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Expanded peripheral $CD4^+CD28^{null}$ T cells and its association with atherosclerotic changes in patients with end stage renal disease on hemodialysis

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ABSTRACT

End-stage renal disease (ESRD) patients, including those on hemodialysis, possess a high risk for cardiovascular diseases, as the first leading cause of death among them. Traditional risk factors do not utterly elucidate this. Throughout the last two decades, $CD4^+CD28^{null}$ T cells; an unusual subset of T lymphocytes, was detected high with excess cardiovascular (CV) mortality. We aimed to investigate the circulating $CD4^+CD28^{null}$ T cells frequency in ESRD patients on hemodialysis and to evaluate their relationship with atherosclerotic changes. High-resolution carotid ultrasonography was done to assess the common carotid artery intima media thickness in a number of ESRD patients, accordingly patients were selected and subdivided into two groups; 30 with atherosclerosis (mean [SD] age, 51.6 [6.3] years) and 30 without (mean [SD] age, 48.9 [5.5] years). Another 30 healthy individuals (mean [SD] age, 48.5 [6.8] years) were enrolled. Analysis of $CD4^+CD28^{null}$ T-cells frequency by flow-cytometry was performed in all studied subjects. $CD4^+CD28^{null}$ T cell percentage was significantly higher in ESRD patients, (mean [SD], 7.3 [2.7] %) compared to healthy individuals (mean [SD], 3.0 [0.8] %), ($p < 0.001$). Additionally, the expansion of these unusual T lymphocytes was significantly higher in ESRD patients with atherosclerotic changes (mean [SD], 9.47 [0.75] %) compared to those without atherosclerosis (mean [SD], 5.22 [2.14] %), ($p < 0.001$). In conclusion circulating $CD4^+CD28^{null}$ T lymphocyte population showed expansion in ESRD patients, and of interest in correlation to preclinical atherosclerotic changes.

1. Introduction

End-stage renal disease (ESRD) is a global devastating health burden with a rising prevalence. Despite improvements in renal replacement therapy (RRT), patients continue to have a high mortality risk, particularly due to atherosclerotic cardiovascular diseases (CVD) [1,2]. Almost 50% of those suffering from established ESRD are unlikely to survive a cardiovascular event. [3–5] Traditional Framingham risk factors, as hypertension, hyperglycemia, and dyslipidemia are highly prevalent in those patients, yet they cannot entirely justify this increased mortality [6].

On the other hand ESRD-patients appear to have premature immunological T cell aging with considerable clinical effect leading to raised morbidity and mortality. This uremia-associated accelerated aging of the immune system, is characterized by increased numbers of $CD28^{null}$ T cells, a pro-inflammatory subset of $CD4^+$ T-lymphocytes lacking CD28 receptor [7,8]. Normally $CD4^+CD28^{null}$ T cell subset constitute a minority in young healthy individuals, accounting for about 0.1–2.5% of the $CD4^+$ T cells. [9] In elderly individuals, this percentage increase and mark immunosenescence [10]. Susceptibility to repetitive infections is increased among ESRD patients, for instance approximately 70% of them are cytomegalovirus (CMV)-seropositive [11]. Previous studies showed that continuous antigenic stimulation of T cells by chronic inflammation or ubiquitous antigens, such as CMV antigens, leads to rapid and extensive T cell replication, during which CD28 costimulatory molecule is downregulated [12,13].

Abbreviations: ESRD, end-stage renal disease; RRT, renal replacement therapy; CVD, cardiovascular diseases; CMV, cytomegalovirus; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; CKD, chronic kidney disease; HC, healthy control; CIMT, carotid intima media thickness; ESC-ESH, European Society of Cardiology-European Society of Hypertension; CRP, C-reactive protein; PBS, phosphate buffered saline

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Over the past two decades, there has been accumulating evidence for age-inappropriate increase in cytotoxic $CD4^+CD28^{null}$ T cells in a variety of chronic inflammatory diseases, suggesting their role in the pathogenesis of these disorders. [14–18] Atherosclerosis is a multifactorial disease, yet chronic inflammation predominates [19,20]. It is by far the most common disease affecting the cardiovascular system. Patients with coronary atherosclerosis that experience myocardial infarction or unstable angina, have excess $CD4^+CD28^{null}$ T cells [16]. Surprisingly, detailed immunological analysis of atheromas revealed the presence of $CD4^+CD28^{null}$ T cells. These cells were detected more in unstable compared with stable atherosclerotic lesions, suggesting that they induce plaque destabilization and consequently life-threatening acute coronary events [21].

$CD4^+CD28^{null}$ T cells possess pro-inflammatory characteristics, compared with their $CD28^+$ counterparts, they produce significantly higher levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and Interlukin-2 [22]. IFN-γ stimulates the recruitment and activation of macrophages and their secretion of metalloproteinases, which has a tissue damaging effect resulting in plaque rupture, superimposed thrombosis and thus raises the risk of acute ischemia [16,23,24]. In addition, they release cytotoxic molecules perforin and granzyme B [25,26], which cause endothelial cells lysis, subsequently dead endothelium mediate plaque instability and rupture [23,27].

To date, there is few data on $CD4^+CD28^{null}$ T cells and preclinical atherosclerosis in patients with ESRD and such a relation still need to be established, especially for hemodialysis patients. In this study, we evaluated the possibility of increased count of peripheral $CD4^+CD28^{null}$ T cells in ESRD patients free of concomitant atherosclerotic disease, and investigated a possible association between their frequency and preclinical atherosclerotic changes.

2. Subjects and methods

2.1. Study participants

We studied 60 adult ESRD patients (35 males/25 females), on maintenance hemodialysis for at least 3 months. Patients were attending Theodor Bilharz Research institute. “End-stage renal disease” is defined as CKD with GFR < 15 ml/min/1.73 m^2 and albuminuria > 300 mg/g [28]. We selected patients free from any coexisting symptomatic or previously established atherosclerotic disease; CAD, CVD or PAD. More than 100 consecutive patients with ESRD were screened for detection of preclinical atherosclerosis by measurement of carotid intima media thickness, patients were chosen accordingly; 30 with preclinical atherosclerotic changes and 30 without. Carotid intima media thickness (CIMT); values > 0.9 mm are considered abnormal according to the criteria of the European Guidelines for the management of Hypertension of the ESC-ESH [29]. Hemodialysis vascular accesses were permanent arteriovenous fistula in most of patients and dialysis catheters in the remaining. We excluded patients above 60 years, smokers, diabetics, those with acute kidney injury, active infection, hyperlipidemia, malignancy, or any autoimmune diseases. Subjects taking antilipidemic or immunosuppressive drugs were not included.

The control group consisted of 30 apparently healthy (HC), both patients and HC were age and gender matched and of the same social class as far as possible. Healthy controls were enrolled with exclusion criteria similar to ESRD patients. Study was approved by the research ethical committee of Ain Shams University and an informed consent was obtained from all participants.

2.2. Clinical and laboratory evaluation

We collected relevant demographic and clinical data including age, gender, duration of hemodialysis, previous kidney transplants, primary kidney disease, medications, and any comorbidities or symptoms suggestive of CAD, CVD, or PAD. Routine biochemical parameters; serum creatinine, serum urea, eGFR, serum cholesterol and triglyceride were done by standard laboratory methods.

2.3. C-reactive protein (CRP) and CMV-serology

CMV serum immunoglobulin G [IgG] antibodies were measured with an enzyme immunoassay [Biomerieux, VIDAS, Lyon, France] and expressed as arbitrary units/milliliter [AU/ml]. A test result exceeding 6 AU/mL was considered positive for the presence of CMV-IgG antibodies. Serum concentrations of CRP were assayed by immunoturbidimetry using kit supplied by Behring Diagnostics (GmbH, Marburg, Germany).

2.4. Flowcytometric analysis of $CD4^+CD28^{null}$ cell T cells

Evaluation of $CD4^+CD28^{null}$ cell expansion on T lymphocytes was done by standard 2-color flow cytometry using the following monoclonal antibodies: Phycoerythrin-labeled anti-CD28 (BD Pharmingen™ CD28 T-cell phenotyping kits) and Fluorescein isothiocyanate-labeled anti-CD4 (BD Pharmingen™ CD4 T-cell phenotyping kits). Heparinized venous blood was collected and peripheral blood mononuclear cells were obtained by density gradient centrifugation. Cell-surface staining was done using anti-CD4 and anti-CD28. After staining, cells were washed once and fixed with 0.5 ml or 1 ml of phosphate buffered saline (PBS) containing 1.5% paraformaldehyde. Data were examined using BECKMAN Coulter flow cytometer and were expressed as percentage.

2.5. Carotid intima- media thickness [CIMT]

High resolution B mode ultrasonography of both the common and internal carotid arteries, was performed using an ultrasound machine [Toshiba Memo 30 scanner] equipped with a 7.5 mHz high resolution transducer. Patients were inspected in the supine position with the head tilted backward. After the carotid arteries were located by transverse scans, the probe was rotated to 90° to obtain a longitudinal image of the common carotid arteries. The maximum CIMT was measured at the posterior wall of the common carotid artery, 2 cm before the bifurcation. Measurement was performed vertically to the arterial wall for accurate measurement of CIMT. Three CIMT measurements were taken and the average measurement was used.

2.6. Statistical methodology

Statistics were analyzed using SPSS© Statistics version 17 [SPSS© Inc., Armonk, NY, USA]. Continuous numerical variables were presented as mean and SD. The between-group differences were estimated using the independent samples *t* test [for two group comparison] or one-way analysis of variance [ANOVA] for multiple group comparison. The Tukey-Kramer test was applied for multiple post hoc comparisons whenever the ANOVA test revealed a statistically significant difference among the groups. Categorical variables were displayed as number and percentage. The differences were compared using Fisher’s exact test [for nominal data] or the chi-squared test [for ordinal data]. Correlations were tested using the Pearson correlation analysis. The correlation coefficient [Spearman rho] is interpreted as follows: < 0.2 very weak, 0.2–0.39 weak, 0.4–0.59 moderate, 0.6–0.79 strong, 0.8–1 very strong.

Receiver-operating characteristic [ROC] curve analysis was used to discriminate between patients with or without atherosclerotic changes according to the value of $CD4^+/CD28^{null}$ T cells. The area under the ROC curve [AUC] is interpreted as follows: 9–1.0 excellent, 0.8–0.89 good, 0.7–0.79 fair, 0.6–0.69 poor, < 0.6 fail.

Multivariable backward binary logistic regression analysis was utilized to identify the relation between CD4+/CD28nullT cells, ESRD, and occurrence of atherosclerotic changes as adjusted for possible confounding factors. P-values < 0.05 were considered statistically significant.

Table 1
Characteristics of the three studied groups.

Variable	ESRD with atherosclerotic changes [n = 30]	ESRD without atherosclerotic changes [n = 30]	Control [n = 30]	p-value
Age [years]	51.6 ± 6.3	48.9 ± 5.5	48.5 ± 6.8	0.07
Gender				
M	19 [63.3%]	16 [53.3%]	17 [56.7%]	0.802§
F	11 [36.7%]	14 [46.7%]	13 [43.3%]	
Duration of hemodialysis [years]	7.6 ± 3.4	6.2 ± 4.8	NA	0.210
Serum urea [mg/dl]	149.6 ± 23.9†	168.2 ± 67.0†	16.8 ± 3.3	< **0.001**
Serum creatinine [mg/dl]	8.7 ± 1.2†,‡	7.7 ± 1.1†	0.8 ± 0.2	< **0.001**
GFR [ml/min/1.73 m^2]	5.9 ± 1.4†	7.3 ± 1.6†	99.9 ± 18.3	< **0.001**
Serum cholesterol [mg/dl]	146.6 ± 14.7†,‡	134.2 ± 21.0†	116.8 ± 20.5	< **0.001**
Serum triglycerides [mg/dl]	110.1 ± 12.4†,‡	140.0 ± 19.3†	88.0 ± 13.1	< **0.001**
CRP [mg/dl]	15.8 ± 5.6†,‡	11.2 ± 3.9†	6.0 ± 4.2	< **0.001**
Positive CMV IgG	8 [26.7%]	3 [10.0%]	0 [0.0%]	**0.005**§
Positive CRP	29 [96.7%]	26 [86.7%]	11 [36.7%]	< **0.001**§

Data are mean ± SD or number [%].
One-way analysis of variance [ANOVA].
† p-value < 0.05 vs. Control group [Tukey-Kramer test].
‡ p-value < 0.05 vs. ESRD without atherosclerotic changes group [Tukey-Kramer test].
§ Fisher's exact test.

3. Results

3.1. Laboratory and demographic characteristics of study subjects

We enrolled 60 patients with ESRD (30 with atherosclerosis vs thirty without) and 30 healthy individuals. Demographic and laboratory variables of ESRD subjects and controls are shown in Table 1. There was no statistical significant difference between studied groups regarding age or gender (Table 1). Serum CRP level was significantly elevated in ESRD patients with atherosclerotic changes compared to the other two studied groups (p < 0.001). Positive CMV IgG serology was detected only in 8 patients with atherosclerosis (26.7%) and 3 patients without (10%).

3.2. $CD4^+CD28^{null}$ T cells frequency

Table 2 showed significantly higher percentage of $CD4^+CD28^{null}$ cells of the total CD4+ cell population, in ESRD patients than in healthy individuals (7.3 ± 2.7% vs 3.04 ± 0.79, P < 0.001). ESRD patients with atherosclerotic changes had the higher peripheral frequency compared to the other studied groups (9.47 ± 0.75% vs 5.22 ± 2.14% vs 3.04 ± 0.79, respectively, P < 0.001). Individual distribution of $CD4^+CD28^{null}$ T cells frequency among the studied groups is shown in Fig. 1.

3.3. Correlation between CIMT, CRP and $CD4^+CD28^{null}$ T cells frequency

Highly significant positive correlation was detected between $CD4^+CD28^{null}$ T cell % and both CIMT (r = 0.795, p < 0.000) and CRP (r = 0.353, p < 0.006) [Figs. 2, 3] besides a highly significant positive correlation between CIMT and CRP (r = 0.4, p < 0.002).

Table 2
Percentage of $CD4^+CD28^{null}$ of the total CD4+ cell population, in ESRD patients in comparison to healthy individuals.

Variable	No ESRD [n = 30]	ESRD [n = 60]	p-value
$CD4^+/CD28^{null}$ [%]	3.0 ± 0.8	7.3 ± 2.7	< **0.001**¶

Data are mean ± SD or number [%].
¶ Unpaired *t* test.

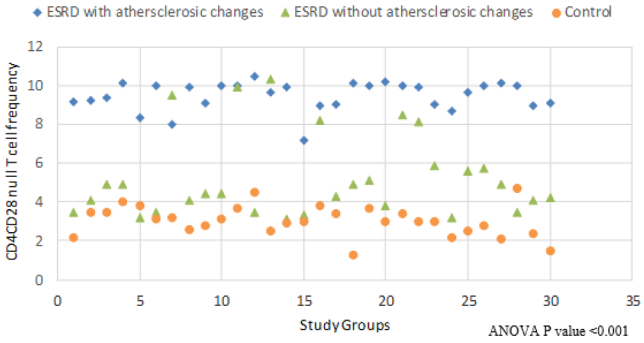

Fig. 1. $CD4^+/CD28^{null}$ T cell frequency in the three studied groups.

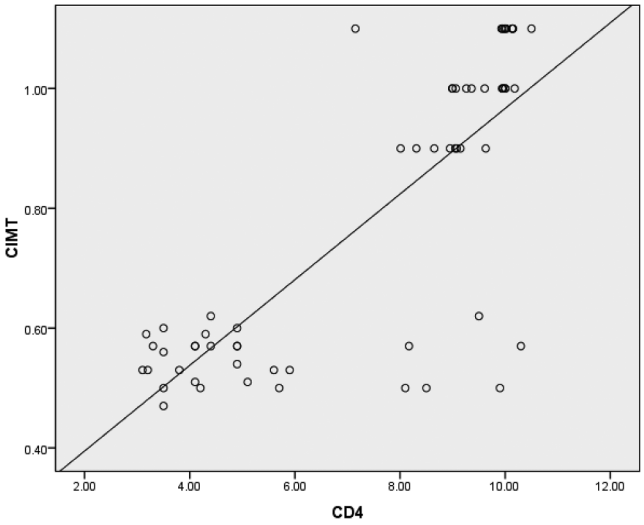

Fig. 2. Correlation between CIMT and $CD4^+CD28^{null}$ T cells (r = 0.795 and P = 0.0001).

3.4. Diagnostic accuracy of $CD4^+CD28^{null}$ T cell % in discriminating ESRD patients with or without atherosclerotic changes

ROC curve analysis for discrimination between ESRD patients with or without atherosclerotic changes using $CD4^+CD28^{null}$ T cell % showed good sensitivity (100%) and specificity (80%), with calculated area under the curve (AUC) of 0.929 and 95% CI (0.832 to 0.979). Best

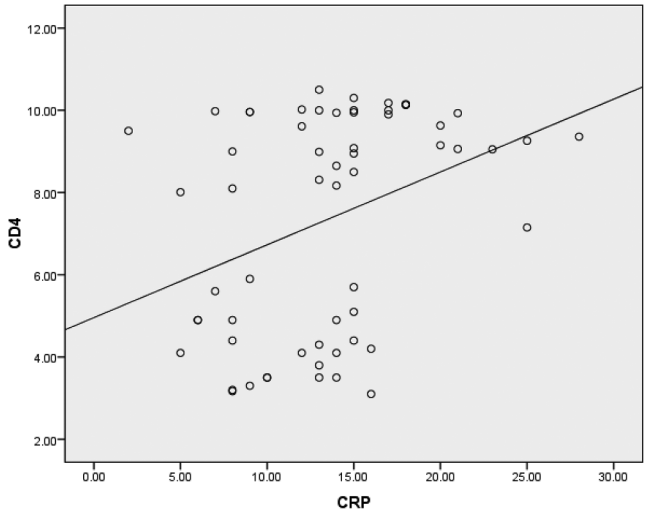

Fig. 3. Correlation between CD4 + CD28 null T cells and CRP (r = 0.363 and P = 0.006).

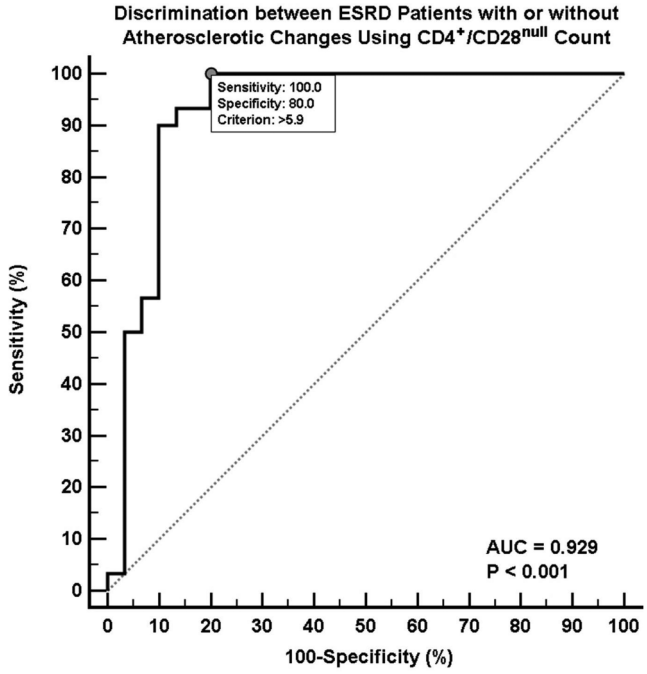

Fig. 4. Receiver-operating characteristic [ROC] curve analysis for discrimination between ESRD patients with or without atherosclerotic changes using CD4 + /CD28^{null} %.

cut-off criterion (%) > 5.9, Positive likelihood ratio (LR +) 5, Negative likelihood ratio (LR −) 0 [Fig. 4]

A Multivariable backward binary logistic regression analysis was performed for the relation between CD4 + /CD28^{null} T cell or ESRD and the occurrence of atherosclerotic changes as adjusted for possible confounding factors is demonstrated in table 3; where CD4^{+}/CD28^{null} T cell (%) (p-value 0.004), positive CMV IgG (p-value 0.038), serum cholesterol (p-value 0.008) and serum triglycerides (p value 0.006) were independent predictors of atherosclerotic changes.

4. Discussion

CD4^{+}CD28^{null} T cell population is a unique CD4^{+}T-cell subsets lacking surface CD28. They are highly cytotoxic, apoptosis resistant with proinflammatory potential [30–32]. These aggressive T-lymphocyte subsets are thought to be a substantial player in the progression of atherosclerotic diseases [21]. They expand in acute coronary syndrome and are related to the ongoing vascular damage [33].

Table 3
Multivariable backward binary logistic regression analysis for the relation between CD4 + /CD28^{null} T cells or ESRD and the occurrence of atherosclerotic changes as adjusted for possible confounding factors.

Variable retained in model*	p-value	OR	95% CI for OR
CD4^{+}/CD28^{null} [%]	**0.004**	6.29	1.81–21.87
Age [years]	0.091	1.24	0.97–1.60
Positive CMV IgG [= 1]	**0.038**	1772.0	1.50–2,099,014.93
Serum cholesterol [mg/dl]	**0.008**	1.17	1.04–1.32
Serum triglycerides [mg/dl]	**0.006**	0.76	0.63–0.93
Constant			
*Variables excluded from the model**			
History of IHD			
Positive CRP			
Serum creatinine			
GFR			
ESRD			

OR, odds ratio; 95% CI, 95% confidence interval.
* Backward regression.

The present study was designed to determine if CD4^{+}CD28^{null} T cells could possibly be involved in atherosclerotic process among ESRD patients on renal replacement therapy (RRT). We analyzed the frequency of peripheral CD4^{+}CD28^{null} T cells in relation to the presence of preclinical atherosclerotic changes in 60 ESRD patients, selected according to CIMT; 30 with atherosclerotic changes and 30 without, in addition to another 30 healthy controls.

We detected findings that confirm and extend multiple previous studies. The frequency of CD4^{+}CD28^{null} T cells was significantly higher in our patients compared with controls, and it is worth mentioning that all our patients were on hemodialysis for at least 3 months. We measured CRP levels in all our studied population as a marker of systemic inflammation, it was found high in all ESRD patients, but those with preclinical atherosclerotic changes had the highest levels compared to the other two studied groups. Significantly elevated serum CRP in group of patients with increased CIMT confirms the inflammatory nature of atherosclerotic plaques; nevertheless atherogenesis remains a complex chronic inflammatory process.

In line with this Betjes et al. detected the highest prevalence of atherosclerotic disease in ESRD patients with high CRP levels [34]. Interestingly in the current study, we further detected a statistically significant positive correlation between CRP and CIMT, between CRP level and CD4^{+}CD28^{null} T cells percentage. Similarly Liao et al. found high-sensitivity-CRP significantly correlated with CIMT among subjects without obvious cardiovascular disorders. CRP is a non-specific inflammatory mediator, speeding up the formation and breakdown of atheromatous plaque [35]. Therefore, it was no wonder that CRP was positively correlated with CIMT.

Positive correlation between both CRP and CD4^{+}CD28^{null} T cells has been shown in a number of studies [36–38] and Pera et al. consistent with this, found the highest count of CD4^{+}CD28^{null} T cells in CMV positive group of patients with high CRP levels, same individuals previously known to have the highest prevalence of atherosclerotic coronary artery disease [39]. Serum levels of CRP increases by at least 25% during inflammatory settings [40]. High CRP in correlation to expanded CD4^{+}CD28^{null} T cell and increased CIMT reflect the high inflammatory potential of these cytotoxic cells, and provide evidence for their contribution in the inflammatory process characterizing atherosclerosis.

Our data regarding circulating CD4^{+}CD28^{null} T cells, are in agreement with Sun et al. who also showed high percentage of these cells and high CRP levels as well in hemodialysis patients compared with healthy participants, besides again a positive correlation between the

percentage of these cells and CRP levels, similar to our observation [41].

High serum CRP is an indicator of chronic inflammation which is very common in uremia and renal replacement therapies. Chronic inflammatory state is due to retained uremic toxins, besides dialysis-induced factors, such as blood and dialyzer bio-incompatibility, endotoxins within dialysis fluid, and infections acquired from vascular access [42]. Reduced renal function per se is associated with an inflammatory response; excessive production of complements, cytokines, increased endothelial cells adhesion molecules and other pro-inflammatory mediators such as TNF-α and CRP [43].

When T lymphocytes are chronically exposed to proinflammatory cytokines, loss of CD28 expression on $CD4^+$ T cells occurs, at the transcriptional level. And this explains raised CRP and $CD28^{null}$ T cells in ESRD patients (whether on hemodialysis or not yet) and positive correlation between them [44–48]. Also peritoneal dialysis solutions have acidic pH, high glucose concentration, glucose degradation products, and all of these enrich the inflammatory process in ESRD patients [42].

Studying $CD4^+CD28^{null}$ T cells in peritoneal dialysis (PD) patients are rare. Only one study by Yadav and Jha found significantly greater number of such $CD28^{null}$ T cells in the peripheral blood of ESRD patients on PD, compared to healthy individuals. However they could not detect statistically significant association of CRP with the $CD4^+CD28^{null}$ T cells in those patients, although this parameter was significantly increased in them [49].

Betjes et al. also reported no significant correlation between $CD4^+CD28^{null}$ T cells and serum CRP levels [50]. And this association has not been consistently found in other conditions either [51,52]. There is an underlying immune derangement in ESRD, involving differentiation and activation status of T cell subsets, these changes could be deepened by dialysis procedures [53,54].

In an attempt to shed more light on this effect we recruited all our ESRD patients while on maintenance hemodialysis, and we detected increased $CD4^+CD28^{null}$ T-cell percentage in all of them as mentioned above. Our findings are consistent with Lisowska et al. who observed reduced expression of CD28; co-stimulatory surface molecule on stimulated $CD4^+$ T lymphocytes of HD patients when compared to predialysis patients and healthy controls [53].

Also previous data obtained by Meijers et al. supported ours; however the influence of dialysis was only detected within the young RRT group with significant higher percentage of $CD28^{null}$ memory T-cell among them. This was justified by longer duration of dialysis treatment than the old RRT group [55].

ESRD patients with long-duration RRT, probably have a greater systemic inflammatory burden that may increase their likelihood of distant effect on the $CD4^+$ T-cell compartment. Chronic inflammatory state which is very common among dialysis patients undoubtedly creates a state of hypercytokinemia. In this context a study by Dumitriu et al. showed that IL7 and IL-15 triggered expansion of $CD28^{null}$ T cells from myocardial infarction patients, moreover, IL-7 and IL-15 activated $CD28^{null}$ T cells and increased their cytotoxic function [56]. On the other hand Zal et al. found increased numbers of $CD4^+CD28^{null}$ cells with no difference; in non-dialysis-dependent chronic kidney disease and hemodialysis dependent ESRD patients [57]. This study however is limited by small number of patients included in each group (n = 15) and it is likely that the results obtained are affected by this. But the same was shown by Betjes et al. who could not find statistically significant differences in T-cell parameters between ESRD patients with or without dialysis, in accordance with previous studies [17,58].

The percentages of $CD28^{null}$ memory T cells were increased in old healthy controls (HC) and young ESRD patients compared with young HCs, although statistical significance was not reached. However, the old ESRD patients showed a significantly higher percentage of $CD28^{null}$ T cells [7].

Overall, their data on circulating T cells indicate that age-related changes observed in old HCs are already present in ESRD patients 30 years younger. The impact of RRT was not clarified by Yadav et al. who showed significantly increased $CD4^+CD28^{null}$ T cells percentage in stage IV-V chronic kidney disease patients, only 48% of them on dialysis, when compared to healthy controls [59].

It is obvious however that loss of renal function is the most dominant factor for premature T cell ageing. Discrepancies in results related to whether RRT could aggravate these changes or not, cannot be fully explained, may be the duration of RRT is an important factor to be considered, and this would be evident on performing further longitudinal studies.

On the contrary other studies detected low frequency of $CD4^+CD28^{null}$ T cells in ESRD patients, related to CMV serostatus and its impact on the expansion of $CD28^{null}$ T lymphocytes. A very low number of $CD4^+CD28^{null}$ T-cells was detected in CMV-seronegative patients compared to CMV-seropositive although they were all ESRD patients [50].

Another study by Betjes et al. showed that $CD4^+CD28^{null}$ T-cell are rarely observed in CMV seronegative individuals; both ESRD patients and healthy controls, but may expand to as much as 65% of the total $CD4^+$ T-cell population in elderly CMV seropositive ESRD patients [17,60]. CMV infection which is common in uremic patients is associated with profound alteration of the global T-cell repertoire, accumulation of large numbers of virus-specific CD4 T cells and $CD4^+CD28^{null}$ T-cells [51].

Yadav and Jha however could not relate the increased frequency of $CD4^+CD28^{null}$ T cells to CMV seropositivity which is highly prevalent in Indian community (95%) and detected in almost all studied CKD patients as well as healthy subjects [49]. And in the current study as well, we could not demonstrate the expansion of $CD28^{null}$ cells in relation to CMV serostatus, due to little number of CMV seropositive patients. Notably, this study is unique in evaluating $CD4^+CD28^{null}$ T-cell in two distinct groups of ESRD patients; one with documented subclinical atherosclerosis (enhanced CIMT) and one without. CIMT could predict cardiovascular events in asymptomatic people with no history of coronary heart disease (CHD) [61].

As expected ESRD patients with atherosclerotic changes had the higher peripheral frequency of $CD4^+CD28^{null}$ T-cell compared to both; patients without atherosclerotic changes and healthy subjects. A highly significant positive correlation between $CD4^+CD28^{null}$ T cell and CIMT was found in all ESRD patients. Our observations parallel previously published data evaluating CIMT among ESRD/CKD patients in association with $CD4^+CD28^{null}$ T cells percentage [41,59,62].

Given the possible association between the frequency of circulating $CD4^+CD28^{null}$ T-cell and the risk of atherosclerosis, we performed a multivariable backward binary logistic regression analysis for the relation between $CD4^+CD28^{null}$ T cell and the occurrence of atherosclerotic changes as adjusted for possible confounding factors; $CD4^+CD28^{null}$ T cell (%), positive CMV IgG, were independent predictors of atherosclerotic changes. A link between CMV seropositivity and increased occurrence of atherosclerotic cardiovascular events was shown in several studies [63–65]. Other reports have shown strong relation between the risk of atherosclerosis in ESRD and CMV serostatus [34]. CMV has been implicated in immunological ageing, with expansion of $CD4^+$ lacking CD28 [66,67].

Based on this, Betjes et al. found significantly higher percentage of $CD4^+CD28^{null}$ T-cell in CMV positive ESRD patients with documented atherosclerotic disease in their medical history. Two to three fold increased prevalence of cardiovascular disease among ESRD patients with the highest and lowest number of $CD4^+CD28^{null}$ T-cell [50].

In a series of studies, $CD4^+CD28^{null}$ T-cell have been found significantly increased in the blood of patients with unstable angina, in extracts from coronary arteries containing unstable plaques [16,21], and are associated with an increased risk of both acute coronary events and ischemic stroke [68,69]. Furthermore, it was shown that an increased frequency of $CD4^+CD28^{null}$ T cells is an independent predictor

of future acute coronary events in unstable angina (UA) patients [36].

To the best of our knowledge, ROC curve analysis for discrimination between ESRD patients with or without atherosclerotic changes using $CD4^+CD28^{null}$ T cell %, was done for the first time in the current study. $CD4^+CD28^{null}$ T cell %, showed an excellent discriminatory power. Therefore we do believe the present data justify the use of CD28 assays, as a definitive clinical tool in identifying ESRD patients with preclinical atherosclerosis.

Of interest, increased number of circulating $CD4^+CD28^{null}$ T-cell with increased prevalence of atherosclerotic disease has been reported in other chronic inflammatory conditions and chronic infections, as HIV and rheumatoid arthritis [70–72]. Gerli et al. described increase in IMT in patients with rheumatoid arthritis who had an increased percentage of $CD4^+CD\ 28^{null}$ T cells [73].

The previous data strongly indicate that this unusual T-cell subpopulation has proatherogenic properties; they express cytotoxic mediators such as perforin, granzyme. $CD28^{null}$ cells isolated from patients with acute coronary syndromes promote damage to human umbilical vein endothelial cells in vitro [23]. Strikingly in ANCA-associated vasculitis, Slot et al. could not detect a difference in CIMT between patients with > 5% $CD4^+CD28^{null}$ T cells and patients with < 5%. In addition, there was no correlation between CIMT and % $CD4^+CD28^{null}$ T cells. Furthermore, they could not detect a difference between patients and controls with or without a previous CMV infection [74].

The reason for this discrepancy is unknown, but it may be explained by different disease entities with different complicated pathologies. However with evidence mounting for the atherogenic potential of $CD4^+CD28^{null}$ T cells, increased focus should be placed on these cells as a therapeutic target for atherosclerosis.

In conclusion, abundant circulating $CD4^+CD28^{null}$ T cells in ESRD patients especially those with enhanced CIMT, support the emerging hypothesis that T cell subsets expressing markers of immunosenescence may be an important CVD mediators among ESRD patients.

Limitations of the current study merit consideration. First, this study has the disadvantage of a being single-center observational. Second, positive CMV IgG was detected in minority, only in 11 ESRD patients. We were not able to relate this few number of patients to $CD4^+CD28^{null}$ T-cells count.

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Conflict of interest

No conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.03.008.

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