RESEARCH ARTICLE | Inflammation and Inflammatory Mediators in Kidney Disease

Markers of endothelial damage in patients with chronic kidney disease on hemodialysis

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Carmona A, Águeda ML, Luna-Ruiz C, Buendía P, Calleros L, García-Jerez A, Rodríguez-Puyol M, Arias M, Arias-Guillen M, de Arriba G, Ballarin J, Bernis C, Fernández E, García-Rebollo S, Mancha J, del Peso G, Pérez E, Poch E, Portolés JM, Rodríguez-Puyol D, Sánchez-Villanueva R, Sarro F, Torres A, Martín-Malo A, Aljama P, Ramírez R, Carracedo J. Markers of endothelial damage in patients with chronic kidney disease on hemodialysis. Am J Physiol Renal Physiol 312: F673–F681, 2017. First published January 11, 2017; doi:10.1152/ajprenal.00013.2016.—Patients with Stage 5 chronic kidney disease who are on hemodialysis (HD) remain in a chronic inflammatory state, characterized by the accumulation of uremic toxins that induce endothelial damage and cardiovascular disease (CVD). Our aim was to examine microvesicles (MVs), monocyte subpopulations, and angiopoietins (Ang) to identify prognostic markers in HD patients with or without diabetes mellitus (DM). A total of 160 prevalent HD patients from 10 centers across Spain were obtained from the Biobank of the Nephrology Renal Network (Madrid, Spain): 80 patients with DM and 80 patients without DM who were matched for clinical and demographic criteria. MVs from plasma and several monocyte subpopulations (CD14+CD16+, CD14+/CD16+), and Ang2-to-Ang1 ratios increased in HD patients with DM compared with non-DM patients. Moreover, MV level above the median (264 MVs/μl) was associated independently with greater mortality. MVs, monocyte subpopulations, and Ang2-to-Ang1 ratio can be used as predictors for CVD. In addition, MV level has a potential predictive value in the prevention of CVD in HD patients. These parameters undergo more extensive changes in patients with DM.

chronic kidney disease; cardiovascular disease; diabetes mellitus; microvesicles; inflammation, hemodialysis

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examined (1) whether inflammation and endothelial damage in CKD patients with diabetes differ from endothelial disease in patients without diabetes and have identified markers of inflammation and endothelial damage in CKD.

The inflammatory state is associated with the activation and apoptosis of endothelial cells (ECs), leading to the release of recently identified circulating biomarkers that are related to endothelial dysfunction, called CD31+/Annexin V+ microvesicles (MVs) (5, 43), which have been proposed to be markers of endothelial damage and dysfunction in several pathologies (15, 17, 26).

Extracellular vesicles (EVs), including exosomes (<100 nm) and MVs (100–1,000 nm), are small, secreted, membrane-enclosed entities that are involved in various biological, physiological, and pathological phenomena. In our study, we will use the term MVs, as our EV detection method allows detection of mostly larger EVs (>400 nm). EVs protect a wide range of biomolecules that originate from secreting cells, and their molecular cargo changes in diseases and other physiological states (25, 34, 49, 58). In conventional flow cytometry, the range of detection of MVs is 400–1,000 nm (38). During inflammation, the number of EVs increases (22, 53). The effects of EVs might be mediated by their support of cell-to-cell crosstalk, because EVs transport microRNA, active molecules, hormones, peptides, and regulator proteins (6, 16, 35), inducing cell-to-cell adhesion, increasing neutrophil adhesion, and apoptosis of endothelial cells (ECs), leading to the inflammation and endothelial damage in CKD. These data have been confirmed in CKD patients, and we have reported that the amounts of these proinflammatory cells correlate with various markers of endothelial damage, including MVs (36, 43).

Angiopoietin 1 (Ang1) stabilizes the endothelium by inhibiting EC apoptosis and activation and decreasing inflammation. In contrast, Ang2 is proinflammatory and promotes EC and epithelial cell apoptosis, increases neutrophil adhesion, and induces cytoskeletal changes to widen interendothelial gaps. The ratio of Ang2 to Ang1 might be a useful prognostic biomarker of endothelial activation (40).

Based on the frequency of cardiovascular complications in patients with Stage 5 CKD who are on hemodialysis (HD), we must improve our understanding of the development of endothelial damage to identify markers that can predict the progression of CVD and establish appropriate targets that might delay disease progression. Therefore, our goal was to measure MV level, monocyte subpopulations, and other soluble markers of endothelial damage, such as Ang, in patients with and without diabetes to determine whether these markers identify CVD patients with HD, whether diabetes modifies their expression profiles in HD, and whether they are prognostic markers in HD patients with and without diabetes.

**MATERIALS AND METHODS**

*Research participants.* Samples from 160 patients on HD were obtained from the Biobank of the Nephrology Renal Network (Madrid, Spain) from a total population of 400 HD patients from whom samples had been collected. The patients underwent HD in various dialysis units throughout Spain. Blood samples were obtained just before the HD session began. The bacterial and endotoxin contaminant levels were below the detection limit in all premixed dialysate samples (<1 bacterial colony-forming unit/ml and <0.03 endotoxin unit).

Data were also gathered on parameters that were related to severe CVD, defined as a cardiovascular event: acute myocardial infarction, cerebrovascular accident, or transient ischemic attack, until completion of the study (5.5 yr).

The study was approved by the Biobank Ethics Committee, and all subjects provided written, informed consent before collection of the samples and their storage in the Biobank.

*Characteristics of the study population.* The algorithm that we used to select patients is shown in Fig. 1. Of the 118 HD patients with DM, we chose 80 HD patients (18 HD T1DM patients and 62 HD T2DM patients) who had undergone at least 6 mo of HD and did not have a history of cardiovascular events. Then we selected 80 HD patients without DM who were matched for center (rate 1:1) and demographics (similar percentage of men and those aged older than 50 yr). The mean age of the study population (n = 160) was 64.23 ± 3.88 yr, and the study sample comprised 95 men and 65 women (Table 1). No differences in C-reactive protein levels were observed. Fifteen healthy subjects (50% men, 25% smoking, no hypertension, no hyperlipidemia) were included as controls. Blood samples were drawn from the arterial line before the start of HD or by venipuncture in healthy individuals. For this, a 21-gauge needle was used (57).

*Isolation and determination of MVs in plasma.* Platelet-free plasma was obtained by centrifugation at 1,500 g for 20 min at room temperature. Next, the supernatant was recovered and centrifuged at 13,000 g for 2 min to separate MVs. The supernatant was discarded, and the pellets were stored at −80°C until use (28, 31, 46, 57). MVs were then resuspended and incubated with 5 μl phycoerythrin (PE)-labeled monoclonal anti-CD31 (Thermo Fisher Scientific, Waltham, MA) using FITC-conjugated Annexin V kits, per the manufacturer’s instructions (Bender MedSystem, Vienna, Austria). CD31 is an adhesion molecule that identifies EVs that are derived from ECs, platelets, and leukocytes. MVs that expressed phosphatidylserine were labeled using fluorescein-conjugated Annexin V in the presence of CaCl₂ (5 mM), per the supplier. As a control for the Annexin V labeling, a sample with fluorescein-conjugated Annexin V, using a CaCl₂-free solution, was established. Isotype controls were included as negative controls for the CD31 labeling. An equal volume of Flow-Count calibration beads (Beckman Coulter, Brea, CA) was added to measure the number of events per microliter. Fluorescence-
activated cell sorter analysis was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter) using CXP (Beckman Coulter).

Before the sample acquisition, the samples were subjected to a separate and combined labeling reaction using all reagents (MAb, Annexin V, and the appropriate negative controls) to compensate for the fluorescence using compensation tools on the flow cytometer. An MV’s gates were established on the FC 500 in preliminary standardization experiments using a blend of size-calibrated beads (Beckman Coulter) with diameters of 0.3, 0.5, and 1.0 μm. The upper and outer limits of the MV’s gate were established just above the size distribution of the 1-μm beads in the forward- and side-scatter area (FSC-A and SSC-A, respectively) settings (log scale). The lower limit was the noise threshold of the instrument (SSC-A), limiting high background noise. The absolute number of MVs was calculated as the following: (MV counts × standard beads/liter)/standard beads counted (FlowCount; Beckman Coulter). Each result (single value) was the average of three independent measurements of the same sample.

**Monocyte subpopulations.** A 10-ml sample of peripheral blood was drawn from HD patients and healthy subjects into tubes that contained EDTA and deposited into the Biobank. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway), washed with PBS (Thermo Fisher Scientific), and supplied with 20% FBS (Thermo Fisher Scientific). After separation, PBMCs were frozen in FBS with 10% DMSO at 80°C for 24 h and transferred to liquid nitrogen until use.

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To identify CD14+/CD16− and CD14++/CD16+ monocytes, PBMCs were incubated with peridinin chlorophyll-conjugated monoclonal anti-CD14 (M5E2) and FITC-conjugated anti-CD16 (3G8). Both antibodies and the appropriate isotype controls were purchased from BD Biosciences (San Jose, CA). Flow cytometry was performed on a FACSCalibur (BD Biosciences) using CellQuest. The percentage of CD14+/CD16− and CD14++/CD16+ monocytes was calculated by subtracting nonspecifically stained cells, as identified in the isotype control histogram.

**Angiogenic factors.** The soluble angiogenic factors Ang1 and Ang2 were quantified by ELISA (R&D Systems, Minneapolis, MN), per the manufacturer’s instructions.

**Statistical analysis.** Continuous data were expressed as means ± SD and as the median (quartile 1 (Q1), Q3) for normal and skewed distributions, respectively. Comparisons between means of healthy subjects vs. HD non-DM, HD T1DM, and HD T2DM were analyzed by ANOVA, followed by Duncan test; χ² test was used for categorical data, which were expressed as percentages. Survival of HD non-DM and HD DM patients was analyzed by the Kaplan-Meier method, and differences in survival between two or more groups were examined by log-rank test, from the collection of blood samples to the start of the follow-up. The influence of MVs on patient survival after stratification by DM or non-DM was analyzed as a categorical variable—divided in two groups (above or below the median value)—and adjusted using traditional cardiovascular risk factors (smoking, hypertension, and hyperlipidemia) by multivariable Cox regression.

**Correlation analysis.** Analysis was performed among the study variables (CD14+/CD16−, CD14+/CD16+, and CD14++/CD16−, and MVs) in each group (HD non-DM, HD DM), separately by Pearson or Spearman test where appropriate. All statistical analyses were performed with SPSS 15.0 (IBM, Armonk, NY). Two-sided P < 0.05 was considered to be statistically significant.

**RESULTS**

**Quantification of MV level (MVs per microliter) in HD patients.** Representative graphs of the flow cytometry analysis of EVs and the number of MVs in HD patients are shown in Fig. 2, A and B. Size-selected events are plotted as a function of their double fluorescence for specific Annexin-PE binding and CD31-FITC for negative control (Fig. 2C) and HD patients (Fig. 2D). We observed that HD non-DM patients experienced a significant increase in MV level (236.4 ± 20.8 MVs/µl) in relation to healthy subjects (26.9 ± 4.1 MVs/µl; P < 0.001). Similarly, HD patients with T1DM had a significantly higher MV level (259.0 ± 34.3) vs. healthy subjects (26.9 ± 4.1 MVs/µl; P < 0.001). HD patients with T2DM had significantly more MVs (321.9 ± 33.5 MVs/µl) compared with healthy subjects (26.9 ± 4.1 MVs/µl; P < 0.001). Moreover, T2DM subjects had a higher MV level than HD patients without DM (321.9 ± 33.5 MVs/µl vs. 26.9 ± 4.1 MVs/µl; P = 0.014; Fig. 2E).

**Monocyte subpopulations in HD patients.** The percentage of CD14+/CD16− monocytes defines the extent of inflammation, which we calculated by flow cytometry (Fig. 3, A–C). The percentage of CD14+/CD16+ monocytes was higher in HD non-DM (8.4 ± 3.5%) and T1DM patients (11.6 ± 3.4%) than in healthy subjects (2.8 ± 0.9%; P < 0.001). Furthermore, the percentage of CD14+/CD16− monocytes was elevated in T1DM patients (11.6 ± 3.4%) compared with HD non-DM patients.
Correlation between inflammation and endothelial damage. In HD patients, we observed a positive correlation between the percentages of CD142+CD16+ monocytes (rho correlation Spearman = 0.544; P < 0.001; Fig. 5A), which also existed in HD patients with DM and in those without DM (rho correlation Spearman = 0.428, P = 0.05 for patients with DM; rho correlation Spearman = 0.599, P < 0.001 in patients without DM).

MV level and the percentage of CD14+CD16+ monocytes correlated in HD patients (Spearman rho correlation = 0.348; P = 0.017; Fig. 5B and Table 2).

Mortality in HD patients with and without DM vs. MV level. We analyzed the relationship between MV level and mortality in HD patients with and without DM after a median of 5.5 yr of follow-up by the Kaplan-Meier method. The patients were divided into two groups, defined by the median level of MVs. HD patients with MV level ≤264 MVs/µl had improved survival vs. those with levels that were above the median (log-rank <0.001; Fig. 6A). HD patients without DM with MV level ≤264 MVs/µl had greater survival than those with higher-than-median levels (log-rank <0.001; Fig. 6B). HD patients with DM and MV level ≤264 MVs/µl also survived longer than patients with MV level that exceeded the median (log-rank = 0.023; Fig. 6C).

Ang2/Ang1 and the CD14+/CD16+ and CD14+/CD16+ subpopulation percentages were not associated with mortality. Cox regression analysis. The hazard ratio for death in HD patients after adjustments by DM and non-DM increased significantly among patients with higher levels of MVs.
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(MVs > 264; 2.364; 95% confidence interval, 1.395–4.008; P = 0.001). The hazard ratio remained significantly higher after adjustments for traditional cardiovascular risk factors (smoking, hypertension, and hyperlipidemia).

DISCUSSION

In this study, we analyzed factors that are related to endothelial damage in patients with HD. Plasma from these patients contained more MVs and had higher Ang2/Ang1 compared with healthy subjects. The extent of endothelial damage was worse in diabetic patients. In contrast to healthy subjects, HD patients experienced an increase in the percentage of proinflammatory (CD14\textsuperscript{+}/CD16\textsuperscript{+}/CD162\textsuperscript{+}) and high cardiovascular-risk (CD142\textsuperscript{+}/CD16\textsuperscript{+}) monocyte subsets.

Parameters of morbidity and mortality were recorded for up to 5.5 yr. MV levels were associated with mortality in HD patients with and without DM. The rise in CD14\textsuperscript{+}/CD162\textsuperscript{+} cells was proportional to the CD142\textsuperscript{+}/CD16\textsuperscript{+} monocyte percentage and MV level.

As described (37), patients on HD harbor more MVs than healthy subjects. MV levels are also higher in other disease states, such as hypertension, DM, and coronary artery disease (9). Consistent with previous results, we noted that patients with HD and DM had higher MV levels than non-DM HD patients, and these levels affected T1DM as much as they did T2DM (39, 41). MV levels have potential value in the diagnosis and therapeutic management of CVD and might indicate worse endothelial damage in HD DM. In patients with coronary heart disease, the number of MVs that bind to Annexin V...
healthy subjects. These monocyte populations are elevated in patients with HD and DM also had a greater percentage of CD14^{+}/CD16^{++} than HD patients without DM, indicating that patients with DM have an increased risk of developing CVD.

The imbalance in Ang1 and Ang2 levels is related to diabetes, CVD, and tumorigenesis (2, 13, 30), and the Ang2/Ang1 might be an early marker of endothelial dysfunction (12, 55). In our study, patients with HD experienced an imbalance in the levels of Ang. Furthermore, the Ang2/Ang1 increased in patients with DM compared with healthy subjects. However, these values had no predictive value with regard to mortality.

Our study showed that the levels of CD14^{+}/CD16^{+} and CD14^{+}/CD16^{++} monocytes rose significantly in HD patients. Both subsets correlated positively, and it is possible that both have important functions in inflammation and CVD. Nevertheless, this association was not significant in HD DM patients, although it appeared to have a relative tendency. We cannot explain this disparate correlation between monocyte subsets in HD–DM compared with HD patients. Thus future studies should establish the events that occur in DM that might be implicated in the changes in monocyte subpopulations. Consequently, the rise in the number of these cells in CKD patients might mediate the development of vascular disease, even though the mechanisms should be examined. Moreover, our studies showed an association between MVs and CD14^{+}/CD16^{+} monocytes in HD patients, which explains the chronic inflammatory status of these patients.

The major limitation of our study is that patients with HD, some of whom could have been lost during the study period, had a high risk of mortality due to CVD. Furthermore, despite being a multicenter study, it was performed in a small sample of patients, necessitating larger prospective studies.

The prevalence of CVD is higher in HD patients with DM and elderly patients (>50 yr), which has significant clinical relevance but is another limitation of our study. We would also be interested in studying younger patients and patients in a less advanced stage of CKD to identify early markers of the disease. Moreover, we cannot exclude that the differences between T1DM and T2DM are attributed to disparities in predictors myocardial infarction and mortality (33). In this regard, we have found an association between the number of MVs and mortality in HD patients. In addition, we have observed that patients with and without DM with an MV level ≤264 MVs/µL experience greater survival than those with an MV level >264 MVs/µL.

In earlier studies, we reported that HD patients have a high percentage of proinflammatory CD14^{+}/CD16^{++} monocytes (35, 43, 45). These cells have been postulated to mediate the ongoing inflammation in such patients, secreting more proinflammatory cytokines than CD14^{+}/CD16^{+} cells (45). Furthermore, CD14^{+}/CD16^{++} monocytes are associated with chronic inflammatory conditions and have significant function in the development of DM (41). In our study, patients with HD had a higher percentage of CD14^{+}/CD16^{++} monocytes than healthy subjects. There is evidence that circulating monocytes in patients with T1DM can be induced to secrete proinflammatory cytokines (7). We also observed an increase in these proinflammatory monocytes in HD patients with DM, the percentage of which depends on whether the diabetes is T1 or T2. Patients with T1DM had a higher percentage of CD14^{+}/CD16^{+} monocytes than those with T2DM.

HD patients had a higher percentage of monocytes that predict cardiovascular risk (CD14^{+}/CD16^{+}) compared with healthy subjects. These monocyte populations are elevated in patients with HD (32, 51). We found that patients with HD and DM also had a greater percentage of CD14^{+}/CD16^{+} than HD patients without DM, indicating that patients with DM have an increased risk of developing CVD.

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Table 2. Correlation analysis

|                      | Non-DM | DM | HD  |
|----------------------|--------|----|-----|
| MVs vs. Ang2/Ang1    | −0.110 | 0.4| −0.005 | 0.9| −0.082 | 0.4|
| MVs vs. CD14^{+}/CD16^{+} | 0.238 | 0.1| 0.428 | 0.07| 0.348 | 0.02|
| MVs vs. CD14^{+}/CD16^{++} | 0.091 | 0.6| 0.211 | 0.4| 0.258 | 0.08|
| Ang2/Ang1 vs. CD14^{+}/CD16^{+} | −0.172 | 0.4| 0.107 | 0.7| −0.165 | 0.3|
| Ang2/Ang1 vs. CD14^{+}/CD16^{++} | −0.227 | 0.3| 0.307 | 0.2| 0.018 | 0.9|
| CD14^{+}/CD16^{+} vs. CD14^{+}/CD16^{++} | 0.599 | <0.001| 0.428 | 0.05| 0.544 | <0.001|

Fig. 5. Correlation between CD14^{+}/CD16^{+} and CD14^{+}/CD16^{++} monocytes in HD patients (A). Correlation between MVs and CD14^{+}/CD16^{++} monocytes in HD patients (B).
glycemic control, which would have an impact on the parameter that is assessed.

The size-detection limits of standard flow cytometry are well known, causing smaller MVs to be overlooked. An upper-size limit of EV detection is likely ~1 μm, as a 0.5-μm polystyrene bead is already reflecting an EV ~1 μm (11). Consequently, an absolute MV count might be under represented. Isolation, purification, identification, and conservation protocols for EVs have advanced significantly. We also believe that MV population might be contaminated with EVs from other origins, such as platelets. Moreover, Annexin V binding by MVs is a calcium-dependent process, and this marker has limited value in assessing apoptotic MVs. However, Annexin V+ MVs remain a well-studied marker of apoptosis-derived MVs from peripheral blood in healthy individuals and HD patients (4).

The results of our study have significant multidisciplinary implications for a wide range of areas in biomedicine, examining a problem that is a component of many chronic conditions. The resulting increase in our understanding of MVs, monocyte subpopulations, and angiogenic factors in CVD can guide the diagnosis and prognosis of the disease and the design of novel drug therapies. In addition, MVs from platelets and leukocytes might also be involved in inflammation, prompting future studies.

There is no consensus on how to detect and preserve MVs. In addition, no single method can characterize these vesicles completely (phenotype, size, count, and image). MVs abound in body fluids, and the detection of MVs in suspension by flow cytometry has attracted strong clinical and scientific interest, but their detection is difficult, because many MVs are small (~<400 nm) — below the limit of resolution of most flow cytometers — causing valuable information on their characteristics to be lost. Other methods (nanoparticle tracking analysis, electron microscopy, resistive pulse sensing) are thus being used to complement flow cytometry (53, 59). Currently, the major challenge for flow cytometry is the identification of single vesicles with a diameter that is less than the present limit of detection.

In conclusion, our findings confirm that patients with HD remain in an inflammatory state and undergo endothelial alterations that can be tracked using early quantifiable markers in peripheral blood. Notably, MVs, measuring 400–1,000 nm, have potential predictive value in the prevention of CVD in patients with HD. In addition, DM alters these inflammatory and endothelial damage factors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.C., R.R., and J.C. conceived and designed research; A.C., R.R., and J.C. performed experiments; A.C., M.L.A., C.L.R., P.B., A.M.-M., P.A., R.R., and J.C. analyzed data; A.C., M.L.A., C.L.R., P.B., A.M.-M., P.A., R.R., and J.C. interpreted results of experiments; A.C., M.L.A., C.L.R., P.B., I.C., A.G-J., M.R.-P., M.A., M.A.-G., G.d.A., J.B., C.B., E.F., S.G.-R., J.M., G.d.P., E. Perez, E. Poch, J.M.P., D.R.-P., R.S.-V., F.S., A.T., A.M.-M., P.A., R.R., and J.C. drafted manuscript; A.C., M.L.A., C.L.R., P.B., L.C., A.G.-J., M.R.-P., M.A., M.A.-G., G.d.A.,
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