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RESEARCH ARTICLE

Monocyte derived large extracellular vesicles in polytrauma

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Abstract

Despite significant progress in the medical field, there is still a pressing need for minimal-invasive tools to assist with decision-making, especially in cases of polytrauma. Our team explored the potential of serum-derived large extracellular vesicles, so called microparticles/microvesicles/ectosomes, to serve as a supportive tool in decision-making in polytrauma situations. We focused on whether monocyte derived large EVs may differentiate between polytrauma patients with internal organ injury (ISS > 15) and those without. Thus, we compared our EV data to soluble biomarkers such as tumour necrosis factor alpha (TNF alpha) and Interleukin-8 (IL-8). From the blood of 25 healthy and 26 patients with polytrauma large EVs were isolated, purified, and characterized. TNF alpha and IL-8 levels were quantified. We found that levels of these monocyte derived large EVs were significantly higher in polytrauma patients with internal organ damage and correlated with the ISS. Interestingly, we also observed a decline in $AnnV^+CD14^+$ large EVs during normal recovery after trauma. Thus, inflammatory serological markers as TNF alpha and as IL-8 demonstrated an inability to discriminate between polytrauma patients with or without internal organ damage, such as spleen, kidney, or liver lacerations/ruptures. However, TNF and IL-8 levels were elevated in polytrauma cases overall when contrasted with healthy non-traumatic controls. These findings suggest that delving deeper into the potential of $AnnV^+$ large EVs derived from monocytes could highly beneficial in the managment of polytrauma, potentially surpassing the efficacy of commonly used serum markers.

KEYWORDS
biomarker, blood, extracellular vesicles, injury severity score, liquid biopsy, microvesicles, trauma, traumatology, triage

1 | INTRODUCTION

An increasing number of recent publications, including some of ours, have suggested that liquid biopsy markers could significantly contribute to assessing diseases, infections, cancer and polytrauma in a minimal-invasive way. Reflecting the state of the secreting cells liquid biopsy markers can be utilized to analyse molecular and cellular changes in the human body and obtain

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valuable information without the need for invasive procedures (Kilgour et al., 2020; Słomka et al., 2022). Polytrauma refers to multiple injuries sustained simultaneously, which can be challenging to assess accurately. Hence, blood born liquid biopsy biomarkers have the potential to detect specific molecular and cellular changes associated with polytrauma at an early stage, allowing for prompt intervention and treatment (Weber et al., 2022; Wu et al., 2021).

One of those exciting liquid biopsy markers might be extracellular vesicles (EVs). Since more than a decade, EVs, large and small EVs, had been associated with diseases (Urban et al., 2020; Yáñez-Mó et al., 2015) but only few dealt with traumatic injuries (Fröhlich et al., 2018; Groven et al., 2023; Kuravi et al., 2017; Weber et al., 2022; Weber, Henrich et al., 2023; Weber, Sturm et al., 2023). Large EVs, to which microvesicles (MVs) belong which may be termed earlier as ectosomes or microparticles (MPs) (Kornek et al., 2012; Willms et al., 2016), are typically plasma membrane derived nano-sized structures comprised of a lipid bi-layer mirroring the donor cell to some extent regarding surface antigens and cytoplasmic molecules as proteins, as miRNA, lncRNA, that are packed in those large EVs but missing cell nucleus (Yáñez-Mó et al., 2015). However, small and large EVs might have different pharmacokinetic behaviours in vivo. Here, we utilized monocyte-derived large EVs to exploit their use as liquid biopsy marker in individuals with polytrauma (ISS > 15) and compared the results to chemokines and cytokines at day 1 post trauma.

2 | METHODS

2.1 | Ethics—human specimens

The second LiBOD pilot study is a non-interventional and retrospective study in agreement to the Clinical Trials Directive (EU) 2001/20/EC and Clinical Trials Regulation (EU) NO 536/2014 and approved by the Ethics Committee of the responsible State Chambers of Medicine in Rhineland-Palatinate, Germany (ANr.:2020-15050). Informed consent was obtained from all patients or their legal representatives. The presented data is part of a study that has been registered on the International Clinical Trials Registry Platform through the German Registry for Clinical Trials (DRKS 00026025; https://drks.de/search/en/trial/DRKS00026025).

2.2 | Human study cohort

Trauma patients treated in the resuscitation bay of the German Armed Forces Central Hospital were included if the injury pattern yielded an Injury Severity Score (ISS) > 15 (i.e., polytrauma). Mechanisms of injury that led to the activation of the trauma team in the resuscitation bay were as follows, in accordance with Level 3 guidelines for the management of patients with severe or multiple injuries (Polytrauma Guideline Update Group, 2018): (i) penetrating injuries to the cervical and/or thoracoabdominal region, (ii) falling from a height of more than 1.5 m, (iii) traffic accident with frontal impact or intrusion of more than 50–75 cm or change in speed of delta > 30 km/h, and (iv) two-wheeler collision: as motorcycle, bicycle, E-bike, and E-Scooter.

Typically, polytrauma patients were excluded if (i) any doubts were given about the patient's or their legal representatives ability to consent or signs of dementia, (ii) patients were below 18 years of age, or (iii) patients were pregnant.

Polytrauma OD (ISS > 15) due to blunt (mainly) or open injury encompasses a variety of trauma patterns, including lacerations and organ rupture to internal parenchymal organs such as the liver, spleen, and kidney, severe lung contusions, aortic vascular dissections, amputation, and various types of bone fractures as summarised in Figure 1. The inclusion of amputation within the internal organ damage category is justified by its severity and the significant challenges it poses to the amputee in terms of blood loss and susceptibility to infection. Additionally, blood parameters as erythrocytes, thrombocytes and leucocytes levels besides medications and drugs given after admission are summarized in Figure 6.

The healthy control group comprised of individuals aged between 21 and 51 years with no history of trauma or known health issues (ISS = 0), with a similar gender distribution.

2.3 | Human cell lines

The cell line THP-1 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, #ACC16) was cultured in growth medium composed of RPMI-1640 GlutaMAX medium (Gibco by Life Technologies, Paisley, UK) supplemented with 10% (v/v) h.i. FBS (Gibco by Life Technologies, Paisley, UK) and 1% (v/v) penicillin-streptomycin (10,000 U/mL, Gibco by Life Technologies, Paisley, UK) in a humidified incubator.

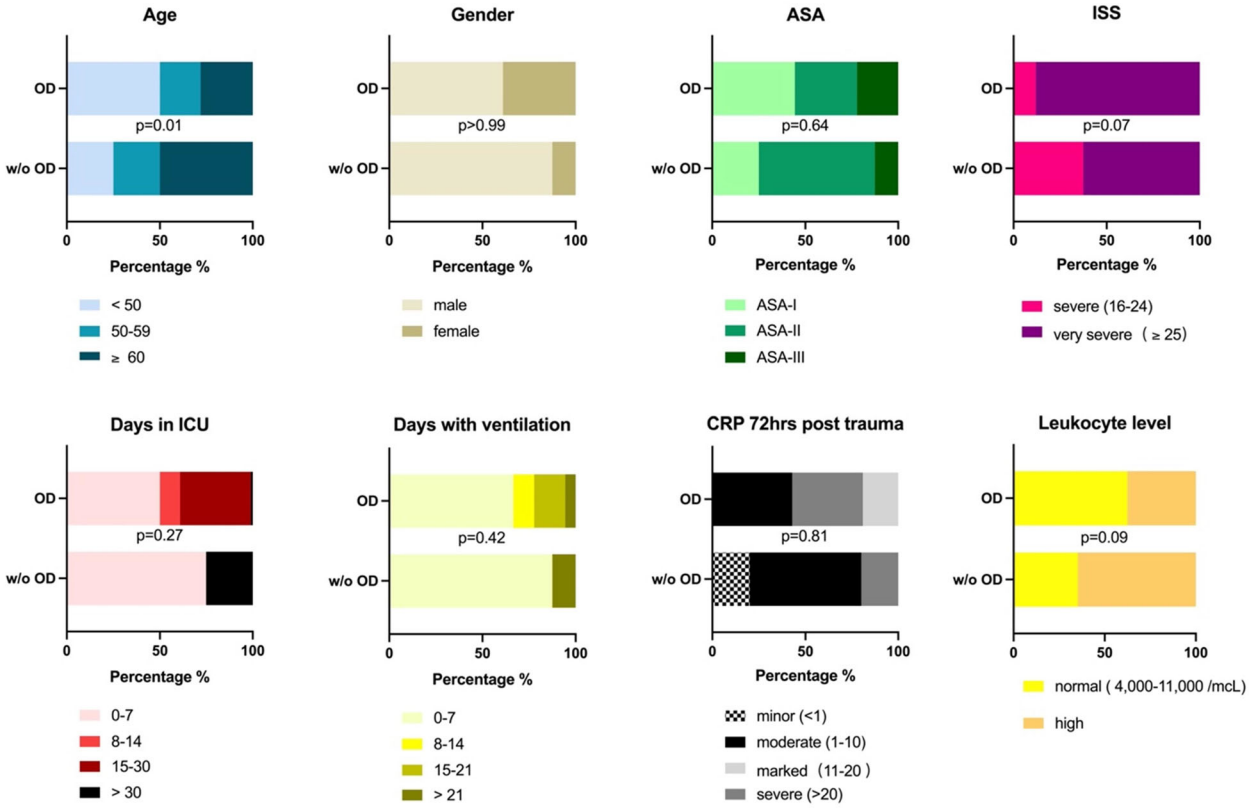

FIGURE 1 **Polytrauma (ISS > 15) Patient's demographics**. Overview of patient's parameters post trauma as age, gender, ASA classification, ISS, days in ICU, days with invasive ventilation, CRP values and leucocytes count. In total 26 polytrauma patients with internal organ damage (polytrauma OD, $n = 8$) and polytrauma patients without internal organ damage (polytrauma w/o OD, n = 18) and 25 healthy controls were enclosed in this retrospective human study. Polytrauma OD due to blunt (mainly) or open injury encompasses a variety of trauma patterns, including lacerations and organ rupture to internal parenchymal organs such as the liver, spleen, and kidney, severe lung contusions, aortic vascular dissections, amputation, and various types of bone fractures. The inclusion of amputation within the internal organ damage category is justified by its severity and the significant challenges it poses to the amputee in terms of blood loss and susceptibility to infection. The healthy control group comprised individuals aged between 21 and 51 years with no history of trauma or known health issues. Statistical significance was calculated taking advantage of the non-parametric Mann–Whitney-test, two-tailed. ASA, American Society of Anesthesiologists (ASA) score; ISS, Injury Severity Score.

2.4 | Differentiation and stimulation of THP-1 monocytes cells in vitro

THP-1 cells were differentiated in vitro according to our previously published protocol (Wang et al., 2023). In brief, THP-1 cells were differentiated into macrophage-like cells by stimulation with phorbol 12-myristate-13-acetate (PMA) (Sigma Aldrich, Missouri, USA; P1585) into M(PMA) or with PMA and lipopolysaccharide (LPS) into M (PMA & LPS) (Merck, Darmstadt, Germany, L3129). 5.0×10^5 THP-1 cells/mL were suspended in growth medium, supplemented with 200 nM PMA and incubated at 37°C, 5% CO_2 for 72 h. Subsequently the cells were maintained in growth medium for 5 days without PMA. If required THP-1 cells were further stimulated with 500 ng/mL LPS for 3 h (Wang et al., 2023) and subsequently kept in fresh growth medium for 72 h. The cellular identity was confirmed by cell surface staining and subsequent fluorescence-activated cell sorting (FACS).

2.5 | Cell surface staining for flow cytometric analysis of THP-1 human cell line

The surface marker analysis was performed according to our previously published protocol (Wang et al., 2023).Typically, 1.5×10^5 cells were suspended in FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Anti CD14 antibody (CD14 APC (170-078-099, Miltenyi Biotec) was added and titrated against their matching isotype (REA Control APC, 130-104-614, Miltenyi Biotec). 7-AAD (BD Pharmingen, New Jersey, USA, 559925) was used for dead cell exclusion. The cells were analysed on a BD FACSCanto™ II system (BD Biosciences, Heidelberg, Germany). Antibody details see Table 1.

TABLE 1 List of antibodies and isotypes used for cell- and cell derived large EV flow cytometric analysis. The antibodies were titrated against their matching isotype controls prior use and applied according to the manufacturers' instructions.

Antibody (Anti-human)	Conjugate	Catalogue#	Vendor	Conc. (µg/µL)	Dilution
CD14 REA	APC	170-078-099	Miltenyi Biotec	0.01	1:50
Isotype REA	APC	130-104-614	Miltenyi Biotec	0.02	1:100

TABLE 2 List of antibodies used for human derived large EV surface marker analysis. The antibody was added according to manufacturer's recommendations and titrated against the matching isotype control prior use.

Antibody (Anti-human)	Conjugate	Catalogue#	Vendor	Conc. (µg/µL)	Dilution
CD14 (mouse IgG2a)	VioBlue	130-094-364	Miltenyi Biotec	0.01	1:50
mouse IgG2a	VioBlue	130-113-839	Miltenyi Biotec	0.06	1:300

2.6 | Isolation of large EVs from cell culture supernatant

Supernatant from THP-1, M (PMA) or M (PMA & LPS) cells was centrifuged at 300 × *g* for 5 min at 4°C to remove cells, cell debris, and larger apoptotic bodies. The supernatant was further purified by centrifugation at 2000 × *g* for 30 min, followed by centrifugation at 20,000 × *g* for 1 h at 4°C. Resulting large EV-enriched pellets were suspended in filtered PBS, pH 7.4 (0.22 µm) (Merck Millipore, Boston, USA) and stored at −80°C until usage.

2.7 | Isolation of serum from human blood

Blood sampling was performed according to the guidelines provided by the European Liquid Biopsy Society (ELBS) and followed the ISO 20186-1:2019 standard for pre-examination processes for venous whole blood in molecular in vitro diagnostic examinations. The sampling procedure was conducted on polytrauma patients with an ISS greater than 15. A total of 10 mL of whole blood was collected from either a venous or arterial access site after the traumatic event in Clotting Activator S-Monocuvettes (7.5 mL, Sarstedt, Nümbrecht, Germany). Blood samples were obtained upon arrival as soon as possible (day 0), at 24 h (day 1) up to 7 days (day 7) post trauma. Full blood was centrifuged at 3000 × *g* for 30 min at room temperature (RT). Sera were aliquotted and stored at −80°C to ensure a minimal number of melting and freezing cycles. Of note, human whole blood was always centrifuged at RT to prevent platelets activation.

2.8 | Isolation of large EVs and subsequent FACS analysis

All large EV isolation and staining procedures were performed according to previously established and published protocols (Julich-Haertel et al., 2017; Lukacs-Kornek et al., 2017). Briefly, 1 mL patient serum was successively centrifuged at 2000 × *g* for 30 min and 20,000 × *g* for 60 min. Resulting large EV-enriched pellets were suspended in filtered PBS, pH 7.4 (0.22 µm) (Merck Millipore, Boston, USA) and stored at −80°C until usage. In agreement with MISEV2018, by us isolated large EV had been characterised according their size and protein composition previously and published (Urban et al., 2020; Wang et al., 2023). Representative electron microscopy (EM) images of large EVs, at that time called S10-MPs by us can be found elsewhere (Kornek et al., 2011). Large EVs were stained with Annexin V (AnnV)-FITC (130-093-060, Miltenyi Biotec) and subsequently stained with anti-CD14-VioBlue (130-094-364, Miltenyi Biotec) (Table 2). For exact staining procedure details please refer to our book chapter on "Multi-Surface Antigen Staining of Larger Extracellular Vesicles" (Lukacs-Kornek et al., 2017). To avoid non-specific antibody binding, Fc receptors on large EVs were blocked with FcR Blocking Reagent (eBioscience™, San Diego, CA, USA) and 0.05% BSA. Used BSA blocking solution was centrifuged at 20,000 × *g* prior to FACS to avoid artefacts due to aggregation. All solutions except antibody containing solutions were centrifuged or filtered (0.2 µm) prior their use to remove contaminations such as possible protein aggregates or particles with similar size as larger EVs. All human large EV samples were analysed using the BD FACSCanto™ II system (BD Biosciences) (Figure 2) and MACSQuant Analyser (Miltenyi Biotec) (Figure 3). Thresholds were set with 200, 500 and 1000 nm green fluorescent beads (F13839, Invitrogen—Waltham, Massachusetts, U.S.).

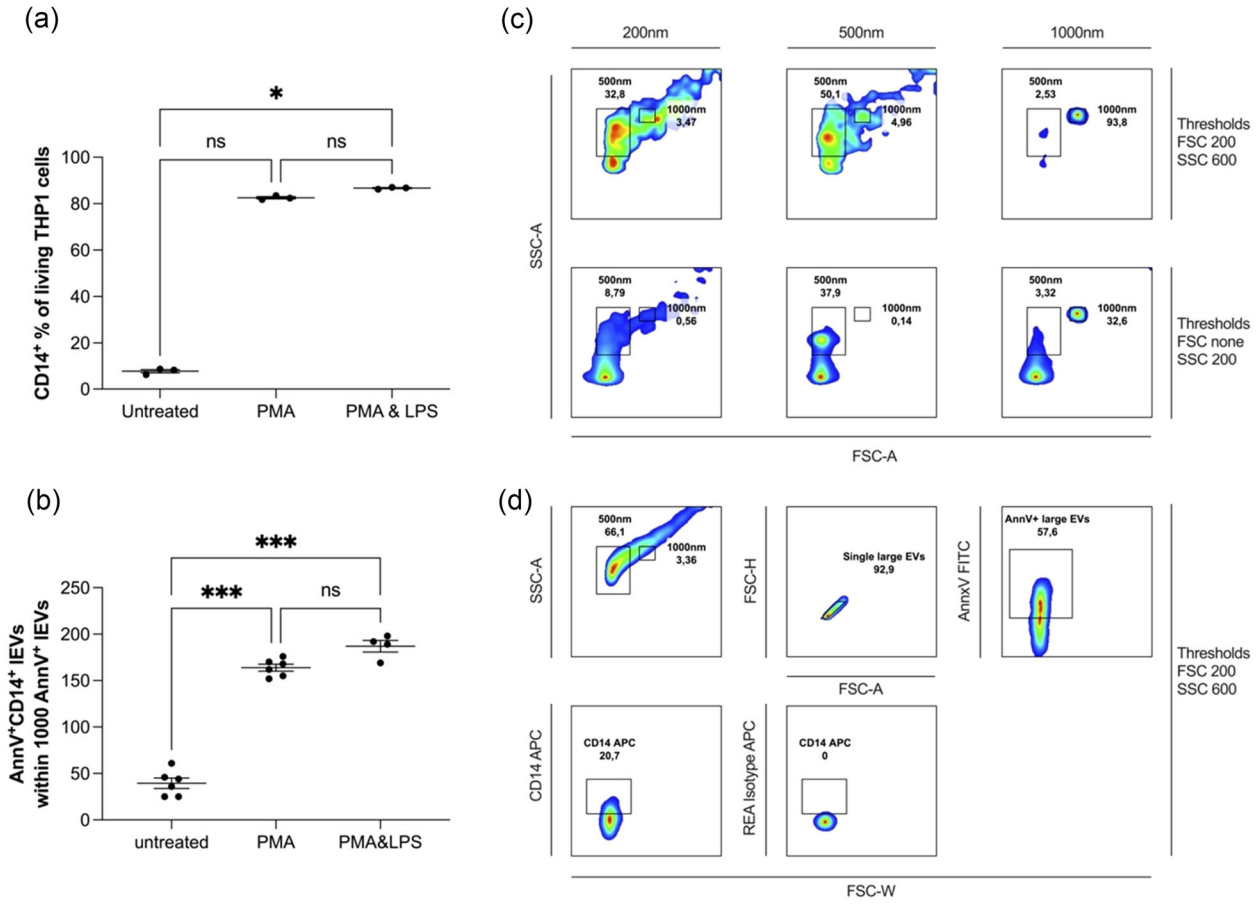

FIGURE 2 **Antibody validation on cultured THP-1 cells for EV detection and general used large EV gating strategy**. (a) In vitro human THP-1 monocyte cells were treated with PMA alone or PMA & LPS (each $n \geq 3$), increasing CD14 expression. (b) Expression of CD14 on surface of AnnV$^+$ large EVs (lEVs) derived from non-stimulated THP-1 cells and from PMA alone or PMA & LPS stimulated THP-1 cells. AnnV is used as a general large EV marker. Values given as mean with SEM. Three column statistical analysis was done by Kruskal–Wallis test including Dunn's post hoc test for multiple comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (c) Thresholds testing for cell culture and serum derived large EV detection and gating. Prior use of flow cytometry gates sensitivity of used flow cytometer was set taking advantage of 1000, 500 and not detectable 200 nm beads. (d) Gating strategy for the detection of CD14-APC on AnnV$^+$ large EVs controlled by matching REA isotype control.

2.9 | TNF alpha and IL-8 ELISA—serum samples

Quantification of tumour Necrosis Factor alpha (TNF alpha) and Interleukin-8 (IL-8) in samples was performed by using enzyme-linked immunosorbent assays (ELISA) (Human TNF-alpha Quantikine ELISA Kit, Catalog #: DTA00 and Human IL-8/CXCL8 Quantikine ELISA Kit, Catalog #: D8000C, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.10 | Statistical analysis

All human data are presented as medians with 95% CI (Figures 3 and 5). In vitro data is given in means with the standard error of mean (SEM). Since n is not even among cohorts/groups and fairly below $n = 200$ and skewness and kurtosis are sometimes above 1.0, hence we assumed a non-parametric approach in agreement with (Fagerland, 2012). Statistical analysis of at least three groups/cohorts was done using the Kruskal–Wallis test including Dunn's post hoc test for multiple comparisons. Thus, respective AUROC, sensitivity, specificity, and associated cut-off values were calculated. The differences between two independent experimental subsets were determined using a two-tailed paired *t*-test (Figure 4a) otherwise non-parametric Mann–Whitney-test was done, two-tailed as indicated. The Spearman (r_{Sp})correlation coefficient was calculated to measure the linear correlations between the two sets of data. Statistical results were considered significant if the *p*-value was <0.05. The experimental strength was calculated using a post hoc analysis (G*Power Version 3.1.9.6). For Day 0 (Figure 3b): Effect size f was calculated from means and SD sigma the average standard deviation (SD) of the groups, $f = 0.3522168$ for $N = 36$ and three groups. α err prob was set to

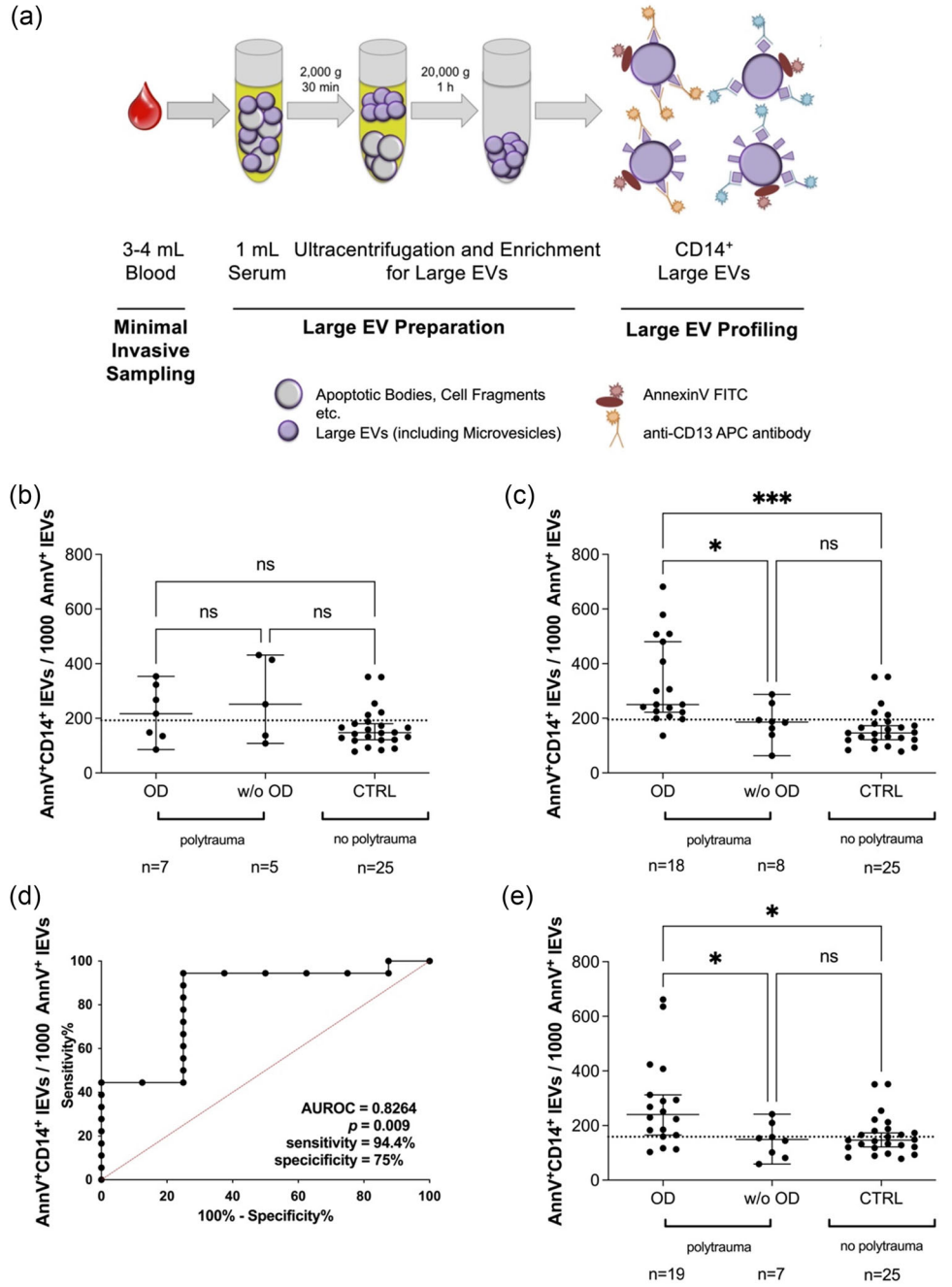

FIGURE 3 **Semi quantification of human serum derived AnnV$^+$CD14$^+$ large EVs**. (a) Workflow SOP for isolation of large EVs (lEVs) from 1 mL of human serum collected from polytrauma patients (ISS > 15, at day 0, at day 1 and day 7 post trauma). (b) AnnV$^+$CD14$^+$ large EVs counts in small EVs in polytrauma (ISS > 15) with internal organ damage (polytrauma OD) versus polytrauma (ISS ≥ 15) without internal organ damage (polytrauma w/o OD) upon admission (day 0). (c) AnnV$^+$CD14$^+$ large EVs counts in polytrauma (ISS > 15) polytrauma OD versus polytrauma w/o OD (ISS > 15) 24 h post trauma (day 1). (d) Day 1, corresponding AUROC, sensitivity, specificity, and cut-off value, respectively. (e) AnnV$^+$CD14$^+$ large EVs counts in small EVs in polytrauma (ISS ≥ 15) polytrauma OD versus polytrauma w/o OD (ISS ≥ 15) 7 days post trauma (day 7). Values given as median with 95% CI. Three column statistical analysis was done by Kruskal–Wallis test including Dunn's post hoc test for multiple comparisons (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; not significant, n as indicated). Horizontal dotted line in panels B, C and E indicates calculated associated cut-off (summarized in Table 3).

0.05 resulting in 0.422 (1-β err prob) for day0 (*F* tests—ANOVA: Fixed effects, omnibus, one-way, post hoc); Day 1 (Figure 3c): $f = 0.7997084$ for $N = 50$ and three groups. α err prob was set to 0.05 resulting in 0.999 (1-β err prob) for day0 (*F* tests—ANOVA: Fixed effects, omnibus, one-way, post hoc). Day 7 (Figure 3e): $f = 0.596751$ for $N = 51$ and three groups. α err prob was set to 0.05 resulting in 0.967 (1-β err prob) for day0 (*F* tests—ANOVA: Fixed effects, omnibus, one-way, post hoc).

FACS data were analysed using FlowJo 10 for MAC OSX (Tree Star Inc., Ashland, USA). Statistical analysis was performed and figures were created using GraphPad Prism 10 (GraphPad Software Inc., La Jolla, USA).

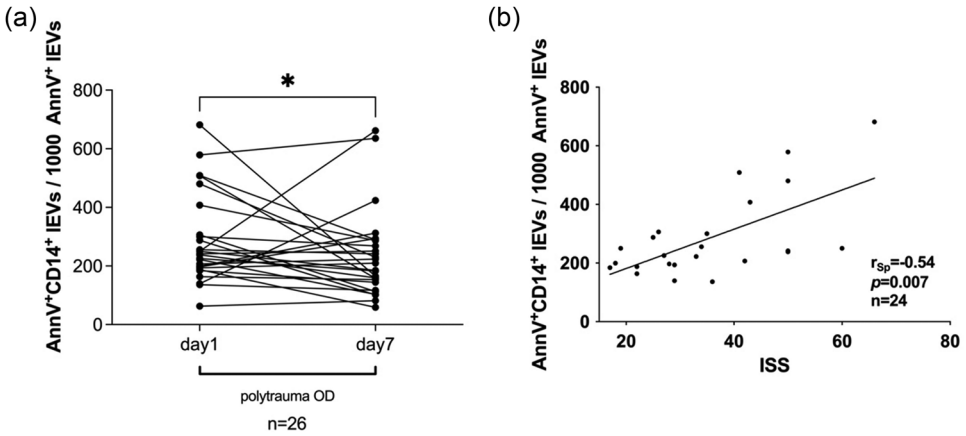

FIGURE 4 **AnnV$^+$CD14$^+$ large EVs kinetics at day 1 and day 7 in polytrauma (ISS > 15) and correlation with the Injury Severity Score (ISS).** (a) depicted are pairs of AnnV$^+$CD14$^+$ large EVs (lEVs) measured at day 1 and day 7 of enrolled polytrauma (ISS > 15). Values given as median. Statistical significance was assessed by two tailed paired *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (b) Spearman (r_{sp}) correlation between percentages of AnnV$^+$CD14$^+$ large EVs and ISS ($n = 24$). p and r_{sp} values as indicated.

3 | RESULTS

3.1 | CD14 surface marker expression on large EV donor cells

Prior to large EV harvest, large EV donor cells were checked for an increased CD14 expression by flow cytometry after being exposed to PMA or PMA & LPS (Figure 2a). Differentiated THP-1 cells towards macrophages (M) and their large EVs served as a positive control for antibody testing of CD14 on large EVs.

3.2 | Large EV populations are present in polytrauma and multiple trauma patients

According to the MISEV2018 guidelines (Théry et al., 2018), the isolated large EVs were characterized by determining their particle count, size distribution and the presence of typical large EV surface markers or the absence of typical markers associated with small EVs or cell debris. The detailed procedure has been previously documented in our published studies. Of note, we detected an increased CD14 expression on AnnV$^+$ large EVs derived from M (PMA) and M (PMA & LPS) cells compared to naive THP-1 cells (Figure 2b). The AnnV$^+$CD14$^+$ large EVs were utilized as positive controls in conjunction with sizing beads of 200 nm, 500 nm, and 1,000 nm to establish gating parameters and appropriate thresholds for flow cytometric analysis of large EVs derived from human serum.

3.3 | Threshold and gating strategy for FACS based analysis of large EVs

Figure 2c depicts how the FSC and SSC gate for large events were set according to beads and various threshold settings. Exemplarily, two different threshold settings are depicted in Figure 2c. The upper horizontal panels show the acquisition of 200, 500 and 1000 nm beads with a threshold setting of FSC 200 and SSC 600. Beads with a 200 nm diameter were not detectable, compared to 500 and 1000 nm beads. Lower horizontal panels represent threshold of FSC 0 and SSC 200 associated with an unknown event population which appears to be independent to the used bead size. In subsequent experiments, the flow cytometer BD FACSCanto™ II system was configured with FSC 200 and SSC 600 parameters. It is important to highlight that the settings of the flow cytometer are unique to each instrument and need to be adjusted accordingly. A machine-specific gate identified at 500 nm was employed to acquire large EVs in FSC and SSC with a size approximating 500 nm for further analysis.

Figure 2d depicts used gating strategy for in vivo derived large EVs from human specimens gating first on approximately size of around 500 nm with indicated thresholds of FSC 200 and SSC 600. Followed by a single particle gate excluding particles aggregates. Next, gating on Annexin V (AnnV) positive large EVs. At last the CD14 APC gate is applied according to the corresponding REA APC isotype control.

3.4 | Monocyte derived large EVs in polytrauma at day 0, day 1 and day 7 post trauma

Human polytrauma large EV specimens (ISS > 15) were divided into two cohorts. The first cohort resembles patients with polytrauma with parenchymal organ damage named 'polytrauma OD'. The second cohort without parenchymal organ damage is called 'polytrauma w/o OD'. The latter cohort included typically polytrauma patients with bone fractures. As negative controls without any trauma and polytrauma served healthy control group (CTRL).

Figure 3b,c and e depicts $AnnV^+CD14^+$ large EV numbers per measured 1000 $AnnV^+$ large EVs representing a semi-quantitative numeration. Upon admission at the ER (day 0, median: 216.9, Figure 3b) $AnnV^+CD14^+$ large EVs were not significantly elevated compared to polytrauma w/o OD (median: 251.9) and to the healthy control group (median: 147.4).

At day 1, defined as 24–28 h post trauma (Figure 3c), $AnnV^+CD14^+$ large EVs polytrauma OD (median: 250.3) were significantly superior by 1.34 times compared to polytrauma w/o OD (median: 186.2) and significantly increased by 1.7 times compared to healthy controls (median: 146.6). The calculated cut-off between polytrauma OD and w/o OD was 195.3, associated with an AUROC of 0.8264 ($p = 0.009$) and a sensitivity of 94.44% and specificity of 75.00% (Figure 3d and summarised in Table 3).

At day 7 post trauma (Figure 3e), $AnnV^+CD14^+$ large EVs polytrauma OD (median: 240.1) were still significantly elevated by 1.6 times compared to polytrauma w/o OD (median: 148.9) and significantly still increased by 1.6 times compared to healthy controls (median: 146.6). Thus, no significant difference could be observed between polytrauma w/o OD (median: 148.9) and healthy controls (median: 146.6). At day 7 the sensitivity between polytrauma OD and w/o OD was reduced to a sensitivity of 83.33% and specificity remained at 75%. The calculated cut-off was now lowered to 158 $AnnV^+CD14^+$ large EV numbers per measured 1000 $AnnV^+$ large EVs (AUROC: 0.8264, $p = 0.009$).

3.5 | Monocyte derived large EVs decrease at day 7 post trauma

Paired statistical analysis was done to assess a possible decline of $AnnV^+CD14^+$ large EV in polytrauma disregarding parenchymal organ damage from day 1 to day 7. Figure 4a depicts a significant decline from median of 240 to median of 197.4 $AnnV^+CD14^+$ large EVs. However, we noticed that some values increased, likely associated with patient's condition as developing pulmonal worsening as systemic inflammatory response syndrome (SIRS), or as acute respiratory distress syndrome (ARDS).

3.6 | Correlation between ISS and $Annv^+CD14^+$ large EVs

The statistical correlation was examined between measured $AnnV^+CD14^+$ large EVs and the ISS. Spearman correlation was chosen because it describes the monotonic relationship between variables (Rovetta, 2020). $AnnV^+CD14^+$ large EVs correlated with $r_{Sp} = 0.54$.

3.7 | Soluble inflammatory chemokines and cytokines

While monocyte derived large EVs were shown to differentiate between polytrauma OD and w/o OD at day 1, next a likely association of known trauma markers such as the inflammatory chemokines and cytokines TNF alpha and IL-8 were exploit in our polytrauma cohort. The TNF alpha and IL-8 did not differentiate significantly between polytrauma OD and w/o OD at day 1 (TNF alpha, polytrauma OD: median: 1.717 [pg/mL], w/o OD: 2.061 [pg/mL]; healthy CTRL: 1.125 [pg/mL]; IL-8 polytrauma OD: median: 41.67 [pg/mL], w/o OD: 64.54 [pg/mL], healthy CTRL: 9.146 [pg/mL]). However, both markers were elevated in polytrauma w/o OD compared to healthy controls (Figure 5a and b).

4 | DISCUSSION

The aim of our study was to investigate whether circulating monocyte derived large EVs may differentiate between polytrauma OD and polytrauma w/o OD and being superior to experimental blood born soluble protein markers, thereby aiding in the prevention of over- and under-triage, as well as potential misinterpretation of polytrauma cases (Schellenberg et al., 2019). Hence, it offers insights into potential constraints associated with monocyte-derived large EVs.

In many Western countries, access to high-quality trauma diagnostics and care is readily available within minutes. However, in exceptional situations such as major conflicts, mass-casualty incidents or global pandemic event, there is a crucial need for an objective trauma assessment to inform decision-making processes and to address the rescue chain or amend the management (e.g., damage control surgery indication, ICU care or transfer to higher-level trauma centres) accordingly (Hirshberg et al., 2001;

TABLE 3 Summarized data FACS of AnnV$^+$CD14$^+$ large EVS in polytrauma including associated SEM, percentage change and statistics at day 1.

Large EV	Median polytrauma OD	SEM	Median polytrauma w/o OD	SEM	Fold change	*p*-value	Cut-off	AUROC (*p*-value)	Sensitivity (%)	Specificity (%)
AnnV$^+$CD14$^+$	250.3	36.98	186.2	24.28	1.34	*	195.3	0.8264 ($p = 0.009$)	94.44	75.00

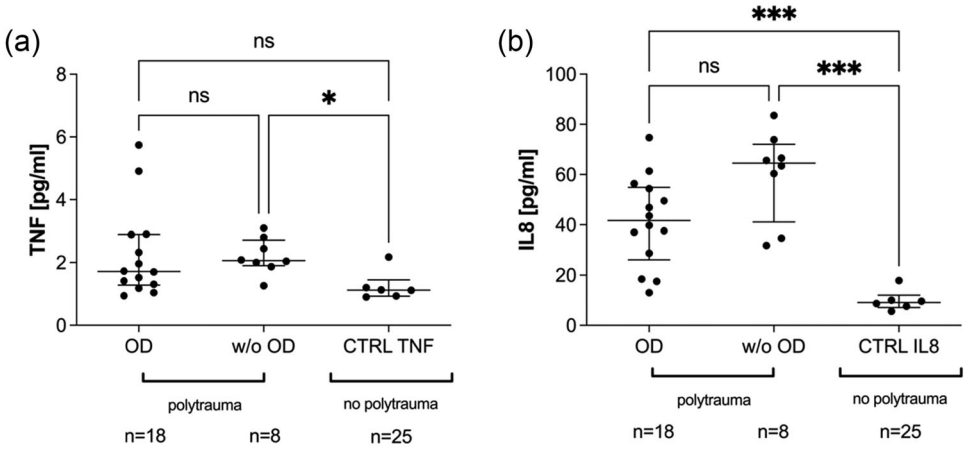

FIGURE 5 **Quantification of representative monocyte/macrophages derived inflammatory cytokine and chemokine at day 1**. (a) Tumour Necrosis Factor alpha (TNF alpha) enzyme-linked immunosorbent assays (ELISA) data in polytrauma (ISS > 15) with internal organ damage (polytrauma OD) versus polytrauma (ISS > 15) without internal organ damage (polytrauma w/o OD) and healthy non-trauma control subjects (CTRL TNF) day 1 post trauma. (b) Interleukin-8 (IL-8) ELISA data in polytrauma (ISS > 15) polytrauma OD versus polytrauma w/o OD (ISS > 15) 24 h post trauma (day 1). Three column statistical analysis was done by Kruskal–Wallis test including Dunn's post hoc test for multiple comparisons, data is given as medians with 95% CI (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: not significant, n as indicated).

Sigfrid et al., 2020; Thomson et al., 2023). This could mend to provide airborne- or advanced ground MedEvac (Medical Evacuation) (Forrester et al., 2019). More effective and personalized medical interventions, reduced risks of over or under-triage, and overall advancements in healthcare delivery (Newgard et al., 2013).

That would require a novel biomarker that should be capable to deliver precise patients status information at the closest time point post trauma (point-of-care diagnostic). In our large EV study we could not detect a trend that monocyte derived could differentiate between polytrauma with parenchymal organ damage as liver-, spleen rupture and polytrauma without organ damages at day 0. Day 0 samples were taken in the resuscitation bay of the German Armed Forces Central Hospital and start of maximal care.

To distinguish between large EVs and other particles like lipoproteins an AnnV staining was used for the membrane protein phosphatidylserine. In general, it is known that this marker is present on the outer leaflet of the large EV bilayer (Arraud et al., 2015; Perez et al., 2023) and is absent on lipoproteins (Dashti et al., 2011). AnnV large EVs may be released from living cell but from apoptotic cells as well during the early phase of apoptosis.

Our study did not specifically target the differentiation between large EVs derived from living or apoptotic monocytes/macrophages. In cases of sterile and non-sterile inflammation, such as in significant trauma, like polytrauma, there is a rapid recruitment of monocytes from the bloodstream which converse into macrophages (Peiseler & Kubes, 2018). Furthermore, macrophages are recruited directly from the perilesional region around the injured organ (Kratofil et al., 2017). Some of those may undergo a slow phenotypic conversion to become tissue-resident macrophages (Mu et al., 2021). We hypnotise that activation of monocytes and macrophages would potentially result in an increased presence of $CD14^+$ EVs in general. Additionally, the injury resolution process is typically associated with apoptosis of the majority of recruited/inflammatory monocytes at the late inflammatory phase due to their short lifespan (Mu et al., 2021), very likely boosting $CD14^+$ EV numbers. In fact, elevated $CD14^+$ EV small EV numbers were reported in polytrauma in human and porcine (Wang et al., 2023). Overall, the identification of large EVs derived from monocytes and macrophages could serve as an important initial marker in polytrauma.

Despite of our reasoning, $AnnV^+CD14^+$ large EVs failed to differentiate between polytrauma w/o OD, polytrauma OD and CTRL at day 0, monocyte derived $CD9^+CD14^+$, or $CD63^+CD14^+$ and $CD9^+CD61^+CD14^+$ small EV were capable to address that within 24 h post trauma as published by us (Wang et al., 2023). Recalling known differences between large and small EVs, and assuming that most large EV are actually cell membrane derived microvesicles and small EVs are mainly exosomes, two major differences are given. 1st, biogenesis and 2nd size/volume (Colombo et al., 2014; Théry et al., 2018). This could lead to differences in generation and clearance (Ayers et al., 2015; Yáñez-Mó et al., 2015). Exosomes are stored prior release 'pre-made' in multivesicular bodies and are formed through inward budding of endosomal membranes giving rise to intracellular multivesicular bodies that later fuse with the plasma membrane, releasing the exosomes to the extracellular space including body fluids (Théry et al., 2018). In contrast, the process of releasing microvesicles is more straightforward compared to exosome secretion, as it entails budding from the plasma membrane without involving exocytosis (Tricarico et al., 2017). If exosomes are the first type of EVs released in various biological processes, while 'pre-made' is still unexplored and that line of argumentation need further support. However, it was shown that cell-specific EV secretion rate estimates were highest for monocytes (45 ± 21 EVs/cell/min) and lowest for erythrocytes (3.2 ± 3.0) × 10−3 EVs/cell/min) as seen in healthy human subjects (Auber & Svenningsen, 2022).

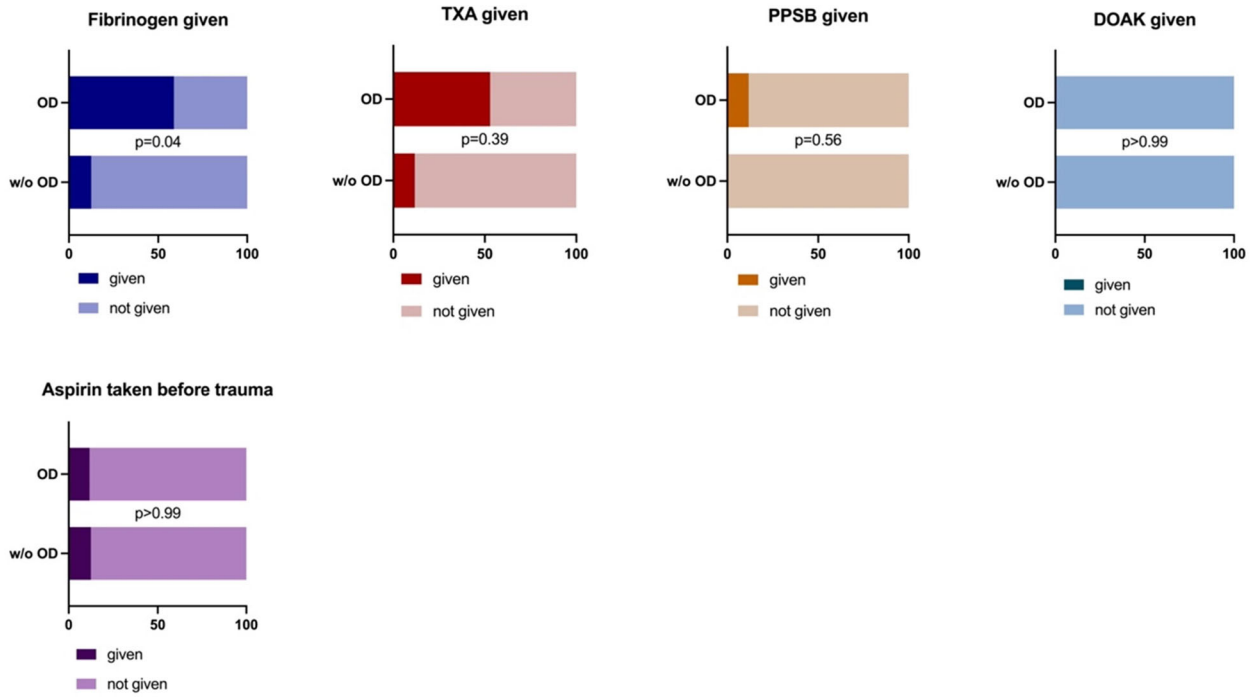

FIGURE 6 **Medications**. Summary of medications administered, including Aspirin as pre-medication used by patients with polytrauma OD and polytrauma w/o OD on a daily basis before the trauma event. Statistical significance was determined using the non-parametric Mann–Whitney test with a two-tailed analysis. The abbreviations used are as follows: TXA for tranexamic acid, PPSB for prothrombin complex concentrate, and DOAK for direct oral anticoagulants.

At day 1 post trauma $AnnV^+CD14^+$ large EVs could differentiate between polytrauma with parenchymal organ damage. Seven days post trauma clearance was observed except if patient circumstance may worsen. A clear correlation between worsening and higher levels at day 7 post trauma has to be elucidated separately in an additional study.

Despite of the discussed limitations of monocyte derived large EVs compared to monocyte derived small EVs, we might argue that soluble inflammatory chemokines as TNF alpha and cytokines as IL-8 should differentiate between polytrauma with parenchymal organ damage at day 1 post trauma compared to EV data. But we did not observe that TNF alpha and IL-8 did not differentiate significantly between polytrauma OD and polytrauma w/o at day 1 post trauma in this study. This is somehow surprisingly, while TNF alpha and IL-8 are not exclusively but also macrophage ($CD14^+$) derived (Baer et al., 1998; Baggiolini et al., 1989). This might lead us again to the discussion if kinetics of releasing 'pre-produced' EVs as exosomes and large EVs as microvesicles are different to TNF alpha and IL-8? Nevertheless, IL-8 was elevated in polytrauma with or without parenchymal organ damage compared to healthy controls going in line with the available literature (Baucom et al., 2023; Namas et al., 2016).

This pilot study was not designed to provide these answers, but may provide the ground for further research on EVs in polytrauma. Notably, monocyte derived large EVs did correlate with ISS which is scored during the anamnesis of the trauma patient with no further influence on provided care. This doesn't imply that large EV, preferably microvesicles, are out of game. It means other microvesicle populations might be more suitable addressing same or different questions around trauma care and diagnosis. Moreover, hypothetically the larger surface area of microvesicles or large EVs compared to exosomes or small EVs allows for detection of a higher number of antigens to be accessible concurrently. This characteristic enables antibody combinations containing more than three different antibodies to potentially bind to large EVs, aiding in the identification of specific rare cell populations, as defined by a unique antibody combination, that release these large EVs. The detection of four to five different antibodies simultaneously on large EVs is do able as shown by us (Julich-Haertel et al., 2017), on small EV limited to max. two to three. In spite of these technical questions, the EV content might be from even bigger interest. Recently, it was shown that large EV-transported miRNAs have a clear systemic involvement in multiple trauma, that could potentially provide additional information regarding the underlying injury pattern and expected inflammatory response (Groven et al., 2023).

We cannot exclude the possibility of coagulation boosters affecting the release of large EVs, especially considering that some of these medications were administered to polytrauma patients (Figure 6). In our polytrauma study focusing on small EVs, we did not detect similar variations; there were no apparent connections between medications and the quantity of monocyte-derived small EVs (Wang et al., 2023). Therefore, we propose that coagulation boosters such as Fibrinogen and others warrant further investigation regarding their potential impact on EVs themselves.

5 | CONCLUSION

Our study with the focus on monocyte derived large EVs supports our previous finding that monocyte derived EVs may bear the potential to assist in quantifying polytrauma whether internal parenchymal organs as the liver, spleen, lung are damaged or not. EVs could potentially serve as a biomarker that—if taken after arrival in hospital care—may support decision making to avoid triage situations. However, the exact time point and EV population that may deliver the most reliable data must be further elucidated. Thus, we delivered first hints that known investigated inflammatory cytokine as TNF alpha and chemokine as IL-8 at day 1 may fall short on differentiating polytrauma with organ damage from polytrauma without.

AUTHOR CONTRIBUTIONS

Aliona Wöhler: Conceptualization (supporting); data curation (supporting); formal analysis (supporting); investigation (supporting); project administration (supporting); resources (lead); validation|(equal); visualization (supporting); writing—review and editing (equal). **Sabine K. Gries**: Data curation (equal); investigation (equal); methodology (equal); project administration (equal); validation (equal). **Rebekka J.S. Salzmann**: Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); project administration (supporting); writing—review and editing (supporting). **Christina Krötz**: Investigation (equal); Methodology (equal); Writing—review and editing (equal). **Bingduo Wang**: Data curation (supporting); formal analysis (supporting); investigation (equal); methodology (equal); writing—review and editing (supporting). **Paula Müller**: Data curation (supporting); project administration (supporting); visualization (supporting); writing—review and editing (supporting). **Angelina Klein**: Data curation (supporting); project administration (supporting); resources (equal); writing—review and editing (supporting). **Ingo G.H. Schmidt—Wolf**: Project administration (supporting); resources (supporting); software (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Sebastian Schaaf**: Formal analysis (supporting); investigation (supporting); project administration (supporting); resources (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Robert Schwab**: Funding acquisition (equal); project administration (supporting); resources (equal); supervision (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Veronika Lukacs—Kornek**: Conceptualization (supporting); formal analysis (supporting); funding acquisition (equal); investigation (supporting); methodology (supporting); project administration (supporting); resources (supporting); software (supporting); supervision (supporting); validation (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Arnulf G. Willms**: Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **Miroslaw T. Kornek**: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); supervision (equal); writing—original draft (lead); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests to disclose.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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