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

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ABSTRACT

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 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 (REDinREN, Madrid): 80 patients with diabetes mellitus (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++) were analyzed by flow cytometry, and the plasma concentrations of angiopoietin (Ang)1 and Ang2 were quantified by ELISA. Data on cardiovascular disease were gathered over the 5.5 years after these samples were obtained. MV level, monocyte subpopulations (CD14+/CD16++ and CD14++/CD16+), and Ang2/Ang1 ratios increased in HD patients with DM compared with non-DM patients. Moreover, MV level above the median (264 MVs/µl) were associated independently with greater mortality. MVs, monocyte subpopulations, and Ang2/Ang1 ratio can be used as predictors for CVD. In addition, MV level have potential predictive value in the prevention of CVD in HD patients. These parameters undergo more extensive changes in patients with DM.

Keywords: chronic kidney disease, cardiovascular disease, diabetes mellitus, microvesicles, inflammation, hemodialysis

INTRODUCTION

Patients with chronic kidney disease (CKD) eliminate toxic compounds from their body less efficiently, resulting in the accumulation of uremic toxins (8, 14, 52, 54) and maintenance of a chronic inflammatory state (18, 20, 21, 27, 39). These events affect endothelial activation and are associated with a higher frequency of cardiovascular disease (CVD), which is one of the main causes of the high morbidity and mortality in these patients (29, 42).

Further, in CKD patients, renal failure is related frequently with other diseases, such as diabetes, that also induce inflammation and endothelial damage (20, 24, 44, 48); the endothelial damage due to diabetes might be another risk factor for CVD in CKD patients (19, 56). Some studies have 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 microvesicles (100-1000 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 (>400nm). 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, 58). In conventional flow cytometry, the range of detection of microvesicles is 400-1000 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), the levels of which rise in patients with CVD, CKD, and type II diabetes mellitus (T2DM) (23, 50). Further, proinflammatory monocytes (CD14+/CD16++) (3, 10, 36, 60, 61) and monocytes that predict cardiovascular risk (CD16++/CD14+) (22, 47, 60, 61) are elevated in the peripheral blood of patients with CVD. 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 endothelial cell apoptosis and activation and decreasing inflammation. In contrast, angiopoietin 2 (Ang2) is proinflammatory and promotes endothelial and epithelial cell apoptosis, increases neutrophil adhesion, and induces cytoskeletal changes to widen interendothelial gaps. The ratio of Ang-2 to Ang-1 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 of was to measure MV level, monocyte subpopulations, and other soluble markers of endothelial damage, such as angiopoietins, 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.

MATERIAL AND METHODS

Research participants

Samples from 160 patients on HD were obtained from the Biobank of the Nephrology Renal Network (REDinREN, Madrid) 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 contamination levels were below the detection limit in all premixed dialysate samples (<1 bacterial colony-forming unit/ml and <0.03 endotoxin units).

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

The study was approved by the Biobank Ethics Committee, and all subjects provided written informed consent prior to 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 Figure 1. Of the 118 HD patients with DM, we chose 80 HD patients (18 HD T1DM patients and 63 HD T2DM patients) who had undergone at least 6 months 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 years). The mean age of the study population (n=160) was 64.23 ± 3.88 years, and the study sample comprised 95 men and 65 women (Table 1). No differences in CRP 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 1500 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 -80ºC until use (28, 31, 46, 57). MVs were then resuspended and incubated with 5 µl of phycoerythrin (PE)-labeled monoclonal anti-CD31 (Caltag Laboratories, Burlingame, CA, USA) using fluorescein isothiocyanate-conjugated (FITC) 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 solution 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 Calibrator beads (Beckman Coulter Inc, Fullerton, CA, US) was added to measure the number of events per microliter. Fluorescence-activated cell sorter analysis was performed on a Coulter Cytomic FC 500 flow cytometer (Beckman Coulter Inc, Fullerton, CA, US) using CXP (Beckman Coulter).

Prior to the sample acquisition, the samples were subjected to a separate and combined labeling reaction using all reactives (monoclonal antibodies, Annexin V, and the appropriate negative controls) to compensate for the fluorescence using compensation tools on the flow cytometer. A MVs gates was established on the FC 500 in preliminary standardization experiments using a blend of size-calibrated beads (Beckman Coulter Inc, Fullerton, CA, US) with diameters of 0.3 µm, 0.5 µm, and 1.0 µm. The upper and outer limits of the MVs gate were established just above the size distribution of the 1-µm beads in the forward (FSC-A) and side scatter (SSC-A) 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: (MVs counted x standard beads/L)/standard beads counted (FlowCount, Beckman Coulter). Each result (single value) was the average of 3 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 ethylenediaminetetraacetic acid (EDTA) and deposited into the biobank. Peripheral blood mononuclear cells (PBMCs) were

isolated by Ficoll density-gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway), washed with PBS (GIBCO, Invitrogen, Carlsbad, BA), and supplied with 20% fetal bovine serum (FBS, GIBCO, Invitrogen). After separation, PBMCs were frozen in FBS with 10% DMSO at -80ºC for 24 h and transferred to liquid nitrogen until the day of processing. The suitability of the samples for processing and labeling was verified using parallel unfrozen control blood. Cell viability was tested by mixing cell suspensions with trypan blue solution (25900048R, CORNING Cellgro, Manassas, VA 20109, USA).

To identify CD14+/CD16++ and CD14++/CD16+ monocytes, PBMCs were incubated with peridinin chlorophyll protein (PerCP)-conjugated monoclonal anti-CD14 (M5E2) and FITC-conjugated anti-CD16 (3G8). Both antibodies and the appropriate isotype controls were purchased from Becton Dickinson (BD Biosciences; San Jose, CA, USA). Flow cytometry was performed on a FACSCalibur (BD Biosciences) using Cell Quest. 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 Ang-1 and Ang-2 were quantified by ELISA (R&D Systems, Minneapolis, Minnesota) per the manufacturer's instructions.

Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD) and as the median (Q1, Q3) for normal and skewed distributions, respectively. Comparisons between means of healthy subjects versus HD non-DM, HD 1TDM, and HD 2TDM

were analyzed by ANOVA, followed by Duncan test. Chi-squared test was used for categorical data. Categorical data were expressed as percentages. Survival of HD non-DM and HD DM patients was analyzed by Kaplan-Meier method, and differences in survival between 2 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 2 groups (above or below the median value)—and adjusted using traditional cardiovascular risk factors (smoking, hypertension, and hyperlipidemia) by multivariable Cox regression.

Correlation analysis was performed between the study variables (CD14++/CD16+, 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. Two-sided P values of less than 0.05 were considered to be statistically significant.

RESULTS

Quantification of MV level (MVs/ µl*) in HD patients*

Representative graphs of the flow cytometry analysis of EVs and the number of MVs in HD patients are shown in Figure 2 A and B. Size-selected events plotted as a function of their double fluorescence for specific annexin-phycoerythrin (PE) binding and CD31-FITC, negative control (C) and HD patients (D).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 significantly higher MV level (259.0±34.3)

versus 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 higher MV level than HD patients without DM (321.9 ± 33.5 MVs/µl vs 236.4 ± 20.8 MVs/µl, p=0.014) (Figure 2E).

Monocyte subpopulations in HD patients

The percentage of CD14+/CD16++ monocytes defines the extent of inflammation, which we calculated by flow cytometry (Figure 3A-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). Further, the percentage of CD14+/CD16++ monocytes was elevated in T1DM patients (11.6 ± 3.4%) compared with HD non-DM (8.4 ± 3.5%, p=0.001) and T2DM subjects (5.8 ±1.8%, p<0.001) (Figure 3D).

The percentage of CD14++/CD16+ monocytes was associated with higher rates of cardiovascular events. Also, this percentage was significantly higher in T1DM versus healthy (5.1 ± 1.1% vs 3.1 ± 0.8%, p=0.009) and HD without DM patients (4.3 ± 2.3%, p=0.043) (Figure 3E).

Ang2/Ang1 ratio in HD patients

The Ang2/Ang1 ratio was calculated in plasma samples from healthy subjects and patients with HD. HD patients without DM had an Ang2/Ang1 ratio of 8.2±4.5, which was significantly higher than in healthy subjects (0.5 ± 0.1, p<0.001). HD patients with T1DM had significantly higher Ang2/Ang1 ratios (2.9±2.5) than healthy subjects (0.5±0.1, p<0.001). Moreover, HD patients with T2DM had

significantly higher Ang2/Ang1 ratios (9.3±5.4) compared with healthy subjects (0.5±0.1, p<0.001) (Figure 4A).

Correlation between inflammation and endothelial damage

In HD patients, we observed a positive correlation between the percentages of CD14++/CD16+ and CD14+/CD16++ monocytes (rho correlation Spearman = 0.544, p = <0.001) (Figure 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) (Figure 5B) (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 years of follow-up by Kaplan-Meier method. The patients were divided into 2 groups, defined by the median level of MVs. HD patients with MV level ≤264 MVs /µl had improved survival versus those with levels that were above the median (log-rank<0.001) (Figure 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) (Figure 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) (Figure 6C).

Ang2/Ang1 ratio 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 (MVs >264, 2.364; 95% confidence interval [CI], 1.395 to 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 ratios compared with healthy subjects. The extent of endothelial damage was worst in diabetic patients. In contrast to healthy subjects, HD patients experienced an increase in the percentage of proinflammatory (CD14+/CD16++) and high-cardiovascular-risk (CD14++/CD16+) monocyte subsets.

Parameters of morbidity and mortality were recorded for up to 5.5 years. MV level were associated with mortality in HD patients with and without DM. The rise in CD14+/CD16++ cells was proportional to the CD14++/CD16+ monocyte percentage and MV level.

As described (37), patients on HD harbor more MVs than healthy subjects. MV level are also higher in other disease states, such as hypertension, diabetes mellitus and coronary artery disease (9). Consistent with previous results, we noted that patients with HD and DM had higher MV level than non-DM HD patients, and these levels affected T1DM as much as they did T2DM (39,41). MV level have

potential value in the diagnosis and therapeutic management of cardiovascular disease and might indicate worse endothelial damage in HD DM. In patients with coronary heart disease, the number of MVs that bind to annexin V predicts 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 MV level ≤264 MVs /µl experience greater survival than those with 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). Further, 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 cardiovascular disease.

The imbalance in Ang 1 and Ang 2 levels is related to diabetes, cardiovascular disease, and tumorigenesis (2, 13, 30), and the Ang2/Ang1 ratio might be an early marker of endothelial dysfunction (12, 55). In our study, patients with HD experienced an imbalance in the levels of angiopoietins. Further, the Ang2/Ang1 ratio 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 can not 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 explain the chronic inflammatory status of these patients.

The major limitation of our study is that patients with HD had a high risk of mortality due to CVD, some of whom could have been lost during the study period. Further, 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 years), 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 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. Upper size limit of EV detection is likely >1μm, as a 0.5 μm polystyrene bead is reflecting already an EV around 1 μm (11) . Consequently, absolute MV count might be underrepresented. 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 a 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 400-1000 nm, have potential predictive value in the prevention of cardiovascular disease in patients with HD. In addition, diabetes mellitus alters these inflammatory and endothelial damage factors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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615 **Table 1. Clinical characteristics of HD patients**

	Healthy subjetcs	Non-DM	1TDM	2TDM	P value
	n=15	n = 80	n=18	n = 62	
Men, n (%)	**8 (53.3)**	**50 (62.5)**	**9 (50.0)**	**45 (56.2)**	**0.8**
eKt/V, mean±SD		**1.6±0.3**	**1.5±0.2**	**1.5±0.3**	**0.8**
PCR, median(Q1, Q3) (mg/dl)		**2.9(1, 9)**	**4.1(1.3,8.9)**	**4(1.1, 10.7)**	**0.6**
Smoking					**0.9**
YES, n (%)	**4 (26.6)**	**33 (41.2)**	**6 (33.4)**	**25 (40.4)**	
NO, n (%)	**11 (73.4)**	**47 (58.8)**	**12 (66.6)**	**37 (59.6)**	
Hypertension, n (%)	**0**	**47 (58.8)**	**13 (72.3)**	**45 (72.5)**	**0.2**
Hyperlipidemia, n (%)	**0**	**41 (51.2)**	**11 (61.5)**	**43 (69.3)**	**0.1**
HD modality (% online hemodiafiltration)	**0**	**12.5**	**10.5**	**16**	**0.9**
HD modality (% low-flux hemodialysis)	**0**	**40**	**55.5**	**35**	**0.6**
HD modality (% high-flux hemodialysis)	**0**	**47.5**	**30**	**49**	**0.65**
Unknown HD modality (%)		**0**	**4**		**0**
CVD					**0.04**
YES, n (%)	**0**	**21 (26.2)**	**7 (38.9)**	**29 (46.7)**	
NO, n (%)	**0 (100)**	**59 (73.7)**	**11 (61.1)**	**33 (53.3)**	

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619 **Table 2. Correlation analysis**

	Non DM		DM		HD	
	Correlation	**p value**	**Correlation**	**p value**	**Correlation**	**p value**
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**

LEGENDS TO FIGURES

Figure 1: Patient flowchart for selection of study population, with inclusion and exclusion criteria.

Figure 2: Quantification of MVs in plasma of HD patients. Fluorescence-gated beads of various sizes for determining gates between 0.4 µm to 1 µm (A). Representative graphs of flow cytometry analysis of EVs in platelet-free plasma. EVs were plotted using forward scatter logarithmic (FS/log)/side scatter logarithmic (SS-log) dot plot histogram. MVs are defined as event numbers with a size of 0.4-1 µm and are gated in a window. It was necessary to use bead counts in each experiment to calculate the concentration of MVs per unit volume of the sample (B). Size-selected events plotted as a function of their double fluorescence for specific annexin-phycoerythrin (PE) binding and CD31-FITC, negative control (C) and HD patients (D). Number of MVs /µl in healthy patients with HD patients no DM, T1DM, and T2DM (E). *p<0.001 vs control; #p=0.014 vs non-DM (ANOVA, followed by Duncan test).

Figure 3: Representative flow cytometry of monocyte subsets. Backgated (R1-monocytic gate) within the FCS height/SSC height (A). The monocyte subsets (M1: CD14++/CD16-; M2: CD14++/CD16+; M3: CD14+/CD16++) within the population were assessed using anti-CD16-FITC/anti-CD14-PerCP dot plot control (B) and HD patients (C). Percentage of CD14+/CD16 ++ monocytes in healthy subsets and non-DM, T1DM, and T2DM patients (D). *p<0.001 vs control; #p=0.001 vs non-DM; and p<0.001 vs T1DM. Percentage of CD14+/CD16 ++ monocytes in healthy

subsets and non-DM, T1DM, and T2DM patients (E). *p=0.009 vs control; #p=0.043 vs T1DM (ANOVA, followed by Duncan test).

Figure 4: Ang2/Ang1 ratio in HD patients. Ang2/Ang1 ratio healthy patients with HD patients without DM and with T1DM and T2DM (A). *p0=0.001 vs control (ANOVA, followed by Duncan test). Ang 1 and 2 were quantified by ELISA in plasma (pg/ml).

Figure 5: Correlation between CD14++/CD16+ and CD14+/CD16++ monocytes in HD patients (A). Correlation between CD14+/CD16++ monocytes and MVs in HD patients (B).

Figure 6: Kaplan-Meier survival curves with regard to MV level (median) over 5.5 years. HD patients (6A). HD patients without DM (6B). HD patients with DM (6C).

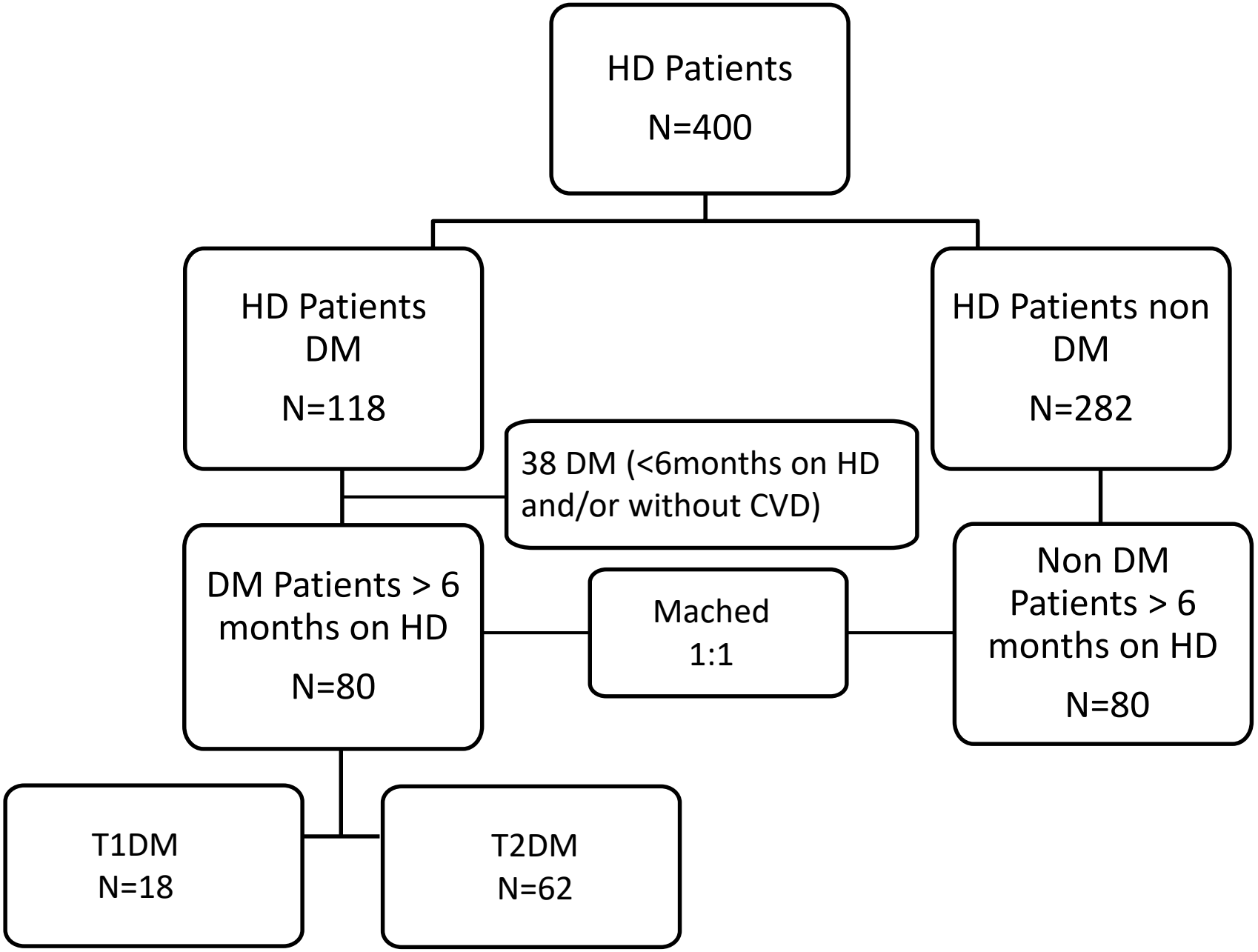

HD Patients
N=400
HD Patients
DM
N=118
HD Patients non
DM
N=282
38 DM (<6months on HD
and/or without CVD)
DM Patients > 6
months on HD
N=80
Mached
1:1
Non DM
Patients > 6
months on HD
N=80
T1DM
N=18
T2DM
N=62

Figure 1

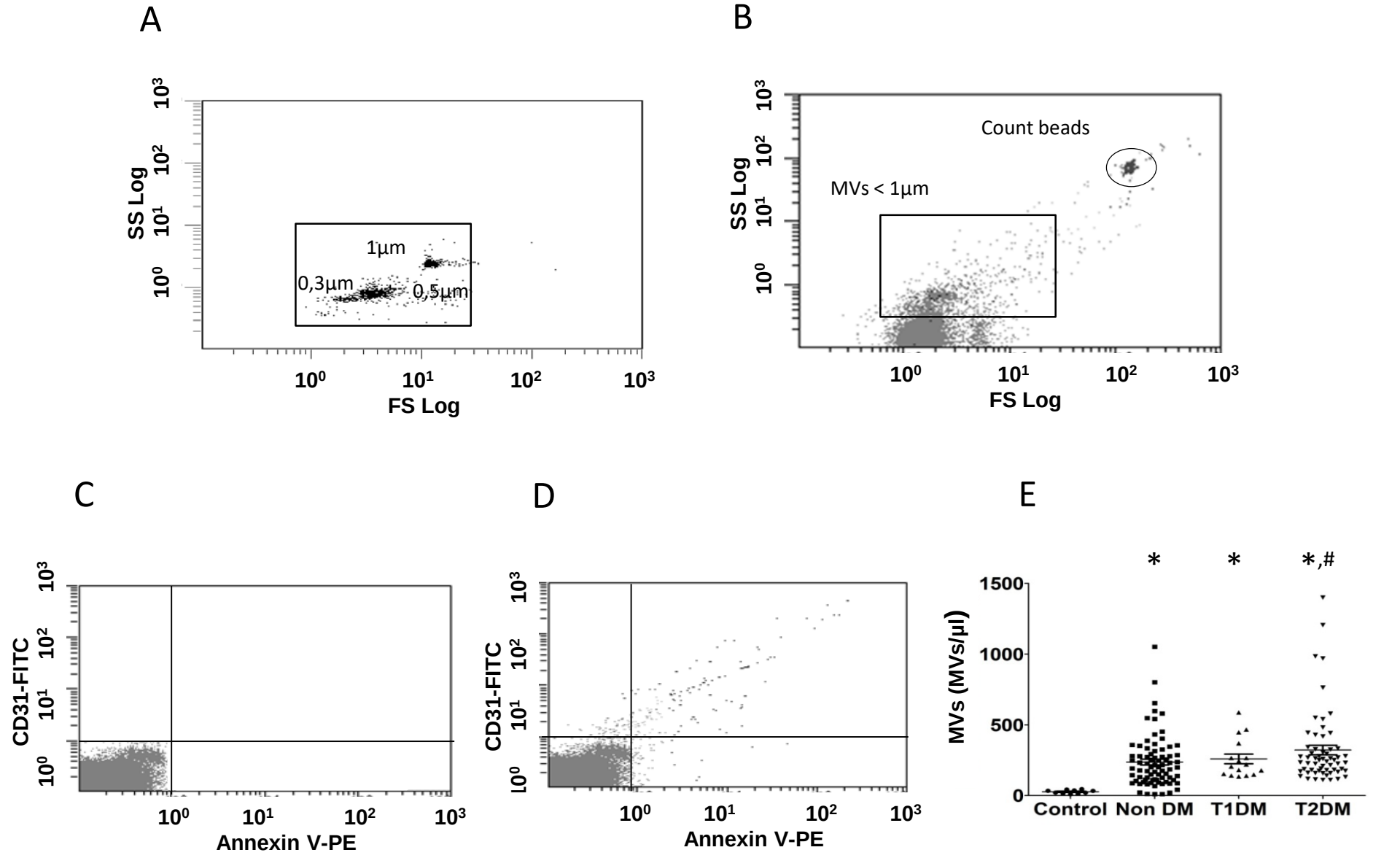

A
SS Log
FS Log
1µm
0,3µm
0,5µm
B
Count beads
MVs < 1µm
C
CD31-FITC
Annexin V-PE
D
E
MVs (MVs/µl)
Control
Non DM
T1DM
T2DM

Figure 2

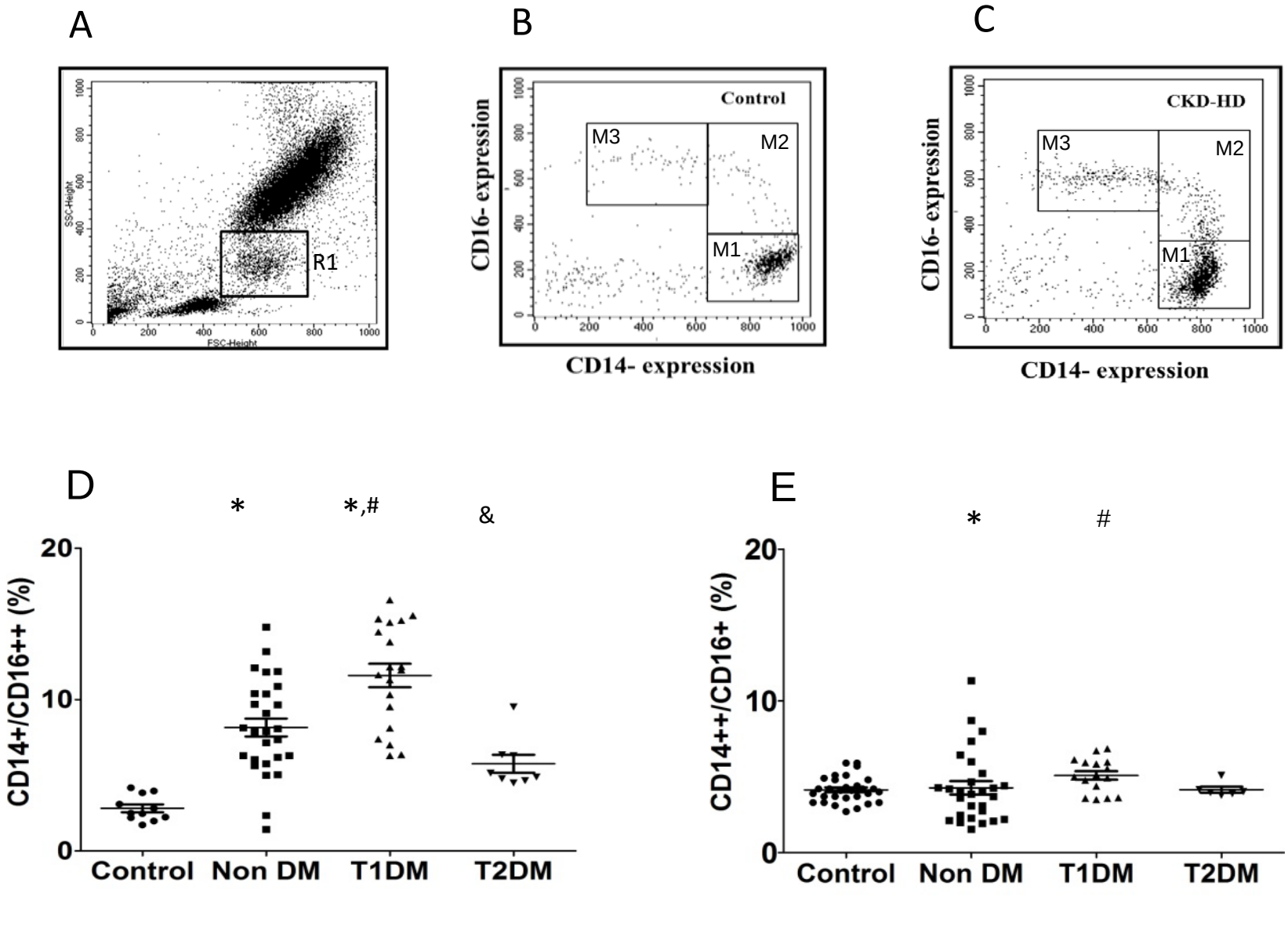

A
SSC-Height
FSC-Height
R1
B
Control
M3
M2
M1
CD16- expression
CD14- expression
C
CKD-HD
M3
M2
M1
CD16- expression
CD14- expression
D
*
*,#
&
CD14+/CD16++ (%)
20
10
0
Control
Non DM
T1DM
T2DM
E
*
#
CD14++/CD16+ (%)
20
10
0
Control
Non DM
T1DM
T2DM

Figure 3

A

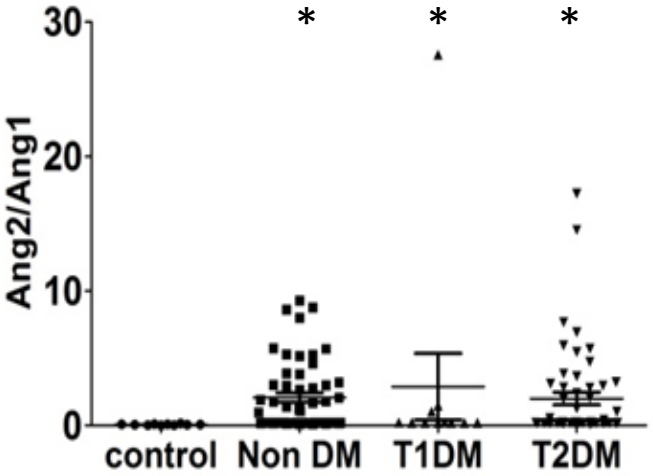


Figure 4

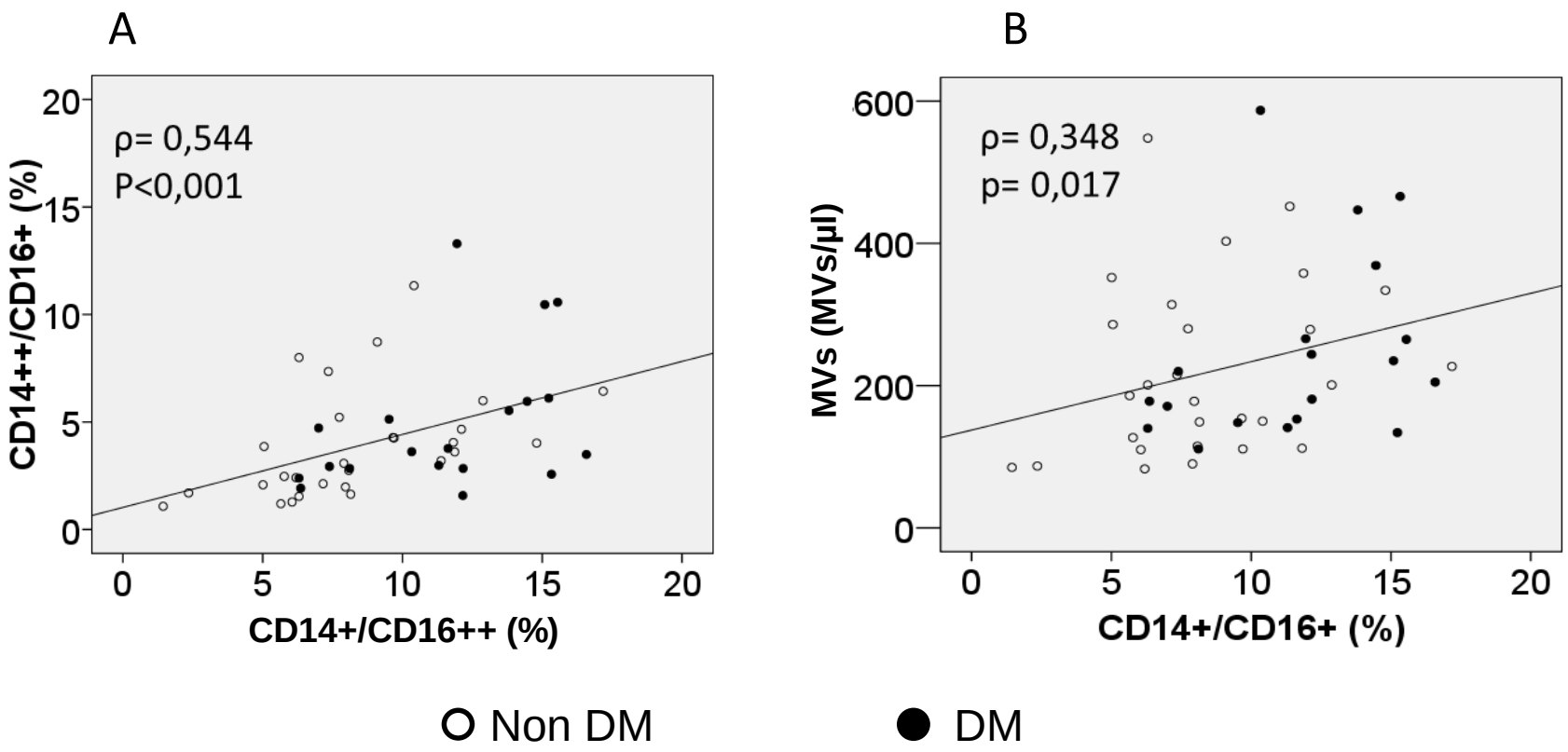
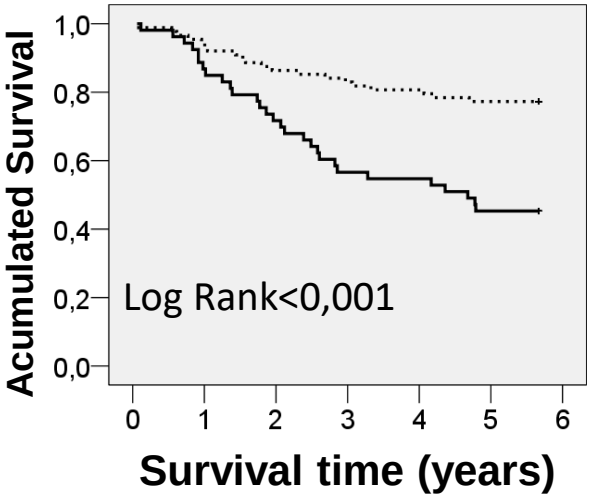
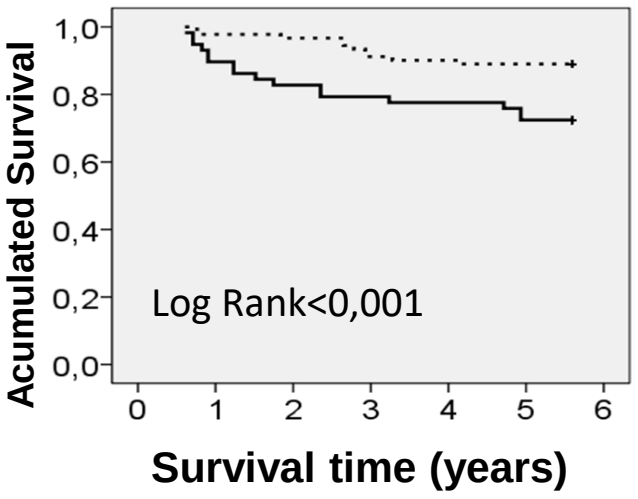

A
ρ= 0,544
P<0,001
CD14++/CD16+ (%)
CD14+/CD16++ (%)
B
ρ= 0,348
p= 0,017
MVs (MVs/µl)
CD14+/CD16+ (%)
Non DM
DM

Figure 5

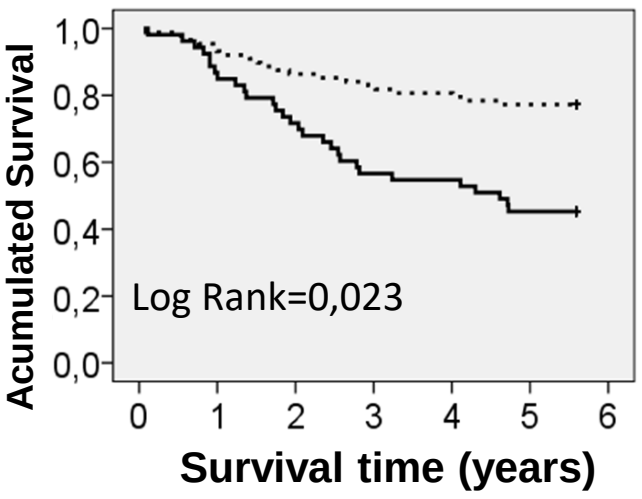
A



B



C



	0	1	3	5
MVs≤264	97	92	82	77
MVs>264	63	55	39	33

	0	1	3	5
MVs≤264	51	49	44	42
MVs>264	29	26	18	16

	0	1	3	5
MVs≤264	46	43	43	33
MVs>264	34	30	22	18

MVs>264 **MVs≤264**

Figure 6