table I. *Clinicopathological features of patients with MGMT analysis.*

Patient characteristics	Number of patients (N=24)	%
Gender		
Female	12	50
Age at diagnosis in years		
Median (range)	53 (31-73)	
Primary tumor		
Pancreas	20	83.3
Bronchus	2	8.3
Gastric	1	4.2
Midgut	1	4.2
Tumor type		
Non-functioning	18	75
Differentiation		
NET	24	100
Grading		
G1	2	8.3
G2	22	91.7
Sites of metastases		
Lymph nodes	13	54.2
Liver	24	100
Bone	4	16.7
Chemotherapy		
DTIC only	9	37.5
STZ-based only	0	0
Both	15	62.5

STZ, Streptozotocin; DTIC, dacarbazine; NET, neuroendocrine tumor.

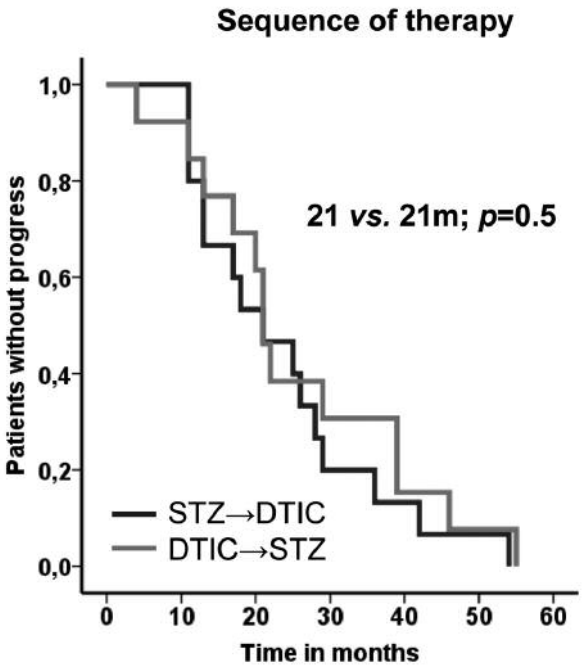

Figure 1. *Time to progression (TTP) for the sequence streptozotocin (STZ) followed by dacarbazine (DTIC) (N=15) or the reverse (N=13). Median TTP was 21 months in both arms (p=0.50).*

Table II. *Baseline patient characteristics.*

Baseline patient characteristics	STZ→DTIC N=15 Group A		DTIC→STZ N=13 Group B	
Parameter	N	%	N	%
Gender				
Female	7	47	7	54
Age at diagnosis in years				
Median (range)	52 (35-70)		55 (33-73)	
Primary tumor				
Pancreas	12	80	13	100
Bronchus	3	20		
Tumor type				
Non-functioning	12	80	9	69
Differentiation				
NET	15	100	13	100
Grading				
G1	2	13	2	15
G2	13	87	11	85
Sites of metastases				
Lymph node	7	47	8	62
Liver	14	93	13	100
Bone	6	40	4	31

STZ, Streptozotocin; DTIC, dacarbazine; NET, neuroendocrine tumor.

had a bronchus NET. These three patients were all assigned to group A. In all other clinical parameters, the two treatment groups were well balanced including patient's age (median: 52 *vs*. 55 years), NET functionality (FNA: 80 *vs*. 69%), grading (G2: 87 *vs*. 85%) and sites of metastases (Table II).

Efficacy results. Median TTP was 21 months in both groups (HR 0.8, 95% CI 0.4-1.7, p=0.5) (Figure 1). The objective response rate during first-line treatment was 47% for group A and 23% for group B (p=0.25); corresponding disease control rates (objective response rate plus stable disease) were 87% and 62%, respectively, (Table III) favoring first-line STZ, however, without significance (p=0.2). Results for TTP after failure of 1st- or 2nd-line therapy demonstrated again a non-superiority of STZ over DTIC (Figure 2).

MGMT expression. A total of 24 tumor blocks were available for immunohistochemistry in patients treated with either DTIC monotherapy or STZ-based combination therapy. Baseline patient characteristics are listed in Table I. Among them, 20 tumors were of pancreatic and 4 of non-pancreatic origin. We additionally grouped the tissue samples of pancreatic NETs according to their anatomical origin in primary tumor tissues and specimens from hepatic metastases (Table IV). Among 24 patients with NETs, 15 (62.5%) were MGMT deficient and 9 (37.5%) MGMT intact

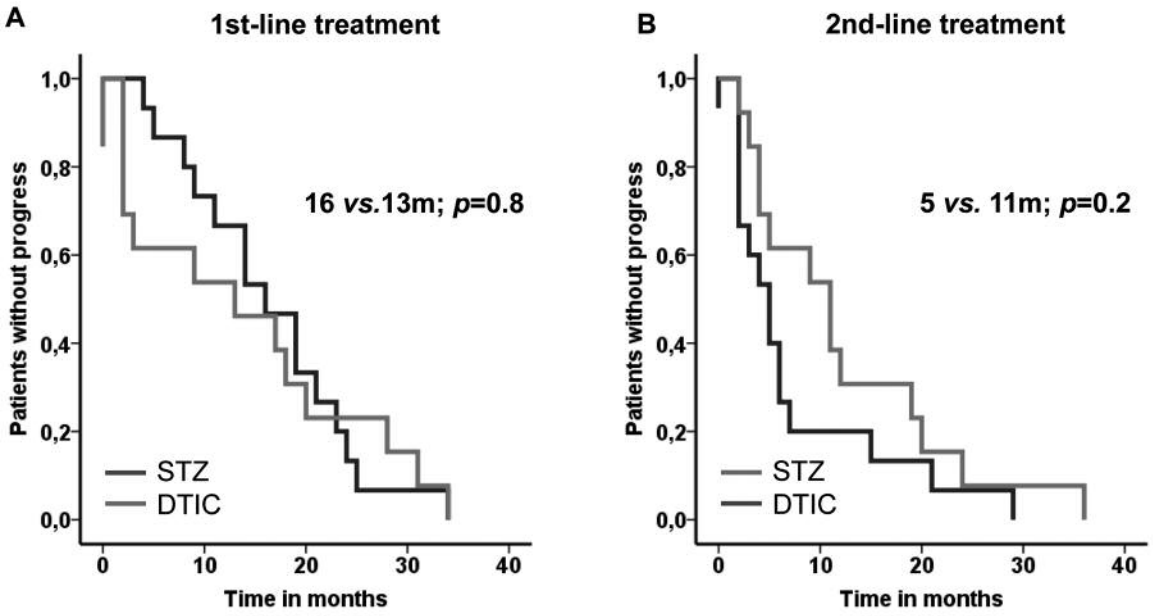

Figure 2. *Median TTP for first- (A) and second-line (B) treatment. Streptozotocin (STZ) vs. dacarbazine (DTIC): 16 vs. 13 months (p=0.8) and DTIC vs. STZ: 5 vs. 11 months (p=0.2).*

Table III. *Treatment efficacy.*

Treatment efficacy	STZ→DTIC N=15		DTIC→STZ N=13	
Parameter	N	%	N	%
1st-line patients				
Complete response	0	0	0	0
Partial response	7	47	3	23
Stable disease	6	40	5	38.5
Progressive disease	2	13	5	38.5
2st-line patients				
Complete response	0	0	0	0
Partial response	5	33	5	38
Stable disease	4	27	2	16
Progressive disease	6	40	6	46

STZ, Streptozotocin; DTIC, dacarbazine.

Table IV. *Correlation of MGMT status to tumor site*

Patient characteristics	Number of patients (%)	MGMT deficient n (%)	MGMT intact n (%)
PNET	20 (100)	11 (55)	9 (45)
Primary site	6 (30)	5 (83.3)	1 (16.7)
Liver site	14 (70)	6 (42.9)	8 (57.1)
Non-PNET	4 (100)	4 (100)	0
Bronchus	2 (50)	2 (50)	
Gastric	1 (25)	1 (25)	
Midgut	1 (25)	1 (25)	
All NET	24	15	9

MGMT, O^6-Methylguanine-DNA methyltransferase; PNET, pancreatic neuroendocrine tumor; NET, neuroendocrine tumor.

(Figure 3). Absence of MGMT staining was observed in 11 of 20 PNETs (55%) and all 4 non-PNETs (100%). In contrast, MGMT expression was present in 9 of 20 PNETs (45%). Of the 20 patients with PNETs we analyzed primary tumor tissues from 6 patients and liver metastases from 14 patients. Five (83.3%) of 6 primary tumors were MGMT deficient and 1 (16.7%) revealed a positive staining. In addition, 6 (42.9%) of 14 liver metastases exhibited no MGMT staining, 8 (57.1%) were positive. There was no statistical correlation of the MGMT status with PNETs *versus* non-PNETs or primary *versus* liver metastatic site. In conclusion, no correlation between patient characteristics and MGMT status was detected (Table V).

Clinical correlation of patients treated with DTIC and STZ to the MGMT status. Overall, 39 treatments with either DTIC or STZ were assessable. Among them, 24 patients were treated with DTIC (9 DTIC only) and additionally 15 patients received

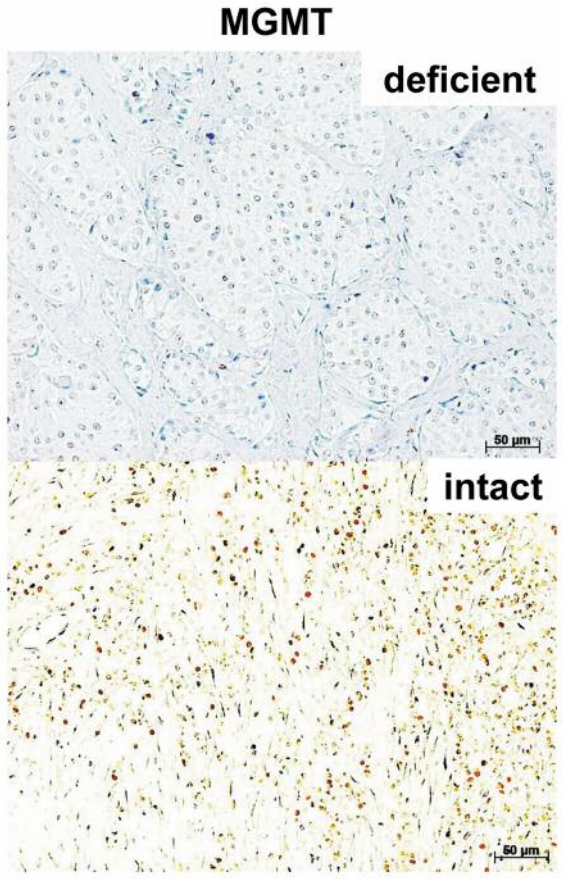

Figure 3. *Representative immunohistochemistry of MGMT in liver metastases in 20-fold magnification.*

Table V. *Correlation of MGMT status to baseline patient characteristics.*

Patient characteristics	Number of patients (N=24)	MGMT intact	MGMT deficient	*p*-Value (Fisher's exact test)
Gender				
Female	12	5	7	0.41
Male	12	8	4	
Age in years				
<60	17	10	7	0.66
≥60	7	3	4	
Tumor Type				
Non-functioning	18	8	10	0.17
Functioning	6	5	1	
Grading				
G1	6	3	3	1.0
G2	18	10	8	
Ki-67				
<10%	13	9	4	0.22
≥10%		11	4	7
SMS status				
Negative	2	1	1	1.0
Positive	18	11	8	
Sites of metastases				
Liver+LN	12	7	5	0.71
+Other	12	6	6	

MGMT, O^6-Methylguanine-DNA methyltransferase; LN, lymph nodes; SMS, somatostatin.

STZ-based therapy during disease progression. In the DTIC group 8 (30.8%) patients had an objective response (PR), all of pancreatic origin. In the PNET cohort, 4 (57.1%) patients with an objective response revealed an MGMT-deficient tumor, whereas the remaining 4 tumors (30.8%) revealed a positive MGMT staining. For STZ-based therapy objective response was achieved in 7 patients (46.7%), and in 5 of the 7 patients MGMT expression was intact (55.6%). Of 5 patients with progressive disease, 2 (40%) had no MGMT expression. Neither response to DTIC nor to STZ-based treatment showed a statistically significant correlation to MGMT status (Table VI). Moreover, MGMT status had no impact on progression-free survival for all or PNET only patients (Figure 4) and was not related to DTIC or STZ first-line therapy (Figure 5).

Discussion

Our study indicated that both investigated sequential therapeutic approaches were equally effective regarding mTTP. Whereas no trend was measured after first-line treatment between both groups, STZ-based therapy was superior in the second-line, however without reaching statistical significance. Overall patient characteristics were well balanced despite the retrospective nature of our study. However, the documentation of the performance status was incomplete in the medical records and thus not analyzable. We assume that only in patients with a very good performance status (PS) STZ combination treatment after failure of DTIC was feasible, possibly explaining the impact of STZ-based combination therapy in the second-line setting. The impact of PS on therapeutic decisions, treatment efficacy and patient outcome is well described (6), particularly for patients who are selected to undergo surgery despite metastatic disease. Resection of the primary tumor in midgut or pancreatic origin is associated with improved survival; however, only patients with an appropriate PS will be candidates for surgical resection (22-25).

Our data on treatment efficacy, as assessed by objective response and disease control are well in line with previously published studies (11, 12). Importantly, our study demonstrates that the switch to another alkylating agent after disease progression during first-line treatment induces objective responses in more than 30% of the patients. This effect was consistent in both treatment arms. Thus, in patients with high

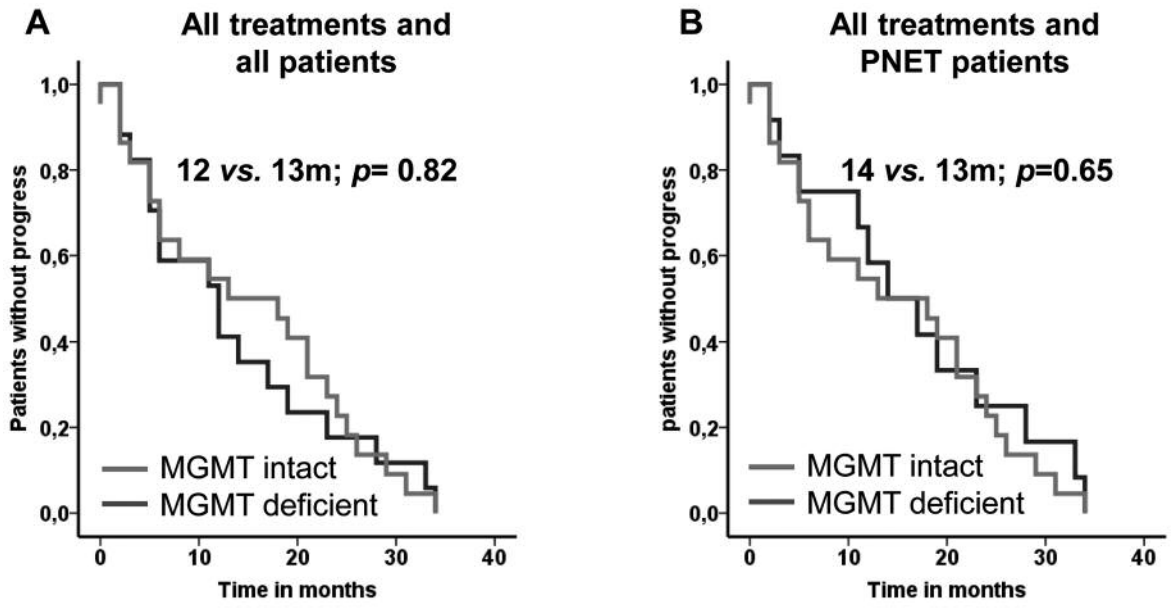

Figure 4. *Kaplan-Meier curves on time to progression with respect to MGMT status (intact, deficient) and primary tumor site. For all patients (A) and only PNET patients (B) no association was described. A: 12 vs. 13 months (p=0.82). B: 14 vs. 13 months (p=0.65).*

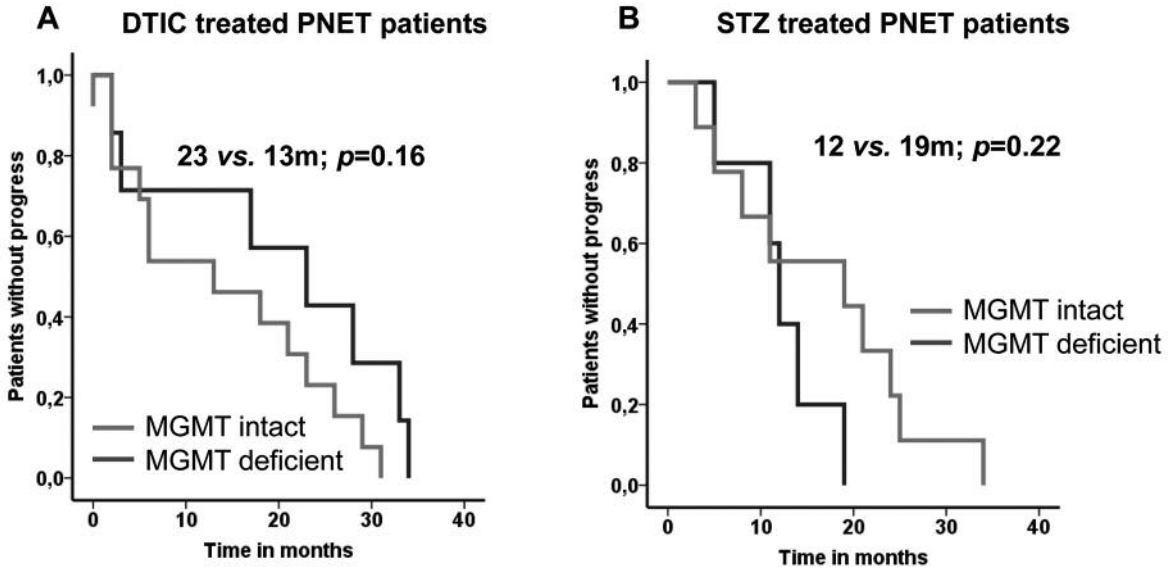

Figure 5. *Impact of MGMT status (intact, deficient) on alkylating compound used. For DTIC treated PNET patients (A) and STZ-based treatment (B). A: 23 vs. 13 months (p=0.16). B: 12 vs. 19 months (p=0.22).*

tumor burden, symptomatic disease and remission pressure after progression of first-line chemotherapy, STZ combinations or DTIC monotherapy are relevant therapeutic options. Our data on therapy sequencing with alkylating agents provide a new concept for treatment in patients with advanced neuroendocrine tumors. Although several treatment options for PNETs have been approved and are in clinical use, randomized phase III trials exist only for lanreotide, sunitinib and everolimus (5, 26, 27). Based on former randomized trials and retrospective evaluations, STZ-based chemotherapy is recommended as the therapy of choice in metastatic disease (6). Since most patients will be treated for many years during

Table VI. *Correlation of MGMT status to treatment response*.

Correlation to response							Fisher's exact test (*p*-value)	
MGMT	PR	SD	PD	ORR %	DCR %	Treatments	OR	DC
All	15	12	12	38.5	69.2	39		
Intact	3	6	3	25.0	75.0	12	0.24	0.66
Deficient	6	4	2	50.0	83.3	12		
DTIC treated	8	9	7	33.3	70.8	24		
Intact	4	6	3	30.8	76.9	13	1.0	0.66
Deficient	4	3	4	36.4	63.6	11		
STZ treated	7	3	5	46.7	66.7	15		
Intact	5	1	3	55.6	66.7	9	0.61	1.0
Deficient	2	2	2	33.3	66.7	6		
DTIC PNET	8	8	4	40.0	80.0	20		
Intact	4	6	3	30.8	76.9	13	0.36	0.66
Deficient	4	2	1	57.1	85.7	7		
STZ PNET	7	3	4	50.0	71.4	14		
Intact	5	1	3	55.6	66.7	9	0.63	1.0
Deficient	2	2	1	40.0	80.0	5		

MGMT, O^6-Methylguanine-DNA methyltransferase; PNET, pancreatic neuroendocrine tumor; NET, neuroendocrine tumor; CR, complete response; PR=partial response; SD, stable disease; PD, progressive disease; OR, objective response; DC, disease control.

the course of their disease (4, 28), most patients will be exposed to a broad variety of therapeutic approaches, including surgery, biotherapy, cytotoxic chemotherapy, loco-regional therapy, targeted therapies and peptide receptor radionuclide therapy (PRRT). It is noteworthy that no comparative trials on optimal therapy sequences are available yet. The SEQTOR trial is an ongoing evaluation of the best sequence of STZ/5-FU followed by everolimus *versus* the reverse sequence. Our study presents promising results about the efficacy of alkylating compounds in sequential therapies. However, these positive results have been acquired retrospectively and are not transferable to other sequential combinations.

As already known, targeted therapies mostly achieve disease stabilization in patients with advanced PNETs, however, induced resistance *via* multiple mechanisms occur potentially driving the disease more aggressive. Preclinical data have very well demonstrated the increase in the development of metastasis during antiangiogenic treatment (29-31). In this context the results of the European multi-center SEQTOR trial are urgently awaited. Furthermore, no studies comparing the impact of targeted therapies, chemotherapy, SSA or PRRT are available. Interestingly, one study is planned to compare PRRT and everolimus in non-resectable progressive and somatostatin receptor-positive PNET patients (COMPETE study). The clinical value of PRRT in advanced NETs was controversial and discussed for a long time, but the NETTER-1 results significantly indicated the superiority of PRRT with lutetium-177 (177Lu)-Dotatate over SSA monotherapy in terms of responses and PFS in midgut patients (14). If these data are reproducible in PNETs, then a comparison with targeted agents is warranted. Selecting the optimal therapeutic sequence for PNET patients also requires predictive markers for treatment stratification and patient selection. There are many studies investigating the potential value of MGMT expression or MGMT promoter methylation to predict outcome in patients treated with alkylating compounds. Our study showed no significant correlation between immunohistochemically-assessed MGMT expression and response to treatment or TTP, neither with STZ nor with DTIC. Alkylating agents such as temozolomide (TEM) and DTIC are frequently used in patients with glioblastomas and melanomas. In these entities MGMT promoter methylation was favorably linked to response (32, 33). Interestingly, many studies have clearly demonstrated that there is no correlation between MGMT protein expression and MGMT promoter methylation (34, 35), the mechanistic basis of this discrepancy still not being completely understood.

In advanced PNET patients, the impact of MGMT status also remains to be elucidated. Kulke *et al*. reported MGMT deficiency in 51% of PNET and 0% of carcinoid tumors, as assessed by immunohistochemistry. This could explain the long known fact that carcinoids frequently are not responsive to chemotherapy in contrast to PNET (16). However, other studies came up with more conflicting results, in particular concerning the frequency of MGMT promoter methylation (36, 37) and response to treatment (38, 39). Inhomogeneous patient cohorts and different therapy regimen may have

complicated the evaluation. Very recently, Schmitt *et al*. published a large series of 141 resected PNETs tested for MGMT protein expression and MGMT promoter methylation (40). In a small subgroup they also investigated the correlation between MGMT protein expression, MGMT promoter methylation and response to TEM chemotherapy. As reported in previous studies, no correlation between MGMT protein expression and MGMT promoter methylation was found. Moreover, response to TEM was predicted by MGMT promoter methylation, but not by MGMT protein expression. Similar results were obtained by Cives *et al*. who described that MGMT expression failed to influence the effect of TEM (41). In addition, in a French cohort reported by Walter *et al*. pyrosequencing was used to assess MGMT promoter methylation status. In their study median PFS was significantly increased in patients with PNETs and methylated MGMT promoter. Multivariate analyses confirmed the benefit for STZ-based treatment and DTIC in this group (17).

Taken together, our data and the majority of reported data in the literature suggest that assessment of the MGMT status by immunohistochemistry is most likely not suitable to predict response to alkylating agents. Determining the methylation status of the MGMT promoter might be more promising according to literature, although also still under investigation. Prospective validation of the optimal detection method of the MGMT status as biomarker is urgently warranted to guide systemic therapy of PNET patients.

Conclusion

In conclusion, our findings indicate a role for sequential approaches with alkylating chemotherapeutics in patients with advanced PNETs. No statistically significant differences were found between STZ-based treatment followed by DTIC monotherapy or the reverse sequence. Both treatment schedules resulted in clinically-relevant objective response rates. MGMT status as assessed by immunohistochemistry failed to select patients for the optimal therapy and was no predictor for treatment efficacy. Further comparative clinical trials must be designed to assess the pivotal challenge of sequential treatment algorithms and to define predictive markers for those treatment strategies to improve the care for PNET patients.

Conflicts of Interest

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki. Collection, storage, and evaluation of patient-related information in our neuroendocrine tumor (NET) database were performed after informed consent and with the approval of the local ethics committee at the University of Marburg.

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