


RESEARCH ARTICLE

Chemotherapy-induced release of ADAM17 bearing EV as a potential resistance mechanism in ovarian cancer

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Abstract

Ovarian cancer (OvCa) is the gynaecological disorder with the poorest prognosis due to the fast development of chemoresistance. We sought to connect chemoresistance and cancer cell-derived extracellular vesicles (EV). The mechanisms of how chemoresistance is sustained by EV remained elusive. One potentially contributing factor is A Disintegrin and Metalloprotease 17 (ADAM17)—itself being able to promote chemoresistance and inducing tumour cell proliferation and survival via the Epidermal Growth Factor Receptor (EGFR) pathway by shedding several of its ligands including Amphiregulin (AREG). We now demonstrate that upon chemotherapeutic treatment, proteolytically active ADAM17 is released in association with EV from OvCa cells. In terms of function, we show that patient-derived EV induce AREG shedding and restore chemoresistance in ADAM17-deficient cells. Confirming that ADAM17-containing EV transmit chemoresistance in OvCa, we propose that ADAM17 levels (also on EV) might serve as an indicator for tumour progression and the chemosensitivity status of a given patient.

KEYWORDS

ADAM17, ascites, chemoresistance, extracellular vesicles, ovarian cancer

Gerrit Hugendieck and Marcus Lettau contributed equally to this work.

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1 | INTRODUCTION

Ovarian cancer (OvCa) is one of the most common gynaecological tumours and a leading cause of female cancer mortality with about 200,000 deaths per year (International Agency for Research on Cancer; Sung et al., 2021). Late stage OvCa and recurrent tumours are often accompanied by ascites formation, representing the main route of metastasis. Ascites contains viable cancer cells and a multitude of cellular constituents, soluble factors and extracellular vesicles (EV), facilitating OvCa metastasis within the peritoneal cavity (Ford et al., 2020; Mitra et al., 2021; Smolle et al., 2014). In most cases the treatment of OvCa involves surgical intervention followed by platinum-based chemotherapy. Despite a good initial response to chemotherapy, most patients relapse and develop acquired resistance to platinum-based treatment (Armstrong et al., 2021; Colombo et al., 2019). Over the last decades, only a few breakthroughs improved the treatment of OvCa such as the introduction of Poly-ADP-Ribose-Polymerase (PARP) inhibition in patients with a mutation in Breast Cancer Gene 1/2 (BRCA1/2) and/or Homologous Recombination Deficiency (HRD) (Moore et al., 2018). Additionally, the anti-angiogenic monoclonal antibody Bevacizumab improved progression-free survival in advanced stages of OvCa (Oza et al., 2015). Nevertheless, tumour recurrence and resistance still represent major clinical problems in OvCa resulting in poor overall prognosis (Armstrong et al., 2021; Kurnit et al., 2021; Stewart et al., 2019).

However, a variety of chemoresistance mechanisms were discovered in the past decades. Interfering with these pathways, for example, by more specific targeting strategies and the design of novel drugs, could sensitize cells to chemotherapeutic treatment (Brasseur et al., 2017; Qiu et al., 2005). Particularly, growth factor receptors like Epidermal Growth Factor Receptor (EGFR) and Human Epidermal Growth Factor Receptor 2 (HER2) were shown to increase chemotherapy resistance to cisplatin and paclitaxel in gynaecological cancers (Hengstler et al., 1999; Qiu et al., 2005; Sasaki et al., 2007). These receptors activate downstream signalling cascades including Mitogen-activated Protein Kinase (MAPK) and serine/threonine kinase AKT (PI3K/AKT), which are involved in tumour cell survival, cell growth and apoptosis signalling (Brasseur et al., 2017). In tumour cells these signalling cascades might be upregulated by either activating mutations or enhanced expression and/or release of receptor ligands.

In our preceding studies we showed that the proteolytic activity of the A Disintegrin and Metalloprotease 17 (ADAM17), which is a major protease responsible for the release of these receptor ligands like Amphiregulin (AREG), is strongly activated upon cisplatin treatment. Thus, this enhanced induction of ADAM17 proteolytic activity facilitates receptor activation and concomitant survival signalling and is therefore regarded as a potential resistance mechanism (Hedemann et al., 2018, 2021).

Intriguingly, in the same study we demonstrated that chemotherapeutic treatment lead to a decrease of ADAM17 cell surface expression on OvCa cells (Hedemann et al., 2018). As we did not observe an increased internalisation of ADAM17, we were interested whether ADAM17 might be released in association with EV upon chemotherapeutic treatment.

EV are a heterogenous group of membrane-enclosed lipid-bilayered particles without replication capability which are secreted by cells under physiological and pathological conditions (Théry et al., 2018). Numerous studies indicate that EV are an essential part of intercellular communication and unfold their activity on recipient cells by their cargo consisting of lipids, DNA, RNA and proteins (Malkin & Bratman, 2020; O'Brien et al., 2020; Pathan et al., 2019; Skotland et al., 2020). Interestingly, this physiological cell communication can be hijacked by cancer cells, which often show an augmented EV release compared to healthy cells (Xu et al., 2018). In OvCa, EV are highly abundant in ascitic fluid and can influence tumourigenesis, metastasis and development of chemoresistance (Crow et al., 2017; Feng et al., 2019; Han et al., 2019; Palma et al., 2012). The tumour-promoting capacity of OvCa-EV has been investigated in numerous studies and their importance in the orchestration of the tumour microenvironment and the formation of pre-metastatic niches is well-documented (Feng et al., 2019; Nakamura et al., 2019). The formation of pre-metastatic niches is an essential step for both invasion and metastasis. EV can promote this process, for example, by suppressing the activity of cells from the adaptive and innate immune system, by promoting angiogenesis and by facilitating the reprogramming of fibroblasts into cancer-associated fibroblasts (CAF). This creates a tumour-promoting microenvironment and fosters dissemination of the primary tumour as a prerequisite of tumour cells to migrate and settle into distant parts of the body (Giusti et al., 2018; Labani-Motlagh et al., 2015; Pink et al., 2015; Samuel et al., 2018; Yi et al., 2015). Particularly in OvCa, where cellular trafficking through ascites fluid represents the major route of metastasis, the presence and function of EV are essential (Ahmed & Stenvers, 2013; Pascual-Antón et al., 2021). Besides promoting metastasis, EV were also identified to play a major role in chemoresistance. For example, Samuel et al. showed that EV derived from cisplatin-treated tumour cells increased the resistance of untreated tumour cells (Samuel et al., 2018). Several studies document an enhanced EV release in response to chemotherapy (Aubertin et al., 2016; Keklikoglou et al., 2018; Vera et al., 2019; Wang et al., 2019).

In the present study, we investigated whether EV-associated ADAM17 plays a role in chemoresistance. Therefore, we isolated and characterized EV from cell culture supernatants released after cisplatin treatment (cis-EV) and from malignant ascites (ascites-EV) of primary and recurrent OvCa patients. Moreover, ADAM17 activity in these EV was analysed as well as their potential to increase substrate release and chemoresistance in responder cells. Thus, we suggested that ADAM17

7-containing cis-EV and ascites-EV play a crucial role in increasing the resilience of OvCa cells against platinum-based chemotherapy.

Understanding the role of EV and the potential transmission of resistance could facilitate the development of improved strategies for the treatment of OvCa. Particularly the identification of resistance-mediating enzymes like ADAM17 could identify novel targetable structures either within a cellular context, the tumour-environment or even in tumour-associated ascites.

2 | MATERIAL AND METHODS

2.1 | Cell generation and patient material

2.1.1 | Ethics statement

This research was approved by the Institutional Review Board of the University Medical Center Schleswig–Holstein, Campus Kiel (AZ-Nr: D563/21), according to the Declaration of Helsinki (World Medical Association, 2013). Written informed consent was obtained from all patients. All patient related information was anonymised.

2.1.2 | Generation of EV-depleted medium

RPMI 1640 medium (PAN Biotech, Germany) supplemented with 20 % fetal calf serum (FCS) Gibco® (Thermo Fisher Scientific, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, USA) was centrifuged at $100,000 \times g$ (Beckman XL-80 centrifuge, SW 32.1 Ti rotor) for 18 h (Théry et al., 2006). The EV-depleted supernatant was diluted with RPMI 1640 medium to a final concentration of 10% FCS. To control for sufficient EV depletion using our protocol, we performed comparable Nanoparticle-tracking analyses of A) FCS-supplemented RPMI 1640 medium, B) EV-depleted FCS-containing RPMI 1640 medium and C) original non-FCS supplemented RPMI 1640 medium. Particle concentrations measured after ultracentrifugation (B) were in a similar range compared to those of original, non-FCS-containing RPMI (C), indicating sufficient EV depletion in the medium used in this study (not shown).

2.1.3 | Cell culture

The human ovarian adeno-carcinoma cell line Igrov-1 wild type (WT) was purchased from American Type Culture Collection (ATCC). The ovarian carcinoma cell line KI-OC-11 was generated from a late stage OvCa patient and characterised as described previously (Oberge et al., 2020). ADAM17 knock-out (KO) Igrov-1 cells (clone B2) were established in our laboratory (see below). Cells were regularly sub-cultivated in fresh medium to ensure optimal growth conditions. OvCa cell clones/line identity was verified by Short Tandem Repeat (STR) DNA profiling analysis (Huang et al., 2016). Mycoplasma contamination was regularly tested with the MycoAlert™ kit (Lonza, #LT07, USA).

2.1.4 | Generation of ADAM17 knock-out cells using CRISPR/Cas9 technology

For the generation of ADAM17 knock-out cells CRISPR/Cas9 technology was used. Two chemically modified guide RNAs (AUCUAAUAUCCAGCAGCAUU; UUUUUCUUACCGAAUGCUGC; Synthego, Redwood City, USA) were complexed with Cas9 protein (PNA BIO, Thousand Oaks, USA) and co-electroporated (Neon, ThermoFisher, Waltham, USA) into Igrov-1 cells to target genomic positions in ADAM17 exon 2 (chr2: 9,543,226; chr2: 9,543,228) for double strand breaks. Single cell suspensions were dispensed into 96-well plates by flow cytometry (FC) sorting (FACS Aria III Fusion, BD). Colony formation of single cells was monitored using the single cell cloning application of the YT-Software® using NYONE® Scientific Imager (SYNENTEC GmbH, Elmshorn, Germany). Using this methodology, we ensured that only colonies derived from single cells were further analysed. Quantification of soluble AREG—as a surrogate for ADAM17 activity—by AREG enzyme-linked immunosorbent assay (ELISA) (#DY262 R&D Systems) allowed a pre-screening of clones with respect to ADAM17 activity. ADAM17 knock-out was analysed using Western blot (WB) (total ADAM17 expression), conventional FC and imaging FC (ImageStream X Mark II (Merck)) (surface ADAM17 expression). Out of the analysed clones clone B2 performed best in all assays and was selected for the present study.

2.1.5 | Cell treatment for EV and lysate generation

Igrov-1-WT were seeded at a density of approximately 4.7×10^4 cells/cm², the ovarian carcinoma cell clone KI-OC-11 at a density of approximately 1.47×10^4 cells/cm². OvCa cell clones/lines were cultivated in EV-depleted medium at 37°C and 5% CO₂. Stimulation with cisplatin was performed 24 h after seeding at approximately 70% confluence after a medium change. After 48 h of incubation with either cisplatin or NaCl solvent supernatants were centrifuged at $300 \times g$, and the pellet, containing cells released into culture supernatants during treatment, were stored together with the scraped cells from the flask. Cell-free supernatants were subjected to differential ultracentrifugation to isolate EV (see below).

2.1.6 | Ascites preparation

Ascites fluid was acquired from late stage, serous OvCa patients during surgery or paracentesis and directly transferred to our laboratory. Peritoneal washings, which were derived from patients undergoing surgical procedures for benign gynaecological diseases such as fibroids were used as benign control. To generate cell-free ascites, the original ascites or flushing fluid was centrifuged ($348 \times g$, 10 min) and stored at -20°C .

2.2 | EV-purification and characterisation

2.2.1 | Extracellular vesicle isolation and storage

EV were isolated by differential centrifugation and ultracentrifugation. Supernatants of treated cells or cell-free ascites (1:4 diluted in $0.1 \mu\text{m}$ filtered PBS) or flushing fluid of controls (not diluted) were centrifuged for 10 min at $300 \times g$, 30 min at $2000 \times g$ and ultracentrifuged for 45 min at $10,000 \times g$ to remove cells and cell debris. EV were pelleted via ultracentrifugation at $100,000 \times g$ for 1.5 h and washed once with $0.1 \mu\text{m}$ filtered PBS again at $100,000 \times g$ for 1.5 h. Washed EV were resuspended in a defined volume of $0.1 \mu\text{m}$ filtered PBS (30–300 μL).

2.2.2 | Nanoparticle tracking analysis

To standardize EV amounts after ultracentrifugation, EV purified from either 3.5×10^6 cells or 1 mL ascites were suspended in 1 mL filtered PBS. EV size and concentration were determined via nanoparticle tracking analysis (NTA) with a NanoSight 300 (NS300) using NanoSight software version 3.40 (Malvern Panalytical, Malvern, UK).

2.2.3 | Western blot (WB)

Cells were detached by scraping and lysed with lysis buffer containing protease inhibitors (Tris: 50 mM (Merck); NaCl: 150 mM (Roth); EDTA: 2 mM (Merck); NaF: 1 mM (Merck); Na₃VO₄: 1 mM (Santa Cruz, Dallas, USA); NP-40 (IGEPAL CA-630): 1% (v/v) (Sigma-Aldrich); Triton™ X-100: 1% (v/v) (Sigma-Aldrich); 1 tablet Complete™ mini (Roche, Mannheim, Deutschland) per 10 mL lysis buffer. The isolated EV in residual PBS were lysed by adding a 2x lysis buffer at a 1:1 ratio. The protein concentration of lysates was determined by Bradford Assay. A total of 1–2 μg of whole cell lysates and 10–25 μL of EV lysates were boiled in 3x SDS-loading buffer under either reducing or non-reducing conditions, electrophoretically separated on SDS polyacrylamide gels and transferred to nitrocellulose membranes. The following primary and secondary antibodies were used: Anti-CD63 monoclonal antibody (mab) (clone TS63, Abcam, #ab59479), anti-HSP70 mab (clone W27, BioLegend, #648002), anti-CD9 mab (clone EPR2949, Abcam, #ab92726), anti-Syntenin-1 mab (clone EPR8102, Abcam, ab133267), anti-Calnexin mab (clone W17077C, BioLegend, #699402), polyclonal anti-ADAM17 extracellular ab (R&D Systems, #AF9301), polyclonal anti-ADAM17 intracellular ab (Abcam, #ab39162), polyclonal anti- β -tubulin ab (H-235) (Santa Cruz, sc-9104), sheep HRP-conjugated anti-mouse ab (GE Healthcare, #NA931), goat HRP-conjugated anti-rat ab (GE Healthcare, #NA935), donkey HRP-conjugated anti-rabbit ab (GE Healthcare, #NA9340). Protein bands were visualised by enhanced chemiluminescence detection reagent (GE Healthcare).

2.2.4 | ADAM17-ELISA

For direct comparison of ADAM17 concentrations in ascites samples, ADAM17 levels were quantified using ADAM17 sandwich ELISA. Therefore, EV lysates, as prepared for WB analysis (Section 2.2.3) were diluted 1:5 in ELISA reagent diluent (1% bovine serum albumin in PBS), and subjected to human ADAM17 DuoSet® ELISA Set (#DY930 R&D Systems). The assay was conducted in 96-well Nunc-Immuno plates (#442404 Thermo Fisher Scientific) following the manufacturer's instructions. Absorbance was determined at 450 nm using a multimode microplate reader (Tecan Spark).

2.2.5 | Transmission electron microscopy

Transmission electron microscopy (TEM) of EV was performed as described previously (Arnold et al., 2014, 2020). EV were transferred to negative-glow discharged continuous carbon grids (Science Service, Munich, Germany) and stained with a half-saturated solution of uranyl acetate. The images were generated using a JEOL-1400-Plus-TEM (JEOL Germany, München) with integrated TemCam-F416 camera (TVIPS, München).

2.2.6 | Flow cytometry and imaging flow cytometry

To investigate ADAM17 surface expression of OvCa cells, 2×10^5 Igrov-1 WT and KO cells were resuspended in PBS and transferred to a 96-well V-bottom plate (Nerbe Plus) and washed once with FACS-wash buffer (1% BSA and 0.1% NaN_3 in PBS). The staining was performed for 30 min at 4°C in the dark using monoclonal mouse anti-ADAM17 ectodomain Phycoerythrin (PE)-conjugated ab (clone 111633, R&D Systems, #FAB9301P) or PE-conjugated IgG1 ab (clone MOPC-21, BioLegend, #400114) as an isotype control. Afterwards, the cells were washed twice before being fixed with 1% PFA in PBS. The cells were stored at 4°C until use.

For imaging FC, the samples were measured with the ImageStream X Mark II (Merck) at 40x magnification. Stained cells were excited with a 562 nm laser and the PE signal was acquired in channel 3 (560–595 nm). Bright field images were displayed in channel 1. The raw images of the channels were collected separately by the integrated software INSPIRE (Amnis). The analysis was done with the IDEAS 6.2 image analysis software (Amnis) after gating on focussed single cells as described previously (Lettau et al., 2020).

To investigate the ADAM17 orientation on EV, 8 μL of ascites-derived EV were incubated with 2 μL of aldehyde/sulfate latex beads (4%, w/v, 4 μm , IDC™ latex particles, Life Technologies) overnight at room temperature. The next day the beads were pelleted and washed once with PBS. The beads were blocked for 45 min in 2% BSA/PBS and washed twice before being stained with a monoclonal mouse anti-ADAM17 ectodomain PE-conjugated ab (clone 111633, R&D Systems, #FAB9301P) or PE-conjugated anti-CD63 mab (clone MEM-259, Abcam, #ab18236) and PE-conjugated IgG1 mab antibody (clone MOPC-21, BioLegend, #400114) as isotype control as described above.

2.3 | Functional assays

2.3.1 | Long-term-EV-stimulation-assay/AREG release assay

To assess the activity of EV-associated ADAM17, 1×10^5 Igrov-1-KO cells were cultivated in a 12 well-plate (Greiner) in 1 mL EV-depleted medium. After 24 h the medium was removed and the cells were washed with PBS. Next, three wells each were incubated with either EV-depleted medium containing 1×10^9 ascites-EV from patient case number 3 (#3) or solvent control (PBS). On each of the following three days 250 μL of the supernatants was removed, stored and replaced by 1×10^9 ascites-derived EV in 250 μL fresh EV-depleted medium or solvent control containing EV-depleted medium. After day 5 the supernatants were stored and later analysed for AREG release with the respective ELISA (see below). To investigate whether the AREG shedding also occurs on the EV, one well without cells was treated with EV every day as control to assess AREG release from EV.

2.3.2 | Enzyme-linked immunosorbent assay

AREG was detected in cell culture supernatants by sandwich ELISA. For this, human Amphiregulin DuoSet® ELISA Set (#DY262 R&D Systems) was conducted in 96-well Nunc-Immuno plates (#442404 Thermo Fisher Scientific) following the manufacturer's instructions. Absorbance was determined at 450 nm using a multimode microplate reader (Tecan Spark).

2.3.3 | ADAM17-activity assay

The enzymatic activity of ADAM17 on EV was analysed using a commercially available quenched fluorogenic peptide substrate (Abz-Leu-Ala-Gln-Ala-Val-Arg-Ser-ser-Ser-Arg-Dap(DNP)-NH₂) (Biozol, #BYT-ORB104351).

For this, 1×10^9 ascites-derived EV were diluted in 100 μ l sterile filtered PBS in a white Corning Costar® 96-well plate. 10 μ M of substrate were added to the EV. The activity assay was carried out in triplicates at 37°C. Fluorescence was measured every 30 s for at least 80 min at 420 nm with an excitation at 320 nm using a microplate-reader (Tecan Spark) and plotted as relative fluorescence units (RFU).

2.3.4 | ApoLive Glo—caspase/viability assay

For the assessment of the cisplatin dose-response of Igrov-1 WT and ADAM17 KO cells 20,000 cells per well were seeded in a 96-well plate (Corning Costar, #3903). After cultivation for 48 h at 37°C the medium was aspirated, and cells were treated with 3 μ M of the ADAM17 inhibitor GW280264X or DMSO as a solvent control. After 2 h of incubation all cells were stimulated with cisplatin concentrations of 1 μ M, 2.5 μ M, 5 μ M, 7.5 μ M, and 10 μ M or NaCl as solvent control in triplicates. 48 h post stimulation the relative caspase activation was determined using the Multiplex Assay ApoLive-Glo™ (Promega #G6411) according to the manufacturer's instructions (TM325). Measurements were performed with a microplate-reader (Tecan Spark). Viability was quantified as relative fluorescence units (RFU, Ex/Em 400/505 nm). Caspase-3/7-cleavage was measured as relative luminescence units (RLU) using the Caspase-Glo® 3/7 assay system. The caspase activity was normalized to the viability as recommended by the manufacturer.

2.3.5 | Short-term-EV-stimulation-assay—evaluation of EV-mediated resistance

Igrov-1 KO (15,000 cells per well) were seeded in 96 well plates (Corning Costar®, #3917). After 24 h cultivation at 37°C and 5% CO₂, medium was aspirated and cells were washed with PBS. One part of the cells was treated with 1.5×10^8 ascites-derived EV per well, and the other half was treated with PBS only. After 24 h the medium was discarded, and all cells were washed with PBS before being treated with cisplatin. After 48 h incubation, viability and caspase activation was determined as described in Section 2.3.4

2.4 | Statistic analysis

Means of at least three independent experiments were calculated using Microsoft Excel (2019), and the Gaussian distribution was tested with the Shapiro–Wilk normality test using GraphPad Prism 9 (GraphPad Software, Inc.). Parametric matched datasets of two groups were analysed using ratio paired t-test. For parametric data of more than two groups, one-way repeated measurement (RM) ANOVA followed by Dunnett's or Tukey's multiple comparison test was calculated, or Friedman's test followed by Dunn's multiple comparison post hoc test (for non-parametric matched datasets) was calculated. P-values of <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Cisplatin induces the release of ADAM17-containing EV from Igrov-1 cells

EV are generally released from cells to mediate intercellular communication and thus play an important role not only during physiological development but also in pathological conditions like infection and cancer (Beer & Wehman, 2017; Kalluri & LeBleu, 2020). Importantly, chemotherapy-induced EV release is considered to promote tumour recurrence and resistance mechanisms which is a major clinical problem in OvCa (Samuel et al., 2018; Tian et al., 2022). To analyse whether cisplatin treatment might induce EV release, we initially investigated the OvCa cell line Igrov-1 which we intensively studied in our laboratory with respect to ADAM17 activity and chemotherapy resistance mechanisms.

We analysed cell survival and the EV content in Igrov-1 cells upon cisplatin exposure (Figure 1). Assessment of particle numbers by NTA demonstrated that EV release significantly increased upon cisplatin treatment in a dose-dependent manner ($p = 0.0031$) (Figure 1a,c) and negatively correlated with cell viability of the cisplatin-sensitive Igrov-1 cells (Figure 1d).

In a next step, EV secreted by Igrov-1 cells were characterised according to the minimal information for studies of EVs 2018 (MISEV 2018) criteria with respect to size, marker protein abundance, and morphology (Figure 1) (Théry et al., 2018). Particularly,

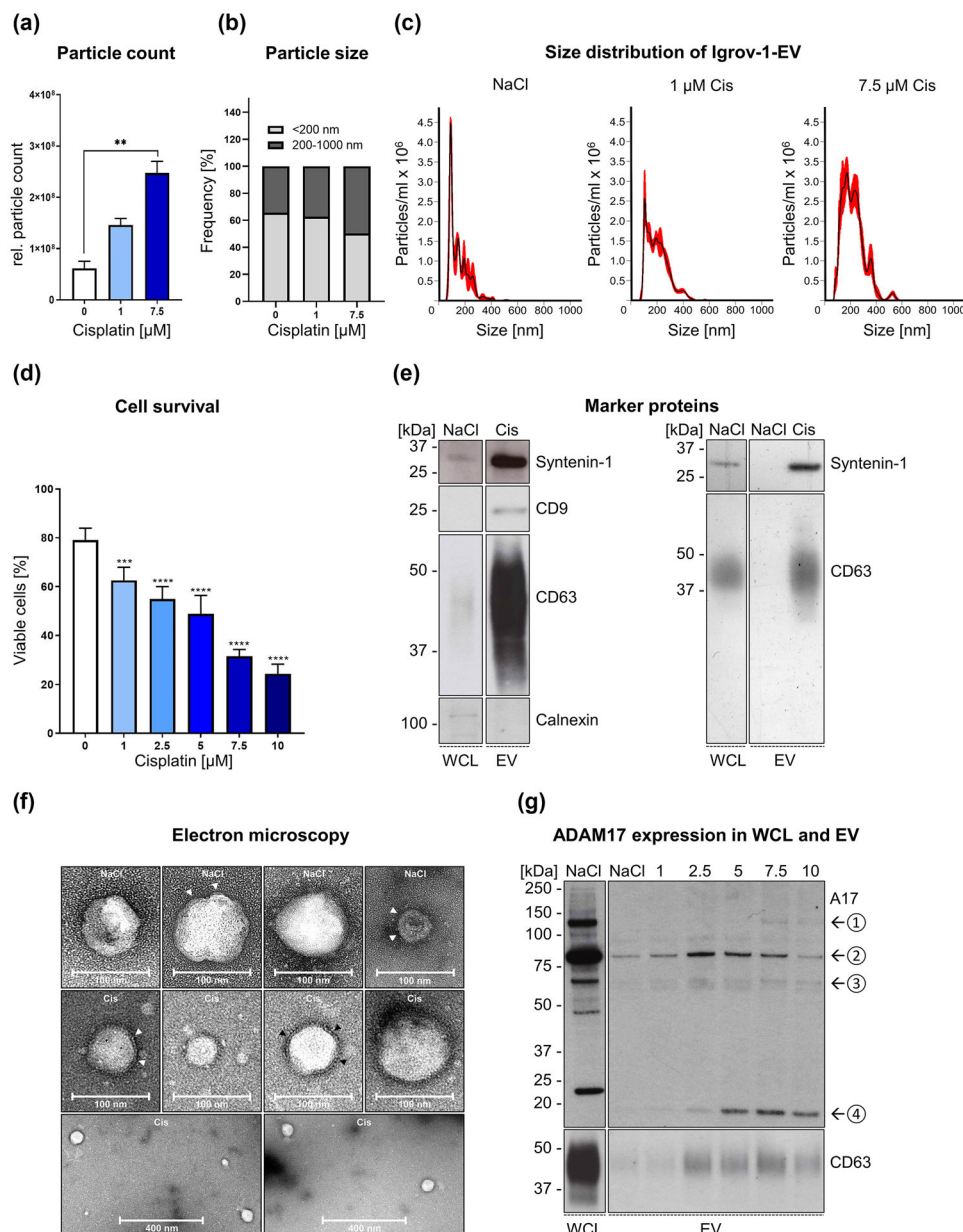


FIGURE 1 Cisplatin induces ADAM17-containing EV release from Igrov-1 cells. Igrov-1 cells were grown in EV-depleted medium and stimulated with 1–7.5 μ M cisplatin or NaCl solvent control (0 μ M). 48 h after treatment, EV were enriched from supernatants and characterised. EV concentration and particle size were assessed by nanoparticle tracking analysis (NTA) and displayed as (a) bar charts of concentration, (b) the relative fraction of EV < 200 nm and 200–1000 nm in size, (c) histograms of size distribution. Size distribution and EV concentration were standardised to the number of seeded cells (3.5×10^6), the corresponding data represent mean values of five independent experiments + SEM. (d) Cell viability was assessed via trypan blue staining and quantified by automatic imaging. (e) Marker protein expression of Syntenin-1, CD9, CD63 and Calnexin of cis-EV and whole cell lysate (WCL) of untreated cells was analysed by WB. The depicted blot is representative of three independent experiments. For WCL, 1 μ g per lane was loaded. For EV samples the maximum of available EV-concentrate was loaded; this varied depending on EV yield of the preceding cell culture experiments. 3.6×10^9 EV were included in the CD63 and CD9 blot, 3.6×10^9 in the Calnexin and 1.8×10^8 in the Syntenin-1 blot (left side). As for restricted material of NaCl-EV, material corresponding to 3.75×10^6 EV were loaded (right blot). For cis-EV, material corresponding to 1.5×10^7 EV was used and 2 μ g WCL per lane were loaded (E, right blot). (f) Electron micrographs of NaCl-EV and cis-EV (7.5 μ M) were investigated by transmission electron microscopy (TEM). White and black arrowheads indicate lipid bilayer. Scalebar = 100 nm (upper rows) and 400 nm (lower row). (g) ADAM17 and CD63 abundance in EV and WCL were analysed by WB. 2 μ g of WCL and the entirety of enriched EV generated by 1.75×10^6 Igrov-1 cells within 72 h were subjected to SDS-PAGE, these corresponded to an average amount of 7.7×10^7 EV per lane with approximately 3×10^7 for NaCl-EV and up to 1.3×10^8 EV for 7.5 cis-EV. The depicted blot is representative of three independent experiments. Pro-form of ADAM17 (1), mature form (2) and fragments at approx. 65 kDa (3) and approx. 20 kDa (4) were detected. These forms are in concordance with the antibody datasheet. Based on the Shapiro–Wilk normality test, statistical significance of five biological replicates was analysed by Friedman’s test followed by Dunn’s multiple comparison test, not normally distributed (a) or RM-one-way-ANOVA following Dunnett’s multiple comparison test, normally distributed (b). Significance is displayed as ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

200–1000 nm EV increased with higher (7.5 μ M) cisplatin concentrations (Figure 1b,c). WB analysis demonstrated the presence of EV-associated Syntenin-1, CD9 and CD63 and the absence of the non-EV marker protein Calnexin in the EV samples (Figure 1e). TEM revealed spherical particles surrounded by a lipid bilayer (Figure 1f, arrowheads).

Next, we aimed to investigate if there could be an association between the metalloprotease ADAM17 and cisplatin-induced EV. Therefore, we enriched EV from supernatants of cisplatin- or control (NaCl)-treated Igrov-1 cells as explained above and assessed the presence of ADAM17 in these EV and a corresponding cell lysate by WB (Figure 1g). Of note, the whole enriched material released by the cells in different conditions was subjected to SDS-PAGE, and thus observed expression levels mainly correlate with the extent of EV release rather than changes in EV protein composition. In concordance with previous publications (Blanchot-Jossic et al., 2005; Groth et al., 2016; Lorenzen et al., 2016; McGowan et al., 2007; Schlöndorff et al., 2000), we identified the pro-form (immature) of ADAM17 at a size of approx. 110–120 kDa in cell lysates, and a mature form—lacking the inhibitory pro-domain—at a size of approx. 75–80 kDa. These are the most prominent ADAM17 forms (Grötzinger et al., 2017; Schlöndorff et al., 2000). Importantly, cis-EV mainly contained an increasing amount of the mature ADAM17, while applying higher cisplatin concentrations (10 μ M) (Figure 1g) induced a decline of ADAM17 protein detected in EV. Strongest ADAM17 signals were detected in EV from supernatants of cells treated within a range of 2.5–7.5 μ M cisplatin, also correlating with CD63 levels. Besides the immature and mature form, smaller fragments of approx. 65 and 20 kDa, were detected in cis-EV. The approx. 20 kDa fragment showed a positive correlation with high cisplatin concentrations (5–10 μ M). Given these results, we continued our experiments using 7.5 μ M of cisplatin.

As an interim result, this data clearly demonstrated that cisplatin treatment strongly induced EV release in the OvCa cell line Igrov-1 and the presence of mature ADAM17 in cis-EV.

3.2 | Cisplatin treatment induces the release of ADAM17-containing EV from patient-derived cells

In a translational approach, we stimulated the in-house established cell-line (KI-OC-11) under identical conditions. The induced EV were characterized as stated before (Figure 2). Notably, KI-OC-11 cells released more EV upon cisplatin treatment than cells treated with NaCl only (Ctrl.) ($p = 0.012$) (Figure 2a).

Remarkably, KI-OC-11 cells produced approximately ten times more EV per cell than Igrov-1 (Figures 1a and 2a). Again, the EV were characterised by WB. We show that following cisplatin treatment KI-OC-11 cells release more EV-associated ADAM17 compared to unstimulated and Igrov-1 cells (Figure 2c). In conclusion, these results demonstrate that the primary OvCa cell line KI-OC-11 also shows an augmented production of ADAM17-bearing EV in response to cisplatin treatment.

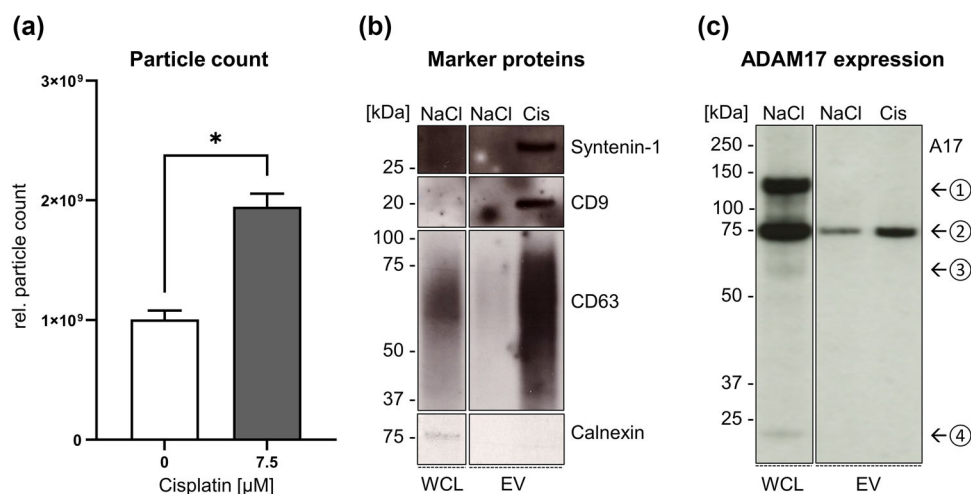


FIGURE 2 Cisplatin treatment induces the release of ADAM17-containing EV from patient-derived cells. KI-OC-11-cells were incubated in EV-depleted medium and stimulated with 7.5 μ M cisplatin or NaCl as solvent control for 48 h. EV were enriched from supernatants by differential ultracentrifugation. (a) EV concentration was determined by NTA. The number of extracted EV was standardised to 3.5×10^6 cells. (b) Marker protein (Syntenin-1, CD9, CD63, Calnexin) and (c) ADAM17 (A17) expression in OC-11-EV (0 μ M (NaCl), 7.5 μ M cisplatin) and cellular lysates (WCL) of vesicle-treated OC11 cells were analysed by WB. Approximately 5×10^8 NaCl-EV and 9.7×10^8 cis-EV were loaded. Pro-form of ADAM17 (1), mature form (2) and fragments at approx. 65 kDa (3) and approx. 20 kDa (4) were detected. These forms are in concordance with the antibody datasheet. The data represents the mean value of three independent experiments + SEM. Based on Shapiro–Wilk normality test, statistical significance of four biological replicates was analysed by ratio paired *t*-test. Statistical significance is displayed as * $p < 0.05$.

3.3 | Native ascites-derived EV contain different amounts of mature ADAM17

Malignant ascites fluid contains a variety of cellular and acellular components—including EV—which could contribute to chemoresistance (Ford et al., 2020). To investigate if ADAM17 is detectable on ascites-derived EV, we isolated and characterised EV from ascites of three OvCa patients (see Table 1). Two patients developed a recurrent tumour including ascites formation which was consequently investigated in this study. In the remaining case ascites from primary diagnosis was sampled—and no recurrence has been detected so far (within 12 months after diagnosis).

TABLE 1 Patient characteristics of ascites-derived EV: Case parameters at the timepoint of ascites sampling. Tumour staging was performed using the FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) classification; chemotherapeutic history indicates if and when the patients were subjected to chemotherapy. The timepoint of tumour relapse is indicated.

Patient Case	FIGO	Chemotherapeutic history	Recurrence
#1	IIIC	Neoadjuvant	22 months after diagnosis
#2	IIIC	Chemo naïve	–
#3	IIIC	Adjuvant	12 months after diagnosis

These samples were analysed as described in Section 3.2 with respect to EV content and marker protein expression as well as ADAM17 levels. Moreover, we investigated the ADAM17 presence and orientation at the EV surface. As non-malignant control samples we investigated flushing fluid derived from patients undergoing surgical procedures for benign gynaecological disease such as fibroids (#4, #5). In WB analyses three different EV marker proteins (Syntenin-1, CD9 and CD63) were detected in all EV-samples (Figure 3d). NTA analyses revealed inter-individual differences in EV quantity with a higher particle count in case #1 and case #3 when compared to chemo-naïve case #2 ($p = 0.0164$ and 0.0428 , respectively) (Figure 3a,b). Of note, concerning the EV size, all ascites samples predominantly contained 200–1000 nm EV (Figure 3c). Again, ADAM17 abundance was analysed as described before. Importantly, ascites of the relapsed cases #1 and #3 showed a higher amount of EV-associated mature ADAM17 in WB analyses, which did not correlate with overall EV marker expression, thus indicating a higher EV-associated ADAM17 content in these patients (Figure 3e). In contrast, even though benign controls showed presence of EV, ADAM17 was not detected on these EV. Note, that amounts of flushing liquid and ascites fluid are barely comparable. For benign samples, we did not perform further dilution steps, as we did for ascites (1:4). EV were purified from the entire flushing liquid of 50 mL and the maximum possible amount of EV was subjected to western blot analysis. Importantly, as the EV marker Syntenin-1 is present in benign samples, even showing a stronger abundance compared to the sample of OvCa patient number #3, we verified the presence of EV in these samples and demonstrated the absence of ADAM17 in benign flushing fluid. As a prerequisite for EV-associated shedding activity, we next investigated whether the ADAM17 ectodomain faces outwards of the EV membrane. ADAM17 orientation on EV was assessed via FC of non-permeabilized EV conjugated to latex beads utilizing an anti-ADAM17 antibody directed against the ectodomain of ADAM17 (Figure 3f). By CD63 staining we confirmed the integrity of EV after conjugation to beads (Figure 3g). The ADAM17 staining indicates that at least a part of EV-associated ADAM17 resides on the EV surface with its catalytic domain facing into the extracellular space. In line with the WB results, the strongest EV surface expression of ADAM17 was detected in the two pre-treated patients (#1 and #3) compared to #2 ($p = 0.0339$), whereas levels of CD63 did not differ significantly. Taken together, mature ADAM17 is also present on ascites-derived EV. However, its surface EV expression/abundance and total ADAM17 levels differ inter-individually.

3.4 | ADAM17-containing EV increase following chemotherapeutic treatment in *ex vivo* patient samples

As proof of concept, reflecting the heterogeneity of patient material and a first longitudinal sample analysis, we performed a direct comparison of ascites derived from an ovarian cancer patient before and after chemotherapy at the timepoint of recurrence, approximately one year after primary diagnosis. Importantly, we detected a twofold increase of EV secretion in the second sample after chemotherapeutic intervention compared to the sample drawn at the timepoint of primary diagnosis (Figure 4a). In addition to NTA analysis, we restricted the analysis to ADAM17 quantification due to material limitation of the primary sample. EV extracted from recurrent disease after chemotherapy contained significantly higher amounts of ADAM17 compared to the sample drawn at the chemo-naïve situation (Figure 4b). We additionally performed ELISA analyses for a quantitative comparison. We found ADAM17 levels to be 8.11-fold increased after chemotherapeutic treatment (Figure 4c). As both NTA analysis and ELISA were performed using comparable volumes of EV solution, this data suggests that EV-associated ADAM17 levels increase following chemotherapeutic treatment in the recurrent situation. Nevertheless, these initial experiments only provide a first indication and thus have to be tested in a larger cohort of patients, which we are currently collecting.

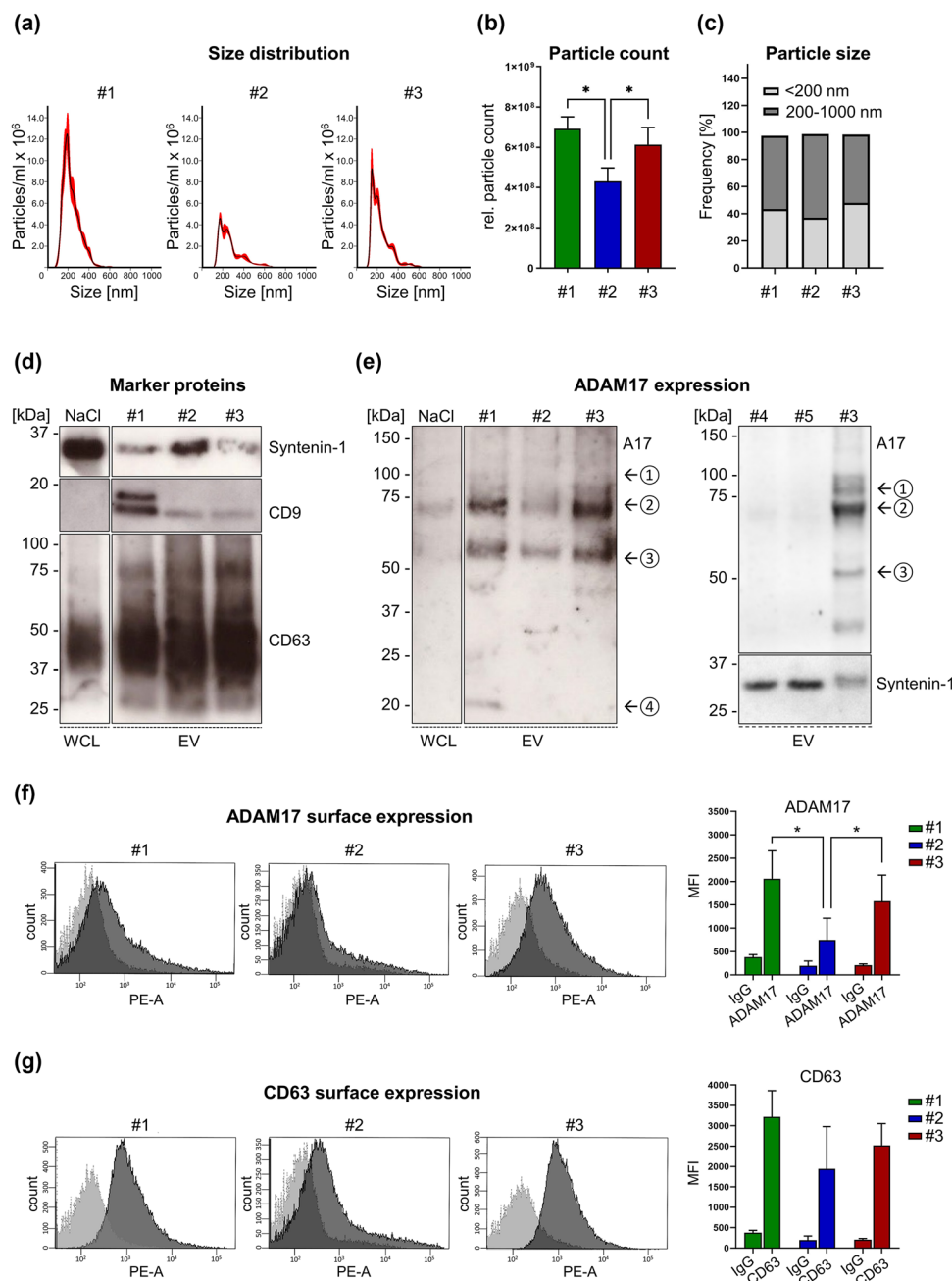


FIGURE 3 Native ascites-derived EV contain mature ADAM17. Ascites-EV derived from three ovarian cancer patients (#1, #2, #3) were isolated by differential ultracentrifugation and characterised. Size distribution and concentration of ascites-EV were determined by NTA and displayed as (a) histograms of size distribution, (b) bar charts of concentration and (c) the relative fraction of EV < 200 nm and 200–1000 nm in size. Size distribution and EV concentration were adjusted to the EV extracted from 1 ml ascites. (d) Marker protein expression of Syntenin-1, CD9 and CD63 was analysed in EV and whole cell lysates (WCL) by WB. (e) Relative ADAM17 abundance in purified EV was assessed by WB analysis. EV purified from 0.5 mL ascites were loaded on the gel. Pro-form of ADAM17 (1), mature form (2) and fragments at approx. 65 kDa (3) and approx. 20 kDa (4) were detected. (f) As benign controls, flushing fluid derived from patients undergoing surgical procedures for benign gynaecological diseases such as fibroids were used (#4 and #5). The maximum amount of EV derived from ~1.4 to 1.9 mL flushing fluid was loaded on the gel. Marker protein and ADAM17 (A17) expression were analysed in comparison to EV derived from patient #3. The marker protein Syntenin-1 and ADAM17 (A17) are displayed and run in the same blot as EV derived from patient #3 for a direct comparison. (g, h) Surface expression of ADAM17 and CD63 on ascites-EV was evaluated via FC using Phycoerythrin (PE)-conjugated anti-ADAM17 and anti-CD63 antibodies. FC data are presented as mean fluorescence intensities (MFI). NTA and FC data are represented as mean + SEM of three independent experiments. Based on the Shapiro–Wilk normality test, statistical significance was analysed by RM one-way-ANOVA following Tukey's multiple comparison test (b, g). Statistical significance is displayed as * $p < 0.05$.

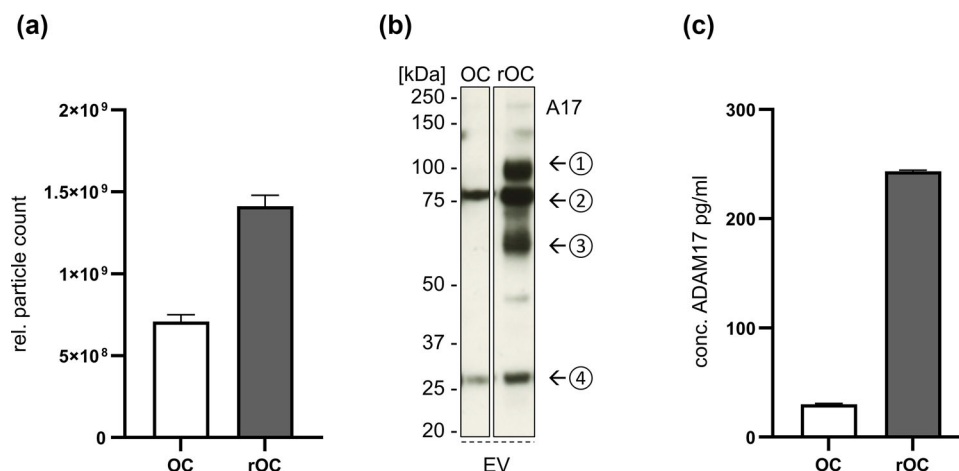


FIGURE 4 Comparison of ex vivo native ascites-derived EV before and after chemotherapeutic treatment of an OvCa patient. Ascites-EV derived from one individual OvCa patient before and after therapy were isolated by differential ultracentrifugation and characterised. The first sample was taken at primary diagnosis (chemo-naïve) (OC) and the second sample after chemotherapeutic treatment at the time of recurrence (1 year later) (rOC)(a). The concentration of ascites-EV was investigated by NTA ($n = 4$ measurements) and displayed as bar charts. (b) EV purified from 0.75 mL ascites, corresponding to 5.32×10^8 EV for the OC sample and 1.06×10^9 for the rOC sample were subjected to gel electrophoresis and relative ADAM17 abundance in purified EV was assessed by WB analysis. Pro-form of ADAM17 (1), mature form (2) and fragments at approx. 65 kDa (3) and approx. 20 kDa (4) were detected. (c) ADAM17 was quantified by ELISA using EV purified from 0.375 mL ascites, which corresponds to a total number of 2.66×10^8 EV for OC and 5.3×10^8 EV for rOC.

3.5 | ADAM17 is active on isolated ascites-EV

To investigate if ascites-EV-associated ADAM17 is proteolytically active and could thus potentially contribute to the ADAM17-dependent establishment of a pro-tumourigenic environment and chemoresistance, we performed a cell-free in-vitro-assay based on the ADAM17-mediated cleavage of TNF- α using a quenched fluorescence probe (Figure 5).

Based on the NTA results, samples with the same EV concentration were created for all three patients. The kinetics of substrate cleavage clearly showed proteolytic ADAM17 activity of EV purified from ascites. The strongest proteolytic activity was observed with EV from patient #3, followed by patient #1, which were also the samples with strongest ADAM17 surface expression (Figures 5a,b and 3f). Concordantly, #2-derived EV with lower ADAM17 surface abundance (Figures 5a,b and 3f) displayed reduced shedding activity ($p = 0.014$ compared to #1 and $p = 0.0027$ compared to #3) (Figure 5b).

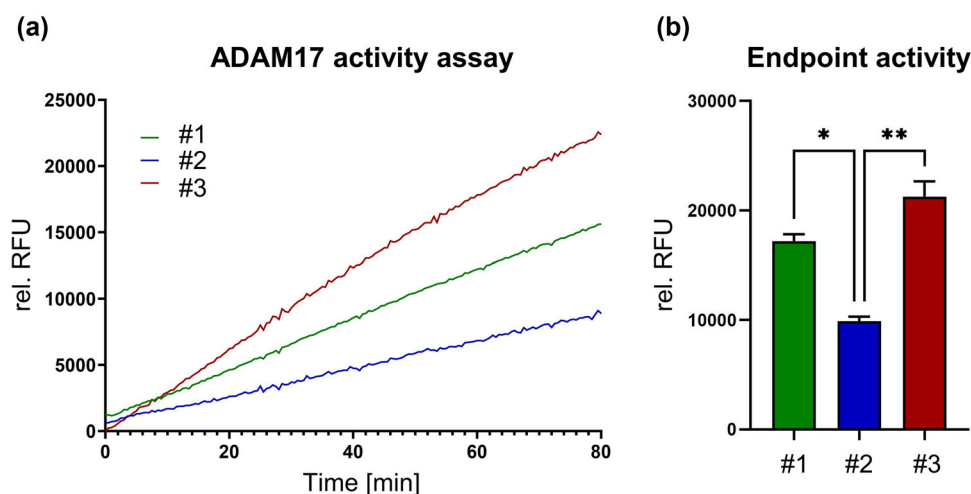


FIGURE 5 ADAM17 is proteolytically active on isolated ascites-EV. ADAM17 proteolytic activity of ascites-EV was quantified using a cell-free ADAM17 activity assay. 1×10^9 ascites-EV of three OvCa patients (#1, #2 and #3) or PBS as a control were incubated at 37°C for 80 min with a fluorogenic substrate. Relative fluorescence units (RFU) were measured every 30 s. RFU of the PBS control was subtracted from the RFU of the EV incubation. (a) Kinetics of ADAM17 activity in ascites-EV, one representative of three biological replicates is shown. (b) Mean RFU (endpoint) of the cleavage product of three biological replicates + SEM is displayed. Based on the Shapiro-Wilk normality test, statistical significance was analysed by RM one-way-ANOVA following Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$.

Summarized, ADAM17 is active on ascites-derived EV. Its proteolytic activity differs inter-individually and correlates with ADAM17 surface expression.

3.6 | Generation and characterization of ADAM17-deficient cells

As we aimed to investigate whether these EV could mediate substrate shedding in a cell-based system, we next established a reliable cell model to test ADAM17 activity. Because ADAM17 is ubiquitously expressed in many tumours including OvCa, we established an ADAM17-deficient cell line using CRISPR/Cas9 technology to investigate if ADAM17 functionality could be mediated by EV (Figure 6) (Blanchot-Jossic et al., 2005; Edwards et al., 2008; Jinek et al., 2012; McGowan et al., 2007; Ni et al., 2013; Ringel et al., 2006; Tanaka et al., 2005). The knockout was verified by the comparative analysis of ADAM17 surface expression of Igrov-1-WT and KO cells via conventional ($p = 0.0053$) and imaging FC and of ADAM17 levels in whole cell lysates via WB. In KO cells, ADAM17 was not detectable in cell lysates nor at the cell surface.

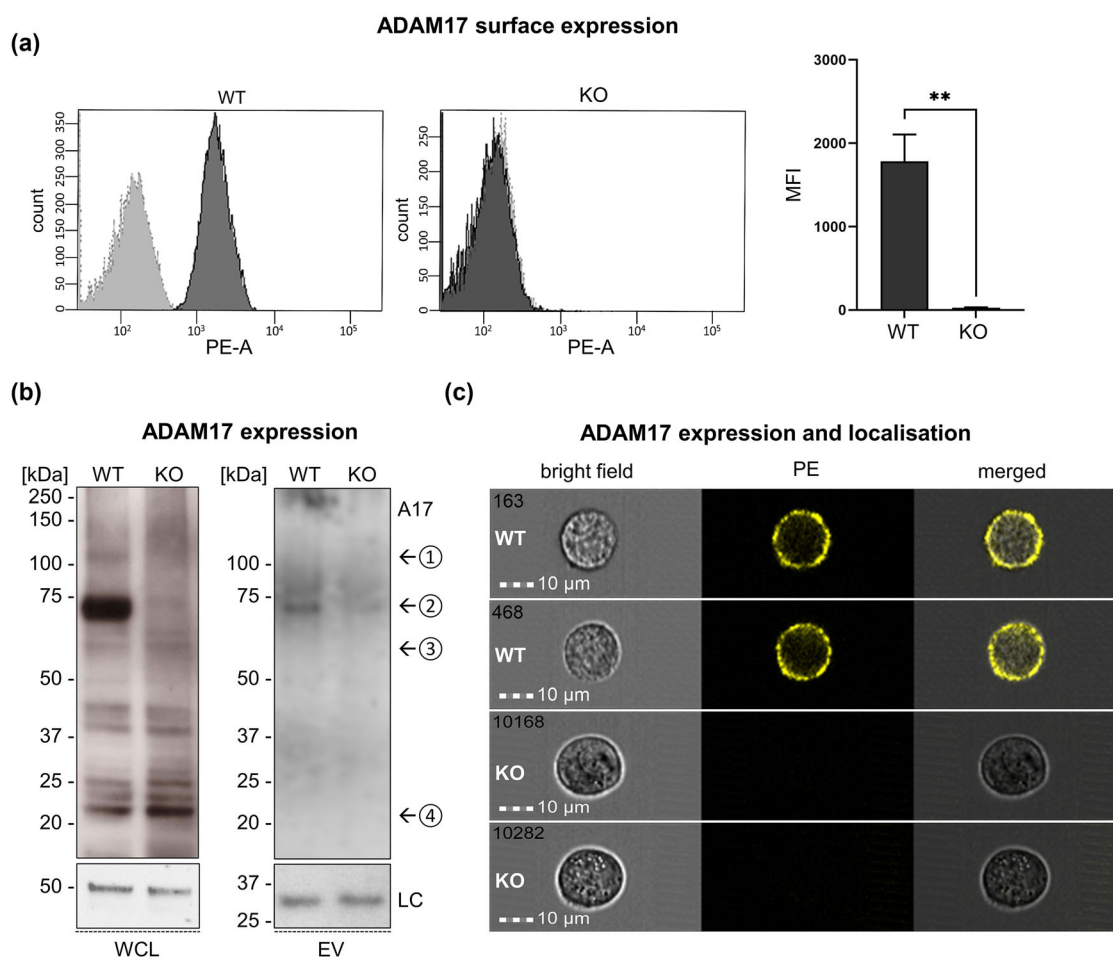


FIGURE 6 Generation and characterisation of ADAM17-deficient cells. ADAM17-deficient cells (KO) were generated using CRISPR/Cas9 technology. ADAM17-positive parental cells (WT) and ADAM17-deficient cells (KO) were characterised in parallel to verify knockout efficiency. (a) Surface expression of ADAM17 on Igrov-1 WT and KO cells was investigated via FC using a Phycoerythrin (PE)-conjugated anti-ADAM17 antibody (black) or IgG control (grey). Data represents the mean value of the fluorescence intensity (MFI) of three independent experiments + SEM. (b) ADAM17 protein levels were evaluated using WB analysis of whole cell lysates (WCL) of Igrov-1 WT and KO cells with β -Tubulin as loading control (LC). Pro-form of ADAM17 (1), mature form (2) and fragments at approx. 65 kDa (3) and approx. 20 kDa (4) were detected. These forms are in concordance with the antibody datasheet. EV of WT and KO cells were quantified by NTA and a particle number of 7.7×10^7 loaded per lane. ADAM17 expression was analysed and EV marker protein Syntenin-1 served as loading control (LC). (c) Surface expression of ADAM17 on Igrov-1 WT and KO cells was investigated via imaging FC using a PE-conjugated anti-ADAM17 antibody. Representative images are shown. Scale bars represent 10 μ m. Statistical significance was analysed by ratio paired *t*-test and statistical significance is displayed as ** $p < 0.01$.

3.7 | ADAM17-ascites-EV induce AREG shedding and mediate resistance in ADAM17-deficient recipient cells

In the next series of experiments, we aimed to analyse if ADAM17 on ascites-derived EV can restore ADAM17 activity with respect to AREG shedding and thus contribute to resistance in ADAM17-deficient cells. Therefore, we investigated if there is any residual ADAM17 activity in KO cells by analysis of AREG shedding (as a surrogate of ADAM17 activity) after cisplatin treatment (Hedemann et al., 2018). ADAM17-deficient cells released significantly lower levels of AREG, which were comparable with levels released by WT cells when incubated with the ADAM17 inhibitor GW280264X ($p < 0.001$) (Figure 7a). Moreover, cisplatin-induced AREG-shedding was only detectable in Igrov-1 WT but not in KO cells, confirming the functional ADAM17 knockout in these cells.

To analyse if ADAM17-deficient cells are more chemo-sensitive than WT cells, we compared caspase activation of both cell lines after cisplatin treatment (Figure 7b). Chemosensitivity in KO cells was even more pronounced compared to WT cells when ADAM17 was inhibited by GW280264X.

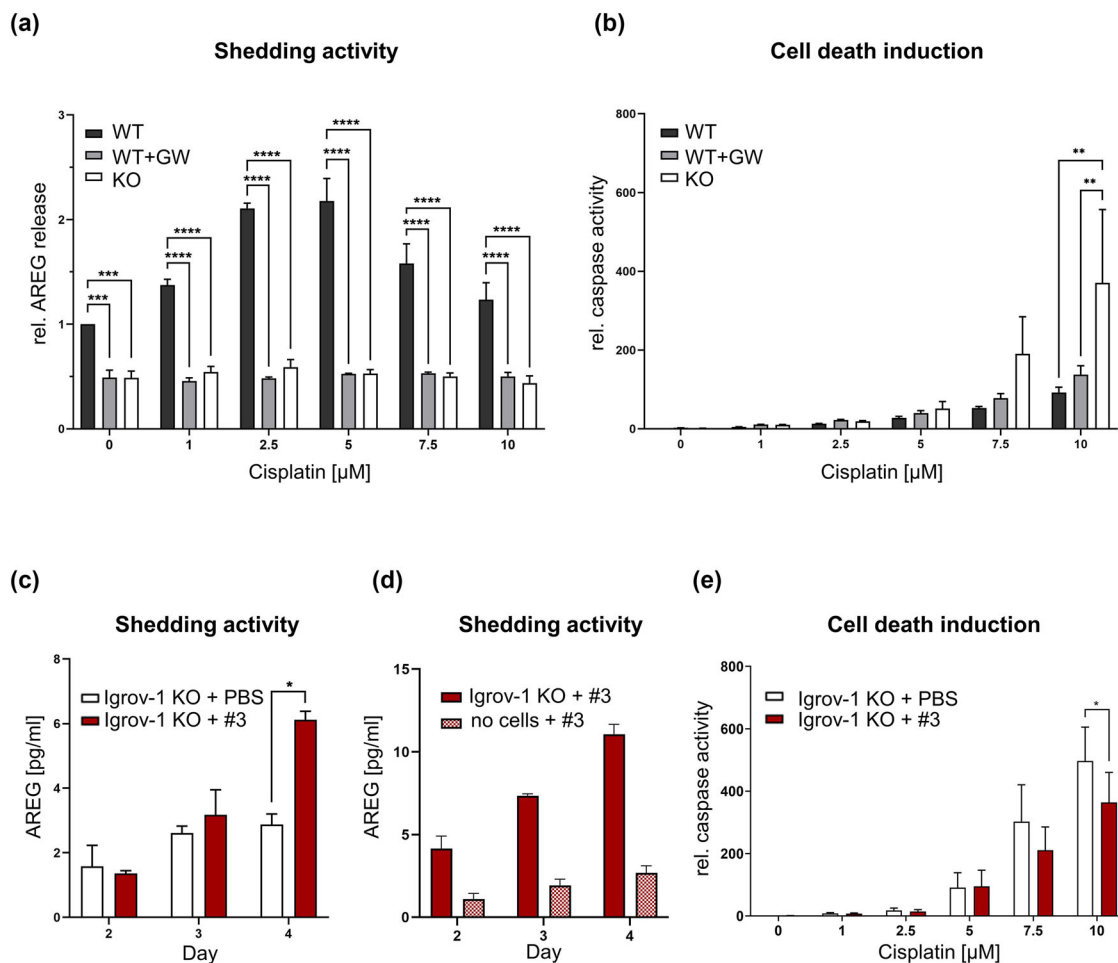


FIGURE 7 ADAM17-containing ascites-EV induce AREG shedding and mediate resistance in ADAM17-deficient recipient cells. Functional characterisation of ADAM17-deficient KO cells compared to ADAM17-positive WT cells. WT cells were treated with indicated concentrations of cisplatin and GW280264X (GW) as a control for ADAM17 activity. (a) AREG release was assessed by ELISA after 48 h of treatment. (b) Caspase activity of Igrov-1 cells was quantified after 48 h of treatment. (c) 1×10^5 ADAM17-deficient cells were seeded in 12-well plates and treated with 1×10^9 ascites-EV of OvCa patient #3 and PBS as a control for 24 h. Thus, the ratio of cells to EV was 10,000 EV per cell. Each day (starting at day 2) 250 μL supernatant were removed from each well and stored for ELISA. The wells were again supplemented with 250 μL medium containing either 1×10^9 ascites-EV or the equivalent volume of PBS. AREG levels were quantified at days 2, 3, and 4. (d) Patient #3-derived EV were cultivated with and without cells to analyse EV-dependent AREG release. (e) ADAM17-deficient cells were seeded at a density of 1.5×10^4 cells per well (96-well plate) and pre-treated for 24 h with 1.5×10^8 ascites-EV and PBS as a control corresponding to a ratio of 10,000 EV per cell. Caspase activity was assessed 48 h after cisplatin treatment. Mean + SEM of three biological replicates is displayed, except for d (technical replicates). Statistical significance was calculated by ANOVA following Tukey's multiple comparison test (a, b) or ratio paired *t*-test (c, d). Statistical significance is displayed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Addressing the complexity of the transmission of cellular information, for example, resistance mechanisms, we conducted a landmark experiment by restoring ADAM17-dependent cisplatin resistance via EV in ADAM17 KO cells. For this we chose the ascites-EV of patient #3, which expressed the strongest proteolytic activity in the cell-free assay (Figure 5). AREG-concentration strongly increased over time and was significantly higher after incubation with ADAM17-containing EV compared to the PBS control ($p = 0.0157$) (Figure 7c). In order to prove that this accumulation was not due to shedding of AREG from the surface of ascites-derived EV, a cell-free control experiment with identical medium changes and incubation times was performed (Figure 7d). Hardly any AREG was detectable in supernatants in the absence of cells (Figure 7d). Finally, we assessed if these ADAM17-mediated effects could contribute to chemoresistance (Figure 7e). Importantly, caspase activity was strongly reduced after incubation with ADAM17-containing EV, indicating a restoration of ADAM17 activity in ADAM17-deficient cells and thus a contribution of EV to chemoresistance ($p = 0.0181$).

These results provide substantial evidence that ADAM17-bearing EV can restore ADAM17 activity in ADAM17-deficient cells and increase the resistance to cisplatin.

4 | DISCUSSION

The most meaningful clinical challenges in OvCa are the distinct chemoresistance potential and high recurrence rates. Among the identified resistance mechanisms, especially the activation of growth factor receptors like EGFR and its downstream signalling elements MAPK and PI3K/AKT play a major role in OvCa (Brasseur et al., 2017). Since ADAM17 is activated by chemotherapeutic treatment, we analysed whether ADAM17 transmitted via EV (ADAM17-bearing EV) subsequently transfers resistance to ADAM17-deficient recipient cells (Hedemann et al., 2018). OvCa is often accompanied by ascites formation, representing one of the major routes of metastasis in this tumour entity with EV contributing to the formation of premetastatic niches (reviewed by Feng et al. (2019) and Nakamura et al. (2019)). We thus purified and characterized EV of ascites from late stage OvCa patients to investigate if ADAM17 is also present in ascites-derived EV.

We conclude that (i) ADAM17-bearing EV are released upon cisplatin treatment; (ii) ADAM17 is proteolytically active on ascites-derived EV; and (iii) ADAM17-containing EV can restore resistance to chemotherapy in ADAM17-deficient cells.

The generic term 'Extracellular Vesicles,' propagated by the International Society of Extracellular Vesicles (ISEV), comprises different subgroups of cell-derived vesicles. Among others, EV can be distinguished regarding their subcellular origin. Exosomes are formed as intraluminal vesicles in intracellular multivesicular bodies (MVB) and are released upon fusion of MVB with the plasma membrane, whereas microvesicles directly bud from the cell membrane (Cocucci & Meldolesi, 2015; Lettau & Janssen, 2021; van Niel et al., 2018). Due to the current difficulty in selectively enriching different EV subgroups, the ISEV promotes standards for the characterisation of EV and the use of a naming system based on the physical or biochemical properties of vesicles rather than their ethology (Théry et al., 2018). Taking these recommendations into account, the vesicles released under chemotherapy treatment (7.5 μ M cisplatin) largely fulfil the ISEV criteria, such as being mostly small with a mean size of 186 nm and displaying positivity for CD63, CD9, CD81, and syntenin-1.

It is known that cisplatin treatment results in apoptotic cell death and consequently also in the release of apoptotic bodies (Barry et al., 1990; Ormerod et al., 1994; Wang et al., 2000). Recently, novel subclasses of EV (<1 μ m) that are generated besides large apoptotic bodies (>1 μ m) during programmed cell death were described. In analogy to their biogenetic origin, these vesicles can be referred to as apoptotic exosomes (ApoExo) and apoptotic microvesicles (ApoMV) (reviewed by Kakarla et al. (2020)). Characteristically, those particles showed a superior membrane integrity compared to traditional apoptotic bodies. This enables them to fuse with recipient cells and consequently improve the exchange of intracellular and membranous components and mediators to orchestrate intercellular communication (Becker et al., 2016; Kowal et al., 2014; Raposo & Stoorvogel, 2013; Stewart et al., 2019).

Notably, with the application of higher cisplatin concentrations (7.5 μ M), the size distribution of particles shifted to larger EV sizes (200–1000 nm) indicative of a transition to a more heterogeneous EV composition under cellular stress. Considering that cisplatin is a strong inducer of apoptosis (at 7.5 μ M 50% cell survival), it is up to discussion if this increased heterogeneity indicates an elevated proportion of apoptosis-associated vesicles (apoptotic bodies, ApoMV and ApoExo). However, OvCa cells display a basal secretion of EV and could also enhance the release of other non-apoptosis-associated vesicles in response to stimulation with cisplatin (Gutwein et al., 2005; Samuel et al., 2018). Representative studies by Gutwein and Altevogt identified two particle types of different densities released by apoptotic OvCa cells that were treated with C2-Ceramide. They were separated by a sucrose gradient and were considered to be exosomes with a lower density and apoptotic blebs with a higher density (Altevogt et al., 2014; Gutwein et al., 2005).

However, due to the lack of specific markers and quantitative immunophenotyping analyses of EV, we cannot define the exact composition of the isolated EV and thus suggest that they represent a heterogeneous mixture of different EV subclasses with a possible increase of the concentration of apoptosis-related particles in response to higher cisplatin concentrations (Kakarla et al., 2020; Menck et al., 2020). Considering the role of chemotherapy-associated EV in chemoresistance transfer, we aimed to figure out if those EV contain ADAM17.

During the last decades, the multifaceted role of ADAM17 in physiological circumstances and disease has been described (Black et al., 1997; Blobel, 2005; Saftig & Reiss, 2011; Sahin & Blobel, 2007; Zunke & Rose-John, 2017). Besides regeneration processes and the initiation of inflammatory reactions, especially its role in cancer progression, invasion and metastasis was elucidated (Düsterhöft et al., 2019). Among the most extensively studied pathways are survival pathways, including receptor tyrosine kinases like EGFR (Maretzky et al., 2011; Zunke & Rose-John, 2017). Sahin et al. identified ADAM17 as the major convertase of the essential EGFR ligands, such as AREG, enabling the activation of EGFR resulting in enhanced proliferation, tumour progression and metastasis in a variety of tumour entities, including OvCa (Ringel et al., 2006; Sahin & Blobel, 2007; Zhang et al., 2012).

Only a small number of groups, including ours, focused on ADAM17-mediated resistance mechanisms. One of the first studies was published by Kyula et al., showing a 5-fluorouracil (5-FU)-dependent ADAM17 activation in colorectal cancer, which lead to an increased shedding of the EGFR ligands TGF- α and AREG that subsequently triggered EGFR-phosphorylation (Kyula et al., 2010). For hepatocellular carcinoma Wang et al. demonstrated an increased EGFR and AKT activation, induced by ADAM17 activation, that ultimately led to a decreased sensitivity towards cisplatin (Wang et al., 2013). Importantly, most therapeutic agents, including platin-derivatives, at least partly induce apoptosis. Intriguingly, apoptosis itself was shown to be associated with concomitant ADAM17 activation, thus suggesting a generalized induction of ADAM17 proteolytic activity upon chemotherapeutic treatment (Chalaris et al., 2010; Sommer et al., 2016; Sommer et al., 2016). In our previous study, we showed an increased ADAM17 substrate release accompanied by enhanced survival signalling in response to cisplatin, which was strongly reduced by the ADAM17/ADAM10 inhibitor GW280264X (Hedemann et al., 2018, 2021).

In the present study, we generated ADAM17-deficient cells using CRISPR/Cas9 technology to further investigate if this chemoresistant phenotype is dependent on active ADAM17 and if it could be restored by ADAM17-carrying EV. The initial characterisation revealed that the functional knockout of ADAM17 in Igrov-1 cells resembled GW280264X inhibitory treatment in terms of cisplatin-sensitivity. This fact provides more evidence that ADAM17, rather than ADAM10, is the protease responsible for the induction of chemoresistance in OvCa. In line with Sahin's findings, AREG shedding from ADAM17 KO cells was completely abolished, and ADAM17-deficient cells become significantly more sensitive to cisplatin treatment than its wild type counterparts (Carvalho et al., 2016; Sahin & Blobel, 2007). Consequently, these ADAM17-deficient cells represent a reliable model system for the detailed analysis of EV-associated ADAM17 activity.

ADAM17 is not only detectable in tissues and sera but also found to be released in association with EV (Blanchot-Jossic et al., 2005; Buchanan et al., 2017; Groth et al., 2016; Hedemann et al., 2021; Ringel et al., 2006; Rogmans et al., 2021; Stoeck et al., 2006; Tanaka et al., 2005). We show that upon platin exposure the EV concentration as well as the particle size increases until a certain platinum concentration is exceeded. Groth et al. (2016) also demonstrated an increased release of ADAM17-carrying EV by Phorbol 12-myristate 13-acetate (PMA) stimulation. Importantly, our platinum-induced EV contained the mature form of ADAM17, and since it was at least partially located at the EV surface it could execute its proteolytic function.

Focusing on the generalised significance of our study, it is important to note that there are several overlapping actions of chemotherapeutic drugs including cisplatin, which could eventually lead to an increased EV release (Lv et al., 2012). Very early in the discovery of resistance mechanisms, Roninson et al. showed a generalised effect of different chemotherapeutic drugs inducing similar downstream mechanisms, for example, the amplification of the same DNA fragments, demonstrating similarities in resistance development (Longley & Johnston, 2005; Roninson et al., 1984). Even though for cisplatin the best understood mode of action is covalent binding to the DNA, followed by initiation of the DNA damage response, a multitude of further resistance mechanisms—including endoplasmic reticulum stress, multi-drug resistance proteins, abnormal cell cycle regulation and signalling pathways—were discovered in the past decades, many of these overlapping with other chemotherapeutic drug resistances, including paclitaxel (Galluzzi et al., 2012; Mandic et al., 2003; Yang et al., 2022; Yusuf et al., 2003).

Similarly, the cisplatin-induced EV release is not restricted to cisplatin but rather a generalized response to cellular stress, which is often induced by chemotherapeutic treatment (Ab Razak et al., 2019). Generation of so-called chemo-EV has been shown for paclitaxel, irinotecan and carboplatin in vitro and even in in vivo studies using bortezomidor, melphalan and doxorubicin (Ab Razak et al., 2019; Bandari et al., 2017; Kreger et al., 2016). Even though basic mechanisms like increased EV release upon chemotherapy could be validated in vivo, significant differences have been documented. E.g. the proteome profile of plasma-derived EV differed substantially between human and mice and lead to contradictory results (Ab Razak et al., 2019; Sandim & Monteiro, 2020). Of note, particularly apoptosis-induced activation and concomitant substrate release of ADAM17 and its close relative ADAM10 underly a strong species-specificity (Garbers et al., 2011). Thus, mouse in vivo models should be regarded with caution in this particular context (Garbers et al., 2011). Therefore, the use of *ex vivo* patient material is of great interest in protease research and particularly in the EV field as it reflects the tumour heterogeneity of the individual patient much better compared to mouse-based in vivo studies (Goričar et al., 2021; Margolis & Sadovskiy, 2019).

Thus, in our study we chose to use *ex vivo* patient material. The great value of *ex vivo* patient material is a potential implementation in longitudinal biomarker research and personalized medicine approaches. Particularly it can be drawn to different timepoints of treatment including chemotherapy cycles (König et al., 2017). Thus, longitudinal sampling helps to monitor changes in tumour heterogeneity and expression profiles of actionable tumour mutations enabling the application of targeted drugs (Chennakrishnaiah et al., 2019; Goričar et al., 2021; LeBleu & Kalluri, 2020; Vasconcelos et al., 2019). In ovarian cancer, one

rich source of EV, which even develops at different time points of disease including disease progression, is malignant ascites (Kim et al., 2016).

Development of malignant ascites is a common complication of OvCa. This fluid compartment is suspected to contain a large variety of metastasis-promoting factors, which are not fully explored yet. One of the components are ascites-EV, representing a heterogenous mixture of vesicles originating from a variety of different cell types, including tumour cells, immune cells, fibroblasts and endothelial cells (Kim et al., 2016). There is accumulating evidence that these EV, along with a variety of cell types and soluble factors, could play a pivotal role in the coordination of the tumour microenvironment as well as in the establishment and maintenance of metastatic niches, as recently proposed by the groups of Nakamura and Feng (Feng et al., 2019; Nakamura et al., 2019). Therefore, patient-derived EV do not only reflect tumour heterogeneity itself but also represent a fingerprint of intercellular communication between the tumour and the tumour microenvironment, including immune cells (LeBleu & Kalluri, 2020; Sandim & Monteiro, 2020).

In a translational approach, we isolated EV from OvCa patients' ascites and assessed EV-associated ADAM17 expression. For this, we isolated and characterized EV from three selected OvCa cases: one very interesting finding is that we observed a generally higher EV abundance in those two patients, who had recurrent diseases and had been exposed to platinum first line therapy already. In contrast, the chemo-naïve patient showed a lower concentration of EV being in line with earlier studies showing increased EV release upon chemotherapy (Aubertin et al., 2016; Bandari et al., 2017; Gutwein et al., 2005). Importantly, in a direct comparison of EV derived from the same patient before and after chemotherapy, we detected a strong increase in ADAM17 protein levels, indicating an association of ADAM17 with disease progression and chemotherapeutic intervention. It has been shown in previous studies, that EV abundance in benign ascites, that is, derived from Meig's syndrome, or borderline tumour, is significantly lower compared to malignant ascites derived from OvCa patients (Im et al., 2014; Mitra et al., 2021; Wang et al., 2022). To investigate if ADAM17 levels differ in samples of benign and malignant ascites, we exemplarily investigated peritoneal washings of two patients undergoing surgical procedures for benign gynaecological disease such as fibroids. In these benign samples ADAM17 was not detected in the EV fraction. Given the limitation of patient material, this initial observation at least supports our findings.

We demonstrate that ascites-derived EV contain mature ADAM17, which is at least partially located at the EV membrane with its ectodomain facing towards the intercellular space. Considering that the transmembrane protease ADAM17 mainly cleaves its substrates in close proximity to the cell membrane, ADAM17 orientation and location at the EV membrane theoretically enables shedding of either EV membrane-associated substrates or substrates on target cells. Using a cell-free shedding assay, we could prove the functional activity of EV-associated ADAM17. This observation is consistent with the research of Altevogt and colleagues, who demonstrated the presence of active proteases on ascites-derived OvCa-EV (Gutwein et al., 2005; Stoeck et al., 2006). Furthermore, Stoeck et al. could demonstrate that EV isolated from cell culture supernatants of OvCa cells contained catalytically active ADAM10 and ADAM17. In the same study, it was shown that these proteases, predominantly ADAM10, were already active in the endosomal compartment and induced shedding of L1 and CD44, which were subsequently released into the extracellular space within exosomes (Stoeck et al., 2006).

In our study, the shedding activity was significantly higher in the EV of those cases with prior chemotherapy treatment and correlated with increased EV numbers and ADAM17 surface levels. Besides higher ADAM17 expression, this increased proteolytic activity might also reflect a preceding activation of ADAM17 by chemotherapeutic treatment.

How and where EV-associated ADAM17 could interact with substrates in or on recipient cells is not known so far. There are several mechanisms of how ADAM17-containing EV could affect recipient cells. Firstly, EV could be taken up by the recipient cell by Clathrin- or Caveolin-dependent endocytosis, by phagocytosis or by micropinocytosis, and guided to intracellular compartments, for example, endosomes, where ADAM17 could shed its substrates within these organelles. Moreover, ADAM17 can be trafficked to the plasma membrane from endosomal compartments via recycling pathways (Kwok et al., 2021; Mathieu et al., 2019; Mulcahy et al., 2014; Shimoda & Khokha, 2017). Secondly, the EV membrane could fuse with the plasma membrane of recipient cells, which allows for a reorganization and integration of membranous parts, thus integrating ADAM17 into the recipient cell's plasma membrane (Kwok et al., 2021; Mathieu et al., 2019; Mulcahy et al., 2014). Thirdly, the EV could adhere to the recipient cells, for example, via integrins, and mediate trans-shedding either on the cell surface of the same or neighbouring cells (Cardeñes et al., 2021; Kwok et al., 2021; Mathieu et al., 2019; Mulcahy et al., 2014). Interestingly, Cardeñes et al. recently showed that ADAM17-containing EV can adhere to recipient cells via direct interaction with integrin- $\alpha 5 \beta 1$ (Cardeñes et al., 2021; Trad et al., 2013). A similar interaction of ADAM17 and integrin- $\beta 1$ on the cellular level was shown in several studies before (Adriaenssens et al., 2004; Gooz, 2010; Gooz et al., 2012; Machado-Pineda et al., 2018). Focusing on tumour/microenvironment interactions, we showed a direct binding of ADAM17 to integrin- $\alpha 5 \beta 1$ of fibroblasts and breast cancer cells, which might thus also contribute to AREG shedding in the context of EV-associated ADAM17 (Trad et al., 2013).

As it is of major clinical interest how chemoresistance develops and gets maintained, we set up a cellular model to investigate if ADAM17-containing EV could play a major role in this context. To test if ascites-derived EV could restore the chemoresistant phenotype of chemosensitive ADAM17-deficient cells, we investigated whether ADAM17-containing EV derived from ascites of the chemoresistant patients are capable to proteolytically cleave substrates from ADAM17-deficient recipient cells. We could show that treatment of KO cells with EV strongly increased AREG release into culture supernatants compared to

unstimulated cells. Since ADAM17 substrates like AREG might also be present on EV, we assessed whether ADAM17 substrate shedding is dependent on the presence of recipient cells or also occurs in a cell-free system (Higginbotham et al., 2011). In the presence of recipient cells, AREG levels increased substantially, whereas hardly any AREG release was observed from EV in the absence of cells. This indicates that EV-associated ADAM17 mainly sheds its substrates from recipient cells rather than shedding substrates located on the EV membrane. To analyse the potential of chemoresistance-transfer through EV-associated ADAM17, the caspase activity of Igrov1-KO cells was measured after incubation with EV and subsequent cisplatin treatment. We showed that caspase activity was strongly reduced after incubation with ADAM17-containing EV. This provides strong evidence—given the limitation of cell-based models and limited access to patient material—that EV-associated ADAM17 can mediate chemoresistance in recipient cells and might serve as a model how resistance can occur and be maintained.

5 | CONCLUSION

Chemotherapeutic resistance is an essential clinical problem in OvCa; the role of EV contributing to this mechanism and potential ways to block intercellular transfer remain elusive. In the present study, we demonstrate that EV are released upon chemotherapeutic treatment and contain active ADAM17. ADAM17-bearing EV are highly abundant in ascites of OvCa cases, and EV numbers correlate with preceding chemotherapeutic treatment. Importantly, treatment of ADAM17-deficient cells with ascites-derived ADAM17-containing EV restored chemotherapy-resistance and substrate shedding in these cells. Consequently, the transport of ADAM17 throughout the peritoneal cavity to distant locations might foster formation of pre-metastatic niches and create a chemotherapy resistance-promoting microenvironment by substrate release and activation of downstream survival signalling. Thus, novel strategies should not only focus on the tumour itself but also on the microenvironment and consider EV as mediators and potential targets to overcome resistance transfer.

AUTHOR CONTRIBUTIONS

Gerrit Hugendieck: Formal analysis; investigation; methodology; supervision; validation; visualization; writing—original draft; writing—review and editing. **Marcus Lettau:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing. **Svenja Andreas:** Formal analysis; investigation; validation; writing—original draft. **Sabrina Neumann:** Formal analysis; investigation; writing—review and editing. **Natalie Reinhardt:** Formal analysis; investigation; writing—review and editing. **Philipp Arnold:** Formal analysis; investigation; writing—review and editing. **Franziska Theilig:** Formal analysis; investigation; writing—review and editing. **Lorenz Bastian:** Methodology; resources. **Christoph Rogmans:** Resources; validation; writing—review and editing. **Jörg P. Weimer:** Investigation. **Inken Flörkemeier:** Validation; writing—review and editing. **Daniela Wesch:** Investigation; writing—review and editing. **Norbert Arnold:** Resources; supervision. **Nicolai Maass:** Funding acquisition; writing—review and editing. **Ottmar Janssen:** Funding acquisition; Methodology; resources; supervision; writing—review and editing. **Dirk Bauerschlag:** Funding acquisition; project administration; resources; supervision; writing—review and editing. **Nina Hedemann:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing—original draft.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

GEOLOCATION INFORMATION

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