

**Journal:** *Annals of Oncology*  
**Article Doi:** 10.1093/annonc/mdw678  
**Article Title:** Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer  
**First Author:** M. M-Rabet  
**Corr. Author:** F. Bertucci

**OXFORD**  
UNIVERSITY PRESS

## INSTRUCTIONS

We encourage you to use Adobe's editing tools (please see the next page for instructions). If this is not possible, (i) print out the proof, mark your corrections clearly in black ink, and fax it to +44 (0)1865 355739 (or scan it and email it to [annonc@oup.com](mailto:annonc@oup.com)), or (ii) send a list of corrections (in an email or Word attachment) listing each change in the following manner: line number, current text, change to be made. Please do not send corrections as track changed Word documents.

Changes should be corrections of typographical errors only. Changes that contradict journal style will not be made.

These proofs are for checking purposes only. They should not be considered as final publication format. The proof must not be used for any other purpose. In particular we request that you do not post them on your personal/institutional web site, and do not print and distribute multiple copies (please use the online offprint order form). Neither excerpts nor all of the article should be included in other publications written or edited by yourself until the final version has been published and the full citation details are available. You will be sent these when the article is published.

- 1. Author groups:** Please check that all names have been spelled correctly and appear in the correct order. Please also check that all initials are present. Please check that the author surnames (family name) have been correctly identified by pink background. If this is incorrect, please identify the full surname of the relevant authors. Occasionally, the distinction between surnames and forenames can be ambiguous, and this is to ensure that the authors' full surnames and forenames are tagged correctly, for accurate indexing online. Please also check all author affiliations.
  - 2. Figures:** If applicable figures have been placed as close as possible to their first citation. Please check that they are complete and that the correct figure legend is present. Figures in the proof are low resolution versions that will be replaced with high resolution versions when the journal is printed.
  - 3. Missing elements:** Please check that the text is complete and that all figures, tables, and their legends are included.
  - 4. URLs:** Please check that all web addresses cited in the text, footnotes and reference list are up-to-date, and please provide a 'last accessed' date for each URL. Please specify format for last accessed date as: Month Day, Year.
  - 5. Funding:** Please provide a Funding statement, detailing any funding received. Remember that any funding used while completing this work should be highlighted in a separate Funding section. Please ensure that you use the full official name of the funding body, and if your paper has received funding from any institution, such as NIH, please inform us of the grant number to go into the funding section. We use the institution names to tag NIH-funded articles so they are deposited at PMC. If we already have this information, we will have tagged it and it will appear as coloured text in the funding paragraph. Please check the information is correct.
-

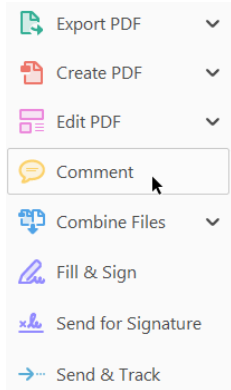
## MAKING CORRECTIONS TO YOUR PROOF

These instructions show you how to mark changes or add notes to your proofs using Adobe Acrobat Professional versions 7 and onwards, or Adobe Reader DC. To check what version you are using go to **Help** then **About**. The latest version of Adobe Reader is available for free from [get.adobe.com/reader](http://get.adobe.com/reader).

### DISPLAYING THE TOOLBARS

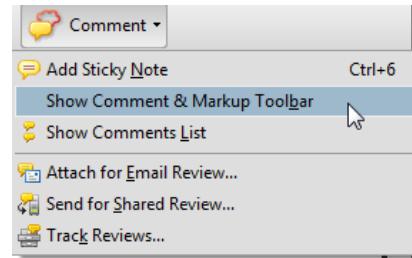
#### Adobe Reader DC

In Adobe Reader DC, the Comment toolbar can be found by clicking 'Comment' in the menu on the right-hand side of the page (shown below).



#### Acrobat Professional 7, 8, and 9

In Adobe Professional, the Comment toolbar can be found by clicking 'Comment(s)' in the top toolbar, and then clicking 'Show Comment & Markup Toolbar' (shown below).



The toolbar shown below will then be displayed along the top.

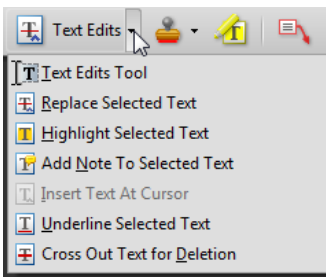


The toolbar shown below will then display along the top.



### USING TEXT EDITS AND COMMENTS IN ADOBE ACROBAT

This is the quickest, simplest and easiest method both to make corrections, and for your corrections to be transferred and checked.



1. Click **Text Edits**
2. Select the text to be annotated or place your cursor at the insertion point and start typing.
3. Click the **Text Edits** drop down arrow and select the required action.

You can also right click on selected text for a range of commenting options, or add sticky notes.

### SAVING COMMENTS

In order to save your comments and notes, you need to save the file (**File, Save**) when you close the document.

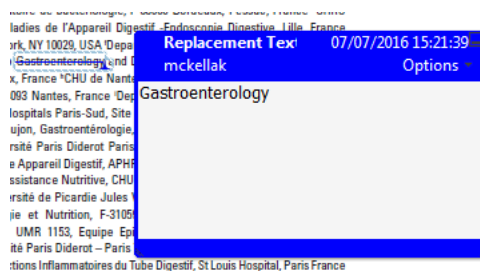
### USING COMMENTING TOOLS IN ADOBE READER

All commenting tools are displayed in the toolbar. You cannot use text edits, however you can still use highlighter, sticky notes, and a variety of insert/replace text options.

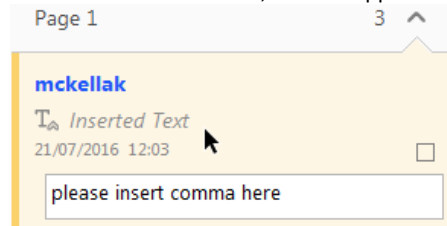


### POP-UP NOTES

In both Reader and Acrobat, when you insert or edit text a pop-up box will appear. In **Acrobat** it looks like this:



In **Reader** it looks like this, and will appear in the right-hand pane:



**DO NOT MAKE ANY EDITS DIRECTLY INTO THE TEXT, USE COMMENTING TOOLS ONLY.**

## Author Query Form

**Journal:** *Annals of Oncology*  
**Article Doi:** 10.1093/annonc/mdw678  
**Article Title:** Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer  
**First Author:** M. M-Rabet  
**Corr. Author:** F. Bertucci

### AUTHOR QUERIES – TO BE ANSWERED BY THE CORRESPONDING AUTHOR

The following queries have arisen during the typesetting of your manuscript. Please click on each query number and respond by indicating the change required within the text of the article. If no change is needed please add a note saying “No change.”

- AQ1:** Please check that all names have been spelled correctly and appear in the correct order. Please also check that all initials are present. Please check that the author surnames (family name) have been correctly identified by a pink background. If this is incorrect, please identify the full surname of the relevant authors. Occasionally, the distinction between surnames and forenames can be ambiguous, and this is to ensure that the authors' full surnames and forenames are tagged correctly, for accurate indexing online. Please also check all author affiliations.
- AQ2:** Please check if the author affiliations have been set correctly.
- AQ3:** Please check that the text is complete and that all figures, tables and their legends are included.
- AQ4:** Please check that all web addresses cited in the text, footnotes and reference list are up-to-date, and please provide a 'last accessed' date for each URL.
- AQ5:** Remember that any funding used while completing this work should be highlighted in a separate Funding section. Please ensure that you use the full official name of the funding body.
- AQ6:** If applicable figures have been placed as close as possible to their first citation. Please check that they are complete and that the correct figure legend is present. Figures in the proof are low-resolution versions that will be replaced with high-resolution versions when the journal is printed.
- AQ7:** We have included the 'key message' text for online-only publication at the end of this proof. Also note that only a maximum of 400 characters, including spaces, are allowed. Please check and revise if necessary.
- AQ8:** Please check if the section “Author ORCIDs” has been set correctly.
- AQ9:** Please provide the volume number and page range.
- AQ10:** Please note there is a charge of £350/€525/\$600 per figure for colour reproduction in print. Please confirm you accept this charge. Alternatively, figures can be published online in colour and printed in black and white free of charge.

## ORIGINAL ARTICLE

# Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer

M. M-Rabet<sup>1</sup>, O. Cabaud<sup>2</sup>, E. Josselin<sup>3</sup>, P. Finetti<sup>2</sup>, R. Castellano<sup>3</sup>, A. Farina<sup>4</sup>, E. Agavnian-Couquiaud<sup>4</sup>,  
G. Saviane<sup>2</sup>, Y. Collette<sup>3</sup>, P. Viens<sup>5</sup>, A. Gonçalves<sup>5</sup>, C. Ginestier<sup>2</sup>, E. Charafe-Jauffret<sup>2</sup>, D. Birnbaum<sup>2</sup>,  
D. Olive<sup>1</sup>, F. Bertucci<sup>2,5\*†</sup> & M. Lopez<sup>2†</sup>

AQ1

AQ2

<sup>1</sup>Equipe Immunité et Cancer; <sup>2</sup>Equipe Oncologie Moléculaire; <sup>3</sup>TrGET Platform; <sup>4</sup>ICEP Platform, Inserm, U1068, CRCM, Institut Paoli Calmettes, Aix-Marseille Université, UM 105, CNRS, UMR7258, Marseille; <sup>5</sup>Département d'Oncologie Médicale, Institut Paoli Calmettes, Aix-Marseille Université, UM 105, Marseille, France

\*Correspondence to: Prof. François Bertucci, Department of Medical Oncology, Institut Paoli-Calmettes, 232 Bd. Sainte-Marguerite, 13009 Marseille, France. Tel: +33-4-91-22-35-37; Fax: +33-4-91-22-36-70; E-mail: bertuccif@ipc.unicancer.fr

†Both authors contributed equally as senior authors.

AQ3

AQ4

AQ5

AQ6

**Background:** Triple-negative breast cancers (TNBCs) are associated with a poor prognosis. In contrast to other molecular subtypes, they have no identified specific target and chemotherapy remains the only available systemic treatment. The adhesion molecule nectin-4 represents a new potential therapeutic target in different cancer models. Here, we have tested the prognostic value of nectin-4 expression and assessed the therapeutic efficiency of an anti-nectin 4 antibody drug conjugate (ADC) on localised and metastatic TNBC *in vitro* and *in vivo*.

**Materials and methods:** We analysed nectin-4/*PVRL4* mRNA expression in 5673 invasive breast cancers and searched for correlations with clinicopathological features including metastasis-free survival (MFS). Immunohistochemistry was carried out in 61 TNBCs and in samples of primary TNBC Patient-Derived Xenografts (PDXs). An anti-nectin-4 antibody eligible for ADC was produced and tested *in vitro* and *in vivo* in localised and metastatic TNBC PDXs.

**Results:** High nectin-4/*PVRL4* mRNA expression was associated with poor-prognosis features including the TN and basal subtypes. High *PVRL4* mRNA expression showed independent negative prognostic value for MFS in multivariate analysis in TNBCs. Nectin-4 protein expression was not detected in adult healthy tissues including mammary tissue. Membranous protein expression was found in 62% of TNBCs, with strong correlation with mRNA expression. We developed an ADC (N41 mab-vcMMAE) comprising a human anti-nectin-4 monoclonal antibody conjugated to monomethyl auristatin-E (MMAE). *In vitro*, this ADC bound to nectin-4 with high affinity and specificity and induced its internalisation as well as dose-dependent cytotoxicity on nectin-4-expressing breast cancer cell lines. *In vivo*, this ADC induced rapid, complete and durable responses on nectin-4-positive xenograft TNBC samples including primary tumours, metastatic lesions, and local relapses; efficiency was dependent on both the dose and the nectin-4 tumour expression level.

**Conclusion:** Nectin-4 is both a new promising prognostic biomarker and specific therapeutic target for ADC in the very limited armamentarium against TNBC.

**Key words:** ADC targeting, biomarker, breast cancer, nectin-4, survival, TNBC

## Introduction

Triple-negative breast cancers (TNBCs) represent about 15–20% of breast cancers, and are characterised by the absence of expression of oestrogen and progesterone receptors and the absence of

HER2 overexpression or gene amplification. They show overlapping genomic characteristics with basal BCs [1–3]. Patients with TNBC have a higher risk of early and visceral relapse than patients with non-TN breast cancer [4]. However, despite recent efforts to find alternative treatments such as hormone therapy and anti-

## Original article

Annals of Oncology

HER2 drugs for, respectively, hormone receptor-positive and HER2-positive breast cancers, there are no efficient targeted therapies for TNBCs and chemotherapy remains the only available systemic treatment [5]. A cell surface protein specifically expressed in TNBC malignant cells and not in normal breast would consequently represent an ideal molecular surface biomarker as well as a potential therapeutic target for antibody–drug conjugate (ADC).

ADCs are monoclonal antibodies conjugated to cytotoxic agents. Antibodies are specific to tumour cell-surface proteins, and thus, have tumour specificity and potency not achievable with traditional drugs [6]. Design of effective ADC for cancer therapy requires the identification of an appropriate target, a monoclonal antibody against the target, potent cytotoxic effector molecules, and conjugation of the monoclonal antibody to cytotoxic agents. Two ADCs recently received approval from the US Food and Drug Administration for treatment of CD30-positive lymphoma (brentuximab-vedotin) and HER2-positive breast cancer (trastuzumab-emtansine), and several new promising ADCs are now in late-phase clinical testing [6, 7].

*PVRL4*/Nectin-4 is involved in the formation and maintenance of adherens junctions in cooperation with cadherins [8–10]. Nectin-4 is a type I transmembrane cell adhesion molecule composed of three Ig-like domains (V–C–C type) in its extracellular region [8]. It is also a receptor for the measles virus, mediating its endocytosis [11]. Nectin-4 is expressed during foetal development, with expression declining in adult life by contrast with the extensive expression of the other nectins in adult tissues [8]. We and others have shown that nectin-4 is re-expressed as a tumour-associated antigen with pro-oncogenic properties in various carcinomas including breast cancer [12–17]. These features associated with its limited expression in the respective normal tissues, suggest that nectin-4 could be a therapeutic target for ADC in these cancers, as recently reported in bladder, breast and pancreatic cancers [17].

Here, we have analysed the mRNA and protein expression of nectin-4 in a series of ~5700 breast cancers and showed that nectin-4 was a cell surface biomarker more frequently overexpressed in TNBCs and basal breast cancers, and that high mRNA expression was an independent poor-prognosis factor in TNBCs. Then, we have developed an anti-nectin-4 ADC and showed its therapeutic efficiency on localised and metastatic TNBC both *in vitro* and *in vivo*, with rapid, complete, and long-lasting regression in nectin-4-positive patient samples.

## Materials and methods

## Patients and breast cancer samples

The clinical samples had been profiled using DNA microarrays for gene expression analyses. Our own data set included 353 cases representing pre-treatment invasive carcinomas from patients non-metastatic at diagnosis. The study was approved by our institutional review board (IRB agreement no. 15-002). Each patient gave a written informed consent for research use. We pooled our series with 17 publicly available data sets comprising at least one probe set representing *PVRL4/nectin-4*. These sets were collected from the National Center for Biotechnology Information (NCBI)/Genbank GEO and ArrayExpress databases, and authors' website (supplementary Table S1, available at *Annals of Oncology*

online). The final pooled set included 5673 non-redundant, non-metastatic, non-inflammatory, primary, invasive breast cancers with *PVRL4* mRNA expression and clinicopathological data available (supplementary Table S2, available at *Annals of Oncology* online).

For protein expression, we analysed a consecutive series of 61 previously untreated TNBC samples collected at the time of diagnosis from women treated at our Institute. Informed consent was obtained for each patient and the study was approved by our IRB (supplementary Table S3, available at *Annals of Oncology* online).

MDA-MB-231 and SUM190 breast cancer cell lines were used in this study. Detailed information is available in supplementary Methods (available at *Annals of Oncology* online).

## Gene expression data analysis

Our own gene expression data set had been generated using Affymetrix U133 Plus 2.0 human microarrays (Affymetrix®, Santa Clara, CA) as described [18]. MIAME-compliant data are deposited in the GEO database (GSE31448). *PVRL4* expression was measured by analysing different probe sets whose identity and specificity were verified using the NCBI program BLASTN 2.2.31+ (supplementary Table S4, available at *Annals of Oncology* online). Additional details are available in supplementary Methods (available at *Annals of Oncology* online).

## Antibody production, immunohistochemistry, western-blot, ELISA and flow cytometry

Different nectin-4 antibodies were produced. Detailed information is available in supplementary Methods (available at *Annals of Oncology* online).

## Production of ADC

The ADC was produced by Concortis (San Diego, CA). Conjugates were produced from purified mab1/N41 mab monoclonal antibody. The linker used was the maleimidocaproyl-L-valine-L-citrulline-*p*-aminobenzyl alcohol *p*-nitrophenyl carbonate (MC-Val-Cit-PAB-PNP) covalently conjugated to monomethyl auristatin-E (MMAE). This cleavable linker was selected because it induced potent bystander killing. The drug-to-antibody ratio was 4.73.

## Animal models

All experiments were done in agreement with the French Guidelines for animal handling and approved by local ethics committee (Agreement no. 01152-01). Additional details are available in supplementary Methods (available at *Annals of Oncology* online).

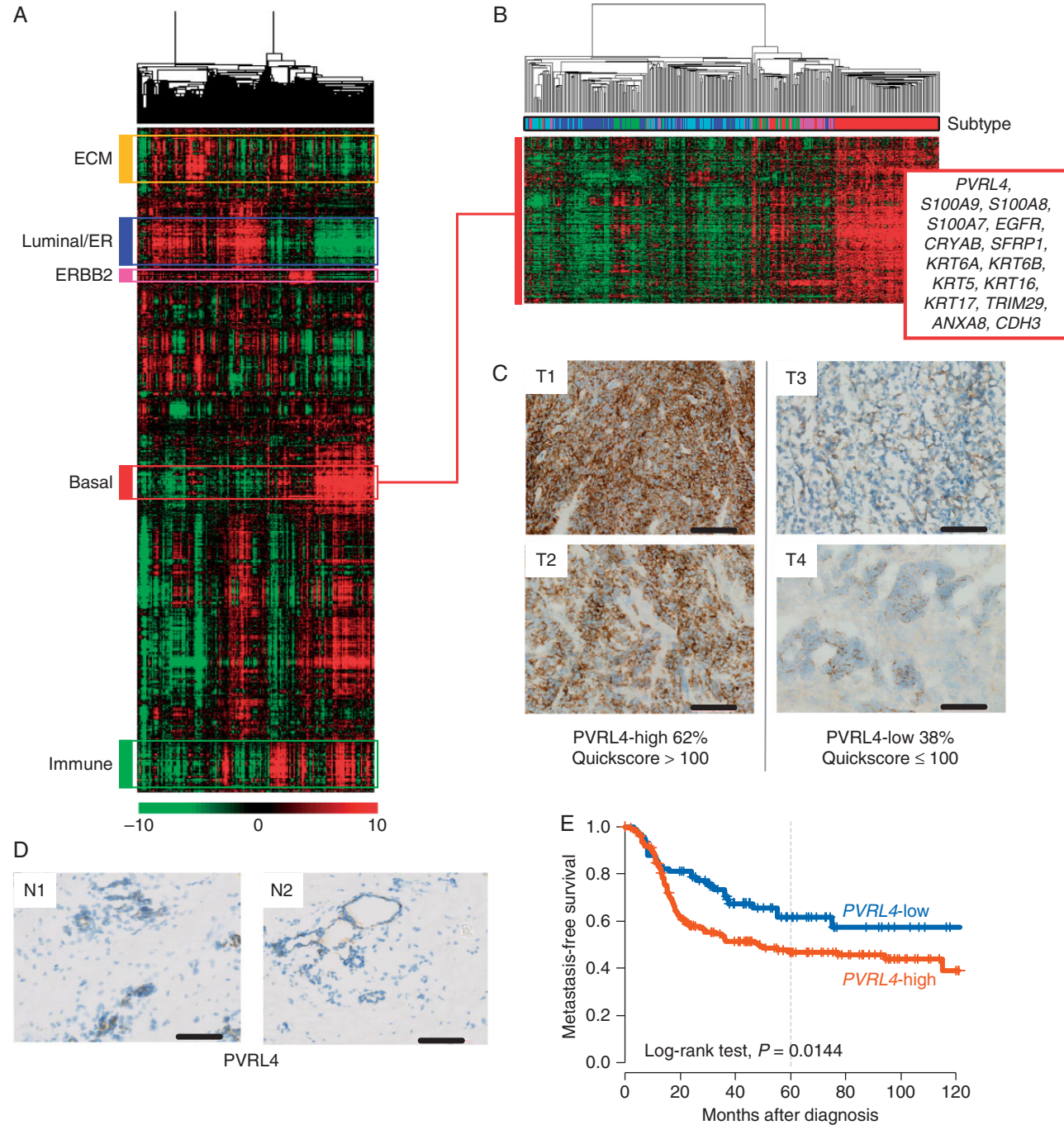
## Statistical analysis

This section is detailed in supplementary Methods (available at *Annals of Oncology* online).

## Results

Nectin-4/*PVRL4* is expressed in TNBC and predicts poor prognosis

We examined *PVRL4* expression in breast cancer. Whole-genome clustering of our 353-sample series showed that *PVRL4* mRNA was in the 'basal' gene cluster and more consistently expressed in basal tumours (Figure 1A and B). We analysed nectin-4 protein expression by immunohistochemistry in 61 TNBCs, 12 of them previously profiled using DNA microarrays.



**Figure 1.** PVRL4/nectin-4 expression in clinical breast cancer samples. (A) Hierarchical clustering of 353 primary breast cancer samples and 12 304 genes based on mRNA expression levels. Each row represents a gene and each column represents a sample. The dendrogram of samples is zoomed in (B). Coloured bars to the left indicate the locations of five gene clusters of interest. (B) Expanded view of the 'basal' gene cluster, which includes the *PVRL4* gene. (C) Nectin-4 expression in TNBCs by IHC on frozen sections using 0.5 mg/ml mab1/N41mab. Staining is prominent at the plasma membrane. T1 and T2 are two TNBCs with QS > 100. T3 and T4 are two TNBCs with QS < 100. Nectin-4 expression is rare in HR+ and HER2+ breast tumours in accordance with mRNA expression (data not shown). Scale bar: 100  $\mu$ m. (D) Absence of expression in the normal human epithelial mammary gland tissue. Scale bar: 100  $\mu$ m. (E) Kaplan-Meier MFS curves according to *PVRL4* mRNA expression in TNBCs ( $n=290$ ).

AQ10

The monoclonal antibody used for this analysis, selected from our screening (see next paragraph), recognised the distal IgV-like domain of human nectin-4 and did not cross-react with the other human nectins or with mouse nectin-4 (supplementary Figure S1, available at *Annals of Oncology* online). Nectin-4 expression was detected at the plasma membrane. Based on the QuickScore (QS) semi-quantitative assessment, we distinguished a 'nectin-4-high

group' (QS > 100) and a 'nectin-4-low group' (QS  $\leq$  100), representing 62% and 38% of TNBCs, respectively (Figure 1C). There was a positive correlation between mRNA and protein expression of nectin-4 (supplementary Figure S2, available at *Annals of Oncology* online;  $P=0.002$ ). Importantly, nectin-4 was detected neither in the normal mammary gland epithelium (Figure 1D) nor in 30 different adult normal tissues except the skin (supplementary

Table 1. Univariate and multivariate prognostic analyses in the TNBC population

Characteristics	Univariate			Multivariate		
	n	HR [95%CI]	P-value	n	HR [95%CI]	P-value
Age, >50 versus <=50	204	1.13 [0.68–1.89]	0.63			
Pathological type, lobular versus ductal	128	1.49 [0.36–6.24]	0.28			
Pathological type, mixed versus ductal		0.00 [0.00–Inf.]				
Pathological type, other versus IDC		0.38 [0.13–1.07]				
Pathological tumour size (pT), pT2–3 versus pT1	107	1.39 [0.56–3.41]	0.48			
Pathological lymph node status (pN), positive versus negative	202	0.75 [0.49–1.15]	0.19			
Pathological grade, 3 versus 1–2	219	1.42 [0.72–2.78]	0.31			
Immune response GES, poor versus good	290	1.82 [1.19–2.80]	6.08E–03	290	1.13 [0.54–2.38]	0.740
LCK GES, poor versus good	290	1.92 [1.34–2.76]	3.80E–04	290	1.55 [1.03–2.35]	3.67E–02
Kinase immune GES, poor versus good	290	1.82 [1.23–2.63]	2.33E–03	290	0.77 [0.40–1.49]	0.439
PVRL4/ <i>nectin-4</i> mRNA expression, high versus low	290	1.65 [1.10–2.47]	1.54E–02	290	1.53 [1.02–2.30]	3.95E–02

Figure S3, available at *Annals of Oncology* online) [10]. These results established nectin-4 as both a new cell surface biomarker and a potential target for TNBCs.

We then analysed a pooled series of 5673 invasive breast cancers profiled using DNA microarrays and five different PVRL4 probes 100% specific. High PVRL4 expression was associated with poor-prognosis features, including both TN and basal subtypes (supplementary Table S5, available at *Annals of Oncology* online). PVRL4 was highly expressed in 61% of TN samples and 62% of basal samples versus 47% of non-TN or non-basal samples. Metastasis-free survival (MFS) data were available for 1037 patients, including 613 without metastatic relapse (median follow-up, 83 months) and 424 with metastatic relapse (median time to relapse, 24 months). The 5-year MFS rate was 61% (95%CI, 0.58–0.65). In the whole population, high PVRL4 expression was associated with shorter MFS ( $P=0.014$ , log-rank test) (supplementary Figure S4, available at *Annals of Oncology* online). Prognostic univariate analysis per molecular subtype showed that high PVRL4 expression was associated with MFS in the TN subtype only, with 5-year MFS of 47% (95%CI, 0.40–0.55) versus 62% (95%CI, 0.51–0.74) in the 'PVRL4-high' and 'PVRL4-low' groups, respectively ( $P=0.014$ , log-rank test) (Figure 1E, available at *Annals of Oncology* online). In multivariate analysis (Table 1), high PVRL4 expression remained significant ( $P=0.039$ , Wald test; HR = 1.53 [1.02–2.30]). Altogether, these results indicated that PVRL4/*nectin-4* is commonly expressed in TN or basal breast cancers, not expressed in the normal breast, and is an independent predictor of shorter MFS in TNBCs.

### ADC-based targeting of nectin-4 *in vitro*

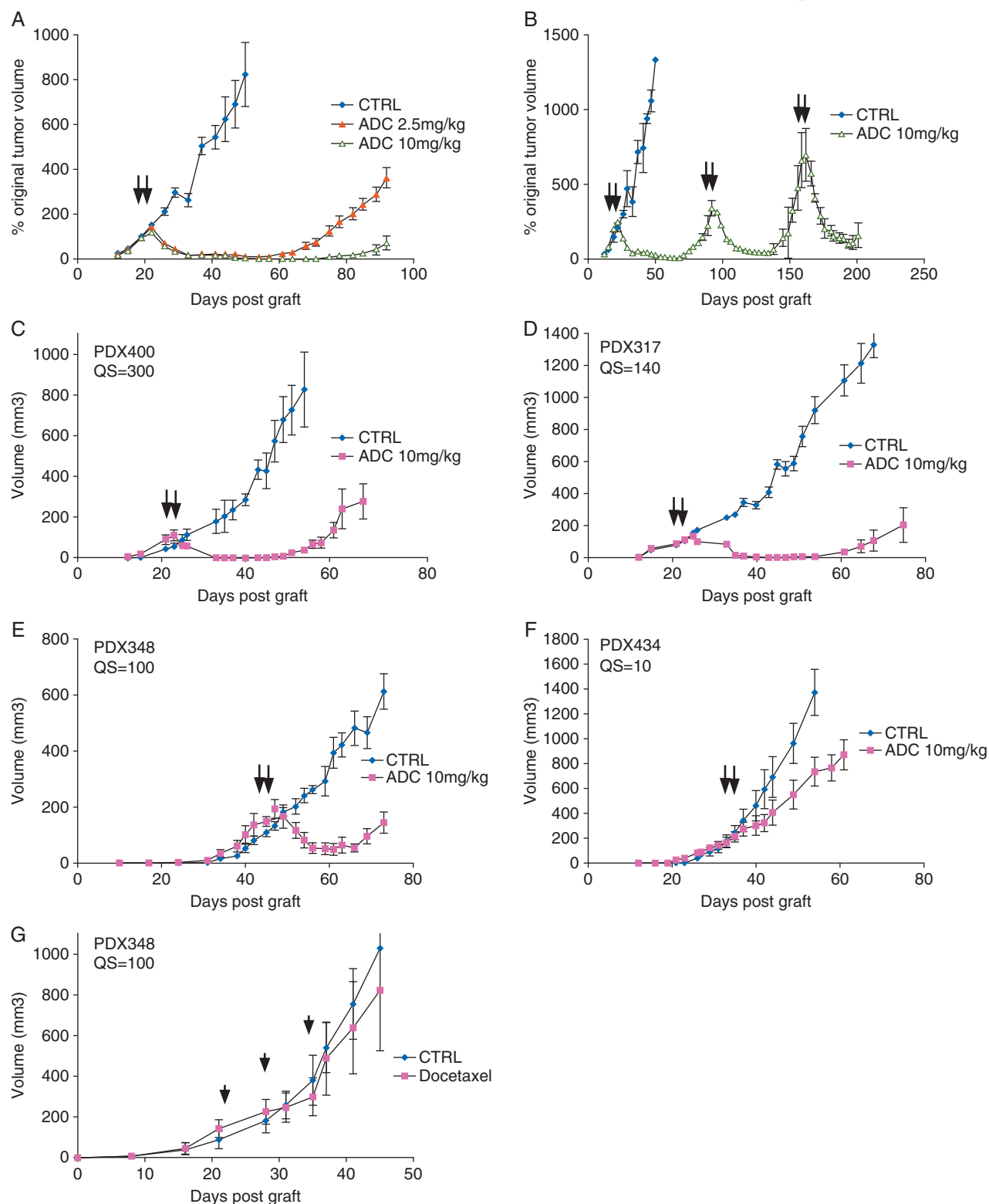
We tested six monoclonal antibodies (mAbs) directed against the IgV-like distal domain of nectin-4 to isolate a mAb able to induce internalisation. MAb were evaluated for EC50, maximum binding capacity, cell surface internalisation and cytotoxicity (supplementary Figure S5, available at *Annals of Oncology* online). Internalisation was tested using ectopically expressed Flag-tagged nectin-4 expressed in MDA-MB-231 cells and FITC-labelled anti-Flag antibody (M2, Sigma-Aldrich, St. Louis, MO) to quantify residual surface nectin-4. Mab1 was the most efficient

antibody. It induced a 60% decrease of cell surface nectin-4 in 24 h and a 60% cell growth inhibition after incubation with a goat anti-mouse monoclonal antibody conjugated to saporin (mab-ZAP kit, ATS-bio, San Diego, CA). Internalisation and cytotoxicity were positively correlated ( $R^2=0.96$ ). Mab1 did not affect cell viability *in vitro* and tumour cell growth *in vivo* (data not shown). Mab1 was then conjugated to monomethyl auristatin-E (MMAE) via a cleavable valine-citrulline (vc) di-peptide linker (thereafter called N41mab-vcMMAE, ADC) to produce an ADC, which was then tested *in vitro* for specificity and efficacy on selected breast cancer cell lines. MDA-MB-231 cells, which express nectin-1, nectin-2, and nectin-3, but not nectin-4, were not sensitive to the ADC. However, ectopic expression of nectin-4 conferred sensitivity with an IC50 = 2 ng/ml (supplementary Figure S6A, available at *Annals of Oncology* online). SUM190 cells, which express endogenous nectin-4, were sensitive with an IC50 = 4 ng/ml (supplementary Figure S6B, available at *Annals of Oncology* online). These data showed the specificity and the efficacy of N41mab-vcMMAE.

### ADC-based targeting of nectin-4 *in vivo*

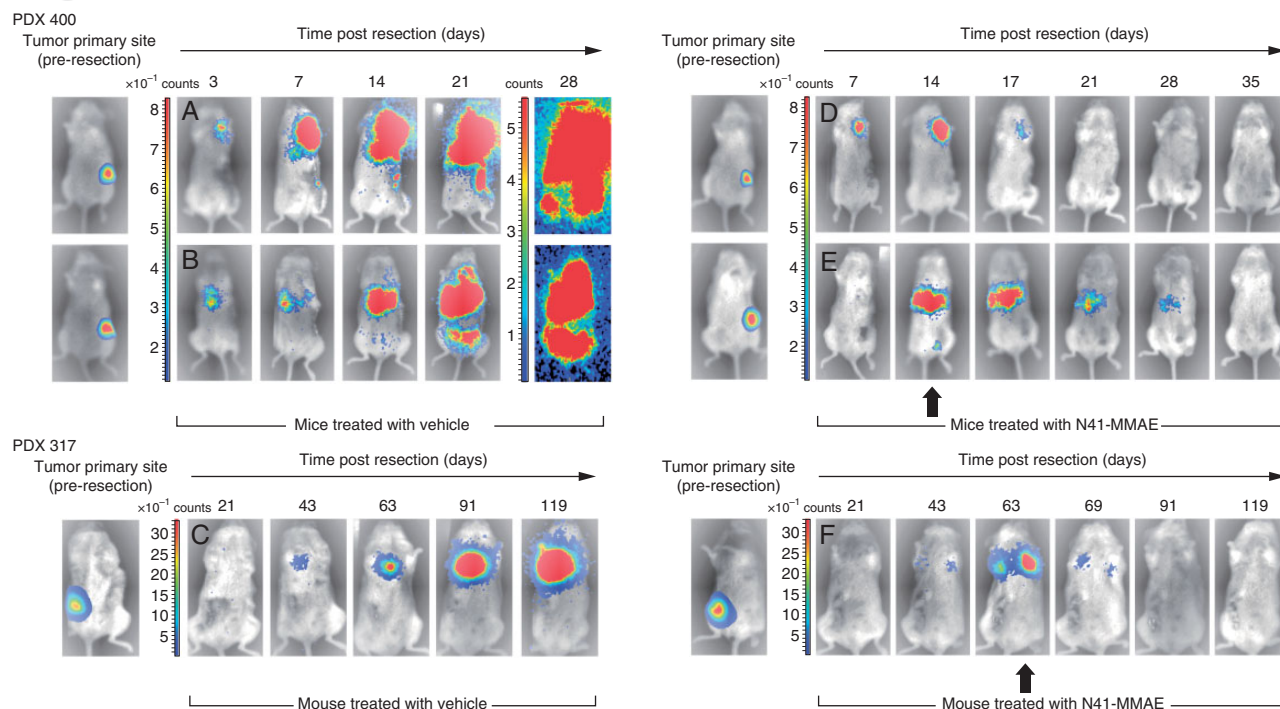
Activity of our ADC was tested in three *in vivo* models of TNBC developed in immunocompromised NSG mice. First, mice xenografted with SUM190 cells were treated with two successive i.v. doses of N41mab-vcMMAE (Figure 2A). These doses were not toxic for mice (supplementary Figure S7, available at *Annals of Oncology* online). N41mab-vcMMAE induced a rapid (4 days) and complete tumour regression, the duration of which was dose-dependent and lasted up to 40 days at the 10 mg/kg dose (Figure 2A). Interestingly, after relapse, tumours still kept their sensitivity to the ADC with tumour regression similar to that obtained after the first treatment, at least over a 6-months period (Figure 2B).

Second, we used patient-derived xenografts (PDX) of nine primary TNBCs. These pre-clinical models recapitulate breast cancer physiopathology [19]. Localisation and levels of nectin-4 expression in PDX were similar to that found in TNBC patients (supplementary Figure S8, available at *Annals of Oncology* online). Nectin-4 expression was prominently found at the plasma membrane in seven of nine PDX (QS > 100). TNBC PDX mice



**Figure 2.** *In vivo* efficacy of the ADC N41mab-vcMMAE in TNBC models. (A) SUM190 cells grown orthotopically as tumours in NSG mice show dose-dependent regression after a single treatment with ADC at the indicated doses (2 i.v. injections (arrows) at 4-days interval). (B) Similar to (A). First arrows: first treatment by 2 i.v. injections at 10 mg/kg; second and third arrows: same treatment at second and third relapses. Tumour regression kinetic after relapse is similar to regression observed after the first treatment. (C–F) TN patient-derived tumour grown orthotopically in NSG mice and treated with ADC at 10 mg/kg (2 i.v. injections at 4-days interval). (C) PDX400 (QS = 3 × 100%); (D) PDX317 (QS = 2 × 70%); (E) PDX348 (QS = 2 × 50%); (F) PDX434 (QS = 1 × 10%). A bystander effect, described for cleavable linkers, may account for the inhibition observed for T317 and T348. (G) Docetaxel treatment (10 mg/kg) of PDX348. Arrows indicate i.v. injection. Error bars represent mean ± SEM.





**Figure 3.** N41mab-vcMMAE treatment of PDX400 and PDX317 mice impacts spontaneous metastatic progression. After surgical resection of primary mammary tumours, spontaneous metastasis occurrence was followed by bioluminescence imaging. Metastatic mice were treated vehicle (left, A, B and C) or with N41mab-vc-MMAE (right, D, E and F) by caudal i.v. injection. Quantification of total bioluminescence signal was measured. Major metastatic localisations observed at autopsy were as follows: PDX400 (A): liver, lymph node, lung; PDX400 (B): liver, lymph node, ovary; PDX400 (D): interscapular mass, lung; PDX400 (E): liver, pancreas; PDX317 (C and F): lung, heart.

with different QS were treated with two successive i.v. doses of ADC. The degree of clinical response was roughly correlated with nectin-4 expression level: a rapid and complete tumour burden regression (and durable up to 35 days) was observed for PDX400 (QS = 300) and PDX 317 (QS = 140), to a lesser extent for PDX348 (QS = 100) (Figure 2C–E), but not observed for PDX434 (QS = 10) (Figure 2F). In contrast, treatment of PDX 348—sensitive to our ADC—by docetaxel (3 times 10 mg/kg i.p.) was ineffective (Figure 2G).

Third, to evaluate ADC treatment efficacy in a more advanced disease, we treated PDX400 and PDX317 mice developing spontaneous metastatic lesions from primary tumours. In both cases, treatment with two successive i.v. doses of ADC led to a rapid reduction and complete disappearance of all metastatic lesions observed by luminescence analysis (Figure 3). Metastatic recurrences were detected at days 42 and 57 for PDX400, and still not observed for PDX317 (ongoing experiment: day 245) (data not shown). These results showed that N4mab1-vcMMAE had a marked anti-tumour activity in both primary and metastatic nectin-4-expressing TNBCs.

## discussion

Nectin-4 has been reported as a new tumour antigen for different carcinomas [12–17]. Here, we have analysed its mRNA and protein expression in a series of ~5700 breast cancers and showed that nectin-4 was a cell surface biomarker more frequently over-expressed in TNBCs and basal breast cancers, and that high mRNA expression was an independent poor-prognosis factor for

MFS in TNBCs. Then, we have developed an anti-nectin-4 ADC and showed its therapeutic efficiency on localised and metastatic TNBC both *in vitro* and *in vivo*, with rapid and long-lasting regression in nectin-4-positive samples. To our knowledge, this work is the first description of a cell surface biomarker eligible for the systemic treatment of TNBCs.

We showed that nectin-4 is a cell surface biomarker more frequently overexpressed in TNBCs and basal breast cancers. Its protein expression, analysed by immunohistochemistry (IHC) using our antibody, was detected at the plasma membrane and positively correlated with mRNA expression. High-protein expression and high mRNA expression were found in the same proportion of cases, nearly two-thirds of TNBCs. This protein expression pattern was confirmed using four other monoclonal antibodies recognising different epitopes in the nectin-4 ectodomain (supplementary Figure S9, available at *Annals of Oncology* online). Importantly, nectin-4 protein was not detected in the normal mammary gland epithelium and 30 other different adult normal tissues except the skin. These results confirmed and extended our previous work done within a limited number of samples [12] (Figure 1). To our knowledge, nectin-4 expression has been studied in clinical breast cancer samples in three other studies using IHC in two series of 197 [20] and 654 [17] cases and using immunocytochemistry in a series of imprint smears of 140 cases [21]. Two studies used the same commercial polyclonal antibody [20, 21]. We tested this antibody on nectin-4 mRNA negative TNBCs and found a cytoplasmic and a nuclear staining by IHC (data not shown). This polyclonal antibody recognised nectin-4 by western-blot but, in contrast to N41mab, gave a high background signal on nectin-4 mRNA negative MDA-MB-231

cells (supplementary Figure 1B, available at *Annals of Oncology* online). In the third study, authors generated their own monoclonal antibody, directed against the extra-cellular domain of human nectin-4, and analysed 654 breast cancers (primary tumours and metastases): 53% of specimens showed moderate to strong staining, but no correlation with clinicopathological features or survival was looked for. In our series, high nectin-4 expression was an independent poor-prognosis factor for MFS in the TNBC patients only. Interestingly, we observed that nectin-4 overexpression increased tumour growth and metastasis formation in mouse xenograft models (data not shown). *De novo* expression of nectin-4 may perturb nectin-supported adherens junctions and increase cell migration.

Nectin-4 presents two interesting properties: an inducible and efficient internalisation, which is hijacked by the measles virus, and a limited expression in healthy tissues. Together, these properties make nectin-4 an attractive target for the development of immune-base therapies especially for TNBCs for which no specific cell surface antigen has been identified so far [22]. ADC directed against nectin-4 has been recently shown efficient in mice models subcutaneously xenografted with cell lines [17]. Based on *in vitro* assessment of EC50, maximum binding capacity, cell surface internalisation and cytotoxicity, we retained the Mab1 for ADC approach. Mab1 was then conjugated to monomethyl auristatin-E (MMAE) *via* a cleavable linker to produce an ADC, thereafter called N41mab-vcMMAE. This ADC was validated *in vitro* for efficiency and specificity.

We then showed its *in vivo* activity in models of TNBC developed in immunocompromised NSG mice. The model based on the SUM190 cell line exhibited rapid and complete response, the duration of which was prolonged and dependent on the dose; furthermore, the relapses remained similarly sensitive to our ADC over the 6-months period tested. We also took advantage of our collection of primary TNBC PDXs phenotypically close to the physiopathology of TNBCs [19]. In these models, patient tumours develop in the mouse mammary gland and spontaneously spread to the same sites as do metastatic TNBCs (Figure 3). High expression of nectin-4 was found in two-thirds of PDXs, a proportion close to that observed in our clinical series. The results showed a marked efficiency of the anti-nectin-4 ADC with complete and durable eradication of both localised TNBC tumours and metastatic lesions of different sites. These data not only confirmed the results previously reported [17] with another anti-nectin-4 ADC (AGS-22M6E) but also showed additional important results. Our ADC induced complete and durable responses on both primary tumours, metastatic lesions and local relapses, and the degree of response was dependent on the nectin-4 tumour expression level. We surmise that tumour shrinkage is due to toxin delivery because N41mab without drug did not have intrinsic anti-tumour growth activity both *in vitro* and *in vivo*. *In vivo* pre-clinical responses with N41mab-vcMMAE were observed for 'nectin-4-high' TN tumours (QS > 100), predicting a good response in most TNBC patients (62% of cases). We also found similar efficacy between N41mab conjugated to emtansine (N41mab-DM1) and trastuzumab-DM1 [23] in SUM190-xenografted mice (data not shown). Because both nectin-4 and HER2 are equally expressed at the surface of SUM190 cells, we can postulate that nectin-4-based ADC is at least as efficient as trastuzumab-DM1 in this model.

Efficacy of drugs used for treatment in humans is closely dependent on the toxicity they induce in healthy tissues. This is also true for ADC-based therapy where expression of the target has to be restricted to the malignant cells. Consequently, the restricted expression pattern of nectin-4 in humans would predict a limited toxicity. The ADC tested here did not induce any toxicity in mice even after repeated treatments. However, this cannot predict an absence of toxicity in humans because our N41mab does not cross-react with murine nectin-4. A phase I study addressing this issue was recently completed for examining the safety of AGS-22M6E ADC as monotherapy in patients with malignant solid tumours expressing nectin-4 (Clinical Trials.gov Identifier, NCT01409135). Another ongoing phase I study is assessing the safety and pharmacokinetics of escalating doses of ASG-22CE given as monotherapy in subjects with metastatic urothelial cancer and other malignant solid tumours that express nectin-4 (Clinical Trials.gov Identifier, NCT02091999).

In conclusion, we show that nectin-4 is a new promising therapeutic target in the armamentarium against TNBC. It is a cell surface biomarker frequently expressed in TNBCs, with independent negative prognostic value, and without expression in normal breast tissue. The anti-nectin 4 ADC that we developed (N41mab-vcMMAE) induced complete and durable responses *in vitro* and *in vivo* on nectin-4-positive samples including primary tumours, metastatic lesions and local relapses; efficiency was dependent on both the dose and the nectin-4 tumour expression level. Evaluation of this ADC in the clinical setting is warranted. Additionally, because resistance to auristatin-based ADC has been reported [24], we are currently developing *in vivo* models aiming to evaluate tumour resistance to our ADC.

## Acknowledgements

We would like to thank Jean-Paul Borg, Françoise Birg and Claude Mawas for their support and helpful discussions. We thank Patrick Gibier and Jean Christophe Orsoni for their helpful assistance in animal facilities.

## Funding

This work has been supported by Inserm, Inserm Transfert, the Ligue Nationale Contre le Cancer (label DB) and SIRIC (INCa-DGOS-Inserm 6038 grant). DO team was labelled 'Equipe FRM DEQ 201 40329534'. DO is Senior Scholar of the Institut Universitaire de France.

## Disclosure

The authors have declared no conflicts of interest

## Author ORCIDs

François Bertucci: <http://orcid.org/0000-0002-0157-0959>.  
Marc Lopez: <http://orcid.org/0000-0002-6573-633X>.

AQ8

105

## References

1. Bertucci F, Finetti P, Cervera N et al. How different are luminal A and basal breast cancers? *Int J Cancer* 2009; 124: 1338–1348.
2. Le Du F, Eckhardt BL, Lim B et al. Is the future of personalized therapy in triple-negative breast cancer based on molecular subtype? *Oncotarget* 2015; 6: 12890–12908.
3. Sorlie T, Perou CM, Tibshirani R et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; 98: 10869–10874.
4. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer* 2009; 9 Suppl 2: S73–S81.
5. Bertucci F, Finetti P, Birnbaum D. Basal breast cancer: a complex and deadly molecular subtype. *CMM* 2012; 12: 96–110.
6. Thomas A, Teicher BA, Hassan R. Antibody-drug conjugates for cancer therapy. *Lancet Oncol* 2016; 17: e254–e262.
7. Tolcher AW. Antibody drug conjugates: lessons from 20 years of clinical experience. *Ann Oncol* 2016.
8. Reymond N, Fabre S, Lecocq E et al. Nectin4/PRR4, a new afadin-associated member of the nectin family that trans-interacts with nectin1/PRR1 through V domain interaction. *J Biol Chem* 2001; 276: 43205–43215.
9. Fabre S, Reymond N, Cocchi F et al. Prominent role of the Ig-like V domain in trans-interactions of nectins. Nectin3 and nectin 4 bind to the predicted C–C′–C″–D beta-strands of the nectin1 V domain. *J Biol Chem* 2002; 277: 27006–27013.
10. Brancati F, Fortugno P, Bottillo I et al. Mutations in PVRL4, encoding cell adhesion molecule nectin-4, cause ectodermal dysplasia-syndactyly syndrome. *Am J Hum Genet* 2010; 87: 265–273.
11. Muhlebach MD, Mateo M, Sinn PL et al. Adherens junction protein nectin-4 is the epithelial receptor for measles virus. *Nature* 2011; 480: 530–533.
12. Fabre-Lafay S, Monville F, Garrido-Urbani S et al. Nectin-4 is a new histological and serological tumor associated marker for breast cancer. *BMC Cancer* 2007; 7: 73.
13. Takano A, Ishikawa N, Nishino R et al. Identification of nectin-4 onco-protein as a diagnostic and therapeutic target for lung cancer. *Cancer Res* 2009; 69: 6694–6703.
14. Derycke MS, Pambuccian SE, Gilks CB et al. Nectin 4 overexpression in ovarian cancer tissues and serum: potential role as a serum biomarker. *Am J Clin Pathol* 2010; 134: 835–845.
15. Pavlova NN, Pallasch C, Elia AE et al. A role for PVRL4-driven cell–cell interactions in tumorigenesis. *Elife* 2013; 2: e00358.
16. Nishiwada S, Sho M, Yasuda S et al. Nectin-4 expression contributes to tumor proliferation, angiogenesis and patient prognosis in human pancreatic cancer. *J Exp Clin Cancer Res* 2015; 34: 30.
17. Challita-Eid PM, Satpayev D, Yang P et al. Enfortumab vedotin antibody–drug conjugate targeting Nectin-4 is a highly potent therapeutic agent in multiple preclinical cancer models. *Cancer Res* 2016; 76: 3003–3013.
18. Bertucci F, Finetti P, Cervera N et al. Gene expression profiling shows medullary breast cancer is a subgroup of basal breast cancers. *Cancer Res* 2006; 66: 4636–4644.
19. Charafe-Jauffret E, Ginestier C, Bertucci F et al. ALDH1-positive cancer stem cells predict engraftment of primary breast tumors and are governed by a common stem cell program. *Cancer Res* 2013; 73: 7290–7300.
20. Lattanzio R, Ghasemi R, Brancati F et al. Membranous Nectin-4 expression is a risk factor for distant relapse of T1-T2, N0 luminal-A early breast cancer. *Oncogenesis* 2014; 3: e118.
21. Athanassiadou AM, Patsouris E, Tsipis A et al. The significance of Survivin and Nectin-4 expression in the prognosis of breast carcinoma. *Folia Histochem Cytobiol* 2011; 49: 26–33.
22. Lopez M, Ghidouche A, Rochas C et al. Identification of a naturally processed HLA-A\*02:01-restricted CTL epitope from the human tumor-associated antigen Nectin-4. *Cancer Immunol Immunother* 2016.
23. Lewis Phillips GD, Li G, Dugger DL et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res* 2008; 68: 9280–9290.
24. Yu SF, Zheng B, Go M et al. A novel anti-CD22 anthracycline-based antibody-drug conjugate (ADC) that overcomes resistance to auristatin-based ADCs. *Clin Cancer Res* 2015; 21: 3298–3306.

**Key Message**

We show that high nectin-4 expression was associated with triple-negative breast cancers with independent negative prognostic value. Nectin-4 was not detected in healthy tissues. We developed an ADC comprising a human anti-nectin-4 monoclonal antibody conjugated to MMAE that induced complete and durable responses of primary tumours, metastases and local relapses in nectin-4-positive

**AQ7** xenograft TNBC samples.