nature portfolio

Peer Review File

Expression of antibody-drug conjugate targets in post-mortem samples of breast cancer metastases and normal tissue

Corresponding Author: Professor Christine Desmedt

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors investigate expression of antibody-drug conjugate targets in breast cancer metastases and normal tissue. They use RNA sequencing to investigate the expression of 72 ADC targets, which are clinically approved or 37 under investigation, in 916 samples (64 primary untreated, 753 metastatic, 99 normal) from 30 patients enrolled in a post-mortem tissue donation program.

The introduction is somewhat lengthy, the detailed characterisations of SG and T-DXd are probably not needed.

From a methodological point of view the use of a large number of different samples from various tumor manifestations and normal tissues is a strength enabled by the postmortem biobank. However, how stable are mRNA expression analyses in post-mortem samples. This should at least be critically discussed and explained. Another problem is the use of only one methodology. While the authors point out in the discussion that correlation with a complementary methods such as immunohistochemistry in the entire cohort would be very resource consuming, IHC validation for select ADC targets of interest would strengthen the manuscript, especially as some results do not seem to reflect prior results, e.g.: HER2 and HER3 are described to be decreased in expression in brain metastases as compared to extra-cerebral tumor manifestations in this manuscript. That seems to contradict prior reports on these molecules. It that a methodological problem? Or how can this be explained?

Minor point: The discussion of T-DXd for brain metastases of HER2-positive breast cancer omits the pivotal trials (done before DESTINYBreast12) DEBBRAH and TUXEDO-1.

Overall, the study is descriptive and relies on only one methodology, which limits the robustness of the results. The presentation and discussion of the data is diffuse and does not achieve to deliver a clear message - can the authors draw any more concrete conclusions than just saying their data may help to inform drug development? Selection of top priority ADC targets and vaidation of those with a complementary method that can also facilitate clinical use (e.g. IHC) may help to make the work more relevant.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This manuscript presents an extensive analysis of RNA expression levels for 72 ADC targets across a large cohort of metastatic breast cancer samples, primary untreated tumors, and normal tissues. Leveraging post-mortem tissue donation, the authors provide insights into inter- and intra-patient heterogeneity, organ-specific expression patterns, and co-expression relationships between ADC targets. This study fills an important gap in our understanding of ADC target prevalence in metastatic breast cancer and contributes valuable data that could inform future ADC development.

Regarding the strengths of the manuscript, the study analyzes 916 breast cancer-relevant samples collected from 30 patients, including a large number of metastatic lesions from diverse organ sites, which enhances the generalizability of the findings. The inclusion of normal tissues allows for an assessment of potential on-target off-tumor toxicities, a critical factor in ADC development. The updated clinical trials search methodology ensures that the study reflects the most current ADC targets under investigation. The authors use modern and validated techniques for RNA extraction, sequencing, and normalization, with validation through external datasets (TCGA), strengthening the reliability of findings. There is definite potential for clinical impact: results provide useful insights into which ADC targets are most promising for breast cancer metastases, an exploration of organ-specific trends in ADC target expression, as well as the feasibility of bispecific ADCs or ADC combinations.

We have a few suggestions to further improve the manuscript

Major Comments

1.Biological and Clinical Relevance of RNA Expression Levels
- While mRNA expression provides valuable insights, protein expression levels are a more accurate representation of ADC target availability. The manuscript acknowledges this limitation but does not provide a clear rationale for why RNA-based analysis alone is sufficient to draw conclusions about target suitability.
- Since HER2 protein expression often differs from mRNA levels, can the authors provide any validation for RNA-protein correlation beyond the Bosi et al. reference? Even targeted immunohistochemistry (IHC) or proteomic data for a subset of samples would strengthen the conclusions.
- Based on the study results, HER2 does not appear as an ideal target. However, empiric experience shows that HER2 represents one of the most successful ADC targets in oncology. Could the authors try to address this apparent contradiction in the discussion?

2.Cohort Characteristics and Potential Biases
- The cohort is heavily skewed toward HR+/HER2- disease (77%), with only 20% TNBC and 3% HER2+ cases. This imbalance raises concerns about how generalizable the findings are, particularly regarding HER2 and TROP2, which are critical in TNBC and HER2+ subtypes.
- The study does not account for potential prior treatment effects on target expression. Although only 4 patients received prior ADCs, other systemic therapies could have influenced target expression, particularly for immune-related targets (e.g., PD-L1). Were there any differences in expression patterns between patients who received prior ADCs and those who did not? What about prior immune therapy in the metastatic setting?

3.Statistical Considerations
- The manuscript states RNA was extracted from some fresh frozen and some FFPE samples – was there correction for this batch effect?
- The choice of p-value threshold (adjusted $p < 0.10$, trend $p < 0.15$) is relatively lenient compared to conventional thresholds. Given the large number of comparisons made, false positives could be a concern. Did the authors explore stricter thresholds or alternative statistical approaches to reduce false discovery rates?
- The weighted Spearman correlation analysis is a useful approach, but the lack of statistical significance values in the wCorr package is a limitation. Did the authors validate the results using a secondary method (e.g., permutation tests)?

4.Interpretation of Co-Expression Findings
- The observed co-expression and mutual exclusivity of ADC targets in metastases provide useful insights for ADC combinations, but the correlation coefficients are moderate (mostly R 0.5–0.6). While the authors suggest that certain targets could be considered for bispecific ADCs, is there any supporting evidence (e.g., known protein-protein interactions or prior co-expression studies) that would justify prioritizing these combinations?

5.Besides target expression, key features of an optimal ADC target include surface localization and adequate internalization. This aspect could be better highlighted in the article.

Minor Comments
1.Figures
- Figure 6 (normal vs. metastatic tissue expression) is important for understanding potential ADC toxicities but does not clearly indicate statistical significance in overlap calculations. Adding p-values or confidence intervals for overlap scores would improve interpretability.

2.Manuscript text
- The manuscript text should be updated to include the FDA approval of datopotamab deruxtecan for metastatic breast cancer (such as in the introduction and discussion).
- The discussion suggests that FN1 and MUC1 are poor ADC targets due to high intra-patient heterogeneity and overlap with normal tissue expression. However, high intra-patient heterogeneity could suggest a need for biomarker stratification rather than complete dismissal of these targets.
- Some terminology should be more precise. For example, "significantly increased expression" in metastases compared to normal tissue is sometimes used without specifying whether the difference is statistically significant or just numerically higher.

3.Statistics
- The use of Spearman correlation thresholds (R > 0.6) to define strong correlation is reasonable, but a sensitivity analysis showing how results change with different cutoffs (e.g., R > 0.5 or R > 0.7) would be informative.
- The regression models used to compare metastatic and primary tumor expression do not explicitly state how confounding variables (e.g., subtype, prior treatment) were handled. If possible, including adjusted effect sizes would strengthen the analysis.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my points of criticism adequately.

(Remarks on code availability)
The authors have addressed my points of criticism adequately.

Reviewer #2

(Remarks to the Author)
I have reviewed the authors' revisions and responses to the previous comments. All concerns have been adequately addressed, and the manuscript has been significantly improved. I am satisfied with the current version and congratulate with the authors for the interesting study.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Response to the reviewers

For the manuscript by Kristien Borremans, Anirudh Pabba, Gitte Zels and collaborators entitled: "Expression of antibody-drug conjugate targets in metastatic breast cancer" with reference NCOMMS-24-85586.

Response to Reviewer #1

The introduction is somewhat lengthy, the detailed characterisations of SG and T-DXd are probably not needed.

RE: We thank the reviewer for this suggestion. The paragraphs concerning SG and T-DXd have been shortened considerably. We did not remove them completely as they illustrate the importance of target expression. Of note, we have also added now in the introduction the 2 ADCs recently given fast track designation by the FDA for patients with metastatic TNBC.

From a methodological point of view the use of a large number of different samples from various tumor manifestations and normal tissues is a strength enabled by the postmortem biobank. However, how stable are mRNA expression analyses in post-mortem samples. This should at least be critically discussed and explained.

RE: We have performed a timepoint analysis to evaluate changes in mRNA expression on samples from 14 patients taken from the same normal or metastatic sample at 1.5h intervals during the post-mortem tissue donation. The methodology has been described in our methods (line 146-149). Statistical analysis was performed as described in our earlier work (Geukens, De Schepper, Van Den Bogaert, et al. NPJ Breast Cancer. 2024, PMID: 38658604). In these samples, the expression of the targets we investigated in this manuscript did not vary substantially with time (Supplementary figure 2A-K, Supplementary Table 5). While these results did not uncover changes with increasing post-mortem interval, we have addressed the limitation of only assessing metastatic samples at death within this study in the discussion.

Another problem is the use of only one methodology. While the authors point out in the discussion that correlation with a complementary methods such as immunohistochemistry in the entire cohort would be very resource consuming, IHC validation for select ADC targets of interest would strengthen the manuscript, especially as some results do not seem to reflect prior results, e.g.: HER2 and HER3 are described to be decreased in expression in brain metastases as compared to extra-cerebral tumor manifestations in this manuscript. That seems to contradict prior reports on these molecules. It that a methodological problem? Or how can this be explained?

RE: We understand the concern of the reviewer and have performed additional exploratory immunohistochemistry analyses to expand on our transcriptomic findings. This methodology has now been added to the methods sections regarding histopathological characterization. Eight targets of interest were selected for immunohistochemical staining of metastatic and normal samples: (i) targets of ADCs being approved for metastatic BC (HER2, TROP2) or having received fast track designation (NECTIN4 and VTCN1), (ii) target with clinical potential in breast cancer based on high mRNA expression in metastatic samples and low mRNA expression in normal samples (HER3) and (iii) targets with high levels of mRNA expression in metastases but also in normal tissues, likely indicative of high risk for toxicities (FN1, MUC1, FGFR2).

Sample selection was performed to consider a range of mRNA target expression across the 6 most common organ sites. A larger number of samples were stained for HER2, TROP2 and HER3, as these were already considered for a separate project on metastatic TNBC. Regarding the normal tissue, 10 samples were taken across liver, lung and axillary lymph nodes. In metastatic samples only staining in tumor cells was considered, while in normal samples staining in every type of cell was considered. Immunohistochemical assessment of HER2 and HER3 was done according to the ASCO/CAP guidelines for HER2 (PMID: 37284804). TROP2 was scored using the H-score, but for our analysis, only the total percentage of cells displaying membranous and/or cytoplasmic staining was considered. Due to lack of guidelines, protein expression of the other 5 targets in metastatic samples was scored as a continuous variable, % of tumor cells, and in normal samples the other 5 targets and HER3 were assessed as the % of all normal cells that showed any level of staining regardless of intensity and cytological localization.

The analysis of the immunohistochemistry data was mainly explorative given the relatively limited number of samples. Our main goal was to compare the expression between metastases and normal tissue samples, to see whether the conclusions we made based on the transcriptomic data were still valid based on the preliminary immunohistochemical data. For the 4 targets from clinically approved ADCs or ADCs having received fast-track designation by the FDA (HER2, TROP2, NECTIN4 and VTCN1), we could confirm a significantly higher expression in metastases as compared to the normal samples (Figure 7A). It should be noted that for these 4 targets, the percentages of stained tumor cells ranged from 0 to 100%, highlighting the heterogeneity in expression. While no staining was detected for HER2 and VTCN1 in the normal tissues, variable percentages of cells expressing TROP2 or NECTIN4 were detected in the normal tissues. Regarding HER3, higher levels of stained cells were observed in normal tissues as compared to the metastases, which showed very low levels of expression. Regarding the last 3 targets, we could not detect any difference in expression between normal and metastatic samples for FN1 and FGFR2. MUC1 was more highly expressed in metastases as compared to normal tissues but with variable percentages of cells expressing MUC1 in normal tissue samples.

While these results should be interpreted with caution since only a proportion of samples were stained, they do validate what we observed in our transcriptomic data for 6 out of the 8 markers regarding differences in expression in metastatic versus normal samples. The results also clearly demonstrate the wide range of protein expression observed for all markers in metastatic BC.

Concerning the question regarding the brain metastases, of the 13 brain metastases in the cohort, 6 were part of the staining cohort. Three metastases were scored as HER2-undetected, 2 as HER2-low (HER2-2+, FISH negative) and 1 as HER2-ultralow. This suggests that while we do observe a lower mRNA expression in brain metastases, the level of HER2 expression might still be sufficiently high at the protein level to achieve therapeutic response with anti-HER2 ADCs. Furthermore, although we have assembled a very large cohort of samples, conclusions based on the mRNA investigation in 13 samples are still exploratory and should be further investigated. Finally, after re-analyses with our updated cohort (see section “other” at the end of the rebuttal), statistical evidence for lower expression of *HER3* in brain metastases was no

longer observed although the estimate was still favoring lower mRNA expression in brain metastases.

Minor point: The discussion of T-DXd for brain metastases of HER2-positive breast cancer omits the pivotal trials (done before DESTINYBreast12) DEBBRAH and TUXEDO-1.
RE: We thank Reviewer 1 for the suggestion of adding these trials. We have added the details of the DEBBRAH and TUXEDO-1 trial to the discussion.

Overall, the study is descriptive and relies on only one methodology, which limits the robustness of the results. The presentation and discussion of the data is diffuse and does not achieve to deliver a clear message - can the authors draw any more concrete conclusions than just saying their data may help to inform drug development? Selection of top priority ADC targets and validation of those with a complementary method that can also facilitate clinical use (e.g. IHC) may help to make the work more relevant.
RE: We thank Reviewer 1 for these relevant and critical remarks. As mentioned above, we have selected 8 targets including 5 top priority targets for further IHC testing. The discussion was thoroughly revised and restructured based on these additional results.

Response to Reviewer #2 and #3

Major Comments

1.Biological and Clinical Relevance of RNA Expression Levels
- While mRNA expression provides valuable insights, protein expression levels are a more accurate representation of ADC target availability. The manuscript acknowledges this limitation but does not provide a clear rationale for why RNA-based analysis alone is sufficient to draw conclusions about target suitability.
- Since HER2 protein expression often differs from mRNA levels, can the authors provide any validation for RNA-protein correlation beyond the Bosi et al. reference? Even targeted immunohistochemistry (IHC) or proteomic data for a subset of samples would strengthen the conclusions.
- Based on the study results, HER2 does not appear as an ideal target. However, empiric experience shows that HER2 represents one of the most successful ADC targets in oncology. Could the authors try to address this apparent contradiction in the discussion?

RE: We thank the reviewers for raising these concerns, which were similar to those raised by Reviewer 1. In this regard we copy the answer provided to Reviewer 1 for your consideration.

To answer the reviewers' concerns, we have performed additional exploratory immunohistochemistry analyses to expand our transcriptomic findings. This methodology has now been added to the methods sections regarding histopathological characterization. Eight targets of interest were selected for immunohistochemical staining of metastatic and normal samples: (i) targets of ADCs being approved for metastatic BC (HER2, TROP2) or having received fast track designation (NECTIN4 and VTCN1), (ii) target with clinical potential in breast cancer based on high mRNA expression in metastatic samples and low mRNA expression in normal samples (HER3) and (iii) targets with high levels of mRNA expression in metastases but also in normal tissues, likely indicative of high risk for toxicities (FN1, MUC1, FGFR2).

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The analysis of the immunohistochemistry data was mainly explorative given the relatively limited number of samples. Our main goal was to compare the expression between metastases and normal tissue samples, to see whether the conclusions we made based on the transcriptomic data were still valid based on the preliminary immunohistochemical data. For the 4 targets from clinically approved ADCs or ADCs having received fast-track designation by the FDA (HER2, TROP2, NECTIN4 and VTCN1), we could confirm a significantly higher expression in metastases as compared to the normal samples (Figure 7A). It should be noted that for these 4 targets, the percentages of stained tumor cells ranged from 0 to 100%, highlighting the heterogeneity in expression. While no staining was detected for HER2 and VTCN1 in the normal tissues, variable percentages of cells expressing TROP2 or NECTIN4 were detected in the normal tissues. Regarding HER3, higher levels of stained cells were observed in normal tissues as compared to the metastases, which showed very low levels of expression. Regarding the last 3 targets, we could not detect any difference in expression between normal and metastatic samples for FN1 and FGFR2. MUC1 was more highly expressed in metastases as compared to normal tissues but with variable percentages of cells expressing MUC1 in normal tissue samples.

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Concerning the question regarding the brain metastases, of the 13 brain metastases in the cohort, 6 were part of the staining cohort. Three metastases were scored as HER2-undetected, 2 as HER2-low (HER2-2+, FISH negative) and 1 as HER2-ultralow. This suggests that while we do observe a lower mRNA expression in brain metastases, the level of HER2 expression might still be sufficiently high at the protein level to achieve therapeutic response with anti-HER2 ADCs. Furthermore, although we have assembled a very large cohort of samples, conclusions based on the mRNA investigation in 13 samples are still exploratory and should be further investigated. Finally, after re-analyses with our updated cohort (see section “other” at the end of the rebuttal), statistical evidence for lower expression of *HER3* in brain metastases was no

longer observed although the estimate was still favoring lower mRNA expression in brain metastases.

2.Cohort Characteristics and Potential Biases

- The cohort is heavily skewed toward HR+/HER2- disease (77%), with only 20% TNBC and 3% HER2+ cases. This imbalance raises concerns about how generalizable the findings are, particularly regarding HER2 and TROP2, which are critical in TNBC and HER2+ subtypes.

RE: We thank the reviewers for this remark. However, as the samples are from a post-mortem tissue donation program, we are limited in the selection of patients. Nonetheless, as an exploratory measure, we thoroughly compared the expression of ADC targets between the subgroup with metastases from HR+/HER2- breast cancer and with metastases from triple negative breast cancer and saw only very limited differences in target expression (Figure 3). Based on this, we have now added a cautionary remark to the discussion about the potential limitations on generalizability for target expression in patients with TNBC.

- The study does not account for potential prior treatment effects on target expression. Although only 4 patients received prior ADCs, other systemic therapies could have influenced target expression, particularly for immune-related targets (e.g., PD-L1). Were there any differences in expression patterns between patients who received prior ADCs and those who did not? What about prior immune therapy in the metastatic setting?

RE: We agree with the reviewers that prior treatment effect could have a potential impact on target expression. As an exploratory measure, we compared the gene expression of targets in patients that received prior ADC and/or immunotherapy. The median expression of targets was only higher in patients who received prior anti-HER2 or anti-MUC1 ADC therapy compared to patients who did not receive ADC. However, since these patients received therapy in their early lines of treatment, we did not proceed with any formal testing and have decided not to report this is in the manuscript. We have added the supporting violin plots as a figure to this rebuttal (Fig 1 rebuttal).

As the number of patients treated with T-DXd and SG is now increasing in Belgium since last summer, we plan to repeat these analyses later in time when we will have analyzed a higher number of patients having received these ADCs.

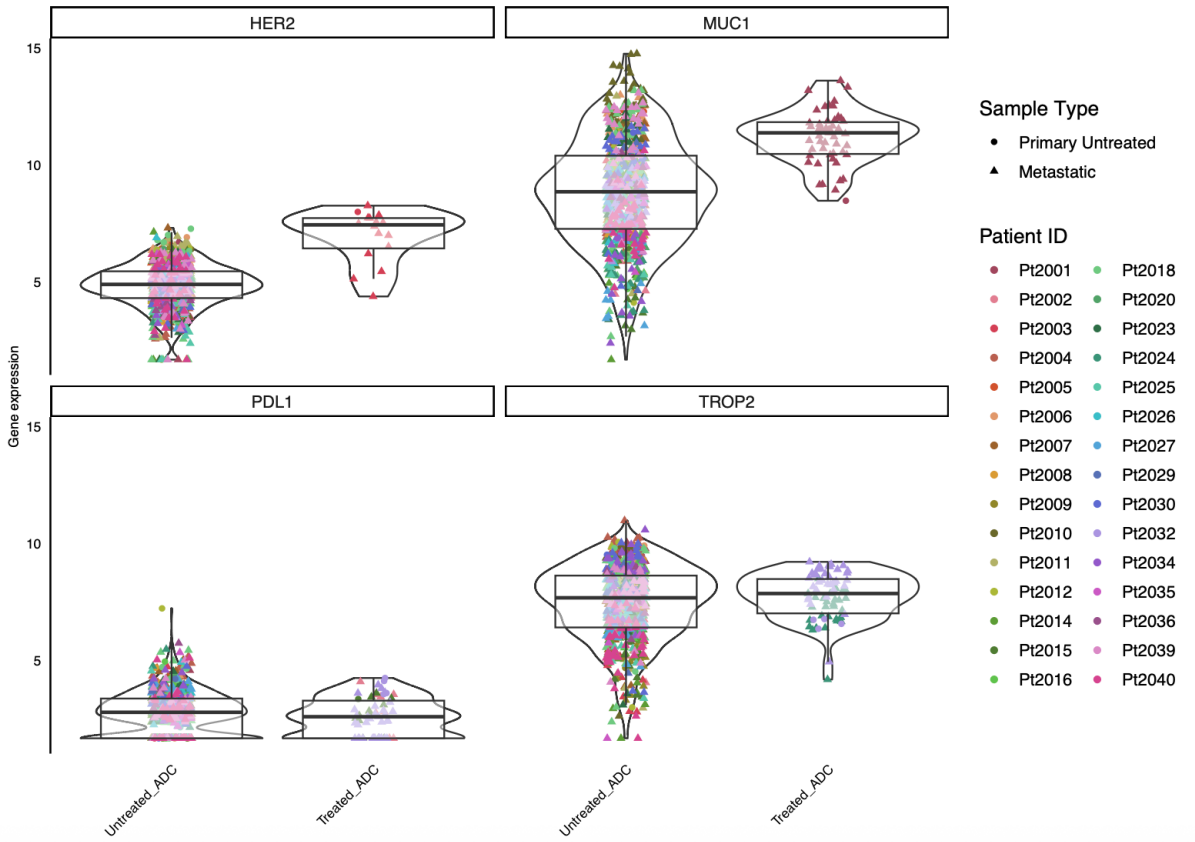

Figure 1 rebuttal: Violin plots comparing the global expression of an ADC target between patient treated and untreated with an ADC specifically for this target. Boxplots represent the median expression of the target. Coloured dots represent the target expression per patient as shown in the legend. Shapes represent samples belonging to primary untreated (circle) and metastases (triangle).

3.Statistical Considerations

- The manuscript states RNA was extracted from some fresh frozen and some FFPE samples – was there correction for this batch effect?

RE: We thank the reviewer for this important point. While we do observe numerically higher reads assigned to FF-OCT samples compared to FFPE, this association is not statistically significant as reported here by the regression model: Estimate: -77616.99, 95%CI: -542500.18-387266.2, p-value=0.74. We therefore did not adjust for this variable in the downstream analysis. The method section now include these elements.

- The choice of p-value threshold (adjusted $p < 0.10$, trend $p < 0.15$) is relatively lenient compared to conventional thresholds. Given the large number of comparisons made, false positives could be a concern. Did the authors explore stricter thresholds or alternative statistical approaches to reduce false discovery rates?

RE: We agree with the comment by the reviewers. We would like to confirm that to account for multiple comparisons, we applied the Benjamini-Hochberg procedure to control the false discovery rate (FDR). This has now been clarified in the methods. As such, since the nature of our work is exploratory, we believe an adjusted p-value of <0.1 is an acceptable threshold to help suggest targets for future hypotheses and validation. This threshold is also based on gene set enrichment analysis tools such as DESeq2, GSEA, GSVA which recommend FDR <0.1 for the

most optimal results to detect gene and gene set enrichment in hypothesis-generating studies (PMID: 23323831, 16199517, 25516281). Additional studies in breast cancer have also experimented with FDR ranging between 0.05 and 0.2 based on the nature of their investigation. PMID: 39090342, 31665365, 36609566). For transparency, both raw and adjusted p-values are provided in Supplementary Table 9, enabling readers to apply custom significance thresholds as needed.

- The weighted Spearman correlation analysis is a useful approach, but the lack of statistical significance values in the wCorr package is a limitation. Did the authors validate the results using a secondary method (e.g., permutation tests)?

RE: We agree with this comment. We have hence validated the correlation values by performing permutation tests with 10,000 iterations for each target pair and per subtype using data described in Figure 5 and Supplementary Figure 6. The permutation reflected evidence of statistical significance for target pairs with correlation coefficient >=0.6 (*HER2-HER3*, *CEACAM5-CEACAM6*, etc.) indicative of a true biological correlation. These p-values were also adjusted using the Benjamini-Hochberg procedure to reduce possibilities for false positives. This data has now been added to the manuscript and the figures and the supplementary tables to complement the results.

4.Interpretation of Co-Expression Findings

- The observed co-expression and mutual exclusivity of ADC targets in metastases provide useful insights for ADC combinations, but the correlation coefficients are moderate (mostly R 0.5–0.6). While the authors suggest that certain targets could be considered for bispecific ADCs, is there any supporting evidence (e.g., known protein-protein interactions or prior co-expression studies) that would justify prioritizing these combinations?

RE: We thank the reviewers for this remark. We looked into combination with interesting targets as identified by our research. To compile this shortlist, we removed targets with no to minimal expression in metastatic samples (10 targets). Next, we removed those targets with higher median expression in normal samples as opposed to metastatic samples which removed 30 targets. *PD-L1* was readded as is sometimes used as immune checkpoint inhibitor in combination with an ADC and not only in a bispecific ADC. Lastly, all targets with 50% or higher overlap of expression between the 3rd and 1st quartile between normal and metastatic samples were removed except for *HER2*. The 9 targets selected as "optimal" were: *CD276, CEACAM6, HER2, HER3, NECTIN4, PD-L1, PTK7, TROP2* and *VTCN1*. The combination HER2-HER3 was the only one with a strong correlation (0.6). Zenocutuzumab is a HER2 x HER3 bispecific antibody used in the treatment of pancreatic adenocarcinomas and non-small-cell lung cancers (NSCLC) carrying *NRG1* gene fusions. Zenocutuzumab in combination with trastuzumab +- vinorelbine or with endocrine therapy has been explored in one breast cancer specific trial (NCT NCT03321981). Only limited results, which do show potential, have been posted. Bispecific ADCs combining HER2 and HER3 are still in the preclinical phase.

In our manuscript, we have now highlighted combinations with a strong negative or positive correlation existing of two targets based on our analysis of mRNA expression in metastatic and normal samples (*CD276, HER2, HER3, NECTIN4, SLC39A6, TROP2, VTCN1*). This includes the combination of HER2-HER3 mentioned above. As our manuscript already has a considerable length and the development of bispecific ADCs is dependent on more than only co-expression

(PMID: 38799638) and as such our data only exploratory (PMID: 38799638), we did not expand our discussion.

5.Besides target expression, key features of an optimal ADC target include surface localization and adequate internalization. This aspect could be better highlighted in the article.
RE: We agreed with this suggestion and have highlighted the importance of surface localization and adequate internalization to the introduction.

Minor Comments
1.Figures
- Figure 6 (normal vs. metastatic tissue expression) is important for understanding potential ADC toxicities but does not clearly indicate statistical significance in overlap calculations. Adding p-values or confidence intervals for overlap scores would improve interpretability.
RE: We thank the reviewer for the comment. We have conducted permutation tests with 10,000 iterations to determine the p-values for the overlaps. These p-values are now presented in Supplementary table 9, and the Methods section has been updated to describe the procedure in detail. Although none of the tests reached statistical significance given the distributions, the overlap scores provided a rationale for selecting potential targets for further protein analysis.

2.Manuscript text
- The manuscript text should be updated to include the FDA approval of datopotamab deruxtecan for metastatic breast cancer (such as in the introduction and discussion).
RE: Dapotamab deruxtecan was now added to the manuscript, specifically to the introduction and discussion.

- The discussion suggests that FN1 and MUC1 are poor ADC targets due to high intra-patient heterogeneity and overlap with normal tissue expression. However, high intra-patient heterogeneity could suggest a need for biomarker stratification rather than complete dismissal of these targets.
RE: We thank the reviewers for this comment. We rather considered these targets of less interest given their high expression in normal tissue. High levels of expression were also seen at the protein level in our new analyses for all normal samples for FN1 and part of the normal samples for MUC1. High intra-patient heterogeneity is indeed less of a problem as it might be circumvented by combining the targeting of this target with another antibody as a bispecific ADC.

- Some terminology should be more precise. For example, "significantly increased expression" in metastases compared to normal tissue is sometimes used without specifying whether the difference is statistically significant or just numerically higher.
RE: We thank the reviewers for this remark. We have adapted our manuscript to be more precise.

3.Statistics
- The use of Spearman correlation thresholds ($R > 0.6$) to define strong correlation is reasonable, but a sensitivity analysis showing how results change with different cutoffs (e.g., $R > 0.5$ or $R > 0.7$) would be informative.

RE: We thank the reviewers for their comment. We have now split the color palette into 10 segments from -1 to 1 to visually identify pairs at a custom threshold, and added p-values to the analysis using permutation tests. Additionally, supplementary table 8 provides detailed rho and p-values, allowing for exploration at custom thresholds.

- The regression models used to compare metastatic and primary tumor expression do not explicitly state how confounding variables (e.g., subtype, prior treatment) were handled. If possible, including adjusted effect sizes would strengthen the analysis.

RE: The regression models were designed to accommodate heterogeneity by accounting for clustering per patient. Since patients received different lines of various treatments prior to tissue donation, treatment could not be added as a confounding variable. Regarding the subtype, we have filtered the dataset for patient subtype prior to regression between metastases and untreated primary tumor per subtype. This enabled us to independently report the effects of subtype on target expression as shown in Supplementary Figure 4B.

Other

Co-first author

To be able to do the requested immunohistochemistry analysis, Gitte Zels, another PhD student of the lab and pathologist in training, performed substantial additional work. She was added as additional co-first author instead of second author. She was already in the previous author list at the same place. This is why we did not ask all authors to sign an author form.

Exclusion of samples

After thorough cross checks, we had to exclude 8 of our samples (7 metastatic and 1 normal) from the cohort due to incomplete metadata annotation and sample duplication during processing. The cohort and the corresponding results have been updated to reflect these changes.