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## Resistance to HER2-targeted anti-cancer drugs is associated with immune evasion in cancer cells and their derived extracellular vesicles

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### ABSTRACT

Neuromedin U (NmU) – a neuropeptide belonging to the neuromedin family – plays a substantial role in HER2-positive breast cancer, correlating with increased aggressiveness, resistance to HER2-targeted therapies and overall significantly poorer outcome for patients. However, the mechanism through which it exerts these effects remains unclear. To elucidate this, initially we used HER2-positive breast cancer cells stably over-expressing NmU. These cells and their released extracellular vesicles (EVs) had increased amounts of the immunosuppressive cytokine TGF $\beta_1$  and the lymphocyte activation inhibitor PD-L1. Furthermore, these cells also showed enhanced resistance to antibody-dependent cell cytotoxicity (ADCC) mediated by trastuzumab, indicating a role of NmU in enhancing immune evasion. All these features were also found in HER2-targeted drug-resistant cells which we previously found to express higher levels of NmU than their drug-sensitive counterparts. Interestingly, EVs from drug-resistant cells were able to increase levels of TGF $\beta_1$  in drug-sensitive cells. In our neo-adjuvant clinical trial, TGF $\beta_1$  levels were significantly higher in EVs isolated from the serum of patients with HER2-overexpressing breast cancers who went on to not respond to HER2-targeted drug treatment, compared with those who experienced complete or partial response. Taken together, our results report a new mechanism-of-action for NmU in HER2-overexpressing breast cancer that enhances resistance to the anti-tumor immune response. Furthermore, EV levels of TGF $\beta_1$  correlating with patients' response versus resistance to HER2-targeted drugs suggests a potential use of EV-TGF $\beta_1$  as a minimally-invasive companion diagnostic for such treatment in breast cancer.

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biomarker; extracellular vesicles; exosomes; immune evasion; neo-adjuvant clinical trial; Neuromedin U

### Introduction



Exosomes and/or ectosomes, collectively termed extracellular vesicles (EVs), are vesicles released from cells and are typically considered to be miniature maps of their cell of origin.<sup>1</sup> Accumulating evidence from our own research and that of others suggests that EVs are useful cargos of biomarkers representing the cells from which they were derived. They also appear to play important roles in cell-to-cell communication and are implicated in tumor growth and transferring drug-resistance.<sup>2,3</sup>


HER2 is overexpressed in ~25% of breast cancers and is associated with poorest prognosis.<sup>4</sup> Therapies targeting HER2 (including monoclonal antibodies such as trastuzumab/Herceptin and small molecules lapatinib and neratinib) have shown encouraging results in the clinic. Unfortunately, not all HER2-overexpressing tumors respond to these therapies and many that initially respond later acquire resistance.<sup>5,6</sup> Predictive companion biomarkers for HER2-targeting drugs are thus urgently needed for improved patient selection and enhanced patient outcome. Ideally, such biomarkers should be extracellular so as

to be easily obtained through minimally invasive-techniques from blood specimens. Of course, novel therapeutic strategies to circumvent resistance are also badly needed.

It has been established that mechanisms that enhance drug-resistance may also result in protection against cancer cell destruction by the immune system.<sup>7</sup> In a study representing mesothelioma, leukemia and prostate cancers, tumor cell-derived EVs were found to possess immunosuppressive properties and are proposed to participate in the transfer of these traits to recipient cells.<sup>8</sup> TGF $\beta_1$  present in EVs was found to be at least partly responsible for the immunosuppressive effects in various cancer types.<sup>9,10</sup> Whether EVs from HER2-targeted drug resistant breast cancer cells have different immunosuppressive properties than those from drug sensitive cells has not been investigated to date.

Neuromedin U (NmU) is a neuropeptide for which several different functions have been ascribed. These include role(s) in feeding, energy balance, smooth muscle contraction, stress response, immune system activation and pain sensing.<sup>11,12</sup> A limited number of studies to date have associated NmU with

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cancer, with conflicting observations depending on the cancer type. In breast cancer, we have shown that NmU expression is increased in cells with innate- or acquired-resistance to HER2-targeted therapies; furthermore, NmU overexpression was also associated with increased migration, invasion and resistance to *anoikis*.<sup>13</sup> Assessing data from approximate 3,500 breast tumor specimens, our work identified association between NmU expression and poor survival outcome for breast cancer patients; particularly those with HER2-overexpressing tumors. Furthermore, our work also found an association between NmU levels and the cell population with cancer stem cell (CSC) phenotype (Martinez et al, unpublished results). Our *in vitro* and *in vivo* NmU knock-down experiments pointed to a role for NmU as a new therapeutic target to help circumvent innate- and acquired- drug resistance, although the precise mechanisms of action remained unexplained.

Here we show that HER2-targeted drug resistance in HER2-positive breast cancer cells correlates with increased levels of the immunosuppressive molecules TGF $\beta_1$  and PD-L1 and resistance to the anti-tumor immune response. Furthermore, these molecules are carried by EVs, which are able to transfer the traits of their cell of origin to drug-sensitive cells. EV-associated TGF $\beta_1$  levels also correlate with response to HER2-targeted treatment in HER2-overexpressing breast cancer patients, suggesting it could be used as a biomarker of response to therapy. We have therefore revealed druggable targets – TGF $\beta_1$  and PD-L1 – to enhance the efficacy of currently used HER2-targeted therapies. Furthermore, we have also shown that circulating levels of EV-associated TGF $\beta_1$  have potential as a predictive biomarker of patients' treatment response.

## Results

### **NmU overexpression increases TGF $\beta_1$ levels**

Our previous studies showed that increased expression of NmU in tumor tissue is associated with poor prognosis in HER2-overexpressing breast cancer patients<sup>13</sup> and with expansion of the CSC population (Martinez et al, unpublished results). As has been shown previously,<sup>14</sup> breast cancer cells with CSC phenotype secrete high levels of TGF $\beta_1$ . We then set out to determine whether NmU-overexpressing cells also showed increased levels of TGF $\beta_1$ . As shown in Fig. 1A and B, TGF $\beta_1$  levels were increased in conditioned media from NmU-overexpressing HCC1954 and SKBR3 cells. As expected, TGF $\beta_1$  levels were also increased in lapatinib-resistant HCC1954 cells compared with their sensitive counterparts; the increase in TGF $\beta_1$  levels was not significant for neratinib-resistant HCC1954 cells (Fig. 1C). TGF $\beta_1$  levels secreted by SKBR3 parent and resistant cell variants were below the level of detection of the ELISA (data not shown). Short-term treatment with NmU did not result in release of TGF $\beta_1$ .

### **NmU overexpression increases cancer cell expression of immunosuppressive mediators**

We have previously reported that NmU overexpression in HER2-positive breast cancer cells causes an enhancement in drug resistance and a more aggressive phenotype, which is

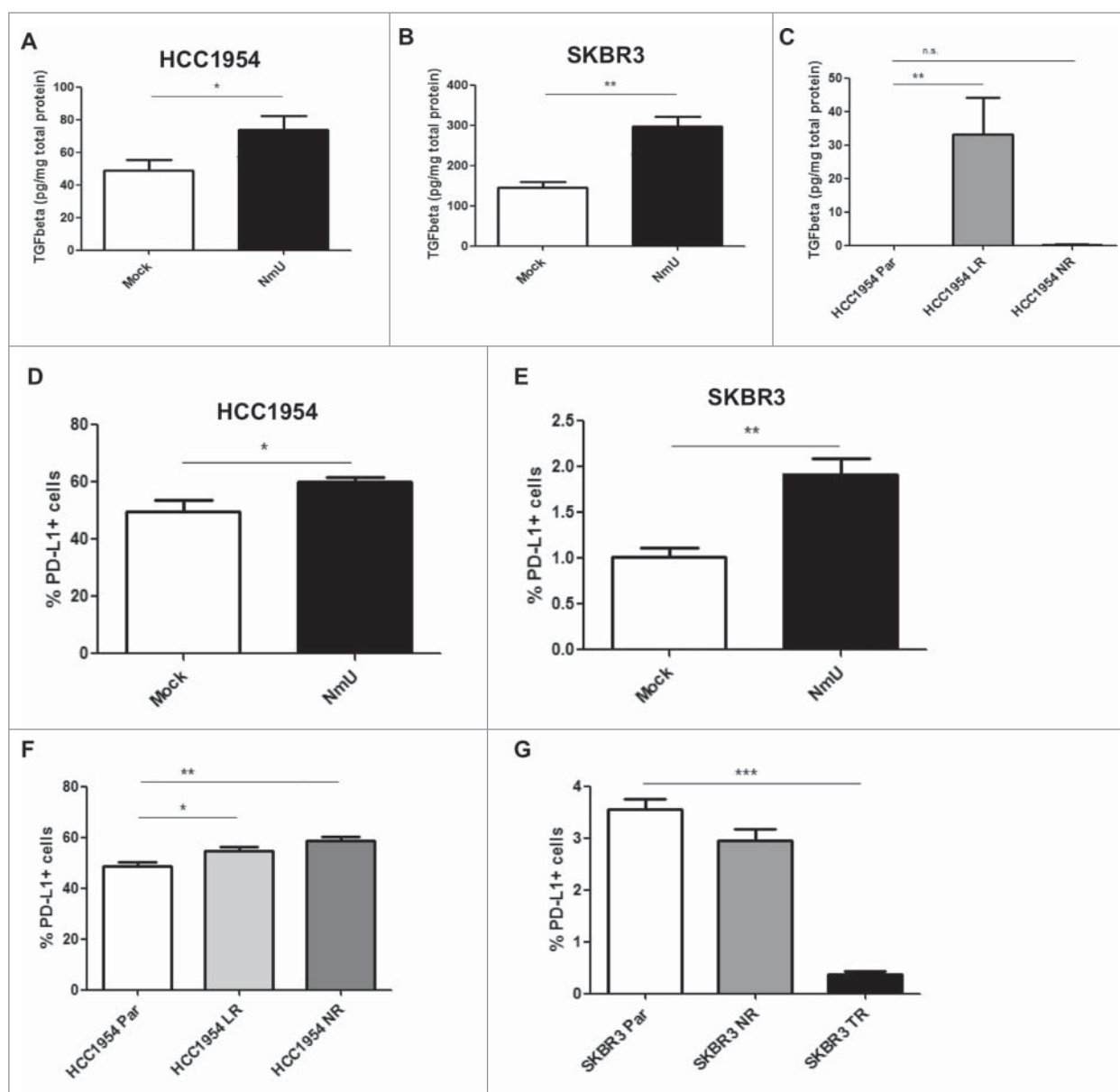
commonly associated with resistance to the anti-tumor immune response.<sup>15-17</sup> Here our results show that NmU-overexpressing cells secrete increased levels of TGF $\beta_1$ , which is a well-known immunosuppressive cytokine. To evaluate the possibility that NmU confers cells the ability to evade destruction by the immune system, expression of cell surface proteins known to inhibit the immune response were assessed by flow cytometry in different cell variants. As shown in Fig. 1D and E, overexpression of NmU in HER2-positive breast cancer cells also increased the expression of the PD-L1 ligand, which has been widely shown to suppress the anti-tumor immune response; the levels of the immunosuppressive receptor CTLA-4, however, were unchanged in both cell line variants (data not shown). HER2-targeted drug-resistant HCC1954 cells also showed increased levels of PD-L1 (Fig. 1F); however, no differences in PD-L1 expression were observed in neratinib-resistant SKBR3 cells, while PD-L1 expression was actually decreased in SKBR3 TR cells (Fig. 1G). Overall, our results show that overexpression of NmU increases the levels of immunosuppressive molecules TGF $\beta_1$  and PD-L1 in HER2-positive breast cancer cells; while the PD-L1 results differed in cells with acquired resistance, depending on the drug in question and the cell line.

### **NmU-overexpressing cells are more resistant to ADCC**

To analyze the functional consequences of NmU-overexpression, we performed antibody-dependent cell cytotoxicity (ADCC) assays in different cell variants. NmU-overexpressing HCC1954 cells were significantly more resistant to ADCC mediated by trastuzumab than control transfected counterparts (Fig. 2A); similar results were obtained with NmU-overexpressing SKBR3 cells, although the differences in this case were not significant (Fig. 2C). On the other hand, lapatinib- and neratinib-resistant HCC1954 cell variants were also more resistant to ADCC than their drug-sensitive counterparts (Fig. 2B): this was not observed in SKBR3 drug-resistant cell variants. These results suggest that, as well as enhancing resistance to HER2-targeted drugs, overexpression of NmU increases the ability of HER2-positive breast cancer cells to evade ADCC, which is one of the main mechanisms of cancer cell death mediated by trastuzumab.

### **EVs from NmU-overexpressing cells have more TGF $\beta_1$ and PD-L1**

EVs are known to transport cargo that is representative of their cell of origin. Since NmU-overexpressing and HER2-targeted drug-resistant cells displayed higher levels of TGF $\beta_1$  and PD-L1, we investigated whether this was also true of their derived EVs. EVs were isolated from cell culture conditioned media by filtration and ultracentrifugation and characterized by specific marker expression by immunoblotting and particle size analysis (Suppl. Fig. 1) and also by transmission electron microscopy (Suppl. Fig. 2). As expected, NmU levels were increased in EVs derived from NmU-overexpressing and drug-resistant HCC1954 cells, particularly in the case of neratinib-resistant cells (Supp. Fig 3A and B). NmU levels were also increased in EVs from neratinib-resistant EFM192A cells (Supp. Fig. 3C),



**Figure 1.** Overexpression of NmU correlates with increased TGF $\beta_1$  and PD-L1 levels. (A), (B), and (C), TGF $\beta_1$  levels were determined by ELISA in serum-free conditioned medium of HCC1954 and SKBR3 cell variants and expressed as pg TGF $\beta_1$  per mg total cell protein. (D) and (F), HCC1954 cell variants were stained with APC-labeled anti-PD-L1, followed by FACS analysis in a FACSCanto II flow cytometer. (E) and (G), Same as in (D) for SKBR3 cell variants. Results represent averaged replicates from at least 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

but not in any of the drug-resistant SKBR3 cell variants (Suppl. Fig. 3D).

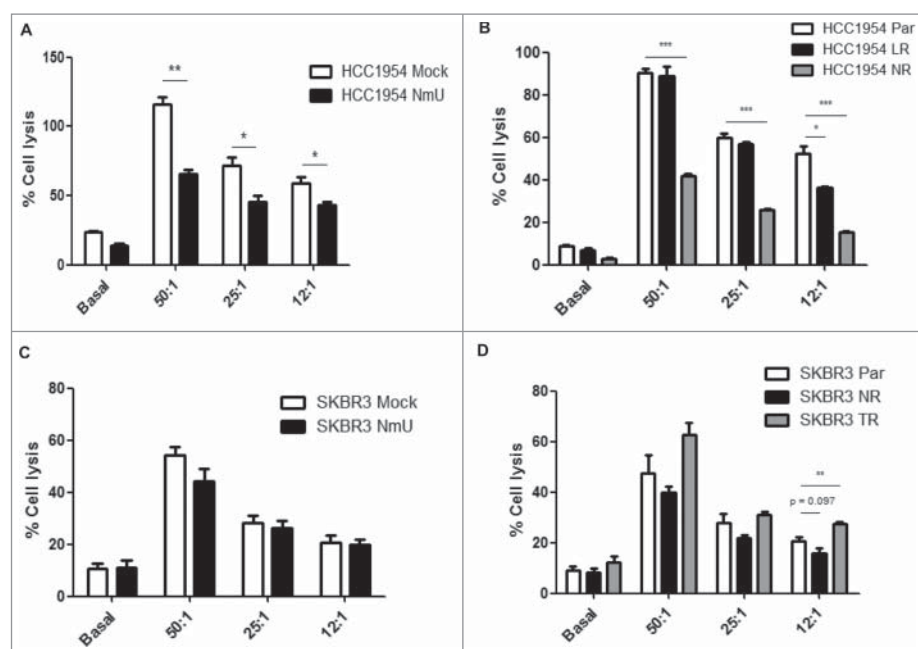
Interestingly, TGF $\beta_1$  levels were increased in intact (unlysed) EVs from lapatinib, neratinib and trastuzumab-resistant HCC1954, EFM192A and SKBR3 cells compared with their sensitive counterparts (Fig. 3A, 3C and 3E); this effect appears to be at least partly mediated by NmU, as EVs from NmU-overexpressing HCC1954 cells also showed significantly increased TGF $\beta_1$  levels compared with EVs from control-transfected cells (Fig. 3A).

Furthermore, EVs from drug-resistant HCC1954 variants also displayed increased levels of PD-L1 (Fig. 3B). This was not observed for EVs from EFM192A and SKBR3 cell variants (Fig. 3D and F); in fact, EVs from drug-resistant SKBR3 cell variants displayed significantly lower levels of PD-L1 compared with EVs from their drug-sensitive counterparts, in agreement

with observations on their cells of origin. The increase in EV-bound PD-L1 in HCC1954 cells did not appear to be mediated by NmU, as EVs derived from NmU-overexpressing cells showed similar levels of PD-L1 as those EVs derived from control transfected cells (Fig. 3B). Our results show that EVs from HER2-targeted drug-resistant cells have higher content of TGF $\beta_1$ , while changes in PD-L1 content are dependent on the particular cell line.

#### **EVs from drug-resistant cell variants can enhance TGF $\beta_1$ secretion and PD-L1 expression in drug-sensitive cells**

EVs have been shown to transfer traits from their cell of origin to other, recipient cells. We then treated drug-sensitive HCC1954, EFM192A and SKBR3 cells with EVs from their drug-resistant variants and measured TGF $\beta_1$  secretion and



**Figure 2.** Overexpression of NmU confers resistance to ADCC. Cells (A) and (C), HCC1954 cells, (B) and (D), SKBR3 cells) were seeded in a round bottom 96-well plate and incubated with trastuzumab (100 $\mu$ g/ml). PBMCs were isolated from healthy donor blood by Ficoll gradient centrifugation and added on top of cells at the appropriate ratios. Cells were incubated at 37 $^{\circ}$ C for a further 6h and cell lysis was then assessed by LDH release. (B) and (D), Same as in (A), but for SKBR3 cell variants. Averaged results from technical triplicates from at least 2 independent experiments with PBMCs from different donors are shown. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . [Note The 100% of cell lysis is calculated based on cancer cells alone and so sometimes the percentage of cell lysis following addition of PBMCs can be over 100% as some PBMCs also die and release their LDH content into the medium].

PD-L1 expression. As shown in Fig. 4B, EVs from drug-resistant HCC1954 cell variants induced an increase in TGF $\beta_1$  secretion by HCC1954 Par cells. This increase did not appear to be mediated by NmU (Fig. 4A). On the other hand, EVs from drug-resistant EFM192A and SKBR3 cell variants did not show the same effect (Fig. 4C and 4D). It should be noted that TGF $\beta_1$  secretion levels were much lower in these cells than in HCC1954 cells.

Expression of PD-L1 was however increased by treatment with EVs from drug-resistant variants from HCC1954 and EFM192A cells (Fig. 5B, C and 5D), even when the EVs themselves did not appear to contain higher levels of PD-L1. In the case of EVs from HCC1954 NR and SKBR3 cell variants the increase was not significant, but an upwards trend was observed. This increase in PD-L1 expression could have been at least partly mediated by NmU, as treatment with EVs from NmU-overexpressing HCC1954 cells increased expression of PD-L1 in HCC1954 Par cells (Fig. 5A). Overall, our results so far show that EVs from HER2-targeted drug-resistant cell variants can affect expression levels of TGF $\beta_1$  and PD-L1 in recipient, drug-sensitive cells.

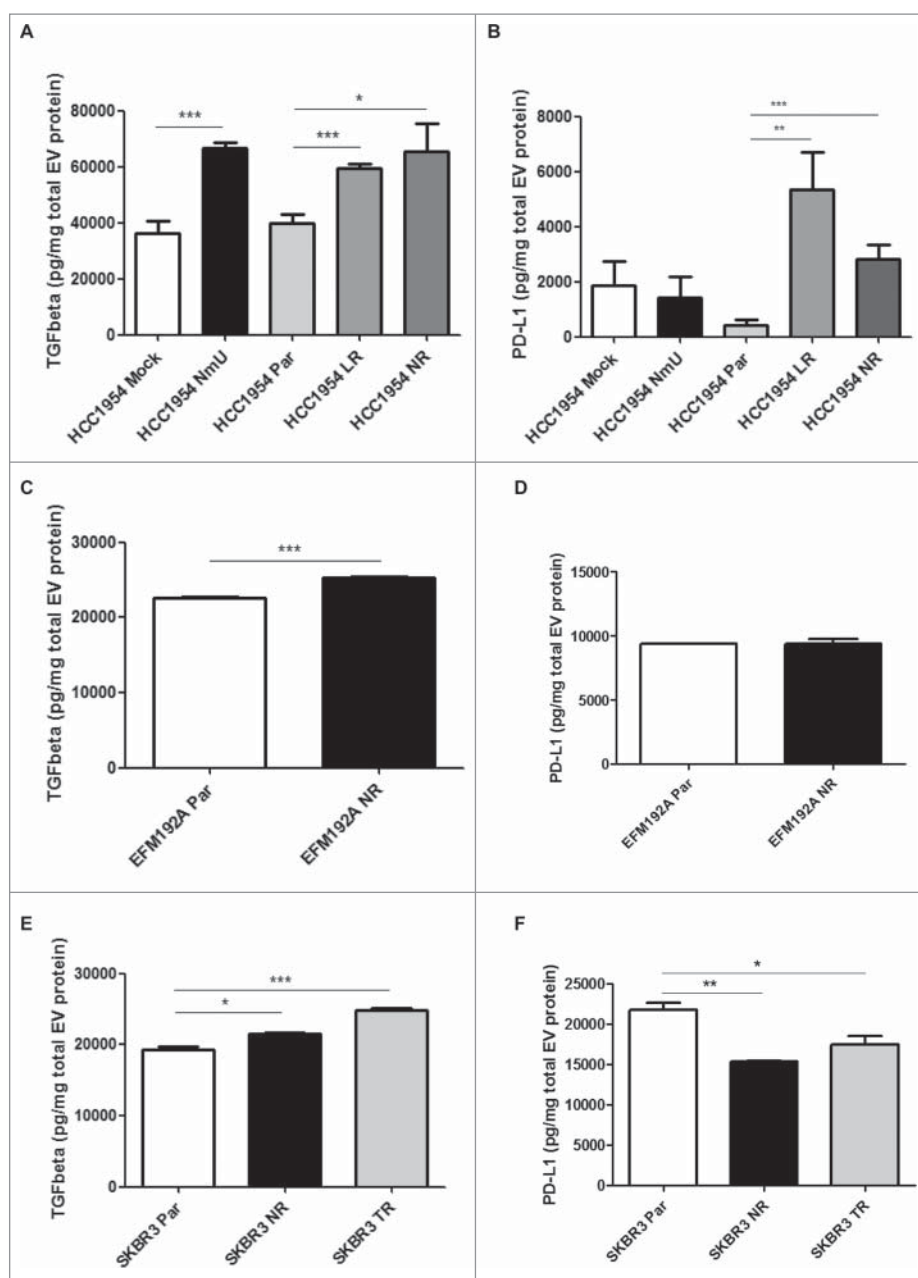
### TGF $\beta_1$ is a potential marker of response for HER2-positive breast cancer treatment

Analysis of EVs from drug-resistant cell variants pointed to a correlation between TGF $\beta_1$  and resistance to HER2-targeted drugs; this suggested a possible use of this molecule as a companion diagnostics/predictive biomarker of response in breast cancer patients with HER2-overexpressing tumors. To evaluate this possibility, EVs were isolated from serum obtained from a blinded study of patients before neo-adjuvant treatment

including trastuzumab with or without lapatinib. Isolated EVs were characterized as described by immunoblotting and particle size analysis (Suppl. Fig. 4). TGF $\beta_1$  and PD-L1 levels were determined on intact EVs by ELISA. Patients were then followed up and classified as complete responders (CR,  $n = 14$ ), partial responders (PR,  $n = 12$ ) and non-responders (NR,  $n = 4$ ). As shown in Fig. 6A, EVs from NR patients showed significantly higher levels of TGF $\beta_1$  than those of CR or PR patients. Of note, no differences in EV quantities were observed from specimens in the CR, PR or NR cohorts, indicating that the higher amount of EV TGF $\beta_1$  is not just a consequence of increased numbers of EVs in patient serum. These results were different from those obtained when measuring TGF $\beta_1$  levels directly in raw serum (Fig. 6B): while CR patients displayed significantly lower levels than either PR or NR patients, there were no significant differences between PR and NR patients, suggesting that serum TGF $\beta_1$  levels cannot distinguish between these 2 groups. No significant differences were observed between CR, PR and NR patient EVs regarding PD-L1 levels, although increased quantities of EV-carried PD-L1 were found for those who were classified as NR vs. PR vs. CR (Fig. 6C). These findings suggest that EV-carried TGF $\beta_1$  –but not raw serum TGF $\beta_1$ –may be able to identify those patients that would benefit from HER2-targeted treatment.

### Discussion

In this study we show that NmU in HER2-positive breast cancer cells promotes evasion of the immune response, increasing the expression of immunosuppressive molecules TGF $\beta_1$  and PD-L1 and functionally affecting ADCC, a key mechanism of action of monoclonal antibodies used in the treatment of breast



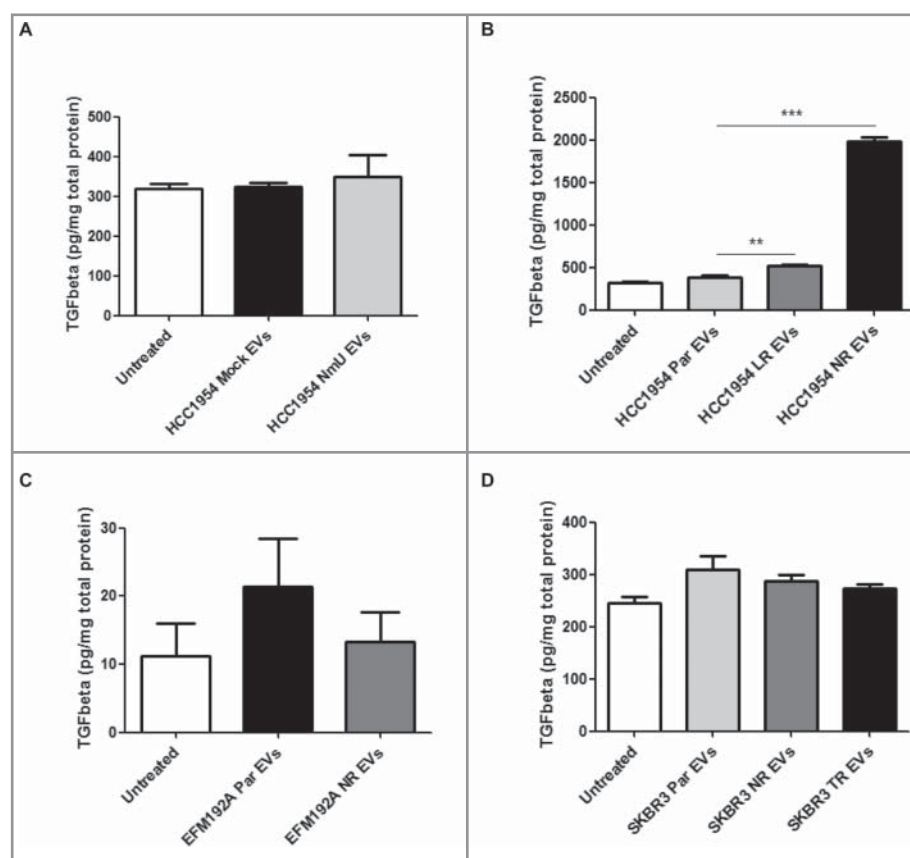
**Figure 3.** EVs from NmU-overexpressing and HER2-targeted drug-resistant variants show altered content of TGFβ<sub>1</sub> and PD-L1. (A), (C), and (E), TGFβ<sub>1</sub> levels were determined by ELISA in intact (unlysed) EVs from HCC1954 (A), EFM192A (C) and SKBR3 (E) cell variants and expressed as pg TGFβ<sub>1</sub> per mg total EV protein. B, D, and F, PD-L1 levels were determined by ELISA in EVs from HCC1954 (B), EFM192A (D) and SKBR3 (F) cell variants and expressed as pg PD-L1 per mg total EV protein. Results represent averaged replicates of at least 3 independent experiments. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

cancer. Interestingly, TGFβ<sub>1</sub> and PD-L1 are also detectable in EVs from HER2-targeted drug-resistant cell variants, which have been found to express high levels of NmU. EVs from these cells can induce an increase in TGFβ<sub>1</sub> secretion in drug-sensitive cells, promoting further immune evasion. Finally, EV-associated TGFβ<sub>1</sub> levels also correlate with response to HER2-targeted treatment in HER2-overexpressing breast cancer patients, suggesting it could be used as a marker of response to therapy.

EVs from cancer cells have been shown to carry immunosuppressive molecules such as FasL, TRAIL, TGFβ<sub>1</sub> and PD-L1,<sup>9,18-19</sup> and also to be involved in immunosuppression at several different stages of the immune response, including T cell proliferation,

activation and apoptosis,<sup>9,20-21</sup> regulatory T cell abundance and function,<sup>23-25</sup> NK cell function,<sup>22,26-27</sup> dendritic cell maturation,<sup>28</sup> induction of suppressive mesenchymal stem cells,<sup>29-30</sup> and macrophage polarization.<sup>31</sup> TGFβ<sub>1</sub> in EVs has been proposed to mediate at least some of the immunosuppressive effects.<sup>9,10</sup>

Our previous work had shown an association between overexpression of NmU and an expansion of cells with CSC phenotype.<sup>32</sup> The CSC phenotype is characterized by increased drug resistance and also by enhanced immune evasion.<sup>33-35</sup> In accordance with this, as well as driving HER2-targeted drug resistance, NmU overexpression was also associated with increased expression of TGFβ<sub>1</sub> and PD-L1, known inhibitors of the immune response. Moreover, HER2-targeted drug-resistant cells were also



**Figure 4.** Treatment with EVs from NmU-overexpressing and HER2-targeted drug-resistant variants enhances TGF $\beta_1$  secretion. (A) and (B), HCC1954 Par cells were treated with EVs from different HCC1954 cell variants ( $20 \mu\text{g EV}/1 \times 10^5$  cells) for 48 hr. Cell conditioned medium was then analyzed for TGF $\beta_1$  content by ELISA, expressed as pg TGF $\beta_1$  per mg total cell protein. (C), Same as in (A) for EFM192A cells. (D), Same as in (A) for SKBR3 cells. Results represent averaged replicates of at least 3 independent experiments. \*\*p < 0.01, \*\*\*p < 0.001.

shown to be more resistant to ADCC mediated by trastuzumab, and this effect was at least partly mediated by NmU. These results show that NmU-overexpressing cells are indeed more capable of avoiding destruction by the immune system.

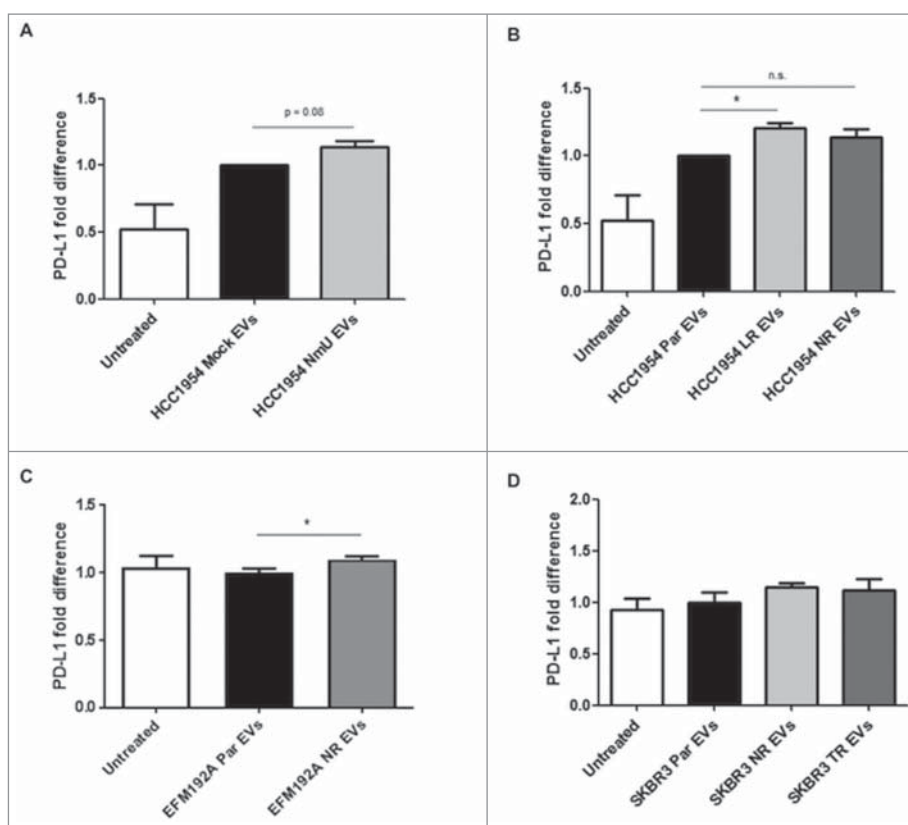
EVs have gathered a lot of interest in recent times due to the understanding that they carry valuable information about their cell of origin. Indeed, we found increased levels of TGF $\beta_1$  in EVs from drug-resistant cell variants, and this increase appeared to be at least partly mediated by NmU. On the other hand, PD-L1 levels were not consistently increased in EVs from drug-resistant cell variants, similarly to what we observed on their cells of origin. To our knowledge, this is the first time that EVs from drug-resistant cells have been shown to possess enhanced immunosuppressive abilities compared with EVs from drug-sensitive cells. Future studies where high levels of NmU in resistant cells are knocked-down with siRNA or shRNA to establish if this would reverse the increased vesicular release of TGF $\beta_1$ /PD-L1 are now warranted. Their success may lead to pre-clinical *in vivo* studies to establish the potential therapeutic relevance of this approach.

Our previous work and that of others has shown that EVs are able to transfer trends from their cell of origin to recipient cells.<sup>1-3</sup> Indeed, EVs from most of the drug-resistant cells tested were able to increase the levels of secreted TGF $\beta_1$  and cell surface PD-L1 in parent, drug-sensitive cells.

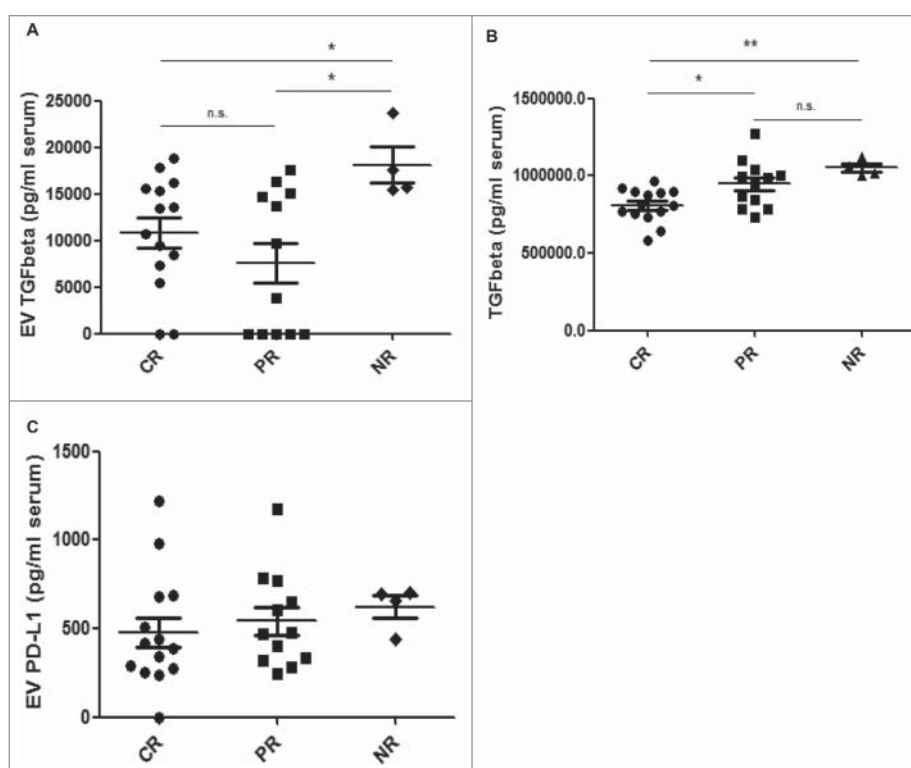
It is noteworthy that HCC1954, SKBR3 and EFM192A -all HER2-positive cell lines- were found to sometimes behave in

different ways under the same conditions. Possible contributing reasons for this may include the fact that HCC1954 cells were originally isolated from a primary breast tumor, while EFM192A and SKBR3 cells were isolated from metastatic sites. Also, their tumor type of origin is different i.e. HCC1954 cells represent a ductal carcinoma, while EFM192A and SKBR3 cells represent adenocarcinoma cells. The 3 cell lines also carry different driver mutations due to the diverse origin of their transformation. The commonality of substantial importance here is that they all represent HER2-overexpressing breast tumors. Crucially, the fact that all 3 cell lines -despite tumor/patient-to-patient differences- display increased TGF $\beta_1$  levels on EVs further highlights the relevance of this finding and how it potentially could be applied to assessing patients whose HER2-overexpressing breast tumors are different.

TGF $\beta_1$  levels in EVs correlated particularly well with HER2-targeted drug resistance displayed by their cells of origin. A study in a small group of patients undergoing neo-adjuvant treatment showed encouraging results, suggesting that high levels of circulating EV TGF $\beta_1$  may indicate that the tumor is resistant to HER2-targeted therapy. Interestingly, the levels of raw serum TGF $\beta_1$  did not correlate as well with response to treatment, indicating that there is an advantage in analyzing EVs. If confirmed, this would point to EV TGF $\beta_1$  as a novel biomarker of response to HER2-targeted therapy in HER2-overexpressing breast cancer. However, these results should be validated in a larger cohort.



**Figure 5.** Treatment with EVs from NmU-overexpressing and HER2-targeted drug-resistant variants increases PD-L1 expression. (A) and (B), HCC1954 Par cells were treated with EVs from different HCC1954 cell variants ( $20 \mu\text{g EV}/1 \times 10^5$  cells) for 48 hr. PD-L1 surface staining was then analyzed by flow cytometry. (C), Same as in A for EFM192A cells. (D), Same as in (A) for SKBR3 cells. Results represent averaged replicates of at least 3 independent experiments and are expressed as fold difference with respect to cells treated with EVs from parent cell variants, which is considered to be equal to 1. \* $p < 0.05$ .



**Figure 6.** EV TGF $\beta_1$  levels correlate with response to HER2-targeted therapy in HER2-overexpressing breast cancer patients. EVs were isolated from  $250 \mu\text{l}$  patients' sera by serial ultracentrifugation. (A), TGF $\beta_1$  was detected on intact EVs isolated from patient serum by ELISA. Values are expressed as pg TGF $\beta_1$  per ml of serum. (B), TGF $\beta_1$  was detected in patients' sera by ELISA. Values are expressed as pg TGF $\beta_1$  per ml of serum. C, PD-L1 was detected on intact EVs by ELISA. Values are expressed as pg PD-L1 per mg total EV protein. \* $p \leq 0.05$ , \*\* $p < 0.01$ .



It may also be interesting, in future studies, to consider the multiple forms of TGF $\beta$ <sub>1</sub>. Our results represent analysis, by ELISA, of the mature form of TGF $\beta$ <sub>1</sub>. However, using an immunoblotting approach, Graner et al.<sup>36</sup> reported that EVs from glioma patient sera have multiple forms of TGF $\beta$ <sub>1</sub> i.e., forms of pro-peptide, LAP, dimer -presumably the active form- and monomer. More recently and also using immunoblotting, multiple TGF $\beta$ <sub>1</sub> forms have been shown on EVs from AML patient plasma (albeit in very small numbers of specimens) and the EVs with more of the “active/mature” form of TGF $\beta$ <sub>1</sub> correlated with greater capacity to suppress NK cell activity.<sup>37</sup> For our studies reported here, we selected the approach (ELISA) that we took because we are not confident that immunoblotting techniques would easily translate into routine clinical utility for a blood-based biomarker (e.g. to predict response to therapy for patients enrolling in a clinical trial), while ELISAs are routinely used in clinics and hospital laboratories. However, undoubtedly using the less quantitative immunoblotting approach could help generate new scientific knowledge -which is also fundamentally important- that could complement the quantitative ELISA data.

NmU is an attractive target for HER2-overexpressing breast cancer, but the multiple physiologic roles it plays would make it difficult to alter its function without side-effects. The identification of downstream druggable mediators of its effects such as TGF $\beta$ <sub>1</sub> and PD-L1 suggest novel ways in which NmU function can be targeted for the treatment of HER2-overexpressing breast cancer. Combination with therapies that block these downstream effectors of NmU could then add value to currently approved HER2-targeted therapies.

## Materials and methods

### Cell culture and treatments

HCC1954, SKBR3 and EFM192A cells, obtained from ATCC, were cultured in RPMI-1640 (Sigma-Aldrich, Ireland) with 10% FCS (PAA Laboratories, UK) and 1% L-glutamine (Sigma-Aldrich, Ireland). Our HCC1954 and SKBR3 NmU and mock-transfected cells have been described previously,<sup>3</sup> as have lapatinib-resistant (HCC1954 LR), neratinib-resistant (HCC1954 NR, SKBR3 NR and EFM192A NR) and trastuzumab-resistant (SKBR3 TR) variants.<sup>13,38</sup> For short-term treatment with NmU peptide, cells ( $1 \times 10^5$  cells) were seeded in a 24-well plate, allowed to attach overnight, fed with serum-free medium and subsequently treated with 1–10  $\mu$ mol/L of NmU-25 (Bachem AG, Switzerland) for 1 hour. Cell lines are authenticated by short tandem repeat profiling (at least every 2 years) and tested for *Mycoplasma* using qPCR methods (quarterly). All experiments are performed within 10 passages of thawing and biologic replicate experiments are performed on consecutive passages numbers of cells. Lapatinib and neratinib were purchased from Sequoia Chemicals Ltd (UK); trastuzumab (Herceptin) was obtained from St. James' Hospital Pharmacy, TCD.

### Immunoblotting

Total cellular proteins (10 $\mu$ g for extracellular vesicles, 30–40  $\mu$ g for cell lysates, depending on the specific protein

abundance but constant for any given protein) were resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Ireland). Primary antibodies used included Grp94 (1:1000, Abcam, UK), PDC61/Alix (1:1000, Abcam, UK) and CD81 (1:200, Abcam, UK). Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Netherlands) and proteins were visualized by chemiluminescence (Millipore, Ireland). Detection was performed with a Chemidoc exposure system (Bio-Rad Laboratories, UK).

### Nanoparticle tracking analysis

Analysis was performed on all EVs with the NTA NS500 system (NanoSight, Amesbury, UK) to determine particle size based on Brownian motion. EV samples were diluted 1:100 in PBS and injected into the NTA system, which obtained 6 60 second videos of the particles in motion. Videos were then analyzed with the NTA software to determine particle size.

### ELISA

ELISA kits for human TGF $\beta$ <sub>1</sub>, PD-L1 (R&D Systems, UK) and NmU (Peninsula Laboratories, CA) were used according to the manufacturer's instructions. For analysis of conditioned media, HCC1954, SKBR3 and EFM192A cell variants were seeded at  $1 \times 10^5$  cells/well in a 24-well plate and left to attach overnight. The following day, cells were washed and fed with serum-free media and incubated for a further 72hr. Supernatant was then collected and analyzed immediately or stored at  $-80^\circ\text{C}$  until analysis. Results were normalized by total cell protein contained in the well. 10 $\mu$ g of protein from lysed or intact EVs, as stated, were tested per well.

### Flow cytometry

HCC1954 and SKBR3 cell variants ( $1 \times 10^5$ /well) were seeded in a 6-well plate and allowed to attach overnight. They were then trypsinised, blocked with 10% FCS in PBS and stained with APC-conjugated anti-CD24 (1:75 dilution, eBiosciences, UK) and FITC-conjugated anti-CD44 (1:800 for HCC1954, 1:400 for SKBR3, eBiosciences, UK) or APC-conjugated anti-PD-L1 antibody (1:800 for HCC1954, 1:400 for SKBR3, eBiosciences, UK) for 30 min at  $4^\circ\text{C}$ . For EV treatment, cells were seeded at  $0.5 \times 10^5$  (HCC1954) or  $1 \times 10^5$  (SKBR3 and EFM192A) cells/well in a 24-well plate and allowed to attach overnight. The following day, cells were washed with serum-free media, fed with media supplemented with extracellular vesicle (EV)-depleted-FBS and treated with EVs for a further 48 hr before staining as above. Staining was assessed in a FACS-Canto II flow cytometer, followed by analysis with BD FACS-Diva software.

### Antibody-dependent cell cytotoxicity (ADCC) assay

HCC1954 cell variants were seeded at  $1 \times 10^4$  cells/well in a round bottom 96-well plate and incubated with trastuzumab (100 $\mu$ g/ml) for 30 min at  $37^\circ\text{C}$ . Whole blood was obtained from healthy donors in accordance with a protocol approved

by the Research Ethics Committee at Trinity College Dublin. PBMCs were isolated from blood by Ficoll gradient centrifugation (Histopaque, Sigma-Aldrich, UK) and added on top of cells at the appropriate ratios. Cells were incubated at 37°C for a further 6 h and cell lysis was then assessed by LDH release (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega, UK).

### Extracellular vesicle isolation from conditioned media

HCC1954, SKBR3 and EFM192A cell variants were seeded ( $1.7 \times 10^7$  cells/flask in a 15 ml volume) in CELLLine Adhere 1000 flasks (Integra Biosciences AG, Switzerland) in complete medium containing 10% extracellular vesicle (EV)-depleted-FBS, 2 mM L-Glutamine, 1% penicillin/streptomycin (Sigma-Aldrich, Ireland), and grown as described previously.<sup>39</sup> Conditioned media (CM) was collected every 7 d and processed for EV isolation. In brief, CM was centrifuged at 400 g for 10 min and 2000 g for 15 min to eliminate cells and cell debris, then filtered through a 0.45  $\mu$ m pore filter and subsequently ultracentrifuged at 110,000 g for 75 min at 4°C, using an SW32.Ti swing bucket rotor. EV pellets were washed in PBS and ultracentrifugation repeated. EVs were then assessed using the BCA assay (Bio-Rad Laboratories, UK) and further characterized by nanoparticle tracking analysis (NanoSight) and immunoblotting for positive and negative markers.<sup>40</sup>

### Extracellular vesicle isolation from patients' specimens

Serum specimens were obtained from patients accrued to the All Cooperative Oncology Research Group (ICORG/Cancer Trials Ireland) translational trial NCT01485926. The procurement and analysis of these specimens was approved by the Ethics Committees of the associated hospitals and in accordance with the Helsinki Declaration of 1975. Specimens were taken before neo-adjuvant treatment that included trastuzumab with or without lapatinib. Following 6 cycles of treatment, response was assessed in accordance with ICORG guidelines, and defined as pathological complete response (CR) by the absence of invasive carcinoma in the breast and lymph nodes. Pathological partial response (PR) was defined as a decrease in either or both the tumor or lymph nodes compared with the pre-treatment tumor or lymph nodes, while having no increase in either the tumor or lymph nodes, while no pathological response (NR) was defined as no apparent change in either the tumor or lymph nodes compared with the clinical (pre-treatment) assignment or an increase in the tumor or lymph nodes at the time of post-treatment pathologic evaluation. Patient information (age, tumor score, lymph node status, metastasis status and overall staging) can be found in Supplementary Table 1. EVs were isolated from 250  $\mu$ l serum diluted in 8 ml PBS, filtered through 0.2  $\mu$ m syringe filter and ultracentrifuged at 110,000 g for 75 min at 4°C, using a 70.1 Ti fixed angle rotor. EV pellets were washed in PBS and ultracentrifugation repeated. EVs were then assessed using the BCA assay (Bio-Rad Laboratories, UK), nanoparticle tracking analysis (NanoSight), immunoblotting for positive and negative markers and transmission electron microscopy (TEM) as described

previously.<sup>11,12</sup> EV pellets re-suspended in 200  $\mu$ l PBS were stored at -80°C until analysis.

### Statistical analysis

One-way ANOVA followed by Tukey's post-test was used for analysis of multiple comparison groups. Two-tailed Student's unpaired t-test was used to compare 2 groups. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., CA). Results are expressed as mean of a minimum of 3 independent experiments (except when noted otherwise)  $\pm$  SEM. Statistical significance was set at \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### Disclosure statement

JC has acted in a consultancy role for GSK and has received honoraria and other remuneration from Roche and GSK. None of these monies were used for any of these studies and no drugs used were received as a gift from any company. There is no other disclosure to report.

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