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Online hemodiafiltration inhibits inflammation-related endothelial dysfunction and vascular calcification of uremic patients modulating miR-223 expression in plasma extracellular vesicles

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1 **On line-hemodiafiltration inhibits inflammation-related endothelial dysfunction and vascular**
2 **calcification of uremic patients modulating miR-223 expression in plasma extracellular**
3 **vesicles**

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26

27 **ABSTRACT**

28 Decreased inflammation and cardiovascular mortality is evident in patients with end stage chronic
29 kidney disease (CKD) treated by on-line hemodiafiltration (OL-HDF). Extracellular vesicles (EV)
30 are mediators of cell-to-cell communication and contain different RNA types. This study
31 investigated whether mixed OL-HDF (mOL-HDF) beneficial effects associate with changes in the
32 RNA content of plasma EV in CKD patients. Thirty bicarbonate hemodialysis (BHD) patients were
33 randomized 1:1 to continue BHD or switch to mOL-HDF. Concentration, size, and microRNA
34 content of plasma EV were evaluated for 9 months; we then studied EV effects on inflammation,
35 angiogenesis and apoptosis of endothelial cells (HUVEC), and on osteoblast mineralization of
36 vascular smooth muscle cells (VSMC). mOL-HDF treatment reduced different inflammatory
37 markers including circulating CRP, IL6 and NGAL. All hemodialysis patients showed higher
38 plasma levels of endothelial-derived EV than healthy subjects, with no significant differences
39 between BHD and mOL-HDF. However, BHD-derived EV had an increased expression of the pro-
40 atherogenic miR-223 in respect to healthy subjects or mOL-HDF. Compared to EV from healthy
41 subjects, those from hemodialysis patients reduced angiogenesis, increased HUVEC apoptosis and
42 VSMC calcification; however, all these detrimental effects were reduced with mOL-HDF in respect
43 to BHD. Cell transfection with miR-223 mimic or antagomiR proved the role of this miRNA in EV-
44 induced HUVEC and VSMC dysfunction. The switch from BHD to mOL-HDF significantly
45 reduced systemic inflammation and miR-223 expression in plasma-EV, thus improving HUVEC
46 angiogenesis and reducing VSMC calcification.

47

48 **INTRODUCTION**

49 Dialysis patients suffer from a high mortality, mostly related to vascular events (1, 2). Recently,
50 several studies demonstrated an improvement of inflammatory parameters and of cardiovascular
51 outcomes in patients with end stage chronic kidney disease (CKD) treated by on-line
52 hemodiafiltration (OL-HDF) (3–5). This cardio-protective effect may be ascribed to an enhanced
53 clearance of middle molecules involved in CKD-associated inflammation, endothelial dysfunction
54 and vascular calcification (2, 6–8).

55 Increasing evidences indicate that plasma extracellular vesicles (EV) contribute to several
56 physiological and pathological processes (9, 10). EV act as intercellular mediators by shuttling
57 lipids, proteins and predominantly extracellular RNAs (11, 12). Indeed, several biological activities
58 of EV may be ascribed to the transfer of microRNAs (miRNAs), small noncoding RNAs able to
59 regulate post-transcriptional expression of gene products (13). EV-carried miRNAs are protected
60 from the activity of degrading enzymes allowing their persistence in biological fluids and their
61 delivery at distant sites. Recent studies have shown that plasma EV exert pro-inflammatory and pro-
62 thrombotic properties and that EV may modulate endothelial function, suggesting a potential role of
63 these microparticles in the pathogenesis of inflammatory disorders and atherosclerosis (14).
64 Moreover, both EV and miRNAs circulating in the bloodstream reflect tissue damage and may be
65 considered as biomarkers of disease activity (11, 15–18). Increased plasma levels of EV have been
66 reported in hemodialysis patients in association with inflammatory parameters such as CRP and IL-
67 6 (19). However, it remains unclear whether the dialysis procedure itself may affect the release of
68 EV and the potential pathogenic role of EV-carried miRNAs in inflammation and vascular damage
69 has not been fully elucidated. We herein hypothesized that circulating EV play a major role in
70 dialysis associated vascular dysfunction and that OL-HDF is beneficial to the vascular system also
71 by modulating the circulating EV content of RNA.

72 The aims of the present study were: 1) to isolate and characterize plasma EV derived from patients
73 with end stage chronic kidney disease (CKD) undergoing high flux bicarbonate hemodialysis

74 (BHD) before and after switching to mixed on-line haemodiafiltration (mOL-HDF); 2) to analyze if
75 different dialysis modalities modulate EV-miRNAs potentially involved in inflammation,
76 endothelial dysfunction, atherosclerosis and vascular calcification.

77

78 PATIENTS AND METHODS

79 Patients

80 Thirty patients undergoing regular high flux bicarbonate hemodialysis (BHD) were enrolled in the
81 study. Written informed consent was obtained. The study was conducted in accordance to Helsinki
82 declaration, approved by the Ethic Committee of the “Città della Salute e della Scienza di Torino”
83 University Hospital (Cod. 0030959, CEI/568) and registered in Clinicaltrials.gov (ID:
84 NCT03202212). Inclusion criteria were: age >18 yrs., hemodialysis from at least 6 months (3 times
85 for week), blood flow rate (Qb) \geq 250 ml/min using arteriovenous fistula (AVF) or permanent
86 central venous catheter (CVC), blood creatinine clearance <5 ml/min, urine output <500 ml/die.
87 Exclusion criteria were: neoplastic diseases, autoimmune diseases, solid organ or bone marrow
88 transplantation. Enrolled patients were randomized to continue high flux bicarbonate hemodialysis
89 (BHD, n=15), or to switch to mixed on-line hemodiafiltration (mOL-HDF using FX 1000 CorDiax,
90 Fresenius Medical Care, Bad Homburg, Germany; n=15) for 9 months (20). All enrolled patients
91 completed the study with valid data (Figure 1). Immediately before the randomization (T0) and at 3
92 (T1), 6 (T2) and 9 (T3) months after study start, the following parameters were evaluated: blood
93 flow rate (ml/min), dialysate flow rate (ml/hr), transmembrane pressure (TMP, mmHg), convective
94 volume exchange (L/session), net ultrafiltration (L/session), dialysis time (minutes) white blood cell
95 count, hemoglobin, hematocrit, C reactive protein (CRP), serum iron, transferrin saturation, ferritin,
96 ERI (Epo units/Kg/week/hemoglobin), β 2-microglobulin, homocysteine, calcium, phosphate,
97 parathyroid hormone (PTH), Neutrophil Gelatinase-Associated lipocalin (NGAL). Dialysis
98 adequacy was defined by eKt/V (according to Daugirdas formula) with a target value of 1.2. In
99 mOL-HDF sessions a total convective volume >25 liters was considered as appropriate.

100 Patients' plasma was collected at the beginning of the second dialysis session of the week at T0, T1,
101 T2 and T3. Harvested samples were used to perform nanoparticle tracking analysis (21), Guava
102 FACS, western blot analysis, cellular and molecular biology studies; plasma drawn from healthy
103 subjects was used as negative control. Clinical and laboratory parameters of the enrolled patients

104 were validated in a further cohort of hemodialysis patients treated by BHD (n=50) or post-dilution
105 OL-HDF (n=30), peritoneal dialysis patients (n=10) and patients with stage IV CKD according to
106 K-DOQI criteria (n=20).

107 **Plasma collection and extracellular vesicle (EV) isolation**

108 Patient and healthy control plasma was obtained by centrifuging peripheral blood in EDTA tubes at
109 6,000 g for 15 minutes at 20°C. To isolate EVs, plasma samples were further centrifuged at 6,000 g
110 for 20 minutes to remove remaining debris and then ultra-centrifuged at 100,000 g for 1h at 4°C.
111 EV were re-suspended in 500 µl of RPMI with 1% DMSO and stored at -80°C.

112 **Nanoparticle tracking analysis**

113 EV preparations were diluted (1:1000) in sterile 0.9% saline solution and analyzed by NanoSight
114 LM10 (Nanosight Ltd., Amesbury, UK) equipped with the Nanoparticle Analysis System & NTA
115 1.4 Analytical Software. The number of total EVs for each patient was obtained by multiplying the
116 value given by the instrument (microparticles/ml) for the dilution made for the analysis and for the
117 number of ml in which EVs were re-suspended.

118 **Guava FACS Analysis**

119 FACS analysis was performed on EV isolated from plasma by ultracentrifugation with GUAVA
120 (GUAVA Easy-Cyte, Millipore) for the following markers: exosomes (CD9, CD63, CD81, CD86),
121 platelets (CD41, CD42b, CD62P), monocytes/macrophages (CD14, CD15), leukocytes (CD45), B-
122 cells (CD5, CD19, CD40), endothelial cells (CD31, CD105, CD144, CD146), T-cells (CD3) and
123 different markers involved in atherosclerosis and vascular senescence (Tissue Factor, C5b-9, CD40-
124 Ligand, ICOS, Fas-Ligand) (22, 23). Briefly, EVs were labeled with fluorescence-conjugated
125 antibodies for 30 min at RT. EVs were washed twice in PBS with 0.5% BSA and resuspended in
126 PBS 0.1% BSA. EVs were analyzed using Guava Incyte™ Software version 3.1.1 (M Millipore).
127 The acquired data files were analyzed firstly by adding a EV-gate based on morphologic
128 characteristics (FSC/SSC) and subsequently by the use of the lineage-specific markers. FITC or PE
129 mouse non-immune isotypic IgG (Beckton Dickinson, USA) were used as controls..

130 **Electronic molecular network generation: Protein Quest**

131 To detect relevant miRNAs, electronic literature screening was performed by the apposite web
132 platform Protein Quest (PQ – Biodigital Valley, Aosta, Italy). PQ retrieves all biological and
133 medical information from PubMed abstract and captions, free articles, US patents and Clinical
134 Trials. Moreover, it classifies terminology by mesh biomedical dictionary and elaborates hierarchic
135 networks displaying term relationships. By using an appropriate research string, we selected all
136 available articles and abstracts regarding uremic vascular dysfunction or calcification; afterwards
137 PQ recognized all described miRNAs and relative pathways.

138 **Western Blot Analysis**

139 EV Proteins from 4 healthy individuals, 4 BHD and 4 mOL-HDF patients were extracted using the
140 RIPA buffer (Sigma), supplemented with proteases and phosphatase inhibitors (Sigma) and then
141 quantified using the Bradford assay (Biorad). Briefly, 10 µg of EV proteins were subjected to SDS-
142 PAGE, transferred onto nitrocellulose membranes and underwent immunoblotting with antibodies
143 directed to anti-CD63 (1:200, Santa Cruz), anti-CD9 (1:2000, Abcam) and normalized to Actin
144 (1:200, Santa Cruz). The same procedure was followed for HUVEC treated with EV from the
145 different experimental groups using IGF1R antibody (1:200, Santa Cruz).

146 **Cell culture**

147 Human umbilical vein-derived endothelial cells (HUVEC) were obtained by ATCC (PCS-100- 010-
148 ATCC, Manassas VA). HUVEC were plated with EBM medium supplemented with 10% fetal calf
149 serum (FCS – GE Health Care, Boston MA) and different endothelial growth factors as previously
150 described (24). Human Vascular Smooth Muscular Cells (VSMC) were obtained by ATCC (CRL-
151 1999, ATCC) and grown in Dulbecco Modified Eagle Medium (DMEM – GE Health Care) with
152 10% of FCS. Both cell types represent an accepted standard to investigate vascular uremic
153 dysfunction (25–28).

154 **RNA extraction and quantitative RT-PCR**

155 Total RNA from HUVEC, VSMC or patients' EV, was extracted using mirVana kit (Life
156 Technologies, USA), and analyzed by NanoDrop1000 spectrophotometer; samples of absorbance at
157 260/280 nm between 1.8 and 2.0 were adopted. We evaluated RUNX2 mRNA expression in VSMC
158 by using High cDNA Reverse Transcription Kit and the Power SYBR Green PCR Master Mix on
159 StepOnePlus Real Time System (Applied Biosystems, USA). We used the web platform Protein
160 Quest (Biodigital Valley, Italy) to electronically screen the literature and to identify miRNA
161 associated with uremic vascular dysfunction. Identified miRNA were detected in patients' EV using
162 miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen): 1ml of blood was
163 used for each quantification experiment. Primers of the selected miRNA (hsa-miR-17-5p, -92-a, -
164 223, -423-5p, -451a), RUNX2 and housekeeping transcripts (actin- β and RNU48) are displayed in
165 Supplemental Table 1. Change in RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The
166 miR-223 content was also analyzed in HUVEC or VSMC after transfection with specific mimic or
167 antagomiR.

168 ***In vitro* angiogenesis of endothelial cells**

169 HUVEC were seeded into Matrigel coated wells ($1,5 \times 10^4$ cells/well) in EBM with or without
170 patients' EVs. VEGF (10 ng/ml) and uremic toxins (ADMA 10 μ g/ml, p-cresyl sulphate 1 μ g/ml
171 and indoxyl sulphate 10 μ g/ml) were used as positive or negative control, respectively.
172 Experimental results were recorded with a Nikon-inverted microscope after 24 hours of incubation
173 with different stimuli at 37° C. Image analysis of capillary-like structures was performed using the
174 ImageJ Analysis System. Results were given as average number of capillary-like structures/field
175 \pm 1SD (magnification x10). In all the assays, cells were stimulated with EV isolated from 1 ml of
176 patients' blood for each 10 ml of medium.

177 ***In vitro* apoptosis of endothelial cells**

178 HUVEC were cultured in 96 flat-bottom microtiter plates at a concentration of 2×10^4 cells/well.
179 Cells were examined after 24 hours of stimulation at 37°C. VEGF and uremic toxins were used as
180 negative or positive control, respectively. HUVEC were re-suspended in 100 μ l of RPMI 1% BSA,

181 then mixed with 100 μ l of Muse Annexin V & Dead Cell reagent, incubated for 20 min RT, and
182 analyzed by the Muse Cell Analyzer (Millipore). In all the assays, cells were stimulated with EV
183 isolated from 1 ml of patients' blood for each 10 ml of medium.

184 **Osteoblastic differentiation of vascular smooth muscle cells (VSMC)**

185 Vascular smooth muscle cells (VSMC) were fixed in 50% ethanol at RT for 10 min, stained with 10
186 mg/ml alizarin red for 5 min and washed twice in PBS. Retained dye was extracted with a solution
187 of 20% methanol and 10% acetic acid; the absorbance at 450 nm was then measured (29).

188 **Cell transfection**

189 Transfection of miR-223 mimic (10 nM) or miR-223 inhibitor (100 nM) was performed on HUVEC
190 or VSMC using HiPerfect Transfection method (Qiagen, Valencia, USA). The expression of miR-
191 223 was evaluated by qRT-PCR. Data were expressed as Log of Rq, normalized to RNU-48.

192 Direct transfection of miR-223 inhibitor was performed co-incubating miR-223 inhibitor and BHD-
193 EV followed by HUVEC or VSMC treatment. Angiogenesis and calcification effects were
194 evaluated as reported.

195 **Statistical analysis**

196 Unless otherwise indicated, all data are shown as mean \pm SEM. Statistical analysis was performed
197 using the unpaired Student's t-test, ANOVA, or Kruskal-Wallis test when appropriate. A two-sided
198 value of $p=0.05$ was considered significant.

199

200 **RESULTS**

201 **Clinical and dialysis parameters**

202 The main clinical characteristics of enrolled patients are described in Table I: no significant
203 differences were found between BHD and mOL-HDF patients in terms of gender, age, prevalence
204 of hypertension, diabetes and cardiovascular diseases, dialysis vintage and vascular access type.

205 As expected from previously published randomized clinical trials, the switch from BHD to mOL-
206 HDF was associated with an improvement of inflammatory parameters: indeed, we observed a
207 significant decrease in plasma CRP ($p=0.05$), ferritin ($p=0.04$), IL6 ($p<0.001$) and NGAL ($p=0.007$)
208 in mOL-HDF patients between T0 and T1 (Figure 2). Consistently, the modulation of the
209 inflammatory state was confirmed by the improvement of erythropoietin resistance index (ERI)
210 ($p=0.05$). A decrease of β 2-microglobulin not reaching statistical significance ($p=0.12$) was also
211 observed. No significant differences were found in hemoglobin levels, transferrin saturation and
212 homocysteine (not shown). By contrast, patients maintained in BHD did not show significant
213 changes in any of the measured parameters. To exclude possible confounding factors, we also
214 analyzed different clinical/therapeutic variables including nutritional and hemodynamic status,
215 serum levels of calcium, phosphate and PTH, intravenous use of iron, treatment with statins, RAAS
216 antagonists or vitamin D (reported in detail in Table II) and intradialytic variables (filter surface,
217 heparin dose, treatment duration, blood and dialysis solution flow, transmembrane pressure, dialysis
218 efficiency and convective volumes reported in details in Table III) at all the time points considered.
219 As expected, mOL-HDF treatment was associated with a higher transmembrane pressure and with
220 an improved eKt/V . All the other variables were comparable in the 2 groups at all considered time
221 points.

222 **Analysis of plasma EV**

223 At study admission (T0), dialysis patients showed higher plasma EV concentration than healthy
224 subjects and EV concentration did not change through the study time points within the groups
225 (Figure 3A). EV size distribution showed similar results among the different groups with a mean

226 size of 170 nm (Figure 3B). Moreover, EV expressed typical exosomal markers such as CD9 and
227 CD63 (Figure 3C). Results concerning EV concentration were confirmed in a further cohort of 80
228 patients undergoing chronic hemodialysis (n=50 in BHD and n=30 in post-dilution OL-HDF), 10
229 patients in peritoneal dialysis and 20 patients with K-DOQI stage IV CKD. Hemodialysis patients
230 were further classified according to vascular access type (AVF vs. CVC): all groups were matched
231 for age and gender. CKD stage IV and all peritoneal and hemodialysis patients showed a 5-fold
232 increase of circulating plasma EV in comparison to healthy subjects (Figure 3D).

233 Guava FACS analysis of EV from dialysis patients at T0 revealed that the majority of circulating
234 microparticles derived from platelets, monocytes/macrophages and endothelial cells; only
235 endothelial EV were upregulated in comparison to healthy subjects (Figure 4A and Supplemental
236 Fig. 1). In both groups, no changes in terms of cell-specific markers were found across the study
237 time points (not shown). Of interest, plasma EV of enrolled patients expressed surface markers
238 involved in inflammation, atherosclerosis, complement and coagulation activation (CD40-Ligand,
239 ICOS, Fas-Ligand, C5b-9, Tissue Factor - Figure 4B and Supplemental Fig. 2). Consistently, an
240 increased percentage of endothelial-derived particles was observed in all the different control
241 groups (stage IV CKD, peritoneal dialysis or hemodialysis independently from vascular access type
242 or dialysis modality - Figure 4C).

243 **Characterization of microRNA content of plasma EV**

244 By Protein Quest analysis (Figure 5A), 5 different miRNAs involved in endothelial dysfunction and
245 vascular calcification were identified (miR-17-5p, miR-92a, miR-223, miR-423-5p, miR-451). The
246 expression of these miRNAs was analyzed through all the study time points by qRT-PCR. The
247 expression of miR-17-5p, miR-92a, miR-423-5p, miR-451 was not significantly different among
248 healthy control, BHD- and mOL-HDF-derived EV (Figure 5B-E). By contrast, EV derived from
249 dialysis patients showed an increased expression of miR-223 at T0 if compared to healthy controls;
250 subsequently, mOL-HDF patients displayed a significant decline of EV miR-223 expression that
251 was not observed in BHD patients and was maintained at all the time points considered (Figure 5F).

252 Patients from the control cohort treated by post-dilution OL-HDF had reduced levels of EV-carried
253 miR-223 if compared to stage IV CKD patients, peritoneal dialysis patients and BHD-treated
254 patients (Figure 5G).

255 **Role of plasma EV-carried miR-223 in endothelial dysfunction**

256 Since we identified a decreased expression of miR-223 in EV derived from mOL-HDF patients in
257 respect to BHD patients, we performed *in vitro* experiments to evaluate the specific role of this
258 miRNA in endothelial dysfunction. In respect to EV collected from healthy subjects, BHD-derived
259 EV reduced the formation of capillary-like structures by HUVEC (Figure 6A). This anti-angiogenic
260 effect was similar to what observed in presence of known uremic toxins (ADMA, p-cresyl sulphate,
261 indoxyl sulphate). HUVEC angiogenesis was significantly higher with mOL-HDF-derived EV than
262 with BHD-EV, even if not reaching the level observed with healthy EV. Similar results were also
263 observed in experiments aimed to evaluate HUVEC apoptosis (Figure 6B).

264 To investigate whether miR-223 was involved in the anti-angiogenic and pro-apoptotic activities of
265 plasma-EV, we evaluated the effect of specific mimic and antagomiR transfection, respectively.
266 HUVEC transfected with mimic miR-223 showed a significant reduction of *in vitro* angiogenic
267 response when challenged with healthy plasma-EV as well as with EV derived from dialysis
268 patients. Conversely, transfection of HUVEC with miR-223-antagomiR significantly restored the
269 angiogenic potential of plasma-EV, particularly in presence of BHD-derived EV (Figure 6C). The
270 levels of expression of miR-223 in all experimental conditions were verified by qRT-PCR
271 (Supplemental Fig. 3). Transfection of AntagomiR-223 was also used in order to inhibit miR-223
272 directly in BHD-EV. As shown in Figure 6D, transfected BHD-EV significantly restored pro-
273 angiogenic properties on target cells indicating that EV can deliver miR-223.

274 **Role of plasma EV-carried miR-223 in vascular smooth muscle cell calcification**

275 We also investigated the role of EV-carried miR-223 in osteoblastic differentiation of VSMC. As
276 shown by red alizarin staining and RUNX2 expression, EV derived from BHD patients induced an
277 increase of VSMC calcification (Figure 7A, B, C). By contrast, EV derived from mOL-HDF

278 patients did not increase VSMC osteoblastic differentiation when compared to healthy subject
279 particles. VSMC transfected with mimic miR-223 showed an increase of osteoblastic differentiation
280 when incubated with healthy plasma-EV as well as with plasma-EV derived from BHD and mOL-
281 HDF patients. Conversely, transfection of VSMC with miR-223-antagomiR significantly reduced
282 the calcification potential of plasma-EV, particularly in presence of BHD derived EV (Figure 7D).
283 Moreover, direct transfection of miR-223-inhibitor in BHD-EV led to a significant decrease of
284 calcification potential induced by BHD-EV (Figure 7E).

285 **Evaluation of miR-223 target genes**

286 To investigate the role of miR-223 in endothelial dysfunction, we analyzed the potential target
287 genes in HUVEC. The network predicted by IPA for miR-223 showed IGF1R as the most likely
288 target (Figure 8A). We then evaluated whether miR-223 delivered by BHD-EV may modulate
289 IGF1R expression in HUVEC. Western blot analysis demonstrated that treatment of HUVEC with
290 BHD-EV significantly reduced IGF1R expression when compared to cells stimulated with BHD-
291 EV + inhibitor-miR-223 (Figure 8B, C). A similar decrease was also observed when HUVEC were
292 incubated with mimic-223, thus confirming miR-223 action on IGF1R. Although a previous study
293 suggested β 1-integrin as a further miR-223 target, we did not find any expression change of this
294 protein in HUVEC (not shown) (30).

295

296 **DISCUSSION**

297 In this study, we evaluated the quantitative and qualitative changes of plasma EV in a population of
298 patients with end stage CKD after switching from high flux BHD to mOL-HDF. We found that
299 mOL-HDF was associated with a modulation of EV miR-223; additionally, our *in vitro* data
300 suggested a role of miR-223 in CKD-related endothelial dysfunction and vascular calcification. As
301 already reported in previous studies (2, 5–7), the use of a mixed convective-diffusive
302 hemodepuration led to a significant decrease of inflammatory parameters such as CRP, ferritin, IL6,
303 NGAL and erythropoietin resistance index (ERI). NGAL is a 25 KDa protein belonging to the
304 calycin family that has been proposed as marker of inflammation and reduced iron bioavailability in
305 hemodialysis patients (31, 32).

306 Systemic inflammation is a hallmark of CKD and plays a key role in the development and
307 progression of cardiovascular diseases. HDF with high convection volumes is currently considered
308 the most effective technique for achieving a better clearance of middle molecules in patients with
309 end stage CKD (5). Several studies have shown that HDF improves intradialytic hemodynamic
310 stability and some CKD complications such as inflammation, malnutrition, erythropoietin resistant
311 anemia, and dialysis-associated amyloidosis. Large observational studies (DOPPS, Euclid,
312 Riscavid) and RCT (Eshol) demonstrated an improved overall and cardiovascular survival in
313 patients treated with HDF vs. BHD (7, 8, 33, 34). Basing on these studies, higher convective
314 volumes are probably responsible for an enhanced clearance of middle/large uremic toxins involved
315 in the development of cardiovascular complications and anemia (35). Post-dilution HDF is the most
316 efficient infusion mode to maximize clearance of small and large solutes; nevertheless, this
317 approach increases the frequency of technical problems due to hemoconcentration and high
318 transmembrane pressure. The simultaneous use of pre- and post-dilution offered by mOL-HDF may
319 avoid the disadvantages of traditional infusion modes. Indeed, in mOL-HDF pre and post-infusion
320 percentage is automatically regulated through transmembrane pressure and ultrafiltration feedback
321 (36). Recent studies compared the clearance of small, medium-sized and protein-bound molecules

322 and the convective volume administered in standard post-dilution OL-HDF vs. mOL-HDF without
323 finding any significant difference. Moreover, mOL-HDF allows achieving a satisfactory convective
324 volume exchange even in patients with suboptimal blood flow rates due to vascular access problem
325 (20).

326 The influence of uremia-related inflammation on circulating plasma EV has been poorly
327 investigated. It is well established that EV represent an important vehicle of intercellular
328 communication due their ability in transporting molecules such as proteins, surface receptors, lipids,
329 mRNA and, particularly, miRNA (11, 37). A significant increase of plasma EV is associated with
330 several inflammatory diseases characterized by endothelial dysfunction and vascular calcification
331 (19). In the present study, we found that CKD patients treated by high flux BHD or mOL-HDF
332 presented higher levels of plasma EV when compared to healthy subjects matched for age and
333 gender. Moreover, the analysis of surface antigens revealed the increase of EV expressing typical
334 endothelial markers, suggesting the presence of ongoing microvascular damage. Furthermore, EV
335 surface protein analysis revealed the presence of molecules involved in coagulation and
336 complement cascades (Tissue Factor, C5b-9) and in the pathogenic mechanisms of inflammation
337 and atherosclerosis: CD40-Ligand, ICOS and Fas-Ligand. These latter receptors are deeply
338 involved in destabilization of atherosclerotic plaques: ICOS/ICOS-Ligand activation has been
339 recently shown to play a key role in the pro-atherogenic activation of the T follicular / B-cell axis
340 (38); similarly, both CD40 and CD40-Ligand are largely expressed in atherosclerotic plaques and in
341 vascular calcifications of uremic patients (22, 39). In addition, the blockade of CD40/CD40-Ligand
342 pathway led to a significant reduction of vascular damage in atherosclerosis-prone mice (40). Last,
343 our research group previously demonstrated that soluble CD40-Ligand levels are predictive of
344 combined cardiovascular morbidity and mortality in dialysis patients (41). Basing on these
345 considerations, one could speculate that CD40-Ligand, ICOS and Fas-Ligand expressed by uremic
346 EV may directly activate the counter-receptors expressed on endothelial cells and smooth muscle
347 cells, leading to plaque destabilization. However, different studies showed that the biological

348 activities of EV are mainly ascribed to the transfer of RNA to target cells (37, 42, 43). In the present
349 study, although we did not detect any significant difference between the two dialysis modalities in
350 terms of concentration, size and surface antigens, we observed that EV isolated after switching to
351 mOL-HDF presented a variation of miRNA content. Of interest, after the switch to mOL-HDF we
352 found a decreased expression of miR-223 which is potentially involved in endothelial dysfunction
353 and vascular calcification (31, 44–49) and is considered a biomarker of atherosclerosis progression
354 (45, 50). Indeed, miR-223 inhibits angiogenesis by targeting β 1-integrin and by preventing insulin
355 like growth factor 1 signaling in endothelial cells (30); moreover, a recent study reported that miR-
356 223 is also involved in osteoblast differentiation of VSMC (51).

357 The decrease of EV miR-223 found in mOL-HDF group was also observed in another cohort of
358 hemodialysis patients regularly treated by post-dilution OL-HDF: this reduction was independent
359 from the vascular access type and not observed in peritoneal dialysis patients and BHD-treated
360 patients. These findings suggest that high volume HDF is responsible for modulation of pro-
361 inflammatory and pro-atherogenic miRNA expression within plasma EV.

362 Furthermore, in our *in vitro* experiments, the increased expression of miR-223 in EV from BHD
363 patients was associated with HUVEC angiogenesis inhibition, increased endothelial apoptosis and
364 enhanced VSMC calcification. Of note, all these detrimental effects were down regulated by mOL-
365 HDF. Consistently, experiments based on miR-223 mimic or antagomiR confirmed the relevance of
366 this transcript in the biological activity of BHD-derived EV. Finally, we found that EV-carried
367 miR-223 may modulate different intracellular pathways involved in endothelial angiogenesis and
368 apoptosis including IGF1R, as suggested by IPA network analysis.

369 In conclusion, the results of the present study indicate that EV derived from hemodialysis patients
370 express surface proteins and carry miRNAs involved in inflammation-related endothelial
371 dysfunction and vascular calcification. Switching from BHD to mOL-HDF significantly decreased
372 the expression of EV miR-223 but not of other miRNAs. The *in vitro* experiments indicated that the
373 reduced expression of miR-223 justifies, at least partially, the decreased detrimental effect of

374 plasma EV from mOL-HDF patients. These results may contribute to explain the protective effects
375 on endothelial dysfunction and vascular calcification observed in OL-HDF clinical studies.

376

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378 None

379 **REFERENCES**

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544

545

546 **FIGURE LEGENDS**

547 **Figure 1: Study flow chart**

548 **Figure 2: Clinical and intradialytic parameters:** Trend lines demonstrate the values of (A) β 2-
549 microglobulin, (B) C-reactive protein - CRP, (C) IL6, (D) serum iron, (E) transferrin saturation, (F)
550 ferritin, (G) Neutrophil Gelatinase Associated Lipocalin - NGAL, (H) hemoglobin and (I)
551 erythropoiesis resistance index - ERI across the study time points. *: $p < 0.05$ vs. T0, BHD: standard
552 bicarbonate hemodialysis, mOL-HDF: mixed on-line hemodiafiltration, T0: study start, T1, 2 and 3:
553 3, 6 and 9 months of treatment start.

554 **Figure 3: Nanosight and western blot analysis of plasma extracellular vesicles (EV).** (A)
555 Plasma extracellular vesicles (EV) concentration at the different study time points in patients
556 randomized to BHD (n=15) or mOL-HDF (n=15) and in a group of healthy subjects (only T0). (B)
557 Representative Nanosight Tracking Analysis plots and medium EV size for each healthy subject,
558 BHD and mOL-HDF patient enrolled. (C) Representative WB analysis of CD9, CD63 and β -actin
559 protein content in healthy-, BHD- and mOL-HDF-EV. (D) EV Quantification in the control cohort
560 of healthy subjects (n=10), stage IV CKD (n=20), peritoneal dialysis (PD) (n=10), BHD
561 hemodialysis (n=50) or post-dilution OL-HDF patients (n=30). Hemodialysis patients were
562 subdivided according to vascular access type (arteriovenous fistula - AVF, n=57; central venous
563 catheter - CVC, n=23). Statistical analysis was performed by ANOVA with Newman-Keuls
564 multicomparison test. *: $p < 0.05$ vs. healthy, BHD: standard bicarbonate hemodialysis, (m)OL-
565 HDF: (mixed) on-line hemodiafiltration, T0: study start, T1, 2 and 3: 3, 6 and 9 months of treatment
566 start.

567 **Figure 4: Guava FACS analysis of plasma extracellular vesicles (EV).** (A) Histograms showing
568 the positivity rate (%) for exosome, platelet, monocyte/macrophage, T-cell, B-cell and endothelial
569 cell markers, (see Material and Methods) in EV derived from healthy subjects, BHD or mOL-HDF
570 patients at study start. *: $p < 0.05$ vs. healthy. (B) Histograms showing the positivity rate (%) for

571 marker of uremia related atherosclerosis and inflammation in EV derived from healthy subjects,
572 BHD or mOL-HDF patients at study start. (C) Quantification of endothelial-derived EV in the
573 control cohort of healthy subjects (n=10), stage IV CKD (n=20), peritoneal dialysis (PD) (n=10),
574 BHD hemodialysis (n=50) or post-dilution OL-HDF patients (n=30). Hemodialysis patients were
575 subdivided according to vascular access type (arteriovenous fistula - AVF, n=57; central venous
576 catheter - CVC, n=23). Statistical analysis was performed by ANOVA with Newman-Keuls
577 multicomparison test. *: $p < 0.05$ vs. healthy, BHD: standard bicarbonate hemodialysis, (m)OL-
578 HDF: (mixed) on-line hemodiafiltration, T0: study start, T1, 2 and 3: 3, 6 and 9 months of treatment
579 start.

580 **Figure 5: Analysis of microRNA content in plasma extracellular vesicles (EV) of hemodialysis**
581 **patients.** (A) Protein Quest web-based analysis identified 5 different miRNAs (miR-17a-5p, miR-
582 92a, miR-223, miR-423-5p, miR-451) involved in endothelial dysfunction and vascular
583 calcification. Squares represent miRNAs, whereas circles represent proteins. Node dimension
584 correlates with literature frequency of displayed molecules; line thickness represents the number of
585 literature co-occurrence between elements. (B-F) Relative quantification by real-time PCR (RT-
586 PCR) for EV expression of the 5 identified miRNAs in healthy controls, BHD- and mOL-HDF
587 patients at the different study time points. (G) qRT-PCR for miR-223 in in the control cohort of
588 healthy subjects (n=10), stage IV CKD (n=20), peritoneal dialysis (PD) (n=10), BHD hemodialysis
589 (n=50) or post-dilution OL-HDF patients (n=30). Hemodialysis patients were subdivided according
590 to vascular access type (arteriovenous fistula - AVF, n=57; central venous catheter - CVC, n=23).
591 Statistical analysis was performed by ANOVA with Newman-Keuls multicomparison test. *:
592 $p < 0.05$ vs. healthy, §: $p < 0.05$ vs. BHD, BHD: standard bicarbonate hemodialysis, (m)OL-HDF:
593 (mixed) on-line hemodiafiltration, T0: study start, T1, 2 and 3: 3, 6 and 9 months of treatment start.

594 **Figure 6: Effect of plasma extracellular vesicles (EV) on *in vitro* endothelial angiogenesis and**
595 **apoptosis.** (A) Histogram showing the quantitative analysis of the *in vitro* HUVEC angiogenesis
596 assay after different stimuli; *: $p < 0.05$ vs. CTRL, §: $p < 0.05$ vs. BHD-EV (B) Histogram showing

597 the quantitative analysis of the *in vitro* TUNEL apoptosis assay on HUVEC subjected to different
598 stimuli; *: $p < 0.05$ vs. CTRL, §: $p < 0.05$ vs. BHD-EV. (C) Quantitative analysis of angiogenesis
599 assay in HUVEC transfected with miR-223 mimic or inhibitor and stimulated with patients EV; *:
600 $p < 0.05$ vs. CTRL (D) Quantitative analysis of HUVEC angiogenesis assay after stimulation with
601 patients EV transfected or not with miR-223-inhibitor; *: $p < 0.05$ vs. BHD EV. BHD: bicarbonate
602 hemodialysis, CTRL: control group, HUVEC: human umbilical vascular endothelial cells, mOL-
603 HDF: mixed online hemodiafiltration, Uremic Toxins: ADMA 10 $\mu\text{g/ml}$ + p-cresyl sulphate 1
604 $\mu\text{g/ml}$ and indoxyl sulphate 10 $\mu\text{g/ml}$, VEGF: vascular endothelial growth factor.

605 **Figure 7: Role of plasma extracellular vesicle (EV)-carried miR-223 in osteoblastic**
606 **differentiation of vascular smooth muscle cells (VSMC).** (A) Representative images and (B)
607 relative quantification of red alizarin staining of VSMC incubated with EV derived from healthy
608 subjects, BHD or mOL-HDF patients; *: $p < 0.05$ vs. healthy EV, §: $p < 0.05$ vs. BHD EV. (C)
609 Quantitative real time PCR for RUNX2 mRNA expression in VSMC incubated with EV derived
610 from healthy subjects, BHD or mOL-HDF patients; *: $p < 0.05$ vs. healthy EV, §: $p < 0.05$ vs. BHD
611 EV. (D) Quantitative analysis of red alizarin staining in VSMC transfected with miR-223 mimic or
612 inhibitor and stimulated with patients EV; *: $p < 0.05$ vs. CTRL, §: $p < 0.05$ vs. mimic. (D)
613 Quantitative analysis of VSMC staining after stimulation with patients EV transfected or not with
614 miR-223-inhibitor; *: $p < 0.05$ vs. BHD EV. BHD: bicarbonate hemodialysis, CTRL: control group,
615 mOL-HDF: mixed online hemodiafiltration.

616 **Figure 8: Analysis of miR-223 target genes: role of IGF1R.** (A) Ingenuity IPA pathway analysis
617 predicted target genes for miR-223. Pointed arrowheads represent activating relationships whereas
618 solid or dotted edges indicate direct or indirect relationships, respectively. Relationship between
619 miR-223 and IGF1R is highlighted. (B) Representative WB analysis and (C) relative quantification
620 of IGF1R protein expression in HUVEC after stimulation with healthy, BHD or mOL-HDF patient
621 EV or after transfection with miR-223 mimic or after stimulation with BHD patient EV transfected
622 with miR-223 inhibitor. *: $p < 0.05$ vs. healthy §: $p < 0.05$ mimic-223 vs. BHD. BHD: bicarbonate

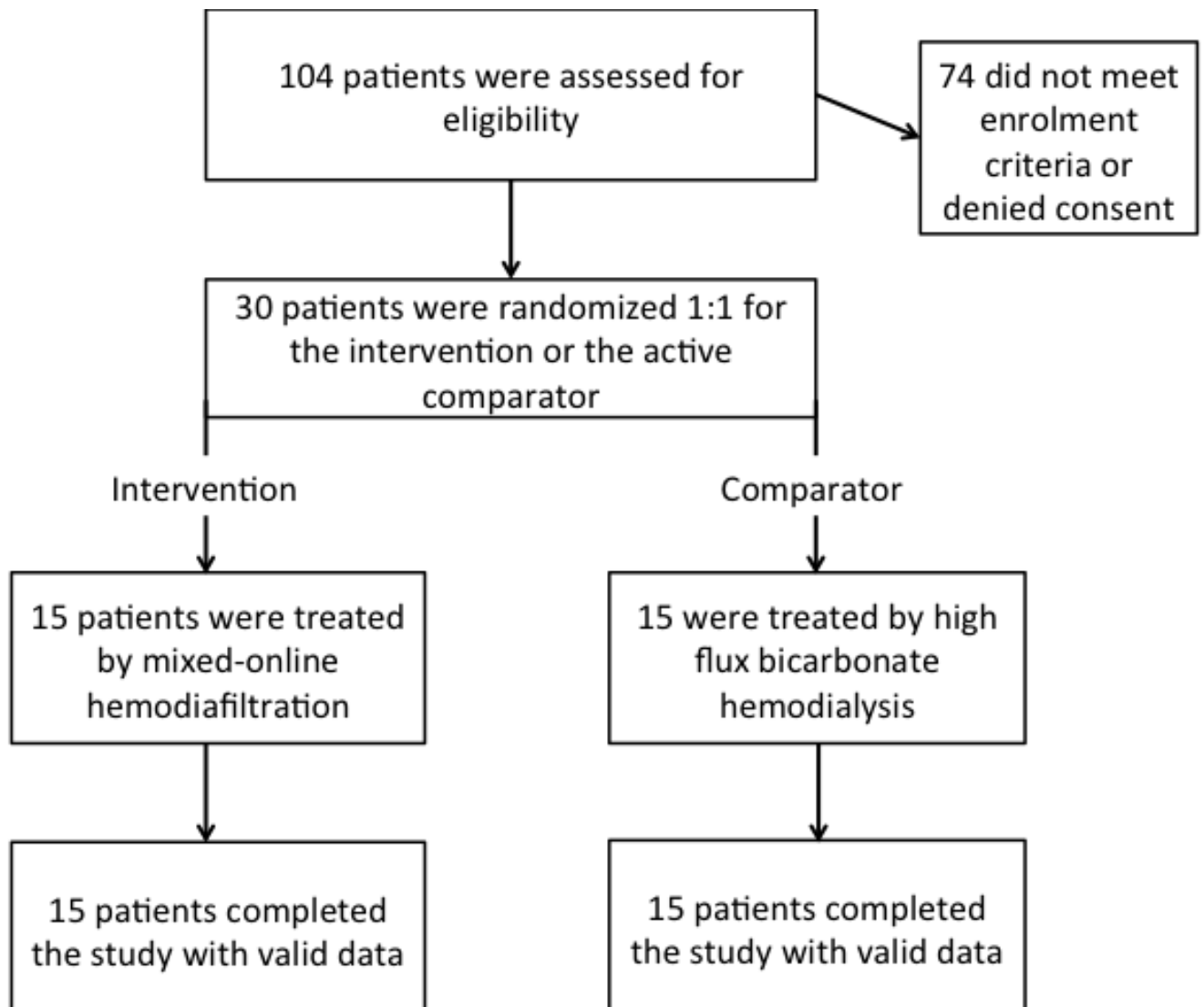
623 hemodialysis, CTRL: control group, EV: extracellular vesicles, IGF1R: insulin like growth factor 1
624 receptor, mOL-HDF: mixed online hemodiafiltration.

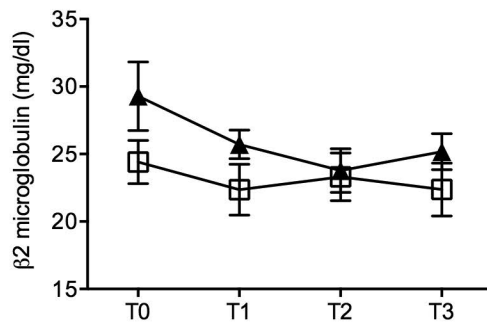
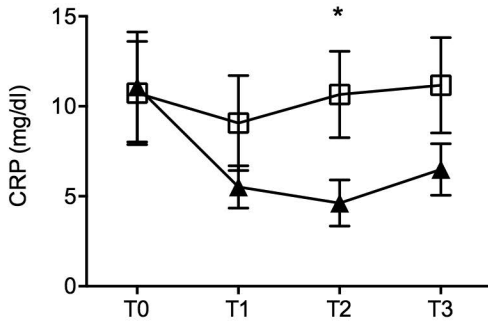
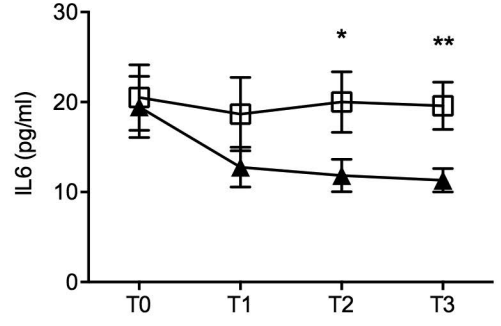
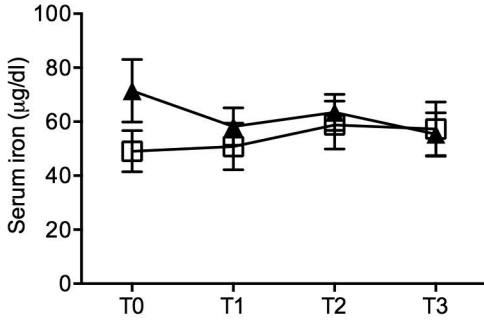
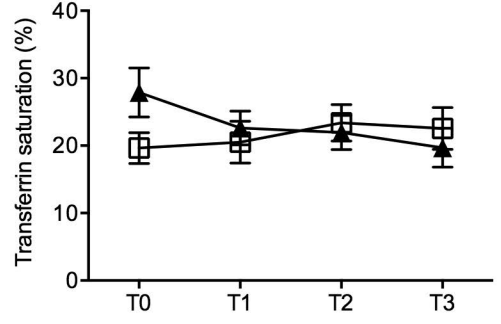
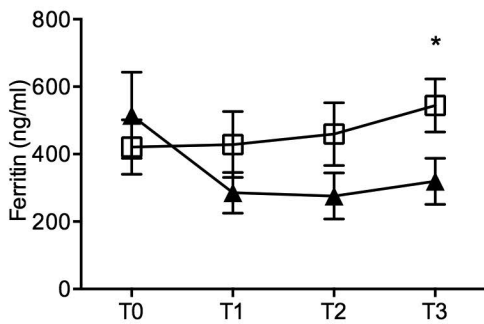
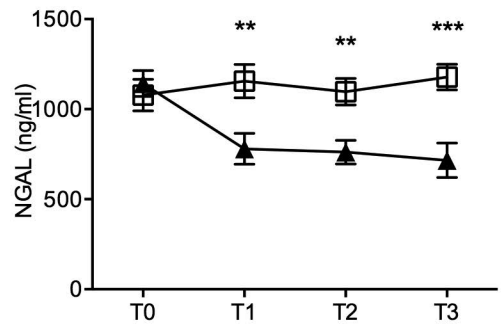
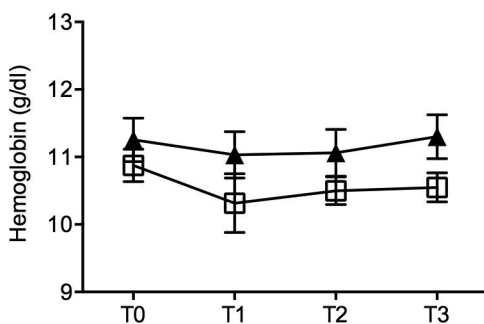
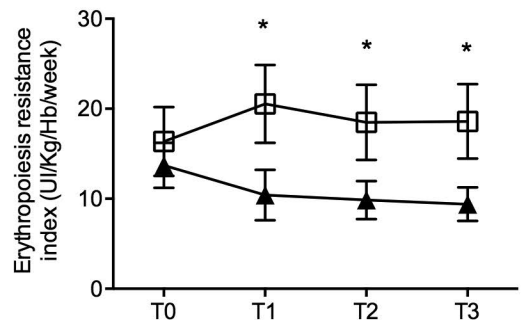
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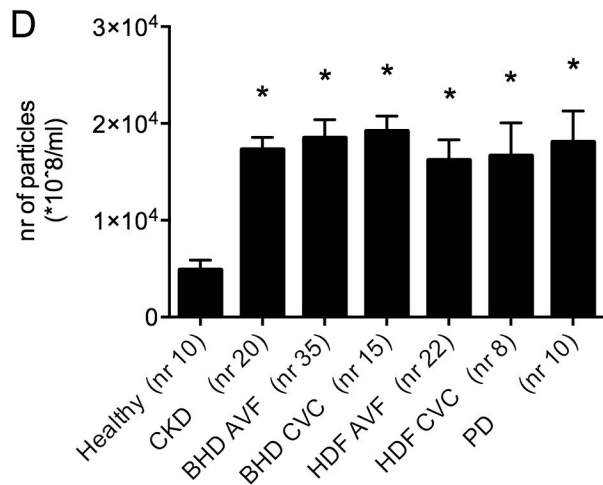
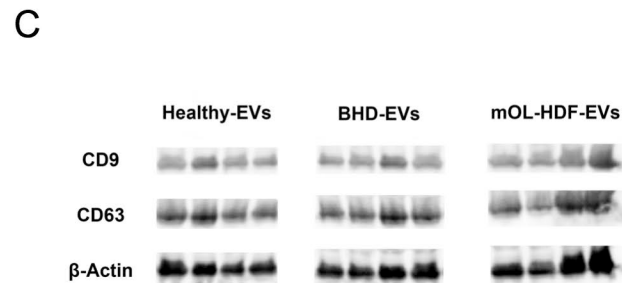
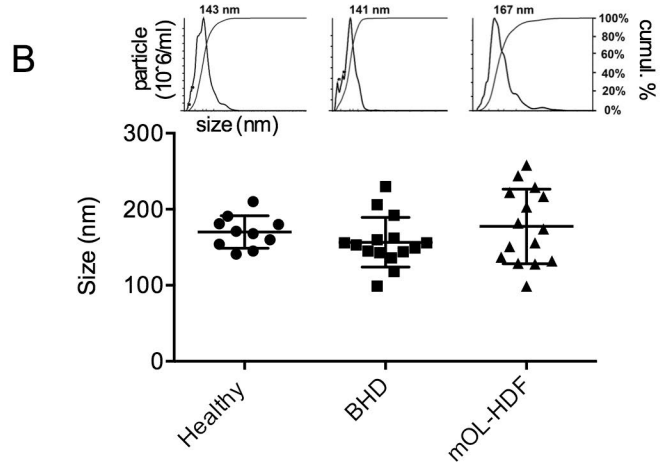
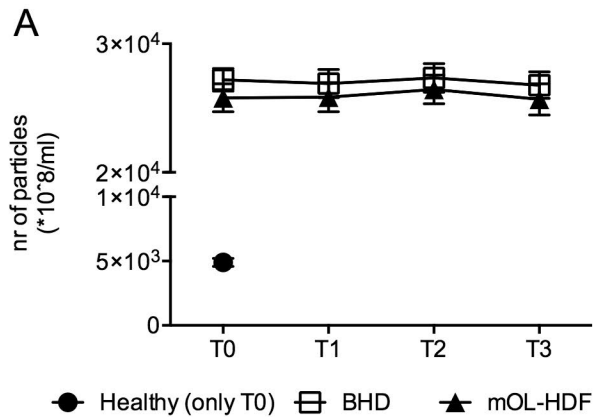
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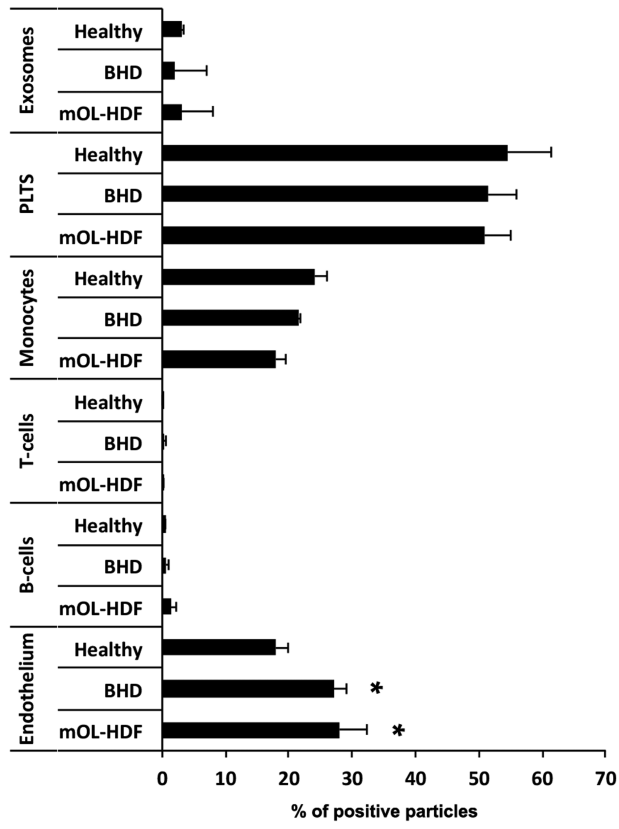
A**B****C****D****E****F****G****H****I**

BHD

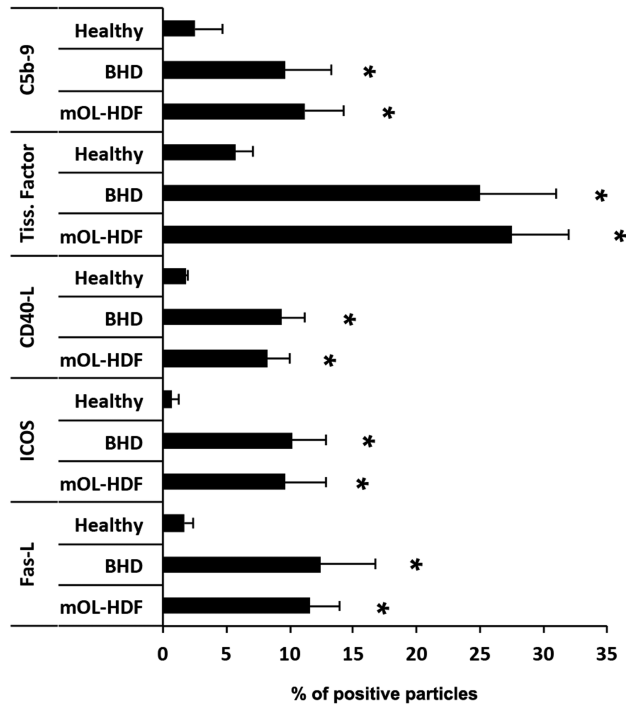
mOL-HDF



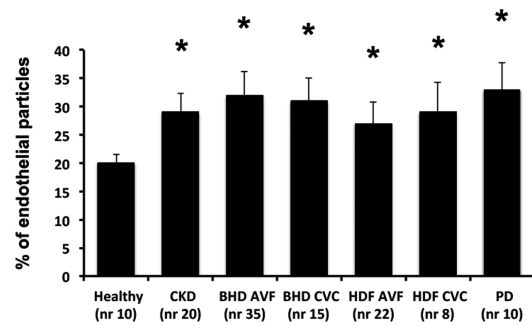
A

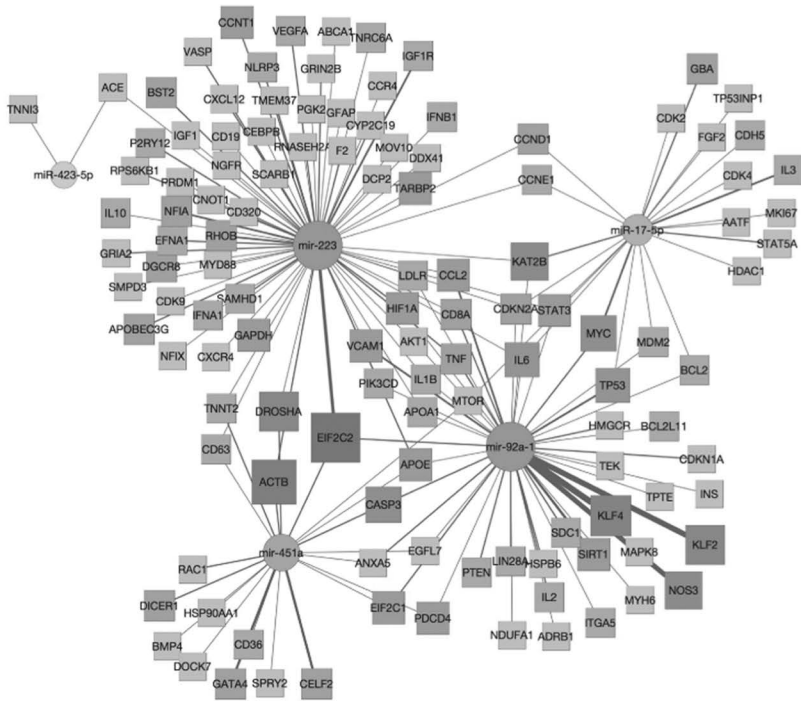
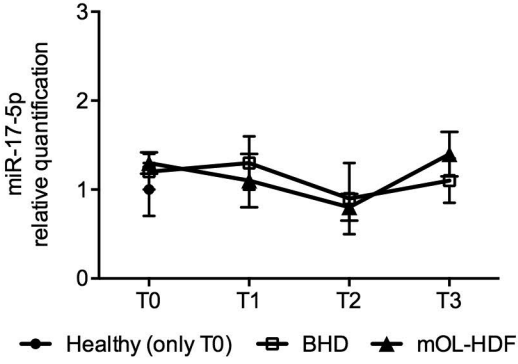
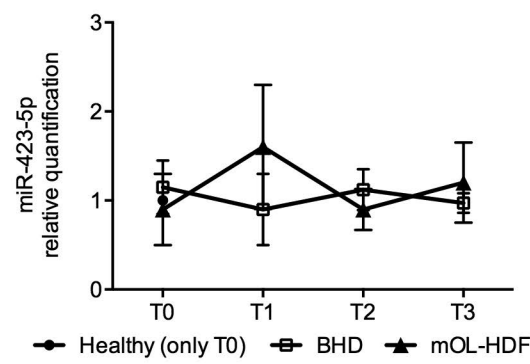
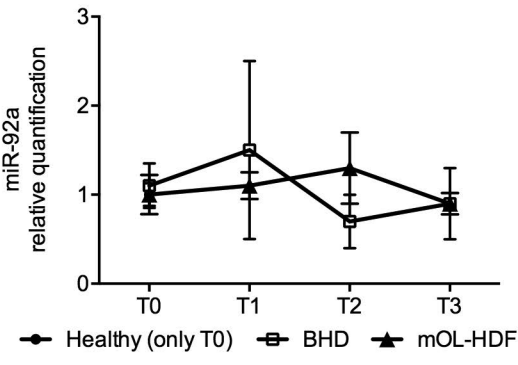
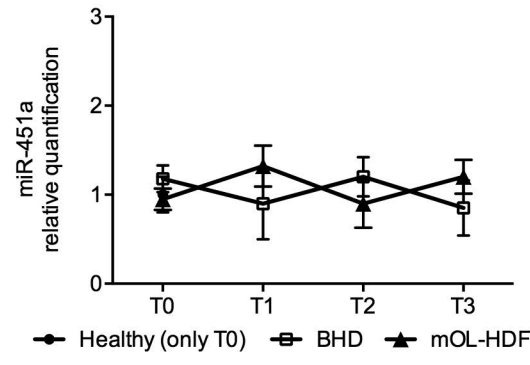
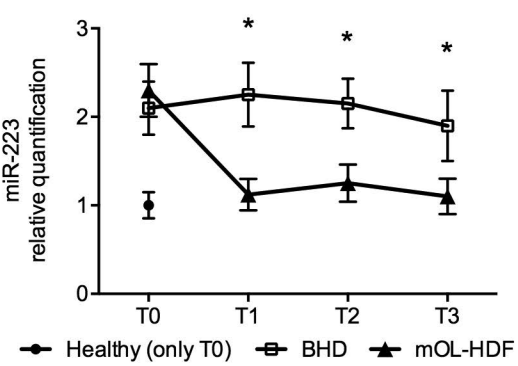
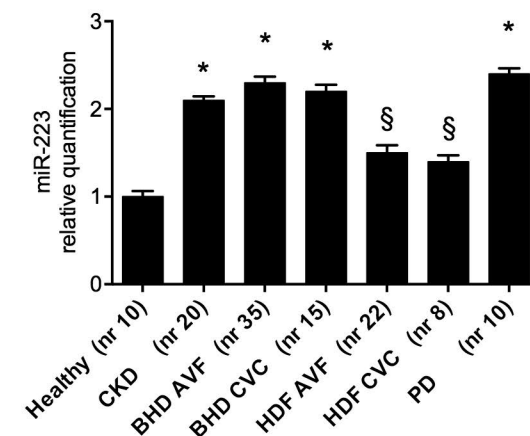


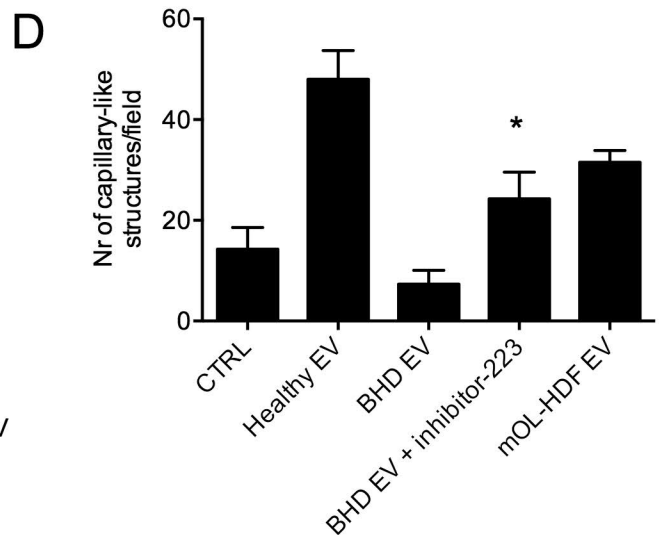
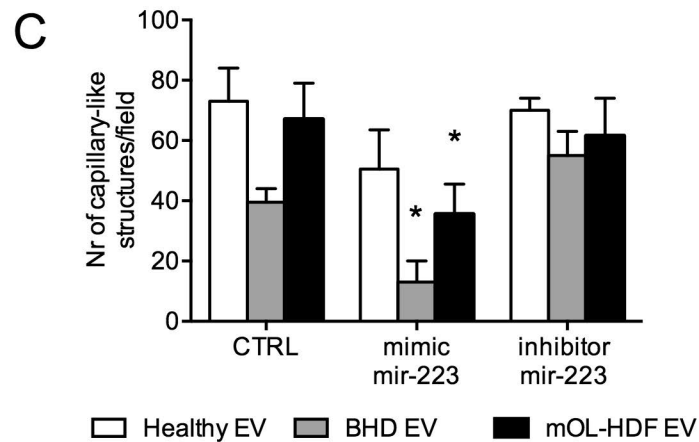
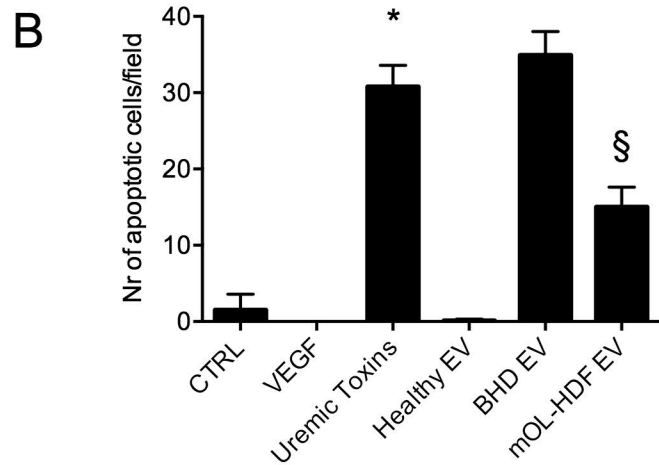
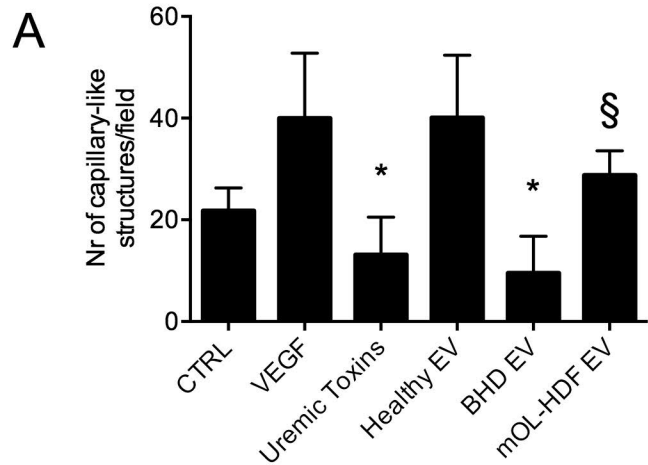
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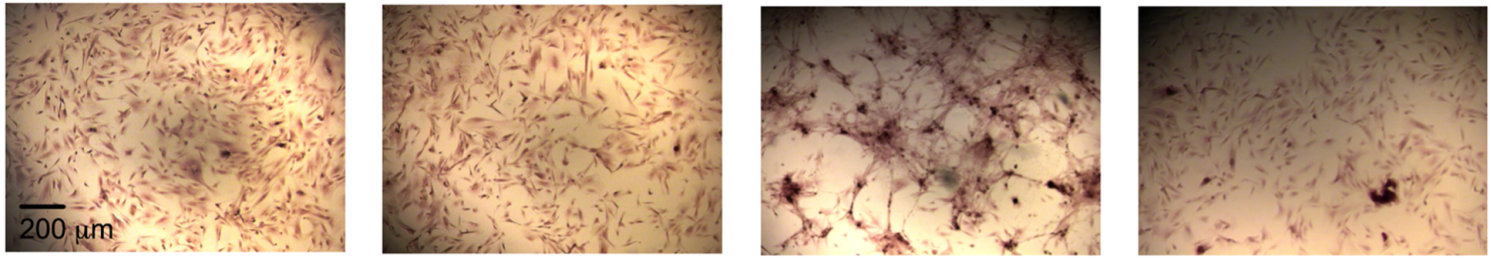


C



A**B****C****D****E****F****G**



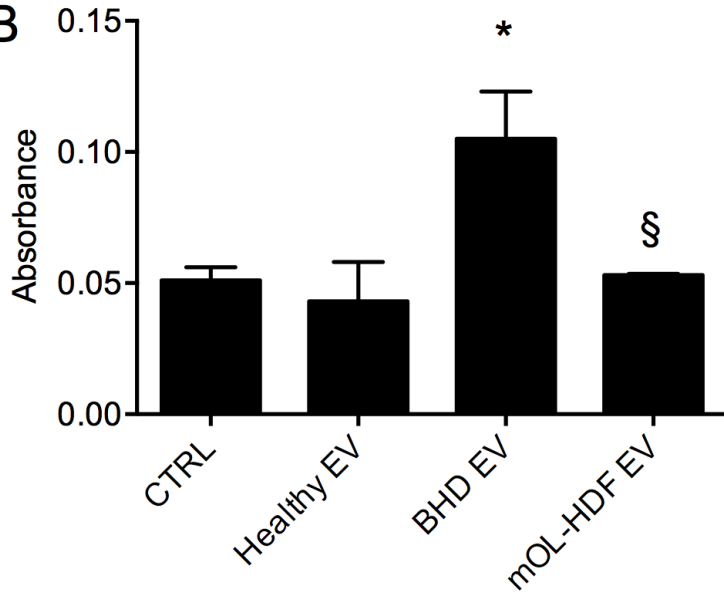
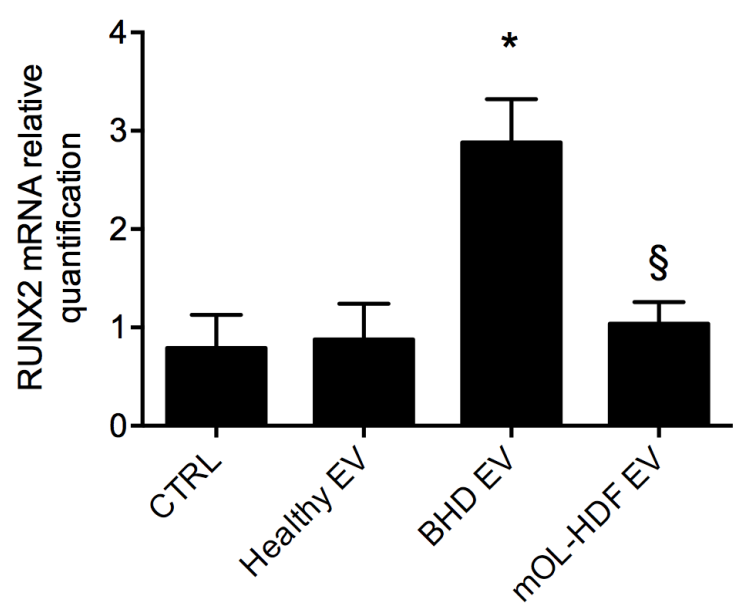
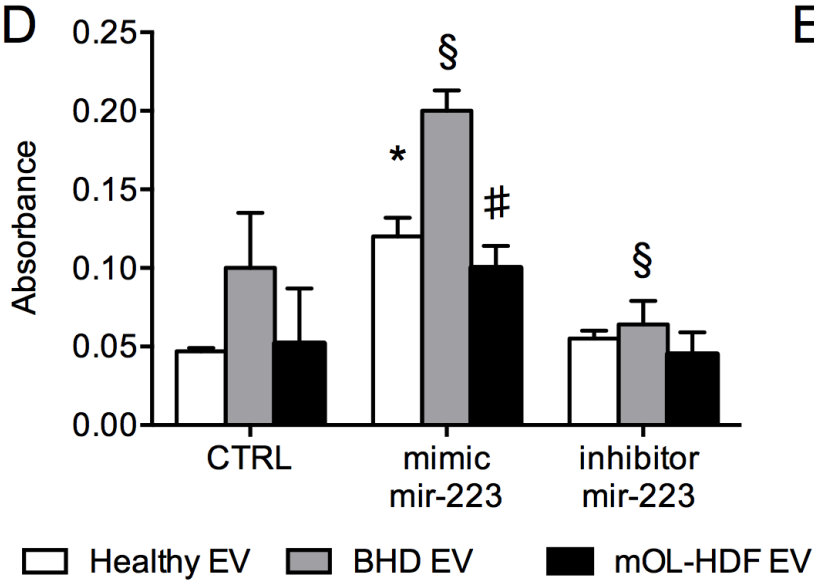
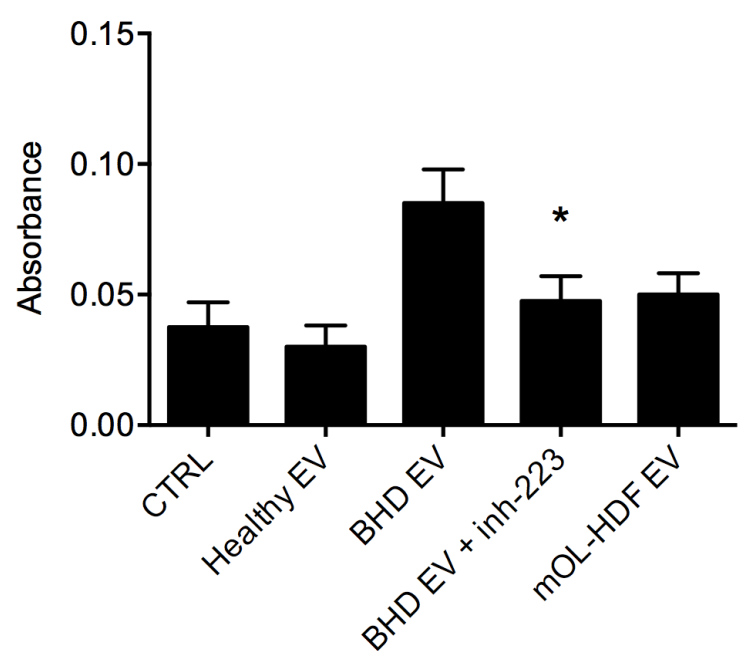
A

CTRL

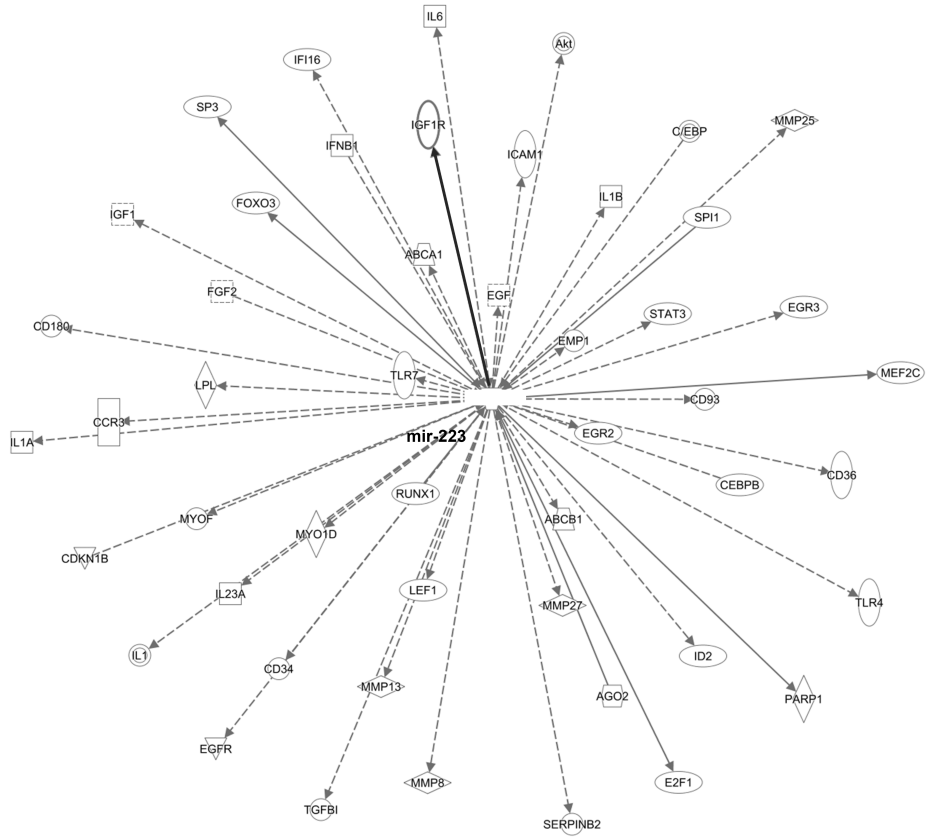
Healthy EV

BHD EV

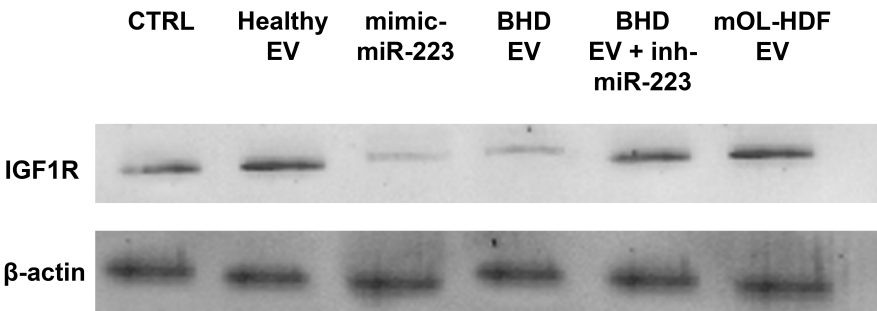
mOL-HDF EV

B**C****D****E**

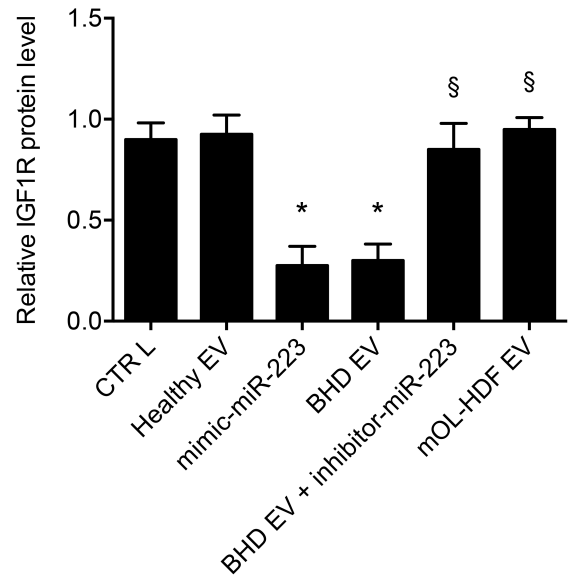
A



B



C



	mOL- HDF	BHD	<i>p</i>
Patients' Characteristics			
Gender (female)	23%	33%	0,2
Age	63,75±11	65,5±16,1	0,92
Hypertension	92%	83%	0,49
Diabetes	31%	25%	0,47
CV disease	31%	58%	0,16
Dialysis age (months)	84±75	82±112	0,93
AVF	92%	83%	0,5

Table I: Demographic characteristics, co-morbidities, dialysis age and access type of enrolled patients. AVF: arteriovenous fistula; BHD: bicarbonate Hemodialysis; CV: cardiovascular; mOL-HDF: mixed on-line hemodiafiltration.

	T0		T1		T2		T3		P (ANOVA or χ^2)		
	mOL-HDF*	BHD	mOL-HDF	BHD	mOL-HDF	BHD	mOL-HDF	BHD	Within mOL-HDF	Within BHD	All
nPCR	1,12±0,14	1,08±0,12	1,16±0,2	1,1±0,15	1,18±0,2	1,2±0,12	1,15±0,1	1,1±0,18	0,92	0,89	0,88
sPhosphate (mmol/l)	1,44±0,6	1,42±0,2	1,48±0,3	1,41±0,2	1,42±0,3	1,38±0,2	1,41±0,4	1,44±0,2	0,97	0,95	0,99
sCalcium (mmol/l)	2,38±0,2	2,36±0,3	2,36±0,2	2,37±0,2	2,40±0,3	2,33±0,4	2,39±0,3	2,38±0,2	0,82	0,79	0,74
sPTH (pg/ml)	289±205	234±156	229±167	267±172	248±164	221±188	230±127	256±201	0,43	0,36	0,28
Number of HT drugs	1,6±1,2	1,42±1,1	1,5±1,1	1,42±1,1	1,6±1,2	1,5±1,2	1,6±1,2	1,42±1,1	0,97	0,94	0,88
Iv. Iron (mg/week)	37±56	35±22	39±51	31±24	42±56	36±24	50±39	41±21	0,82	0,76	0,88
Statin use	15%	33%	38%	33%	46%	33%	46%	33%	0,31	1	0,41
RAAS blocker	23%	15%	31%	15%	31%	25%	31%	25%	0,82	0,84	0,76
Vitamin D use	100%	100%	100%	92%	100%	92%	100%	92%	1	0,92	0,88
Pre-HD PA≤140/90	69%	75%	69%	66%	84%	75%	84%	66%	0,76	0,65	0,82

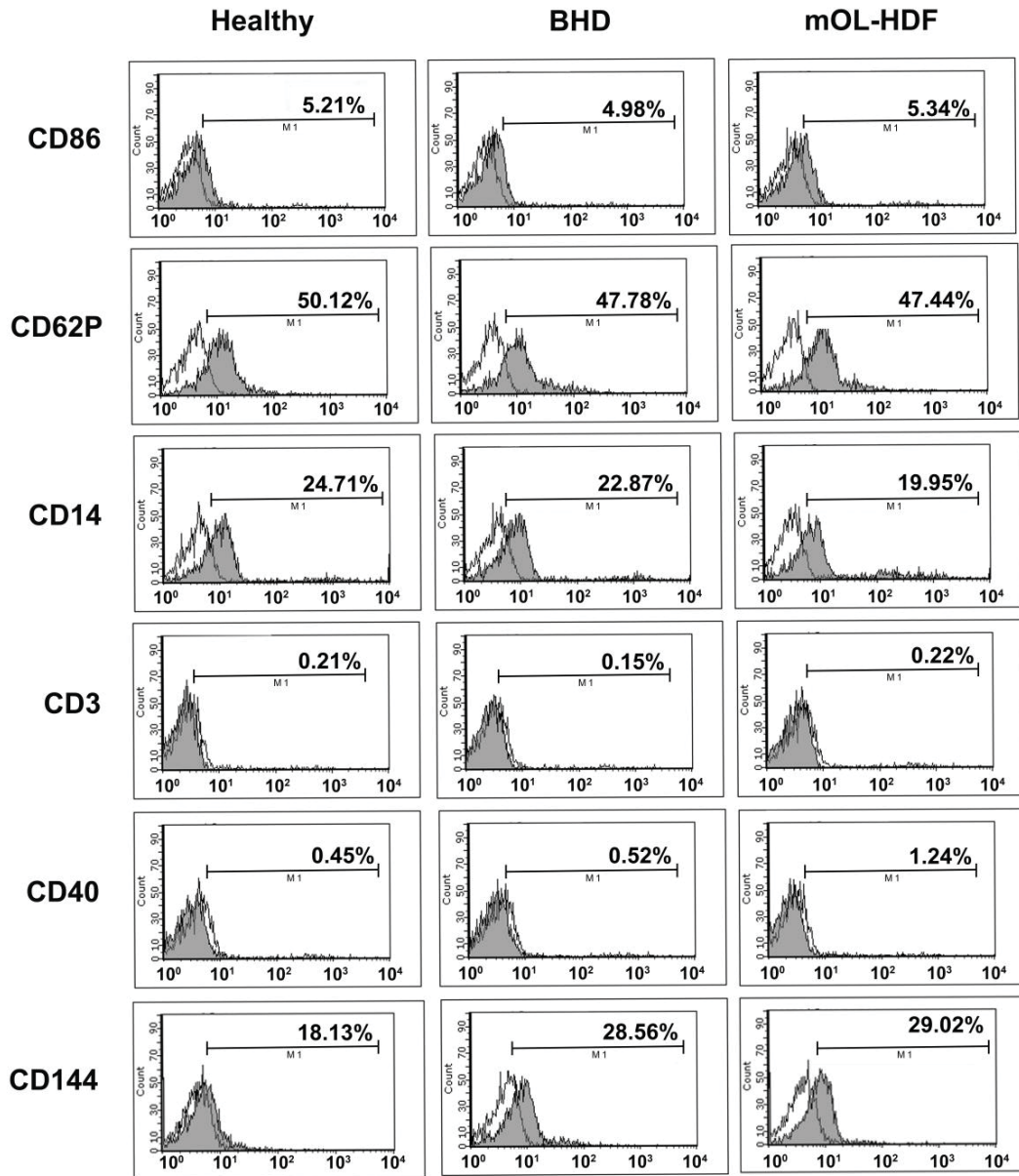
Table II: Patients' clinical variables at all considered time-points. BHD: bicarbonate Hemodialysis; HT: hypotensive; Iv: intravenous; mOL-HDF:

mixed on-line hemodiafiltration; nPCR: normalized protein catabolic rate; RAAS: renin-angiotensin-aldosterone system. *At T0, mOL-HDF group was still treated by BHD. Statistical analysis was performed by one way ANOVA or χ^2 test when appropriated: $p < 0.05$ was considered statistically significant.

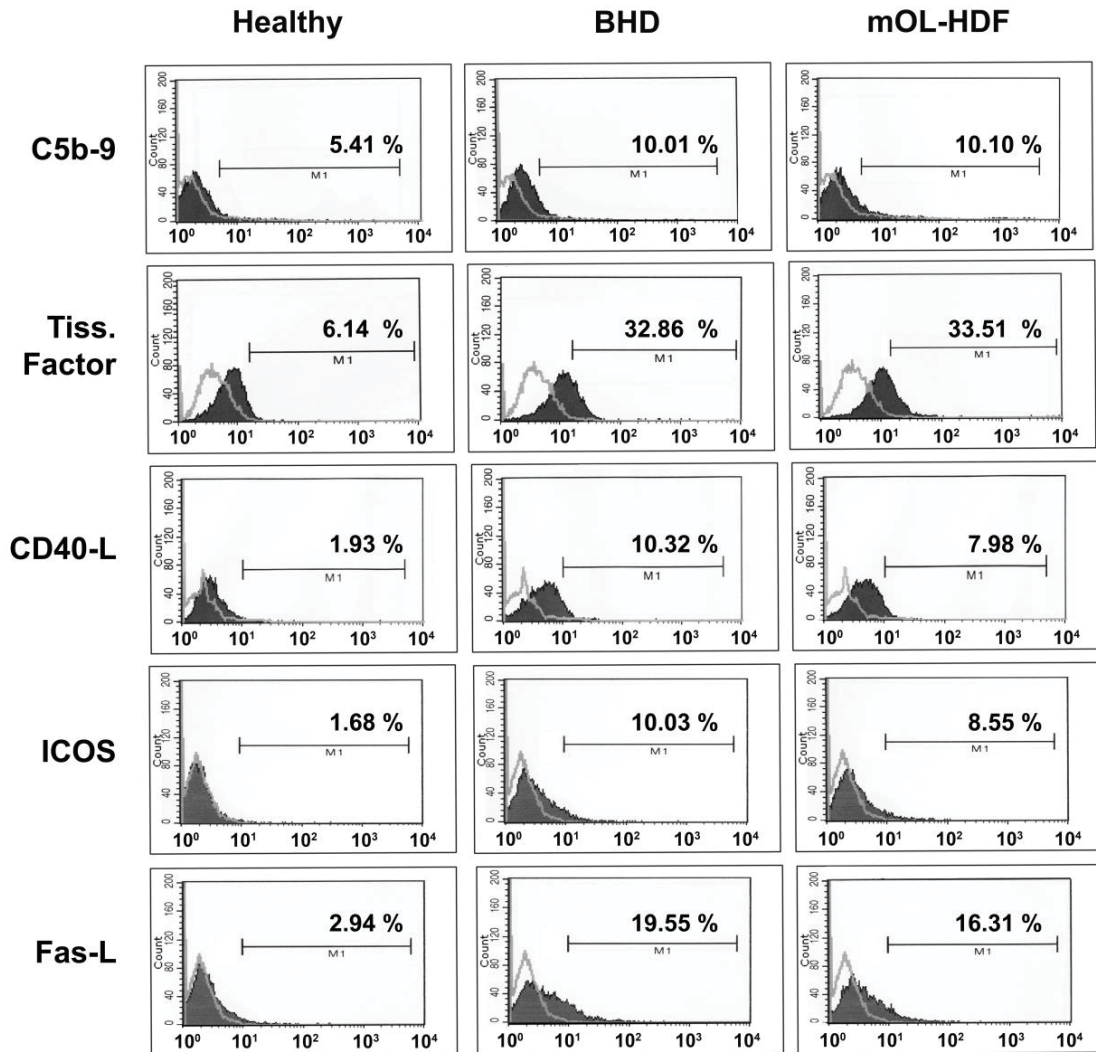
	T0		T1		T2		T3		P (ANOVA or χ^2)		
	mOL-HDF*	BHD	mOL-HDF	BHD	mOL-HDF	BHD	mOL-HDF	BHD	Within mOL-HDF	Within BHD	All
Dialyzer Surface (m²)	2±15	2,0±0,2	2±15	2,0±0,2	2±15	2,0±0,2	2±15	2,0±0,2	1	1	0,92
Heparin start dose (IU)	1250±210	1458±486	1250±210	1458±486	1250±210	1458±486	1250±210	1458±486	1	1	0,31
Heparin maintenance dose (IU/h)	307±166	333±144	307±166	312±136	307±166	312±136	307±166	333±144	1	0,91	0,68
HD duration (min)	237±8	240±0	237±8	240±0	237±8	240±0	237±8	240±0	1	1	0,63
Qb (ml/min)	317±15	315±18	320±13	322±21	318±14	313±16	319±15	324±26	0,96	0,76	0,65
Qd (ml/min)	500±0	500±0	468±66	500±0	459±67	500±0	466±71	500±0	0,88	1	0,08
TMP (mmHg)	114±13	115±18	268±12	118±12	265±11	112±10	265±12	108±8	0,99	0,56	<0,01
Convective Volume (L)	NA	NA	35,1±4,6	NA	33,8±4	NA	34,5±4,2	NA	0,92	NA	NA
Net UF (L)	2,88±0,52	2,75±0,47	2,76±0,54	2,92±0,58	2,85±0,49	3±0,64	2,84±0,43	2,76±0,5	0,78	0,47	0,65
eKt/V	1,34±0,23	1,38±0,24	1,48±0,27	1,36±0,24	1,68±0,34	1,37±0,26	1,48±0,28	1,38±0,22	0,04	0,98	0,03

Table III: Intradialytic variables at all considered time-points. BHD: bicarbonate Hemodialysis; HD: hemodialysis; mOL-HDF: mixed on-line hemodiafiltration; Qb: blood flow; Qd: dialysis solution flow; TMP: transmembrane pressure. *At T0, mOL-HDF group was still treated by BHD. Statistical analysis was performed by one way ANOVA or χ^2 test when appropriated: p<0.05 was considered statistically significant.

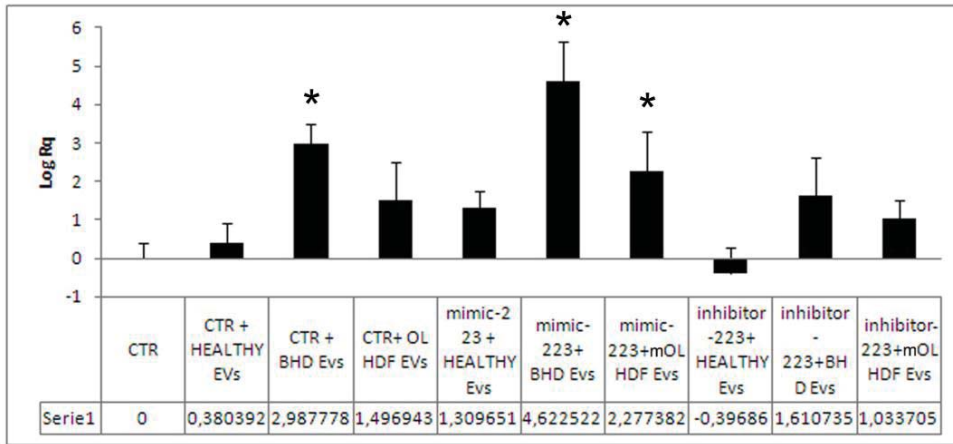
Supplementary Figures



Supplementary figure 1: representative healthy, BHD- and mOL-HDF-EV FACS Guava analysis of the main markers for exosomes (CD86), platelets (CD62P), monocytes/macrophages (CD14), T-cells (CD3), B-cells (CD40) and endothelial cells (CD144). Staining with the different antibodies (gray-filled curves) was compared to internal control (bright-line curve) represented by appropriate secondary isotype incubation



Supplementary figure 2: representative healthy, BHD- and mOL-HDF-EV FACS Guava analysis of molecules involved in inflammation, atherosclerosis, complement and coagulation activation: Membrane Attack Complex/ Terminal Complement Complex (C5b-9), Tissue Factor (Tiss. Factor), CD40-Ligand (CD40-L), Inducible T-cell COStimulator (ICOS), and Fas-Ligand (Fas-L). Staining with the different antibodies (gray-filled curves) was compared to internal control (bright-line curve) represented by appropriate secondary isotype incubation



Supplementary figure 3: Levels of expression of miR-223 in all experimental conditions in the *in vitro* angiogenesis and apoptosis assays. Results are expressed as mean \pm 1SD of three different experiments. AntagomiR-223 = inhibitor.

Table 1S (Supplementary Material): list of primers used for miRNA identification in plasma EV and for RUNX2 mRNA quantification in VSMC.

PRIMER	SEQUENCE
hsa-miR-17a-5p	5'- CAA AGT GCT TAC AGT GCA GGT AG -3'
hsa-miR-92a	5'- TAT TGC ACT TGT CCC GGC CTG T -3'
hsa-miR-223	5'- TGT CAG TTT GTC AAA TAC CCC A -3'
hsa-miR-423-5p	5'- TGA GGG GCA GAG AGC GAG -3'
hsa-miR-451	5'- AAA CCG TTA CCA TTA CTG AGT T -3'
hsa-RNU-48	5'- AAC TCT GAG TGT GTC GCT GAT G -3'
RUNX2 forward	5'-GAG TCC GGC CCC TCC AT-3'
RUNX2 reverse	5'-GCA ACT AAG TCA TAG TCC GCC TAG A-3'
Actin-β forward	5'-GAG TCC GGC CCC TCC AT-3'
Actin-β reverse	5'-GCA ACT AAG TCA TAG TCC GCC TAG A-3'