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## Predictive value of the decrease in circulating dendritic cell precursors in stable coronary artery disease

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**ABSTRACT**

Dendritic cells are present in atherosclerotic lesions leading to vascular inflammation. The number of vascular dendritic cells increases during atherosclerosis. Recently, we showed that circulating dendritic cell precursors (DCP) are reduced in acute coronary syndromes through vascular recruitment. In our present study, we investigated whether DCP might be reduced in stable coronary artery disease (CAD) too. The levels of circulating myeloid (mDCP), plasmacytoid (pDCP), and total DCP (tDCP) were flow cytometrically investigated in 290 patients with suspected stable CAD. The coronary angiogram was used to evaluate for each patient a CAD Score: 1. excluded CAD (n=57), 2. early CAD (n=63), 3. moderate CAD (n=85), and 4. advanced CAD (n=85). Compared to controls, patients with advanced stable CAD had lower HDL cholesterol ( $p = 0.03$ ) and higher creatinine ( $p = 0.003$ ). In advanced CAD a significant decrease of circulating mDCP, pDCP, and tDCP was shown (each  $P < 0.001$ ). A significant inverse correlation was observed between the CAD Score and mDCP, pDCP, or tDCP (each  $P < 0.001$ ). Patients who required a percutaneous coronary intervention or coronary artery bypass grafting had less circulating mDCP, pDCP, and tDCP than controls (each  $p < 0.001$ ). Multiple stepwise logistic regression analysis proved mDCP, pDCP, and tDCP as independent predictors of a CAD. In conclusion, we show that patients with stable CAD have significantly lower levels of circulating DCP than healthy individuals. Their decrease seems to be an independent predictor of the presence and consecutive therapeutical procedure in stable CAD.

**Keywords:** atherosclerosis, coronary artery disease, immune system, dendritic cells

**Short title:** Circulating DC precursors in stable CAD

## INTRODUCTION

Atherosclerosis is a chronic inflammatory disease characterized by the invasion of immune cells such as T cells, monocytes, dendritic cells (DC), and mast cells into the vascular wall [1]. A decade ago, DC were firstly described in atherosclerotic lesions as well as healthy vascular intima [2, 3]. DC are professional antigen-presenting cells with the unique ability to stimulate naïve as well as memory T cells [4]. Therefore, DC play a critical role in many inflammatory conditions, particularly in autoimmune diseases [5]. DC patrol the blood and the peripheral tissues to detect foreign, potential harmful antigens, able to initiate an immune response against them [6]. Two lineages of DC can be differentiated: Myeloid DC ( $CD11c^+$ ,  $CD11b^+$ ) which respond to bacteria and fungi, releasing IL-12, as well as plasmacytoid DC ( $CD303^+$ ) which release interferon- $\alpha$  upon viral infection [6]. Both lineages can be detected in blood as DC precursors (DCP) which possess a high expression of HLA-DR, but lack the expression of costimulatory molecules, so that they are not able to activate T cells. After invasion of (inflamed) tissue they change their morphological appearance, and are enabled to take up potential antigens (immature DC), subsequently acquiring the ability to stimulate in an antigen-specific manner T cells through upregulation of costimulatory molecules (mature DC).

In recent times, it was suggested that myeloid DC are critically involved in the progression and destabilization of atherosclerotic plaques [7-10]. In the vessel wall, myeloid DC are activated by different atherogenic factors, such as oxidized low density lipoprotein (LDL) cholesterol [11], nicotine [12], or advanced glycation end products [13], enabling them to stimulate T cells, and probably leading to vascular inflammation. Proving this concept, Han et al. recently demonstrated in a vascular model that myeloid DC are the most potent T cell activators in atherosclerosis and crucial for the immune reactions which occur in the vessel wall microenvironment [14].

Whereas scientific investigations had focussed on the role of myeloid DC in atherosclerosis so far, Niessner et al. recently described that plasmacytoid DC are present in atherosclerotic plaques too, and that they stimulate T cells against viral antigens there [15].

During the progression of atherosclerosis, the number of mature myeloid and plasmacytoid DC significantly increases in atherosclerotic lesions, and two independent mechanisms are responsible for that. First, in atherogenic areas of the vascular wall an increased recruitment of circulating DCP was observed with accumulation particularly at the upstream shoulder of atherosclerotic plaques [16, 17]. Certain atherogenic factors such as hypoxia, oxidized LDL cholesterol, or tumor necrosis factor (TNF)- $\alpha$  seem to enhance the transmigration of DCP over the endothelium into the intima [18]. Second, recent studies showed that the emigration of DC from atherosclerotic lesions into the regional lymph nodes is reduced by certain pro-atherogenic conditions such as the presence of oxidized LDL cholesterol or platelet-activating factor [19, 20]. Activated platelets were shown to induce both vascular recruitment and maturation of circulating DCP [21].

In two recent studies it was firstly described that the level of circulating DCP is significantly reduced in blood of patients with acute coronary syndrome (ACS), indicating the possibility of their increased recruitment into the vascular wall [16, 22]. Patients with stable coronary artery disease (CAD) seemed to have a reduction of circulating DCP too [16], implicating that their decrease might be not only a transient effect in ACS but could also represent a permanent state in stable CAD. Beyond alterations in the frequency of circulating DCP it was recently shown that in patients with CAD, particularly those with ACS, cultivated DC from blood monocytes had a significantly higher expression of costimulatory molecules, suggesting a higher ability of them to stimulate T cells [23]. It was suggested that differentiation of monocytes into myeloid DC might be an alternative way for their increase in the atherosclerotic altered vascular wall [23].

The aim of our present study was to analyze in a larger-scale clinical investigation whether circulating myeloid or plasmacytoid DCP are significantly reduced in stable CAD, which factors are associated with their reduction, and to determine if the analysis of circulating mDCP or pDCP can predict the presence and stage of a CAD.

## **METHODS**

### **Study population**

Between December 2005 and July 2007, 290 patients with suspected CAD who underwent a coronary angiography at our clinic were enrolled in this study. Exclusion criteria were acute coronary syndromes (unstable angina pectoris, new or dynamic ST segment changes, and/or troponin-I > 0.5 ng/mL) and non-cardiac diseases that could interfere with our analysis: acute or chronic infections, malignancies, autoimmune diseases, hyperthyroidism, and medication with immunosuppressive agents. Routine blood analyses were performed in our hospital laboratory according to clinic standard. Standardized blood sampling for flow cytometrical analysis was carried out at admission before coronary angiography and in some of the patients after 24h. Immediately after blood withdrawal, samples were flow cytometrically analyzed. The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Informed consent was obtained from all patients and the study was approved by the institutional ethics committee.

### **Evaluation of the CAD Score and subdivision into the study groups**

For each patient, a CAD Score was calculated. For this purpose, the coronary angiogram was analyzed by two experienced investigators, and for each of 15 coronary segments the maximum grade of stenosis was assessed as previously described: left main coronary artery (S5), the proximal (S6), middle (S7), and distal (S8) left anterior descending (LAD), the first (S9) and second (S10) diagonal branch of the LAD, the proximal (S11), middle (S13)

and distal (S15) circumflex artery (CX), the first (S12) and second (S14) obtuse marginal branch of the CX, and the proximal (S1), medial (S2), and distal (S3 & S4) right coronary artery (RCA). According to the maximum grade of stenosis, each segment received a point value: 0% (0 point), 1-25% (1 point), 26-50% (2 points), 51-75% (3 points), and  $> 75\%$  (4 points) [24]. The CAD Score was calculated for each patient by counting up the point values in each coronary segment. According to the CAD Score, patients were subdivided into the following groups: *Score 0* (excluded CAD, n=57), *Score 1-5* (early CAD, n=63), *Score 6-10* (moderate CAD, n=85), and *Score  $> 10$*  (advanced CAD, n=85). Additionally, patients were subdivided according to the therapeutical recommendation into the following groups: 1. no therapy required (n=48), 2. medication (n=123), 3. percutaneous coronary intervention (PCI, n=80), or 4. coronary artery bypass grafting (CABG, n=44).

#### **Serum analysis of high-sensitivity CRP.**

The serum concentration of high-sensitivity (hs)CRP was measured using an immunonephelometric assay on a BN II analyzer (Dade Behring, Marburg, Germany).

#### **Identification of DC precursors by fluorescence activated cell sorting (FACS)**

The technique for the analysis of circulating mDCP and pDCP was developed by Ito et al. [25] and Dzionek et al. [26] who showed that FACS sorting of BDCA-1<sup>+</sup> and BDCA-2<sup>+</sup> cells led to the isolation of cells who express mDC- and pDC-specific markers, respectively, and possess certain functions which are characteristic of DC. This technique is provided in the *Blood Dendritic Cell Enumeration kit<sup>TM</sup>* (BDCA kit, Miltenyi Biotec, Bergisch Gladbach, Germany) which was used in our study.

Fresh blood samples were collected on EDTA. Three color-staining and FACS analysis were performed as previously described [16]. Briefly, 300  $\mu$ L of blood was mixed with 20  $\mu$ L of the *anti-BDCA-Cocktail* for DCP analysis and another 300  $\mu$ L of blood was

mixed with 20  $\mu\text{L}$  of the *control cocktail* for the isotype control. Subsequently, 10  $\mu\text{L}$  of *dead cell discriminator* was added, and the samples were incubated for 10 min. under a 60W light bulb. Erythrocytes were removed using the *red blood cell lysis solution*, following the fixation of the samples with the *fix solution*.

FACS analysis was performed using the FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). Since circulating DCP comprise only 0.1 to 1% of white blood cells (WBC), a special gating strategy was used to analyze mDCP, pDCP, and tDCP accurately (*Fig. 1*). For each sample,  $2 \times 10^5$  WBC were registered in region (R)1 defined by forward scatter (FSC) and side scatter (SSC). In R2, granulocytes were excluded according to their high SSC, and monocytes, lymphocytes, and dead cells were excluded according to their CD14-, CD19-, and propidium iodide-staining, respectively. In R3 and R4, circulating mDCP and pDCP were detected according to their specific staining for BDCA-1 and BDCA-2, respectively, and tDCP were the sum of cells in R3 and R4. The relative cell numbers of circulating mDCP, pDCP, and tDCP were assessed as a percentage of WBC (% WBC). The absolute cell numbers (cells/ $\mu\text{L}$ ) were calculated using their relative cell numbers multiplied by the white blood cell count.

### Statistical analysis

Statistical analyses were performed using the SigmaStat 2.03<sup>®</sup> software of the SPSS incorporation (Chicago, U.S.A.). Data were expressed as median or as proportions (%).  $P < 0.05$  was considered statistically significant. The non-parametric Mann-Whitney rank sum test was used to compare numerical data (e.g. mDCP, pDCP, or tDCP values) between the study groups as well as for the subgroup analysis. Categorical clinical data (e.g. sex, atherogenic risk factors, medication) were compared between the study groups by Chi-square test. Correlation analyses, e.g. circulating DCP with the CAD Score, were performed using the non-parametric Spearman rank order test. The predictive value of different

parameters for a CAD was analyzed using univariate and multivariate logistic regression analysis. For this purpose, CAD was encoded binary for each patient (all coronary stenosis < 50%: "0"; one or more coronary stenoses > 50%: "1"), and was analyzed as dependent variable compared with other factors (e.g. mDCP, pDCP, or tDCP values) as independent variables. Quantitative variables such as DCP values, creatinine, or age, were binary encoded for the logistic regression analysis too using the median calculated for all patients (individual value < median: "0"; individual value > median: "1"). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Since mDCP, pDCP, and tDCP showed a high correlation with each other, for each of them a separate multiple stepwise logistic regression analysis was performed including all parameters of the univariate analysis.

## RESULTS

### Baseline characteristics

Clinical characteristics of patients with coronary artery disease (CAD) are reported in *Table 1*. Compared to individuals without CAD, patients with CAD were significantly older ( $p < 0.001$ ), more often treated with statins ( $p < 0.001$ ) or aspirin ( $p = 0.03$ ), and had lower high density lipoprotein (HDL) cholesterol ( $p = 0.03$ ) and higher creatinine ( $p = 0.003$ ). Additionally, more often diabetes mellitus and hyperlipidemia as well as a trend for higher hsCRP and leukocytes were observed in patients with a CAD. Regarding the therapeutical procedure suggested in the coronary angiogram report, patients with mild CAD (Score 1-5) rarely required a PCI (16%), PCI was performed in 41% of patients with moderate CAD (Score 6-10), whereas only 8% of them were treated with CABG. In advanced CAD (Score > 10) nearly equal numbers of patients underwent a PCI (39%) or CABG (44%).

### **Decrease in circulating DCP according to the stage of CAD**

Comparing patients with mild (CAD Score 1-5), moderate (CAD Score 6-10), or advanced CAD (CAD Score > 10) with coronary healthy individuals (CAD Score 0), a stage-dependent significant decrease in circulating mDCP, pDCP, and tDCP was observed. (Fig. 2A-F, for exact values see Table 1). Since relative as well as absolute numbers of circulating DCP were reduced, we were able to exclude the possibility that their decrease was caused by a dilution phenomenon due to an increase of another WBC population.

In a part of the study patients, a second DCP analysis was performed after 24h, showing that coronary angiography (n = 51) or PCI (n = 38) itself did not cause any alteration in the level of circulating DCP (data not shown).

### **Decrease in circulating DCP according to the required therapeutical procedure**

In a second model, patients were subdivided into four different study groups according to the therapeutical procedure suggested in the coronary angiogram report: (1) Excluded CAD with no necessity of any therapy compared to CAD to be treated with (2) medication, (3) PCI, or (4) CABG. Compared with control individuals or CAD patients suggested to medication, CAD patients requiring a CABG or PCI had significantly reduced relative and absolute numbers of circulating mDCP, pDCP, and tDCP (Fig. 3A-F).

### **Effect of atherogenic risk factors or current medication on circulating DCP levels**

In a subgroup analysis (table 2), we investigated whether different clinical factors or pre-existing medical therapy might influence the levels of circulating DCP. Regarding age and gender of the patients, no significant changes were observed. In patients with hypertension or hyperlipidemia significantly lower levels of circulating pDCP and tDCP, and a trend for lower mDCP, were observed. Patients with diabetes mellitus had a trend for lower levels of mDCP, pDCP, and tDCP too. No significant changes were observed for smoking or family

history of a CAD. Regarding current medication of the patients, a trend for lower DCP levels was observed for statins, aspirin, and  $\beta$  blockers. Different serum parameters such as LDL cholesterol, HDL cholesterol, hsCRP, or creatinine were not associated with any changes in circulating DCP. As expected, higher blood leukocytes were associated with higher DCP levels.

#### **Correlation of circulating DCP and further serum parameters with the CAD Score.**

To analyze if circulating DCP and other serum parameters are directly associated with a CAD, correlation analyses were performed. These analyses showed a significant inverse correlation between the CAD Score and circulating mDCP% (Fig. 4A), pDCP% (Fig. 4B), and tDCP% (Fig. 4C). Further parameters which were significantly, but weaker correlated with the CAD Score were: HDL cholesterol (Fig. 4E) and creatinine (Fig. 4F). For hsCRP, only a trend for correlation with the CAD Score was observed (Fig. 4D). However, an inverse correlation was observed between hsCRP and circulating mDCP ( $r = -0.27$ ;  $p < 0.001$ ), pDCP ( $r = -0.19$ ;  $p < 0.002$ ), and tDCP ( $r = -0.25$ ;  $p = 0.001$ ). Comparing the different DC subtypes, circulating mDCP and pDCP showed a strong significant correlation with each other ( $r = 0.55$ ;  $p < 0.001$ ).

#### **Circulating DCP as independent predictors of the presence of a CAD**

As shown in Table 1 and Fig. 4, different factors seemed to be associated with the presence of a CAD. Thus, we investigated next which factors might have a predictive value for a CAD. Univariate analyses showed that significant predictors of a CAD were age  $> 66$  years, diabetes mellitus, HDL cholesterol  $< 46.5$  mg/dL, and hsCRP  $> 1.97$  mg/dL (Fig. 5A). Additionally, the reduction of circulating mDCP  $< 0.21\%$  or  $< 15.2/\mu\text{L}$ , pDCP  $< 0.10\%$  or  $< 7.9/\mu\text{L}$ , and tDCP  $< 0.33\%$  or  $< 24.8/\mu\text{L}$  were also predictive of a CAD, and actually seemed to be the strongest predictor (Fig. 5A). Male gender, hypertension, and

creatinine > 1.04 mg/dL might be predictive of a CAD too, but failed to reach statistical significance. To analyze if the decrease in circulating DCP is a predictor of a CAD independent of the other variables shown in the univariate analysis, multiple stepwise logistic regression analysis was performed. Since circulating mDCP, pDCP, and tDCP showed a high correlation with each other, separate multiple stepwise logistic regression analyses were performed for their relative and absolute values in a model together with age, gender, diabetes mellitus, hypertension, HDL cholesterol, creatinine, and hsCRP (*Fig. 5B*). In these analyses, reduction of circulating DCP either as relative (mDCP, pDCP, and tDCP) or absolute (pDCP and tDCP) values remained the only independent predictor of a CAD.

## DISCUSSION

Myeloid DC were implicated in the inflammatory processes related to atherosclerosis [16, 27]. It was shown that they are present in atherosclerotic plaques, that their number increases in the vascular wall depending on the stage of atherosclerosis, and that they may promote plaque destabilization [7-9]. Apart from that, recent studies independently showed that the level of circulating DCP is reduced in blood of patients with a CAD, particularly of those with ACS [16, 22]. The aim of our present study was to investigate whether the level of circulating DCP is altered in stable coronary artery disease too. Compared to previous studies [16, 22], circulating DCP were analyzed in a higher number of patients with angiographically exactly evaluated stable CAD, allowing us to investigate whether the decrease in circulating DCP is associated with the stage of CAD, or any other clinical factors, and if their decrease is an independent predictor of coronary artery stenoses.

In the present study, we show a stage-dependent decrease in circulating mDCP, pDCP, and tDCP in patients with a CAD compared to individuals with similar age, coronary risk factors, and angiographically excluded CAD. In contrast to our previous study [16], where we described a predominant decrease in circulating mDCP in unstable CAD and

acute ST-elevation myocardial infarction (STEMI), we now found in patients with exclusively stable CAD a comparable decrease in both circulating mDCP and pDCP, as reported by van Vre et al. recently [22]. The explanation for the discrepancy concerning the DCP results in stable CAD and STEMI seems to lie in the different migratory pattern of mDCP and pDCP. Whereas mDCP are recruited to the sites of inflammation in response to inflammatory chemokines, pDCP are attracted steadily by homeostatic chemokines into the lymphatic tissue [28]. Proving this concept, in a previous study in patients with dengue fever stable levels of circulating pDCP but a significant reduction of circulating mDCP due to enhanced emigration into inflammatory lesions was shown [29]. Indeed, we recently found in necrotic myocardium of patients with STEMI a dense infiltration with mDC but not pDC (unpublished data). In the study of Van Vre et al. [22], patients with STEMI were excluded similar to our present investigation, explaining the comparable DCP decrease pattern observed in both these studies. In a recently published work, Van Vre et al. described that mDC are activated by hsCRP leading to DC maturation and increased DC-induced T cell stimulation, and that mDC are colocalized with hsCRP in atherosclerotic lesions [22]. Corresponding to that, we observed in the present study a significant correlation between circulating DCP, particularly mDCP, and serum hsCRP, indicating that hsCRP might be involved in mDCP recruitment into atherosclerotic lesions in CAD.

Regarding the therapeutical procedure, we compared CAD patients who required CABG or PCI with those appointed to medication, or no need for any treatment. Patients who required CABG or PCI had significantly lower levels of circulating mDCP, pDCP, and tDCP compared to the other groups, indicating that their level might be predictive of the targeted therapy after coronary angiography. Skowasch et al. described a recruitment of mDCP into the treated lesion after PCI, suggesting that they are responsible for in-stent restenosis [30]. However, in our study we were not able to detect any differences in the level of circulating DCP in PCI-treated patients before and 24 h after PCI.

Compared to other previously published studies [16, 22], the higher number of patients in our present study allowed us to perform a detailed subgroup analysis. We showed significantly lower levels of circulating pDCP and tDCP, and a trend for lower mDCP, in patients with hypertension or hyperlipidemia. Patients with diabetes mellitus had a trend for lower mDCP, pDCP, and tDCP too. An important aspect of the subgroup analysis is that patients with a family history of a CAD had no reduced levels of circulating DCP, indicating that lower levels of DCP are not a genetically determined factor for the development of a CAD. A trend for lower DCP levels were observed in patients treated with statins, aspirin,  $\beta$  blockers. However, regarding *table 1* a significantly higher number of CAD patients than controls were treated with aspirin or statins, so that the higher percentage of CAD patients in the corresponding treatment groups (*table 2*) is most likely to be responsible for the observed slightly lower levels of circulating DCP in those groups, and a drug-induced effect can be excluded.

Several factors were described to be predictive of the presence of significant coronary stenoses in patients with suspected CAD and of the cardiovascular risk in patients with known CAD. It was shown that lipoprotein(a) [31, 32], lipoprotein-associated phospholipase A2 [33], total and differential leukocyte counts [34], leptin [35], serum glycated albumin [36], and hsCRP [37] are significantly associated with and predictive of a stable CAD. In those studies it was shown in univariate analyses that certain clinical and serological factors such as age, male sex, diabetes mellitus, hypertension, HDL cholesterol, creatinine, and hsCRP were predictors of the presence of a stable CAD [31, 34, 36, 37]. However, most of those factors are associated with each other, so that even established atherogenic markers such as hsCRP, interleukin (IL)-6, and TNF- $\alpha$  lost their independent predictive value for a stable CAD in multivariate analysis after adjusting for conventional CAD risk factors [38]. In contrast, large-scale clinical studies showed that hsCRP, IL-6,

and several other factors are well established predictors of future ischemic cardiovascular events in patients with higher risk for or a known CAD [39, 40].

In our study, we investigated whether the decrease in circulating DCP is predictive of the presence of a stable CAD. In the first step, we investigated which factors are associated with a stable CAD using correlation analysis. Compared to hsCRP, HDL, and creatinine, we showed a higher significant correlation of circulating mDCP, pDCP, and tDCP with the CAD score. Subsequently, we performed logistic regression analyses to investigate if the decrease in circulating DCP is a predictor of the presence of significant coronary artery stenoses. In univariate analysis, several factors such as higher age, diabetes mellitus, low HDL cholesterol, high hsCRP, and reduced relative and absolute levels of circulating mDCP, pDCP, and tDCP were predictors of a CAD. Male gender, hypertension, and elevated creatinine seem to have a predictive value too, but did not reach statistical significance. In multivariate analyses only the reduction of circulating mDCP, pDCP, or tDCP remained as independent predictors of a significant CAD.

The reason for the decrease in circulating DCP could not be clarified in our study. Several explanations are possible such as (1) reduced production of DCP in the bone marrow, (2) increased apoptosis of circulating DCP, or (3) enhanced recruitment and higher turnover of circulating DCP in atherosclerotic lesions. There is no evidence for the first or the second explanation as other leukocyte subpopulations are not reduced in blood of CAD patients. There exist several studies supporting the third explanation. Firstly, it was shown that mDC and pDC accumulate in vascular lesions dependent on the stage of atherosclerosis [7-9]. Secondly, it was suggested that mDCP recruitment from blood into the intima is induced by several atherogenic factors like oxidized LDL cholesterol, hypoxia, TNF- $\alpha$ , or nicotine [12, 18]. Thirdly, recirculation of mDC from the vascular wall into blood is suppressed by lysophosphatidic acid or platelet-activating factor in atherosclerosis [19].

In conclusion, the results of our present study show a significant decrease in circulating mDCP, pDCP, and tDCP in patients with a CAD with stable clinical manifestation. The extent of their reduction is associated with the stage and with the recommended therapeutic procedure of CAD. Atherogenic risk factors such as hypertension or hyperlipidemia might affect the level of circulating DCP too. In multiple stepwise logistic regression analysis we prove that in contrast to other classical predictors only the decrease in circulating mDCP, pDCP, and tDCP remains as an independent predictor of a significant CAD. Thus, analysis of circulating DCP might be useful for the diagnosis of CAD. Future prospective studies will be necessary to investigate if the decrease in circulating DCP is predictive also of future ischemic cardiovascular events in patients with high risk for or known CAD.

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#### DISCLOSURES

None

## FIGURE LEGENDS

**Figure 1** Representative dot plots showing the gating strategy used for the identification of circulating myeloid DC precursors (mDCP), plasmacytoid DC precursors (pDCP), and total DC precursors (tDCP) by flow cytometry (upper panel: DCP analysis, lower panel: isotype control). In region **R1** peripheral blood mononuclear cells (WBC) were separated from debris and platelets according to their typical forward (FSC) and side scatter (SSC). In region **R2** excluded were: granulocytes by side scatter, B lymphocytes by CD19-staining, monocytes by CD14-staining, and dead cells by propidium iodide-staining. In region **R3** and **R4** circulating mDCP and pDCP were detected according to their specific BDCA-1- and BDCA-2-staining, respectively. The number of circulating tDCP represents the sum of cells detected in **R3** plus **R4**.

**Figure 2** Frequency of circulating mDCP, pDCP, and tDCP in different stages of coronary artery disease (CAD): **Score 0** (excluded CAD), **Score 1-5** (early CAD), **Score 6-10** (moderate CAD), and **Score > 10** (advanced CAD). Relative numbers of circulating mDCP (**A**), pDCP (**B**), and tDCP (**C**) shown as a percentage of peripheral blood mononuclear cells (% WBC). Absolute numbers of circulating mDCP (**D**), pDCP (**E**), and tDCP (**F**) shown as cells per microliter (cells/ $\mu$ L). The *box plots* indicate the median (line inside box), 25<sup>th</sup> and 75<sup>th</sup> percentile (upper and lower boundary of box), and 10<sup>th</sup> and 90<sup>th</sup> percentile (whiskers outside box); ns = not significant.

**Figure 3** Relative and absolute frequency of circulating mDCP, pDCP, and tDCP according to the required therapeutical procedure in firstly diagnosed CAD: Control/No (excluded CAD, no therapy), CAD/Med (CAD to be treated with medication), CAD/PCI (CAD requiring a percutaneous coronary intervention), CAD/CABG (CAD to be treated with coronary artery bypass grafting).

**Figure 4** Correlation of circulating DCP and other factors with the CAD Score. **Upper panel:** A significant inverse correlation was observed between the percentage of circulating mDCP (**A**), pDCP (**B**), and tDCP (**C**) and the CAD Score. **Lower panel:** Between hsCRP and the CAD Score, there was only a trend for correlation (**D**). HDL cholesterol (**E**) and creatinine (**F**) were significantly correlated with the CAD Score.

**Figure 5** Predictive value of the decrease in circulating DCP for the presence of a CAD.

**A. Univariate Analysis:** Several factors such as age, diabetes mellitus, HDL cholesterol, hsCRP, relative and absolute numbers of circulating mDCP, pDCP, and tDCP were identified to be predictive of the presence of a CAD (coronary stenosis/es > 50%). Other factors such as male gender, hypertension, and creatinine seemed to be associated with a CAD, but missed statistical significance

**B. Multiple stepwise logistic regression analysis:** Relative and absolute numbers of circulating mDCP, pDCP, and tDCP were separately analyzed in multiple stepwise logistic regression analyses each together with the factors of the univariate analysis (their results shown for the tDCP % analysis). In contrast to those factors, relative (mDCP, pDCP, and tDCP) and absolute (pDCP and tDCP) numbers of circulating DCP remained as independent predictors of a CAD. Red color: statistically significant. Black color: not significant. On the right of each figure: odds ratio, 95% confidence interval (CI), and P value.

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**Table I Baseline characteristics of patients with coronary artery disease (CAD) and control subjects**

Score	0	1-5	6-10	>10	P
<b>No. of patients</b>	57	63	85	85	
<b>Age (years)</b>	57.5	66.5	68	67	< 0.001
<b>Male gender (%)</b>	52	57	52	54	ns
<b>Atherogenic risk factors (%)</b>					
Diabetes mellitus	14	16	25	21	ns
Hypertension	68	87	71	64	ns
Smoking	18	19	14	13	ns
Hyperlipidemia	58	78	58	74	ns
Family history of CAD	32	27	19	21	ns
<b>Current Medication (%)</b>					
Statins	38	73	88	84	< 0.001
Aspirin	47	70	61	67	0.03
β Blocker	60	62	49	47	ns
ACE inhibitors	53	64	52	55	ns
<b>Therapeutical procedure (%)</b>					
PCI	0	16	41	39	< 0.001
CABG	0	0	8	44	< 0.001
<b>Serum parameters</b>					
LDL cholesterol (mg/dL)	137	139	116	129	ns
HDL cholesterol (mg/dL)	55	47	45	45	0.03
Leukocytes (cells/ $\mu$ L)	6500	7455	7235	7535	0.06
hsCRP (mg/L)	0.26	1.68	2.20	2.17	0.16
Creatinine (mg/dL)	0.97	1.05	1.03	1.09	0.003
<b>Circulating DCP values (% / cells/<math>\mu</math>L)</b>					
mDCP	0.27 / 17.8	0.22 / 15.6	0.21 / 15.0	0.18 / 13.4	< 0.001
pDCP	0.15 / 9.4	0.12 / 8.7	0.10 / 6.8	0.09 / 6.0	< 0.001
tDCP	0.42 / 29.0	0.36 / 26.9	0.33 / 25.5	0.26 / 21.9	< 0.001

Clinical data were compared for different stages of CAD: Score 0 (excluded CAD), Score 1-5 (early CAD), Score 6-10 (moderate CAD), and Score > 10 (advanced CAD). Values are reported as median or %. Statistical analysis was performed using the Mann-Whitney rank sum test.

**Table II Subgroup analysis of DCP values compared to clinical data or medication**

	mDCP			pDCP			tDCP		
	-	+	P	-	+	P	-	+	P
<b>Age &gt; 66 years</b>	15.6	15.5	NS	8.9	7.9	NS	27.3	25.9	NS
<b>Male gender</b>	15.0	15.5	NS	7.3	8.2	NS	23.9	25.7	NS
<b>Atherogenic risk factors (%)</b>	x			x			X		
Diabetes mellitus	15.3	14.8	NS	8.0	7.0	NS	24.8	23.9	NS
Hypertension	16.6	15.1	NS	<b>9.5</b>	<b>7.3</b>	<b>0.001</b>	<b>27.4</b>	<b>24.3</b>	<b>0.01</b>
Smoking	14.8	14.8	NS	7.1	8.4	NS	17.2	19.1	NS
Hyperlipidemia	16.1	15.1	NS	<b>8.7</b>	<b>7.4</b>	<b>0.03</b>	<b>28.9</b>	<b>24.2</b>	<b>0.02</b>
Family history of CAD	15.3	15.6	NS	7.8	7.8	NS	25.1	25.6	NS
<b>Current Medication (%)</b>									
Statins	18.1	17.8	NS	9.0	8.6	NS	29.1	27.9	NS
Aspirin	15.7	15.1	NS	9.2	8.3	NS	27.3	25.3	NS
β Blocker	16.7	16.0	NS	8.5	8.3	NS	27.3	26.1	NS
ACE inhibitors	15.0	15.4	NS	8.0	7.7	NS	25.9	24.7	NS
<b>Serum parameters</b>									
LDL cholesterol > 130mg/dL	14.4	15.5	NS	7.4	8.3	NS	23.5	26.1	NS
HDL cholesterol > 45 mg/dL	14.5	16.2	NS	7.2	8.3	NS	18.2	20.5	NS
hsCRP > 2.0 mg/L	16.8	14.5	NS	8.0	7.9	NS	26.7	25.4	NS
Creatinine >1.0 mg/dL	14.9	15.4	NS	7.7	7.9	NS	24.4	24.8	NS
Leukocytes > 7300 cells/μL	<b>13.9</b>	<b>16.8</b>	<b>0.002</b>	<b>6.9</b>	<b>8.5</b>	<b>0.002</b>	<b>23.2</b>	<b>28.2</b>	<b>&lt; 0.001</b>

Absolute numbers of mDCP, pDCP, and tDCP (cells / μL) are calculated for the presence (+) or absence (-) of a clinical feature or medication. Values are reported as median. Statistical analysis was performed using the Mann-Whitney rank sum test.

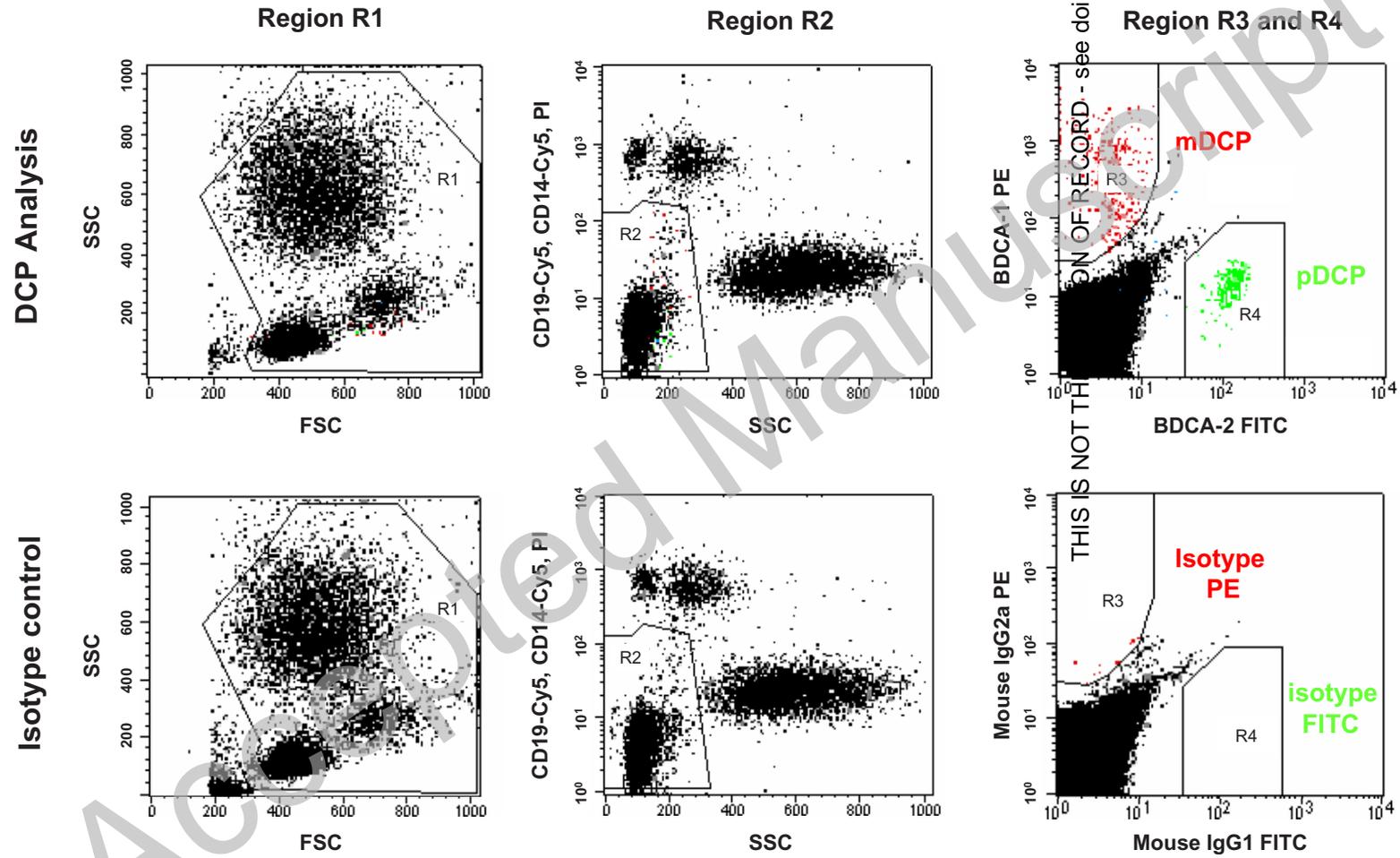


Figure 1

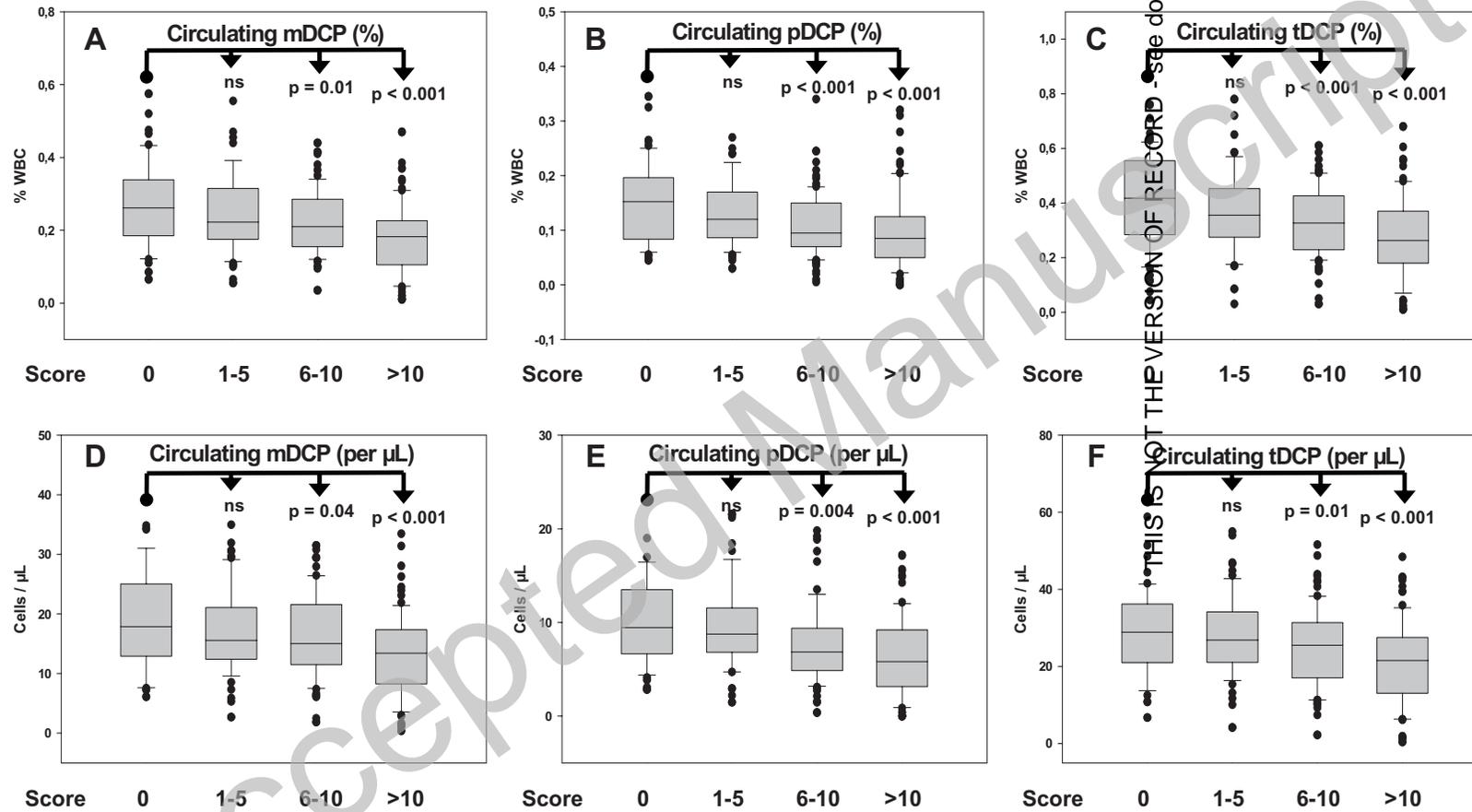


Figure 2

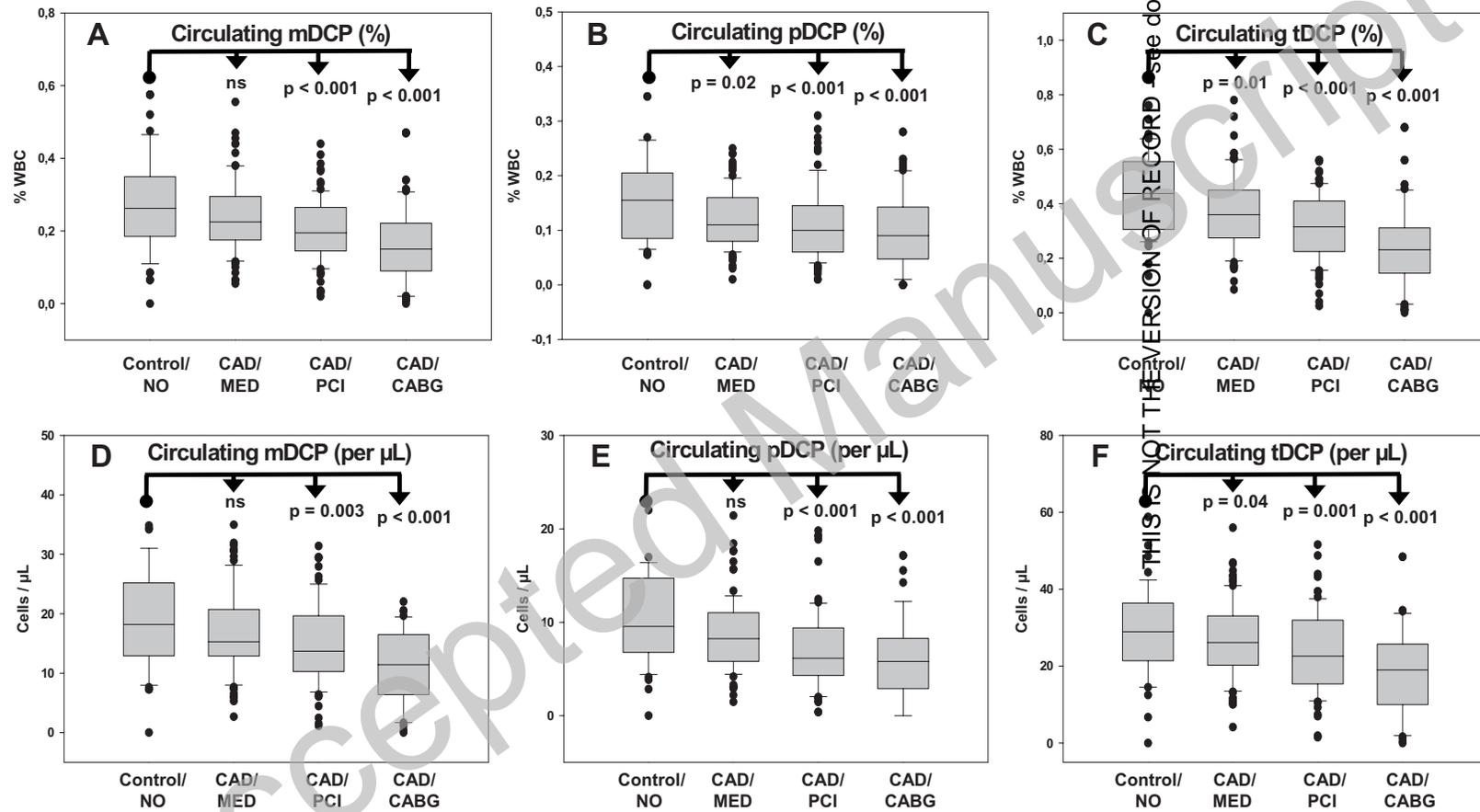


Figure 3

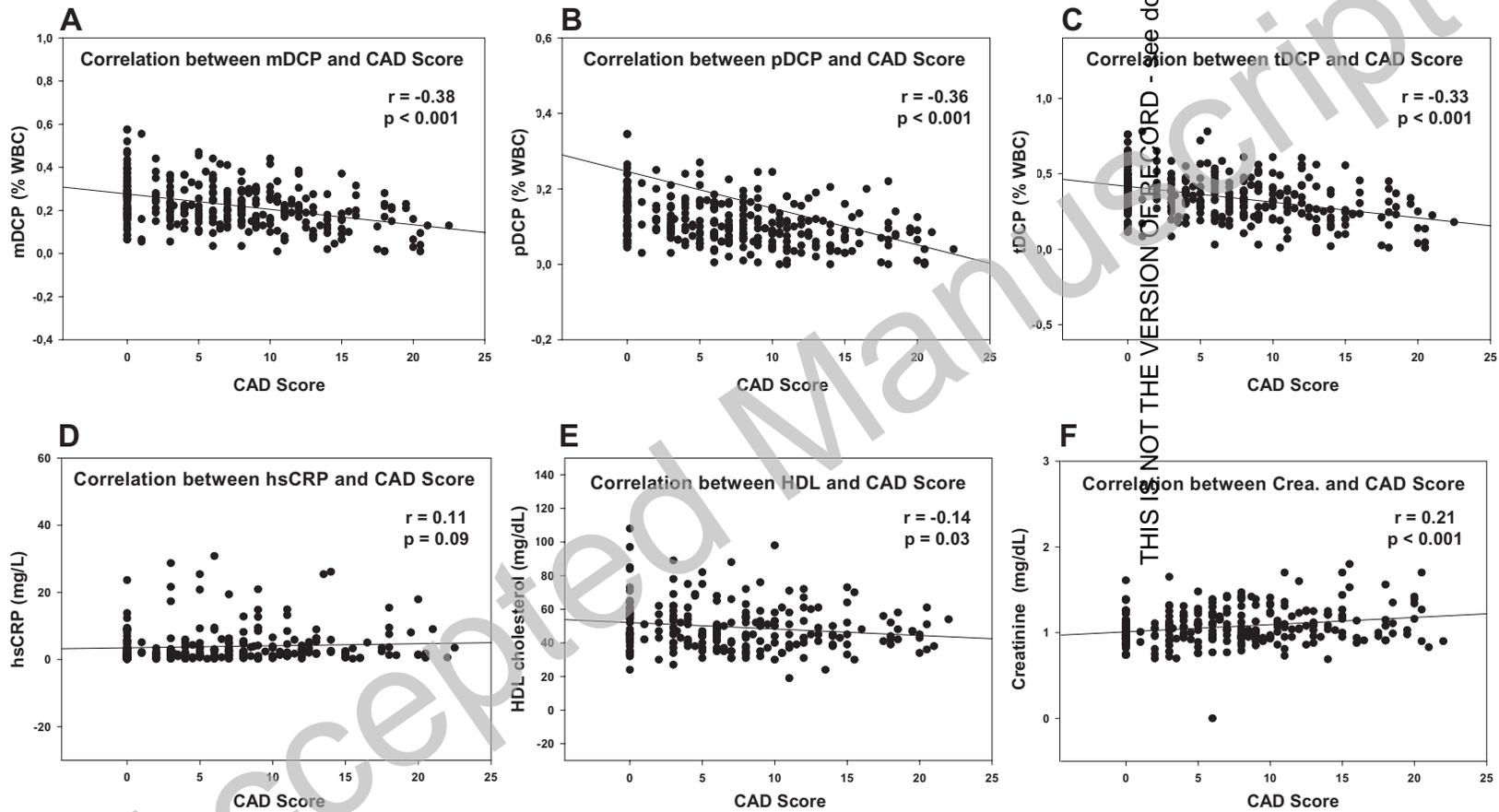


Figure 4

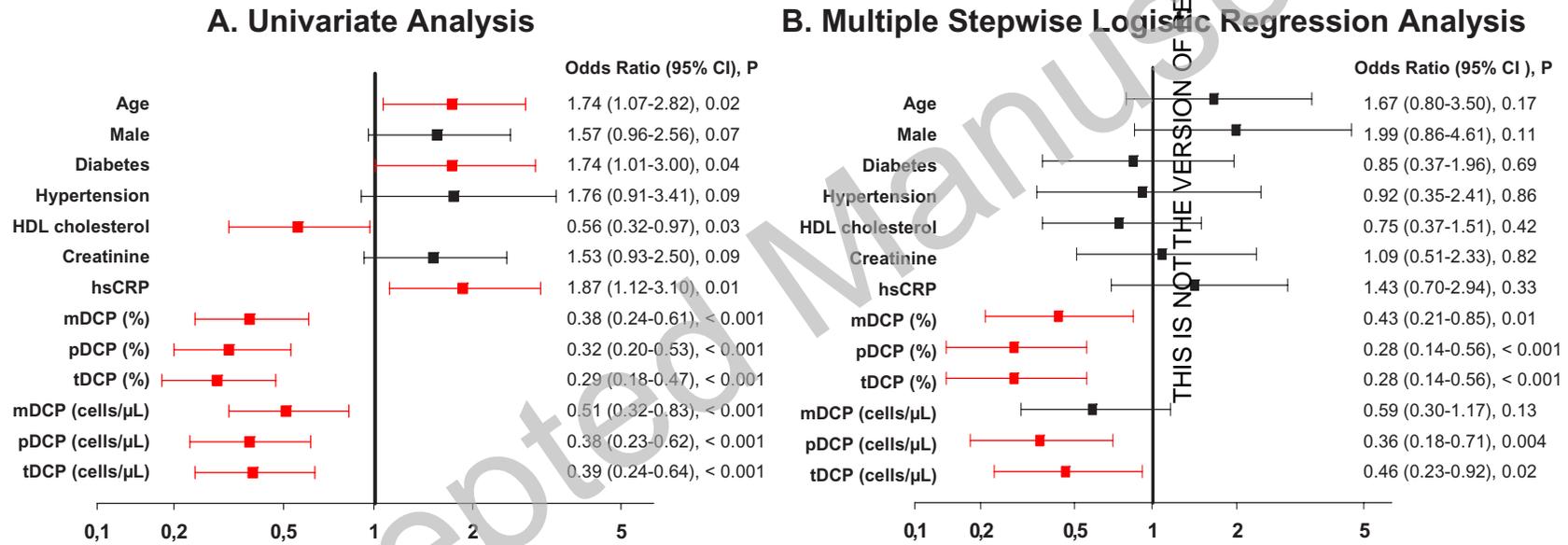


Figure 5