Membranous NECTIN-4 Expression Frequently Decreases during Metastatic Spread of Urothelial Carcinoma and Is Associated with Enfortumab Vedotin Resistance

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ABSTRACT

Purpose: The antibody–drug conjugate enfortumab vedotin (EV) releases a cytotoxic agent into tumor cells via binding to the membrane receptor NECTIN-4. EV was recently approved for patients with metastatic urothelial carcinoma (mUC) without prior assessment of the tumor receptor status as ubiquitous NECTIN-4 expression is assumed. Our objective was to determine the prevalence of membranous NECTIN-4 protein expression in primary tumors (PRIM) and patient-matched distant metastases (MET).

Experimental Design: Membranous NECTIN-4 protein expression was measured (H-score) by IHC in PRIM and corresponding MET ($N = 137$) and in a multicenter EV-treated cohort ($N = 47$). Progression-free survival (PFS) after initiation of EV treatment was assessed for the NECTIN-4–negative/weak (H-score 0–99) versus moderate/strong (H-score 100–300) subgroup. The specificity of the NECTIN-4 IHC staining protocol was validated by establishing CRISPR-Cas9–induced polyclonal NECTIN-4 knockouts.

Results: In our cohort, membranous NECTIN-4 expression significantly decreased during metastatic spread (Wilcoxon matched pairs $P < 0.001$; median H-score = 40; interquartile range, 0–140), with 39.4% of MET lacking membranous NECTIN-4 expression. In our multicenter EV cohort, absence or weak membranous NECTIN-4 expression (34.0% of the cohort) was associated with a significantly shortened PFS on EV (log-rank $P < 0.001$).

Conclusions: Membranous NECTIN-4 expression is frequently decreased or absent in mUC tissue. Of note, the clinical benefit of EV strongly depends on membranous NECTIN-4 expression. Thus, our results are of highest clinical relevance and argue for a critical reconsideration of the current practice and suggest that the NECTIN-4 receptor status should be determined (ideally in a metastatic/progressive lesion) before initiation of EV.

See related commentary by Aggen et al., p. 1377

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Translational Relevance

Enfortumab vedotin (EV) releases a cytotoxic agent into the urothelial carcinoma (UC) tumor cell via binding to the tumor surface protein NECTIN-4. Although EV is approved in the metastatic disease stage, the expression of its target in metastatic tissue is insufficiently studied. Here, we demonstrate that NECTIN-4 expression decreases substantially during metastatic evolution and is absent in more than one third of patients with metastatic UC. Further, in our multicenter EV-treated cohort, membranous NECTIN-4 expression predicts EV response and outcomes. Our data argue against the common practice of EV treatment without prior assessment of target protein expression and suggest that NECTIN-4 receptor status should be determined in a metastatic lesion before initiation of EV.

Introduction

The therapeutic landscape of metastatic urothelial carcinoma (mUC) has undergone substantial changes in recent years (1). Alongside the broad implementation of immune checkpoint blockade and FGFR inhibition, in particular antibody–drug conjugates (ADC) have moved into the clinical focus (2, 3). ADCs consist of a monoclonal antibody conjugated to a cytotoxic payload via a linker sequence, thereby combining the selectivity of a targeted antibody therapy with the cytotoxic potential of conventional chemotherapy representing an innovative and emerging oncological therapy approach (4–6).

The anti–NECTIN-4 ADC enfortumab vedotin (EV) has been approved for patients with mUC by the FDA in July 2021 and just recently by the European Medical Agency (EMA) and is now being adopted into routine uro-oncology practice (2, 3).

However, new therapeutic options require reconsideration and adaptation of established therapeutic sequences. There is an yet uncharacterized patient population with mUC that does not benefit from EV therapy (e.g., in the EV-301 ∼30% of patients; ref. 2). Identifying patients who will not benefit from EV is of greatest clinical interest, as it can prevent severe, potentially life-threatening toxicity and significant costs. However, currently, there is no validated biomarker that can robustly predict EV treatment failure.

EV exerts its antineoplastic activity by delivering the highly cytotoxic monomethyl auristatin E into the tumor cell via antibody binding of the tumor surface protein NECTIN-4.

Preclinical data indicate that lack of NECTIN-4 expression is associated with EV resistance (7, 8), which is intuitive, because targeted therapy cannot work without the presence of the target. However, there are no clinical data yet on whether the tumoral NECTIN-4 expression pattern affects the response to EV, probably because it is currently assumed that almost all urothelial carcinoma cells express this receptor at sufficient levels.

The assumption that NECTIN-4 is ubiquitously expressed in UC is based on data from the phase I EV-101 trial (9). In EV-101 almost all tumors ($N = 147/152$; 96.7%) were reported to display strong protein expression of NECTIN-4 (median H-score 290), defined as H-score >150, which led to a protocol amendment to omit assessment of NECTIN-4 expression before enrollment in the subsequent pivotal EV-301 trial (2). Hence, EV is currently applied without prior evaluation of NECTIN-4 expression.

However, other studies reported conflicting data with mostly less profound NECTIN-4 expression in UC, especially in variant histology (8–11). In addition, it has to be emphasized that most of the current data is based on gene expression data whereas independent studies on the prevalence of specific membranous NECTIN-4 protein expression, which represents the biological prerequisite for EV binding, in large cohorts are lacking until today. Further, the expression of NECTIN-4 in primary tumors (PRIM) and patient-matched distant metastases (MET) has not yet been systematically studied, although metastatic burden determines morbidity and mortality in patients with mUC.

In this study, we therefore aimed to investigate how membranous NECTIN-4 protein expression changes in a well-characterized UC progression cohort by comparison of patient-matched PRIM and syn- or metachronous MET. In addition, we examined whether expression of membranous NECTIN-4 predicts EV response in a multicenter cohort of EV-treated patients with mUC.

Materials and Methods

Patient cohort and case report

We investigated a well-characterized retrospective UC progression cohort with syn- or metachronous metastatic disease treated at the Department of Urology of the University Hospital of Erlangen and the Technical University Munich ($N = 137$). $N = 96$ were specimens from radical cystectomy and $N = 41$ were TURBT specimens. Clinicopathologic data of the cohort are summarized in Supplementary Table S1. None of the patients whose samples were included in the PRIM cohort received neoadjuvant systemic therapy. The biopsies of the metastases of our UC progression cohort were all obtained before the start of first-line therapy for metastatic disease stage. Patients included in our multicenter EV cohort were treated as part of routine clinical care ($N = 47$). Patients' oncology treatments were independent of this study. All patients received platinum-based chemotherapy, and 97.9% ($N = 46/47$) of patients received an immune checkpoint inhibitor prior to initiation of EV therapy, consistent with the current EMA approval for EV. Baseline characteristics are depicted in Supplementary Table S2. $N = 21$ samples of the EV cohort were distant MET, the remaining $N = 26$ samples were PRIM or locoregionary lymph node MET from cystectomy. Tissue samples were systematically reviewed by 2 uropathologists (F. Erlmeier, M. Eckstein) according to the 8th TNM- [Union International Contre le Cancer, Geneva, Switzerland, 2017) and 2016 WHO-classification for genitourinary tumors. The study was approved by the ethical review board of the Friedrich-Alexander-University Erlangen-Nürnberg (approval number: 329_16B and 97_18Bc) and the ethical review board of the Medical Faculty of the University of Bonn (approval number: 372/21). The study was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent.

IHC

IHC staining of the NECTIN-4 protein was performed on a VENTANA BenchMark ULTRA autostainer (Ventana) according to an accredited staining protocol in a routine IHC facility accredited and certified according to DIN EN ISO/IEC 17020. The monoclonal anti–NECTIN-4 primary antibody (clone EPR15613–68, #ab251110, Abcam, dilution: 1:100, incubation time 32 minutes at 37°C, antigen retrieval: Cc1 buffer from Ventana with incubation time of 64 minutes at 91°C) was used, which was previously used in the literature (8, 11, 12). The staining was established employing the known specific membranous NECTIN-4 protein expression on normal bladder urothelium.

The final titration of 1:100 was identified by a dilution row (1:25; 1:50; 1:75; 1:100; 1:150; 1:200; 1:300; 1:400; 1:500) where the 1:100 primary antibody dilution revealed a strong (intensity 3+) specific membranous NECTIN-4 protein expression in normal urothelium without any detectable unspecific background staining. The surrounding smooth muscle tissue of bladder specimens served as an internal and an additional tonsil tissue (lymphocytes/leukocytes are consistently negative) as external negative control. Membranous NECTIN-4 expression was evaluated by experienced (board-certified) uro-pathologists (M. Eckstein/A. Hartmann/F. Erlmeier). Specific NECTIN-4 immunoreactivity was localized in the cell membrane and cytoplasm of tumor cells, but only specific membranous expression as the biological prerequisite for EV binding was assessed. Samples were then classified as negative (H-score 0–14), weak (H-score 15–99), moderate (H-score 100–199), and strong (H-score 200–300), as previously described (10, 11). The distribution of NECTIN-4 expression was compared with the data of Challita-Eid and colleagues and the EV-101 study cohort (9, 10). For comparison with EV-101, a NECTIN-4 H-score cutoff of 150 was applied, because in EV-101 it was reported that 5/152 samples had a NECTIN-4 H-score < 150.

Validation of NECTIN-4 IHC staining protocol

The specificity of the NECTIN-4 staining protocol was validated by establishing CRISPR-Cas9-induced polyclonal NECTIN-4 knockouts (KO) in the urothelial carcinoma cell line HT1376. The KO allele (out-of-frame indel) frequency of our polyclonal approach was assessed via targeted amplicon next-generation sequencing (NGS) using the MiSeq Gene & Small Genome Sequencer (Illumina) according to the manufacturer's instructions. HT1376 KOs were stained using the above-described staining protocol and their NECTIN-4 protein expression was compared with the HT1376 wild-type control (CTRL). Further, differential NECTIN-4 protein expression of NECTIN-4 KOs versus CTRL was compared by Western blot (anti–NECTIN-4, AF2659, R&D) and FACS (anti–NECTIN-4, #130–116–028, Miltenyi Biotec).

Cell culture

The HT1376 (RRID:CVCL_1292) cell line was obtained from ATCC (Manassas, VA) and was cultured in DMEM media (Gibco, ThermoFisher Scientific) supplemented with 10% FBS and 1% penicillin and streptomycin (Gibco, ThermoFisher Scientific). Cells were regularly tested for *Mycoplasma* contamination by PCR.

Generation of NECTIN-4 single-guide RNA CRISPR-Cas9 plasmids

pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/addgene:48138; RRID:Addgene_48138; ref. 13). The generation and cloning of the NECTIN-4 single-guide RNA (sgRNA) CRISPR-Cas9 KO plasmids was performed following our established STAR protocol (14). PX458 was digested with the restriction enzyme BbsI (New England Biolabs, catalog no: R0539L). Two different sgRNA sequences targeting the *NECTIN4* gene were cloned into PX458. The following DNA oligos were used for cloning (*NECTIN4* targeting sequences in uppercase letters):

NECTIN-4_KO1_TS: caccgTGGGACACCACACAAGTCAG
NECTIN-4_KO1_BS: aaacCTGACTTGTGTGGTGTCCCAc
NECTIN-4_KO2_TS: caccgCTCCCTCATACAACTGGACA
NECTIN-4_KO2_BS: aaacTGTCCAGTTGTATGAGGGAGc

Establishment of polyclonal NECTIN-4 KO cultures

HT1376 cells were transfected with the NECTIN-4 KO plasmids using FuGENE Transfection Reagent (Promega, catalog no: E2311) with a FuGENE to DNA Ratio of 4:1 (14). Seventy-two hours after transfection, the 15% strongest GFP-positive cells were sorted via the Flow Cytometry Core Facility (FCCF; https://btc.uni-bonn.de/fccf/) of the UKB using the FACS Aria III (BD Biosciences) to obtain polyclonal HT1376 NECTIN-4 KO cultures.

Western blot

HT1376 CTRL and polyclonal KO cells were seeded on a 6-well plate. At a confluency of 80% to 90%, cells were collected and lysed in RIPA lysis buffer containing protease inhibitors (#11423129; R-Biopharm, Roche). The Protein concentration of the lysates was determined using the BCA protein assay (#23225, Pierce BCA Protein Assay Kit). A 4X SDS Sample Loading Buffer [Tris-HCl (0,2 mol/L), DTT (0,4 mol/L), SDS (277 mmol/L), 8.0% (w/v) Bromophenol blue (6 mmol/L), Glycerol (4,3 mol/L)] was added and samples were cooked at 95°C for 5 minutes right before loading of the 8% SDS-Gel. After running the Gel proteins were blotted to a 0,2 µmol/L Nitrocellulose membrane (#10600004; AmershamTM ProtranTM Premium). After transfer, the membrane was blocked in 1x Tween-20 TBS containing 5% BSA for 60 minutes. Then, the membrane was incubated with primary antibodies against NECTIN-4 (1:500, AF2659, R&D) and beta-Actin (1:1,000, sc47778, SCB) in TBS containing 2,5% BSA overnight at 4°C. Secondary antibody incubation was performed applying fluorescence labeled anti-mouse and anti-goat antibodies (#926–68072 and #926–32214, LICOR,) for 1 hour in in TBS containing 2,5% BSA. Fluorescence signal was detected using the Odyssey CLx Imaging System (LICOR).

FACS

Immunostainings were performed according to standard protocols. HT1376 single-cell suspensions were stained with the anti-human NECTIN-4 antibody (1:100; APC, REAfinity, Miltenyi Biotec, #130–116–028) and Zombie NIR (1:400, BioLegend, #423106). Data were recorded on an AURORA flow cytometer and analyzed using FlowJo software (FlowJo v10 Tree Star. Inc., https://flowjo.com/).

IHC on HT1376 cell pellets

At a confluency of 90% to 100%, HT1376 cells were collected and pelleted at 400×*g* for 5 minutes. Cells were resuspended in 50-µL collagen matrix (Cellmatrix Type I-A, #637–00653, Fujifilm Wako Chemicals) and supplemented with 10x Ham's F-12 and reconstitution buffer at a 8:1:1 ratio. The collagen-cell preparations were incubated at 37°C for 30 minutes until solidification. The collagen-cell pellet was then transferred into a plastic cassette with a swamp and fixed in ROTIHistofix 4% (P087.5, Carl Roth) for 2 hours at room temperature. Afterwards the samples were washed twice with tap water for 30 minutes, then transferred to 70% Ethanol and afterwards embedded in FFPE according to standard protocols. Subsequently, IHC staining of NECTIN-4 was performed using the VENTANA BenchMark ULTRA autostainer (Ventana) as described above.

Targeted amplicon NGS

Genomic DNA (gDNA) from cultured cells was extracted using the Nucleo Spin Tissue Kit (#740952.250, Macherey & Nagel) according to the manufacturer's instructions. A two-step PCR protocol was performed to generate targeted PCR amplicons prior to NGS. Two NECTIN-4 gene-specific primer pairs encompassing the sgRNA target site were used for the first PCR with additional adapter sequences for the second PCR. (*NECTIN4* specific sequences in lowercase letters, adapter sequences in uppercase letters):

MiSeq_hsNECTIN-4-KO1_fw: ACACTCTTTCCCTACACGACGCTCTTCCGATCTgacacggaggtcaaaggcacaac

MiSeq-hsNECTIN-4-KO1_rev:

TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTggacctgtggcatctcaccaatc

MiSeq_hsNECTIN-4-KO2_fw

ACACTCTTTCCCTACACGACGCTCTTCCGATCTggcatcattccattctttctcc

MiSeq_hsNECTIN-4-KO2_rev

TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgctcagaatatgttgtcccattc

For the second PCR, adapter-specific universal primers were used that contained barcode sequences and Illumina adapter sequences P5 and P7 (combinations of primers D501–508 and D701–712).

In the first PCR, the genomic region of interest was amplified with 22 cycles using approximately 20 to 50 ng of gDNA in a 15-μL mixture with Q5 polymerase (M0491L, New England Biolabs) according to the manufacturer's protocol. Subsequently, 2 μL was transferred to the second PCR. This product was amplified with an additional 22 cycles in a 25-μL reaction mixture with Q5 polymerase. NGS was performed using the MiSeq Gene & Small Genome Sequencer (Illumina) according to the manufacturer's standard with a single-end read and 300 cycles (MS-102–2002, MiSeq Reagent Kit v2 300 cycles).

Indel detection

For indel detection, we used the web-based program outknocker (http://www.outknocker.org/outknocker2.htm; ref. 15). FASTQ files were imported and the sequence of the NECTIN-4 PCR amplicon was used as a reference sequence for alignment. This analysis was performed for the polyclonal HT1376 NECTIN-4 KO1+2 versus control cells (CTRL).

Statistics

Statistical analyses were performed with R studio (Version 1.4.1106) and GraphPad Prism (Version 9.4.0). Descriptive statistics (mean, median, range) and frequencies were presented for the distributions of continuous variables. Comparisons between two groups were statistically tested with the nonparametric Mann–Whitney test, and comparisons between multiple groups with the nonparametric Kruskal–Wallis test. Comparisons between paired samples (PRIM with corresponding MET) were performed with the nonparametric Wilcoxon matched-pairs signed rank test. Progression-free survival (PFS) after EV start was estimated by univariable Kaplan–Meier regressions and statistical significance was assessed with the Log-rank test. The H-score cutoff of 100 was applied to divide patients from the EV cohort into the subgroups with negative/weak versus moderate/strong membranous NECTIN-4 expression, as previously described (10, 11). Pearson χ^2 test and Fisher exact test were applied to compare baseline characteristics of both subgroups. Univariable and multivariable Cox regression analyses were performed to compare the prognostic value of membranous NECTIN-4 expression with baseline patient characteristics [age, sex, Eastern Cooperative Oncology Group (ECOG), prior systemic therapies] in relation to PFS after initiation of EV. PFS was defined as the time from EV initiation to radiologic progression or death from any cause. All *P*-values were calculated two-sided, and a $P < 0.05$ was considered statistically significant.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

We investigated a well-characterized retrospective UC progression cohort with PRIM and matched syn- or metachronous metastatic disease ($N = 137$) for NECTIN-4 expression by IHC. A detailed characterization of this cohort is depicted in Supplementary Table S1.

Decrease of NECTIN-4 expression during metastatic spread is common

NECTIN-4 was heterogeneously expressed in both, the PRIM [median H-score = 110; interquartile range (IQR), 25–200], and the corresponding MET (median H-score = 40; IQR, 0–140). In our cohort, 19.7% (27/137) of PRIM were classified as NECTIN-4–negative. In addition, 28.4% of PRIM showed weak, 26.3% moderate and only 25.5% strong NECTIN-4 expression. Of note, NECTIN-4 expression decreased significantly during metastatic spread (Wilcoxon matched pairs $P < 0.001$; **Fig. 1A** and **B**), with 39.4% (54/137) of MET lacking NECTIN-4 expression. Further, 24.8% of MET showed weak, 19.7% moderate and 16.1% strong membranous NECTIN-4 expression. In 59.1% of cases, NECTIN-4 expression decreased during metastatic spread, whereas increased expression was observed in only 19.0%. Exemplary cases are shown in **Fig. 1C–E**. Consistent with previous reports (11), PRIM with conventional UC [not otherwise specified (NOS)] have higher membranous NECTIN-4 expression than histologic UC subtypes. The same applies to MET, although variant histologies show similar NECTIN-4 expression to NOS (Supplementary Fig. S1A and B). Further, there was no significant difference between the changes in membranous NECTIN-4 expression during metastatic spread in the histologic UC subtypes (Supplementary Fig. S1C). Of note, there was also no temporal relationship between differential NECTIN-4 expression in PRIM and the corresponding MET in our cohort (**Fig. 1F**), even when comparing syn- (<1 month between PRIM/ MET sampling) and metachronous MET (Mann–Whitney, $P = 0.7$). Thus, regardless of UC histomorphology and even in the presence of synchronous metastasis, prediction of metastatic NECTIN-4 expression status based on PRIM is not reliable, suggesting that decreased membranous NECTIN-4 expression during metastatic spread is a tumor biological process.

Comparison of NECTIN-4 expression with the literature

Next, we aimed to compare our NECTIN-4 expression data with two published datasets (9, 10). In the study by Challita-Eid and colleagues (10), which is affiliated with the company that developed EV, NECTIN-4 expression in a UC cohort of in total 524 patients (including 25 metastases) was studied. The distribution of NECTIN-4 expression in the Challita-Eid and colleagues cohort and our PRIM cohort did not differ significantly and showed a comparable frequency for absence of membranous NECTIN-4 expression (17% versus 19.7%; **Fig. 2A**). Compared with the reported NECTIN-4 expression of the EV-101 study cohort, both the study by Challita-Eid and colleagues and our work report significantly lower NECTIN-4 expression (**Fig. 2B**).

Validation of NECTIN-4 IHC staining protocol

Considering that our staining protocol results in significantly lower NECTIN-4 expression in UC tumor tissue compared with EV-101, we next wanted to validate the specificity of our NECTIN-4 IHC staining protocol. Therefore, we established CRISPR-Cas9-induced polyclonal NECTIN-4 KO cultures of the urothelial carcinoma cell line HT1376, that is reported to express high levels of NECTIN-4 (13). We designed two independent nonoverlapping NECTIN-4 sgRNAs for the CRISR-Cas9

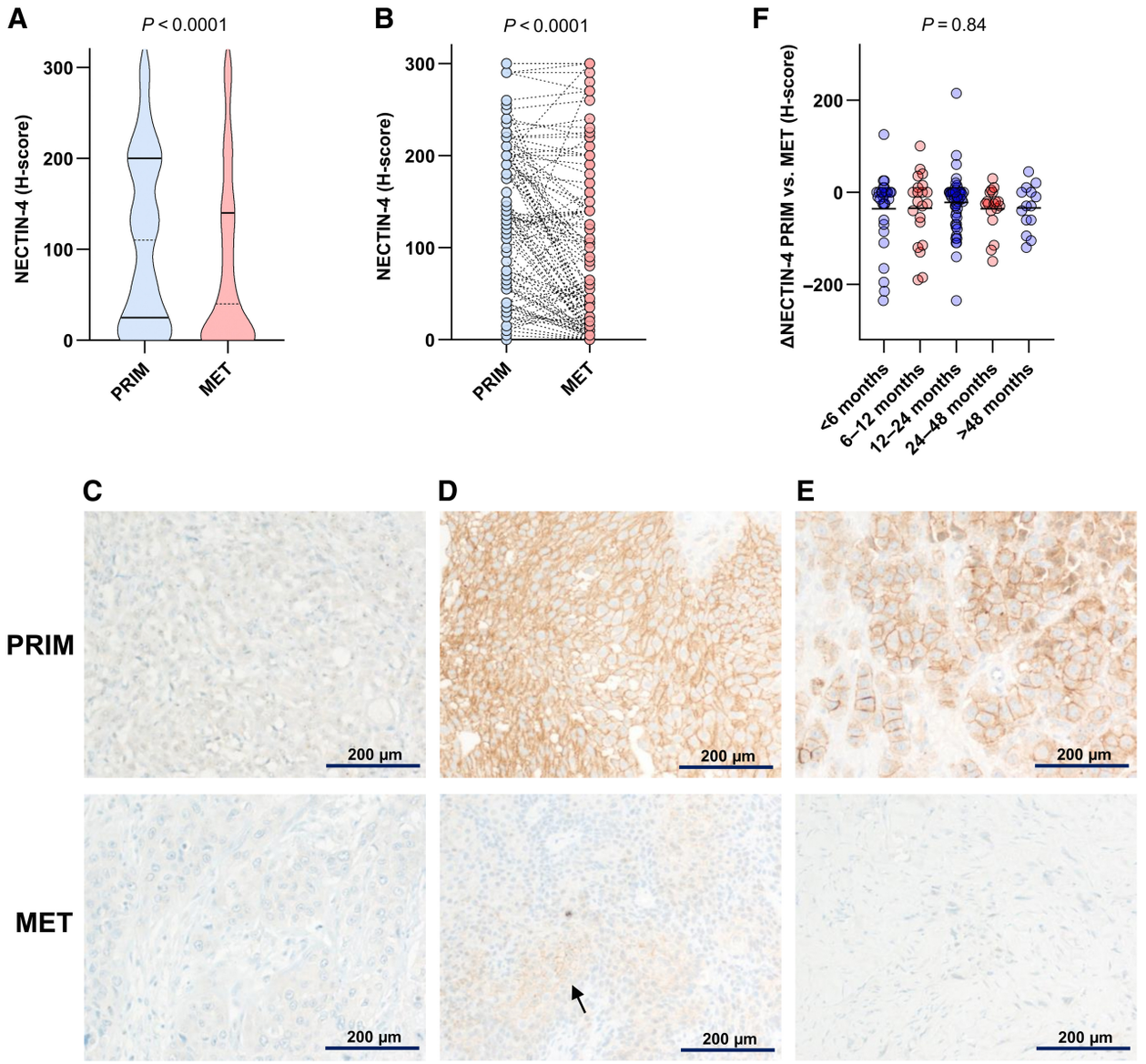

Figure 1.

Membranous NECTIN-4 protein expression patterns assessed by IHC. **A** and **B,** Membranous NECTIN-4 expression decreases significantly during metastatic spread of urothelial carcinoma, with 39.4% of MET lacking NECTIN-4 expression. *P* values were calculated by a nonparametric Mann–Whitney test. **C–E,** Representative IHC stainings for NECTIN-4. Each panel resembles pairs of matched PRIM and distant MET. **C,** Concordantly negative PRIM and MET (membranous H-score 0). **D,** Strongly and homogenously positive PRIM (membranous H-score 300) with strong reduction of membranous expression in corresponding MET (membranous H-score 20; positive tumor cells marked with arrow). **E,** Strongly but inhomogenously positive PRIM (membranous H-score 270) with negative corresponding MET (membranous H-score 0). All pictures were taken on an Axio Imager A1 microscope (Carl Zeiss AG, Jena, Germany) under 200× magnification using a Gryphax Arktur camera (Jenoptik, Jena, Germany). **F,** The differential expression of membranous NECTIN-4 is not dependent on the time interval between PRIM and MET biopsy. Intergroup comparison was calculated by nonparametric Kruskal–Wallis test.

induced KO, KO-1, and KO-2, respectively. Via Western blot, we confirmed a decrease in NECTIN-4 expression for both polyclonal HT1376 NECTIN-4 KO cultures (KO-1 and KO-2) compared with parental HT1376 cells (CTRL; **Fig. 2C**). By FACS, we confirmed a high KO efficiency for KO-1 (>80%), whereas the polyclonal KO-2 culture exhibited a 50%/50% distribution of membranous NECTIN-4 expression (**Fig. 2D**). Next, we determined the KO allele (out-of-frame indel) frequency of the polyclonal HT1376 KO-1 and KO-2 cultures by targeted next-generation amplicon sequencing (NGS) and confirmed a high out-of-frame (KO allele) frequency of about 84% for KO-1 compared with only 55% for KO-2 (**Fig. 2E**). We then validated the specificity of our NECTIN-4 staining protocol on HT1376 CTRL versus KO-1 + KO-2. Consistent with WB and FACS data, HT1376 showed strong membranous NECTIN-4 expression, polyclonal KO-1 culture showed only isolated tumor clones with membranous NECTIN-4 expression, while KO-2 showed some NECTIN-4–positive cell colonies (**Fig. 2F**).

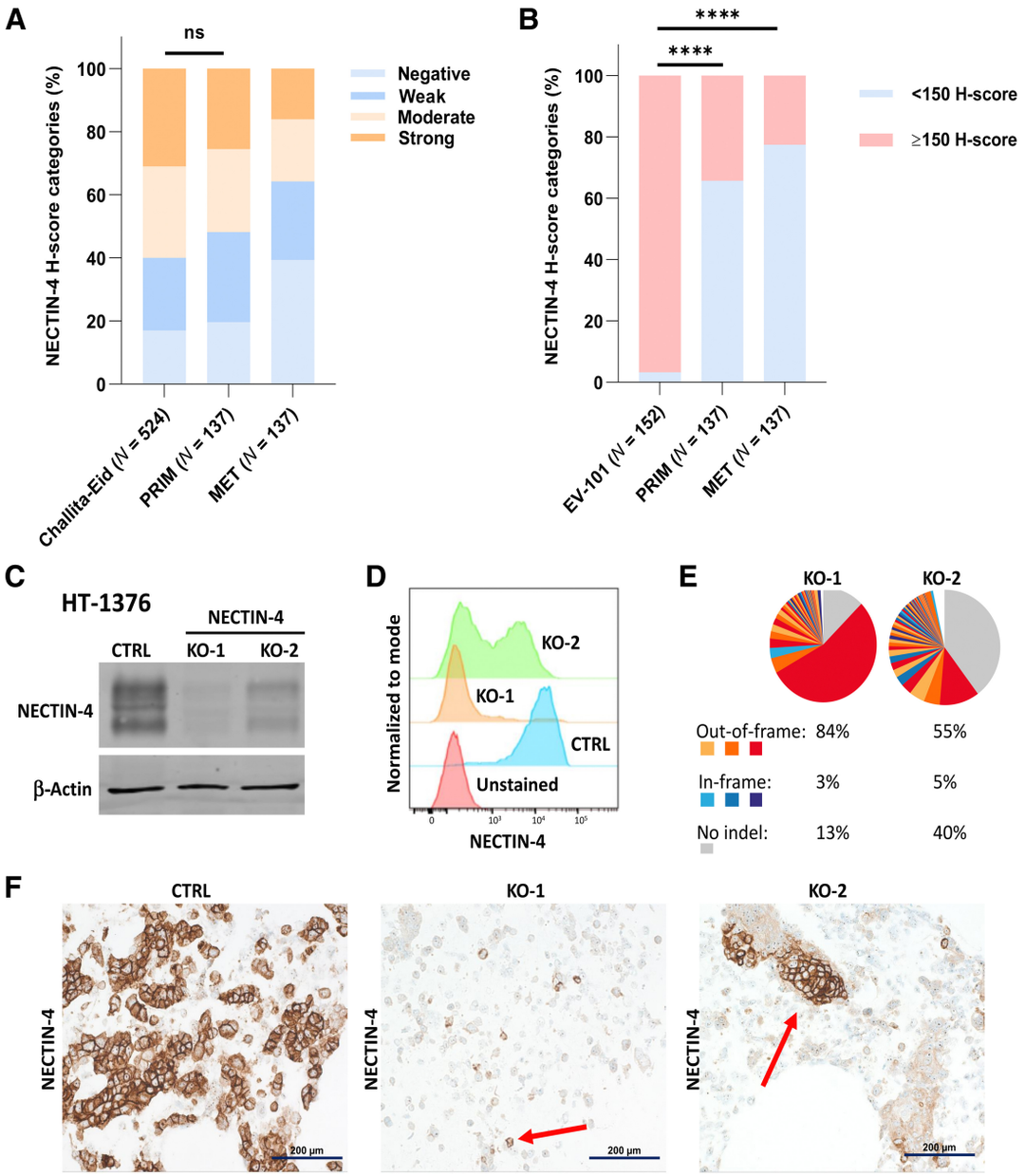

Figure 2.

Comparison of NECTIN-4 expression with previously published UC cohorts and validation of NECTIN-4 IHC staining protocol. **A,** Comparison of expression data obtained from our cohort with the UC cohort reported by Challita-Eid and colleagues including a total of 524 patients (including 25 metastases). **B,** NECTIN-4 expression in our PRIM and MET subcohort is significantly lower than in the phase I EV-101 trial (Cutoff NECTIN-4 H-score 150), where only 5/152 cases had a NECTIN-4 expression < 150. **C,** Western blot comparing NECTIN-4 protein expression in polyclonal HT1376 NECTIN-4 KO cultures (KO-1 and KO-2) compared with control (CTRL). Detection of β-actin served as loading control. **D,** Normalized histogram illustrating membranous NECTIN-4 expression detected by flow cytometry in HT1376 CTRL, KO-1, and KO-2 cultures (plus unstained control as reference). **E,** Pie charts summarizing out-of-frame (KO), in-frame indel, and no-indel (WT allele) frequencies of the polyclonal HT1376 KO-1 and KO-2 determined by targeted amplicon NGS. **F,** Membranous NECTIN-4 protein expression patterns of our HT1376 cell culture models (CTRL vs. KO-1 vs. KO-2) assessed by IHC. The red arrow depicts single isolated NECTIN-4–positive clones of HT1376 KO-1 and a strong NECTIN-4–positive cell cluster of HT1376 KO-2.

Absence or weak membranous NECTIN-4 predicts shortened PFS on EV

We next evaluated the predictive and prognostic value of membranous NECTIN-4 expression in a multicenter EV-treated cohort of $N = 47$ patients with mUC. Baseline characteristics are depicted in Supplementary Table S2. Patients who achieved stable disease or objective responses (partial response or complete response) on EV exhibited significantly higher membranous NECTIN-4 expression than patients with progressive disease (PD), which indicated that low/absent membranous NECTIN-4 expression predicts EV resistance

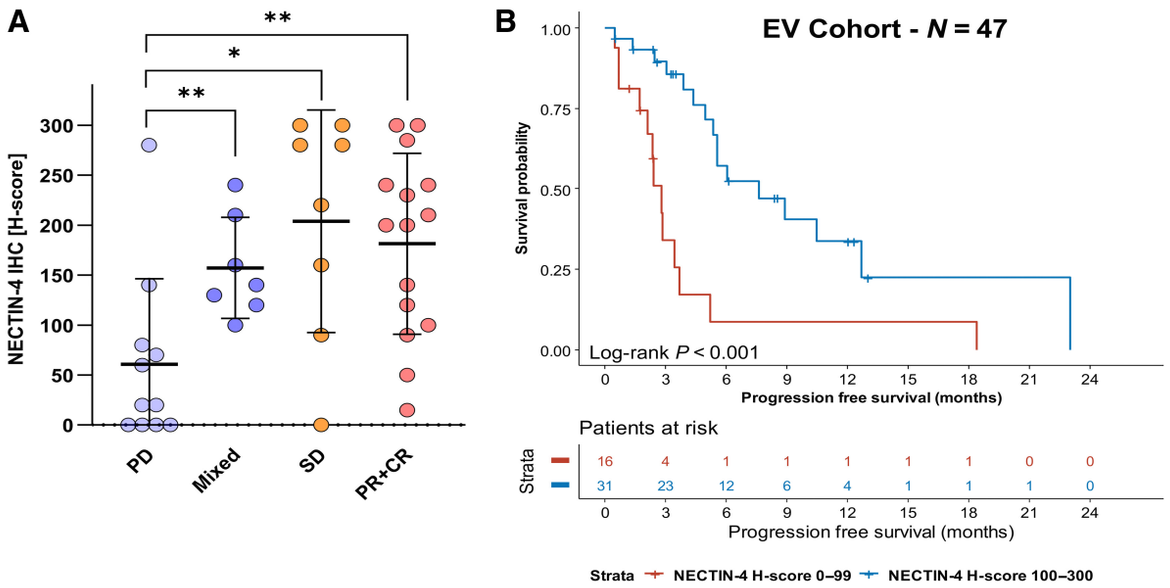

Figure 3.

Membranous NECTIN-4 predicts response and outcomes for patients with mUC treated with EV. **A,** Membranous NECTIN-4 expression predicts EV response (mixed, mixed response; SD, stable disease; PR, partial response; CR, complete response); radiologic response data were available for $N = 41$. *P* values were calculated by a nonparametric Mann–Whitney test. **B,** Absence or weak membranous NECTIN-4 expression (H-score 0–99) is associated with shortened PFS on EV.

(**Fig. 3A**). However, there are also patients with disease control who have low tumoral NECTIN-4 expression. In addition, approximately 15% of our multicenter EV cohort showed a mixed response on EV. This subgroup also exhibited higher NECTIN-4 expression compared with PD.

In univariable Cox analysis, membranous NECTIN-4 expression as a continuous variable was significantly associated with PFS on EV [HR, 0.99; 95% confidence interval (CI), 0.99–1.00; $P = 0.019$]. Next, we separated the EV cohort into patients with negative/low versus moderate/strong tumoral NECTIN-4 expression. The subgroups had similar baseline characteristics (Supplementary Table S2). Of note, absence or weak membranous NECTIN-4 expression (H-score 0–99; 34.0% of the cohort) was associated with significantly shortened PFS on EV (**Fig. 3B**). In addition, patients with absent or weak tumoral NECTIN-4 expression showed a multivariable-adjusted (age, sex, ECOG, prior systemic therapies) 4-fold increased risk of progression on EV compared with patients with moderate/strong NECTIN-4 expression (HR, 4.26; 95% CI, 1.55–11.70; $P = 0.005$; **Table 1**). Overall, our data highlight that membranous NECTIN-4 expression predicts EV response and outcomes.

Discussion

Our study demonstrates for the first time that the membranous expression of the EV target protein NECTIN-4 decreases frequently during metastatic spread of UC and predicts EV response and outcomes in patients with mUC. In essence, NECTIN-4 expression decreases in more than 50% of the cases during metastatic progression, with more than one third of MET being NECTIN-4–negative. Of highest clinical relevance, in our multicenter EV-treated cohort, we demonstrate that absence or low membranous NECTIN-4 expression predicts EV resistance, which translates into unfavorable outcomes. As EV is currently administered without prior determination of NECTIN-4 expression, we believe that our results argue for a reconsideration of this clinical practice and suggest performing metastatic biopsies prior to initiation of EV for improved patient selection.

The assumption that NECTIN-4 is ubiquitously expressed in UC making a pre-treatment assessment obsolete is based on the data of the phase I EV-101 study (9). In EV-101 nearly all cases (96.7%) were reported to strongly express NECTIN-4, defined as an H-score >150 (9). In contrast, but in line with our data, Challita-Eid and colleagues found 17% of cases to be NECTIN-4–negative in a cohort of $N = 524$ UC (including only $N = 25$ MET, not matched to PRIM; ref. 10). Of note, the publication by Challita-Eid and colleagues is affiliated with the company that developed EV. When comparing the results from different studies, the use of different antibody clones must be considered. We, as others (8, 11, 12), used the commercially available monoclonal anti–NECTIN-4 antibody EPR15613–68 (#ab251110, Abcam). Our staining protocol was established in an accredited and certified academic uropathology referral center and validated using our CRISPR-Cas9 induced polyclonal NECTIN-4 KO model. Cohorts from EV-101/ 103 and Challita-Eid and colleagues were analyzed with two different anti–NECTIN-4 antibodies, namely clones M22–321b41.1 (EV-101 and EV-103) and M22–244b3 (Challita-Eid and colleagues) both by Agensys Inc. (not commercially available, patent ID CA3065514A1; refs. 9, 10, 16). Thus, there is an apparent discrepancy in the reported prevalence of NECTIN-4 expression between the initial work by Challita-Eid and colleagues and the EV-101/103 study which may result from the use of different IHC antibody clones. Further, it is unclear why the EV patent holder used different IHC antibody clones. Apart from considerations regarding detection thresholds and dynamic ranges of the different NECTIN-4 antibody clones for IHC, our work unambiguously demonstrates that the reduction of NECTIN-4

Table 1. Uni- and multivariable Cox regression analyses in multicenter EV cohort.

Characteristic	HR (95% CI)	*P* value
Univariable Cox		
Membranous NECTIN-4		
H-score ≥100	—	
H-score <100	3.93 (1.79–8.64)	<**0.001**
Age		
<75 years	—	
≥75 years	0.41 (0.14–1.19)	0.10
Sex		
Female	—	
Male	0.89 (0.37–2.11)	0.8
ECOG		
0	—	
1	2.43 (0.89–6.65)	0.082
≥2	21.1 (3.28–136)	**0.001**
Previous systemic therapies		
1–2	—	
≥3	1.13 (0.53–2.40)	0.7
Multivariable Cox		
Membranous NECTIN-4		
H-score ≥100	—	
H-score <100	4.26 (1.55–11.7)	**0.005**
Age		
<75 years	—	
≥75 years	0.39 (0.11–1.35)	0.14
Sex		
Female	—	
Male	1.09 (0.39–3.06)	0.9
ECOG		
0	—	
1	1.66 (0.60–4.59)	0.3
≥2	34.8 (4.56–266)	<**0.001**
Previous systemic therapies		
1–2	—	
≥3	0.43 (0.15–1.23)	0.12

expression is a frequent event occurring during metastatic progression of UC and that our NECTIN-4 staining protocol detects clinically relevant thresholds in our multicenter EV-treated cohort. Thus, our findings indicate that we currently overestimate the prevalence of therapeutically relevant membranous NECTIN-4 expression in mUC. Of note, the proportion of NECTIN-4–negative MET in our UC progression cohort (39.4%) is comparable to the proportion of patients who did not benefit from EV in the phase III EV-301 trial, as the reported disease control rate in the EV-treated cohort was 71.9% (95% CI, 66.3–77.0; ref. 2).

However, there are sparse clinical data yet on whether membranous NECTIN-4 expression pattern in PRIM or MET affects EV therapy response. In our multicenter EV-treated cohort, we now demonstrate that absent or weak membranous NECTIN-4 expression predicts shortened PFS on EV. Consistent with our data and the concept that ADC cannot act without their respective target on the tumor cell surface, Chu and colleagues demonstrated that CRISPR-Cas9-induced loss of NECTIN-4 leads to EV resistance in the HT1376 cell culture model (8). Patients lacking tumoral NECTIN-4 expression may therefore be more likely to benefit from other therapies, such as the anti-Trop2 ADC Sacituzumab govitecan (3, 7). However, this needs to be investigated in prospective, ideally biomarker-driven clinical trials (17), e.g., with choice of ADC after consideration of respective target expression on the tumor surface in the sense of precision oncology.

In contrast, in EV-103, where only 1 of 39 evaluable patients exhibited absent tumoral NECTIN-4 expression, treatment responses were independent of NECTIN-4 (and PD-L1) expression. However, this could be due to the fact that EV-103 studied the combination therapy of EV plus pembrolizumab compared with EV monotherapy in our cohort (16). Consequently, prospective validation is needed to investigate the predictive value of NECTIN-4 expression also for EV therapy combinations.

Of note, approximately 15% of our multicenter EV cohort showed a mixed response on EV. The frequent rate of mixed response may indicate interlesional heterogeneity of NECTIN-4 expression among different metastases, e.g., due to different metastatic niches. However, in cases of multiple metastases to different organ systems, only one metastatic manifestation was available for expression analysis, so possible interlesional heterogeneity in individual patients could not be investigated. Therefore, this question should be addressed in further studies. One promising approach to correlate differential NECTIN-4 expression and EV response could be molecular imaging by NECTIN-4 PET/CT, which has already been preclinically tested in a mouse model (18). Considering the decrease of membranous NECTIN-4 expression during metastatic spread, the implementation of EV in earlier therapy lines, e.g., intravesical EV for non-muscle invasive bladder cancer (NCT05014139) or EV in the neoadjuvant/perioperative setting, seems reasonable as well.

We strongly believe that our data justify performing metastatic biopsies to determine membranous NECTIN-4 status before EV treatment to prospectively confirm that patients with NECTIN-4–negative tumor burden do not benefit from EV. To date, repeat biopsies are not standard of care in patients with mUC, whereas they are common practice in the treatment of patients with metastatic breast cancer, e.g., to monitor the loss or increase of target receptors such as Her2 during tumor evolution (19, 20). Our data now provide, for the first time, a compelling rationale for performing pre-EV metastatic biopsies to achieve the primary goal of rational patient selection in the era of individualized precision oncology. This would constitute a paradigm shift in the treatment of patients with mUC, as metastatic biopsies were taken in < 10% of mUC clinical trials between 1995 and 2020 (unpublished meta-analysis by our BRIDGE consortium).

Despite notable strengths of our study, the main limitation is, that we used retrospectively collected patient cohorts, so the data must be considered in the context of its retrospective design. In addition, we used a NECTIN-4 H-score cutoff of 100 to divide patients into absent/low versus moderate/strong membrane NECTIN-4 expression as previously described (10, 11). Whether this is the optimal clinical cutoff for EV therapy stratification needs to be investigated in prospective studies.

However, our data argue that the uro-oncological field needs to reconsider the previous assumption of ubiquitous NECTIN-4 expression in UC. Consequently, this merits a critical review of the current approval of EV without prior determination of NECTIN-4 receptor status to prevent unnecessary toxicity and cost. In conclusion, we propose the novel concept of taking metastatic biopsies to determine membranous NECTIN-4 receptor status before starting EV to ensure optimal use of this promising new drug. In addition, we envision that patients with NECTIN-4–negative metastatic burden may be more likely to benefit from alternative therapies, so our data support the initiation of future biomarker-stratified clinical trials.

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The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Authors' Disclosures

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Note

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