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Original Article

ORIGINAL ARTICLE

Intradialytic cardiovascular injury is lowest in high-volume haemodiafiltration: a randomized cross-over trial in four intermittent dialysis strategies

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ABSTRACT

Background. Intradialytic hypotension (IDH) and subsequent tissue damage may contribute to the poor outcome of chronic haemodialysis (HD) patients. While the IDH-incidence is lower in high-volume haemodiafiltration (HV-HDF) than in standard HD (S-HD), survival is better in HV-HDF. Tissue injury, as measured by extracellular vesicle (EV)-release, was compared between four modalities.

Methods. Forty chronic patients were cross-over randomized to S-HD, cool-HD (C-HD), low-volume HDF (LV-HDF), and HV-HDF. Blood pressure was recorded every 15 minutes. EVs from circulating blood-cell-elements (bio-incompatibility-related) and cardiovascular (CV) tissues (CV-related), were measured before and after dialysis. The influence of modalities and IDH on the rate of change of EVs was assessed. Both crude and haemoconcentration-adjusted analyses were performed.

Results. Leukocyte and erythrocyte-derived EVs increased in all modalities. Platelet-derived EVs increased more in LV-HDF and HV-HDF (68.4 respectively 56.1 $\times$ 10^6/ml) than in S-HD (27.5 $\times$ 10^6/ml), *P* values for interaction were <.01 respectively .06. Endothelial-derived CD144$^+$ (2.3 $\times$ 10^6/ml in HV-HDF and 9.8 $\times$ 10^6/ml in S-HD) and cardiomyocyte-derived Connexin-43$^+$ (12.0 respectively 31.9 $\times$ 10^6/ml) EVs increased less in HV-HDF than in S-HD (*P* for interaction .03 respectively .06). Correction for haemoconcentration attenuated all changes, although the increase in platelet-derived EVs remained significant in LV-HDF and HV-HDF, and CD144$^+$ and Connexin-43$^+$ EVs increased most in S-HD. EV release was similar in patients with varying IDH susceptibility and in sessions with and without IDH.

Conclusions. Most EVs increase during HD and HDF. Regarding platelet-derived EVs, HDF appears less biocompatible than HD. Considering CV-related EVs, tissue injury seems less pronounced in HV-HDF. The finding that EV release is IDH-independent needs confirmation.

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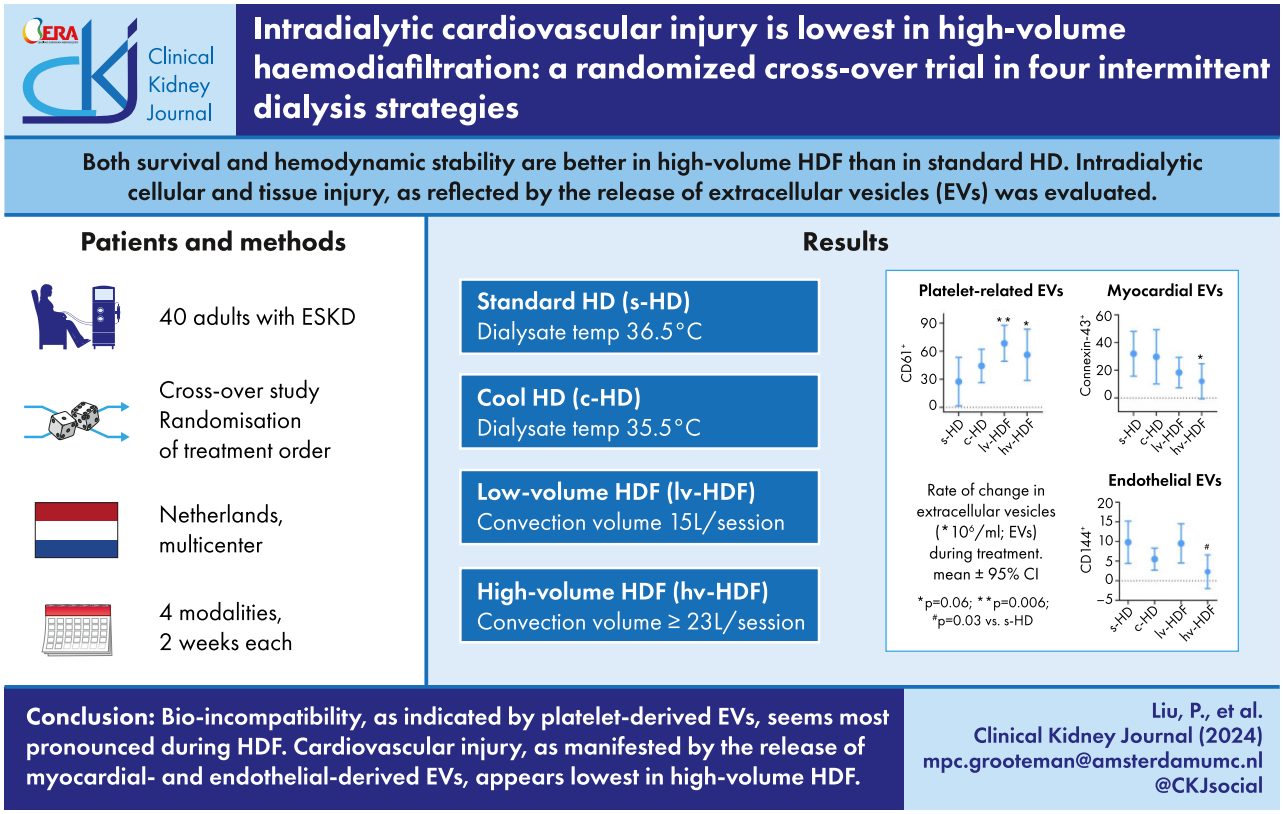
GRAPHICAL ABSTRACT



Keywords: cross-over study, extracellular vesicle, haemodiafiltration, haemodialysis, hypotension

KEY LEARNING POINTS

What was known:

- Extracellular vesicles (EVs) are key mediators of intercellular communication in tissue injury.
- Conflicting data exist regarding the effect of dialysis on EV concentrations.

This study adds:

- Most EVs increase during dialysis.
- While the increase in platelet-derived EVs is higher in haemodiafiltration (HDF) than in standard haemodialysis (S-HD), the rise in cardiovascular-(CV)-tissue-related EVs is lowest in HDF with a high convection volume (HV-HDF).
- EV-increments seem largely independent of intradialytic hypotension (IDH).

Potential impact:

- These findings indicate that intradialytic bio-incompatibility-related injury is greatest in HDF and CV-related tissue injury is lowest in HV-HDF.
- Studies on the kinetics of HD(F)-induced EVs are required.

INTRODUCTION

Despite important advances in the last decades, the outcome of haemodialysis (HD) patients remains poor [1–3]. Although vital for survival, HD itself has serious drawbacks, such as rapid osmolality and electrolyte shifts [4]. In addition, the removal of fluid may evoke recurrent drops in blood pressure (BP) [5].

Intradialytic hypotension (IDH) is the most common complication of HD [6]. Depending on its definition, the incidence ranges from 4% to 69%/session [7, 8]. Briefly, IDH occurs when removal of fluid from the blood and refill from the interstitial space do not occur in parallel and/or compensatory mechanisms fail [9]. IDH has been associated with myocardial, cerebral, and mesenteric ischaemia, loss of residual kidney function, and mortality [10–12]. From a large study on eight IDH definitions, it appeared that intradialytic SBP <90 or <100 mmHg, depending on pre-dialytic SBP <160 or ≥160 mmHg, respectively, is

Table 1: Identification of cellular origin of EVs

EV label	Cellular origin
Lactadherin$^+$	General EV marker [56]
CD45$^+$	Leucocyte
CD61$^+$	Platelet
CD61$^+$CD62p$^+$	Activated platelet
CD235a$^+$	Erythrocyte
CD144$^+$	Vascular endothelium
CD62e$^+$CD144$^+$	Activated vascular endothelium
Connexin-43$^+$	Myocardium

CD = cluster of differentiation

most strongly associated with mortality [5]. Although IDH has been associated with adverse outcomes, direct evidence is lacking that its prevention reduces tissue damage. Reliable studies showing associations between IDH and biomarker-release, such as creatinine-kinase myocardial-band (CK-MB) and/or troponins, are lacking. Molecules that are smaller than the pore size of high-flux (HF) devices, such as troponins, may leave the blood compartment and, hence, escape from detection [13]. As for CK-MB, experimental studies have shown that its release occurs several hours after an injurious event, implying that cardiac damage will not be detected when samples are only taken twice during 4 hours of dialysis [14].

Other, perhaps more trustworthy, substances that may arise as a result of tissue injury are 'extracellular vesicles' (EVs), which contribute to physiological and pathological processes in human health and disease [15, 16]. As key mediators of intercellular communication, they are shed on cellular activation or apoptosis in response to various stimuli and carry different molecular cargo depending on their origin and physiological states [17, 18]. Consequently, their potential roles as non-invasive biomarkers were recently extensively explored in human diseases [19]. An important aspect in dialysis research is their size, which is too large (>700 kDa) [20] to remove by convection.

In the present study, EVs originating from circulating blood-cell-elements (CD45$^+$, CD61$^+$, CD61$^+$CD62p$^+$, and CD235a$^+$, respectively from leukocytes, platelets, activated platelets, and erythrocytes) were distinguished from EVs originating from cardiovascular (CV) tissues ['CV-related': CD144$^+$, CD62e$^+$CD144$^+$ (endothelial origin), and Connexin-43$^+$ (cardiomyocytic origin)] (Table 1). Because it is plausible that the first group results from bio-incompatibility (BI), this group is further arbitrarily denoted 'BI-related'.

As earlier suggested by two meta-analyses [21, 22] and recently confirmed in a large randomized controlled trial (RCT) [23], survival in high-volume haemodiafiltration (HV-HDF) is superior to HD. Recently, we showed that the IDH-incidence is lower in HD with cool dialysate (C-HD) and HV-HDF than in low-volume HDF (LV-HDF) and standard HD (S-HD) [24]. Because IDH may induce cellular injury, the survival benefit of HV-HDF over S-HD may be due to less intradialytic organ damage. Therefore, the aim of this study was to assess cell and tissue injury, as measured by the rate of change of various EVs, during four intermittent dialysis strategies (S-HD, C-HD, LV-HDF, and HV-HDF). Furthermore, potential associations between EV release and IDH, assessed both at the patient and the session level, were evaluated *post hoc*.

MATERIALS AND METHODS

Study design

The HOLLANT study (ClinicalTrials.gov identifier NCT03249532) was an open, cross-over, multi-centre RCT as described elsewhere [25]. In short, dialysis patients were randomized to: (i) S-HD [dialysate temperature (Td) 36.5°C], (ii) C-HD (Td 35.5°C), (iii) LV-HDF (Td 36.5°C, convection volume 15 l/session), and (iv) HV-HDF (Td 36.5°C, convection volume ≥23 l/session), all for 2 weeks. Study duration was 10 weeks. Blood samples were drawn in the last session of each modality. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guideline and approved by the Medical Ethical committee (METc) of VU University Medical Center (METC: 2017.581/NL61210.029.17). All patients gave written informed consent.

Study population

Between July 2018 and February 2021, patients were recruited from three centres in the Netherlands: Niercentrum aan de Amstel, Amstelveen; Amsterdam UMC, location VU University Medical Center, Amsterdam; Sint Antonius Ziekenhuis, Nieuwegein. Inclusion criteria were: (i) treatment with HD(F) three times a week for 4 hours for ≥2 months, (ii) ability to understand the study procedures, (iii) willingness to provide informed consent, (iv) dialysis single-pool $Kt/V_{urea} \geq 1.2$, and (v) blood flow rate feasibility of ≥350 ml/min. Exclusion criteria were a life expectancy <3 months and severe non-compliance to dialysis and accompanying prescriptions.

Dialysis prescription and equipment

Dialysis prescription and equipment are described extensively in the study design paper [25]. In short, treatments were performed with Xevonta 23 high-flux dialysers (membrane material: Amembris, i.e. polysulfon-based membrane with polyvinylpyrrolidone; B.Braun Avitum AG, Melsungen, Germany) on Dialog iQ dialysis machine including the captive lines Diastream (both B.Braun Avitum AG, Melsungen, Germany). Ultrapure dialysis fluids were mixed using Sol-Cart Bicarbonate cartridge and acidic dialysate. Substitution fluid was prepared from the dialysis fluid by one additional ultrafiltration (UF) step with a dialysis fluid filter (Diacap Ultra, B.Braun Avitum AG, Melsungen, Germany), before infusing into the blood. Apart from the modality, all prescriptions and devices were unchanged during the study. Routine patient care was performed according to national and international quality of care guidelines [26].

Data collection

Clinical measurements

Demographics, primary renal diagnosis, co-morbidity including CV disease (CVD), medication, and dialysis-related parameters were recorded at baseline.

Haemodynamic monitoring

During the last three sessions of each modality, BP was measured pre-treatment and every 15 minutes thereafter, using an automated manometric cuff device connected to the dialysis machine (Dialog iQ, automatic BP monitor, B. Braun Avitum AG, Melsungen, Germany). IDH episodes, defined by a systolic BP (SBP) <90 or <100 mmHg (providing a pre-dialysis SBP <160 or ≥160 mmHg respectively) independent of symptoms and interventions [5], were recorded.

EV measurements

Blood sampling and preparation. Blood samples were drawn from the arterial line before dialysis (after administering low-molecular weight heparin) and after 4 hours, using a 21-Gauge needle, and collected in plastic vacuum tubes [2.7 ml of trisodium citrate; final concentration 0.109 mol/l (BD Vacutainer®, USA)]. Platelet-depleted plasma was prepared by double centrifugation using a Rotina 380-R equipped with a swing-out rotor and a radius of 155 mm (Hettich Zentrifugen, Tuttlingen, Germany). Citrated blood samples were centrifuged for 15 minutes at 2500*g*, 20°C, no brake. Next, the EV-containing supernatant was isolated and centrifuged again (15 minutes at 2500*g*, 20°C, no brake). For freezer storage, samples were transferred to 1.5 ml microtubes (Sarstedt AG & Co., Germany), immediately frozen, and stored at −80°C. Before staining, samples were thawed for 1 minute at 37°C.

EV isolation and assessment. EVs were stained with antibodies to identify their origin (Table 1). Prior to staining, antibodies were diluted in Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 18 890*g* for 5 minutes to remove aggregates. Each sample was single labelled with CD45-APC (BioLegend, CA, USA), CD235a-PE (Agilent Technologies Inc., CA, USA), and Connexin-43-APC (R&D System, MN, USA) or Lactadherin-FITC (Prolytix, VT, USA). Furthermore, samples were double labelled with CD61-APC (Thermo Fisher Scientific Inc., MA, USA) and CD62p-PE (Beckman Coulter, IN, USA), as CD61 is present on all platelet-derived EVs and P-selectin (CD62p) on a subpopulation of platelet-derived EVs that originate from activated platelets only. Similarly, samples were double labelled with CD144-APC (Thermo Fisher Scientific Inc., MA, USA) and CD62e-PE (BD Biosciences, CA, USA) as CD62e is only present on EVs derived from activated endothelium, which is a subpopulation of $CD144^+$ EVs that derived from all endothelial tissues. To stain, 20 μl of pre-diluted plasma sample was incubated with 2.5 μl of antibodies or isotype controls and kept in the dark for 2 hours at room temperature. To decrease background fluorescence from unbound reagents, 200 μl of DPBS was added. All samples were measured with Apogee A-60-Micro (Apogee Flow systems, Hemel Hempstead, UK) for 2 minutes at a flowrate of 3.01 μl/min. Fluorescence signals were calibrated and expressed as units of molecules of equivalent fluorochromes, side scattering was related to EV-diameter in nm using Rosetta Calibration (Exometry, The Netherlands). Custom-build software (MATLAB R2018b, Mathworks, Natick, MA, USA) was applied to generate data summaries. The concentrations reported describe the number of particles (i) that exceeded side scatter threshold, (ii) with diameter <1000 nm, and (iii) exceeding the fluorescent threshold corresponding to the used labels/ml. The experiments fulfil the criteria of the framework for standardized reporting of EV flow-cytometry experiments, MIFlowCyt-EV, which incorporates the latest MISEV-guidelines [27, 28]. MIFlowCyt-EV, containing all details required to reproduce the experiments, can be found in the online supplementary material (Supplementary Appendix 1).

Statistical analyses

Baseline characteristics are summarized as mean (standard deviation), median (interquartile range), or number (percentage), as dictated by data type. Differences in the rate of change of EVs were assessed (i) between modalities, (ii) in subcategories of IDH susceptibility, and (iii) during sessions with and without IDH. In all cases, model assumptions were checked and not violated. For all linear mixed models (LMM), a random intercept, random slope or both were used, depending on the lowest Aikaike's information criterion.

Differences between modalities

First, we visualized the intradialytic rate of change of every EV as stratified by modality. Next, we evaluated whether pre-dialytic EV values were similar in all modalities. To this end, pre-dialytic values were log-transformed given their non-parametric distribution. We used a repeated-measures-analysis of variance (ANOVA) to evaluate potential differences. If the assumption of sphericity as determined by Mauchly's test was violated, we used the Greenhouse–Geissner correction. To assess potential differences in the rate of change between modalities per EV, LMMs were fitted with an interaction term between modality and time. Next, the rate of change in EV per modality was determined using stratified LMMs.

Correction for haemoconcentration

In separate analyses, the influence of increased haemoconcentration on the intradialytic change in EVs was evaluated. Post-dialytic values were corrected for UF volume. To this end, we first calculated adjusted post-dialytic EV values using the serum haematocrit (Ht) in available patients ($n = 11$) with the formula [29]: corrected post-dialytic EV_{tx} = crude post-dialytic $EV_{tx} \times [Ht_{t0}/(1 - Ht_{t0})] \times [(1 - Ht_{tx})/Ht_{tx}]$. These data were then used to calculate a UF-based correction factor for all patients ($n = 40$) with the formula: $(1 - [(UF/1000) \times (1 - \text{average Ht/average UF})]$.

Differences between tertiles of IDH susceptibility

In the second week of each modality, all BP measurements of every patient were used (maximum 192 BP readings) to assess IDH susceptibility. We identified how many BP readings met the definition of Flythe *et al*. [5]. Next, we calculated the ratio between the number of IDH episodes and the total amount of BP measurements. We then divided the patients into tertiles, according to their IDH susceptibility (IDH-resistant, IDH-intermediate, IDH-prone). We calculated average pre- and post-dialytic values of every EV/patient. Hereafter, we plotted graphs of the change in every EV/IDH-tertile. To determine whether the pre-dialytic values of the groups (unpaired data) were similar, we used one-way ANOVA. To this end, we log-transformed the average pre-dialytic EV values given its non-parametric distribution. Thereafter, we evaluated whether the rate of change between the IDH-tertiles differed using LMMs with an interaction term between IDH-tertiles and time. IDH-resistant patients were used as reference category. Finally, stratified rates of change of every EV per IDH-tertile were calculated, using LMMs.

Differences between dialysis sessions with and without objectified IDH

Last, we evaluated potential differences in the rate of change of EVs between sessions with and without IDH. For this analysis, only the sixth session, in which EVs were sampled, of every modality was included. Next, we again evaluated potential differences in the pre-dialytic values between sessions with and without IDH, using unpaired *t*-tests after log transformation of the data given its non-parametric distribution. Hereafter, LMMs with an interaction between the dichotomous variable of the presence or absence of IDH and time were fitted to evaluate whether the rate of change of every EV differed between

Table 2: Baseline characteristics of study participants

Demographics	$n = 40$
Sex (male)	30 (75%)
Age (years)	69.7 ± 13.5
Ethnicity: Caucasian/African/Asian	28/10/2 (70%/25%/5%)
Clinical characteristics	
BMI (kg/m^2)	26.7 ± 4.2
Smoking status: never/former/current	14/18/8 (35%/45%/20%)
Systolic BP, pre-dialysis (mmHg)	145 ± 23
Diastolic BP, pre-dialysis (mmHg)	81 ± 13
Residual kidney function[1]	24 (60%)
Residual kidney function (ml/min)[2]	1.9 (1.0–2.5)
Medical history	
Dialysis modality: HD/HDF	23/17 (58%/42%)
Dialysis vintage (years)	3.0 (1.0–5.8)
History of kidney transplantation	3 (8%)
Primary cause of ESKD	
Glomerulonephritis	10 (25%)
Renal vascular disease	9 (23%)
Diabetic nephropathy	15 (38%)
Cystic kidney disease	1 (3%)
Other/unknown	4 (10%)/1 (3%)
Diabetes mellitus	19 (48%)
Hypertension	28 (70%)
History of CVD	29 (73%)
Medication	
ACE-I/ARB	10 (25%)
Beta-blockers	25 (63%)
Calcium antagonists	10 (25%)
Diuretics	11 (28%)
ESA	32 (80%)
Laboratory data	
Haemoglobin (mmol/l)	7.1 ± 0.7
Creatinine (μmol/l)	865 ± 229
Sodium (mmol/l)	138 ± 4
Potassium (mmol/l)	5.1 ± 0.6
Phosphate (mmol/l)	1.6 ± 0.5
Albumin (g/l)	38.6 ± 4.5
PTH (pmol/l)	28.2 (15.1–48.3)
Dialysis parameters	
Vascular access: AVF/Graft/CVC	32/4/4 (80%/10%/10%)

Values are number (*n*) (%) for categorical variables and mean ± standard deviation or median (interquartile range) for continuous variables. Laboratory data are pre-dialytic values. [1]Residual diuresis >100 ml/24 hours.[2]In patients with diuresis >100 ml/24 hours.

BMI = body mass index; ESKD = end-stage kidney disease; ACE-I = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; ESA = erythropoiesis-stimulating agent; PTH = parathyroid hormone; AVF = arteriovenous fistula; CVC = central venous catheter.

groups. Finally, stratified models were fitted to calculate the rate of change per group.

RESULTS

Patient and treatment characteristics

Forty-five patients were included, as five dropped out before randomization (Supplementary Figure S1). Tables 2 and 3 show baseline and dialysis characteristics. Most patients were male (75%), mean age 69.7 ± 13.5 years, diabetes mellitus was present in 48%, and CVD in 73%.

Missing data

Out of 40 patients who completed the study, two were not exposed to HDF but completed S-HD and C-HD. Two other patients withdrew their consent after completing 75% and 50% of the study. The percentages missing EV values were: CD235a$^+$ and Connexin-43$^+$:9.4%, CD45$^+$:8.1%, CD144$^+$ and CD62e$^+$CD144$^+$:7.5%, CD61$^+$, and CD61$^+$CD62p$^+$:6.9% and lactadherin$^+$-labelled EVs:7.2%. The exact number of specimens collected for each EV phenotype is reported in Supplementary Table S1.

Extracellular vesicles

Table 4 shows the pre- and post-dialytic plasma EV concentrations.

Rates of change of EVs and differences between modalities

Regarding BI-related changes, both leukocyte and platelet-derived EVs increased in all modalities. The rise in platelet-derived EVs was more pronounced during both LV-HDF and HV-HDF (68.4 and 56.1 × 10^6/ml; *P* for interaction <.01 respectively 0.06) than during S-HD (27.5 × 10^6/ml). Considering EVs from activated platelets, a significant increase was noticed during LV-HDF only (Fig. 1 and Table 4). Considering CV-related EVs, the release of CD144$^+$ (2.3 respectively 9.8 × 10^6/ml, *P* for interaction .03) and Connexin-43$^+$ (12.0 respectively 31.9 × 10^6/ml, *P* for interaction .06) was lower during HV-HDF as compared to S-HD.

Analyses corrected for haemoconcentration

As expected, correction for haemoconcentration attenuated the increase in all EVs, as shown in Supplementary Table S2. However, a higher increase in platelet-derived EVs (CD61$^+$) in both HDF modalities (29.9 and 24.0 × 10^6/ml; $P < .001$ and .04 for LV-HDF respectively HV-HDF), compared to the two HD modalities (3.0 and 10.4 × 10^6/ml; $P = .77$ and .13 for S-HD respectively C-HD) was still observed.

As for CV-related changes, endothelial-derived EVs (CD144$^+$) increased significantly in S-HD and LV-HDF (5.1 and 4.5 × 10^6/ml; $P = .02$ respectively 0.04) and remained stable in C-HD and HV-HDF (−0.2 and −0.7 × 10^6/ml; $P = .93$ respectively 0.66). The differences between S-HD and both C-HD and HV-HDF were significant (*P* for interaction .05 respectively .03). Despite a non-significant interaction, a noticeable difference in intradialytic cardiomyocyte-derived EVs (Connexin-43$^+$) change was observed between HV-HDF and S-HD (−0.1 and 13.6 × 10^6/ml; $P = 0.98$ respectively <.01).

Rates of change of EVs and the patients' susceptibilities to IDH

Data on IDH are extensively described elsewhere [24]. Median IDH-episode/BP-measurement was 1% (IQR 0–4). According to their IDH susceptibility, patients were divided into tertiles (proportion of IDH episodes of all BP measurements): group I, IDH-resistant: 13 patients (<0.5%); group II, IDH-intermediate: 14 patients (≥0.5%–2.7%); and group III, IDH-prone: 13 patients (≥2.7%). All EVs increased in the IDH-resistant group. As shown in Supplementary Table S3, IDH-prone patients did not show any rise in CV-related EVs and exhibited a lower increase in EVs from activated platelets ($P = .01$) and cardiomyocytes ($P = .05$) than IDH-resistant patients.

Table 3: Dialysis characteristics

Modality	Blood flow (ml/min)	Dialysate flow (ml/min)	UF (l/session)	Convection volume (l/session)
S-HD	339 ± 33	505 ± 11	2.3 ± 0.7	N/A
C-HD	332 ± 41	505 ± 13	2.4 ± 0.7	N/A
LV-HDF	339 ± 36	590 ± 19[a]	2.3 ± 0.6	15.1 ± 1.3
HV-HDF	347 ± 27	594 ± 18[a]	2.3 ± 0.7	22.6 ± 1.1

Mean ± standard deviation for blood flow, dialysate flow, UF volume and convection volume. N/A = not applicable. [a]Includes substitution flow in LV-HDF and HV-HDF

Table 4: Pre- and post-dialysis plasma concentration and rate of change of EVs

EV[a] (×10^6/ml)	Pre	Post	*P* pre[b]	Change (%)	Rate of change (95% CI)	*P*	*P* for interaction[c]
Lactadherin$^+$							
S-HD	332.0 (180.5–499.5)	558.3 (330.1–846.5)	.15	68	249.5 (138.5–360.4)	<.001	Reference
C-HD	312.6 (174.4–499.7)	532.4 (316.3–816.0)		70	355.1 (205.5–505.7)	<.001	.68
LV-HDF	276.4 (168.5–477.4)	523.5 (343.5–850.4)		89	480.9 (136.3–825.4)	.007	.20
HV-HDF	369.8 (161.3–670.8)	545.4 (342.8–793.3)		48	192.5 (120.6–264.5)	<.001	.69
CD45$^+$							
S-HD	23.5 (15.7–40.3)	40.4 (26.1–69.7)	.69	72	21.9 (10.3–33.4)	<.001	Reference
C-HD	25.9 (18.0–42.8)	38.8 (27.6–74.2)		50	27.0 (15.3–38.6)	<.001	.87
LV-HDF	22.8 (13.4–34.5)	35.4 (24.6–60.6)		55	28.4 (12.3–44.5)	<.001	.51
HV-HDF	26.5 (13.3–39.7)	43.5 (26.1–56.9)		64	14.6 (9.2–20.1)	<.001	.41
CD61$^+$							
S-HD	64.9 (34.8–95.3)	95.7 (66.1–147.6)	.55	48	27.5 (1.6–53.4)	.010	Reference
C-HD	52.5 (32.0–98.9)	112.7 (67.2–131.9)		115	44.3 (26.4–62.2)	<.001	.27
LV-HDF	54.6 (38.7–100.5)	133.8 (77.5–171.8)		145	68.4 (49.2–87.7)	<.001	.006
HV-HDF	57.5 (34.6–122.7)	127.23 (85.0–194.0)		121	56.1 (28.7–83.4)	<.001	.06
CD61$^+$CD62p$^+$							
S-HD	0.9 (0.4–1.8)	0.8 (0.5–3.0)	.38	11	−0.2 (−0.9–0.5)	.55	Reference
C-HD	0.6 (0.3–1.2)	1.1 (0.4–2.3)		83	0.5 (−0.1–1.0)	.09	.12
LV-HDF	0.7 (0.2–1.5)	0.8 (0.3–2.4)		14	0.5 (0.2–0.9)	.008	.06
HV-HDF	0.9 (0.3–1.9)	1.1 (0.3–2.0)		22	0.3 (−0.2–0.8)	.21	.27
CD235a$^+$							
S-HD	32.5 (17.9–51.5)	43.3 (31.3–80.5)	.13	33	23.7 (12.3–35.0)	<.001	Reference
C-HD	29.2 (19.5–54.8)	48.9 (33.4–71.3)		68	28.5 (16.9–40.1)	<.001	.96
LV-HDF	29.9 (20.9–39.8)	47.7 (34.1–76.5)		60	31.2 (16.8–45.6)	<.001	.23
HV-HDF	39.2 (19.7–49.6)	43.7 (30.5–72.1)		12	15.4 (7.7–23.2)	<.001	.27
CD144$^+$							
S-HD	5.2 (2.2–10.7)	13.0 (5.0–25.3)	.36	150	9.8 (4.4–15.2)	<.001	Reference
C-HD	6.7 (2.9–13.3)	13.6 (4.9–21.9)		103	5.5 (2.8–8.3)	<.001	.14
LV-HDF	5.5 (2.4–11.6)	9.5 (5.1–21.6)		73	9.5 (4.6–14.5)	<.001	.96
HV-HDF	5.3 (2.0–17.7)	11.0 (4.8–20.1)		108	2.3 (−1.9–6.6)	.28	.03
CD62e$^+$CD144$^+$							
S-HD	0.1 (0–0.3)	0.1 (0–1.2)	.11	0	0.7 (0–1.5)	.05	Reference
C-HD	0.1 (0–0.4)	0.2 (0–0.6)		100	−0.1 (−0.5–0.4)	.81	.19
LV-HDF	0.1 (0–0.4)	0.1 (0–0.4)		0	0.8 (−0.1–1.7)	.09	.93
HV-HDF	0.2 (0–1.1)	0.3 (0–0.6)		50	−0.4 (0–0.3)	.26	.07
Connexin-43$^+$							
S-HD	18.1 (5.7–51.4)	49.3 (17.5–74.1)	.73	172	31.9 (15.8–48.1)	<.001	Reference
C-HD	15.7 (7.1–43.6)	44.2 (18.9–82.1)		182	29.7 (10.3–49.1)	.003	.63
LV-HDF	15.2 (7.1–51.9)	30.8 (13.6–58.9)		103	18.3 (7.4–29.3)	.002	.22
HV-HDF	12.7 (5.6–36.2)	38.9 (15.2–71.3)		206	12.0 (−0.7–24.8)	.06	.06

Pre- and post-EV concentration are shown as the median with interquartile range. [a]As determined by size (<1000 nm). [b]*P* for difference in pre-dialytic value between modalities. Rate of change of EVs (change in concentration in 4 hours) shown as mean with 95% confidence interval. [c]*P* for difference in the rate of change of respective modality in reference to LV-HDF.

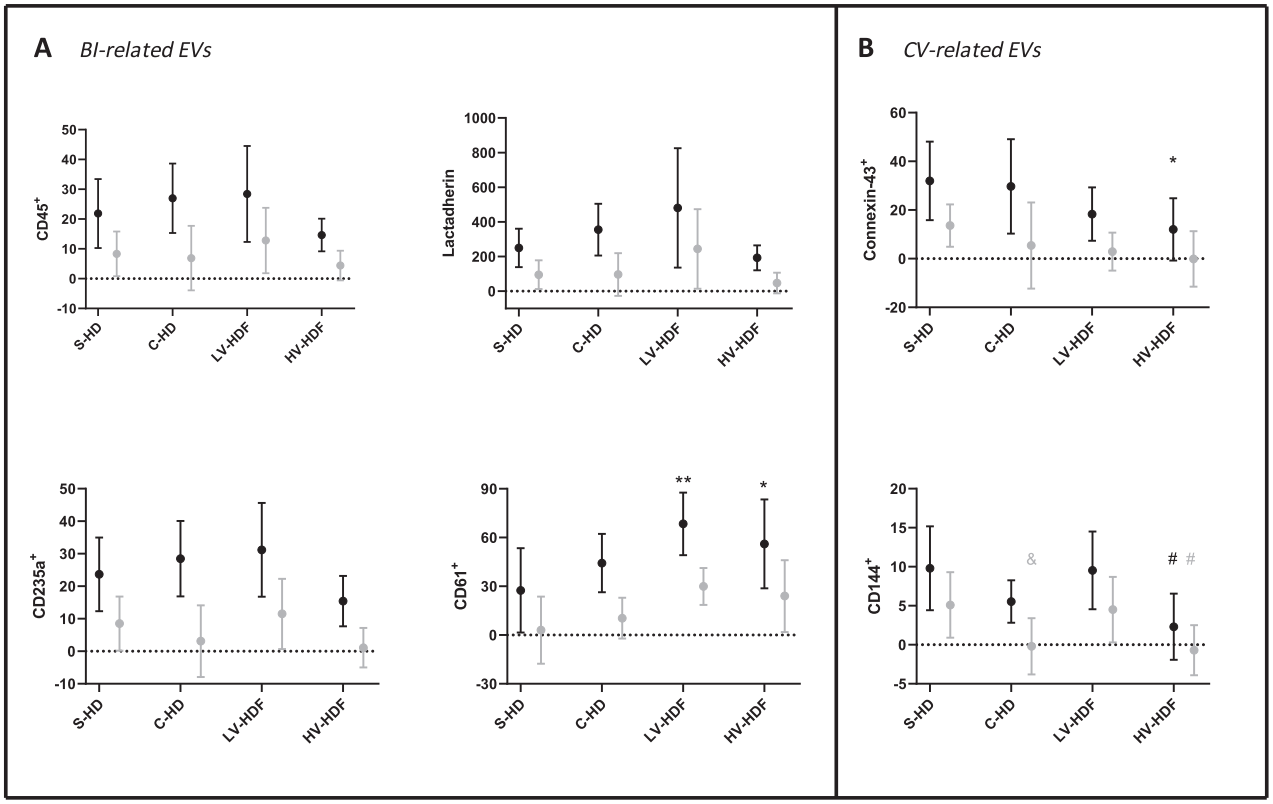


Figure 1: Rate of change of plasma EVs in modalities. Rate of change (mean ± 95% CI) of EVs ($\times 10^6$/ml) during 4 hours of HD/HDF (black) and after correction for haemoconcentration (grey). *P* values for interactions (significance as compared to S-HD = reference): $^{*}P = 0.06$, $^{**}P = 0.006$, $^{\#}P = 0.03$, $^{\&}P = 0.05$. *P* values of rate of change during treatment with each modality in Tables 4 and S2 (significant if 95% CI does not cross the 0 line).

Rates of change of EVs and IDH on session level

To assess potential associations between EV-changes and IDH, two groups were created consisting of sessions with (group I) and without IDH (group II). As shown in Supplementary Table S4, differences were not observed.

DISCUSSION

The current study shows that most EVs increase during dialysis. While the increase in platelet-derived EVs is higher in HDF, the rise in CV-related EVs is less pronounced in HV-HDF. Before reflecting on our results, it should be noted that the study was not designed to investigate which pathophysiological mechanisms underlie intradialytic EV release, but rather to assess the origin of HD(F)-induced EV release, to evaluate whether EV-levels are different between HD and HDF and to explore a potential association between Connexin-43$^+$ EVs and IDH. Furthermore, the subdivision in BI-related (derived from circulating blood-cell-elements) and CV-related (derived from endothelial cells and cardiomyocytes) EVs was not random. Whereas BI arises due to contact between blood and the extra-corporeal circuit, CV-related injury is supposed to result mainly from IDH. In addition, our analytical approach consisted of two parts: a crude analysis and a correction for haemoconcentration.

Accordingly, three sets of findings were obtained. For all EVs, the increase, as observed in the crude analysis, was considerably weakened by correction for haemoconcentration. Yet, the noticeably higher increase in platelet-derived (CD61$^+$) EVs in the two HDF modalities persisted. Considering CV-related EVs, in the crude analysis, endothelial-derived EVs (CD144$^+$) remained unaltered in HV-HDF but increased in all three other modalities. After correction for haemoconcentration, not only a difference between S-HD and HV-HDF could be demonstrated, but also between S-HD and C-HD. Regarding cardiomyocyte-derived EVs (Connexin-43$^+$), in the adjusted analysis only S-HD showed an increase, whereas C-HD, LV-HDF and HV-HDF remained unaltered. *Post hoc*, EV changes were analysed both at the patient and the session level. Whereas all EVs increased in the IDH-resistant group, CV-related EVs remained unaltered in IDH-prone patients. Compared to IDH-resistant individuals, EV-increments from activated platelets and cardiomyocytes were lowest in IDH-prone patients. Differences between sessions with and without IDH were not found.

As for BI-related EV changes, beforehand we speculated that changes would be most noticeable in HDF due to the high transmembrane pressure [29]. Indeed, while platelet-derived (CD61$^+$) EVs increased significantly in both HDF modalities, these particles remained unaltered in the two HD strategies. Yet, the finding that, even after correction, the increase in CV-related endothelial-derived EVs (CD144$^+$) was significantly less pronounced in both HV-HDF and C-HD than in S-HD, is highly intriguing in view of the lower mortality rate in HV-HDF [23, 30]. As for cardiomyocyte-derived EVs (Connexin-43$^+$), the results are less clear, although the rise was only significant in S-HD.

Actually, these findings support our initial concept that especially HV-HDF and C-HD induce only scant intradialytic CV-tissue damage due to the low IDH-frequency in these modalities

[24]. Yet, whether our results are indeed caused by differences in IDH is unclear, as outlined before. Other mechanisms, which might play a role in dialysis induced EV release, include rapid changes in electrolyte concentrations, activation of the coagulation cascade, adsorption of inhibiting factors to the dialyser, etc., as cardiac injury seems not exclusively related to ultrafiltration induced IDH [31]. As for HV-HDF, our data is in line with the recent confirmation that survival in this modality is superior to S-HD [23]. Considering C-HD, however, two recent large studies did not show any survival benefit over S-HD [32, 33]. Notably, however, in these studies Td in C-HD was only slightly below Td in S-HD and haemodynamic differences were either absent or limited. Because neither study was randomized at the patient level and several other weaknesses were acknowledged before [34–36], it remains unclear whether C-HD, on certain terms and conditions, prolongs survival.

Nonetheless, our results are largely in line with prior studies, showing that endothelial-derived EVs ($CD31^+$/$Annexin^+$) [37] and pro-atherogenic EVs (increased miR-223 expression) [38] are substantially lower in HDF than in S-HD. Furthermore, our findings seem in accordance with prior reports showing an increase in EVs [39–41], non-confirmative to investigations showing no effect [39, 40, 42], and contrary to studies reporting drops [42–44]. Clearly, the available literature is conflicting due to the many different antibodies used [41]. Whereas most studies utilized $CD31^+$, $CD66e^+$, or $CD146^+$ for endothelial-derived EVs [41], we used $CD144^+$. Differences in the pre-analytical phase may have led to major variations downstream [45]. Comparison of EV values between different laboratories and studies is complicated. Standardization and calibration, including EV reference values based on healthy controls, are currently on their way. As the analysis performed in our study complies to recent international standards [46], comparison of our data with healthy controls and other patient groups will be possible in the near future. Anyhow, it seems justified to conclude that even modern dialysis techniques evoke a rise in most EVs.

Regarding our *post hoc* analyses, the data are puzzling. Differences in EV release were not only absent between patient groups with varying IDH susceptibility, but also between sessions with and without IDH. It should be realized, however, that most IDH episodes will be missed when BP is only assessed four times per hour and, hence, unknown during the remaining treatment. Moreover, EVs were only measured twice. Therefore, this analysis should be considered explorative rather than conclusive.

To the best of our knowledge, this is the first investigation comparing intradialytic EV changes between two HD and two HDF modalities. Important strengths are its randomized crossover design, the meticulous data collection, BP assessments every 15 minutes, and the use of a state-of-the-art method to measure EVs. Since patients served as their own controls, inter-subject variability is eliminated, enabling us to investigate causality. Besides strengths, this study also has limitations. The number of EVs derived from activated platelets and activated vascular endothelium are low after dilution of plasma samples, affecting the reliability of $CD61^+CD62p^+$ and $CD62e^+CD144^+$ EV concentrations. As we investigated the origin of HD-induced EVs in this study, we only measured specific EV-markers and not their functionality or RNA transfer. Hence, further research is mandatory to confirm the assumption that intradialytic cardiac damage is reduced by HV-HDF. Regarding our correction for haemoconcentration, which was an extrapolation of only 11 patients in whom Ht was available, it should be admitted that errors may arise. As for the clinical limitations, just measuring EVs before and after dialysis may be too simplistic. After all, EVs may not only arise from IDH and BI [47], but also from electrolyte and osmolality shifts. As peak BI occurs early [48] and the frequency of IDH increases towards the end of sessions [49], it is plausible that differences are not captured when samples are only taken twice. Moreover, EVs may not only be cleared physiologically, but also by adsorption onto the dialyser membrane [43, 50]. Because T½ of the various EVs is unknown, multiple samples should be taken to assess their levels more reliably [51].

In summary, most BI- and CV-related EVs increase during dialysis. Yet, whereas the changes in BI-related EVs were not unexpected, our findings on CV-related EVs are intriguing. The diverging changes in HV-HDF and S-HD match our previous observation that especially cardiovascular survival is prolonged by HV-HDF [30]. At the patient and session level, it seemed that differences were not observed among groups of IDH susceptibility. Because the groups were small and confounding cannot be excluded, it seems premature to draw firm conclusions. As the IDH-incidence increases towards the end of dialysis [52] and cardiac hypoperfusion has been observed shortly after the start [31] when both inflammation and complement consumption are high [48, 53–55], future studies should take frequent blood samples for the concurrent measurement of EVs and the activation status of these processes.

SUPPLEMENTARY DATA

Supplementary data are available at *Clinical Kidney Journal* online.

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AUTHORS' CONTRIBUTIONS

M.P.C.G. and M.J.N. designed and edited the study protocol. C.L.M.R.Z. wrote the statistical part of the protocol. P.R. and G.W. collected the data. P.L. and C.L.M.R.Z. did the statistical analysis. P.L. and P.R. wrote the manuscript. M.P.C.G., M.J.N., C.L.M.R.Z., C.M.H., R.N., and G.W. were responsible for the critical revision of the manuscript. The final version of the manuscript was seen and approved by all authors.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

CONFLICT OF INTEREST STATEMENT

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