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ΝΕΦΡΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

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ΝΕΦΡΟΛΟΓΟΣ, MD

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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2. Νάκα Αικατερίνη, Καθηγήτρια Καρδιολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
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4. Μιχάλης Λάμπρος, Καθηγητής Καρδιολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
5. Μηλιώνης Χαράλαμπος, Καθηγητής Παθολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
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Η Αναπληρώτρια Γραμματέας του Τμήματος

Ιωάννα Ντούβλη

Dedications

To my mother, Tefta,

for incepting in me the intellectual quest

In memory of my father, Theodore,

for teaching me critical reasoning

In memory of my grandmother, Chrisanthi,

for shaping me

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Prologue

Chronic kidney disease (CKD) has become a major global health issue, affecting millions of people worldwide and with a high socioeconomic impact. The prevalence of CKD is continually rising, driven by an aging population as well as an increasing incidence of diabetes, hypertension, and cardiovascular diseases (CVD). Cardiovascular disease and CKD are closely linked. The burden of CVD in chronic kidney disease is significant and multifaceted, leading to increased cardiovascular morbidity and mortality in this patient population, particularly as kidney function progressively exacerbates. Cardiac remodeling is a hallmark of chronic kidney disease (CKD) manifesting as myocardial fibrosis, left ventricular hypertrophy (LVH), impaired myocardial strain and eventually left ventricular diastolic and systolic dysfunction. Kidney transplantation is associated with significant improvements in left ventricular size and function as well as regression of LVH otherwise known as reverse remodeling. Nevertheless, subclinical abnormalities of myocardial function may be observed in kidney transplant recipients (KTRs) and remarkably, heart failure following kidney transplantation remains responsible for the majority of adverse cardiovascular events in KTRs. During the last two decades, the term cardiorenal syndrome (CRS) has been introduced and established to mark the tight interaction between the heart and the kidneys. The pathophysiology of uremic cardiomyopathy, in general, involves complex intertwining pathways, including hemodynamic changes, neurohumoral activation, oxidative stress amplification, and chronic inflammation as well as the participation of immune system components, including cytokines, toll-like receptors, and innate and adaptive immune system cells.

Maladaptive activation of the immune system plays an essential role in the pathogenesis of CKD and CVD. A significant body of data from animal and human research indicates that inflammation and immunological pathways are implicated in all aspects of CVD phenotypes and have been well-established until now in atherogenesis, viral myocarditis, and inflammatory cardiomyopathy. Chronic kidney disease progression itself is associated with complex alterations in innate and acquired immunity and disruption of immune regulatory processes. The chronic inflammatory state of CKD is mediated and perpetuated by an intricate interaction of multiple immune mediators as well as cellular components of the innate and adaptive immune systems. Inflammatory and immune pathways appear to participate in the pathogenesis of the entire spectrum of CVD in CKD, including accelerated atherosclerosis, left ventricular hypertrophy, and heart failure. The chronic inflammatory state and immune system activation are considered as significant mediators in the pathogenesis of myocardial dysfunction in CKD. Yet, the role of immune system components in the development of myocardial remodeling in CKD and kidney transplantation remains an open question. In specific, there is scarce clinical evidence available regarding the implication of immune cell subsets in the development of CKD associated cardiomyopathy.

Accordingly, in this study we investigated the expression of a selected panel of immune cell subpopulations in the peripheral circulation of CKD patients and KTRs without

established CVD and examined their relation to subclinical indices of myocardial dysfunction. Furthermore, we focused on the alterations of specific immune cell subsets in the setting of cardiorenal syndrome type 2 that is CKD in the setting of heart failure.
By elaborating on the current knowledge of immunological pathways mediating the crosstalk of the damaged kidney with the heart, the aims of our study were to create a background to support future endeavors, including the elucidation of the pathophysiological implication of immune cell subpopulations in the development of CKD related CVD, their role as potential prognostic biomarkers and the development of targeted interventions.

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General part

General part

1. Chronic kidney disease and kidney transplantation – an overview.

Chronic kidney disease (CKD) is a progressive disease with no specific cure which carries a high morbidity and mortality burden, commonly affecting the general adult population (1). CKD remains a major, global public health problem, involving nearly 10% of the global population, with the burden of CKD in terms of prevalence, morbidity, mortality and expenditures continually increasing (1-3). It is estimated that by 2040, CKD will emerge as the fifth leading cause of death in the world (3). CKD comprises a heterogeneous group of disease pathways which are characterized by disorders in kidney structure and function, and which display various manifestations according to the underlying etiology and the degree of severity of disease (1). Risk factors for CKD include genetic or epigenetic determinants, or the presence of diseases which can trigger and promote kidney damage, such as obesity, diabetes, and hypertension (1). The diagnosis of CKD is based on establishing a chronic reduction in kidney function and structural kidney damage (4). The definition and classification of CKD have changed and evolved during the last two decades with current international guidelines defining CKD as decreased kidney function shown by GFR of less than 60 mL/min per 1.73 m^2, or markers of kidney damage, or both, present for at least 3 months duration, regardless of underlying cause (3, 4). Kidney damage refers to pathologic abnormalities, which are identified either by kidney biopsy or imaging studies or otherwise detected in the setting of abnormal markers such as urinary sediment abnormalities or elevated urinary albumin excretion rates (1-4). With regard to decreased kidney function, it refers to a decreased glomerular filtration rate (GFR), which remains the best available indicator of overall kidney function, equaling the volume of fluid filtered through all of the functioning nephrons per unit of time (1-4). GFR is usually estimated (eGFR) using serum creatinine and or serum cystatin and the recommended method for estimating GFR in adults from the National Kidney Foundation, is the 2021 CKD-EPI equation (3). CKD is classified based on the cause, GFR category (G1–G5) (table 1A) and albuminuria category (A1–A3) (Table 1B) (3, 4). Kidney failure is defined as severely reduced kidney function and implies eGFR decline to less than 15 mL/min per 1.73m^2 (category G5, table 1), signifying that a person has reached end stage kidney disease at which point kidney function is no longer able to fulfill the metabolic and is thus incompatible with life in the long term (2). Management options for patients with ESKD include kidney replacement therapy (KRT) in the form of dialysis or kidney transplantation (1, 2). The purpose of CKD staging is to guide therapeutic strategies, including stratification of risk for CKD progression and for development of CKD related complications (3). All three components of the CKD classification system bear a critical significance for the evaluation of patients with CKD and further guide the ascertainment of CKD severity and associated risk. Based upon the CKD staging system, a "heat map" has been generated that classifies patients with CKD into three broad risk categories, that is moderate, high, and very high risk, depending on the probability of developing future kidney and cardiovascular complications (Figure 1) (4). Accordingly, patients with CKD are five to ten times more likely to die prematurely than they are to progress to kidney failure. Thus, the increased risk of death augments exponentially with CKD progression and is mainly ascribed to death due to cardiovascular causes (1-3). Other commonly recognized CKD related complications include hormonal and metabolic one, such as anemia and hyperparathyroidism, increased risks for systemic drug toxicity, infections, cognitive impairment, and impaired physical function (1, 2).

Kidney transplantation is the treatment of choice for kidney failure as it significantly reduces the mortality risk as well as improves the quality of life for most patients when compared with dialysis (1, 2). Recent advances, including novel immunosuppressants, improved organ allocation policies, and better medical care of transplant recipients, have led to an increased number of transplants with improved overall outcomes (2). Although great progress in the development of the currently established immunosuppressive regimens has been associated with a significant reduction in episodes of early acute rejection episodes together with excellent 1-year allograft survival exceeding 95%, this has not translated into improved long-term graft prognosis (2). Decreased long-term patient and graft survival have been attributed at large to the increased medical complexity that characterizes these patients, including the presence of multiple comorbidities in the setting of or as a cause of kidney failure as well as to the complex immunosuppressive regimens that increase their susceptibility to infections, malignancy, and cardiovascular complications (2). Accordingly, these patients require close follow-up after transplantation. Apart from the lack of improvement in long term transplant outcomes, reduced organ availability and the long-term morbidity of transplant candidates with CKD, remain major obstacles to be tackled. Overall, after successful transplantation, establishing an equilibrium between the chronic adverse effects of immunosuppression with chronic immune damage remains a major clinical challenge for transplant physicians today (2).

Table 1. Classification of CKD, according to international guidelines. A. Classification of CKD according to eGFR and classification of CKD according to albuminuria levels.

A.

	GFR descriptors and range	**Range (ml/min/1.73 m^2)**
G1	Normal or high	≥90
G2	Mildly decreased	60-89
G3a	Mildly to moderately decreased	45-59
G3b	Moderately to severely decreased	30-44
G4	Severely decreased	15-29
G5	Kidney failure	<15

B.

Persistent albuminuria categories, descriptors and ACR range		
Normal to mildly Increased (<30 mg/g)	Moderately increased (30–300mg/g)	Severely increased (>300 mg/g)

CKD, chronic kidney disease; GFR, glomerular filtration rate; ACR, albumin creatinine ratio.

KDIGO: Prognosis of CKD by GFR and albuminuria categories

GFR categories (ml/min/1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	<15			

Figure 1. Prognosis of CKD by eGFR and albuminuria categories.
Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red: very high risk. GFR, glomerular filtration rate. Adapted from the KDIGO 2012 Clinical practice guideline for the evaluation and management of chronic kidney disease.

1.1 The burden of cardiovascular disease in chronic kidney disease

Cardiovascular disease (CVD) remains by far the principal cause of morbidity and mortality in patients with CKD (7). Thus, even though the risk of various adverse outcomes is significantly increased in CKD patients, CVD is responsible for up to half of deaths in patients with CKD before they reach kidney failure, as compared to only 26% in people with normal kidney function (7-11). Even though the cardiovascular risk in patients with severe CKD is lower compared to patients undergoing kidney replacement therapy, it is still significantly higher than in the general population (8). CVD in CKD manifests with various adverse outcomes, including CHD, arrhythmias, heart failure (HF) as well as stroke and peripheral artery disease (7, 9). The strong association of CKD with CVD is attributed to the involvement of complex pathophysiological mechanisms which lead to vascular damage, to shared traditional risk factors including diabetes mellitus (DM) and arterial hypertension, as well as to several non-classical risk factors including among others anemia, CKD mineral bone disease (CKD-MBD), fluid overload, oxidative stress, chronic inflammation and uremic toxins (7).

Many studies in various populations have reported that low eGFR and raised albuminuria are associated with cardiovascular disease (10-17). Accordingly, data from the CKD prognosis consortium that included 629 746 patients, indicate that cardiovascular mortality increases during the early stages of CKD, when the eGFR decreases below 60 to 75 mL/min per 1.73 m^2 with the risk tripling with CKD stage 3b. Strikingly, increases in the urine albumin-creatinine ratio (UACR) are associated with augmented cardiovascular mortality, which dramatically escalates with UACR levels above 300 mg/g (12).

In line with the above a large meta-analysis, including more than 100 000 individuals with UACR measurements and 1·1 million participants with dipstick albumin measurements from 21 general population cohorts from 14 countries and 4 continents, showed an independent correlation of eGFR and albuminuria with all-cause mortality and cardiovascular mortality independently of each other and of traditional cardiovascular risk factors (10). Notably, an exponential increase in mortality risk was observed at low levels of eGFR (10). Thus, the risk became evident at eGFR levels of 60 mL/min/1·73 m² and doubled at eGFR levels of 30–45 mL/min/1·73 m² as compared to eGFR levels between 90–104 mL/min/1·73 m². The association of eGFR to excess mortality risk was independent of albuminuria or other potential confounding factors. With regard to albuminuria, its association with mortality was linear on the log-log scale, with a two times higher risk within the UACR of moderately increased albuminuria when compared with the optimum UACR level, independently of eGFR and other conventional risk factors. In addition, the excess mortality risk conferred by increased UACR was more than two-fold within all ranges of eGFR, except for the lowest eGFR category, suggesting that albuminuria provides additional prognostic information apart from eGFR alone (10). Thus, the association of albuminuria with cardiovascular risk does not display a threshold effect, even after adjustment for traditional cardiovascular risk factors and eGFR, indicating that the presence of albuminuria even at the upper limits of the normal range (threshold 30 mg/g) carries cardiovascular risk. Likewise, results from a subsequent meta-analysis showed that eGFR and albuminuria independently improved the prediction of incident cardiovascular events beyond traditional risk factors (11). The additional prognostic value provided was greater with UACR than with eGFR or dipstick proteinuria and was more evident for cardiovascular mortality and HF than for CHD and stroke. It should be noted that UACR was superior to most of the traditional cardiovascular risk factors for predicting cardiovascular mortality, HF, and stroke in the general populations. The prediction improvement shown by markers of kidney disease was more pronounced in specific patients' subsets such as diabetics and patients with hypertension whereas UACR alone improves prediction for cardiovascular mortality and HF in patients without either of these conditions as well. Specifically, in the CKD population, the combination of eGFR and UACR surpassed the modifiable traditional risk factors as well as the combination of traditional lipid variables for the prediction of all cardiovascular outcomes (11).

Considering that as eGFR declines, cardiovascular events and infectious complications are the main culprits responsible for increasing mortality rates, the Alberta

Kidney Disease Network conducted a study in residents of Alberta, Canada who died between 2002 and 2009 in order to shed light on common causes of death in people with CKD. EGFR was calculated using the CKD Epidemiology Collaboration equation. An inverse association between eGFR and specific causes of death, including specific types of cardiovascular disease, infection, and other causes, were shown in this cohort (13, 18). In specific, the most common cause of death for individuals with eGFR≥60 ml/min per 1.73 m2 and without proteinuria was cancer (38.1%) whereas the most common cause of death for those with eGFR<60 ml/min per 1.73 m2 was CVD. Furthermore, the unadjusted proportion of patients who died from CVD increased as eGFR decreased. The proportions of deaths from HF and valvular disease as well as deaths from infectious and other causes specifically increased with declining eGFR, whereas the proportion of deaths from cancer decreased (13, 18).

Strikingly, in patients with stages 3A and 3B CKD, the incidence of cardiovascular mortality is much higher than the incidence of progression to kidney failure (10,, 15), with less than 1 % of patients with CKD ultimately reaching the end-stage. Instead, most patients with moderate to advanced CKD will prematurely die, and this with CVD as the main cause of death (19). These data indicate that the true burden of disease in patients with CKD is related to the increased risk of CVD rather than the risk of reaching kidney failure requiring renal-replacement therapy (RRT) (17-19). Only in patients with CKD stage 4 and beyond does the risk of kidney failure exceed that of the occurrence of cardiovascular events (17-19). Mortality rates due to CVD peak in dialysis patients being several times higher than the general population. Compared to the general population in Europe as a reference, the age-adjusted cardiovascular mortality in dialysis patients was 8.8 times higher (16, 19). Overall, nearly half of patients with CKD stage 4–5 suffer from CVD and approximately 40%–50% of them die due to cardiovascular causes (16, 19). Dialysis patients aged 25 with 34 years display an annual cardiovascular mortality risk that is 500 to 1.000 times higher compared to healthy individuals of the same age (20). However, the relative prevalence of the various types of cardiac disease differs in patients undergoing RRT compared with the non-dialysis population. Accordingly, 80 % of CVD deaths in patients on dialysis are presumably due to arrhythmic events whereas less than 10 % can be ascribed directly to CHD (19).

Overall, the above evidence highlights the immense burden of CVD in patients with CKD (21-27). In specific, various CVD phenotypes are associated with impaired kidney function. Accordingly, the risk is increased for atherosclerotic CVD and atrial fibrillation, with the risk of HF being approximately double in patients with eGFR less than 60 mL/min per 1·73 m2 compared to normal individuals.

1.2 Atherosclerotic CVD in CKD

Despite the overall agreement on the association of CKD with an increased incidence of cardiovascular events, there is less information on the prevalence and progression of atherosclerosis in CKD (28, 29). Postmortem studies have shown that CKD patients display a

significant degree coronary artery atherosclerosis, which is characterized by a predomination of calcified plaques, greater media thickness as well as the presence of medial calcification in comparison with patients without CKD (30, 31-33). The association between kidney disease and carotid atherosclerosis has been confirmed by several studies including patient cohorts from the general population (34). Thus, reduced renal function is strongly associated with increases in carotid intima media thickness (cIMT) (35). The latter is a significant adverse prognostic factor for ischemic cardiovascular events as well as long-term mortality in both in non-dialysis-CKD patients and in hemodialysis patients (35-37). Recent data indicate that the atherosclerosis risk actually increases early in CKD, however as CKD progresses, the burden of atherosclerosis adds less to the overall cardiovascular risk (29). According to recent evidence from a Canadian study, the rate of carotid plaque progression was lower in patients with advanced CKD (eGFR category G4) compared to patients belonging to eGFR categories G2-3a) (28, 30).

The incidence and prevalence of coronary heart disease in the dialysis population vary across different studies and cohorts mainly due to heterogeneities of the definition that is used (38). In line with the above, even though the burden of CVD is much higher than that observed in the general population, the precise contribution of CHD to cardiac mortality in the dialysis population remains to be clarified. The unadjusted prevalence of CHD in 2020 was 43 and 37 percent among patients on hemodialysis and peritoneal dialysis, respectively (17). Additionally, 16 % of patients on hemodialysis and 13 % of patients on peritoneal dialysis had a history of acute myocardial infarction (MI) (17). With regard to mortality, CVD accounted for more than 38 % of all deaths in hemodialysis patients (17, 37) with less than 10% of those being due to atherosclerotic heart disease and over 80 % being attributed to arrhythmia and cardiac arrest. The latter mainly occurred in the setting of nonischemic etiologies such as myocardial fibrosis and left ventricular hypertrophy LVH (37).

1.3 Left ventricular remodeling in chronic kidney disease

Cardiac remodeling is a hallmark of the systemic nature of CKD manifesting as myocardial fibrosis on histology, increased left ventricular (LV) mass and LVH**,** impaired myocardial strain and eventually development of LV diastolic and systolic dysfunction (39, 40). The spectrum of cardiomyopathic alterations in the setting of CKD is included under the umbrella term of uremic cardiomyopathy. Various imaging modalities studies, including echocardiography and cardiac magnetic resonance imaging (CMRI) display a high prevalence of uremic cardiomyopathy, which increases with CKD progression (41). Thus, recent evidence from imaging studies using echocardiography and CMRI have defined the key features of uremic cardiomyopathy including abnormal myocardial deformation, which is considered a surrogate of myocardial fibrosis, LVH, left atrial (LA) dilatation, and diastolic dysfunction which are exhibited by CKD patients with mild to moderate renal dysfunction before progressing to advanced CKD and before the development of overt systolic dysfunction and HF (42).

Considering that diastolic dysfunction and myocardial strain have emerged as predictors of mortality in the general population, they have been examined as well in experimental and clinical studies of uremic cardiomyopathy so as to clarify their significance as clinically relevant outcomes in patients with CKD (43). Although diastolic dysfunction is usually considered a result of the development of LVH, available evidence indicates that myocardial fibrosis leads to myocardial thickening and augmented ventricular stiffness as well. Thus, experimental data indicate that impaired myocardial strain and diastolic dysfunction in mice with CKD were observed before the development of LVH on the echocardiogram (43). In like manner, diastolic dysfunction appears to come before the increases in blood pressure (BP) and the establishment of LVH in the setting of essential hypertension (43).

Myocardial fibrosis is caused by increased production and deposition of extracellular matrix by myocardial fibroblasts. The development of myocardial fibrosis is considered a key and defining pathophysiological process underlying CKD-associated cardiomyopathy, ventricular stiffening and impaired LV diastolic function (41). Both autopsy studies and clinical studies conducting endomyocardial biopsies have shown that CKD patients display a greater burden of extracellular matrix deposition including collagen compared to their counterparts without CKD (2, 44). Myocardial fibrosis emerges since the initial stages of CKD and its extent as determined by the percentage of total myocardial tissue occupied by collagen fibers, defined as collagen volume fraction, becomes prominent with disease progression (44-47). Notably, CKD is associated with myocardial fibrosis independent of other potential confounding variables (46). The location of interstitial myocardial fibrosis in CKD differs from post-MI fibrosis in terms of its distribution affecting not only the perivascular areas as occurs in the latter (45-48). Patterns of fibrous deposits can include micro scars, thick deposits surrounding intramural coronary arteries and arterioles and bands surrounding the cardiomyocytes sheaths (46). In a study of LV endomyocardial biopsies of hemodialysis patients, 42% of them carried cardiac fibrotic deposits (49). A study of endomyocardial biopsy samples from hemodialysis patients with HF showed that the collagen volume fraction amounted to more than 20% of the entire myocardial tissue (46, 48). Furthermore, it has been suggested that in patients with kidney disease, perivascular fibrosis may contribute to cardiac microvascular disease via extrinsic compression of intramyocardial vessels (50). It should be noted that endothelial dysfunction in the setting of arteriolar remodeling and reduced capillarization has been associated with reduced coronary flow reserve (CFR), which in turn correlates with cardiovascular mortality in CKD patients (46).

Since the seminal observation of Richard Bright and the experimental studies of Traube nearly 2 centuries ago, arterial hypertension and cardiac hypertrophy have been recognized as CKD major features (54). Autopsy studies of CKD patients have revealed increased cardiac cell size in the LV wall as well as an increased myocardial wall (48). The prevalence of LVH across the CKD stages is inversely related to the severity of kidney dysfunction. Cardiac imaging studies utilizing echocardiography or CMRI have shown that

increased LV mass and LVH affect approximately 40% of non-end stage CKD patients and its prevalence rises to over 75% in patients undergoing RRT (50-53). However, there is a relatively large heterogeneity of the prevalence of LVH in CKD as reported by different studies, according to the characteristics of the population studied, the method chosen to estimate the GFR and the definition used (50-56). Furthermore, LVH and increased left ventricular mass index (LVMI) are regarded as a strong, independent predictor of mortality in CKD, displaying a graded association with adverse cardiovascular outcomes and mortality in these patients (39, 57-62). Accordingly, an analysis of 1,249 patients with pre-dialysis CKD showed that LVH was associated with a mortality risk of 25 deaths per 1000 person-years, thus constituting the largest increase among traditional risk factors (41, 57). Moreover, both the severity and persistence of LVH are adversely associated with prognosis in CKD patients. Strikingly, a 10% decrease in LV mass has been associated with a 28% reduction in cardiovascular mortality risk in a cohort of hemodialysis patients (58, 61). Imaging studies, including echocardiographic studies have confirmed that LVH as assessed by echocardiography bears prognostic significance regarding the occurrence of adverse cardiovascular and renal outcomes (63, 64). Apart from LVMI, other echocardiographic indices, such as estimation of the relative wall thickness (RWT) are utilized to identify abnormal LV geometric patterns (65, 66). Studies involving patients from the general population and patients with essential hypertension have shown that abnormal patterns of LV geometry adversely affect prognosis, with concentric LVH portending a higher risk of cardiovascular events and all-cause mortality (65-67). With regard to patients CKD patients, both concentric and eccentric LVH is associated with either more rapid progression to kidney failure or higher cardiovascular risk, respectively (62, 63, 68). Accordingly, in non-dialysis CKD patients, the presence of LVH was associated with a two- to threefold increase in the risk of adverse cardiovascular outcomes, progression to kidney failure, and all-cause mortality, independently of the specific LV geometric pattern involved (92). A secondary analysis of the CREATE Study showed that both concentric and eccentric LVHs were associated with adverse cardiovascular events although but there was no data provided with regard to kidney outcomes (68). In addition, higher LVMI is associated with de novo development of HF in patients with CKD in a graded fashion (69, 70). Results from the Chronic Renal Insufficiency Cohort (CRIC) study which enrolled individuals with CKD and without baseline HF, showed that LVMI was a strong, predictor of incident HF and death, independently of known cardiac risk factors in CKD, such as eGFR, troponin T, brain natriuretic peptide (BNP), and FGF23 (69).

Diastolic myocardial dysfunction is common finding in CKD, with studies reporting an incidence of 71% in non-dialysis CKD and up to 85–90% in patients undergoing RRT (41). Excess myocardial deposition of collagen affects the elastic properties of the cardiomyocytes, subsequently causing increased ventricular stiffness, impaired myocardial relaxation and diastolic recoil (41,71). Diastolic dysfunction, increased LV mass and LVH, as well as myocardial fibrosis are tightly linked with each other and all are associated with increased mortality (41, 72). Accordingly, the American Society of Echocardiography and the

European Association of Cardiovascular Imaging consider LVH as a marker of diastolic dysfunction (73). On the other hand, ejection fraction (EF) remains preserved in the majority of patients with CKD. Thus, overt LV systolic dysfunction, manifested by a reduced LV EF**,** is not a common finding in pre-dialysis CKD patients, with a reported prevalence of 8% and interestingly it does not bear an association with eGFR (21, 74). Accordingly, data from several studies show that less than a third of patients with kidney failure display detectable systolic dysfunction. Nevertheless, it should be noted that although EF is an established predictor of outcome, especially in patients with HF, it is only a crude marker of impaired systolic function. With regard to CKD, observational studies have demonstrated an association between reduced EF with a greater risk of cardiovascular and all-cause mortality. Although the complex nature of myocardial remodeling affects the performance of LVEF for evaluation of the systolic function, technical limitations of EF measurement may also play a role. On the other hand, recent studies have shown that changes in myocardial deformation exist even during the initial stages of CKD, thus indicating the presence of subclinical LV systolic dysfunction (75, 76). As a result, the quest is open for other non-invasive and more objective means for assessment of LV function.

This is of relevance to the CKD cohort who undergoes progressive cardiac remodeling. Modification of the LV geometry with development of concentric hypertrophy occurs in various pathophysiological settings including CKD, in which the magnitude of LV mass substantially surpasses the level required to sustain its normal performance which is otherwise labelled ''inappropriate LV mass'' (71). Concentric LVH is associated with echocardiographic markers of abnormal myocardial relaxation and increased myocardial stiffness (71). Concentric LVH as defined by the European Guidelines on Arterial Hypertension and based on the LVMI, is consistently related to echocardiographic markers of abnormal LV relaxation, including E/A ratio with dominant A velocity (71, 77). Ventricular diastole involves tightly linked and overlapping phases that include the elastic recoil, active relaxation, passive late diastolic filling and LA contraction. Every abnormality of LV filling affects LA dimensions and function, because the LA is extremely sensitive to changes in pressure, due to its thin wall thickness. Accordingly, in the absence of significant mitral regurgitation, LA dilatation with increased LA volume and LA volume index (LAVI) would be a surrogate marker of any abnormalities related to atrial emptying, characterizing diastolic dysfunction (71, 73). Doppler echocardiographic examination of mitral and aortic flow velocities, together with pulmonary vein flow and tissue Doppler of the mitral annulus are of paramount importance for assessing LV diastolic function. There are several doppler parameters associated with diastolic dysfunction (71, 73). Accordingly, the ratio of mitral inflow early peak velocity (E) to the late peak velocity, induced by atrial systole (A), measurable at the level of the mitral valve leaflet (E/A ratio) provides significant data regarding myocardial relaxation and LV filling pressure. LV filling pressure, an index of passive late diastolic filling, can be estimated by the ratio between transmitral E velocity and tissue Doppler E’ velocity recorded at the mitral annulus (71, 73). CMRI is a highly reliable method for the accurate assessment of biventricular function, ventricular geometry and

mass, as well as myocardial structure, including identification and quantification of myocardial scars and inflammation (71). Although CMRI is a useful tool for assessing diastolic dysfunction, its utilization in routine clinical practice is limited due to the cost, availability and lack of clear advantages compared with Doppler echocardiography (71).

Non-invasive and widely available diagnostic methods are required in order to detect subclinical myocardial abnormalities in CKD patients so as to identify those at higher risk for CVD. Echocardiography remains an essential tool for the assessment of cardiac structure and function in CKD patients, with various echocardiographic indices being related to adverse cardiovascular outcomes. Yet, classic echocardiographic indices of LV systolic and diastolic function may not be sensitive enough in detecting early myocardial deterioration in CKD patients (53). Thus, as already discussed previously, although LVEF is a widely used surrogate marker for LV systolic function, it is subject to influence by LV loading conditions plus it tends to increase with LV concentric remodeling. In addition, increased afterload may reduce the LVEF despite preserved LV contractility. In this regard, LVEF may not be an adequate measure to assess myocardial intrinsic contractility in patients with kindey failure, in whom changes in preloads and afterload status occur frequently. In this respect, LV global longitudinal strain (GLS) assessed by speckle-tracking echocardiography (STE) has been reported to more sensitively assess subclinical LV systolic dysfunction with better reproducibility compared to LVEF.Two-dimensional speckle tracking echocardiography (2DSTE) is a sensitive semi-automated modality for the evaluation of the LV systolic as well as diastolic function in an operator-independent manner (53). This relatively new echocardiographic technique, which is used along with conventional echocardiographic measures, examines regional LV function by tracking acoustic markers or speckles within the myocardium from frame to frame in B-mode images (78). However, the evaluation of myocardial motion by classical echocardiograph is affected by translational and tethering effects. On the other hand, 2DSTE analysis determines deformation and removes the effect of tethering and translational motion of the whole heart, thus outperforming traditional echocardiographic imaging with regard to detection of early changes of myocardial function (78). Furthermore, as 2DSTE makes possible the assessment of the deformation of the LV myocardium via strain and strain rate analyses in the longitudinal, circumferential, and radial myocardial axes. Available data suggest that 2DSTE may provide further insight into the pathophysiology of specific myocardial pathological states, including cardiac ischemia and infarction, hypertrophic or diabetic cardiomyopathy. In addition, 2DSTE may be a useful tool for the identification of areas of myocardial fibrosis in the setting of uremic cardiomyopathy (78). Peak global longitudinal strain (GLS) has emerged as the most important load-independent index that provides an accurate measurement of LV systolic and diastolic function with many prognostic implications (53). GLS which is equal to the negative ratio of the maximal change in LV longitudinal length in systole to the original length reflects the longitudinal contraction of the myocardium (79). There are several other advantages of GLS including it being operator independent, more reproducible compared to EF, easily measured and integrated to standard echocardiography (79). The accuracy of GLS

detecting and quantifying subtle disturbances in LV systolic function has been validated against tagged CMRI (79).

GLS was shown to be a superior, robust predictor of cardiac events and all-cause mortality compared to EF, both in the general population as well as in patients with MI, cardiomyopathy and HF (79). Myocardial deformation analysis data from STE studies have revealed subclinical abnormalities of the LV myocardial relaxation and contractile function in CKD patients in the absence of established CVD (47, 48). Similarly, 2DSTE studies in CKD patients have shown that parameters such as peak GLS, peak systolic longitudinal strain rate, and peak GCS and GRS, were independent risk factors for cardiovascular mortality (78). Several studies described a reduction of LV strain parameters in patients with CKD or dialysis patients despite preserved LVEF (79). Accordingly, even patients with CKD stages 2–3 display impaired systolic strain and strain rate values as assessed by tissue Doppler imaging, whereas the classical echocardiographic indices of LV function, such as EF do not display any differences compared with healthy controls (79). In specific, LV GLS has emerged as a more sensitive predictor of mortality compared to EF in CKD patients as well (78, 80, 81). Abnormal GLS was independently associated with both all-cause and cardiovascular mortality in patients with CKD and those undergoing hemodialysis, even in the setting of preserved LV function (78, 80, 82). In line with the above, diastolic tissue velocity by tissue Doppler imaging is an independent predictor of cardiovascular events in patients with CKD (83). Thus, a more accurate assessment of systolic function may significantly improve the detection of early subclinical myocardial systolic dysfunction in patients with kidney disease and subsequently identify those at increased risk of future HF or other major cardiovascular events.

Patients with CKD with diastolic LV dysfunction are at increased risk of progression of CKD as well. Thus, among patients with non-dialysis CKD, increased early diastolic mitral inflow velocity/early diastolic mitral annulus velocity ratio (E/E’), measured by echocardiography, was significantly associated with progression of CKD, defined as a >50% decrease in eGFR from baseline, doubling of serum creatinine, dialysis initiation, and/or kidney transplantation (84, 85).

The counterclockwise rotation of the LV apex with respect to base clockwise rotation is referred to as LV twist or torsion which represents a mechanistic link between systole and diastole (86). The orientation of the myocardial fibers changes continuously during ejection from a right-handed helix in the sub-endocardium to a left-handed helix in the subepicardial region resulting in LV twist (87). The outer epicardial layer dominates the overall direction of rotation due to its larger radius of rotation. Available data suggest that nearly 40 % of the LV stroke volume is ascribed to ventricular twist dynamics. LV preload, afterload and contractility affect the extent of LV twist, which increases with higher preload and increasing contractility and decreases increased afterload (86). Twist during ejection predominantly deforms the subendocardial fiber matrix, resulting in storage of potential energy, which is released during subsequent recoil of twist deformation, thus contributing to LV diastolic relaxation and early diastolic filling (86). Longitudinal LV mechanics, which are at large

driven by the subendocardial region, are the most vulnerable and sensitive to the presence of myocardial disease. Thus, the mid-myocardial and epicardial function may not become impaired during the initial stages of myocardial dysfunction, with GCS and twist remain normal or displaying exaggerated compensation in order to preserve LV systolic performance (87). LV twist is evaluated by STE and CMRI and has proven to be a more sensitive marker of subtle myocardial dysfunction when compared with conventional echocardiographic methods, including EF (86, 88). LV twist is a potential marker of subclinical LV systolic dysfunction in CKD patients with normal EF, with abnormal twist values detected observed as early as CKD stage 3 (89). Notably, STE assesses global and segmental myocardial deformation, thus making possible the characterization of myocardial tissue (47, 52, 53). Accordingly, the amount of myocardial fibrosis has been related to the degree of reduction in myocardial GLS in patients with HF (81, 90). Molecular imaging methods such as single-photon emission computed tomography and positron emission tomography as well as CMRI are very promising techniques for the accurate identification and quantification of collagen deposition as a hallmark of myocardial fibrosis in patients with CKD (46, 81, 90). (62) Finally, DIPSE is used to non-invasively measure the CFR, thus assessing both the potential existence of main coronary artery stenosis as well as the coronary microcirculation in the left anterior descending artery (LAD) territory. Accordingly, a CFR value less than 2 is associated with significant microvascular dysfunction and is a strong predictor of the presence of epicardial coronary artery stenosis (53). Impaired CFR has been also advocated as an adverse prognosticator for CVD.

1.4 Heart failure in CKD and the cardiorenal syndrome

The 2016 European Society for Cardiology guidelines for managing HF define it based on signs and symptoms due to structural and/or functional cardiac abnormalities, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress (91). Heart failure is classified into specific subsets of HF with regard to EF levels and specifically HF with preserved EF (HFpEF), ≥50% ; reduced EF (HFrEF), <40%; and mildly reduced EF (HFmrEF), 40% to 49% (91). The presence of comorbidities such as CKD further complicates the diagnosis, as volume overload in the setting of CKD further contributes to the pathogenesis and manifestations of HF. In specific, HF as the primary syndrome can lead to development of kidney disease, and vice versa, or both states can exist simultaneously due to shared risk factors or systemic disorders which affect both organs (92, 93). However, the differential diagnosis of which disease comes first, and which is secondary may be complex.

The term cardiorenal syndrome (CRS) comprises a spectrum of disorders involving both the heart and kidneys in which acute or chronic dysfunction in 1 organ may induce acute or chronic dysfunction in the other organ (94). CRS was first defined by the Working Group of the National Heart, Lung, and Blood Institute in 2004, as the result of interactions between the kidneys and other circulatory compartments that increase circulating volume,

which exacerbates the symptoms of HF and disease progression (95). The uttermost manifestation of cardiorenal dysregulation leads to CRS, in which therapy to relieve congestive symptoms of HF is hindered by exacerbation of the renal function (95). Yet, taking into the consideration of the complex pathophysiological and clinical relationship between these two organs, there is a wider clinical spectrum of disorders that may belong to the dysregulation of the cardiorenal axis. Thus, the Acute Dialysis Quality Initiative outlined a consensus approach in 2008 that classified CRS into 2 major groups, cardiorenal and reno-cardiac syndromes, based on the instigating factor of the disease process (94, 96). Subsequently, there were recognized 5 subtypes of the CRS with regard to disease onset and duration as well sequential organ involvement (Table 2) (94, 96). Type 1 CRS is characterized by a rapid impairment of cardiac function, leading to acute kidney injury (AKI). Type 2 CRS is characterized by chronic cardiac dysfunction such as chronic congestive HF causing progressive CKD. Type 3 CRS is characterized by a sudden worsening of kidney function leading to acute cardiac dysfunction including HF or ischemic heart disease. Type 4 CRS is characterized by the development of chronic pathological cardiovascular phenotypes, including LVH, diastolic and systolic myocardial dysfunction. Finally, type 5 CRS is characterized by the presence of both cardiac and renal dysfunction due to other acute or chronic systemic disease.

Table 2. Classification of CRS Based on the Consensus Conference of the Acute Dialysis Quality Initiative

Phenotype	Nomenclature	Description	Examples
Type 1 CR	Acute CRS	HF resulting in AKI	ACS resulting in cardiogenic shock and AKI, AHF resulting in AKI
Type 2 CRS	Chronic CRS	Chronic HF resulting in CKD	Chronic HF
Type 3 CRS	Acute renocardiac syndrome	AKI resulting in AHF	HF in the setting of AKI from volume overload
Type 4 CRS	Chronic renocardiac syndrome	CKD resulting in chronic HF	LVH and HF from CKD-associated cardiomyopathy
Type 5 CRS	Secondary CRS	Systemic disease resulting in HF and CKD	Sepsis, cirrhosis, amylodosis

ACS, acute coronary syndrome; AHF, acute heart failure; AKI, acute kidney injury; CKD, chronic kidney disease; CRS, cardiorenal syndrome; HF, heart failure; LVH, left ventricular hypertrophy.

HF and CKD may occur as a bidirectional process with considerable overlap. The incidence of de novo HF in the setting of CKD is approximately 20% (91, 92). The development of HF varies according to the stage of CKD as well as the modality of RRT and

kidney transplantation. The prevalence of HF increases with CKD progression. Thus, among patients undergoing hemodialysis, approximately 10% have HFpEF, 13% have HFrEF, and 21% display unspecified HF (92). The simultaneous occurrence of HF and CKD is frequent with 30 to 60 % of patients with HF having a GFR less than 60mL/min per 1.73 m2 (97-103). The Acute Decompensated Heart Failure National Registry (ADHERE) database which reported data on over 100,000 patients with HF requiring hospitalization showed that approximately 30% had a diagnosis of CKD defined as a serum creatinine greater than 2.0 mg/dL (103). Furthermore, the mean eGFR in this cohort was 55 mL/min/m2, whereas only 9% of patients had an eGFR above 90mL/min/1.73 m2 (100). A systematic review of 16 studies including more than 80,000 hospitalized and non-hospitalized patients with HF, showed that moderate to severe kidney impairment as defined by either eGFR criteria, serum creatinine or serum cystatin C levels, was present in 29% of patients (99). A large meta-analysis including patients with HF with reduced EF (HFrEF) and HF with preserved EF (HFpEF) showed that more than half of the patients had eGFR less than 60 ml/min per 1.73 m^2, with a stepwise increase in mortality risk depending on the stage of CKD (97). In the Atherosclerosis Risk In Communities (ARIC) study, the incidence of HF was three times higher in people with an eGFR less than 60 mL/min/1.73 m^2 compared to those with a preserved kidney function (22, 98). The prevalence of kidney function decline in patients with HF was illustrated by a study of 3,570,865 United States veterans with an eGFR ≥60 mL/min/1.73m2 of which 156,743 were diagnosed with HF (100).

Heart failure patients had greater than two times the risk of incident CKD, a composite of incident CKD or mortality, as well as rapid eGFR decline when compared to those without (100). Data from the ADHERE study show that nearly one third of the patients hospitalized for acute decompensated HF, display acute kidney disease (AKD) or CKD as well (103). Furthermore, the prevalence of CKD is slightly higher in patients with acute HF compared to those chronic HF (103). Kidney impairment is a predictor of a poor prognosis in patients with HF and it has a strong association with worse outcomes (98, 104). In addition, albuminuria per se constitutes a prognostic for adverse outcomes in HF, although its contribution is inferior to the role of eGFR (92, 104-106). The presence of CKD more than doubles the mortality risk in patient with HF (106). Reduced eGFR is associated with increased risk of all-cause mortality, cardiovascular mortality, and hospitalizations in patients with established HFpEF or HFrEF (104-106). In line with the above, data from the CHARM program which included 2680 patients with chronic HF followed for a median of nearly three years showed that all-cause mortality increased significantly when the baseline eGFR was below 75 mL/min/1.73 m2, with an adjusted hazard ratio (HR) 1.09 (95% CI 1.06-1.14) for every 10 mL/min/1.73 m2 decline in eGFR (104). Furthermore, the adjusted HR increased from 1.20 at eGFR levels between 60 and 75 mL/min/1.73 m2 to 2.92 when eGFR fell below 45 mL/min/1.73 m2. Notably, the effect of kidney function on all-cause mortality was independent of the LVEF (104).

In the meta-analysis by Smith et al, when compared to HF patients with normal kidney function, the mortality rate at a follow-up of one year or more was increased by

more than 50% in HF patients with mild reduction in eGFR and it more than doubled in those with moderate to severe reduction in eGFR (99). It was estimated that mortality increased by approximately 15% for every 0.5 mg/dl increase in creatinine and 7% for every 10 mL/min reduction in eGFR (102). A meta-analysis of more than 18,000 patients with HF, both hospitalized and outpatients, showed that the mortality risk increased nearly 50% when eGFR declined by 11 to 15 mL/min/1.73 m2 and tripled when the eGFR declined by more than 15 mL/min/1.73 m2 (107). It should be noted that not only cardiac dysfunction, but treatment of acute or chronic HF as well frequently leads to kidney function decline in the acute or chronic setting, thus fulfilling the criteria for type 1 or type 2 CRS (107-112). Worsening renal function among hospitalized HF patients which usually occurs in the first three to five days of hospitalization, has been reported by several series, with approximately 20 to 30 % of patients fulfilling criteria for AKI by developing an increase in serum creatinine of more than 0.3 mg/dL (107-112). Risk factors for worsening kidney function during admission for HF include a prior history of HF, diabetes or uncontrolled hypertension as well as already increased serum creatinine at admission (107-112). An analysis of the PROTECT trial identified multiple different trajectories of kidney function during hospitalization for acute HF including a transient rise in serum creatinine affecting 19 % of patients, a sustained increase affecting 17.6%, whereas 14.5 % displayed a decrease (112). However, after multivariable adjustment, none of the trajectories of change showed any prognostic correlation with patients' outcomes thus raising a question regarding the significance of changes in kidney function during acute HF (112). Furthermore, the cause of worsening of eGFR in HF influences its prognostic significance (107, 108). Thus, even though the degree of eGFR decline during treatment of HF has often been associated with a progressive risk of increased mortality by various clinical studies, there is evidence suggesting that patient outcomes may be improved with aggressive fluid removal even if it is accompanied by a rise in serum creatinine.

2. Epidemiology of CVD in kidney transplantation

The survival benefit displayed by kidney transplant recipients (KTRs) is largely attributable to the reduction of CVD burden, however, continue to remain at higher risk for CVD-related morbidity and mortality when compared with the general population (113-115). CVD remains the leading cause of morbidity and mortality after kidney transplantation with death from CVD being the most common cause of graft loss. KTRs have a lower risk of cardiovascular events compared to hemodialysis patients on the waitlist for kidney transplant; still the risk is considerably higher compared with the general population (113, 114). CVD is responsible for 40 to 60 % of deaths following kidney transplantation and accounts for 30 % of graft loss from death overall, with the greatest rates early following transplantation (116-121). The annual risk of cardiovascular events rate in KTRs is 3.5–5% (122). When compared to age matched control subjects, KTRs carry a three- to five-fold higher risk of CVD (123). Strikingly, the higher odds of cardiovascular death in KTRs reach a

nearly 50-fold increase in patients in the fifth decade of life (124, 125). Nevertheless, it should be noted that the incidence of cardiovascular death after transplantation is currently displaying a declining trend despite the increased age and comorbidities among KTRs, which should be ascribed to improvements in the management of KTRs as well as competing risks of cancer and infection (117). Indeed, recent data from the UK Renal Registry demonstrate that the annual mortality directly attributable to CVD in KTRs has fallen over the previous decade (126). It should be noted that the large number of kidney failure patients with diabetes, who are at markedly increased cardiovascular risk contributes to the high rate of cardiovascular deaths in KTRs. Accordingly, a study including 933 predominantly living-donor KTRs, showed that CVD was the most common cause of death among diabetic KTRs whereas most deaths among patients without diabetes occurred due to infection, malignancy, or other causes (118). In spite of these, the cardiovascular risk among KTRs without diabetes as a cause for CKD, is still higher than in the general population. There are various components leading to the increased cardiovascular risk in KTRs, including traditional risk factors for CVD as well as non-traditional risk factors, including the adverse effects of the immunosuppressive medications and risk factors related to CKD. Furthermore, CVD is associated with an increased rate of hospital admissions in these patients, accounting for about 30% of these hospitalizations and with the respective mortality rate reaching 4 % (127). The clinical phenotypes of CVD in KTRs resemble those observed in non-transplanted individuals. Yet, it should be noted that similar to dialysis patients, more than half of cardiovascular deaths in KTRs are sudden and presumed to be related to arrhythmic events (123), highlighting the significant burden of non-atherosclerotic CVD, such as myocardial fibrosis and LVH in these patients as well (123).

2.1 Atherosclerotic CVD following kidney transplantation

Atherosclerotic CVD remains a major contributor to death with a functioning allograft (115). This was shown in the results of the Folic Acid for Vascular Outcome Reduction in Transplantation (FAVORIT) study cohort where the long-term survival of KTRs was mainly determined by infectious, malignant and cardiovascular complications (128). Despite competing causes of adverse outcomes and mortality, coronary artery disease (CAD) is an important cause of morbidity and mortality among KTRs, which seems relevant when one considers the comorbidities of CKD (128). The incidence of ischemic heart disease in the post-transplant population is approximately 1 per 100 person-years at risk whereas the incidence of MI is nearly six-fold higher than observed in the general population with rates of 5.6% and 11.1% at 1 year and 3 years post-transplantation, respectively (116). Mortality in KTRs after hospitalization for acute coronary syndrome (ACS) may be as high as 24% at 1 year to 45% at 4 years (122). Recipient characteristics associated with post-transplant MI include older age, history of angina, peripheral vascular disease, dyslipidemia, and pretransplant MI and arrhythmia (122).

2.2 Heart failure following kidney transplantation

The development of cardiomyopathy prior to transplantation impacts outcomes in KTRs, yet a functioning kidney graft influences the extent and progression of preexisting cardiomyopathy. Kidney transplantation is associated with remarkable improvements in LV size as assessed by LV diastolic diameter and function as well as regression of LVH as assessed by LV mass, otherwise known as reverse remodeling (129-132). Moreover, positive changes are observed in LV diastolic function and right ventricle (RV) systolic pressure in the post-transplantation period (130-132). Accordingly, although LVH is observed in approximately 50% of patients following transplantation, regression of LVH reduces the risk of cardiovascular events up to nearly 60% in KTRs (130-133). Still, LVH remains common in KTRs, related mainly to hypertension and anemia and it is an independent risk factor for congestive cardiac failure and mortality in these patients (130-133). Data from a randomized trial indicate that preservation of allograft function and adequate control of BP are the main factors associated with regression of LVH in this patient population (134).

In line with the above, available evidence indicates that restoration of kidney function and reversal of the uremic milieu have a positive influence on myocardial mechanics and function in patients with established cardiomyopathy prior to transplantation. Several studies report improvement in EF over time in most patients following kidney transplantation (130-132), This is most prominent in dialysis patients with underlying LV systolic dysfunction who showed substantial, stable improvements in LVEF following kidney transplantation, with a mean increase of 15% in those with a baseline LVEF lower than 40% (132). Accordingly, in a cohort of 103 patients with HFrEF who had a median of two hospitalizations due to HF before transplantation and no inducible ischemia, the mean EF increased from 32% prior to transplantation to 52% at 1-year post-transplant, with more than two thirds of the KTRs displaying an EF above 50% (130). Likewise, results from another study showed that graft outcomes and survival of KTRs with baseline mean EF of 35% did not differ from those with normal EF, which was largely attributed to subsequent improvements in EF post transplantation (135).

On the other hand, data regarding changes in subclinical indices of diastolic and systolic myocardial dysfunction following transplantation are less straightforward. Data using echocardiographic strain measurements suggest that subtle abnormalities in GLS, a sensitive measure of LV function, exist after transplant even among individuals with normal LVEF (136, 137). Thus, a study of children with CKD under maintenance dialysis who underwent kidney transplantation showed GLS improvements post-transplant (136). Similarly, results from a multi-center cohort with a long hemodialysis vintage showed improvements in LV GLS with reverse remodeling and improvements in LV systolic function being most prominent in patients with severely reduced LV GLS compared to those with mildly or moderately reduced LV GLS (137). However, an analysis of biventricular strain in dialysis patients prior to and following kidney transplantation showed that strain abnormalities persisted post-transplant even with preserved EF, thus suggesting that

subclinical abnormalities in myocardial mechanics may persist whilst other classical indices of myocardial function such as EF are within normal values (138). Yet, it is possible that the length of time needed for the recovery of strain abnormalities may require a long-term follow-up as well as the utilization of targeted therapies so as to further improve cardiac performance. Reduced GLS peri-transplant has been linked to an increased risk of cardiovascular events or death following transplantation even after adjusting to other correlates of adverse outcomes including older age, history of CAD, hypertension and diabetes as well as a higher E/E' ratio (139). In conclusion, subclinical abnormalities in the biventricular strain may be observed in KTRs even when other classical indices of myocardial function such as EF are normal (131, 138-140). Larger studies are needed in the future to define the incremental predictive value of myocardial strain and deformation indices over clinical and other echocardiographic parameters for adverse CVD events following transplantation.

Evidence from single center studies as well as large database analyses indicate that both preexisting and de novo HF follows kidney transplantation adversely affect post-transplant outcomes. Overt HF following kidney transplantation remains a significant cause of hospitalizations related to CVD, accounting for 16% of all hospitalizations in these patients (129, 141-142). Notably, even though the absolute rates of major adverse cardiovascular events (MACE) in KTRs display a stable trend during the last two decades, 78% of all MACE in this patient population were ascribed to HF (141, 142). Despite the improvement in EF over time in most KTRs, rates of de novo HF are as high as 10%–18% at 12 and 36 months after transplant, and de novo HF is independently associated with higher mortality and graft loss (140). However, the incidence de novo HF after transplantation according to the USRDS significantly declined over the period of 1998–2010, with no apparent change in subsequent mortality (141). In conclusion, although improvements in LV systolic and diastolic volume and reduction in ventricular masses are observed after transplant, the effects of potential pathological cardiorenal crosstalk before and following transplantation contribute to ongoing myocardial dysfunction in KTRs.

3. Pathogenesis of CVD in CKD and kidney transplantation

3.1 Traditional and novel atherosclerosis risk factors in CKD

CKD is considered as a major risk factor for development of CAD. Experimental animal models of CKD, performed in either ApoE or LDL receptor null mice or in rabbits, with 5/6 nephrectomy or with unilateral nephrectomy, support the link between CKD and accelerated atherosclerosis (30). In addition to the high prevalence of traditional CAD risk factors, such as diabetes and hypertension, patients with CKD also carry other nontraditional CVD risk factors, which are related to the uremic milieu, including chronic inflammation, endothelial dysfunction, oxidative stress, uremic toxins and abnormal calcium-phosphorus metabolism among others (7). As a result, in patients with CKD, the

increased cardiovascular risk is multifactorial and to a certain degree a direct consequence of the pathophysiological mechanisms specific to CKD, thus rendering CVD prevention by traditional strategies difficult in this setting (Figure 2).

It is well-known that individuals with CKD have a high prevalence of traditional risk factors such as hypertension and diabetes, which contribute to the increased cardiovascular risk in these patients. Noticeably, as already presented above, the associations of kidney function and albuminuria with cardiovascular risk are independent of these traditional cardiovascular risk factors (7). Hypertension and CKD are bidirectionally related as arterial hypertension is an established and strong risk factor for development of CKD whereas CKD itself is the most common cause of secondary hypertension (7, 143). The prevalence ranges from 60% to 90% depending on the stage of CKD and its cause (143). Hypertension is a hallmark of CKD and even during its early stages; CKD can cause hypertension, which is likely to further augment cardiovascular risk in affected patients (7, 61).

Type 2 DM as well as the metabolic syndrome is the main risk factors responsible for CKD in economically developed and developing countries (61). According to a great body of evidence, a significant percentage, ranging from 20% to 40% of individuals diagnosed with DM is likely to develop diabetic nephropathy (144). Hyperglycemia induces microvascular and macrovascular complications through various mechanisms including enhanced glycoxidation, intracellular generation of reactive oxygen species (ROS), and accumulation of glycosylated proteins, which promote mainly via epigenetic changes a chronic inflammatory state (61). Thus, inflammation is a key common denominator in the pathogenesis of diabetic kidney disease and its cardiovascular complication (145). Risk factors for obesity, type 2 diabetes, and CKD-related risk factors for CVD overlap in patients with diabetic kidney disease.

CKD causes both quantitative and qualitative changes in circulating lipid levels that can be atherogenic (146). Dyslipidemia in CKD patients is characterized by elevated triglycerides levels, low levels of HDL (high density lipoprotein) cholesterol, variable levels of LDL (low density lipoprotein) cholesterol and high lipoprotein a (LP(a)) levels (147). In CKD, the quantitative changes in serum lipids are not particularly proatherogenic with triglyceride levels being associated with subclinical atherosclerosis in CKD G3, whereas in CKD G4-5 only total cholesterol levels showed a weak association with the presence of atherosclerosis (30, 148). Notably, the predictive value of LDL-cholesterol levels with regard to CAD risk in CKD is lower compared to the general populations (149). However, qualitative changes in lipid profile observed in CKD could be associated with a higher atherogenic profile (30). Available evidence indicates that CKD is linked to modifications in the metabolism of triglyceride-rich LDL or otherwise very low-density lipoprotein (VLDL) particles, intermediate-density lipoprotein, and LDL with excessive oxidation of LDL cholesterol and the generation of small dense LDL particles, which enhance their atherogenic potential (147). Furthermore, reverse cholesterol transport, a process which is mediated by HDL cholesterol is impaired in CKD as well (61, 147). Additional striking qualitative changes in the composition of lipoproteins include chemical modifications in LDL and HDL, such as glycation, oxidation, and

carbamylation, which are associated to activation of pathogenic pathways and receptors such as the proinflammatory lectin-like oxidized LDL receptor (30, 148-151). Carbamylated LDL caused oxidative stress, accelerated senescence and death in endothelial and endothelial progenitor cells, potentially contributing to the decreased endothelial regenerative ability that characterizes CKD (150, 151). LDL carbamylation favors atherosclerosis in animal models (150). Furthermore, Lp(a) levels are increased in CKD and Lp(a) overexpression in uremic mice increased the extent of atherosclerosis (30).

Apart from the traditional cardiovascular risk factors, several non-classical risk factors, related to CKD have come at the spotlight for their links to the pathogenesis of CVD in CKD.

CKD patients and especially those undergoing maintenance hemodialysis patients display significantly accentuated sympathetic activity when measured by sympathetic microneurography (61, 152). Notably, increased sympathetic activity does not regress after kidney transplantation but is abolished with bilateral nephrectomy (61). There are several causes of sympathetic nervous system (SNS) overactivity in CKD patients including activation by afferent renal nerves in diseased kidneys and comorbidities such as sleep apnea, obesity or concomitant CVD (61). Activity of renalase, an enzyme produced by the kidney that inactivates catecholamines, is lowered in individuals with CKD (9). This high sympathetic activity in CKD patients is associated with concentric LVH and a high risk of cardiovascular complications and death in dialysis patients (61). Increased activity of renin-angiotensin-aldosterone system (RAS) in CKD is a pivotal mechanism within the complex pathways involved in the pathogenesis of CVD in these patients. Accordingly, angiotensin, among others, stimulates sodium and water retention, production of superoxide, interleukin 6 and other cytokines and induces endothelial dysfunction (61).

Endothelial dysfunction is a hallmark of CKD as well as a risk factor for CVD. Although endothelial function is established in advanced CKD, there are other culprits that promote endothelial dysfunction early in CKD including DM and hypertension (153, 154). Impaired nitrous oxide production due to the accumulation of endogenous inhibitors of nitric oxide (NO) synthase, like asymmetric dimethyl arginine (ADMA) together with a parallel rise in endothelin levels are the main features of endothelial dysfunction, further inducing hypertension, inflammation and oxidative stress in CKD (155-158). Bioavailability of NO, which affects the growth and function of vascular smooth-muscle cells, platelet aggregation and leucocyte adhesion to the endothelium, is decreased in CKD (7). Concentrations of ADMA increase with decreasing kidney function are associated with concentric LVH and predict mortality and cardiovascular complications in patients with CKD (156-158). In specific, ADMA through inhibition of NO generation, reduces cardiac output, and augments systemic vascular resistance and as a result BP. Abnormal endothelium-dependent vasodilation, manifested as impaired brachial artery reactivity, is a predictor of cardiovascular events and mortality in CKD patients independently of arterial stiffness and LVH (153). Endothelial cell apoptosis facilitates atherosclerotic plaque formation and increased levels of sFas, a marker of apoptosis, are independent predictors of future

adverse cardiovascular event in patients with CKD (153). Moreover, albuminuria per se can be regarded both as marker and as a contributor to impaired endothelial function (7). Considering that oxidative stress is considered a central pathway in the pathogenesis of atherosclerosis and HF, there is great research interest regarding the implications of oxidative stress markers as CVD risk factors in patients with CKD (159). CKD enhances oxidative stress in the organism with ensuing cardiovascular damage (159).

Oxidative stress in uremia is the consequence of higher reactive oxygen species (ROS) production, whereas attenuated clearance of pro-oxidant substances and impaired antioxidant defenses play a complementary role (159). Enhanced oxidative stress in the setting of the uremic milieu promotes enzymatic modification of circulating lipids and lipoproteins, protein carbamylation, endothelial dysfunction via disruption of NO pathways, and activation of inflammation, thus accelerating atherosclerosis (159). NADPH oxidase activation, xanthine oxidase, mitochondrial dysfunction, and NO-ROS are the main oxidative pathways leading to LVH and the cardiorenal syndrome (159). Finally, the activity of a subset of antioxidant enzymes, the paraoxonases is reduced in CKD which has been linked to increased burden of CVD in these patients (159).

Abnormal mineral metabolism as occurs with CKD mineral bone disorder (CKD-MBD) promotes arterial calcification, and arterial stiffness, which may in turn lead to LVH and potentiation of atherogenesis (61, 160, 161). Coronary artery calcifications are considered as biomarkers of increased cardiovascular risk in CKD patients and especially in those on maintenance dialysis (160, 161). Vascular calcifications are a hallmark of CKD with vascular calcification in the large arteries being associated to the presence of atheroma plaques, whereas in small arteries medial calcification without plaque is often observed. The pathogenesis of vascular calcifications in CKD is mainly linked to the instability of calcium and phosphate ions in the circulation plus the abnormal differentiation of vascular smooth muscle cells (VSMC) which acquire an osteoblast and chondroblast-like phenotype. Furthermore, CKD is associated with disequilibrium in the expression of promoters and inhibitors of soft tissue calcification (61, 160, 161). However, it remains to be clarified whether medial vascular calcification often observed on patients with CKD can be considered as an atherosclerotic equivalent. An additional mechanism of vascular calcification relates to calcium deposition in the intimal layer of the artery (162) with the instability of calcium salts in the circulation in the setting of a dysfunctional endothelium being regarded as the main promoter of intimal calcification (30). Increased inorganic phosphate levels cause endothelial dysfunction, characterized by phenotypic changes, decreased viability, and senescence (163). Moreover, vitamin D metabolites regulate endothelial function, by modulating the synthesis of endothelial vasoactive factors and the interaction with circulating leukocytes (30).

The accumulation of uremic toxins in the circulation and in tissues is considered to play a significant part in the progression of CKD and the development of CVD in this setting (164). The complex interactions between uremic toxins, RAS activation, inflammation and oxidative stress may all contribute to the high cardiovascular risk of CKD. High levels of

uremic toxins, such as indoxyl sulphate, and paracresyl sulphate, are linked to the pathogenesis of endothelial dysfunction and vascular damage (164). However, among the great number of uremic toxins identified so far, very few have been extensively studied whereas the pathophysiological mechanisms of cardiovascular damage induced by uremic toxins is a subject of current investigation. Thus, highly protein-bound uremic toxins stress endothelial cells, induce oxidative damage and expression of adhesion molecules, and inhibit the proliferation and endothelial progenitor cell-dependent neovascularizarion (30, 165).

Inflammation is now considered one of the main mechanisms of atherosclerosis. Finally, a chronic state of low-grade inflammation is common in CKD and almost a ubiquitous finding in dialysis patients (61). Mechanisms of increased systemic inflammation in CKD are a subject of ongoing investigation, with several pathogenic factors implicated, including among others oxidative stress, immune disorders and a propensity to infection, intestinal dysbiosis, metabolic acidosis, as well as reduced renal clearance of cytokines. Chronic inflammation in CKD patients and patients maintained on chronic dialysis portends a high risk for all-cause and CV death (61, 166). Actually, C-reactive protein (CRP) levels were associated with CAD in the general population and with the presence of atherosclerotic plaque in CKD patients. Several novel risk factors related to decreased kidney function might interact with the renal and systemic immune system mediators involved in renal injury and repair to participate in accelerated atherogenesis which is otherwise known as Immune Inflammation-Renal Injury-Atherosclerosis or the IRA Paradigm (167).

In conclusion, classical atherosclerosis risk factors do not have the same predictive value in patients with CKD, particularly in advanced CKD, as in the general population and underlying CKD-related alterations in the uremic milieu contribute to the phenotype of accelerated atherosclerosis that is observed in these patients (30, 168).

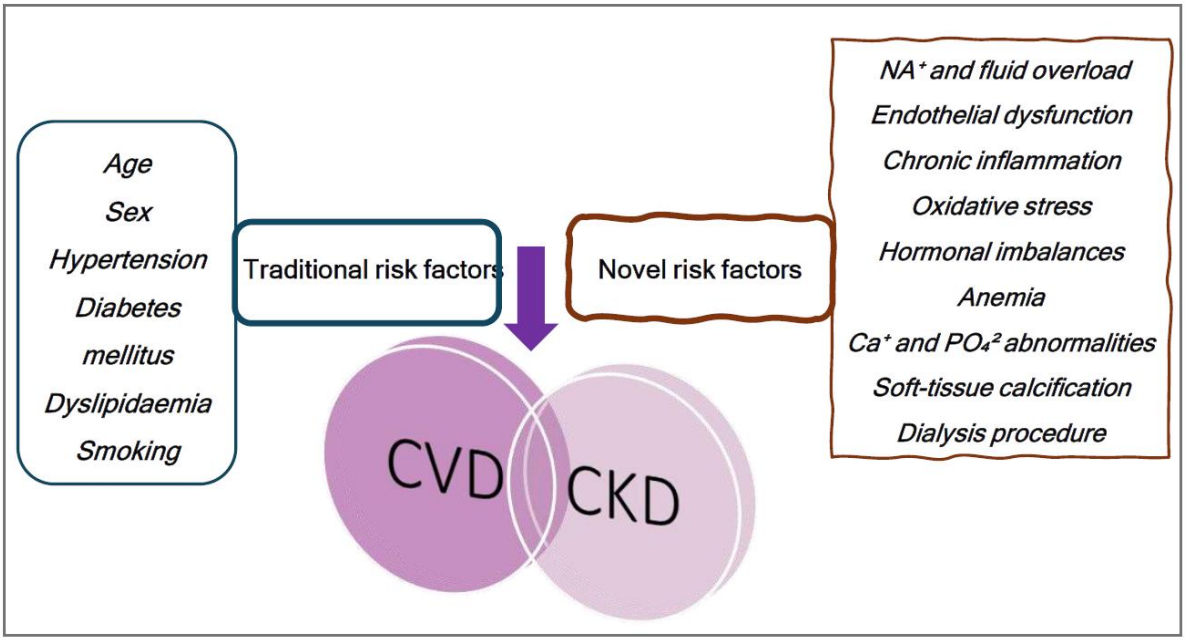

Figure 2. Traditional and nontraditional risk factors for CVD in CKD.

3.2 Pathophysiology of LVH in CKD

Several intertwining mechanisms are involved in the pathophysiology of LVH development in CKD patients, with factors unique to CKD amounting to a great degree to the pathogenesis of LVH (169). Accordingly, among the classical implicated factors are those related to preload, characterized by increases in systemic arterial resistance and afterload which include increased arterial BP and reduced large-vessel compliance (170).
Arterial hypertension is a major determinant of LVH in CKD. Multiple pathways are involved in the pathophysiology of hypertension in CKD, including reduced nephron mass, increased sodium retention and extracellular volume expansion, SNS overactivity, stimulation of the RAS and endothelial dysfunction (61). Systolic hypertension and elevated pulse pressure are strongly associated with LVH in patients with advanced CKD, suggesting that fluid overload and increased arterial stiffness play a role in LVH even before the start of dialysis therapy (169).

Volume overload is increasingly common from stage 3 to stage 5 CKD and is tightly related to cardiovascular complications such as LVH, hypertension and HF (61, 71-173). In CKD patients, an increased sodium intake and fluid overload are directly and independently associated with the incident risk of CVD and death (61, 171). Strikingly, volume overload doubles the risk of death in dialysis patients, independent of hypertension and other risk factors (173). An interesting fact is that sodium in CKD also is deposited in the muscles and the skin without simultaneous water retention, which has been associated with the severity of CKD (61). In experimental models, skin sodium reservoirs induce inflammatory mechanisms in the local macrophages (61,174, 175). Furthermore, volume expansion in CKD

promotes the production of endogenous cardiotonic steroid compounds, including ouabain and ouabain-like steroids, which raise BP by impairing vasodilatory mechanisms (61). RAS activation induces hyperaldosteronism and promotes cardiac fibrosis through the generation of signals leading to profibrotic transforming growth factor (TGF) production (169).

All components of the CKD-MBD axis appear to be involved in the pathogenesis of LVH. Dysregulation of fibroblast growth factor (FGF23), a central component of CKD-MBD promotes myocardial hypertrophy and its levels are strongly associated with the LVMI in CKD patients (176). FGF23 has been shown to play a role the regulation, growth, and differentiation of cardiac myocytes, exerts paracrine functions in the kidneys due to its phosphaturic properties as well as blocks vitamin D 3 synthesis and inhibits reabsorption in the proximal nephron reabsorption (169, 177). Serum levels of FGF23 increase gradually as kidney function decreases. FGF23 levels are often two to five times higher than normal during the initial stages of CKD whereas they peak in advanced CKD by reaching levels more than 200 times the normal values (169, 178-180). FGF23 is able to induce cardiomyocyte hypertrophy in vitro as well as LVH when administered to mice, whereas inhibition of the FGF receptor in rats subjected to subtotal nephrectomy reduced LVH that developed in the setting of CKD (21). Moreover, the results of several clinical trials suggest a close relationship between FGF23 and LVH. Accordingly, in hemodialysis patients the FGF23 levels are independently associated with the degree of LVH (178, 179). Results from the CRIC study showed that higher C-terminal FGF23 levels were independently associated with reduced EF, greater LVMI and increased prevalence of both eccentric and concentric LVH (180). Results from large meta-analyses show that FGF23 is a strong predictor of CVD and mortality in non-dialysis CKD patients as well as in dialysis patients (177).

Likewise, parathyroid hormone (PTH) is related to LV mass in dialysis patients and elevated serum PTH levels are associated with a high risk of cardiovascular events within all the spectrum of CKD severity (176). Similar associations have been described with low vitamin D levels and experimental data suggest that the vitamin D pathway may play a role in modifying myocardial structure and function (181). Recent data indicate that vitamin D receptor (VDR) Bsm I gene polymorphism is independently related to LVH progression in patients with advanced CKD and in dialysis patients (182). Available evidence links higher plasma phosphate to diastolic dysfunction and myocardial fibrosis in CKD patients (169). Accordingly, patients with increased levels of phosphorus display increased diastolic and mean BP, a higher cardiac index, higher heart rates, and an increased stroke index compared to normophosphatemic patients (183).

Anemia as a major complication of CKD is robustly associated with an increased risk of arteriosclerosis, LVH, cardiovascular hospitalizations and mortality in non-dialysis CKD as well as in dialysis patients (184-187). Due to long term augmentations in cardiac output to compensate for reduced oxygen delivery to peripheral tissues, anemia leads to LV remodeling including initial dilation from the increase in preload, with subsequent hypertrophy in an attempt to decrease the high wall tension of the dilated LV (184-187).

Finally, miscellaneous factors, like the presence of arteriovenous fistulas which lead to myocardial cell lengthening and eccentric or asymmetric LV remodeling, have been as well implicated in the pathogenesis of LVH in dialysis dependent CKD patients (169).

3.3 Pathophysiology of HF in CKD

An array of hemodynamic and metabolic factors, inflammatory processes, oxidative stress, as well as the accumulation of uremic toxins, lies at the core of the pathophysiologic mechanisms underlying CKD-associated cardiomyopathy (2, 21).
Impaired natriuresis and abnormal salt and water retention in CKD occur as a result of diminished sodium filtration due declining GFR together with disordered sodium absorption by the nephron segments in the setting of inappropriate RAS activation (188, 189). Yet, it should be noted that the CKD-associated cardiomyopathy is not altogether BP dependent (2, 190). The elucidation of the signals that trigger these mechanisms in response to kidney disease and of their systemic consequences is of major importance (23). The classical paradigm of kidney injury due to inability of the failing heart to generate forward blood flow, thus resulting in prerenal hypoperfusion, has been considered for many years the fundamental pathophysiological mechanism of type 1 and type 2 CRS (188). Thus, insufficient blood flow in afferent renal arterioles activates the RAS axis, the SNS, and arginine vasopressin secretion, leading to fluid retention, increased preload, and further worsening of HF (188). However, evidence from the ADHERE registry showed that the incidence of rising serum creatinine was similar among patients with acute HF and reduced versus preserved EF (103). In line with the above, many patients hospitalized with acute CRS display normal or increased BP as well as preserved LV EF (191). As a result, another key component, the central venous pressures (CVPs), has been introduced by experimental models and by clinical studies in patients with acute HF using invasive hemodynamic monitoring (188). Increased CVP results in renal venous hypertension, increased renal resistance, and impaired intrarenal blood flow, with subsequent neurohumoral activation causing decreases in intraglomerular pressures and reduced GFR (188). Furthermore, the enhanced activation of the neurohumoral axis results in increased proximal tubular sodium and water reabsorption further worsening congestion (188). Notably, the low-resistance nature of the renal vasculature and the very low pressure of oxygen in the outer medulla render the kidneys vulnerable to hypotension-induced injury. Thus, data from cardiovascular patients undergoing right-sided heart catheterization showed that increased CVP was associated with reduced GFR and all-cause mortality (192). Finally, elevated intra-abdominal pressures in the setting of acute HF may further exacerbate renal dysfunction by causing renal compression and reduced perfusion (192, 193). Type 4 CRS is characterized by a chronic state of volume expansion and depending on the severity of CKD, 60% to 90% of patients have arterial hypertension (23). Chronic activation of the RAS and SNS as well as of mineralocorticoid receptor overactivation play a central role in the pathogenesis of type 4 CRS, via hemodynamic, pro-inflammatory, pro-oxidant and pro-fibrotic effects (23, 188).

Notably, the inhibition of RAS by angiotensin-converting enzyme (ACE) inhibition or angiotensin receptor blockers (ARB) has beneficial effects to the heart in uremic cardiomyopathy, independent of blood pressure reduction (23). Thus, the inhibition of Angiotensin II type 1 receptor (AT1) by losartan in a model of subtotally nephrectomized rats prevented or hindered the progression if uremic cardiomyopathy, including CKD-induced diastolic dysfunction, LVH, cardiac fibrosis and cardiac inflammation independently of significant BP effects (194). These beneficial effects were more prominent with early administration during the course of CKD (194). Additionally, the genetic knock out of the angiotensin 1 (AT1) receptor in mice yielded similar effects (195). Notably, treatment of mice with CKD after unilateral urinary obstruction for 3 weeks with enalapril but not with hydralazine, apart from improvements in BP, cardiac hypertrophy and cardiac fibrosis, resulted in inhibition of the pro-fibrotic transforming growth factor beta (TGF-β) signaling (196).

The role of the SNS is essential for blood pressure control and hemodynamic stability (188). CKD-induced activation of the β-adrenergic receptors by catecholamine neurotransmitters is associated with adverse cardiac outcomes (23). On the other hand, inhibition of catecholaminergic signaling appears to be cardioprotective in the setting of CKD as experimental models have shown that treating nephrectomized rats with b-blockers reduced blood pressure, cardiac hypertrophy, and cardiac fibrosis as well as inhibited cardiac apoptotic signaling pathways (197). It is well-established that mineralocorticoid receptor expression is present in renal tubular epithelia as well as in endothelial cells, SMC, cardiomyocytes, fibroblasts and immune cells (188,189). Activation of the mineralocorticoid receptor stimulates multiple pathogenic pathways which lead to increased production of plasminogen activator inhibitor- 1, TGF-β, interleukin (Il)-6, and monocyte chemoattractant protein (MCP)-1 which further promote inflammation and fibrosis (189). Induction of myeloid cells by the activation of the mineralocorticoid receptor causes increased release of profibrotic molecules and chemotaxis of other inflammatory cells. Additionally, mineralocorticoid receptor activation stimulates generation of ROS by activation of nicotinamide adenine dinucleotide phosphate (NADP) (23, 189). The end results are increased myocardial stiffness and impaired LV relaxation in the heart as well as glomerular and interstitial fibrosis in the kidneys. Mineralocorticoid antagonists, like spironolactone and finerenone have shown pleiotropic beneficial cardiovascular effects in experimental models of CKD including reduction in systemic and vascular inflammation, inhibition of vascular calcifications, restoration of diastolic dysfunction and attenuation of the CKD-induced decrease of LV fractional shortening and EF (189, 198). The FIDELIO-DKD (Finerenone in Reducing Kidney Failure and Disease Progression in Diabetic Kidney Disease) and the FIGARO-DKD (Finerenone in Reducing Cardiovascular Mortality and Morbidity in Diabetic Kidney Disease) trials demonstrated benefits for finerenone, a novel non-steroidal mineralocorticoid, antagonist (nsMRA) in improving adverse kidney and cardiovascular outcomes in patients with type 2 diabetes and CKD (199, 200). Ongoing research shall clarify

in the near future the potential of nsMRAs to reduce risk for CKD progression as well risk for CVD in non-diabetic kidney disease.

The close relationship between FGF23 and LVH has been described above. Apart from LVH, FGF23 has been reported to contribute to cardiac calcium mishandling. Cardiomyocytes isolated from subtotal nephrectomized mice or from mice treated with FGF23 showed a slower increase in cytosolic calcium levels during systole as well as a slower decline in cytosolic calcium during diastole compared with controls (201). Notably, FGF23 induced depression of cardiomyocyte contractility via intracellular Ca2+ mishandling can be blocked by soluble Klotho via unclear mechanisms (23, 201). Heterozygous Klotho deficient mice with CKD display more prominent cardiac hypertrophy and fibrosis as well as more severely impaired cardiac dysfunction compared with wild-type CKD mice (23, 202). However, delivery of soluble Klotho to Klotho deficient CKD mice appears to improve the cardiac phenotype without significant effects on kidney clearance function (202). Hyperphosphatemia has been associated with a substantially higher incidence of mortality from CVD and in specific HF in CKD patients (203). An echocardiographic study which correlated the serum phosphorus levels LV size and to HF in 3,300 CKD patients, showed that serum phosphorus was positively related to the internal mass of the LV and systolic dysfunction. Furthermore, development of HF during long term follow-up was directly and independently associated with increased serum phosphorus levels (203).

Uremic toxins might be involved by both direct and indirect pathways in the pathogenesis of uremic cardiomyopathy. The most studied protein-bound uremic toxins in CRS are indoxyl sulphate and p-cresyl sulphate which display pro-inflammatory properties (23). First of all, uremic toxins have been strongly implicated in the induction and maintenance of chronic inflammation in CKD, by modulating several mediators such as CRP, cytokines and transcription factors as well as induction of oxidative stress (23). Indoxyl sulphate in cardiomyocytes increases the production of pro-inflammatory cytokines such as interleukin 1 beta (IL1-β), IL-6, and tumor necrosis factor (TNF), a process mediated by NF-kB interaction (204-206). Moreover, indoxyl sulphate promotes hypertrophic effects in cardiomyocyte cultures by activating the signaling of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) pathways (206). In addition, indoxyl sulphate increases the proliferation and senescence of VSMCs as well as apoptosis of endothelial cells while decreasing cell to cell contacts (23). Notably, the classical uremic toxins, creatinine and urea have been linked to cardiomyocyte contractile injury and increases in myocardial oxygen consumption by lowering norepinephrine and causing insulin resistance (23).
The interaction of iron deficiency, anemia, HF and CKD, now known as the cardiorenal iron deficiency syndrome, has been at the center of the spotlight during the last years both in terms of pathophysiological implications as well as regarding treatment strategies that might benefit both organs (189). Although most of the studies have included patients with HFrEF, iron deficiency is a common comorbid condition in HFpEF also and is associated with decreased exercise capacity and quality of life (189).

With regard to the chronic inflammation, it is a common denominator of other diseases such as obesity and DM Inflammation, which in turn are independent risk factors for CKD (189). Moreover, inflammation is a key component in both HFrEF and HFpEF; however, while the inflammatory response in HFrEF is the result of cardiomyocyte damage from infection, ischemia, or toxicity, inflammation in HFpEF occurs as of result of systemic pathological states, such as CKD, obesity, DM, and hypertension. Taking into consideration the shared background, CKD interacts synergistically with obesity and DM to further promote the cardiac inflammation by reducing NO bioavailability, cyclic guanosine monophosphate (cGMP) content, and protein kinase G (PKG) activity in cardiomyocytes (189). Furthermore, microvascular changes and endothelial dysfunction further ensue (189). During the progression of CKD, alterations in nuclear factor erythroid 2 (Nfr2), a regulator of cellular resistance to oxidants are observed. Nfr2 is responsible for encoding and modulating the activity of various enzymatic antioxidants, including superoxide dismutase (SOD) and NADPH oxidase (NOX) (23, 159). In hypertensive and diabetic nephropathy, Ang II inhibits Nrf2 leading to reduced glutathione cycle activity (23). Ischemic injury, venous congestion, and inflammation further exacerbate oxidative stress in the CRS (207). An experimental study in mice with ischemia reperfusion injury (IRI) and development of type 3 CRS, showed that eight days after kidney injury induction, there were observed lipid oxidation and increased NO levels in the heart (208).

3.4 Risk factors for CVD in KTRs

The risk for CVD is influenced by traditional and non-classical risk factors, with a part of those being present prior to transplantation and others emerging during the posttransplant period. Accordingly, KTRs carry a significant burden of preexisting CVD risk factors due to the prolonged exposure to traditional risk factors as well as the accumulation of risk factors specifically related to CKD and kidney replacement treatment (125, 142). The prevalence of post-transplant hypertension in KTRs approaches 80%–90% as reported by a retrospective cohort of 1666 KTRs followed for 5 years after transplantation, with immunosuppressive medications further exacerbating it (125, 209). Similarly, in the FAVORIT cohort including 4110 stable KTRs, a follow-up study showed that each 20-mmHg increase in systolic BP was associated with a 32% higher risk of CVD (hazard ratio [HR], 1.32; 95% CI, 1.19 to 1.46) whereas diastolic BP levels lower than 70 mmHg were associated with higher risk of CVD and death (125, 210).

Dyslipidemia is frequent following kidney transplantation due to the coexistence of comorbid conditions like obesity, DM, and metabolic syndrome. Furthermore, immunosuppressive medications, including mammalian target of rapamycin inhibitors (mTORI), calcineurin inhibitors (CNIs), and steroids exacerbate lipid disorders in KTRs (125). Notably, the association between dyslipidemia and CVD is strong in KTRs with the risk of ischemic heart disease doubling when serum cholesterol levels exceed 200 mg/dL or triglycerides levels exceed 350 mg/dL (211). Abnormal glucose metabolism is usual following

transplantation, in the absence of preexisting diabetes and represents a spectrum of disorders of impaired fasting glucose, impaired glucose tolerance, and post-transplant DM (PTDM) (125). Up to 45 % of KTRs display impaired fasting glucose early in the post-transplant period whereas post-transplant diabetes develops in 16% at 1 year and 24% at 3 years (125). Abnormal glucose metabolism increases approximately threefold the risk of fatal and nonfatal cardiovascular events (140) as compared with nondiabetic patients (125, 212, 213). Established risk factors for PTDM include deceased donor graft, older recipient age, recipient ethnicity and race as well as the presence of hypertension and obesity. Use of CNIs and steroids also contribute to PTDM risk by suppressing insulin secretion and increasing insulin resistance (123).

A functioning kidney allograft attenuates the influence of some of the nontraditional factors. The post hoc analysis of the FAVORIT study suggests no association with incident CVD or all-cause mortality above a GFR threshold of 45 ml/min/1.73 m^2, with each 5 ml/min/1.73 m2 increase in eGFR above this cut-off being associated with a 15% reduction in CVD and mortality (214). Proteinuria is a common finding in KTRs with approximately 20% of them having proteinuria of greater than 1 g daily (125). Similar to its role as a CVD risk factor in the general population, proteinuria is associated with CVD in KTRs with persistent proteinuria doubling the risk of CVD and all-cause mortality in KTRs (215). The presence of LVH, a common finding in KTRs linked to hypertension and anemia, is an independent risk factor for congestive cardiac failure and mortality in KTRs (141, 216). A well-functioning graft and adequate BP control are associated with regression of LVH in KTRs (125, 133). Finally, despite the improved endothelial function post-transplantation, inflammation and oxidative stress are also associated with vascular disease and endothelial dysfunction in KTRs, with CRP shown to be independently associated with cIMT, CVD and mortality in these patients (61, 125, 217).

4. Chronic inflammation and immune system abnormalities in CKD

4.1 Chronic inflammation in CKD

CKD is considered to be a classical paradigm of inflammatory disease and premature ageing (61, 218). Thus, a persistent state of inflammation is a CKD hallmark, and it is more prominent in patients with kidney failure (61). The coexistence of impaired immune responses with persistent stimulation of the immune system, perpetuate the low-grade systemic inflammation that characterizes the uremic environment, and which may translate into increased risk for vascular disease (61). Notably, the level of inflammatory mediators increases with CKD progression (219, 220). Thus, as demonstrated in 3,939 patients enrolled in the CRIC study, eGFR and cystatin C levels as well as albuminuria strongly correlated with the levels of IL-6, TNF, inverse acute phase reactants such as albumin, and fibrinogen, a mediator that links the inflammatory and the coagulation system (220). Likewise, levels of serum fetuin A, a major inverse acute phase reactant, the most prominent inhibitor of

calcium phosphorus precipitation as well as an inhibitor of insulin sensitivity, decline as renal function deteriorates (219). Although the role of a large number of pro- and anti-inflammatory cytokines is considered of major pathophysiological importance in CKD related inflammation, available data suggest that the anti-inflammatory cytokine IL-10 and two key proinflammatory cytokines IL-6 and TNF-a, play an essential part in the development of immune cells imbalance, CVD and wasting in the setting of the uremic milieu (221).

The pathogenesis of microinflammation in CKD is complex and multifactorial. The kidneys play a major role in clearing proinflammatory cytokines and bacterial antigens from the circulation with the kidney being the main site of metabolic degradation. Thus, as a result of declining kidney function, the reduced clearance of inflammatory mediators may augment the overall inflammatory responses (222). Notably, the serum half-lives of TNF-a and IL-1 are greater in animals without kidney function than those with kidney function (221, 222). Furthermore, a study of hemodialysis patients showed that serum IL-6 was strongly correlated to cytochrome c levels, a marker of mitochondrial damage-associated molecular patterns (DAMPs) (223). These findings suggest that circulating mitochondrial DAMPs, which are released during cell necrosis or apoptosis, may be a causative factor for the inflammation that characterizes hemodialysis patients (223). It should be noted that there is vast literature available on cytokine alterations in both dialysis and non-dialysis patients with kidney failure reporting elevated, yet conflicting results regarding the concentrations of both IL-6 and TNF-a in this population (221). Similar to Il-6, kidney function impairment is associated with a significant increase in TNF-a activity (221), with correlations between eGFR and TNF-a as well as its soluble receptors demonstrated in CKD patients (221). Additionally, the Tamm-Horsfall glycoprotein might be involved in the regulation of TNF-a activity, thus further strengthening the link between the kidneys and TNF-a handling (224). However, it should be noted that TNF-a has a short half-life and is subject to local tissue degradation apart from kidney clearance (221). Thus, there is a need to further identify other potential causes of increased circulating TNF-a levels in the kidney failure, including among others insulin resistance or volume overload (221).

Gut microbiota dysbiosis is involved in the pathogenesis of chronic inflammation in CKD. Altered gastrointestinal permeability due to vascular congestion in CKD patients may permit the translocation of gut-derived toxic products such as endotoxins and bacterial DNA fragments into the systemic circulation, which may in turn stimulate immune cells and the release of proinflammatory cytokines (225, 226). Furthermore, the uremic environment in the gastrointestinal tract disrupts the balance of the normal gut microbiota, which might be associated with increased risk of specific bacteria invading the body (227). Accordingly, overgrowth of bacterial DNA has been detected in the blood of nearly 20% of non-dialysis kidney failure patients (228). Moreover, significantly higher levels of CRP and IL-6 were found in these patients compared to those without detectable bacterial DNA (228). The overgrowth of gastrointestinal bacterial families that produce indole and p-cresol-forming enzymes in CKD, promotes the production of the two gut-derived uremic toxins p-cresol sulfate and indoxyl sulfate. These toxins activate white blood cells (WBC), stimulate

oxidative stress and increases leukocyte-endothelial interaction to induce inflammatory reactions (229).

Oxidative stress and inflammation, as well as their interaction, are considered as the main pillars in the pathogenesis and progression of CKD (159). Oxidative stress promotes inflammation via formation of proinflammatory oxidized lipids, advanced oxidation protein products (AOPPs) and advanced glycation end-products (AGEs). Nuclear factor κB (NFκB) transcription factor, the master orchestrator of the inflammatory response, is activated in the pro-oxidant milieu and promotes the expression of proinflammatory cytokines as well as recruitment and activation of WBC and other resident proinflammatory cells (159, 230). Accordingly, in a cohort of 176 patients with varying CKD severity, serum levels of high sensitivity (hs)-CRP, interleukin-6, and malondialdehyde were significantly increased and inversely related to the GFR, whereas serum levels of SOD and glutathione peroxidase were significantly decreased (2331). Notably, IL-6 and hs-CRP were positively correlated with malondialdehyde and negatively associated with SOD and glutathione peroxidase, further supporting the relationship between inflammation and oxidative stress in CKD (159, 231). AGEs, which result from carbonyl stress, have been associated with several markers of inflammation, including hsCRP, IL-6, serum fibrinogen, and soluble vascular cell adhesion molecule (sVCAM-1), in patients with kidney failure (230, 232). The interplay between uremic toxins, such as indoxyl sulphate, activation of inflammatory pathways and induction of oxidative stress creates a vicious circle of propagation of inflammation (230, 232). The anti-inflammatory properties of Nrf2 mediated by suppression of pro-inflammatory genes, including those encoding MCP-1 and VCAM-1, have been shown by several studies. CKD leads to dysfunction of Nrf2 activation, leading to accumulation of hydroperoxides and lipoperoxides, which are potent activators of NF-κB (159). Studies conducted in animals with CKD have shown marked decreases in nuclear Nrf2 content, thus suggesting an impaired regulation of the feedback antioxidant mechanism in the setting of oxidative stress and inflammation, which under normal conditions induce Nrf2 activation (159).

High phosphorus levels activate the NF-κB signaling pathway, thus leading to the release of IL-1β, IL-6, IL-8, TNF-α and other cytokines (233). In line with the above, there is a positive correlation between high phosphorus levels and inflammatory markers such as C-reactive protein (CRP) in CKD whereas the administration of phosphate binders reduced CRP levels in CKD patients in several prospective studies (233, 234). FGF23 interaction with its receptor promotes the secretion of CRP, IL-6 and TNF- α and vice-versa; inflammation may be a potent stimulator of FGF23 production in CKD (235). Additionally, inhibition of FGF23 signaling in a mouse model of diabetic nephropathy led to improvement of chronic inflammation (235).

Other factors implicated in the genesis of chronic inflammation in CDK include high SNS activity, presence of periodontal disease, increased susceptibility to infections and frequent comorbidities (61). Furthermore, conditions specific to the hemodialysis procedure may augment the inflammatory processes such as exposure to dialysis tubing and the less biocompatible dialysis membranes, poor quality of dialysis water and back-filtration or back-

diffusion of contaminants and endotoxins as well as the presence of foreign bodies contained in arteriovenous grafts or intravenous catheters (61, 236). Similarly, patients undergoing peritoneal dialysis are not immune to chronic inflammation with peritoneal dialysis catheter-related infections and exposure to peritoneal dialysis solution being some of the predisposing factors to generation of inflammation in this setting (237).

As it will be presented subsequently, immune cell dysfunction occurs in CKD, with increased activation as well as apoptosis rates in monocytes, lymphocytes, and dendritic cells, which might perpetuate the systemic low-grade inflammation (238-241).

4.2 Immune system dysfunction in CKD

Immunophenotyping of patients with CKD and kidney failure reveals findings compatible with a state of secondary immunodeficiency and impaired immune effector functions simultaneously with stigmata of persistent immune cell activation (243). Overall, immune system dysfunction in the setting of the uremic milieu is characterized by suppressed innate immune responses such as decreased phagocytic capability of immune cells to clear pathogens, reduced antigen-presenting ability of macrophages and dendritic cells to T and B cells, as well as enhanced immune cell activation by upregulation of cell surface receptors and release of proinflammatory cytokines. Furthermore, impaired adaptive immune responses include a reduction in the number and function of lymphoid cells, disordered maturation and activation of T lymphocytes or reduced antibody production by plasma cells (243). Notably, despite the increased interactions of immune cells with endothelial, the migratory ability of leukocytes is impaired due to a downregulation of integrins (243). Impaired urinary clearance of immunoregulatory factors, increased production of immunoregulatory proteins and persistent immune activation, extrinsic immunosuppressors, and a disrupted gastrointestinal barrier with alterations in the functional profile of the intestinal microbiota further augment immune system dysfunction in CKD (243). Subsequently, the dysregulation of the innate and adaptive immune system contributes to bacterial overgrowth, infections, and persistent inflammation. Notably, the chronic inflammatory state and immuno-activation have come at the spotlight as potential contributing causes of premature CVD in CKD (243).

The profile of immune components in kidney failure shares similar features with healthy elderly individuals displaying increased numbers of specific proinflammatory subsets of T cells and monocytes, thus suggesting the presence of premature immunological ageing in these patients. It should be noted that the cellular composition of the immune system does not become normal after successful kidney transplantation despite diminished inflammation and oxidative stress (241).

The immune system is composed of multiple intertwining pathways and several compounds including cellular and soluble factors which provide defense against pathogens, eliminate cancer cells and respond to tissue damage. The immune system is classically divided into two main interacting branches, the innate and adaptive immune system with

the first conducting a direct and nonspecific reaction to infection and tissue injury and the second being responsible for antibody production and generation of immunological memory (241, 242). The main cellular elements of the innate immune system are granulocytes, monocytes, dendritic cells and natural killer (NK) cells which recognize, phagocytose and digest pathogens, induce inflammation and present antigens to the lymphoid cells (241, 242). Innate immune cells express various molecular pattern recognition receptors (PRR) which enable the cells to respond to specific pathogen-associated molecular patterns (PAMPs) including bacterial and viral proteins, and fragments of damaged cells (241, 242). PRRs are classified into three main groups according to their mode of action. Thus, secreted PRRs which are represented by the mannose-binding lectin family, function as opsonins by binding to pathogens so as to make possible their recognition by the complement system and phagocytic cells. Endocytic PRRs are expressed on the surface of phagocytes and following PAMPs recognition, they mediate the uptake of pathogens into lysosomes. Signaling PRRs, with the Toll like receptor (TLR) family being the most extensively studied among them, recognize PAMPs, and activate signal-transduction pathways such as NF-kB, resulting in the induction of the expression of several immune response genes, such as those encoding cytokines (244). NOD-like receptors (NLRs) are expressed in the cytoplasm of macrophages and other inflammatory cells where they form multimeric complexes, called inflammasomes (241, 242).

The adaptive immune cells, B lymphocytes and T lymphocytes provide an elaborate and specific response to antigens. T cells that express CD4 on their cell surface are defined as helper T cells as they are responsible for the activation of cytotoxic effector T cells which express CD8 and the differentiation of B cells. T cells express the T-cell receptor (TCR) that recognizes the antigen when presented as a processed peptide bound to a human leukocyte antigen (HLA) molecule which is usually expressed by specialized antigen-presenting cells (APC), such as dendritic cells. Yet, in order to become activated, T cells require an additional signal from the innate immunity system, the costimulatory signal mediated by CD80 and CD86 molecules on APCs. Accordingly, naive T cells in the thymus gland and other reservoirs are activated by the antigenic stimuli bound to major histocompatibility complex (MHC) molecules on the APCs which convert them into either killer T cells or helper T cells. Antigen specific B cell receptors consist of immunoglobulin bound to the cell surface and after antigen binding as well as further activation by T helper (Th) cells, they differentiate into immunoglobulin producing plasma cells and memory B cells which makes possible the generation of a robust immune response once the pathogen is detected again (241, 242).

4.3 Alteration of the cellular components of the innate immune system in CKD

4.3.1 Polymorphonuclear leukocytes

The number of circulating (polymorphonuclear cells) PMNs, their basal activation state otherwise known as neutrophil priming as well as the expression of TLR2, TLR4 and integrins by these cells gradually increases with CKD progression (241, 245, 246). Thus,

PMNs display increased degranulation and ROS production after being stimulated by increased concentrations of proinflammatory cytokines or by AOPs resulting from amplification of oxidative stress (241). On the other hand, evidence from in vitro studies of PMNs of kidney failure patients indicates impaired migratory and phagocytic function as well as increased sensitivity to Fas-ligand mediated apoptosis and decreased ability of neutrophils to form neutrophil extracellular traps (NETs) when primed with their autologous uremic serum (241, 247-249). As a result, PMNs display decreased bactericidal capacity leading to impaired host defense in patients with kidney failure. Notably, the hemodialysis procedure results in further activation and degranulation of neutrophils, whereas kidney transplantation is associated with improved neutrophil function (285, 250).

4.3.2 Monocytes

Monocytes originate from common myeloid progenitor cells in the bone marrow that after circulating in the peripheral blood for 1-3 days, they subsequently differentiate into tissue macrophages or dendritic cells (251). Although monocytes were considered to represent a single cell population, extensive research during the last two decades, has shown that at least three phenotypically and functionally distinct human monocyte populations are recognized, distinguished by the expression of CD14 and CD16 surface antigens (252). CD14 acts as a co-receptor for TLR4 and mediates lipopolysaccharide signaling, whereas CD16 is a low affinity type III –A receptor for the invariable Fc region of immunoglobulin gamma (IgG) (253, 254). Accordingly, the classical monocytes do not express the CD16 antigen (CD14++CD16−) and represent 65-85% of all monocytes, whereas nonclassical monocytes express CD16 on their cell surfaces (CD14+CD16++) and account for 10-20% of all circulating monocytes (251, 252). The recent identification of an intermediate monocyte population denoted CD14++CD16+ has further advanced our understanding of monocyte subtypes in disease settings (255, 256). In specific, monocytes expressing CD16 are in fact a heterogeneous population, which can be subdivided into intermediate CD14++CD16+ monocytes and nonclassical CD14+CD16++ monocytes (255, 256). As of 2010, 3 distinct monocyte populations have been officially recognized, theCD14++CD16− (classical), the CD14++CD16+ (intermediate) and the CD14+CD16++ (non-classical) monocytes (255). The classical CD14++CD16- monocytes are important scavenger cells which display high phagocytic capability and increased production of antimicrobial proteins (251). Classical monocytes have the potential to directly invade inflamed tissues, where they differentiate into macrophages, whereas in the peripheral circulation, they differentiate into intermediate CD14++CD16+ monocytes (257, 258). Intermediate CD14++CD16+ monocytes are labelled “proinflammatory monocytes” as they produce high levels of ROS, TNF and IL-1β and substantially express HLA—DR isotype and CD74, which enable antigen presentation (257, 258). Their marked inflammatory capacity is additionally characterized by the expression of genes such as TGF- β1, allograft inflammatory factor 1 (AIF1) which is a highly conserved, inflammation-responsive scaffold protein that regulates the expression of inflammatory mediators such as cytokines, chemokines and inducible NO synthase (iNOS)

and protein tyrosine phosphatase non-receptor type 6 (PTPN6), a prominent regulator of cell proliferation and signaling of the innate and adaptive immune systems (255). Finally, since they express the angiopoietin markers, endothelium-specific receptor tyrosine kinase Tie-2 and endoglin (CD105) an accessory receptor for transforming growth factor beta (TGF-β), they are considered to play a role in the regulation of angiogenesis (255). Finally, intermediate monocytes also differentiate into nonclassical CD14+CD16++ monocytes, which express high levels of the adhesion related receptor CX3CR1, a transmembrane protein and chemokine involved in the adhesion and migration of these cells (251, 259). CD14+CD16++ monocytes have been suggested to remove damaged cells and debris from the vasculature (251, 259).

Most studies describe normal numbers of monocytes in the circulation together with a tendency towards lymphopenia in patients with CKD (261). However, various alterations in the phenotype and function of monocytes are observed in CKD, which contribute to immune dysfunction (260). Activated monocytes in the setting of CKD are more prone to react to the inflammatory cytokines and they display augmented adherent and migratory abilities to the endothelium by expressing integrin alpha M (CD11b) as well as chemokine receptor expression of CCR2 and CX3CR1 (261). Monocytes from hemodialysis patients have an activated profile with increased expression of integrin and TLRs and increased secretion of proinflammatory cytokines in response to basal and activated conditions (241, 261-263). However various studies provide contradictory results in hemodialysis patients, with some showing suppressed TLR4, particularly those with longer dialysis vintage, whereas another study has reported increased TLR2 and TLR4 expression and activity in monocytes in these patients (245, 251, 264). On the other hand, augmented expression of TLR2 in CD14+ monocytes have been directly correlated with IL6 levels in hemodialysis patients, whereas diminished expression of TLR2 has been reported in patients undergoing peritoneal dialysis (264). Non-kidney failure patients display reduced TLR4 expression on monocytes together with a diminished cytokine response of these cells to endotoxin stimulation (241, 265). Moreover, augmented oxidative stress in the setting of uremia can generate potential TLR ligands, such as the oxidized phospholipids formed during the oxidation of LDL (241). Many of the morphological and functional alterations of monocytes are found in both non-end-stage CKD and in dialysis patients with uremic toxicity being a shared causal factor. However, potential specific dialysis related mechanisms affect the phenotypic and functional features of monocytes subsets which might be ascribed to inflammatory activation through infectious stimulation by colonized central venous catheters, cell free DNA, or endotoxin leaking into the blood stream through the dialyzer membrane, dialysis fluid blood-membrane interactions and biocompatibility issues (260). Notably, dendritic cells and macrophages have a reduced antigen-presenting capability to activate T cells, which might be due to an abnormal expression of TLR4 or costimulatory molecules CD80 and CD86 (260, 265, 266).

Even though several earlier studies analyzing supernatants from unfractionated leukocytes described enhanced secretion of monocyte-derived cytokines, such as TNF- α, IL-

1ß, IL-1-receptor antagonist, IL-6, and soluble IL-6 receptor, IL-8, and the regulatory cytokine IL-10, when stimulated by endotoxin, these cells display a lower capacity of cytokine production compared to healthy donors which might ascribed to a state of exhaustion (258, 260). Both experimental and clinical evidence reveal suppressed endocytosis capacity and impaired maturation properties of monocytes and monocyte-derived cells when they are cultured with uremic serum or when obtained from patients with kidney failure (260, 265, 266). Similarly, both ROS production in response to Staphylococcus aureus and phagocytic capacity of monocytes are diminished in dialysis patients (260, 267). Additionally, overexpression of ACE by monocytes may be considered as a CKD related feature for producing inflammatory mediators (260). Thus, a recent study showed that serum from patients with renal failure induces high expression of ACE on monocytes, an effect tightly linked to the induction of the micro-RNA miR-421 in monocytes by the uremic toxins, including indoxyl sulphate and p-cresyl sulphate (268, 269). Monocytes incubated with uremic plasma display marked inhibition of iNOS, an effect attributed to a uremic toxin inhibiting monocyte phagocytosis, which was identified following fractionation of the hemofiltrate and analysis by gas chromatography and mass spectrometry (270, 271). Monocytes from patients with CKD and undergoing RRT show higher apoptosis rates compared to monocytes from healthy controls which further exacerbates their phagocytic function over time (260, 268). Accordingly, uremic plasma has been shown to nearly double the apoptosis rates of monocytes in cell culture, with this effect however being attenuated when the plasma donors are treated by high flux compared to low flux dialysis (260, 272).

Monocytes subpopulations in uremia display changes in relative numbers as well as altered respective characteristics (260). Overall, monocytes subsets shift to a CD16+ phenotype in the uremic environment, whereby they display a smaller size together with accentuated inflammatory, adhesive, and senescent properties (260). Patients with kidney failure typically display an increased number of circulating proinflammatory monocytes compared with healthy controls (241). Even though infectious stimuli contribute to the increased numbers of inflammatory CD16+ monocytes in patients with kidney failure, other complex mechanisms might be at play and remain to be elucidated (241, 273). Interestingly, low concentrations of circulating TLR ligands stimulate the release of the murine equivalent of these cells, Ly6ChighCCR2+ monocytes, from the bone marrow in a process mediated by stromal mesenchymal stem cells (241). The numbers of circulating inflammatory CD16+ monocytes remain high even after kidney transplantation, irrespective of the immunosuppressive regimen and the transplantation vintage (241, 274).

The expansion of CD14++CD16+ monocytes in patients with CKD reaches levels up to 18% of total monocytes number, compared to healthy individuals where this population accounts for nearly 8% of the monocytes (275, 276). The presence of DM seems to further contribute to the expansion of this cell population in patients with CKD (260). CD16+ monocytes display increased attaching capacity to endothelial cells in coculture experiments and are associated with endothelial damage in vivo as demonstrated by increased levels of CD31+Annexin+ microparticles in the blood of CKD patients (260, 277, 278). Studies

examining the effect of uremic plasma on peripheral blood mononuclear cells (PBMC) have yielded controversial results regarding induction of CD14++CD16+ cells, with shifts between subsets mainly occurring due to cells sequestration or release from the bone marrow and not by subsequent differentiation (260, 279, 280). However, the implication of uremic toxins in the expansion for CD14++CD16+ monocytes has been suggested by the findings of several studies. Accordingly, patients with preserved residual renal function seem to have lower counts of the intermediate CD14++CD16+ monocytes (281). With regard to different dialysis modalities, even though online-hemodiafiltration was reported to reduce the intermediate monocytes subsets as well as the production of TNFα and IL-6, prospective studies of high cut-off hemodialysis filters did not show any effects (282). Hemodialysis patients display higher levels of proinflammatory CD14+CD16+ monocytes together with elevated apoptotic endothelial microparticles and serum vascular endothelial growth factor (VEGF) concentrations compared with patients undergoing peritoneal dialysis thus suggesting an improved inflammatory and endothelial function profile in peritoneal dialysis (251, 278). Additionally, it appears that a longer dialysis vintage is directly related to a higher percentage of intermediate monocytes in the blood (283). Kidney transplantation modulates monocytes subpopulations mainly in the setting of immunosuppression, with CD14+CD16+ monocytes decreasing early post-transplant. Accordingly, methylprednisolone increases CD14++CD16− and CD14++CD16+ monocyte counts in vitro, findings which are replicated in patients receiving steroids when compared to those not taking steroids whereas mycophenolate, CNIs and mTOR inhibitors do not appear to affect monocyte subset counts (284, 285).

4.3.3 Natural killer cells and natural killer T cells

NK cells and natural killer T (NKT) cells are two fundamental cell subsets involved in innate immunity pathways as well as important regulators of adaptive immune responses (251, 286). NK cells are large granular lymphocytes with the bone marrow being the principal site of NK cell development, even though these cells may as well develop in secondary lymphoid tissue (286, 287). NK cells are a heterogenous population marked by a variety of receptors and functions (251, 287). Thus, they express the cell surface receptors, CD56 and CD16, but do not express the TCR complex, CD3. Accordingly, the CD56+CD3- NK cell subpopulations can be defined based on the relative expression of the markers CD16 and CD56 on their surfaces and CD27, a member of the TNF receptor superfamily, is an additional marker that identifies specific NK cell subsets (251, 286-288). Human NK cells can be divided into three functional subsets, respectively the NK cytotoxic, NK tolerant and NK regulatory cells, with the last two subsets, which are CD56bright CD16- NK cells, being expanded in several disease states (251). NK cells are normally regulated by cytokines, principally IL-2 and IFNγ via receptors such as gamma-chain receptor family, also known as IL-2Rγ whereas infectious stimuli activate these cells via antigen-specific receptors (287). NK cells conduct an immediate immunological response that is regulated by the expression of various activating and inhibiting receptors, following recognition of respective ligands on

virus-infected cells or tumor cells (241). Following activation, NK cells secrete cytokines and chemokines that reinforce the antigen presentation process by dendritic cells (241).
In the peripheral blood, invariant NKT (iNKT) cells are a subset of lymphocytes that develop in the thymus gland and undergo positive and negative selection (294, 288). iNKT cells produce several cytokines and display both proinflammatory and anti-inflammatory properties as well as immunoregulatory features and at times have a tendency toward autoreactivity (251, 288). iNKT cells express markers that are characteristic of the NK cell lineage, such as the activating NK cell receptor CD161, together with surface markers characteristic of T lymphocytes, including CD25, CD69 and CD122 (251, 288). Although iNKT cells express TCR, these receptors are semi-invariant and interact with a restricted group of lipid and glycolipid antigens presented in association with CD1d molecules on APCs, thus bearing resemblance to the innate immune system PRRs (251, 288). In line with the above, iNKT cells, like other innate immune cells, display immediate reactions to stimuli and do not possess immunological memory. Thus, iNKT cells are currently considered to serve as a bridge between the innate and adaptive immune systems (251, 289).

Studies addressing the number and function of NK cells in patients with kidney failure are few and of poor quality and have yielded controversial results. Early studies conducted in the 1980s showed that NK cell activity is lower in hemodialysis patients compared with healthy control individuals (288). Furthermore, NK cells in patients with kidney failure appear to display reduced responsiveness to Il-2, with guanidino compounds contained in uremic sera suppressing the NK cell activity (290). However, a study matching patients receiving hemodialysis and healthy control subjects for NK cell counts, found unaltered NK cell functions and expression of specific cell receptors, except for expression of the activation markers CD69 and NKp44 which were increased on NK cells from patients with kidney disease (291). Nevertheless, in vitro evidence of the suppressive effect of uremic serum on healthy donor NK cells, indicates that factors related to uremia might decrease NK cell cytotoxicity (290-296). Apart from the uremic toxins, chronic inflammation and oxidative stress may further affect the properties of NK cells in kidney failure. Thus, the upregulated inflammatory state could be responsible for NK cell zeta-chain downregulation and ensuing decreased NK cell activity in hemodialysis patients (293, 294). Another study showed decreased expression of the pivotal activating receptor, NKG2D, on NK cells in dialysis patients which might be caused directly by ROS or indirectly by ROS induced upregulation of the NKG2D ligand, MHC class I polypeptide-related sequence A (292). Accordingly, catalase significantly reversed the ability of serum from kidney failure patients to reduce NKG2D expression on NK cells whereas it had no effect on NK cells incubated with control serum (292, 293). Still, all studies indicate that in patients with advanced CKD, the absolute number of NK cells is markedly decreased by 20–32% and lower NK counts are directly associated with diminished creatinine clearance, thus suggesting that the uremic milieu is the main factor underlying the low NK cell counts in advanced CKD (291, 293). Contrary to the above, studies of patients with non-end-stage CKD have shown increasing NK cell percentage (293, 295). Even though, defective overall NK cell activity in patients with

kidney failure might at least in part be considered as one of the potential causes for increased susceptibility to infection and malignancy in these patients, yet there is no available evidence in support of this hypothesis.

Factors related to the hemodialysis procedure itself may also influence both the count and activity of NK cells. In patients exposed to non-biocompatible cuprophan membranes, the proportion of NK cells and activated NK cells as well as their cytolytic activity is lower than in patients treated with bio-compatible membranes (251, 297-299). On the other hand, switching patients from cuprophan to biocompatible membranes leads to recovery of NK cytotoxic activity to normal levels (299). Although the expression of receptors modulating NK cytotoxicity does not change in patients receiving hemodialysis, the activation markers CD69 and NKp44, CD94 and the chemokine receptor CXCR4 SDF1 display augmented expression whereas the chemokine CX3CR1-fractalkine receptor is suppressed in these patients (299, 300). Following kidney transplantation, NK cell lymphopenia below the 10th percentile appears to persist in nearly half of the patients (301). Additionally, KTRs show dose-dependent inhibition of NK cell function in the setting of immunosuppressive treatment (302). Low NK cells numbers have been associated with the development of squamous cell carcinomas whereas NK cells diminished cytotoxicity has been associated with the occurrence of infectious complications in KTRs (298, 301, 302). There are few data regarding iNKT cell counts in CKD, with some studies suggesting diminished numbers, particularly in kidney failure as are the expressions of CD56 and CD161 by iNKT cells in these patients. Kidney transplantation appears to restore iNKT cells counts to normal (303).

4.4 Alteration of the cellular components of the acquired immune system in CKD

4.4.1 T lymphocytes

Patients with advanced CKD and kidney failure display decreased total circulating T cells count together with significant alterations in T cell subsets constitution, including reduced CD4/CD8 ratio, increased Th1/ Th2 ratio and exhaustion of naïve and central memory CD4+ and CD8+ T cells (304-308). Furthermore, the remaining T cells appear to have an aberrant activation status (308). Notably, a nearly linear decline in the total number of T cells, affecting both the CD4+ and the CD8+ T cell subsets is observed with CKD progression from the early stages until kidney failure, mainly concerning naive T cells (241, 304, 307, 308). Reduced thymic output of naïve T cells, decreased circulating IL-7 levels and augmented apoptosis of both naïve and central memory CD4+ and CD8+ T cells are responsible for the reduction in naïve and memory T cells in these patients (304). Thus, compared with healthy individuals, thymic output of naive T cells as indicated by the amount of the genomic DNA remnants produced during TCR rearrangements or otherwise TCR excision circles is substantially decreased in kidney failure patients, regardless of age (309). Flow cytometry analyses data have revealed that in CKD patients and mainly in those undergoing RRT, a high percentage of T-lymphocytes express simultaneously the activation

marker CD69 and the apoptosis markers annexin and Fas (CD95). These T-lymphocytes are prone to apoptosis after stimulation with phytohemagglutinin or anti-CD3, with the apoptosis concerning mainly the naive and the central memory T-lymphocytes, but not the effector memory T-lymphocytes (307, 310). Apart from the dialysis procedure itself which further exacerbates, activation-induced apoptosis and reduces the proliferative capacity of T cells, other CKD related factors have been associated with the magnitude of the naïve and central memory CD4+ and CD8+ T cell depletion that include the severity of uremia, oxidative stress, increased phosphorus levels and secondary hyperparathyroidism, iron overload and inflammation (304, 307, 309).

Incubation of uremic serum added to PBMC and to CD4+ T-lymphocyte cultures from healthy individuals as well as from hemodialysis patients led to impaired MHC II expression on monocytes following stimulation, together with decreased TCR density on CD4+ T-lymphocytes by 40% (311). Available evidence indicates that the expression of the initial costimulatory ligand CD86 is decreased in dialysis patients, but not in patients with non-kidney failure, whereas the CD80 ligand does not differ between hemodialysis patients and healthy individuals (311, 312). In line with the above, the addition of monocytes from healthy individuals or anti-CD28 monoclonal antibodies in cultures of PBMC from hemodialysis patients restores T-lymphocyte proliferation (311, 312). These data suggest that a disturbance in binding of CD80 and CD86 ligands with CD28 might play a role in the impaired acquired immunity of kidney failure (310, 311). Furthermore, peripheral T cells isolated from kidney failure patients have been shown to markedly display markers of acute and chronic activation, including HLA-DR, CD57, and CD69 compared to similar-aged healthy individuals (304, 307, 309), whereas soluble markers of T-lymphocyte activation like sCD25 are as well elevated. A T-cell immunophenotyping study including CKD and kidney failure patients, showed increased frequency of exhausted $CD4^+$ T cells ($CD4^+KLRG1^+PD1^+CD57^-$) and $CD8^+$ T cells ($CD8^+KLRG1^+PD1^+CD57^-$), as well as anergic $CD4^+$ T cells ($CD4^+KLRG1^-PD1^+CD57^-$) and $CD8^+$ T cells ($CD8^+KLRG1^-PD1^+CD57^-$) in these patients, suggesting that in the setting of chronic stimulation, possibly by uremic toxins or other stimuli, T cells become dysfunctional (313). Although the total percentage of follicular helper T cell (T_{FH}) was similar amongst groups, kidney failure patients had reduced frequency of TFH1 ($CCR6^-CXCR3^+CXCR5^+PD1^+CD4^+CD8^-$) which have a putative protective role against viral infection, but increased frequency of the pro-inflammatory TFH2 cells ($CCR6^-CXCR3^-CXCR5^+PD1^+CD4^+CD8^-$) (313). Decreased IL-2 production from T-lymphocytes in hemodialysis patients leads to the suppression of differentiation pathways into Th1 and Th2 lymphocytes. Even though hemodialysis patients appear to display a Th1 predominant phenotype, there is contradictory evidence available with one study which analyzed cytokines in the supernatants of CD4 T-lymphocyte cultures from hemodialysis patients showing preferable differentiation of naive CD4+ T-lymphocytes to Th2 cells after stimulation (310). On the other hand, suppression of the Th2 lymphocyte differentiation pathway, which via IL-4 production stimulates the humoral arm of the acquired immune system, leads to impaired B-lymphocyte function and decreased antibody production (314).

Naive T cells from patients with kidney failure when compared to those from matched healthy controls, display higher proliferating rates, are more activated and show an increased susceptibility to activation-induced apoptosis as recognized by pronounced expression of the proapoptotic molecule CD95 (306, 307, 309). In addition, naive T cells have higher frequency expression of CD25, that is the IL-2 receptor, of the activation marker, CD69, as well as of the inflammatory chemokine receptors, CXCR3 and CCR5 thus suggesting an abnormal activation pattern (304, 306, 309).

In patients with kidney failure, the memory T cells are as well affected and evidence from several studies indicates the accumulation of CD4+CD28null cells within terminal effector memory T cell (TEMRA) subsets in these patients. Thus, there are observed increased counts of terminally differentiated CD4+ and CD8+ T cells that do not express the costimulatory molecule CD28 or that express the CD45RA, which is under normal conditions selectively expressed by the naive T cells (241, 315). In addition, the terminal differentiation sate of memory T cells is characterized by a marked decrease in T cell telomere length, suggesting increased numbers of previous cell divisions (241, 309). The terminally differentiated CD4+CD28− cells possess pronounced inflammatory features, such as increased expression of IFN-γ and TNF upon activation and secretion of pro-inflammatory cytokines even under basal conditions,they contain perforin and granzyme as well as they degranulate following stimulation, thus showing cytotoxic traits (309, 315).

A number of studies have shown that reduced numbers together with impaired proliferation of circulating T cells persist kidney transplantation (241). Available evidence indicates that thymic output does not improve in many KTRs however it remains to be further clarified whether these features are due to permanent changes in thymic function, epigenetic marks established in T cells themselves, alterations in bone marrow precursor cells, or due to immunosuppressive medications utilized during induction therapy as well subsequently with maintenance therapy (308). Accordingly, a prospective study showed that that low naive T cell counts, accumulation of memory T cells, and T cell function did not improve following transplantation, suggesting imprinting of uremia-induced T cell changes (316). Yet the confounding effects of immunosuppression were not taken into account. Another study found that approximately 5% of KTRs had reduces CD4+ T-cells with absolute counts < 300/mm^3 at 10 years following kidney transplantation which was associated with reduced thymic emigration (308, 317). Furthermore, dialysis vintage, but neither recipient age nor the immunosuppressive medications used for induction therapy, was the only significant risk factor for long-term lymphopenia in this cohort (317). Similar findings are confirmed in the pediatric population where CD28null CD4+ and CD8+ T -cells counts do not improve but remain increased compared to healthy individuals one year after transplantation, even in the absence of induction therapy (308, 316, 318). On the other hand, there is evidence indicating that accumulation of CD28null T lymphocytes results in immune senescence and thus carries a lower risk of rejection, as suggested by expanded CD28null CD4+ T cells subset in KTRs on CNI immunosuppression with long term graft survival (319). Likewise, patients with the highest tertile of pre-transplant CD8+CD28null

frequencies display the lowest rates of acute rejection at 1 year following transplantation in the setting of basiliximab induction and standard triple-therapy immunosuppression even after adjusting for the number of HLA mismatches (320). On the other hand, increased frequency of CD27−CD28− CD8+ TEMRA cells in stable KTRs was associated with a doubling of the risk for graft dysfunction during long term follow-up (321).

4.4.2 T regulatory cells

Regulatory T cells (Tregs) are derived either from the alternative differentiation of naïve T cells or as a distinct lineage maturing in the thymus (322). The first are otherwise known as adapted regulatory T cells whereas the second are named natural regulatory T cells, also known as CD4+CD25+ forkhead/winged helix transcription factor (Foxp3)(+) Treg cells (322). Naive CD4+ T lymphocytes differentiate into Th1, T Th2, TFH, Th17, and peripherally derived Tregs under the effects of different cytokines, co-stimulation, and antigen stimulation. Notably, the Th17 T-cells and Tregs exhibit distinct functions as the former are inflammatory while Tregs are suppressive, yet their differentiation pathways are related and both are capable of transdifferentiating into one another (308). Accordingly, the presence of TGF-beta alone leads to Treg polarization, while TGF-beta in the presence of IL-6 results in Th17 polarization. Foxp3 was identified as the key transcription factor that characterizes the lineage of thymically derived Tregs (322). Tregs, which were initially identified as CD4+CD25+ T lymphocytes based on experimental models of autoimmune disease, constitute 5% to 10% of peripheral CD4+ T lymphocytes in healthy humans and play an essential role in immune homeostasis and in the suppression of unwanted inflammatory responses to self-antigens, including prevention of autoimmune disease (322). Tregs suppress T cell proliferation and cytokine production by regulating target T cells and APCs (322). Thus, Tregs suppress the inflammatory cells via various mechanisms such as cell surface interactions using negative costimulatory molecules, cell-contact-independent mechanisms such as the production of inhibitory cytokines and deprivation of IL-2 and ATP or ADP, production of anti-inflammatory cytokines, that have strong immunosuppressive properties, including TGF-β and IL-10, competition for interleukin Il-2 or APC interactions and as a result protect against tissue injury (322). Available data support the suppressive role of Tregs on antibody production by B lymphocytes through inhibition of class switch recombination and induction of apoptosis by perforin and granzyme (322). Moreover, the interaction of programmed cell death protein 1 (PD-1) on autoreactive B cells with PD-1 ligand on Tregs prevents the activation and proliferation of self-reactive B cells in vivo (322).

Several experimental data suggest that depletion of Tregs exacerbates kidney disease in a variety of model systems. Additionally, $CD4^{+}Foxp3^{+}$ Tregs, are at the center of ongoing experimental research, for their role as potential therapeutic targets for several inflammatory and autoimmune diseases, including CKD and transplant rejection (322-324). CKD is associated with alterations in Tregs populations however additional research is needed to further clarify potential ambiguous points of currently available data, including patient and protocol heterogeneity. Thus, in some studies patients with kidney failure were

shown to have reduced CD4+CD25+ Tregs together with impaired regulatory function whereas other studies of dialysis patients showed an increased effector T cell to Tregs ratio (308, 325). Increased Tregs apoptosis rates have been demonstrated in patients with advanced CKD as well as in patients undergoing peritoneal dialysis and hemodialysis (326, 327). Furthermore, the observed depletion of Tregs is accompanied by their compromised ability to inhibit the proliferation of CD4+ T cells induced by phytohemagglutinin, thus suggesting the diminished anti-inflammatory capacity (326, 327). Accordingly, while the proliferation of the PBMC from kidney failure patients upon stimulation with alloantigen was affected, it appeared that there was also a defect in regulation by CD4+CD25bright+ T-cells which did not adequately perform their suppressive function in the direct pathway of allorecognition, especially in dialysis patients (326). The degree of Tregs cell depletion and dysfunction was most pronounced in hemodialysis patients, followed by peritoneal dialysis patients and finally non-dialysis CKD patients (326, 327). Notably, incubation of isolated Tregs from healthy individuals with uremic plasma or even oxidized LDL, led to a Tregs number decline together with an impaired suppressive capacity of these cells, thus highlighting the pathogenic implications of the uremic milieu as well as the interconnection between oxidative stress and lipid disorders with immunological abnormalities in CKD (240). In vitro data from CD4+ T cells isolated from the whole blood of hemodialysis patients which were submitted to independent T-cell mitogen stimulation, showed that the proportions of CD4+CD25+FOXP3+ Tregs and CD4+GATA3+ Th2 cells as well as Il-4 levels were significantly lower in dialysis patients compared to healthy controls, while the proportion of CD4+T-bet+ Th1 cells and Il-10 did not differ (328). Moreover, levels of IFN-γ levels were higher in supernatants from hemodialysis patients, thus indicating a suppressed anti-inflammatory Tregs, and Th2 cells profile together with an accentuated pro-inflammatory IFN-γ profile in hemodialysis patients (328). However, a relatively recent study evaluating isolated CD4+CD25+FOXP3+ T regs from both non-dialysis and dialysis dependent kidney failure patients showed similar frequencies of CD4+CD25+FOXP3+ Tregs present in the circulation of kidney failure patients either on dialysis or not (329). Additionally, the isolated CD4+CD25+FOXP3+ Tregs responded adequately to expansion via allogeneic mature monocyte-derived dendritic cells whereas the demethylation status of the Treg-specific demethylated region which is a hallmark of CD4+CD25+FOXP3+ Tregs as well as key to their suppressive function, was maintained in these patients (329).

Available evidence indicates that peritoneal dialysis may be more effective compared to hemodialysis regarding improvements in Tregs number. Accordingly, a longitudinal study of kidney failure patients initiating hemodialysis or peritoneal dialysis which evaluated the longitudinal changes in Treg before and one month following dialysis initiation showed that the proportion of total lymphocytes and CD4+ T lymphocytes did not change significantly after the start of dialysis in both groups whereas Tregs cells identified as $CD25^{+}FOXP3^{+}$, $FOXP3^{+}$, or $CD25^{+}CD127^{-}$, showed a significant increase following initiation of peritoneal dialysis but not hemodialysis (330).

A meta-analysis of five studies involving dialysis patients showed that the Tregs to CD4+ T cells ratio was lower in the non-dialysis kidney failure patients compared to healthy subjects whereas no significant differences were observed between non-dialysis kidney failure patients and dialysis patients (331). However, when all kidney failure patients were compared to healthy controls, the difference in the Tregs to CD4+ T cells ratio was lost, which most probably should be ascribed to the suppressive effect of hemodialysis on all CD4+ T-cells subsets, thus as a result maintaining the fraction Tregs to CD4+ T cells unaltered, despite reduced absolute Tregs values (331).

Hemodialysis patients exhibit an imbalance of Treg versus Th17 function when compared to healthy individuals, displaying increased peripheral Th17 cells frequency together with elevated Th17-related cytokines such as interleukin IL-17, IL-6 and IL-23 as well as increased expression of the DNA binding transcription factors RAR-related orphan receptor gamma (RORγ) RNA. Additionally, decreased Tregs frequency and Treg-related cytokines such as IL-10 and TGF-beta1 as well as reduced Foxp3 mRNA levels have been observed (332).

Considering that IL-2 targets the master gene FoxP3, thus controlling the function of CD4+CD25+FoxP3+ T-cell function, the relatively lower inducibility of IL-2 is in accordance with the already reported lower amount of IL-2 protein in the supernatant of stimulated PBMC from kidney failure patients as well as the high level of soluble IL-2 receptor which would bind any amount of circulating Il-2 (333). Upregulation of both IL-6 and TGF-β occurs in kidney failure, thus inducing effector Th17-cells which inhibit the function of CD4+CD25+FoxP3+ T-cells (334, 335).

Several experimental transplant models have demonstrated that Foxp3+Tregs play an essential part in the induction and maintenance of renal allograft tolerance. Accordingly, in specific MHC mismatched murine models, development of kidney transplant tolerance depends on Foxp3+ Tregs and specific deletion of Foxp3+Tregs in mice hemizygous for the depletion of regulatory T cells (DEREG), led to kidney allograft rejection in a DBA/2 (H-2d) to C57BL/6 (H-2b) model (324, 336). Small, single center studies using various of immune staining definitions to identify the Tregs populations have shown that low pre-transplant frequencies of CD4+CD25+CD127- Tregs subsets characterized as bearing TNF receptor 2 (TNFR2) have been associated with delayed graft function (337). A recent, multi-center study performed a longitudinal analysis of Tregs populations in 75 KTRs and found decreased absolute number and proportions of CD4+CD25+CD127-FOXP3+ Tregs and activated CD4+CD25+CD62L+CD45RO+ Tregs following transplantation (337). Patients suffering acute rejection within the first year of transplantation had higher proportions of activated Tregs prior to transplantation thus raising questions regarding the role of Tregs in preventing kidney transplant rejection and potentially in promoting transplant tolerance as well as regarding the significance of Tregs subset levels in the prediction of kidney transplantation outcomes (337). Several other observational studies point to a role for Tregs in kidney allograft survival, with high levels of $FOXP3^+$ Tregs in the peripheral blood and in specific IFN-γ producing, $CXCR3^+$ Tregs and HLA-$DRhigh^+$CD45RA Tregs, being associated

with graft function and outcomes (3338). A recent study assessed the impact of dialysis and maintenance immunosuppression on CD4+CD25+FOXP3+ Tregs expression and peripheral survival in patients undergoing RRT and in KTRs at regular intervals up to 20 years after kidney transplantation. Study results showed that CD127 expressing $CD4^{+}$CD25+FoxP3+ Tregs were detectable at increased frequencies in dialysis patients with no negative impact on the Tregs end product quality and therapeutic usefulness of the ex vivo expanded Tregs whereas immunosuppression or rejection episodes had only mild effects on Tregs maturation and did not prevent Tregs survival in vivo (339).

4.4.3 B lymphocytes

Similar to T cells, CKD progression is associated with a gradual decline in the number of circulating B lymphocytes, eventually leading to pronounced B-cell lymphopenia in patients with kidney failure, mainly affecting the naive and memory B cell subsets (241). Several studies have demonstrated significant B lymphopenia in patients with kidney failure with or without RRT (340-342). Additionally, children with stage 5 CKD exhibit reduced populations of CD5+ innate B cells and CD27+ memory B cells (342). Furthermore, the number of transitional B cells is significantly reduced in dialysis patients. Transitional $CD19^{+}CD24^{high}CD38^{high}$ B cells are the most immature subtype of B cells in the blood, which progressively decrease with aging (343-345). Additionally, transitional B cells might play a regulatory role as they primarily inhibit T cell responses by producing IL-10 (343-345). The proportion of transitional B cells starts to decrease since early in CKD and continues to gradually decrease as the disease progresses, with both dialysis patients and KTRs exhibiting lower levels of these cells compared to normal individuals (343, 346, 347). $CD5^{+}$ B cells primarily produce IgM-type antibodies and are tightly involved in maintenance of tissue homeostasis, protection from autoimmune diseases and infections, whereas recently their beneficial role in atherogenesis has been highlighted. $CD5^{+}$ B cells appear to be significantly reduced within the entire spectrum of CKD stages as well as in hemodialysis patients, both in terms of percentage and absolute count (343).

Apart from decreased output from bone marrow of B lymphocytes, diminished responsiveness to TNF ligand superfamily member 13B (BAFF) and increased activation-induced apoptosis of B lymphocytes are the other two main culprits responsible for the B-cell lymphopenia in patients with kidney failure. Accordingly, patients with kidney failure display elevated serum levels of the B cell growth and proliferation factors such as IL- 7, a cytokine that facilitates conversion of pre-B cells to B cells and BAFF, findings which indicate that it is not the shortage of these essential factors the reason underlying diffuse reduction of B lymphocytes in these patients (340). On the other hand, down-regulation of BAFF receptor expression has been observed on B cells from patients with kidney failure, which may at least partly contribute to B-cell lymphopenia because of augmented resistance to the effects of BAFF as a potent B lymphocytes differentiation and survival factor (340). It should be noted that the observed reduction of BAFF receptor expression concerns only transitional B lymphocytes, whereas BAFF receptor expression appears unchanged in

circulating mature CD19+CD10− B lymphocytes. Even though spontaneous apoptosis of B lymphocytes was not increased when measured directly ex vivo, it was significantly increased following cell culture which was associated with decreased expression of the anti-apoptotic molecule Bcl2 (341). Overall, the uremic environment may promote apoptosis and cause dysregulation of the maturating process of transitional B cells to mature B cells by promoting resistance to BAAF-mediated differentiation and survival signals (340). However, it is worth mentioning that the clinical studies examining of the effect of CKD on B cell subsets have mainly evaluated cells from peripheral blood samples. In order to fully assess the influence of CKD on B lymphocytes, additional studies are required so as to assess the tissue distribution of these cells and in specific, the bone marrow and lymphoid tissues, which are the major sites of maturation and functional development of B lymphocytes. Furthermore, the effects of uremia on B cell precursors in the bone marrow as well as on the downstream signal transduction pathways responsible for B lymphocytes growth, differentiation and survival remain to be further evaluated. Overall, uremia-induced naïve and memory B cell lymphopenia is associated with a defective humoral response to infections, vaccination and immune memory despite normal immunoglobulin levels including serum IgG isotypes, and both IgM and IgA production in dialysis patients (240).

5. The implication of immune system mechanisms in the pathogenesis of CVD in CKD

5.1 Inflammation as the common denominator of CVD phenotypes in CKD

5.1.1 The cytokines hypothesis and CVD in CKD

The perpetuation of chronic inflammation is a common denominator in the pathogenesis of CKD and CVD with genetic studies as well as large epidemiological studies linking inflammation with CKD progression and cardiovascular events (348). Thus, development of strategies targeting the complex inflammatory pathways, hold great promise for therapeutic advance in CKD and CVD (348). Pivotal experimental studies nearly four decades ago demonstrated beyond any doubt the fundamental role of inflammation as a critical component of the atherogenesis process (349, 350). Furthermore, large cohort studies both in the general population and in patients with CVD have established a strong association between various biomarkers of inflammation and cardiovascular outcomes with leukocytosis, CRP and IL-6 representing the best predictors of cardiovascular risk among the proinflammatory mediators (351,352). In line with the above, abundant evidence suggests that inflammation plays a significant part in the pathophysiology of HF (352, 353). Accordingly, the levels of inflammatory cytokines are increased in patients with LV dysfunction and furthermore they carry prognostic implications (398). Increased levels of IL-6 in HF are associated with a poor prognosis whereas TNF levels correlate with New York Heart Association (NYHA) class in chronic HF and are found to be elevated earlier during disease progression as compared with the natriuretic peptides which tend to be elevated only in severe disease (354).

Chronic inflammation is considered as one of the most significant non-traditional risk factors accounting for the increased risk of CVD in CKD and both inflammation and CKD predict cardiovascular events (355-357). However, the systemic nature of inflammation should be taken into consideration in this setting as well as its complex nature characterized by the interplay of several mediators, including cytokines, complement factors, and WBC subsets. Thus, Il-6, Il-1 or TNF-a are required for upregulation of CRP as an acute inflammatory phase reactant, whereas fibrinogen, another acute-phase reactant displaying a delayed but sustained increase, requires Il-6 for upregulation, whereas it is inhibited by Il-1 and TNF-a (358). Similarly, other markers of the acute phase responses, such as leukocytosis and hypoalbuminemia, increase early following inflammatory stimuli and their respective changes are maintained in chronic inflammatory states (358). Among the multiple potential pro-inflammatory stimuli identified in CKD, ischemia, infections, the uremic environment and accentuated oxidative stress are the most notorious (159). Several analyses of large cohort studies examining inflammation and subsequent kidney disease outcomes have demonstrated an association between inflammatory markers and prognosis in CKD populations thus suggesting that inflammation may be the unifying mechanism between disease and accelerated CVD in this setting (359-362).

In order to explore the influence of CKD, inflammation, as well as the synergy between CKD and inflammation on cardiovascular events, markers of inflammation including fibrinogen, albumin and WBCs were evaluated in 20413 individuals including 1649 with non-end stage CKD, from the two well-known cohorts of the Cardiovascular Health Study and the ARIC Study (356, 357). Inflammation, defined by the presence of at least two of three criteria including highest quartile of fibrinogen, the lowest quartile of albumin, and the highest quartile of race-specific WBC was identified in 3594 patients. Even though both inflammation and CKD were associated with increased risk of a composite of cardiac events, stroke and mortality as well as components of this composite, their interaction was non-significant, thus further establishing the association between markers of inflammation with mortality and cardiovascular outcomes in a community-based population regardless of the presence or absence of reduced kidney function. Inflammation was associated with increased hazards for stroke, cardiac events, or death after adjustment for conventional risk factors that ranged between 35% and 50% whereas in patients with CKD, the hazard ratio (HR) was similarly elevated between 15% and 25% (356, 357). Overall, when compared with patients who did not display either inflammation or CKD, those with CKD alone had an increase in hazards of hard cardiovascular end points by 20%–60%, those with inflammation alone had an increase in risk by 40%–60%, and those with both inflammation and CKD had an increase in risk by 40%–100% (57, 356, 357). In addition, regardless of kidney function, a composite of reduced serum albumin, increased fibrinogen, and increased WBC count, and a composite of only albumin and WBC count, both predicted adverse events in an elderly population to a degree similar as CRP (357). These findings are in accordance with previous evidence from the Cardiovascular Health Study cohort which compared traditional and novel risk factors as predictors of cardiovascular mortality in a total of 5808 persons,

including 1249 patients with CKD, aged 65 years or older living in 4 communities in the United States. During an average follow-up of 8.6 years, CKD patients displayed an almost double cardiovascular mortality risk rate compared with those without it. Apart from the classical risk factors such as, diabetes, systolic hypertension, smoking, low physical activity, nonuse of alcohol and LVH, only CRP and Il-6 among the novel risk factors, were associated with the outcome as linear predictors (359). Additionally, in the same cohort, older adults with elevated levels of CRP, factor VII, fibrinogen, WBC count and hemoglobin or low levels of albumin showed a greater risk of CKD progression as assessed by a yearly eGFR change of more than 3 mL/min/1.73 m^2 (364).

On the other hand, a prospective, nested case-control analysis from 244 women without history of CVD, who were participants in the Nurses' Health Study, demonstrated a direct correlation of inflammatory markers, including CRP, IL-6, and soluble TNFRI and TNFRII, with greater coronary artery disease risk among participants with eGFR lower than 75 ml/min/1.73 m^2 but not in those with a GFR higher than 75 ml/min/1.73 m^2 (363). Thus, higher inflammatory biomarkers levels were significantly associated with coronary events only in women with reduced kidney function. Potential contradictory points in the results generated by each of these studies mainly rely in their design, cohort characteristics as well as definitions of cardiovascular outcomes (357, 359, 363).

Data from the CRIC Study, which included participants with CKD but without a history of CVD at study entry, have provided fundamental insights into the risk factors associated with progression and outcomes of CKD and CVD in these patients (365). Accordingly, a baseline panel comprised of inflammatory biomarkers including IL-1β, IL-1RA, IL-6, TNF-α, TGFβ, high-sensitivity (hs) CRP, fibrinogen, and serum albumin, were independently associated with incident MI, peripheral arterial disease, stroke and death in this patient cohort (365). A composite inflammation score incorporating Il-6, TNF-a, fibrinogen and serum albumin was associated with a graded augmentation in the risk for the composite outcome of atherosclerotic cardiovascular events and death (365). Traditional cardiovascular risk estimates such as the Pooled Cohort Equation probability, a gender- and race-specific tool for estimating 10-year absolute rates of atherosclerotic cardiovascular disease events in a primary prevention population, could be further improved by adding markers of inflammation and indices of kidney function (365).

IL-6 has complex atherogenic properties, including effects on the endothelium, on platelets, as well as on coagulation factors and its mRNA is abundantly expressed in atheromatous arteries and is found together with macrophages in vulnerable rupturing plaque areas (366). High serum IL-6 is associated with history of CVD and independently predicts overall and cardiovascular mortality in patients across all eGFR stages (367). Moreover, it is a significantly better predictor of mortality than CRP, albumin or TNF-a (367). Accordingly, the value of IL-6 as a robust and independent predictor of the progression of carotid atherosclerosis and mortality in patients with non-end-stage kidney disease before the initiation of dialysis treatment has been demonstrated nearly 3 decades ago (368). Studies in several patient groups with CVD, such as those with carotid atherosclerosis,

abdominal aortic aneurysm or undergoing coronary artery bypass grafting (CABG) have identified potential links between genetic polymorphisms of Il-6 with CVD (369). Similarly, meta-analytic data from two genetic consortia exploring the effect of a polymorphism in the IL-6 receptor on the risk of CAD showed the allele that attenuated IL-6 signaling was significantly associated with reduced risk of CAD (370). Strikingly, a parallel relationship of the −174 G/C functional polymorphism in the promoter of IL-6 gene with history of CVD and incident CV events has been discovered which suggests that IL-6 may play a direct causal role in the pathogenesis of CVD complications in this specific patient population (371). Accordingly, in a multicentric cohort of 755 patients with CKD stages G2–G5 from southern Italy, patients homozygous for the risk allele (C) of the −174 G/C polymorphism had higher levels of IL-6 than did those with other genotypes. Additionally, homozygous CC patients had double the risk of displaying a CVD history as well as an 87% higher rate of incident cardiovascular events compared with the other genotypes (371).

Regarding dialysis patients, a great body of experimental and clinical evidence suggests that inflammation is a risk major factor for increased mortality as well as exerts detrimental effects in the cardiovascular system, with cytokines and other inflammatory proteins being not only mediators of cardiovascular damage in experimental models but predictors of cardiovascular complications as well (372-375).

A study assessing the predictive value of CRP, of the main proinflammatory cytokines and of two adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) **i**n dialysis patients, showed that IL-6 adds significantly greater predictive power for all-cause and CV death to statistical models based on traditional and nontraditional risk factors in these patient population with the gain in the prediction power attributable to IL-6 being approximately two times higher compared to CRP (372). On the other hand, the gain in prediction power associated with TNF-alpha, IL1-beta, IL-18, ICAM-1, and VCAM-1 was marginal. It should be noted that, the cardiovascular mortality risk estimates of patients with high serum IL-6 were not significantly different from that of patients with increased serum CRP, thus indicating that the later might be a more readily available alternative for clinical practice utilization compared to IL-6 (372). A study of 280 stable hemodialysis patients revealed that nearly half the patients displayed an activated acute phase response, characterized by an increase of CRP and serum amyloid A (SAA) with CRP remaining a powerful independent predictor of both overall mortality and cardiovascular death together with age, during a prospective follow-up of two years (374). Moreover, the acute phase reactants were positively associated with the atherogenic plasma proteins Lp(a) as well as fibrinogen and inversely correlated with antiatherogenic HDL cholesterol and apo A-1 (374).

Evidence from experimental studies indicates that cytokines regulate cardiac remodeling pathways and contractile function (376, 377). In vitro data from a model of adult ventricular cardiomyocytes cultured with cardiac fibroblasts showed that addition of antagonist antibodies against IL-6 or against its transducer gp130 induced a significant decrease in expression of atrial natriuretic peptide (ANP) and beta-myosin heavy chain in

cardiomyocytes as well as diminished fibroblast proliferation (376). Furthermore, angiotensin II secreted by cardiac fibroblasts induces IL-6 secretion by cardiomyocytes whereas the hypertrophic and proliferative effects of IL-6 on cardiomyocytes and fibroblasts were inhibited by addition of the angiotensin-1 receptor antagonist, losartan (376). Similarly, IL-6 and leukemia inhibitory factor mediated the genesis of angiotensin II-dependent LVH in two hypertensive rat models. Overall, cytokines influence cardiac remodeling via various mechanisms including stimulation of the synthesis of protein found in sarcomeres, induction of fetal gene expression, alteration of extracellular matrix degradation as well as promotion of apoptosis (377, 378).

The association between inflammatory biomarkers and cardiac geometry were evaluated in 3,939 CRIC study participants which demonstrated independent and consistent associations of elevated hs-CRP and IL-6 with LVH and systolic dysfunction as well as an inverse correlation of serum albumin with LVMI and eccentric hypertrophy. Overall, among the pro-inflammatory biomarkers studied, circulating IL-6 was associated with the presence of both concentric and eccentric hypertrophy, thus best reflecting the inflammatory status as well as the relation with adverse cardiac remodeling in CKD patients (379).

The overexpression of proinflammatory cytokines in patients with CRS has been well characterized thus highlighting the role of immune-mediated inflammatory markers in the heart and kidney pathophysiological crosstalk (380-383). Accordingly, 5 times higher plasma levels of proinflammatory cytokines such as IL-6 and IL-18 have been detected in CRS type 1 patients compared to patients with acute HF (382). Additionally, a positive correlation between myeloperoxidase (MPO), an enzyme stored in azurophilic granules of neutrophils and macrophages, involved in inflammatory and oxidative processes and neutrophil gelatinase associated lipocalin (NGAL), an AKI marker particularly for proximal tubular cell damage, with inflammation markers has been described in these patients (382). Studies conducted on CRS have shown that IL-6 and IL-1β hinder the expression of suppressor of cytokine signaling-3 (SOCS3), a protective factor against cardiomyocyte hypertrophy and apoptosis and increase neutrophil recruitment (384, 385). Additionally, IL-6 is an upstream signal for myocardial growth factor receptor-bound protein 2 (Grb2), a major factor involved in myocardial diastolic dysfunction, which acts through inhibition of the Akt/mTOR signaling pathway thus causing disruption of the mitochondrial metabolism (381, 386). IL-6 stimulates epithelial sodium channels in the distal renal tubules, impairing natriuresis and as a result leading to volume expansion and aggravation of congestion in CRS (387).

During the recent years novel agents including monoclonal antibodies that target inflammatory cytokines aiming to inhibit the inflammatory axis which mediates the heart-kidney interaction, have been evaluated by numerous studies (388-393). Valuable clinical data for the potential cardiovascular benefit, including atherosclerotic cardiovascular events as well as HF hospitalizations, of the treatment of inflammation in patients with CKD with a history of MI and system inflammation were derived with canakinumab, a monoclonal antibody targeting IL-1β (392). Finally, cardiovascular safety and efficacy of ziltivekimab, , was tested in a phase 2 RCT published in 2021, the RESCUE trial. In a total of 66 subjects

with moderate to severe CKD and residual inflammatory risk included in the RESCUE trial, treatment with ziltivekimab a fully human monoclonal antibody directed against the IL-6 ligand, every four weeks, up to 24 weeks, compared to placebo, resulted in a significant decrease in hsCRP levels in a dose-dependent manner, which remained stable over THE treatment period without any major safety issues (393).

5.1.2 The role of the inflammasome and Toll-like receptors

The nucleotide-binding domain family pyrin domain containing 3 (NLRP3) inflammasome is the best characterized among the inflammasome complexes (394). Thus, NLRP3 is an intracellular signaling molecule that acts as a danger signal sensor with tissue injury, metabolic stress, and infection being the main triggers for its activation (394). Following activation, NLRP3 proteins oligomerize and subsequently recruit and couple to an adaptor protein, apoptosis associated speck-like protein containing a caspase- activation and-recruitment domain (ASC), interact with caspase-1 and in the end cleave precursors of Il-1β and Il-18 and convert them into their active forms of secreted cytokines (394). NLRP3 is overexpressed in atherosclerotic plaques, particularly in unstable plaques (429). Experimental studies in murine models have shown contradictory results with some indicating overexpression of NLRP3 in atherosclerotic plaques with deletion of NLRP3 leading to attenuation of inflammation and atherosclerosis, while no such effect have been confirmed by other studies applying the same intervention (394).

Recent data suggest that inflammasome-related cytokines IL-1β and IL-18 may modulate the cardiac remodeling process after injury and impair myocardial function (395, 396). Results from experimental models of cardiac ischemia have revealed that ischemia induced mitochondrial damage together with the ensuing IRI induced increase in ROS, lead to upregulation of NLRP3, caspase-1 activity, IL-1β, and IL-18, all resulting in cell membrane damage, edema and cell death (397). On the other hand, experiments involving abolishment or inhibition of NLRP3 signaling indicate diminished myocardial infiltration by inflammatory cells, as well as attenuation of fibrosis and LV dysfunction in this setting (397). Available evidence implicates the NLRP3 inflammasome pathway in the pathogenesis of HF with prominent expression of the inflammasomes in WBCs, fibroblasts, endothelial cells as well as cardiomyocyte together with increased IL-1β and IL-18 levels in the plasma of these patients (398, 399).

Kidney injury often triggers an intense inflammatory response with upregulation of cardiac NLRP3 inflammasome components that induces cardiac dysfunction and pathological remodeling. Thus, in a remnant kidney model, NLRP3 was significantly upregulated at the transcript and protein level in the hearts of animals submitted to $5/6^{th}$ nephrectomy together with a significant increase of circulating IL-1β levels, as compared to sham-operated control animals. Additionally, pathological remodeling with cardiac fibrosis, myocyte hypertrophy and wall thickening, was observed in $5/6^{th}$ nephrectomized animals (400). NLRP3 inflammasome activity also increases in atrial cardiomyocytes of patients with both paroxysmal and chronic atrial fibrillation (394). Taking into consideration the link

between CKD and increased risk of atrial fibrillation, NLRP3 activity was enhanced in THE atria of patients with CKD and IL-1β levels were elevated in the circulation of patients with CKD who had AF compared to patients in sinus rhythm. Moreover, NLRP3 activity was enhanced in atria of patients with CKD (400). In order to investigate the role of NLRP3 and IL-1β signaling in the pathogenesis of CKD-induced atrial fibrillation, NLRP33-knockout and wild type mice with subtotal nephrectomy were compared with regard to susceptibility to pacing-induced atrial fibrillation as well to atrial fibrillation remodeling (401). NLRP3 knock-out mice displayed significantly reduced incidence of atrial fibrillation, lower IL-1β levels, normalized left atrial dimensions, as well as less fibrosis compared to wild type mice with CKD, thus suggesting that CKD promotes atrial fibrillation development at least in part by activation of the NLRP3 inflammasome in atria, leading to structural and electrical remodeling (401).

Similar to NPLRP3-inflammasome complex, the activation of TLRs by PAMPs and DAMPs induces the expression of several genes encoding proinflammatory cytokines and initiates a series of processes eventually promoting atherosclerosis, MI, and HF (394). TLR2 and TLR4 are undoubtably the most abundantly expressed TLRs in atheromatous plaques, with their knockdown in atherosclerosis-prone, apolipoprotein E deficient mice, leading to attenuation of the severity of the atherosclerotic lesion, thus highlighting their substantial role in the inflammatory response of the vascular wall (403, 404). Myocardial ischemia and infarction cause release of endogenous DAMPs, such as heat shock proteins, high-mobility group box 1 protein, and DNA from ischemic myocardial cells, which signal and activate TLR receptors (394). Accordingly, experimental models have shown that increased heat shock protein-60 in mice cardiomyocytes in the setting of ischemic HF activates TLR4, leading to TNF-α-mediated apoptosis and myocardial injury (405). Furthermore, in the same model, myocardial necrosis after hypoxia and reoxygenation is mediated at least partly by TLR2 and TLR4 through rapid activation of protein kinase C alpha (PKCα), followed by increased expression of NOX2, a process which was inhibited by antibodies against TLR2 and TLR4 (405). Heart failure models in rats have revealed that TLR2 is upregulated in cardiomyocytes, and vascular endothelial cells whereas TLR2 double knockout mice do not exhibit progression of HF (406). Mice deficient in toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP), an adaptor molecule that is essential for TLR2 signaling, display significantly greater recovery of postischemic myocardial contractile performance (406). Furthermore, antibodies against TLR2 prevent angiotensin II-induced myocardial fibrosis through inhibition of macrophage infiltration in the heart (407).

Several endogenous ligands for TLRs have been identified in CKD which together with increased expression of TLRs on leukocytes contribute to the development of the chronic inflammatory state as well as the development of arterial hypertension, the promotion of vascular calcifications and atherogenesis (394).

In the CKD population, HDL is transformed from its physiological form to an abnormal lipoprotein, via symmetric dimethylarginine (SDMA), with the modified HDL particle mimicking a DAMP and subsequently activating endothelial TLR-2 via a TLR-1- or

TLR-6-coreceptor-independent pathway. As a result, the modified LDL promotes endothelial proinflammatory activation through NF-κB-dependent cytokine release, induces endothelial superoxide production and impairs endothelial repair through substantially reduced NO bioavailability, and by that increases arterial blood pressure.

TLRs are involved in the pathogenesis of cardiac hypertrophy in the setting of pressure overload (410-413). TLR2 stimulation directly promotes cardiomyocyte hypertrophy in vitro via NF-κB pathway activation and TLR2 knockout mice failed to respond to elevated heat shock protein 70 after transverse aortic constriction (TAC), thus exhibiting impaired adaptive cardiac hypertrophy (410, 411). On the other hand, stimulation of TLR9 affects the NF-κB pathway and upregulates IL-10 secretion, leading to attenuation of cardiac hypertrophy and cardiac systolic dysfunction following TAC (410, 411). Similarly, blunted NF-κB pathway activity is observed in TLR4 deficient mice, which are protected from the development of cardiac hypertrophy and from cardiomyocyte loss after aortic ligation (412, 413).

5.2 The implication of cellular components of the innate immune system in the pathogenesis of CVD in CKD

The participation of immune and inflammatory mechanisms in both acute and chronic cardiovascular conditions has recently come to the spotlight with the significance of cardiovascular immunology being currently in a similar position to that of lipidology during the nineties (414, 415). Thus, it is well established that both innate immune pathways and adaptive immune effectors including various subtypes of mononuclear phagocytes as well as of T and B lymphocytes play a significant part in atherosclerosis and its complications. Similarly, cardiac remodeling and fibrosis, a shared outcome by many myocardial diseases, involve innate and adaptive immunity immune cells which critically regulate the clearance of injured cells and repair responses in the setting of injury (414, 415). Attention to the involvement of cellular innate and acquired immune mechanisms in the accelerated phenotypes of CVD in CKD has generally lagged and understanding of these complex mechanisms can aid to further develop novel diagnostic and therapeutic strategies in this field (251, 414, 415).

5.2.1 Monocytes

Increased monocyte count has been associated with a higher risk of cardiovascular events and mortality in individuals without CKD with the monocyte to lymphocyte ratio (MLR) emerging as a novel inflammatory parameter that is associated with cardiovascular risk in the non-CKD populations (416-418). Taking into consideration the link between elevated monocyte numbers and increased circulating levels of proinflammatory mediators, monocyte count might represent a marker of inflammation that is involved with the increased risk of CVD (419). On the other hand, bearing in mind that participation of monocytes in the inflammatory reaction process and the regulatory role of lymphocytes in

immune pathways, an increased MLR may indicate accentuated inflammation together with an impaired immune response (420). Notably, compared individuals without CKD, the CKD population have higher levels of MLR. There is a limited number of studies with small samples sizes which have examined the potential association of monocyte counts and MLR with adverse cardiovascular outcomes in the CKD population. Data from prospective studies including small dialysis as well as non-dialysis dependent patient cohorts suggest that MLR can serve as an easy and effective clinical indicator for predicting mortality risk in CKD patients (421-424). Accordingly, in patients undergoing chronic hemodialysis the highest tertile of monocyte count compared with the lowest tertile was associated with double the risk of CVD death during a follow-up of 40 months, whereas a higher MLR was associated with a nearly seven-fold increase in cardiovascular mortality during a median follow-up of 51 months (421, 422). Similarly, the highest tertile of MLR compared with the lowest tertile was associated with a 45% higher risk of CVD death in patients undergoing peritoneal dialysis during a median follow-up of 31 months (423). Analysis of data from the National Health and Nutrition Examination Survey 2003-2010, indicate that in the non-end stage CKD population, MLR is positively correlated with the risk of death and displays a higher predictive efficacy for mortality risk than other clinical indicators such as the CRP (424). In line with the above, data from the CRIC study show a graded association of the monocyte count and MLR with a higher risk of CVD, cardiovascular death, and all-cause death in non-dialysis CKD patients (425).

5.2.1.1 Monocyte subpopulations and atherosclerosis— a clinical perspective

Monocytes are the predominant, key cells in atherogenesis and the sequence of events has been well characterized from the adhesion of circulating monocytes to the endothelium, subsequent transmigration into the subendothelial space to their differentiation into scavenger cells that internalize oxidized lipoprotein, foam cell formation and the development of fatty streaks (426, 427). Monocytes within atherosclerotic plaques produce cytokines and growth factors, which further induce the recruitment and activation of other inflammatory cells and plaque growth whereas plaque fibrous cap weaking by macrophage-derived matrix metalloproteinases (MMP) leads to plaque rupture and ACS (251, 414, 426). On the other hand, there are few data regarding the regulation and accumulation of monocytes subsets or regarding their roles in atherosclerosis. Monocyte subsets are customarily characterized by differential expression of specific cell surface markers including chemokine receptors that regulate their function such as chemokine C-C motif receptor 2 (CCR2), CX3C chemokine receptor 1 (CX3CR1) and C-C chemokine receptor type 5(CCR5), as well as adhesion molecules and integrin receptors (426). Thus, classical CD14++CD16- monocytes display high CCR2 and low CX3CR1 expression and in the other end of the spectrum non-classical CD14+CD16++ monocytes do not express CCR2 whereas they highly express CX3CR1 (427) (Figure 3). Accordingly, the high affinity for the endothelium due to surface expression of chemokine receptors and adhesion molecules relevant to atherosclerosis plaque progression such as CX3CR1 and CCR5, their propensity

for prompt deployment from their marginating pool following stimulation as well as their capacity to recruit T-lymphocytes and additional monocytes, support the potential implication of CD16+ monocytes in atherosclerosis and CVD in general (426, 427). Experimental studies which have provided invaluable evidence regarding the roles of monocytes subsets in various CVD phenotypes, have utilized mouse models with a correspondent classification of their monocytes based on lymphocyte antigen 6 family member C (Ly6C) and CD43 expression with Ly6C++CD43+ considered equivalent to CD14++CD16- and Ly6C+CD43++ considered equivalent to CD14+CD16++ (427). Relatively recent data indicate that in fact human CD14++CD16– and CD14++CD16+ monocytes are clustered with murine $Ly6C^{high}$ monocytes, whereas only CD14+CD16++ monocytes correspond to $Ly6C^{low}$ monocytes. Atherosclerosis is inhibited by a selective absence of the monocyte chemokine receptor CCR2, CX3CL1 or its receptor CX3CR1 in atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mice (167). Similarly, mutations that result in decreased function of CX3CR1 and monocyte adhesion in humans are protective against CVD (167). Recent studies have shown that in $ApoE^{-/-}$ mice fed a high-fat diet, monocyte counts in the circulation were elevated and skewed toward the pro- inflammatory monocytes subtype which further required CX3CR1 in addition to CCR2 and CCR5 to accumulate within plaques (167).

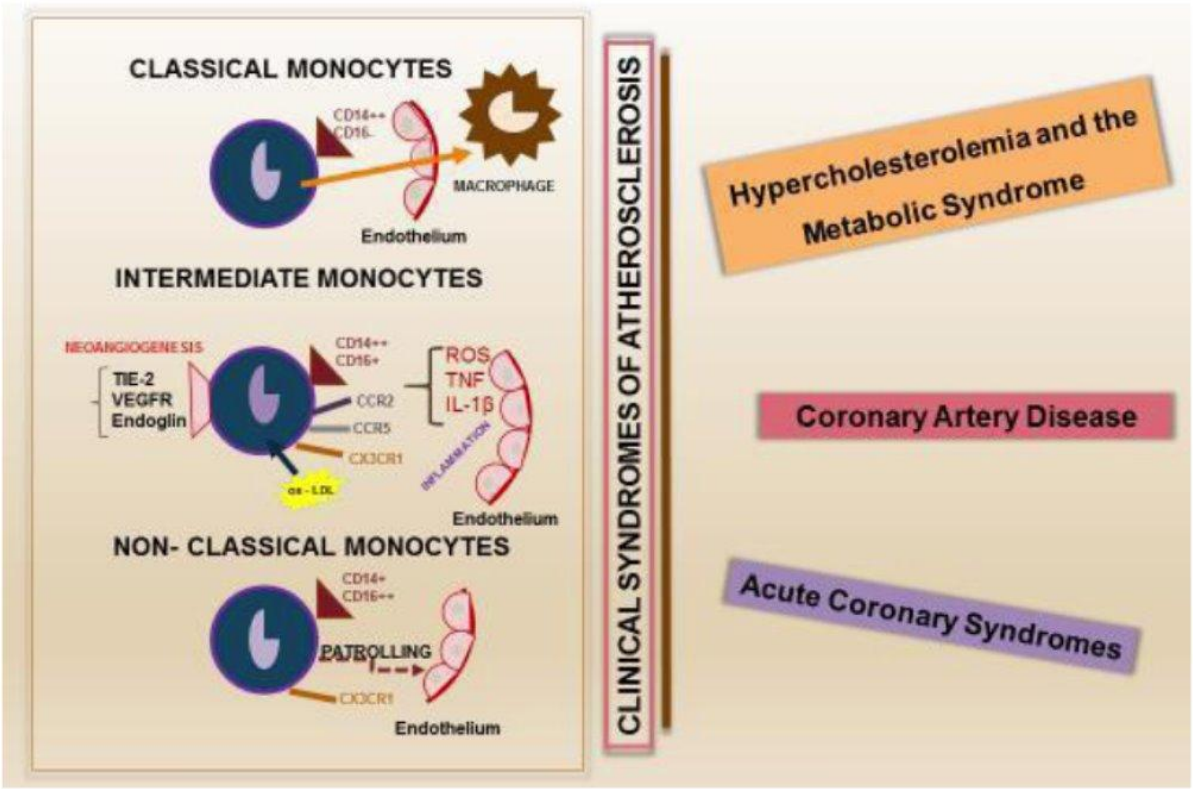

Figure 3. Monocyte subpopulations and atherosclerosis. CCR, CC chemokine receptors; CD, Cluster of Differentiation; CX31,CX3C chemokine receptor 1; IL-1β, Interleukin 1-β; ROS, reactive oxygen species; TNF, tumor necrosis factor; VEGFR, Vascular Endothelial Growth Factor Receptor (251).

Increased expression of the β2 integrin subunit (CD18) and CD11b in classical monocytes has been described in hemodialysis patients. As the integrin subsets expressed by monocytes mediate interactions with cell adhesion molecules on the endothelial cell surface, such as intercellular adhesion molecule (ICAM) 1-3, they may be involved in the formation of atherosclerotic plaques (428-430). In patients with CKD, CD14+CD16+ monocytes express a proatherogenic profile of chemokines and adhesion molecules, such as CX3CR1 and ICAM-1 compared to classical CD14++CD16- monocytes (277, 431). Expression of the proatherogenic chemokine receptors CCR2 and CCR5, and the fractalkine receptor (CX3CR1), is elevated in proinflammatory CD14++CD16+ monocytes (432). Patients receiving hemodialysis and carrying a deletion variant of the CCR5 gene (CCR5Δ32) causing deficiency of the respective receptor, show improved cardiovascular outcomes, thus supporting a clinically relevant role of CCR5 in modulating inflammation and atherosclerosis (433). Increased levels of CX3CR1 on CD14++CD16- monocytes and decreased levels on CD16+ monocytes have been found before hemodialysis, whereas a notable depletion in CX3CR1 expression occurs in all monocyte subsets after dialysis treatment, a phenomenon interpreted to be a consequence of the adherence of monocytes overexpressing this chemokine receptor to the activated endothelium during hemodialysis (434). The percentage of CCR2 positive CD14+ monocytes in patients with HD is greater than that in healthy controls (434, 435) and pilot transcriptomic data suggest that genes encoding CD16 and CX3CR1 in peripheral blood monocytes may be upregulated regardless of CVD (435).

The link between the pro-inflammatory monocytes and endothelial dysfunction in CKD is reinforced by the positive association of increased CD16+ monocytes count and the presence of apoptotic endothelial microparticle in CKD patients as well as by in vitro findings indicating that CD16+ monocytes collected from patients with CKD adhere more strongly to a human umbilical vein endothelial cell monolayer compared to classical monocytes (277, 278). Furthermore, experimental studies of CD14++CD16+ monocytes collected from CKD donors exhibit preferential lipid accumulation such as marked oxidized LDL uptake capacity together with high expression levels of CD36, a scavenger receptor with high affinity for tissue uptake of long chain fatty acids and CD68 a scavenger receptor that modulates platelet mediated oxidized LDL plaque deposition as well as low cholesterol efflux due to diminished expression of the cholesterol transporter ATP-binding cassette A1 (ABCA1) (431, 436). Conversely, low levels of cholesterol efflux mediators such as Apo-1 and HDL cholesterol are associated with increased CD14++CD16+ monocyte counts in patients with CKD (436). TLR-4 induces the expression of several genes encoding proinflammatory cytokines that are actively involved in myocardial inflammation, ischemia-reperfusion injury, MI and HF (437) however in the sole study testing the relationship between TLR4 expressions on monocytes with the incidence of cardiovascular events in kidney failure no such relationship was found (438).

The ACE expression on monocyte-derived cells is a hallmark of their pro-atherogenic phenotype and has been detected within advanced atheromatous plaques (439). Marked

expression of ACE or CD143 on monocytes, macrophages and foam cells within the intimal vascular wall induces the activation of the local RAS, thereby resulting in the progression of atherosclerotic plaques (439). In vitro preconditioning of healthy monocytes with uremic serum results in the upregulation of ACE expression and angiotensin II and angiotensin 1-7 receptor, thus stimulating endothelial adhesion and transmigration (440). Intermediate CD14++16+ monocytes from patients with established CVD undergoing dialysis show increased ACE expression (441), and high ACE expression in these monocytes is associated with atherosclerotic cardiovascular disease and mortality (440-443).
CD40, a type 1 transmembrane proteine found-on APCs and required for their activation is considered to play a prominent role in the development and progression of atherosclerosis (431, 444). Increased counts of classical and intermediate monocytes highly expressing CD40+ are observed in CKD patients with CVD (445). Furthermore, the proportion of CD40+ intermediate monocytes in these patients displays higher levels of the inflammatory markers CD86, HLA-DR, CD11b, CD49d, CCR2, CCR5 and Cx3CR1, compared to CD40−/CD14+ monocytes (445).

Despite the unequivocal evidence of the direct involvement of monocytes in the pathogenesis of atherosclerosis, initial epidemiological studies did not find a significant association between differential counts of WBCs, including monocytes and CAD (251, 446). However, subsequent flow cytometric studies have shown that the pro-inflammatory CD16+ monocytes subsets are elevated in dyslipidemias such as ApoE gene variants, conferring susceptibility to atherosclerosis and in obesity whereas they show an inverse association with HDL cholesterol levels (251, 447). To further reinforce these observations, cross sectional studies have shown that in patients treated with statins, CD16+ monocyte counts are lower compared to patients not receiving these drugs (251, 448, 449).

Patients with stable CHD appear to have diminished expression of CD14 together with elevated expression of IL-6 receptors on both classical and nonclassical monocytes as well as altered responses to stimulation with endotoxin, thus suggesting altered immune cell regulating mechanisms (450). However, classical monocytes CD14++CD16− appear to be the predominant subtype in patients with stable angina (251, 451). On the other hand, experimental MI in mice has revealed various time patterns in the activation and mobilization of monocyte subsets in the myocardium, a process mediated by differential cytokine expression (452). Accordingly, Ly6C high monocytes, the analogues of classical monocytes in humans, are the first monocytes to perform phagocytic, proteolytic and proinflammatory functions via CCR2, whereas LyC low monocytes, which correspond to CD16+ monocytes in humans, are recruited at a later stage via CX3CR1 and mediate the healing phase through collagen production and angiogenesis (431). Peak levels of classical CD14+CD16- monocytes have been inversely associated with the extent of both myocardial salvage and recovery of LV function after MI while the development of coronary collaterals is associated with increased CD14++CD16- levels (453, 454). Thus, in patients with ST-elevation MI, post-reperfusion increases in CD14+CD16- monocyte levels are predictive of microvascular obstruction, an impaired LVEF and larger infarct size (455, 456). Direct renin

inhibitors combined with ACEIs or ARBs have been shown to improve the extent of myocardial salvage after MI, an effect associated with a decrease in circulating CD14+CD16- monocytes (457). Taking into consideration that angiotensin II stimulates migration of the mouse equivalent to human CD14+CD16− monocytes from the spleen into the circulation, the therapeutic implications of these studies remain to be tested in clinical trials (458).

The count and activity of intermediate CD14++CD16+ monocytes, in terms of NF-κB expression, increase immediately after acute coronary syndromes (ACS) with peak counts correlating with cardiac enzymes and plasma cytokine levels as well as LV function (451). The cardiovascular arm of the Malmo Diet and Cancer study showed that classical monocytes (CD14++CD16−) were directly associated with the incidence of cardiovascular events independently of other risk factors in a randomly selected population of 700 subjects followed up for 14 years (459). It should be noted that in this study flow cytometric analysis was performed on thawed cells that were stored frozen at −140°C for up to 15 years, thus potential alterations in cells over this time period could not be ruled out. However, at baseline, the proportion of monocytes expressing CD16, but not classical monocytes, was associated with the extent of carotid atherosclerosis, measured as IMT (459). In contrast, another recent large cohort study evaluating the association of human monocyte subsets with cardiovascular outcomes in a broad patient population, showed that intermediate monocytes CD14++CD16+ were the sole subset independently associated with cardiovascular events during a mean follow-up period of approximately 2.6 years (460).

Yet, it should be taken into consideration that a number of the studies, particularly initial ones did not distinguish between intermediate CD14++CD16+ monocytes from nonclassical CD14+CD16++ monocytes.

Whereas intermediate and nonclassical monocyte counts are elevated in dialysis patients, classical monocyte counts are lower than those in healthy subjects with higher counts of the intermediate CD14++CD16+ monocytes being associated with established CVD as well as with incident cardiovascular events and death in these patients (460, 461). Even though CD14++CD16+ monocyte counts are considerably lower in pre-dialysis CKD than in patients receiving hemodialysis, these counts also have a direct relationship with cardiovascular outcomes in these patients (462, 463). However, a J-shaped relationship may exist between CD16+ monocyte subsets and adverse outcomes in patients receiving hemodialysis, such that both high and low CD16+ counts confer an increased risk of all-cause and cardiovascular mortality (251, 464). Moreover, the dialysis procedure itself increases inflammation, as indicated by the temporary sequestration of monocytes adhering to the activated endothelium and to cellulosic dialysis membranes, but this phenomenon is fully reversed after hemodialysis (280, 465, 466). Paradoxically, patients with a less marked decrease in CD14++CD16+ monocytes during hemodialysis have poorer cardiovascular outcomes than patients with major decreases (467). Albeit speculative, a possible explanation for this phenomenon is that CD16+ monocytes with diminished adhesive properties also have reduced migratory properties, and monocytes may thus become confined in atherosclerosis lesions and worsen vascular damage (251, 467). Even though the

cardiovascular risk in patients receiving dialysis is attenuated after kidney transplantation, the atherosclerotic disease remains a major cause of death in KTRs with the independent association of CD14+CD16+ monocytes with subclinical atherosclerosis also persisting in this patient population (468).

Vascular calcification as a component of CKD-MBD, is a major contributor to CVD and excess cardiovascular mortality in patients with CKD (469). As already described previously, intimal calcification generally accompanies atherosclerotic plaques and is thus associated with ischemic events whereas medial calcification, preferentially develops along the tunica media elastic fibers, leading to increased arterial stiffness and subsequent development of LVH, diastolic dysfunction and HF (469). High circulating levels of inflammatory cytokines, such as TNF-α and IL-6, have been associated with increased rate, burden and progression of vascular calcifications, with related pathogenic mechanisms including direct effects in vascular cells as well as indirect ones through inhibition of fetuin-A expression by the liver and α-Klotho expression by the kidneys (469). Accordingly, elevated serum IL-6 levels correlate with aortic intimal and medial calcification and predict risk of cardiovascular death in hemodialysis patients. Furthermore, IL-6 is associated with progression of coronary artery calcification and mortality in incident dialysis patients (469, 470).

Inflammation favors the CKD-associated osteochondrogenic transition of VSMC, matrix vesicle release and formation of apoptotic bodies acting as complexes of calcium and phosphate nanocrystal nucleation points (469). Nanocrystals induce VSMC osteochondrogenic transition and promote the production of pro-inflammatory cytokines by resident macrophages, thus creating a vicious circle which further expands the calcification process (469, 471). Furthermore, the pro-apoptotic, pro-oxidative and pro-osteogenic effects of TNF-α on VSMC calcification depend on IL-6 secretion and TNF-α activation of the NF-κB pathway promotes IL-6 production by VSMC (469, 472).

Accordingly, in vitro data from VSMCs incubated with samples of uremic serum indicate that the effects of TNF-α are mediated by extracellular signal-regulated kinase (ERK) modulation of c-Fos, also known as activator protein 1 (AP-1), a transcription factor that controls gene expression in response to a variety of stimuli, including cytokines, growth factors and bacterial and viral infections (472). Thus, increased TNF-α in the uremic serum upregulates Il-6 via ERK and AP-1 mediated signaling, with Il-6 subsequently activating the Wnt-β-catenin-dependent VSMC osteochondrogenic transition (472).

The expression of the calcium-sensing receptor (CaSR) expression and function have been extensively studied in the parathyroid gland and in vascular tissues in CKD patients. The monocyte CaSR exerts pleiotropic biological effects, including both proinflammatory and anti-inflammatory actions, thus it could be involved in the complications associated with CKD (473-476). Accordingly, the CaSR has been shown to synergize with chemokines and to favor monocyte migration whereas on the other hand, monocyte CaSR activation has been shown to switch the proinflammatory M1 macrophage phenotype to the anti-inflammatory M2 phenotype (473-476). Both total and surface CaSR expression in PBMC progressively decrease with the degree of severity of CKD, suggesting a potential

pathophysiological role (473). The uremic environment potentially inhibits CaSR expression in blood monocytes of CKD patients with an independent association found between total monocyte CaSR expression and serum p-cresyl sulphate concentration in CKD patients (473). Notably, surface monocyte CaSR expression is associated with CKD independently of total monocyte CaSR expression suggesting the existence of impaired mechanisms of CaSR maturation, trafficking to the cytoplasmic membrane, and recycling in these patients (502, 503). The decrease in CaSR by monocytes isolated from patients with CKD was associated with a reduction in their ability to inhibit calcium deposition in rat carotid arteries in vitro whereas pretreatment with a calcimimetic agent of PBMC significantly improved monocyte capacity to reduce carotid calcification (473). Additionally, monocytes from CKD patients display an impaired resorptive capacity as demonstrated by in vitro functional assays, which may be ascribed at least partly to the decrease in CaSR expression since preincubation of human monocytes isolated from patients with advanced CKD with calcimimetic agents restores this action (473). It should be highlighted that phosphorus, indoxyl sulphate and oxidized LDL have been shown to hinder the differentiation of monocytes into osteoclasts in vitro as well as inhibit their bone resorptive capacity, thus preventing the resorption of cardiovascular calcium and phosphate nanocrystals (431). These findings suggest a potential role of the of CaSR in monocytes of CKD patients in the promotion of vascular calcifications and evaluating this alteration or monitoring of monocyte CaSR expression as a potential marker, might become a useful clinical therapeutic target regarding arterial calcifications in CKD.

Direct effects of uremic toxins on monocyte phenotype and function might further influence their implications in the pathogenesis of CVD in this setting. Phosphate and indoxyl sulphate induce both monocytes and endothelial cells to express adhesion molecules promoting monocyte adhesion, rolling and migration into cardiovascular tissues as well as subsequent polarization toward a pro-inflammatory phenotype characterized by increased expression of TNF-α, IL-1β, IL-6, and MCP-1 (431). Indoxyl sulphate also promotes IL-10 and TGF-β expression by monocytes and macrophages, thus contributing to a profibrotic inflammatory macrophage phenotype (431). On the other hand, unpolarized macrophages in the setting of high serum phosphate levels, display protective properties against calcifications, mediated by the greater availability of extracellular ATP and disodium pyrophosphate, greater antioxidant synthesis, and lower levels of tissue-nonspecific alkaline phosphatase, suggesting the existence of a balancing mechanism (431).

5.2.1.2 Monocyte subsets and the inflammatory paradigm of HF: implications for CKD

Maladaptive activation of the immune system is the basis for the inflammatory paradigm of HF and monocytes are an essential component of the inflammatory cascade in this setting (353, 477, 478). Potential triggers of monocyte activation in HF include lipopolysaccharide or viral stimuli, heat shock proteins, hypoxia and tissue ischemia, as well as augmentation of LV filling pressure (377, 478). Activated monocytes and macrophages are the major source of cytokines implicated in the pathophysiology of HF, such as Il-6 and TNF-α, but represent as

well a cellular target of these proinflammatory mediators, thus amplifying the cascade of monocyte activation and recruitment into the failing myocardium (251). Increased levels of monocytes activation markers have been found in HF, including TLR4, which represents as well an index of disease severity (251, 479, 480).

The remodeled myocardium has a higher count of TLR4+ monocytes compared with the healthy myocardium, thus creating a proinflammatory environment with TLR4- deficient mice demonstrating a lower inflammatory burden as well as reduced cardiomyocyte apoptosis in the setting of acute ischemia (481). There are several mechanisms responsible for monocyte activation in HF, including increased gut permeability due to fluid overload and augmented sympathetic activity (481, 482). The endotoxin-cytokine hypothesis which is a common denominator for CKD and HF refers to the bacterial lipopolysaccharide transition into the circulation via a permeable bowel membrane in the setting of venous congestion. An alternative mechanism to the introduction of bacteria into the circulation in HF involves activation of the sympathetic system; a common feature of HF. Sympathetic overactivity activity redistributes blood flow away from the splanchnic circulation, causing transient bowel ischemia and subsequently increased endothelial permeability (481, 482).

Following activation, monocytes release cytokines and ROS and migrate into the myocardium, where they are considered to exert both detrimental and beneficial effects such as stimulation of angiogenesis and tissue repair among others (353).
HFpEF, as already described previously, is a clinical syndrome characterized by diastolic dysfunction due to myocardial inability for adequate relaxation and which is especially common in CKD and associated conditions such as hypertension and diabetes (483). Even though, the majority of pathophysiological evidence for monocytes in HF is based on HFrEF, monocytosis and activation of the proinflammatory CD14++CD16+ monocyte subset has been associated with LV diastolic dysfunction in preserved EF (484, 485). Angiotensin II promotes cardiac fibrosis principally through the activation of cardiac fibroblasts through the macrophage related TGF-β/Smad2/3 pathway (486, 487). TLR2 expression has been detected in macrophages infiltrating the heart and its inhibition suppresses myocardial infiltration and inflammatory cytokine production, as well as the NF-κB pathway (488). Both monocyte derived cytokines, IL-6 and TNFα, have been implicated in the high cardiovascular risk of CKD, including the risk of HF in patients with CKD and in patients receiving dialysis (371, 489-491). Patients with ischemic HF have similar number of classical monocytes with patients with stable CAD without HF, however the number of classical monocytes increase with HFdecompensation (492). On the other hand, the non-classical CD14+CD16++ monocytes role remains ambiguous in HF as both decreased counts and no changes have been reported (492). Proinflammatory CD14++CD16+ monocyte counts are elevated in patients with both acute and stable chronic HF and have been associated with mortality and rehospitalization after an episode of HF decompensation (493-495). Defective regulation of monocyte apoptosis has been described in patients with acute HF and kidney dysfunction including patients with already advanced CKD and CD14++CD16+ monocytes are elevated in patients with acute HF and a concomitant decline in kidney function (496-499). In vitro

models suggest that medications used for the treatment of HF might also modulate the inflammatory mechanisms. Thus, enalapril inhibits the release of monocytes from the splenic reservoir and decreases their recruitment into the healing infarcted tissue in a mouse model of permanent coronary ligation (500). Eplerenone downregulates the TNF-α converting enzyme and reduces theTNFα concentrations in cultured monocytes from patients with HF (501). Likewise, carvedilol significantly dampens in vitro lipopolysaccharide induced TNFα synthesis in isolated human monocytes (502).

Rapidly following tissue damage or infection, monocytes are recruited to the affected site, where they can differentiate into macrophages with diverse properties and functions, however the relationship between different monocyte subsets and macrophage phenotypes has not been thoroughly clarified and remains under investigation (503-505). Tissue macrophages can undergo transformation into distinct functional phenotypes depending on the pathophysiological stimuli with Th1 cytokines such as IFN-γ promoting a switch towards the classically activated proinflammatory M1 phenotype and Th2 cytokines including Il-4, Il-10 and Il-13 directing their polarization towards the alternatively activated, anti-inflammatory M2 phenotype (505, 506). Thus, the polarization of macrophages is a tightly regulated process involving specific signaling pathways both at the transcriptional and posttranscriptional levels. Accordingly, the NF-κB pathway and signal transducer and activator of transcription (STAT) 1 driven activation polarize macrophages toward the cytotoxic M1 phenotype which releases pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, whereas STAT3 and STAT6 signaling polarizes macrophages towards the M2 phenotype, with wound healing properties (506). The cardiac tissue macrophages include mixed phenotypes identified by the expression of MHCII and CCR2 with two major groups identified, respectively fetally derived self-renewing cells native to the heart and those arising from bone marrow originating monocytes (50-509). Furthermore, a compromised myocardium provides multiple stimuli (492). The presence of tissue hypoxia and ischemia or excessive LV and atrial stretch as shown in experimental studies of hypertensive mice with HFpEF represent potential stimuli for myocardial resident macrophages to signal the release monocyte chemoattractants such as MCP-1 and interleukins thus causing monocyte recruitment in patients with HF (492).

Available evidence implicates macrophages in the pathogenesis and progression of both CKD and HF and ongoing research aims to evaluate their role in the development of the cardiorenal syndrome. In the setting of cardiac injury, monocyte-derived macrophages and resident macrophages participate both in the pro-inflammatory pathways mediating HF progression and development of cardiomyopathy as well as in the healing process inducing scar formation (507-509). The specific features of macrophage populations involved in the pathogenesis of uremic cardiomyopathy remain to be elucidated. Both the native subsets as well as circulating monocytes contribute to the expansion of cardiac macrophages in the setting of reduced kidney function (503). The increase of resident cardiac macrophages in CKD appears to be dependent upon CXCL10 signaling, but no association has been found with hypertension or with the severity of kidney dysfunction. On the other hand, plasma

CXCL10 is elevated in CKD with experimental models showing that human induced pluripotent stem cells (iPSC) derived cardiomyocytes and primary cardiac fibroblasts are the main source of CXCL10 production following incubation with uremic serum from CKD donors (510). Furthermore, CXCL10 blockade by specific antibodies reduced the macrophage infiltrate in the failing heart of CKD mice. In addition, the expansion of CD14++CD16+ monocytes was an independent factor correlated with brachial-ankle pulse wave velocity in diabetic CKD patients (504).

Severe myocardial dysfunction models in animals, as occurs in the setting of MI or TAC, point to CCR2-derived macrophages as the predominant cells in the heart following injury (507, 511). Likewise, in validated models of diastolic myocardial dysfunction, such as hypertension induced by drinking salty water with unilateral nephrectomy and chronic exposure to aldosterone (SAUNA) and in the physiological aging model, cardiac macrophage expansion has been shown to rely on CCR2, as macrophage numbers decreased in CCR2 knockout mice exposed to SAUNA (512). In a CKD experimental model of mice with folate induced nephropathy, CCR2 knock-out animals were protected from cardiomyocyte hypertrophy and cardiomyopathy, thus suggesting a contribution of circulating monocytes to the development of uremic cardiomyopathy (510).

Dysfunction of the circadian clock has been studied with relation to the pathogenesis of CVD (513). The periodic activation and repression of CLOCK gene products control the transcription of elements associated with regulation of the circadian oscillation in the molecular clock and also the variations in output physiology at 24-hour basis. Interestingly, CKD induced cardiac inflammation and fibrosis are attenuated in CKD mice with a mutated and dysfunctional Clock gene despite elevated BP and increased serum angiotensin II levels. Apparently, expression of Gpr68 was not induced in Clock mutated mice whereas under natural conditions, high-GPR68-expressing monocytes release TNFα and IL-6, thus representing a risk factor and revealing an uncovered role of monocytic clock genes in CKD-induced HF (513).

In addition, the role of monocyte function in CRS still is unclear (499). A cross-sectional study compared markers of systemic inflammation including peripheral blood monocyte status in incident hospitalized patients with acute CRS, with kidney failure or due to hypertensive emergency (499). Patients with acute CRS, including those with and without significant fluid overload, display a higher proportion of CD14++CD16+ activated monocytes as compared to hypertensive controls whereas in vitro stimulation of monocytes using opsonized E. coli bacteria was found to be reduced in CRS, which is suggestive of an already established in vivo monocytic activation of higher degree (499). These results are in accordance with data from a previous study showing that an enhanced expression by monocytes of the membrane-bound adhesion molecule CD11b, a sign of enhanced cellular activity, reduces the ability for in vitro stimulation of monocytes and vice versa (514). Furthermore, systemic inflammatory indices were higher in patients with acute CRS, regardless of volume state compared to patients with hypertension; however Il-6 levels were higher in CRS patients with fluid overload compared to those without (497).

Accumulating evidence from experimental models, genetic data, and cross-sectional human studies suggest that increased proportions of selected immune cell subsets may be related to hypertension with monocytes showing associations with blood pressure, vascular remodeling as well as vascular inflammation (515-518). Assays of immune cells from cryopreserved samples collected at the baseline examination from 1195 participants from the MESA study showed that a 1-standard deviation (SD) increment in classical CD14++CD16− monocytes was associated with 2.01 mmHg lower average systolic blood pressure whereas a 1-SD increment in non-classical CD14+CD16++ monocytes was associated with a 1.82 mmHg higher level of systolic blood pressure (515).
Excessive monocyte and macrophage cardiac recruitment leads to a vicious circle of myocardial damage and remodeling with cardiac fibrosis being a consequence of this cascade (492). The increase in the number of monocytes and macrophages leads to increase in collagen deposition in the myocardium and conversion of cardiac fibroblasts to myofibroblasts (545). Cardiac fibroblasts comprise the majority of cells within the extracellular matrix and even though they are relatively scarce in the healthy adult heart, cardiac injury leads to an expansion of this cell population, a process mediated by Il-1b as well as by an increase in the rate of differentiation of precursor cells including monocytes, endothelial progenitors, pericytes, and bone marrow circulating progenitor cells, into fibroblasts (492). The process by which monocytes and their surface receptors are stimulated to promote cardiac fibrosis involves the complex interplay of systemic and local cascades of inflammatory pathways, with monocytes related cytokines such as MCP-1, TNFa, and TGF-b being actively involved (492). Notably, MCP-1experimental models of heart failure involving MCP-1 knockout mice studies have shown significant suppression of fibrosis and macrophage activation (492, 520). It is well known that monocyte related TNFa induces production of the iNOS and promotes uncontrolled oxidative stress, and, consequently, apoptosis and tissue necrosis (487). Likewise, increased numbers of intermediate monocytes highly expressing TGF-b have been observed in human cardiac tissues from patients with HF (492, 521). Furthermore, data from studies of hypertensive animals have shown cardiac upregulation of M2 macrophages, which contribute to cardiac fibrosis in hypertension whereas healthy monocytes incubated with serum form patients with HFpEF differentiate into an M2 profibrotic phenotype (485, 492, 522).

Pro-inflammatory and pro-fibrotic cytokines such as IL-1, IL-6, TNFa, and MCP-1 are all upregulated in atrial fibrillation thus promoting the electrical and structural remodeling with fibrotic changes, which are characteristic of atrial fibrillation (492, 523). Furthermore, the intermediate CD14++CD16+monocytes might play a role in the pathogenesis of atrial fibrillation and especially in the remodeling of the eft atrium. A case–control study of 30 atrial fibrillation patients without systemic diseases referred for catheter ablation, and 30 healthy age-matched controls showed that atrial fibrillation patients had a higher proportion of circulating intermediate CD14++CD16+monocytes than the healthy controls and the proportion of intermediate CD14++CD16+monocytes was the sole factor independently associated with the presence of atrial fibrillation (523). The mean proportion

of intermediate CD14++ CD16+monocytes was higher in the persistent AF patients than in the paroxysmal AF patients (523). Additionally, intermediate CD14++CD16+monocytes were associated with a slower left atrial appendage flow during sinus rhythm, potentially reflecting its influence on the atrial systolic function, as well as with higher BNP levels (524). Thus, monocyte activation, might contribute to the mechanoelectrical remodeling process in the genesis of atrial fibrillation with effects on cell death processes, atrial fibrosis expansion and changes of the atrial conduction features.

A study of patients with MI explored the dynamic changes in monocyte subset counts as well as their phagocytic and NFκB activity as reflected by intracellular levels of inhibitory κB kinase β, with regard to MACE and LVEF (524). More prominent dynamic reduction in the levels of intermediate CD14++CD16+ monocytes 2 weeks following MI was independently associated with reduced risk of MACE by approximately 40% whereas on the contrary, a less prominent reduction of the intermediate monocytes 1 month following MI was independently predictive of LVEF at six month and recovery of cardiac contractility (524). Furthermore, neither any of the other monocyte subsets nor their phagocytic or the NFκB activity were associated with changes in survival from MACE (524).

In accordance with the above, data form a study of patients suffering a ST elevation MI, showed that the intermediate CD14++CD16+ monocytes are not only predictive of MACE following MI, but they are also strong predictors of the occurrence of post-MI HF, with the highest quartile of the intermediate monocytes being associated with fivefold increase in the development of HF (525). Notably, ROC analysis showed that the inclusion of the CD14++CD16+ monocytes significantly improved predictive value of troponin T and creatinine kinase for MACE and new-onset HF in these patients (525). On the other hand, elevated intracellular levels of the inhibitory κB kinase β, the cytoplasmic marker of activation of the NFκB signaling, were associated with tenfold lower risk of HF, thus suggesting that the functional characteristics of the intermediate CD14++CD16+ monocytes, including dysregulated NFκB and phagocytic activity, may be responsible for potential beneficial functions of the intermediate monocytes (526-526). Furthermore, intermediate monocytes significantly correlated with MMP9 levels as well as with the degree of post-MI myocardial scaring on CMRI, thus indicating their participation in post-STEMI remodeling and their influence on post-MI cardiac contractility (525, 527).

It should be noted that experimental studies involving mice have shown that following MI, dynamic changes are observed in monocytes subsets and distinct monocyte subtypes with different roles appear to participate in specific stages of recovery after MI. In mouse models, CCR2hiLy6C+ monocytes, the equivalents of human classical monocytes appear to predominate in the myocardium early following MI, whereas CXCRIhiLy6C− monocytes, which are analogue to the human non-classical monocytes, become the major monocyte subset to be recognized later on during the chronic phase (526, 528). Overall, CCR2hiLy6C+ exhibit phagocytic activity and appear to be associated with tissue damage, while CXCRIhiLy6C− principally display anti-inflammatory as well as pro-angiogenic properties and they enhance the healing process by promoting myofibroblast accumulation

and collagen deposition (526, 528). Similar to classical monocytes, intermediate monocytes which are poorly represented in mice, appear to be more functionally active during the first days following MI. However, even though the classical monocytes increase following MI and might be related to future cardiovascular events, the dynamic changes in classical monocytes in this setting do not appear to hold a prognostic significance. On the other hand, dynamic changes in intermediate monocytes during the first week following MI displayed a significant association with survival from MACE, with adequate decrease of intermediate monocytes counts being crucial for MACE risk reduction as well as for improved myocardial performance as demonstrated by a higher LVEF (524, 529).
CD14++CD16+ monocytes during the acute MI setting have been shown to correlate with peak plasma troponin and cytokines levels as well as with increased blood levels of VEGF following coronary intervention for MI (451, 524). At the same time, Mon2 are enriched in the region of the myocardial infarct damage. Finally, non-classical monocytes do not appear to correlate with outcomes following MI, including risk of MACE and survival, which might be reasonable considering the profile of non-classical monocytes which display a weak phagocytic and inflammatory activity, attenuated production of pro-inflammatory cytokines in response to lipopolysaccharide stimulation of CD14 as well as a lower rate of aggregation with platelets (524, 530). A study of patients with CAD undergoing CABG showed an association of classical and non-classical monocytes with endothelial dysfunction, manifested by NO bioavailability which was assessed using ex vivo isometric tension studies in segments of internal mammary arteries. Endothelial dysfunction was associated with higher expression of activation marker CD11c selectively on $CD14^{+}CD16^{++}$ monocytes which as well exhibited increased vascular superoxide production (531). Notably, following fibrotic stimuli, Neuregulin-1 (NRG-1), a cardioactive growth factor released from damaged endothelial cells in the endocardium, activates ErB4, a member of the epidermal growth factor (EGF) receptor and downregulates the PI3K/Akt pathway and the phosphorylation of STAT3 thus reducing the release of proinflammatory mediators as well as the recruitment of new monocytes (528). However, even though the non-classical $CD14^{+}CD16^{++}$ monocytes might be linked to endothelial dysfunction, the impact of this association on atherogenesis as well as on myocardial remodeling and heart failure remain an open question.

The myocardial remodeling process following MI undergoes three phases with distinct profile of immune cells involved during each of them. Accordingly, the initial phase includes the pro-inflammatory response, the second phase is characterized by monocytes and macrophages returning to baseline, whilst macrophages predominance is the hallmark of the last phase of chronic myocardial remodeling or the reparative phase (452, 532). Macrophages have been shown to directly contribute to scar collagen and fibronectin production as well as increase the expression of α-smooth muscle actin, a marker of the mature myofibroblasts in zebrafish and mouse models of heart injury (528).
The accumulation of a great number of macrophages at the site of cardiac injury is a key part of the cellular host response to tissue damage together with a set of diverse humoral and connective tissue elements (528). Fibroblasts present in sites of tissue injury generally

have been considered to originate from the surrounding connective tissue, yet part of the human cardiac infiltrating fibroblasts is suggested to derive from a circulating monocytic subset of CD14+ cells known as “fibrocytes”, that are characterized by a distinctive collagen+/vimentin+/CD34+ phenotype (528, 533). Increased numbers of circulating fibrocytes have been observed with cardiac fibrosis, in response to the augmented circulating levels of CC chemokine subfamily that preferentially acts on mononuclear cells, including MCP-1/CCL2, CCL4, and CCL3, thus indicating that stimulation of chemokine receptors promotes fibrogenesis by causing a recruitment of myofibroblast progenitors to the injured site (528, 533-535). Available experimental evidence indicates that CCL2 is the principal orchestrator of fibrosis through mobilization of monocytic-derived fibrocytes in the myocardium (528). Thus, augmented CCL2 expression has been shown to correlate with increased macrophage infiltration, fibrosis and LV dilation in murine cardiac muscle, whereas CCL2-null mice appeared to be protected from mineralocorticoid-induced cardiac fibrosis (528, 536, 537). Among others, macrophages secrete MMPs, which degrade the extracellular matrix and thus further enhance LV remodeling, whereas MMPs also regulate macrophages phenotype and functions (528, 538). Particularly, iMMP9 expression is significantly increased in both animal and clinical MI models where it has been linked with inflammation, extracellular matrix degradation and synthesis as well as upregulation of LV remodeling whereas MMP14 has been associated with reduced survival and myocardial function in mice following MI (528, 539).

5.2.2 Natural killer cells and NKT cells

Natural killer cells and NKT cells are suspected to participate in atherogenesis (250, 540, 541). NK cells appear at an average of 1–2 cells per plaque lesion section and they are abundant in the necrotic cores of atherosclerotic plaques where they infiltrate via the production of perforin and granzyme (250, 541, 542, 543). Yet, it remains to be further clarified whether NK cells exert harmful or protective effects toward the arterial system because of the relative lack of representative mouse models of NK cell deficiency, at least until recently (Figure 4). Indeed, experimental and clinical data are controversial with depletion of functional NK cells decreasing the atherosclerosis burden in atherosclerosis-susceptible LDL receptor null mice whilst both NK cell counts and NK cytotoxic activity are diminished in patients with coronary artery disease, a phenomenon which has been ascribed to persistent low-grade inflammation (542- 551). Obese patients with metabolic syndrome had lower levels of NK cells compared to obese patients without metabolic syndrome (552). CAD patients when compared to healthy subjects, apart from decreased absolute number and percentage of total NK cells, display as well reduced counts of the CD3-CD56dim cytotoxic NK subsets as well as a trend towards a lower percentage of CD3-CD56+IFN-gamma+ cells and of the CD3-CD56bright regulatory NK subset which might be attributed to their migration into the atherosclerotic arterial wall (545). This is supported by evidence of an abundance of $CD56^{bright}$ NK cells in atherosclerotic plaques compared with autologous peripheral blood, where they enhance inflammation by stimulating TNF-α

production by monocytes (553). Notably, the CD56^{bright} NK cell subset is even more enrichened in atherosclerotic plaques of symptomatic patients with CAD where they exhibit a higher production of IFN-γ, thus further reinforcing the possible role of CD56^{bright} NK cells in plaque instability (554). Data concerning the peripheral blood NK cell number in patients with ACS are controversial with most studies indicating a decline in the peripheral blood NK cell number and only two studies demonstrating an elevated percentage of NK cells in ACS patients compared to healthy controls (555-557). Increased levels of IL-6 were also observed in patients who failed to restore NK cell levels in this setting, which may point towards a lower NK cell proliferation rate due to persistent low-grade inflammation whereas a higher percentage of IL-10 positive NK cells found in AMI patients was associated with a negative correlation with the severity of the infarction (543).

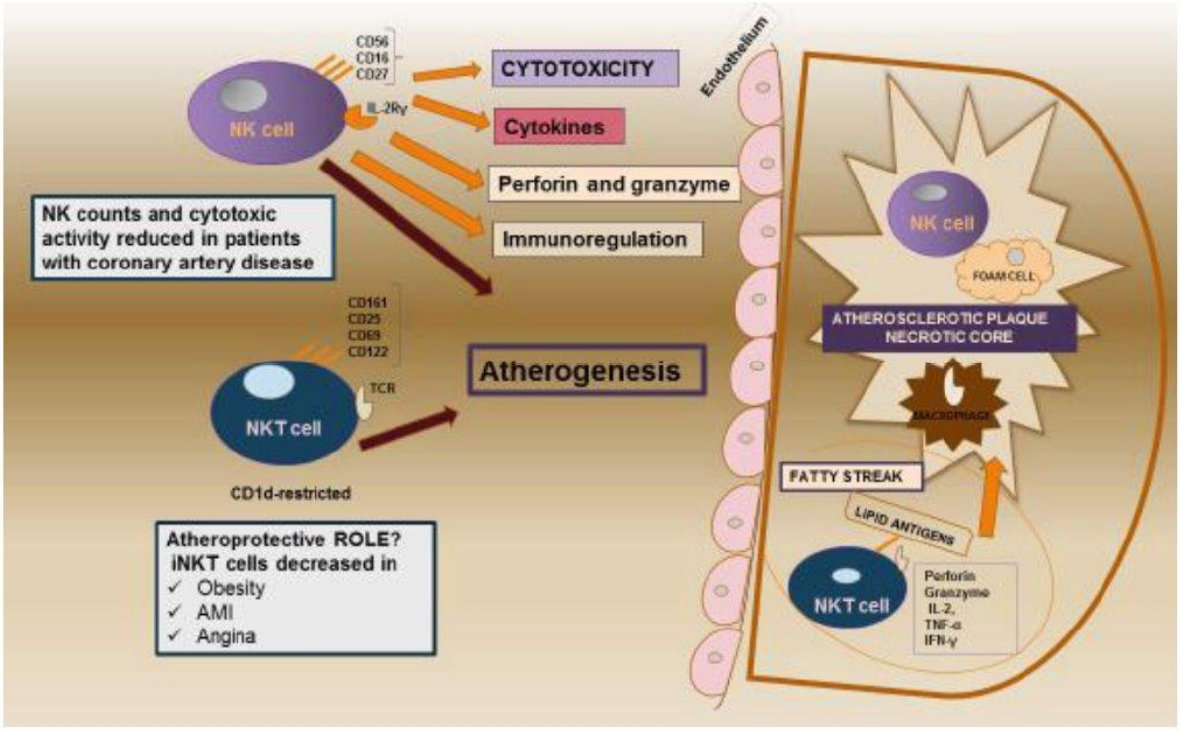

Figure 4. Implications of NK and NKT cells in atherogenesis. CD1d-restricted NKT cells produce cytokines, are autoreactive cells and are involved in immunoregulation. NK, Natural Killer; NKT, Natural Killer T; CD1d, Cluster of differentiation 1d (251).

Genetic models of mice with NK cell deficiency on one hand and models with hyper-reactive NK cells on the other indicate that neither of these states affects the atherosclerotic process except for conditions of modeled chronic viral infection, in which NK cell deficiency was shown to protect against atherosclerosis (558). In addition, NK cytotoxic activity in elderly patients has been shown to correlate with a history of severe infections or death due to infection (559). In line with this, an expansion of NKG2C+ NK cells, a population of NK cells expressing adaptive NKG2C- activating receptor that exhibit augmented cytokine response to antibody-dependent cellular cytotoxicity, seems to be associated with the loss

of plaque stability in patients with chronic cytomegalovirus infection and CAD (560). These findings altogether suggest a relevant proatherogenic role of NK cells only under specific conditions and might bear implications for the pathogenesis of CAD in patients with chronic infections (543).

NK cells can be a potential source of soluble Fas ligand as cytokine-induced apoptosis of NK cells results in the marked release of Fas ligand, with the latter further regulating the apoptotic susceptibility of NK cells. Apoptosis of NK cells is increased in patients with CAD, as indirectly indicated by the finding of elevated plasma Fas ligand, which has been significantly associated with NK cell apoptosis ex vivo in these patients (561). Taking into consideration that with regard to CVD high levels of soluble Fas in plasma seem to indicate an increased risk while elevations in soluble Fas ligand represent a profile of low cardiovascular risk, the influence of NK cell apoptosis on atherosclerosis merits further investigation (543, 552).

Experimental data have shown that NK-cell-derived IFN-γ stimulated the differentiation of circulating monocytes to macrophages and inflammatory dendritic cells as well as the replacement of tissue mononuclear phagocytes with circulating monocytes which further differentiated into macrophages or inflammatory dendritic cells (543, 563). On the other hand, macrophages and dendritic cells produce Il-12 and Il-18 which stimulate NK cells to secrete IFN-γ and augments their proliferation and cytolytic activity, thus highlighting the crosstalk between cells and the positive feedback mechanism that augments the early innate inflammatory response in the tissues (543, 563). Considering that the expression of IL-18 receptor is associated with augmented expression of the IFN-γ, the increase of circulating NK cells expressing the Il-18 receptor in ACS is suggestive of a more activated state of NK cells in ACS (543, 556, 564).

Interestingly, elevated monocyte expression of MHC class I polypeptide- related sequence A (MICA), an HLA antigen related molecule and marker of inflammation induced by cellular stress, has been detected in patients receiving dialysis and high circulating levels of MICA are associated with low NK cell counts in patients receiving dialysis (565). Even though MICA may be implicated in atherosclerosis, the clinical implications of these findings remain undefined (566).

There are few data available regarding the effects of NK cells on cardiac remodeling and HF (567). Mouse models of myocarditis indicate that NK cells play a vital role in limiting cardiac viral infection and reducing cardiac eosinophilic infiltration (568). Thus, according to the limited available data, circulating NK cells have lower counts and display impaired cytolytic activity in patients with HF compared to healthy individuals (250). The suppressed NK cell status in this setting may be attributed to IL-6, which is substantially elevated in HF and regulates pathways that may induce cell dysfunction and energy (569). Taking into consideration the physiological properties of NK cells, such as the production of IFNγ and other anti-inflammatory mediators, they may further promote the expression of anti-inflammatory chemokines and thus protect against the development of cardiac fibrosis while simultaneously favoring neovascularization (570, 571). NK cells express and produce

high levels of MMP-9 and seem to act protectively against the development of cardiac fibrosis by preventing the accumulation of specific inflammatory populations in the heart as well as by directly limiting collagen formation in cardiac fibroblasts (568). The RAS axis appears to influence the innate immune system cells and particularly NK cells in mice with arterial hypertension through the stimulation or inhibition of the production of chemokines and cytokines by mononuclear cells (572, 573). IFN-$\gamma^{-/-}$ mice appear to be partially protected from angiotensin II induced vascular endothelial and SMC dysfunction thus indicating that angiotensin II induced vascular damage is to a certain extent NK-cell-dependent (573). IFN-γ receptor knockout mice did respond to angiotensin II induced hypertension and displayed reduced cardiac hypertrophy, reduced cardiac macrophage and T-cell infiltration, less fibrosis, as well as less arrhythmogenic electric remodeling independent of blood pressure changes (574).

Results from an exploratory analysis of data from the MESA study showed an association between increased proportions of NK cells and levels of systolic blood pressure (515). Thus, taking into consideration these associations as well as the fact that NK cells are a major source of IFN-γ, their role in arterial hypertension in humans, merits further attention.

A study utilizing a murine model of type 1 CRS induced by cardiac arrest and cardiopulmonary resuscitation, aimed to investigate the AKI to CKD transition and identify potential immune drivers of renal fibrosis in this setting (575). The study results showed that renal inflammation occurs during CRS, which correlates with mesenchymal cell expansion. Accordingly, the kidney immune cell phenotypes infiltrating the kidney which were profiled by flow cytometry analysis and immunofluorescence, consisted primarily of innate immune cells, including monocytes and macrophages, neutrophils and NK cells, with the latter immediately preceding mesenchymal cell expansion (575). Notably, immune cells colocalized with mesenchymal cells, accumulating in the areas of fibrosis. In specific, NK cells peaked at three days following the acute event, suggesting these cells may play a role in myofibroblast formation but they declined as mice developed CKD (575). Granzyme A produced by NK cells appears to mediate the immune to mesenchymal communication as well as the mesenchymal cell growth and proliferation in type 1 CRS (575). Thus, in vitro administration of granzyme A to fibroblasts induced cell growth and proliferation whereas pharmacologic blockade of granzyme A signaling in vivo attenuated fibrosis and improved renal function in mice with type 1 CRS (576). Similar conclusions have been provided by AKI models of ischemia–reperfusion injury with NK cells accumulating in the kidneys and contributing to CKD progression (576-578). Although depletion together with modulation of the phenotypic and cytotoxic characteristics of NK cells may contribute to the immune dysfunction in advanced CKD, the role of these alterations in the pathogenesis of CKD associated CVD remain currently hypothetical and require further investigation.

Several studies have shown that NKT cells can promote atherogenesis, with numerous pathways being implicated in their activation, including both endogenous and exogenous phagocytosed lipids (250, 579, 580). Furthermore, the control of circulating

monocyte levels by the same cells, rather than macrophage recruitment into plaques, appears to be their key involvement into the atherogenic process (250, 571). However, it should be noted that iNKT cells have been implicated in the development of the atherosclerotic fatty streak lesions during the early phases of atherosclerosis (579 582). In the experimental ApoE knock-out mouse model, iNKT cells in atherosclerotic plaques following activation through the CD1d- NK pathway by lipid antigens, such as α-galactosylceramide 1, synthesize various cytokines including IL-2, TNFα and IFNγ (583-585). After lipid recognition, iNKT cells release IL-8 which may further promote the recruitment of macrophages, SMC and cytotoxic lymphocytes, neoangiogenesis and destabilization within the atheromatous plaques (583, 586). Accordingly, experimental data from ApoE-deficient mice fed a high-fat diet indicate that NKT depletion decreases macrophage accumulation, apoptosis, necrotic cores and inflammatory cytokines, whereas transfer of NKT cells into lymphocyte-deficient, ApoE-deficient mice aggravates atherosclerosis by perforin and granzyme B-mediated apoptosis of macrophages, SMC and endothelial cells, thus leading to plaque inflammation, accumulation of necrotic cells within the lesion and necrotic core formation (587). Nevertheless, some studies hint to potential atheroprotective properties of NKT cells (588). A relevant paradigm would be the decreased number of iNKT cells observed in the obesity, a phenomenon regarded to be a compensatory response to the proinflammatory milieu in this atherogenic state (589). Available clinical data indicate that iNKT absolute number and cell fraction are lower in patients with AMI, unstable angina and stable angina compared to control subjects (590, 591).

At present, the implications of NKT cells in pathways of cardiac hypertrophy and remodeling remain unknown at large and need to be clarified by future research. Experimental data from CD1d knockout mice deprived of activated NKT cells have revealed augmented inflammatory responses induced by angiotensin II, enhanced activation of the NF-κB and TGF-β1/Smad2/3 pathways as well as aggravation of the ensuing myocardial hypertrophy and fibrosis. On the other hand, these effects appear to be reversed after the induction of NKT cells in the same mice treated with α-galactosylceramide (592). Accordingly, mice with MI undergoing NKT cell stimulation with α-galactosylceramide, displayed increased myocardial infiltration of iNKT cells, attenuated LV cavity dilatation and dysfunction, decreased myocyte hypertrophy, interstitial fibrosis, and apoptosis as well as improved survival compared to mice which received only phosphate-buffered saline (593). Thus, it appears that iNKT cells might play a protective role against LV remodeling and failure following MI through the enhanced expression of cardioprotective cytokines such as IL-10 (593).

A study investigating the pathophysiological role of iNKT cells in HF caused by pressure overload showed that myocardial infiltration of iNKT cells was increased in mice following TAC (594). Notably, iNKT cell-deficient Jα18 knockout mice displayed greater myocyte hypertrophy and a greater increase in interstitial fibrosis as well as hastened transition to HF compared with wild type mice after TAC (594). Furthermore, the phosphorylation of extracellular signal-regulated kinase was significantly increased whereas

expressions of Il-10 and TNF-α mRNAs as well as their ratio in the LV after TAC were decreased in iNKT deficient mice compared with wild type mice, allowing us to speculate that the disruption of iNKT cells causes an imbalance between Th type 1 and type 2 cytokines including diminished Il-10 levels (594). In conclusion, the protective role of iNKT cells against HF caused by pressure overload has been shown to include a shift from Th1 towards a Th2 pattern as well as the induction of the immunosuppressive cytokine IL-10 and targeting the activation of iNKT cells might be a promising candidate as a new therapeutic strategy for HF (595).

To sum up, there is a paucity of data regarding the implication of NK cells and NKT cells in the pathogenesis of CVD phenotypes in CKD, however taking into consideration that these cells provide the basis for the transition from immunologically chaotic conditions to ordered conditions and orchestrate tissue inflammation, future research is necessary to elucidate their implication in this setting.

5.3 The implication of cells of the acquired immune system in the pathogenesis of CVD in CKD

5.3.1 T-cells

Considering the chronic inflammatory nature of atherosclerosis and the associated autoimmune component with LDL and ApoB peptides as the most notorious self-antigens driving the autoimmune response in the atherosclerotic plaque, T-lymphocytes have been well studied in this context and are considered to be significantly involved in atherosclerosis (596). Yet, it should be noted that the antigen specificity of the various T cell subsets participating in the atherosclerotic lesion remains unknown in almost all studies in atherosclerosis. The development of high-dimensional, parametric single-cell analyses, including single-cell RNA sequencing and mass cytometry studies, provided insight in the immune composition of mouse and human atherosclerotic plaques, revealing that up to 40 % of all leukocytes are $CD3^+$ T cells (597, 598). A study using single-cell proteomic and transcriptomic analyses in plaques obtained from carotid endarterectomy of patients with clinically symptomatic disease such as recent stroke or transient ischemic attack has shown that T cells account for the majority of immune cells in human atherosclerotic plaques with $CD4^+$ effector memory T cells being particularly enriched in this setting (599). Moreover, genetic or antibody-mediated depletion of $CD4^+$ T cells protects from atherosclerotic lesion development in mice (600, 601). Type 1 Th cells are enriched in atherosclerotic lesions compared with PBMC, with experimental data from studies in mice indicating a pro-atherosclerotic role of type 1 Th cells whereas the role of the other Th cell subtypes and $CD8^+$ T cells remains unclarified, with various studies yielding discrepant results (596, 599). In line with the above, several experimental studies have shown that type 1 Th cells are the most prominent T-lymphocyte subset in the atherosclerotic plaque whereas human studies have shown that type 1 Th cells are increased in the plaques from patients with recent cerebrovascular events compared with patients with asymptomatic atherosclerosis (596,

599). Type 1 Th cells express the chemokine receptors like CXCR3 and CCR5 as well as the defining T-box transcription factor TBX21, also known as T-bet, which is responsible for the induction of Th1 cells and the suppression of Th2 cell from naive T lymphocytes and they secrete INF-γ (602, 603). Furthermore, many $CD4^+$ T cells in the atherosclerotic plaque express other pro-inflammatory cytokines associated with type 1 Th cells such as IL-2, IL-3, TNF and lymphotoxin, which further activate macrophages, T cells as well as other plaque cells and thus promote the inflammatory response (596, 604). With regard to IL-4 as a type 2 Th cell related cytokine, it has been shown to antagonize type 1 Th responses and reduce atherosclerotic lesion formation in ApoE deficient mice (596). On the other hand, some studies have shown that depletion of IL-4 has atheroprotective effects in LDL receptor knock out mice fed with a high-fat diet whereas administration of exogenous IL-4 did not decrease atherosclerosis in ApoE deficient mice with angiotensin II-induced atherosclerosis (596). Apart from Il-4, available data indicate a clear atheroprotective role of other type 2 Th cell cytokines, including IL-5 and IL-13 (596). Clinical studies have shown a significant inverse correlation of IL-5 with cIMT change over time in the common carotid segment even after adjustments for traditional risk factors of atherosclerosis, such as age, gender, smoking, systolic BP, LDL, and body mass index (596, 605). The immunization of LDL receptor knock-out mice with an oxidation-specific epitope of modified LDL has atheroprotective effects and leads to a classic adaptive type 2 Th cells response with Th2 cells producing large amounts of IL-5, which in turn stimulated B lymphocytes to secrete IgM antibodies (596, 606). In line with the above, a significant positive association between plasma IL-5 levels and antibody titers with the two most commonly used models of oxidized LDL, the copper oxidized LDL and malondialdehyde-modified LDL was found in 1011 Finnish middle-aged subjects (607). Likewise, IL-13 administration to LDL receptor knock-out mice fed with a high-fat diet compared with mice treated with phosphate-buffered saline, appears to exert regulatory effects on the established atherosclerotic lesions by increasing the amount of plaque collagen and reducing VCAM1 expression, thus leading to suppressed macrophage infiltration in plaques (608).

The significance of the contribution of T cells to cardiovascular remodeling are still poorly understood (609). Despite the emerging association between HF and inflammation, the role of T cells, key players in chronic inflammation, has only recently come to the center of attention. So far, there is clear evidence regarding the role of adaptive cell–mediated immunity in the pathogenesis of inflammatory heart diseases such as viral or autoimmune myocarditis with T-lymphocytes being significant contributors to myocardial damage following their activation in the setting of antigen presentation by dendritic cells (609). Experimental models of lipopolysaccharide induced myocardial inflammation have established a direct link between T-cell activation and recruitment to the heart, in a heart-trafficking process including CD4CXCR3CCR4 T cells. Moreover, the adoptive transfer of purified T lymphocytes from mice with active myocarditis successfully transferred the disease and induced inflammation and fibrosis into recipient mice, thus highlighting the significance of these cells in cardiac dysfunction (609, 611). Several lines of experimental

evidence have recently involved T lymphocytes in the process of cardiac remodeling following MI with effects on the formation of mature collagen matrix and promotion of fibrosis, facilitation of early wound healing whereas T cell ablation appears to affect the initial postinfarction healing and remodeling response (612). Experimental data evaluating the participation of T lymphocytes in cardiac remodeling and in the transition from hypertrophy to HF in the setting of TAC, indicate high density of both $CD4^+$ and $CD8^+$ T-cells in the heart mediastinal draining lymph nodes and an accumulation of T lymphocytes and activated effector CD4+ T cells within the myocardial tissue (609). TCR knock-out mice displayed preserved LV systolic and diastolic function, reduced LV fibrosis, hypertrophy and inflammation, and improved survival compared with wild-type mice in response to TAC (613). In accordance with the above, overexpression of chemokines associated with the recruitment and homing of T lymphocytes in injured tissues such as CX3CL1, CCL17, CXCL10, and CXCL16 has also been found in HF (609). Notably, cytokines produced by T cells isolated from the mediastinal nodes following TAC and stimulated ex vivo with anti-CD3ε and anti-CD28 antibodies, revealed a significant increase in INF-γ and a decrease in Il-4, thus indicating a Th-1 polarization (609). In line with the above, clinical studies have shown a positive association between INF -γ and Il-18 expressing T-cells and LV myocardial dysfunction in patients with ischemic and idiopathic dilated cardiomyopathy (614). Although mice models of ischemic cardiac remodeling and heart tissues from patients with myocarditis and advanced HF exhibit a Th2-biased inflammatory response, there is no direct evidence showing the involvement of Th2 cells in models of pressure overload induced cardiac hypertrophy (609, 613). Even though there is evidence linking Th2 cells to mechanisms enhancing fibrosis, available data are few and remain controversial (615). Secretion of Th2 cytokines such as IL-4 and IL-13 appears to promote recruitment of monocyte-derived M2 macrophages thus indirectly influencing cardiac fibroblasts (615). The striking effect of T cells in the fibrotic response induced by pressure overload is further reinforced by the induction of a vigorous fibrotic response following T-cell transfer in T-cell deficient mice, a process which is independent of procollagen gene expression and prevented in mice lacking the CD4+ T-cells but not by the absence of CD8+ T cells (609). Interestingly, the transfer of T cells into T lymphocyte deficient mice was accompanied by enhanced collagen fiber density with simultaneous upregulation of the expression of lysyl oxidases (LOX), which are responsible for the assembling of collagen into final fibers in heart tissue (609, 615). These findings strongly support the contribution of T lymphocytes in cardiac collagen cross-linking through the induction of LOX expression and as a result in the potentiation of myocardial fibrosis in chronic pressure overload (609, 616). Thus, taking into consideration the available evidence highlighting the relevance of T lymphocytes in the myocardial fibrotic response, we may infer their beneficial implications during the cardiac repair processes following MI and detrimental effects in the setting of chronic pressure overload. The participation of T cells in cardiac remodeling is further supported by studies in animal models of hypertension, showing that sustained angiotensin II infusion in mice induced myocardial hypertrophy and increased the number of activated $CD4^+$ T subsets in

the circulation (609). Mice deficient in CD4+ T cells do not display a downregulation either of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a), which mediates the rate of myocardial relaxation through regulation of Ca^{2+} re-entry from the cytoplasm to the sarcoplasmic reticulum or of the MYH6 gene, which encodes the alpha heavy chain subunit of cardiac myosin, the cytoskeletal motor protein found in the cardiac muscle cells, following TAC (609). Similarly, expression of ANP and BNP is less pronounced in mice deficient in CD4+ T cells compared with wild type mice following TAC, thus further supporting the role of $CD4^+$ T cells in promoting HF (609).

In order to investigate whether T-cell recruitment to the LV participates in the development of HF and particularly nonischemic HF, a study utilizing real-time video microscopy of T cells from mice with pressure overload HF induced by TAC as well as of human T cells and LV tissue from nonischemic end-stage HF patients, revealed augmented T cell adhesion to activated vascular endothelial cells under physiological flow conditions in vitro compared with T cells from sham mice and healthy individuals respectively (613). T cells activation in the mediastinal lymph nodes and T cell infiltration into the LV myocardium together with the activation of the intramyocardial endothelium following TAC correlated directly with the development of LV myocyte hypertrophy, fibrosis and systolic dysfunction (613). Identification of T-cell recruitment into the LV as a negative contributor to pathological cardiac remodeling in HF suggests that reduction of T-cell infiltration might represent a novel translational target in HF (613). Antigen recognition by T cells seems to be essential for inducing the pathology associated with TAC, since blockade of T-cell costimulatory molecules on APC and depletion of bone marrow-derived CD11c dendritic cells significantly reduce LV fibrosis and hypertrophy in the setting of TAC (613). Various antigens have been examined with regard to their role in activation of T cells in pressure overload induced cardiac hypertrophy. Thus, mice with transgenic TCR, which have CD4+ T cells recognizing chicken ovalbumin, did not develop cardiac dilation following TAC (609). However, when these mice were crossed with mice which express ovalbumin on cardiomyocytes, the presence of ovalbumin-specific T helper cells plus ovalbumin antigens on myocytes increased T cells infiltration and activation in heart and accelerated cardiac hypertrophy progression to HF after TAC (617). On the other hand, even though available evidence strongly supports the participation of T-cells and effector T-cell subsets in the pathogenesis and progression of HF, the role for potential peptide antigens resulting from cardiac cell death and stimulating T-cell responses in this setting is unlikely, considering that cardiac apoptosis following TAC occurs at a significantly later time point compared to the timing of T cell infiltration to the heart which occurs as early as 2 weeks post TAC (613). Nevertheless, specific antigens involved in the development of cardiac hypertrophy in pragmatic pathophysiologic conditions like pressure overload remain to be identified. Disruption of immune tolerance mechanisms in the setting of pressure overload might be a plausible mechanism, allowing self-cardiac antigens such as α-myosin which is not expressed in the thymus, to induce a T-cell immune response as occurs in autoimmune myocarditis (613, 618). In addition, mechanisms involving Th1 lymphocytes related release of cytokines

altering myocyte and fibroblast function might be further involved in the induction of cardiac fibrosis and hypertrophy, since a significant upregulation of the Th1 signature cytokine, IFNγ and T-bet, the key transcription factor that directs the differentiation and activation of Th1 lineage cells, was noted in wild type mice but not in TCR knock-out mice (574, 613, 619). Of note, adoptive transfer of Th1 cells into mice lacking the αβ T cell receptor partially reconstituted myocardial fibrosis and dysfunction, supporting a central profibrotic role for Th1 cells (631). Abatacept, the cytotoxic T-lymphocyte-associated protein 4 (CTLA- 4) immunoglobulin fusion protein, which binds to CD80/86 on APCs and disrupts the interactions between co-stimulatory molecules CD28 on T cells and CD80/86 on APCs, thus suppressing T cells functions, showed impressive beneficial effects by attenuating cardiac hypertrophy and cardiac systolic and diastolic dysfunction compared to phosphate buffered saline-treated controls in the TAC mouse models (620, 621). Overall, pressure overload might exert mechanical force on the heart and cause the release of DAMPs and subsequent activation of the TLR signaling pathway in APCs such as macrophages and dendritic cells which would eventually result in cytokines secretion, stimulation of T cells activation and recruitment to the heart (622). T cells interact with endothelial cells and penetrate into the inflammatory sites with the mediation of cell adhesion molecules (622). Upregulated expression of E-selectin, VCAM-1 and ICAM-1 in the endothelial cells of the LV intramyocardial vessels together with more T cells infiltrating into the left ventricle were detected in a TAC mouse model (623). In contrast, mice lacking ICAM-1 exhibited attenuated T cell accumulation in the heart as well as less severe cardiac fibrosis and cardiac systolic dysfunction than wild type mice in response to TAC, thus underscoring the significant role of cell adhesion molecules in T cells infiltration to the heart (622). Overall, these findings further suggest that ICAM-1 normally mediates the ability of activated T cells to bind to the endothelium and infiltrate into the pressure overloaded LV to induce functional and structural abnormalities. Thus, pressure overload induces systemic changes in T-cell activation state and infiltration potential rendering them more capable of adhering to the cardiac endothelium with T-cells isolated from mice after TAC displaying enhanced adhesion to endothelial cells in ex vivo adhesion assays under flow conditions, directly identifying that pressure overload induces primary changes in these cells (613). The dominant role of T cells in the process of LVH hypertrophy is further emphasized in results from analyses of human LV tissue obtained from patients with HF due to aortic stenosis, which showed significantly higher accumulation of T cells compared to healthy myocardial tissues (622). With the use of a transgenic mouse model that reports the expression of both CXCL9 and CXCL10, called REX3 or Reporting the Expression of CXCR3 ligands, REX3 mice, it was demonstrated that cardiac fibroblasts and myeloid cells are sources of CXCL10 and less so of CXCL9, which attract CXCR3 Th1 cells to the heart in a lymphocyte function-associated antigen 1 (LFA-1), an integrin and ICAM-1-dependent manner in response to TAC (624). Notably, CXCR3 mice did not develop cardiac fibrosis and dysfunction in response to TAC as well as had reduced T-cell infiltration in the heart. On the other hand, CCR2 macrophages, which precede T-cell infiltration to the heart were detectable in the heart and produced

CXCL10 and CXCL9, thus supporting that early recruitment of CCR2 macrophages post-TAC induces subsequent T-cell recruitment to the heart (624). Considering that Th1 cells are drivers of cardiac fibrosis associated with TAC, their marked expression of the chemokine receptor CXCR3 as well as the fact that they do not infiltrate the heart in CXCR33 mice, the CXCR3-CXCL9 and CXCR3CXCL10 pathway is considered to directly influence T-cell infiltration in response to cardiac pressure overload.

Hypertension is another example of a strong pressure overload stimulus that induces cardiac hypertrophy and remodeling with angiotensin II infusion via subcutaneous osmotic minipumps as well as deoxycorticosterone acetate salt treatment failing to induce cardiac hypertrophy in Rag1−/− mice with no mature T or B cells, thus suggesting an essential role of the immune cells in hypertension development as well (625-627). On the other hand, adoptive transfer of T cells but not B cells restored the hypertensive responses to Angiotensin II infusion in Rag1−/− mice, thus highlighting the pathogenic implication of T cells (625-627).

CD4+ T cells are considered to exert a more substantial effect on cardiac hypertrophy and remodeling compared to CD8+ T cells. Several lines of experimental evidence underscore the dominant role of CD4+ T cells in the process of pathological pressure overload induced cardiac hypertrophy. Accordingly, a more significant reduction of activated CD4+ T cells than CD8+ T cells were observed in mouse hearts following conditional genetic depletion of the mineralocorticoid receptor in T cells, which was associated with improved cardiac remodeling profile as well as systolic and diastolic function in the setting of abdominal aortic constriction, indicating a more significant role of CD4+ T cells in promoting cardiac hypertrophy (628). Moreover, an increasing number of studies have demonstrated that interactions of tumor necrosis factor receptor superfamily, member 4 (OX40), a potent costimulatory receptor which accentuates TCR signaling on the surface of CD4+ and CD8+ T-lymphocytes and which leads to their augmented activation proliferation, survival, differentiation and cytokine production, with its ligand, affect the development of ACS as well as the survival of patients with ACS (629). Transferring only CD4+ T cells from spleens of donor wild type mice into mice lacking OX40 abolished the protection against cardiac hypertrophy and cardiac systolic and diastolic dysfunction offered by the OX40 gene knockout in the setting of aortic banding (629). In accordance with the above, mice unable to develop activated CD4+ T cells due to absence of MHC II expression following disruption of H2-Ab1 genes, which encode the light chains of the mouse MHC II, displayed less severe cardiac systolic and diastolic dysfunction in response to TAC (630). On the other hand, knockout mice without functional CD8+ T cells due to deletion of the gene encoding the α chain of CD8 displayed the same degree of hypertrophy and myocardial dysfunction as wild type mice after TAC (630). Still, evidence regarding the implication of CD8+ T-cells in the pathogenesis of LVH are controversial with single-cell RNA sequencing analysis from a mice model of TAC induced pressure overload, showed that CD8+ T cell depletion increased the degree of hypertrophy during the early compensatory phase but provided late-stage protection from HF (631). Additionally, CD8+T cells regulated the

macrophage expression of growth factor genes such as amphiregulin (Areg), a member of the EGF family, Oncostatin M (Osm), and insulin growth factor 1 (IGF1), which are considered to play an essential part in the myocardial adaptive response after cardiac pressure overload (631).

Cardiac hypertrophy pathways involve activation of pro-hypertrophic transcription factors, such as the nuclear factor of activated T cells (NFAT) which is calcium and calcineurin regulated, myocyte enhancement factor (MEF) and GATA-4 that mediate expression of hypertrophy-related genes in cardiomyocytes and initiate hypertrophy in response to pressure overload (632). NFAT1 is the predominant NFAT isoform in resting T cells and TCR activation causes the influx of calcium and the activation of calcineurin, which in turn dephosphorylates NFAT and drives its nuclear translocation to activate the expression of downstream genes (632). Modulation of cardiac fibroblasts-to myofibroblast transition could be one of the profibrotic mechanisms of Th1 cells. Joint cultures of CD4+ T cells purified from mediastinal lymph nodes of mice subjected to TAC with cardiac fibroblasts have shown that IFN-γ+Th1 cells were the subset that adhered to and stimulated TGF-β expression by cardiac fibroblasts as well as their transition from to myofibroblasts (633).

Several studies examining the mechanisms of acquired immunity in the pathogenesis of myocardial fibrosis have shown that T-cell infiltration in the myocardium is a both a prerequisite and a determinant factor for generation of LV fibrosis (615, 634). On the other hand, models of ischemic cardiomyopathy have shown different patterns of T-cells behavior compared to pressure overload models. It is well established that myocardial scarring after infarction does not simply represent replacement fibrosis of necrotic tissue with extracellular matrix and it is an accepted fact that T cells play a central role in orchestrating this process with T-cell infiltration to the myocardium directly modulating fibroblast phenotype and function during all the all stages of the process. Furthermore, in addition to their effects on the early phases of infarct scar formation, T cells within the heart appear to direct the remote fibrosis and scarring process throughout the LV during chronic remodeling. Cardiac repair mechanisms following MI involve a series of integrated events initiated by the first proinflammatory phase involving among others immune cell infiltration, including T cells, in response to cardiomyocyte necrotic death which is followed by the reparative phase in which a different array of immune cells and T cells promote healing of myocardial injury albeit at the expense of accentuated fibrosis (634). Taking as well into consideration the implication of Th1 T lymphocytes in the atherosclerotic plaques, one might infer that T cells are orchestrators of several responses, from destabilization of the atherosclerotic plaque inflammation and precipitation of ACS to the remodeling process post-MI (634). The specific pathways involved in the triggering of T-cell activation and recruitment to the heart post- MI, as well as the specific role for T cells during the different phases post-after MI are not thoroughly understood. The specific mechanisms underlying T-cell trafficking to the postischemic myocardium continue to be examined in different experimental models. Culprit antigens eliciting the T-cell response might include cardiac-

specific myosin heavy chain isoform or troponin-I, released by dying myocytes following MI, which are not expressed in the thymus and may be reactive to T cells that escaped central and peripheral tolerance (634). Thus, following necrotic injury, APCs internalize DAMPS released from the necrotic tissue and migrate into the lymph nodes where they activate T-cells into tissue-homing effector cells via a multistep activation process that includes upregulation of MHC class I and II to display cognate peptides to TCR, induction of surface expression of co-stimulatory signals such as CD28 and CD80/86, and production of cytokines including IFNγ for effector T-cell activation and polarization (615, 634). Antigen-mediated activation was proven to play an essential part for the protective function of CD4+ T-cells after MI in a transgenic mouse model expressing the mouse alpha-chain and beta-chain TCR that pairs with the CD4 coreceptor specific for chicken ovalbumin (OT-II) that inhibits TCR interactions with its ligand, cross-presentation of antigens, and central and peripheral T-cell tolerance and induction (612). Thus, the OT-II mice displayed a looser distribution and disarray of collagen fibers one week following MI compared to wild type mice whereas no differences were detected regarding collagen mRNA expression or MMP activity indicating that impaired processing of matrix proteins at the post-transcriptional levels are most probably responsible for the observed phenotype (612). Overall, a marked reduction of the Th1/Th2 ratio, an augmentation of the Th17/Treg ratio, and upregulation of Th2 cytokines were reported in the ischemic failing heart (634). The presence of Th1 cells in the setting of myocardial necrosis is considered to diminish fibroblast activation and limit fibrosis through both direct and indirect mechanisms via macrophage-dependent fibroblast activation (604). Th1 polarization is associated with enhanced cardiomyocyte apoptosis, dysregulation of extracellular matrix turnover and expression of collagen-I and -III mRNA as well as altered myofibroblast proliferation and differentiation, leading to cardiac rupture following ischemic necrosis of the heart muscle (634). IFNγ as the representative marker of Th1-mediated inflammation inhibits fibrosis by blocking TGF-β (604). In addition, IFNγ inhibits Th2-mediated fibroblast activation and indirectly regulates fibrosis through activation of macrophages with administration of IFNγ neutralizing antibodies reversing the TGF-β induction of α-smooth muscle actin expression, thus demonstrating that Th1 cells may be indirectly regulating fibrosis (635, 636). Experimental evidence indicates a prolonged Th1 response after MI with Th1 cells remaining elevated in the LV and in the circulation for two months post-MI (637). Similarly, clinical data have shown that Th1 cells increase in the circulation of patients with acute MI and unstable angina within 24 hours following the onset of symptoms, however in post-MI patients Th1 cells remained elevated for a longer time following hospitalization compared to patients with unstable angina (638).

The percentage of circulating CD4+ T cells expressing inflammatory cytokine such as IFN-gamma is increased in patients with ischemic HF compared with those with stable angina or healthy subjects and has been positively correlated with left ventricular dysfunction (610). Notably, even though myocardial CD4+ T cells are associated with infarct size expansion, experimental studies also support a requirement of CD4 cells for healing postinfarct. Still, the exact role that T-cells play to coordinate the resolution phase has not

been explored in depth to date (634). Even though CD4+ T cells are necessary to promote wound healing in the experimental model of permanent ischemia, in the ischemia-reperfusion injury model, CD4+ T cells appear to have deleterious effects by amplifying inflammation and promoting myocardial damage in the early stage of postischemic reperfusion (634). Thus, the extent of myocardial injury immediately post reperfusion is decreased in RAG mice, which lack T cells and B cells, compared with wild-type mice and antibody depletion of CD4+ T cells, but not of CD8+ T cells in wild-type mice before reperfusion, abolished post-reperfusion injury thus demonstrating that CD4+ T cells are responsible for this pathologic entity (634). Initial findings to support T-cell extravasation from the blood vessels to the heart are supported by observations of increased T lymphocytes in the myocardium within 2 minutes of reperfusion in parallel with a decrease in peripheral blood T lymphocytes as early as one hour after reperfusion post-MI, suggesting accelerated T-cell recruitment (638).

Although CD4+ T-cells may promote healing of myocardial injury in the acute phase post MI, they are involved in the pathogenesis of LV remodeling during the development of ischemic HF in the chronic setting. Thus, the question arises as to whether CD4+ T-cell activation is responsible for this pathological response, or whether distinct T-cells subsets are activated during ischemic necrosis as compared to HF. Notably, cardiac CD4+ T-cells exhibit temporally distinct patterns of activation with the first underlying a prompt CD4+ T-cell response peaking at the third day following MI and the second wave of $CD4^+$ T-cell activation and transmigration observed at 8 weeks following MI with these biphasic CD4+ T cell kinetics including all T-cell subsets such as Th1, Th2, Th17, and T-regs (639). Furthermore, depletion of CD4+ T-cells during chronic HF blunted the development of progressive end-systolic and end-diastolic volume increase and EF reduction from 4 to 8 weeks post-MI (639). These data indicate potentially divergent roles of CD4+ T-cell during HF and during MI (639).

The role of CD8+ T-cell response in post-MI remodeling is complex and involves both detrimental effects together with beneficial cardiac wound healing properties (615). Furthermore, CD8+ T-cell regulation of CD4+ T-cell recruitment might also affect collagen scar formation (615). In a mouse model of permanent ischemia, levels of CD8+ T-cell increase immediately during the first day following MI and remain elevated for two weeks (634, 640). Moreover, plasma levels of the CD8+ T-cells secreted granzyme B, were found to correlate with LV end-diastolic diameter, suggesting that CD8+ T-cells may be involved in adverse post-MI LV remodeling (640). Even though the absence of functional CD8+ T-cells in mice has been associated with improved myocardial performance and survival compared to wild-type animals during the subacute phase following MI, yet animals that died did so due to cardiac rupture, most probably due to delayed removal of the necrotic debris (641). On the other hand, administration of monoclonal antibodies causing depletion of CD8+ T-cells markedly increases both wound-breaking strength and collagen synthesis in mice (642). Strikingly, a decrease in circulating CD8+ T-cells following percutaneous coronary

intervention (PCI) was associated with poor prognosis, thus suggesting that effects from CD8+ T-cells vary according to a temporally-dependent pattern (643).

T-cell alterations accompany HFrEF. The first clinical data regarding the role T-cell activation specifically in HFrEF, derived from studies of patients with dilated cardiomyopathy, who demonstrated increased circulating levels of T-cell-related cytokines such as IL-2 and IL-10 compared with asymptomatic counterparts (634, 644). Additionally, the relative proportion of T-cell subsets appears to shift toward a general proinflammatory T-cell activation state in the setting of HFrEF and peripheral T cells isolated from patients with HF demonstrated increased expression of mRNA for TNFα, INF, Il-18, and Il-10 as well as increased surface expression of the T-cell activation markers such as CD25 and CD69 compared with healthy controls (614, 645). Human studies have revealed CD3+ T-cells infiltration in LV specimens from nonischemic cardiomyopathy patients with end-stage HF compared with nonfailing controls, with T-cells demonstrating enhanced adhesive properties to activated endothelial cells (613). Notably, CXCL9 and CXCL10 positively correlate with Th1 type cytokines in the peripheral blood of patients with symptomatic HF and CXCR3 T cells are infiltrated in the hearts of end-stage nonischemic HF patients (614).

In summary, while the explicit mechanisms of T cells homing to the myocardium in the setting of pressure overload or ischemic injury are not adequately clarified, several experimental models and multiple lines of clinical evidence support the role of T-cells and specifically CD4+ T cells as the primary cell type infiltrating the myocardium and producing LV fibrosis and dysfunction. Furthermore, it appears from these combined studies that the mechanisms of T-cell infiltration and promotion of fibrosis may differ between pressure overload or ischemia/reperfusion injury and infarction. Future research with the aim to elucidate the precise mechanisms of T-cell recruitment to the LV in these different pathological states may generate immune-specific therapeutic strategies in these patients (634).

The fundamental role of immune mechanisms within the kidney and heart in the pathogenesis of hypertension, which is mediated by T cells and their subsets such as γδ, CD4+ and CD8+ T cells, has been demonstrated by numerous studies (646). Thus, models of salt-sensitive rats with hypertension and CKD have shown that T cells infiltrate the kidney whereas administration of the immunosuppressant tacrolimus abolishes T-cell renal invasion, prevents renal damage and lowers BP (647). On the other hand, the adoptive transfer of T cells re-establishes the hypertensive response to angiotensin II in immunodeficient mice (648). In the setting of arterial hypertension, CD4+ T cells as well as γδ T cells produce cytokines such as IFNγ and IL-17A and facilitate the activation of CD8+ T cells (646). In particular, CD8+ T cells apart from participating in cytokine production within the general inflammatory responses, they also interact directly with the nephron structures to increase sodium retention through stimulation of the sodium chloride cotransporter in the deoxycorticosterone acetate and salt model of hypertension (649). IFNγ is the key cytokine in the process of CD8+ T-cells infiltration in the kidneys, where it stimulates the IFNγ receptor expressed within the distal convoluted tubule to promote the interaction

between the CD8+T cells and the tubule (650-652). IFN-γ and IL-17A production regulate the pressure natriuretic response in proximal tubule sodium transport and IFN-γ participates in mechanisms enhancing distal sodium reabsorption in the setting of angiotensin II hypertension, with mice lacking INFγ and Il-17 production displaying an abolished BP response (653). These findings have been replicated by clinical evidence revealing that the hypertensive stimulus of angiotensin II promotes accumulation of human T cells in the kidney, aorta, and lymph nodes of humanized mice, with CD8+ T-cell IFNγ production and CD4+ T-cells IL-17 production being significantly elevated (654). Furthermore, pro-inflammatory immunosenescent CD8+T cells with accentuated expression of perforin, granzyme B, IFNγ, and TNFα are increased in the peripheral blood of hypertensive patients (655).

Emerging evidence supports the causal implication of T cells in the pathogenesis of uremic cardiomyopathy phenotypes, including LVH, diastolic dysfunction and worsened myocardial strain. An experimental model of uremic cardiomyopathy in 5/6 nephrectomized mice assessed the expression of markers of T cell memory or activation as well as lymphocyte capacity for cytokine production and compared them to controls (656). Mice with CKD appeared to accumulate T cells expressing markers of memory differentiation such as CD44, a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration as well as activation markers such as PD-1, killer cell lectin-like receptor subfamily G member 1 (KLRG1), and OX40, as well as exhibited augmented cytokine secretion capacity in vitro (656). Additionally, flow cytometry analysis of immune cells isolated from mice heart tissue showed activated T-cells infiltrating the heart as early as two weeks following the establishment of uremic cardiomyopathy whereas next-generation RNA sequencing of uremic hearts identified enrichment for genes in pathways required for T cells recruitment, priming, and maturation (656).

During the last decade, a unique cytotoxic CD4+ T cell subset, identified by the loss of the costimulatory cell surface marker CD28, the CD4+CD28- T cells, has been identified, which display pronounced proinflammatory properties, possess the functional characteristics of professional killer lymphocytes and can increase from less than 1% to over 50% of the total CD4+ T cell population (657, 658). Accumulating evidence suggests that these cells are capable of infiltrating atherosclerotic plaques and probably play an important role in their destabilization, thus explaining, at least partly, the association of CVD with the inflammatory milieu (657, 658). Several recent studies have shown that the population of CD4+CD28- cells is expanded in patients with established atherosclerotic CVD and correlates with the recurrence of cardiovascular events (657). Notably, low CD4+ T-cell counts combined with the expansion of the CD28- T cells are a common feature of the elderly in general and of end-stage CKD patients (658, 659). A study examining whether elevated numbers of circulating CD4+CD- T cells may represent a risk factor for CVD in end-stage CKD analyzed data from 240 cytomegalovirus-seropositive patients. Apart from the traditional cardiovascular risk factors, both the percentage and absolute number of CD4+CD28- T cells were significantly associated with the presence of atherosclerotic disease (658). Thus, the

findings of this study suggest that circulating CD4+CD28- cells may represent a novel non-traditional risk factor in end-stage CKD patients.

Considering that sustained exposure to exogenous antigens induces the accumulation of the hyperactivated, proinflammatory and pro-atherogenic CD57+CD28-CD8+ T cells, persistent alloimmune responses in the setting of kidney transplantation may promote immune activation and contribute to posttransplant atherosclerosis. A single-center cohort study of 577 KTRs with a mean follow-up of 7 years showed that the cumulative incidence of atherosclerotic adverse events increased with the number of HLA mismatches whereas the recipients of a well-matched kidney exhibited a substantially reduced risk of adverse events (660). Notably, a significant association was observed between HLA mismatch numbers and levels of circulating CD57+CD28-CD8+ T cells, therefore suggesting that chronic allogeneic stimulation contributes to the accelerated atherosclerosis observed following transplantation (660).

5.3.2 T regulatory cells

Multiple lines of evidence from experimental and clinical studies have brought Tregs at the spotlight during the last years with regard to their important role in protecting against CVD, with a special focus on atherosclerosis, hypertension, MI, HF and abdominal aortic aneurysm, with a reduced number and impaired function of Tregs cells being present in a variety of cardiovascular diseases (661). Experimental in vivo models using the apolipoprotein E deficient mice have extensively examined the role of Tregs in atherosclerosis. Thus ApoE–/– mice displayed a significantly lower number of Tregs compared with their wild-type counterparts, whereas depletion of peripheral Tregs by anti-CD25 monoclonal antibodies was associated with expansion of the atherosclerotic lesion and increased plaque vulnerability (661, 662). On the other hand, adoptive transfer of Tregs to Apoe–/– mice led to a reduction in atherosclerotic lesion size and enhanced plaque stability and was associated with a decreased incidence of plaque rupture as well as a significantly lower accumulation of macrophages and T cells but with preservation of SMCs and collagen contents compared to plaques of control mice (661-664). The atheroprotective mechanisms via which Tregs might attenuate the cardiovascular risk remain a subject of ongoing investigation. Accordingly, a study found that the beneficial effects of T on the endothelium and on vulnerable atherosclerotic plaques were independent of effects on lipid status (661, 662). Thus, administration of Tregs in ApoE deficient mice significantly suppressed inflammatory cell accumulation, generation of foam cells and secretion of proinflammatory cytokines as well as stimulated M1 macrophages to convert to M2 macrophages (661, 662, 665). Tregs diminished the expression of two receptors responsible for the internalization of modified lipoproteins by macrophages, respectively scavenger receptor A (SRA) and CD36 (665). Inhibition of MMP-2 and MMP-9 mediated by increased secretion of anti-inflammatory cytokines such as TGF-β, IL-10, and IL-5, improved the stability of vulnerable plaques by increasing collagen content in atherosclerotic lesions (661, 662). The beneficial effects of Tregs on the resolution of atherosclerotic injury and plaque

reversal were examined through the use of multiple independent mouse models of atherosclerosis regression (666). Accordingly, increased levels of Tregs in plaques is a hallmark of regressing plaques, with single-cell RNA-sequencing of plaque immune cells showing that Tregs in these plaques did not express neurophilin 1 (Nrp1), a marker of thymus-derived natural Tregs, suggesting that they derive from a local expansion of Tregs from naïve T cells which can traffic between the circulation and the vessel wall (666). In contrast, Tregs from progressing plaques expressed markers of natural Tregs derived from the thymus as well as higher levels of memory T-cell markers, such as integrin B1 (ITGB1) and CD28, which have been associated with the reduction of Tregs during atherosclerosis progression (666, 667). To test whether Tregs are required for resolution of atherosclerotic inflammation and plaque regression, Tregs were depleted using CD25 monoclonal antibody in atherosclerotic mice during apolipoprotein B antisense oligonucleotide-mediated lipid lowering. Furthermore, Tregs appear to promote macrophage expression of receptors for specialized pro-resolving mediators which are molecules enzymatically derived from essential fatty acids, limit acute responses and mediate the clearance of tissue pathogens (666, 668).Thus, macrophage receptors such as G protein-coupled receptor 18 (GPR18), N-formyl peptide receptor 2 (FPR2), and Chemerin Receptor 23 (ChemR23), initiate signaling pathways to augment macrophage phagocytosis of apoptotic cells, suppress proinflammatory cytokine production, and increase anti-inflammatory cytokine production (666). On the other hand, morphometric analyses revealed that depletion of Tregs abolished plaque regression in the atherosclerotic mice undergoing aggressive reduction of hypercholesterolemia (666).

Polymerase chain reaction (PCR) measurements of the CD4+CD25+FOXP3+ marker of Tregs, have revealed its presence in the atherosclerotic plaques of human coronary arteries however the FOXP3+ Tregs count was low in all the stages of human atherosclerotic lesions, as evaluated in surgical or biopsy samples (669). In line with the above, the frequency of FOXP3+ Tregs is reduced in the peripheral circulation of patients with carotid artery plaques and a decreased Tregs count directly correlated with carotid atherosclerotic plaque vulnerability and inversely correlated with the infiltration of mature dendritic cells (670, 671). Clinical studies have reported conflicting results regarding the Tregs status in patients with CAD. Accordingly, patients with vulnerable coronary arterial plaques exhibit lower levels of Tregs compared with healthy individuals and likewise a smaller number and less efficient Tregs have been observed in patients with ACS compared with healthy controls (672-674). Decreased Tregs numbers are found in patients with MI, with Tregs counts displaying an inverse association with risk for MI (675, 676). Amplified rates of spontaneous apoptosis of Tregs, as demonstrated by decreased levels of the mRNA level of the antiapoptotic gene Bcl-2 and markedly higher levels the proapoptotic gene Bak, have been shown in purified Tregs from patients ACS and are at least partly deemed responsible for the decreased number of Tregs in this setting together with the impaired thymic output (674). Moreover, oxidized LDL has been shown to induce apoptosis of Tregs (674). Still heterogeneities in Tregs subsets examined in the published studies as well as in the flow

cytometry protocols utilized, might account for the inconsistent results regarding circulating Tregs numbers in patients with ACS. Thus, as an example, Tregs have been found to be reduced in patients with non-ST-segment elevation MI and increased in patients with ST-segment elevation MI (677).

Tregs appear to be implicated in the process of ischemic myocardial remodeling as occurs following MI, however available studies have shown contradictory results particularly regarding Tregs counts in this setting (661). In mouse models of permanent coronary occlusion, Th1 and Treg cells are among the predominant subsets which are found expanded in the mediastinal lymph nodes and the spleen. Overall, Tregs promote myocardial healing after MI through effects on monocyte and macrophage differentiation, suppression of inflammation, regulation of extracellular matrix degradation, and as a result prevention of adverse remodeling (661). Increased counts of Tregs but with impaired functional suppressive capacity have been observed in mice undergoing experimental MI, with Tregs being recruited to the infarcted mouse myocardium, where they modulate fibroblast phenotype and function (678, 679). Tregs co-cultured with cardiac fibroblasts led to reduced cardiac fibroblast transformation to myofibroblasts, decreased SMC actin and MMP-3 expression and diminished the contraction of fibroblast populated collagen pads, thus supporting a direct cell to cell contact mechanism involved in Tregs regulation of fibroblast function and matrix preservation (679). Two Tregs related cytokines have been considered to participate in the complex regulation of myocardial fibrosis, respectively TGF-β, a pro-fibrotic molecule and Il-10 which inhibits Th17-mediated fibrosis and downregulates VEGF expression thus regulating neovascularization in response to ischemia (615, 678). Transfer of Tregs attenuated interstitial fibrosis, MMP-2 activity and cardiomyocyte apoptosis through both direct effects on cardiomyocytes and indirect anti-inflammatory effects in a rat model of MI (680). Thus, infiltration of neutrophils, macrophages and lymphocytes as well as expression of TNF-α and IL-1β by cardiomyocytes were significantly suppressed as were CD8+ cytotoxic T lymphocyte responses (680). Additionally, Treg activation induces an M2-like macrophage polarization with depletion of Tregs being associated with an increased pro-inflammatory M1 macrophages response and impaired M2-like differentiation (681). In vitro, co-culture of Tregs with macrophages have shown that Tregs increase the expression of genes associated with healing such as osteopontin and arginase-1, which favor the formation of a solid collagenous scar (681). MMP2 is mainly expressed by cardiac fibroblasts in the heart and Treg transfer in a myocardial infarction model attenuated the augmented MMP-2 activity following coronary artery ligation and prevented adverse ventricular remodeling (661). Treg expansion induced by administration of CD28 antibodies significantly improved survival and reduced cardiac ruptures during the first week following MI, which should be principally ascribed to diminished MMP-mediated degradation of collagen within the infarcted tissue (681). In summary, increases in Tregs after MI appear to promote extracellular matrix deposition and scar formation, with Tregs displaying beneficial effects in ischemic cardiomyopathy through the suppression of excessive inflammatory responses and promoting stable scar formation

in the early stage of heart injury (661, 681). Treg-depleted mice exhibited reduced survival rates, reduced fractional shortening, and accentuated myocardial dilation following MI (681). Notably, in contrast to the healing role in the setting of acute ischemic injury, Tregs appear to acquire a proinflammatory phenotype in mice with chronic ischemic HF, thus promoting fibrosis and Tregs depletion in this setting had beneficial effects and reversed cardiac fibrosis as well as improved cardiac function in ischemic chronic HF (682).

Experimental models of hypertension in mice have shown a direct link between increased BP and low levels of FOXP3 expression, reduced Tregs number, and altered functional properties of Tregs (661). Tregs have been shown to protect endothelial function, whereas on the other hand reduced Tregs numbers are considered to be causally implicated with endothelial dysfunction and the development of hypertension (661, 683). Accordingly, small vessels such as mesenteric resistance arteries from hypertensive mice incubated with conditioned media of cultured Tregs displayed significantly improved endothelium-dependent relaxation responses which appear to be mediated by IL-10 (683). On the other hand, blockage of Il-10 signaling pathways abolishes the beneficial effect of Tregs on the regulation of endothelium-dependent relaxation. One of the putative mechanisms through which Tregs and IL-10 control in a paracrine mode the vascular endothelium-dependent relaxation is the attenuation of oxidative stress through the inactivation of NOX (683, 684). Furthermore, mice with endothelial dysfunction, adverse vascular remodeling and hypertension in the setting of aldosterone infusion, displayed attenuated aldosterone-induced increase in BP and arterial injury when injected with Tregs prior to aldosterone infusion (685). Similarly, clinical evidence indicates that an increase in the number of Tregs and related cytokines such as TGF-β and IL-10 was associated with reduced levels of BP in hypertensive patients treated with telmisartan, an AT1 receptor antagonist for 3 months (686). Furthermore, considering that the kidneys are key to regulation of BP as well as a principal site of action of Tregs, the hypothesis that the beneficial effects of Tregs on hypertension might also involve mechanisms related to the kidneys appears rationale (685). Thus, angiotensin II infusion for 2 weeks resulted in a 43% reduction in FOXP3+Tregs in the renal cortex of mice, as shown by immunofluorescence staining, whereas adoptive transfer of Tregs improved cell infiltration in the kidneys, attenuated inflammation and subsequent renal damage as well as rehabilitated the impaired endothelium-dependent relaxation of arteries and abolished the development of arterial hypertension (687). Arterial hypertension models of target organ damage also shed light on the role of Tregs on cardiac remodeling. Accordingly, Tregs transfer appears to alleviate cardiac hypertrophy, fibrosis and arrhythmogenic electric remodeling as evaluated by connexin 43 gap junction protein localization in angiotensin II-infused hypertensive mice, independently of BP lowering effects (688). Decreased proportions of Tregs among the CD4+ T cells together with elevated BP have been observed in stroke-prone spontaneously hypertensive rats compared to normotensive rats, whereas administration of IL-2 and anti-IL-2 monoclonal antibodies complex selectively induced Treg cells in vivo, delayed the development of hypertension and attenuated cardiomyocyte hypertrophy, in this way further reinforcing the pathological

implication of reduced Tregs during the hypertensive process (689). Similarly, scurfy mice, which are genetically deficient in Treg cells because of a mutation in the FOXP3 gene, exhibit exaggerated Angiotensin II infusion-induced resistance artery endothelial dysfunction and remodeling, oxidative stress, inflammation and finally hypertension (690). Furthermore, RAG1-/- T and B-cell deficient mice undergoing adoptive transfer of Scurfy T cells, displayed an augmented response to Angiotensin II including proinflammatory polarization of monocyte and macrophages, compared with RAG1-/- mice undergoing adoptive transfer of wild-type T cells, which contain Tregs, thus strongly supporting the protective role of Tregs in hypertension and LVH via the modulation of immune responses (690). Adoptive transfer of Treg cells attenuated MMP-2 expression in the aortic tissue of Angiotensin II induced abdominal aneurysm model and if we take into consideration that MMP2 knockout and MMP2 downregulation by long noncoding RNA intervention in mice is associated with reduced cardiac fibrosis and improved myocardial diastolic dysfunction in TAC models of pressure overload, we may conclude that Tregs mediate their effects on myocardial remodeling at least in part through effects on MMPs (691, 692). Overall, an inverse association Tregs counts has been reported to be inversely correlated with the severity of myocardial remodeling whereas transfer of Tregs appears to attenuate myocardial fibrosis, ventricular hypertrophy, and electrical remodeling in mouse models of hypertension (693, 694).

Tregs display alterations in both their phenotypes and functions in established chronic HF with acquisition of a Th1-like proinflammatory phenotype as well as expression of antiangiogenic and profibrotic properties, which might be related to HF decompensation (695). Additionally, the suppressive function of Tregs on conventional T cells and the secretion of soluble fibrinogen-like protein 2, a novel effector factor of Tregs in HF, are reduced in the setting of HF (696-698). However, the culprit mechanisms implicated in the modification of the characteristics of Tregs in various pathological states, including HF, merit further investigation. Clinical studies have shown decreased frequency of circulating Tregs and suppressed Tregs function in patients with chronic HF compared with healthy subjects with increased apoptosis as well as impaired thymic output being suggested as potential mechanisms (696, 699, 699). Furthermore, Tregs have been associated with the severity of left ventricular dysfunction and decreased Tregs counts might be utilized as an independent adverse prognostic indicator for rehospitalization and reduced survival in patients with HF (696). Accordingly, a ratio of Tregs to CD4+ T cells lower than 6% correlated with an increased incidence of recurrent hospitalization for worsening heart failure (700).
There is very little evidence available regarding the role of Tregs in the accelerated atherosclerosis and left ventricular remodeling in the uremic milieu. A study evaluating the balance between Tregs and Th17 cells and its significance with relation to CVD in maintenance hemodialysis patients showed that hemodialysis patients displayed a reduced of Treg to Th17 ratio compared to the healthy individuals. These patients had increased peripheral Th17 frequency and reduced Tregs frequency as well as elevated Th17-related cytokines such as IL-17, IL-6 and IL-23 but diminished Tregs-related cytokines including IL-10,

and TGFb1 (701). In contrast, no differences were observed either in Th17 cells or in Tregs between the dialysis patients and non-end stage CKD patients (701). Moreover, inflammatory markers such as CRP and IL-6 displayed a positive association with Th17 cells but they were inversely correlated with Tregs (701). Notably, hemodialysis patients with NYHA III–IV HF exhibited an increase in Th17 to Tregs cell ratio compared to patients with NYHA I–II HF (701).

5.3.3 B lymphocytes

B cells have emerged as essential immune cell subsets in the regulation of the atherosclerotic process and their effects are mediated by antibodies and cytokines in a subset specific manner (702). Research is ongoing in order to elucidate the mechanisms via which specific B cells subtypes participate in atherosclerosis which might be translated in the future into potential B cell–linked therapeutic strategies, such as immunization and B cell–targeted biologic treatments which have already taken center stage in clinical research and clinical practice for other diseases (702).

Landmark experimental studies have provided evidence on the protective role of B cells against atherosclerosis development (703, 704). Accordingly, splenectomy in apolipoprotein E gene knockout mice was associated with atherosclerosis progression whereas subsequent transfer of B cells to these mice hindered atherosclerosis exacerbation (703). In line with the above, LDL receptor–deficient mice displaying suppressed B-cell populations in the bone marrow to levels less than 1%, exhibited a 30% to 40% increase in the atherosclerotic lesion area (704).

By the same token, mice models of atherosclerosis have shed light on the pathogenic implication of specific B cells subpopulations in this setting (705-707). Thus, the $CD20^{+}CD27^{+}CD43^{+}$ B1 lymphocytes secrete IgM, which binds to oxidation-specific epitopes on LDL, thus preventing lipid uptake by macrophages as well as subsequent inflammatory cytokine production and as a result prevent the formation of foam cells and suppress inflammation (702). Likewise, IgM secreted from B1 lymphocytes binds epitopes on apoptotic cells thus facilitating their clearance (702). According to their expression of specific cell surface markers, murine B1 lymphocytes are further divided into CD5+ and CD5- which produce atheroprotective IgM in a T cell independent manner (702). On the other hand, B2 cells are considered to promote atherogenesis through production of pathogenic IgG, activation of T cells, and induction of proinflammatory cytokines such as IFN-γ (705, 708). Finally, both B1 and B2 cells may generate regulatory B cells which produce the anti-inflammatory IL-10 cytokines. Yet, even though there are abundant experimental data regarding the role of B-cell subtypes in animal models of atherosclerosis, clinical evidence remains limited at present. In accordance with the experimental evidence presented above, clinical data from a cohort of 504 patients undergoing coronary angiography, showed an inverse association trend of IgM to oxidation specific epitopes with angiographically significant coronary stenoses whereas IgG to oxidation specific epitopes displayed a positive correlation with angiographically significant coronary stenoses (709). Even though the

associations were lost at multivariate analysis, results of this study provide a framework for future investigations on the benefits of passive immunization with antibodies to oxidized LDL for atherosclerosis inhibition. A network-driven analysis involving whole-blood gene expression profiles and CAD single nucleotide polymorphism analysis constructed from 188 subjects with CAD and 188 control subjects matched for age and gender from the Framingham Heart Study with Bayesian networks pointed out the B cells genes relationship to CAD pathogenesis among the top 20 CAD key driver genes (702, 710). Furthermore, gene ontology enrichment analysis recognized B-cell activation, B-cell differentiation, and B-cell receptor signaling pathways to be significantly accentuated in CAD (702, 710). Increased numbers of unswitched memory B lymphocytes which express more IgM on their cell surface, have been associated with fewer cardiovascular events including death due to CVD, stroke, MI, percutaneous interventions for CAD or peripheral artery disease (711). In addition, the expression of chemokine receptor CXCR4 on circulating human CD20+CD27+CD43+ B1 cells has been inversely correlated with coronary atherosclerotic burden and plaque necrosis in intravascular ultrasound measurements IVUS with virtual histology, which coincided with increased protective IgM titers (712). Considering these data in combination with experimental results from mice displaying increased atherosclerosis in the setting of B-cell CXCR4 absence, we may reach the conclusion that B-cell CXCR4 exerts a casual role in atheroprotection (713). B2 cells in humans are capable of producing high-affinity IgG antibodies which have been shown to correlate with coronary artery stenosis in some human studies (702, 714). In this regard, IgG autoantibodies to malondialdehyde-modified LDL independently associate with new MACE in a multiethnic cohort of patients from the Dallas Heart Study (714).

Mature B cell recruitment and accumulation to the site of myocardial injury is observed following MI (716, 717); nevertheless, the roles of B cells in MI remain contradictory, a phenomenon which can be explained at least partly by the existence of distinct B cells subpopulations. Experimental models using a combination of in vitro and cell-specific reconstitution studies of MI in mice have shown that upon activation via TLR–mediated pathways, mature B lymphocytes selectively produce Chemokine (C-C motif) ligand 7 (CCL7)**,** a major CCR2 ligand, which mediates the signaling required for monocyte mobilization from the bone marrow and recruitment to the injured tissues such as the infarcted myocardium where they increase tissue injury and lead to impairment of myocardial function (717). On the other hand, genetically deficient mice for B-cell activating factor (BAFF), a major cytokine that regulates survival, maturation and differentiation of B2 lymphocytes, or mice administered antibodies against mature B lymphocytes or BAFF displayed suppressed CCL7 production and monocyte mobilization and consequently attenuation of myocardial damage and dysfunction (717). Mice models with an increased number of B cells exhibited a greater degree of adverse myocardial remodeling following MI with B lymphocytes coordinating the involvement in this process of diverse immune cell types from the heart pericardial adipose tissue (718). Pirfenidone, an anti-fibrotic agent has been shown to exert a cardioprotective role in murine hearts following MI by decreasing

$CD19^{+}CD11b^{-}$ lymphocytes expression and modulation of the gene expression changes induced by acute ischemic injury in myocardial B1 and B2 cells (719). Ligation of LAD artery so as to establish acute MI in B lymphocytes knock-out mice and wild type mice, showed that B-cell deletion reduced the expression of the cytokines TNF-α, IL-1β, IL-6, and TGF-1β as well as decreased the levels of mRNA from genes involved in collagen metabolism (720). Thus, B lymphocytes knock-out mice displayed reduced myocardial collagen synthesis, less myocardial fibrosis, lower LV end-diastolic and end-systolic diameter as well as maintained LVEF compared to wild type control mice (720). These experimental data have been replicated in human studies where patients with MI who had detectable concentrations of CCL7 in the circulation at the time of admission displayed substantially higher risk of death and recurrent MI after 2 years of follow-up compared to patients with no detectable CCL7, even after adjustment for several multivariable risk factors (717). Likewise, increasing levels of BAFF in the circulation were independently associated with increased risk of death and recurrent MI (717). In summary, ischemic signaling from the myocardium appears to trigger circulating B cells which sense damage and further generate a B cell–derived chemotactic signal to induce monocyte recruitment and inflammation in the injured cardiac tissue (716, 717). Yet, the mechanisms via which B are activated in the setting of myocardial ischemic injury as well as their role in the infarct area itself are currently obscure and remain to be elucidated. In contrast, to B cells subsets which produce cytokines that accentuate the inflammatory response early following MI, IL-10–producing B cells promote the resolution of inflammation in the setting of myocardial injury, thus attenuating myocardial injury and improving the outcome of acute MI (721). The cytokine IL-33 and the chemokine CXCL13 which appear to be preferentially expressed in pericardial adipose tissue, promote the expansion of IL-10-producing $CD5^{+}$ B cells (721). On the other hand, B cell-specific deletion of IL-10 in the setting of acute MI is associated with deleterious consequences, including exacerbation of myocardial damage, myocardial dysfunction and delayed resolution of inflammation (721). Of note, B1 lymphocytes secrete GM-CSF, which regulates IL-23 secretion by dendritic cells and IL-17 production by T cells, which mediate the healing myocardial responses following MI (722). Similarly, intramyocardial injection of bone marrow-derived immature B lymphocytes into the early post-ischemic myocardium of rats proved to be beneficial to cardiac function because it reduced in situ cell apoptosis and helped maintain the EF (723). A significant body of evidence supports the participation of various B cells subsets in the process of chronic cardiac remodeling (716). In line with above, cardiac B cells and splenic marginal zone B cells accentuate cardiac remodeling whereas IL-10-producing B cells display a beneficial role (716). With regard to regulatory B cells, they have been shown to exert beneficial effects on ventricular remodeling with decreased scar size and attenuated interstitial fibrosis in murine MI models (724). Accordingly, adoptive transfer of B regulatory cells suppressed the expression of CCR2 in monocytes and reduced pro-inflammatory monocytes infiltration in the hearts of mice (723) whereas administration of Il-10 antibodies abolished the cardioprotective effects of B regulatory cells (724). Elaborate phenotyping of B cells subsets expressed in infarcted hearts and mediastinal

lymph nodes draining the myocardium of mice with MI, revealed both polyclonal B cells with no antigen-specificity infiltrating the heart after MI via the CXCL13-CXCR5 axis, as well as a distinct subset exclusively found in the heart that expressed high levels of Cd69 as an activation marker, C-C-chemokine receptor type 7 (CCR7), CXC-chemokine receptor type 5 (CXCR5) and TGF-ß1 Investigating the mechanistic basis of B cells recruitment to the injured myocardium, Heinrichs et al. found that the CXCL13-CXCR5 axis might be a key driver of B cell cardio-tropism post-MI (725).

Even though most data regarding the implication of B cells in myocardial injury process stems from models of ischemic damage, it appears that toxin induced myocarditis as occurs in a genetic model of cardiac injury induced by diphtheria toxin administration as well as acute pressure overload in the setting of TAC lead to an increase in the number of myocardial B cells, thus supporting their role in different types of myocardial injury regardless of the type of the noxious stimuli (719, 726, 727). Experimental studies have shown that B cells participate in the development and progression of HFrEF via various mechanisms including antibody independent pathways (727). Nevertheless, until now published data have yielded heterogenous results. Patients with HFrEF display a greater prevalence of circulating CD19+ B lymphocytes, a higher prevalence of replicating B lymphocytes as well as higher counts of B lymphocytes expressing inflammatory cytokines (728). On the other hand, a marked reduction of both absolute and relative B-cell counts with a trend towards more differentiated B-cell subsets was observed in a cohort of 92 patients with HF in the setting of ischemic and dilated cardiomyopathy (728). Additionally, in these patients, the prevalence of TNF-α positive B lymphocytes correlated with the extent of myocardial fibrosis as evaluated by cardiac MRI (728). However, administration of medications for HF such as sacubitril/valsartan restored the B-cell lymphocytes counts towards normal levels (729). An interesting finding is that increased levels of circulating anti-inflammatory B regulatory lymphocytes producing IL-10 have been observed in patients with non-ischemic cardiomyopathy but not in patients in patients with ischemic HF (729). Furthermore, in vitro cultures of mononuclear cells isolated form patients with dilated cardiomyopathy contained fewer IL-10 secreting B cells and B regulatory lymphocytes counts were inversely correlated with HF severity (730). Accordingly, the aforementioned results further reinforce the hypothesis that different subsets of B lymphocytes possessing different properties participate with diverse and potentially contrasting mechanisms on the injured and failing myocardium via non-antibody dependent mechanisms.

B cells appear to be involved in HFpEF, however available evidence is limited. In a murine model of cardiomyopathy induced by the use of l-NAME and NaCl in the drinking water and angiotensin-II infusion, it was shown that B lymphocytes were required for the full-blown expression of the cardiomyopathy phenotype, independently of the hypertensive response (731). On the other hand, depletion of B lymphocytes reduced cardiac hypertrophy and collagen deposition as well as preserved LV function (731). In contrast, animals with normal B lymphocytes expression exhibited deposition of IgG3 in the myocardium and expression of Bax, a proapoptotic molecule, thus supporting the possibility

of cell injury being mediated by nonspecific activation of Fc gamma receptor and triggering of apoptotic pathways (731). On the contrary, B regulatory lymphocytes were shown to reduce myocardial remodeling in a murine model of acute pressure overload-induced myocardial hypertrophy and fibrosis (621). Even though clinical evidence is limited regarding the role of B lymphocytes in HFpEF, proteomic analysis of serum from patients with HF showed that patients with HFpEF upregulate serum markers compatible with activation of B cells (732).

Overall, the antibody independent mechanisms via which B cells promote myocardial injury and the development of HF involve immune cells chemotaxis and activation (733-735). Up-regulation of TGF-β and IL-6 and perpetuation of the pro-inflammatory state via TNF-α, IL-1β, and IL-6 production by B lymphocytes induce recruitment of monocytes and differentiation to pro-fibrotic macrophages, and increased expression of TGF-β, collagen-I, and IL-1β by fibroblast and macrophages (733-735). Furthermore, with regard to their role as APCs, activated B cells can activate $CD4^+$ T cells and promote their differentiation into the Th1 phenotype which in turn stimulate the transition of cardiac fibroblasts to to TGF-β and collagen-producing myofibroblasts as already described previously (733-735).
Data regarding the implication of B lymphocytes in the progressive atherosclerosis of CKD and the cardiorenal syndrome are scarce at present. A study aiming to characterize the cellular immune cell response in the kidney and heart tissue following AKI induced by renal ischemia and reperfusion showed a decrease in cardiac B lymphocytes together with a pronounced inflammatory profile in the heart tissue influenced by IL-17RA and IL-1β (736). On the other hand, a significant increase in CD4+ and CD8+ T cells as well as in M1 macrophages was observed in the renal tissue, where the repair response was characterized by characterized by Foxp3 activation (736). The results of this study suggest that only B cells contributed to the generated cardiac injury in the setting of type 3 CRS with the recruitment of B cells to the inflamed myocardial tissue probably being the consequence of specific modifications in the endothelium (736, 737).

A study of elderly patients with moderate-to-severe CKD showed that that the levels of CD19+CD5+ B lymphocytes were significantly decreased in moderate-to-severe CKD patients compared to non-CKD controls and displayed a significant independent association with IMT, which was increased in those with the lower levels of CD19+CD5+ B lymphocytes (738). Furthermore, Kaplan-Meier analysis showed that patients with lower levels of CD19+CD5+ and CD19+CD5- B lymphocytes exhibited worse survival (738). In line with the above, CD19+CD5+ and CD19+CD5- B lymphocytes have also been negatively correlated with myocardial remodeling-related echocardiographic indices in elderly patients with advanced CKD (739). Accordingly, CD19+CD5+ B lymphocytes were negatively correlated with LV end-diastolic dimension (LVDD), LV end-systolic dimension (LVSD) and LVM whereas LVEF was positively correlated with both CD19+CD5+ and CD19+CD5- B lymphocytes (739). Moreover, patients with higher CD19+CD5+ B lymphocytes levels displayed lower levels of pro-BNP, high sensitivity troponin (hsTn), interventricular septum (IVS), LVSD and LVM (739). On the other hand, patients with higher levels of CD19+CD5- B cells also displayed

lower levels of pro-BNP, hs-TN and LVSD, but higher levels of LVEF (739). A prospective study conducted in a cohort of prevalent hemodialysis patients showed that patients with low CD19+ B lymphocytes counts had higher all-cause and cardiovascular mortality (740).

6. Aims of the study

Cardiac remodeling is a hallmark of CKD, manifesting early during disease progression as myocardial fibrosis, LVH, impaired myocardial strain and eventually left ventricular diastolic and systolic dysfunction. Kidney transplantation is associated with significant improvements in left ventricular size and function as well as regression of LVH, otherwise known as reverses remodeling. Nevertheless, subclinical abnormalities in the biventricular strain may be observed in KTRs even when other classical indices of myocardial function such as the EF are normal. Maladaptive activation of the immune system plays an essential role in the pathogenesis of CKD and CVD. A significant body of experimental data and human research indicate that immunological pathways are implicated in all aspects of CVD phenotypes and have been well established in atherogenesis, viral myocarditis and inflammatory cardiomyopathy. The potential involvement of immune pathways in the pathogenesis of HF has come under the spotlight especially during the last decade, with the deleterious role of proinflammatory cytokines in the myocardium underlying the inflammatory paradigm of HF. The chronic inflammatory state, a CKD hallmark, is mediated and perpetuated by an intricate interaction of immune mediators and cellular components of the innate and adaptive immune systems. CKD progression itself is associated with complex alterations in innate and acquired immunity and disruption of regulatory immune processes. The roles of the cellular immune system components in the development of myocardial remodeling in CKD and kidney transplantation remain at present an open question. Accordingly, the intermediate CD14++CD16+ monocytes have been directly associated both with the atherosclerosis burden as well as with the occurrence of adverse atherosclerotic cardiovascular events in patients without and with CKD, however the role of the monocyte subsets in the LV remodeling of CKD and the CRS is unknown. About NK cells, although the few available data indicate that reduced circulating NK cells, with impaired activity, are found in patients with HF and kidney failure, studies on NK cells in CKD are limited. Regarding the acquired immune system cells, the role of T-lymphocytes subpopulations in CKD as well as their contribution to the development of LVH and subsequent progression to HF has recently gained increasing attention. Thus, taking into consideration the suggested links between the accumulation of pro-inflammatory T-cells to myocardial dysfunction in CKD and of the CD4+ T cells to the transition from hypertrophy to HF during chronic pressure overload the association of T cells subsets with subclinical indices of myocardial dysfunction in CKD merits further investigation. On the other hand, Tregs which are active players in maintenance of immune homeostasis and tolerance, display reduced numbers and impaired function in CKD as well as in various form of CVD including atherosclerosis, hypertension, and LV remodeling following MI; however, data

regarding their implication in CKD associated myocardial dysfunction are scarce. B-lymphocytes are diffusely depleted in the uremic milieu but there are no data available with respect to their role in LV remodeling and HF in CKD. Overall, the role of monocyte subsets as well as of T-lymphocytes and B-lymphocytes as putative factors causally implicated in myocardial dysfunction in the setting of CKD remains an open question. Particularly, there is a paucity of data regarding the involvement of immune cell subsets in the development of myocardial remodeling in CKD before the establishment of overt CVD. Likewise, the role of cellular immunity in the myocardial remodeling process following kidney transplantation remains unknown. Regarding the CRS, available data until now provide insight mainly into the cardiovascular complications of AKI and especially CKD, that is type 3 and type 4 CRS. On the other hand, scarce evidence has been generated regarding the potential alterations of the cellular components of the immune system in patients with CKD due to HF as occurs in type 2 CRS. Accordingly, considering the scant clinical evidence regarding the implication of immune cells subsets in the development of CKD associated cardiomyopathy and their role in CRS, this study investigated the expression and alterations of specific immune cell subsets in the peripheral blood of patients with non-end-stage CKD, kidney transplantation and CRS type 2. In particular, we focused on the identification of potential associations between immune cells subsets and indices of subclinical myocardial dysfunction before the development of overt CVD as well as on the evaluation of the prognostic significance of immune cells in these patient groups.

The primary aims of our study were to investigate:

1. Potential associations between a pre-specified panel of immune cells subpopulations in the peripheral circulation of non-dialysis CKD patients without established CVD with classical and novel, subclinical indices of myocardial performance as evaluated by two-dimensional STE.
2. Potential associations between a pre-specified panel of immune cells subpopulations in the peripheral circulation of KTRs without established CVD with classical and novel, subclinical indices of myocardial performance as evaluated by two-dimensional STE.
3. The prognostic value of a pre-specified panel of immune cells subpopulations in the peripheral circulation in patients with CRS type 2 with respect to overall and cardiovascular mortality.

The secondary aims of our study were to investigate:

1. The differences in the expression of the pre-specified immune cell subsets between the patients' subgroups and with healthy individuals. In particular, the following comparisons were contacted:

A) between CKD patients and a control group of healthy individuals.
B) between KTRs and a control group of healthy individuals.
C) between patients with type 2 CRS and a control group of healthy individuals.
D) between CKD patients and KTRs.

E) between patients with type 2 CRS and a selected group of patients from the CKD study group who were matched for gender and eGFR to patients with type 2 CRS.

2. The correlations of the pre-specified immune cell subsets in the peripheral blood of patients with non-dialysis CKD, kidney transplantation and CRS type 2 to important clinical and laboratory indices.
3. In a longitudinal follow-up sub-study, the potential clinical correlations of immune cell subsets in CKD patients and KTRs.

Specific Part

7. Study Methods

The study design included two arms, the cross-sectional arm and the prospective arm. Study design is depicted in Figures 5 and 6.

Figure 5 represents the flowchart of the study in CKD patients and KTRs. As presented above, the main objective of this cross-sectional arm was to investigate the expression of immune cell subsets in CKD patients and KTR and examine the correlations between the immune cell subsets with classical and novel indices of myocardial function. In addition, comparisons regarding the expression of immune cells subsets were made between CKD patients and KTRs, and the associations of immune cell subsets with clinical and laboratory parameters were investigated

Figure 6 represents a flowchart of the study design in patients with the CRS type 2.

With regard to patients with the CRS type 2, the cross-sectional arm of the study investigated the potential association of immune cell subsets with clinical and laboratory parameters in these patients and compared CRS type 2 patients to a group of CKD patients matched for age and eGFR, who were selected from the study's CKD cohort.

The prospective arm of the study had different objectives in CKD patients and KTRs on one hand and CRS-2 patients on the other, considering that among the three sub-groups, only CRS-2 patients were the ones with already established CVD and accordingly had the highest risk for adverse outcomes. Thus, the main objective of the prospective arm of the study was a follow-up analysis of the changes in the expression of immune cells subsets in the circulation at specific time points (at baseline and 24 months later), in CKD patients and KTRs and their potential associations to clinical outcomes including eGFR and proteinuria changes. With regard to CRS-2 patients, the main objective of the prospective arm of the study was to investigate the potential prognostic role of the immune cell subsets to the combined outcome of all-cause mortality and cardiovascular mortality. For this study arm CRS-2 patients, after baseline evaluation, were followed until the end of the established observation period or until the study endpoint occurred (which came first).

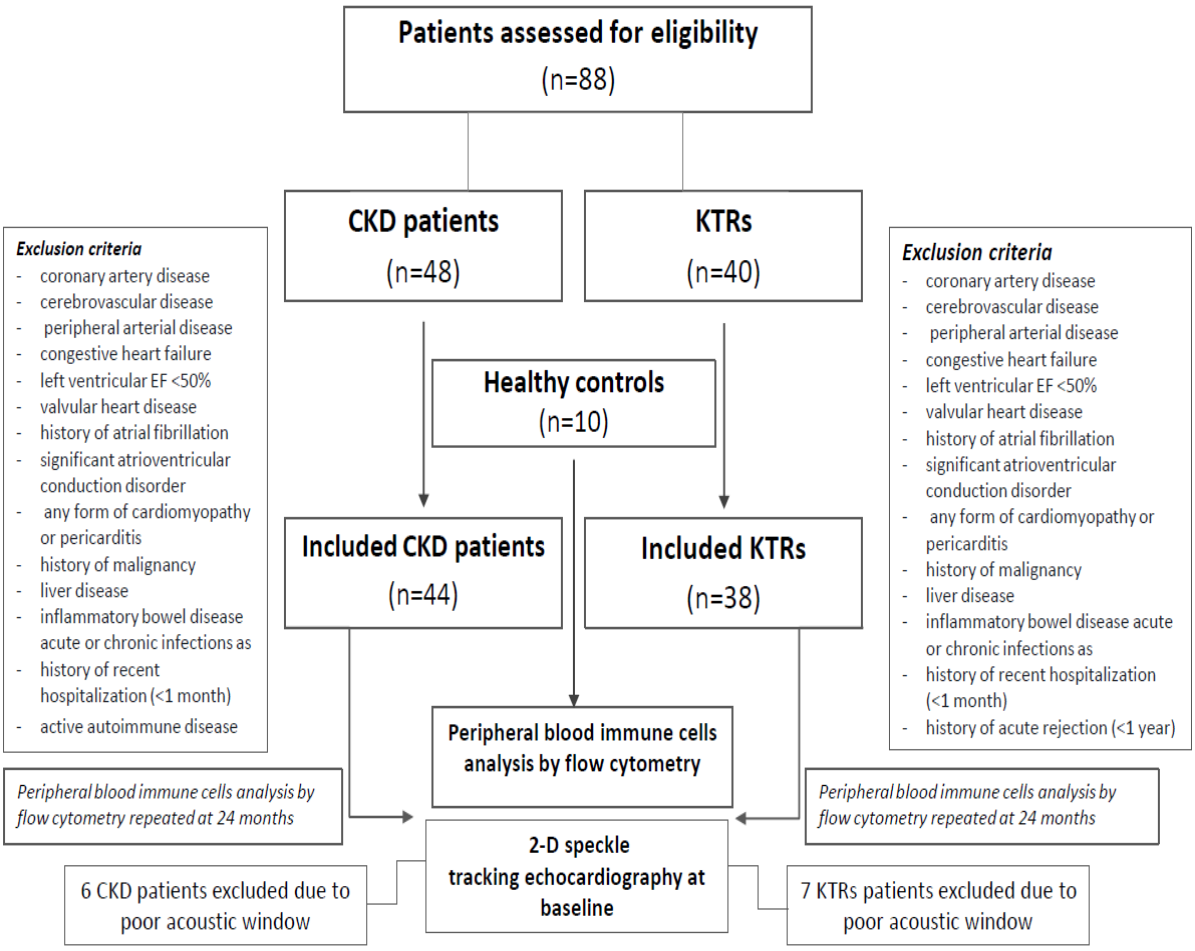

Figure 5. Flowchart of the study design in CKD patients and kidney transplant recipients.

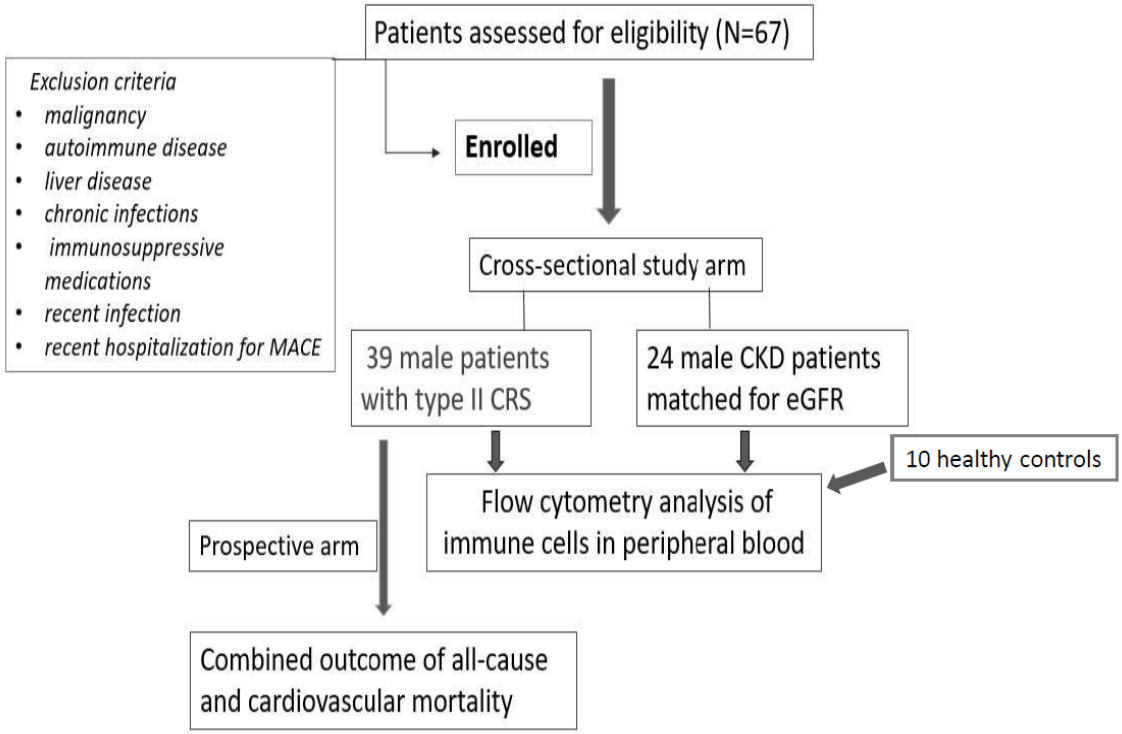

Figure 6. Flowchart of the study design in patients with type 2 CRS.

7.1 Study population

The single-center observational cohort study included:

- 44 consecutive patients with CKD who were under regular follow-up by the outpatient nephrology clinic of the Nephrology Department of the University Hospital of Ioannina (UHI).
- 38 KTRs who were under regular follow-up the kidney transplant unit of UHI
- 39 stable male patients with CRS type 2 under regular follow-up by the outpatient HF clinic and the CKD outpatient clinics of UHI
- control group including 10 healthy adults with no co-morbidities

7.1.1 Inclusion criteria

Participants were eligible to be included in the study only if all the following criteria apply:

1. Participants should be ≥18 years of age at the time of enrollment in the study.
2. Patients with CKD and patients with CRS type 2 should have an eGFR (CKD-EPI formula) ≥15 and ≤60 mL/min/1.73 m2 at the time of study enrollment.
3. Type 2 CRS was defined as chronic abnormalities in heart function (CHF-CHD) leading to kidney injury and/or dysfunction according to classification of cardiorenal syndrome (CRS) as proposed in the consensus conference on cardio-renal syndromes held in Venice Italy, in September 2008 under the auspices of the Acute Dialysis Quality Initiative (ADQI) (94).

4. Capable of giving signed informed consent

7.1.2 Exclusion criteria

The exclusion criteria applicable for patients with CKD, KTRs and patients with CRS-2 are presented in Table 3, Table 4 and Table 5 respectively. In specific, with regard to CKD patients and KTRs, the presence of established CVD was a major exclusion criterion.

Table 3. Exclusion criteria for patients with chronic kidney disease

Presence of established cardiovascular disease
History of atherosclerotic cardiovascular disease such as coronary artery disease, cerebrovascular disease or peripheral arterial disease
History of congestive HF or reduced left ventricular EF <60%
The presence of moderate-severe valvular heart disease
History of atrial fibrillation or significant atrioventricular conduction disorder
Any form of cardiomyopathy
Pericarditis
II. Active autoimmune disease requiring current treatment with steroids and/or other immunosuppressive medications
III. Any systemic immunosuppression therapy within 3 months prior to the baseline visit
IV. Other medical conditions
History of malignancy
Hepatic impairment corresponding to Child-Pugh B or C or other significant liver disease (e.g., acute hepatitis, chronic active hepatitis, cirrhosis as indicated by e.g. AST or ALT >3x ULN or total bilirubin >2x ULN) at study enrollment
Inflammatory bowel disease
Acute or chronic infections
History of recent hospitalization less than 1 month prior to study enrollment
Other condition limiting life expectancy to less than12 months

Table 4. Exclusion criteria for kidney transplant recipients

I. Presence of established cardiovascular disease
History of atherosclerotic cardiovascular disease such as coronary artery disease, cerebrovascular disease or peripheral arterial disease
History of congestive HF or reduced left ventricular EF <60%
The presence of moderate-severe valvular heart disease
History of atrial fibrillation or significant atrioventricular conduction disorder
Any form of cardiomyopathy
Pericarditis
II. Active autoimmune disease
III. History of acute rejection less than 1 year from enrollment in the study

IV. Other medical conditions
History of malignancy
Hepatic impairment corresponding to Child-Pugh B or C or other significant liver disease (e.g., acute hepatitis, chronic active hepatitis, cirrhosis as indicated by e.g. AST or ALT >3x ULN or total bilirubin >2x ULN) at study enrollment
Inflammatory bowel disease
Acute or chronic infections
History of recent hospitalization less than 1 month prior to study enrollment
Other condition limiting life expectancy to less than12 months

Table 5. Exclusion criteria for patients with type 2 cardiorenal syndrome

I. Recent hospitalization less than 1 month for any major adverse cardiovascular event including acute myocardial infarction, stroke, hospitalization for unstable angina or revascularization procedures and/or or heart failure decompensation
II. Active autoimmune disease requiring current treatment with steroids and/or other immunosuppressive medications
III. Any systemic immunosuppression therapy within 3 months prior to the baseline visit
IV. Other medical conditions
History of malignancy
Hepatic impairment corresponding to Child-Pugh B or C or other significant liver disease (e.g., acute hepatitis, chronic active hepatitis, cirrhosis as indicated by e.g. AST or ALT >3x ULN or total bilirubin >2x ULN) at study enrollment
Inflammatory bowel disease
Acute or chronic infections
History of recent hospitalization less than 1 month prior to study enrollment
Other condition limiting life expectancy to less than 12 months

The Ethical Committee of the University Hospital of Ioannina approved the study protocol, and all participants provided fully informed consent.

7.2 Evaluation of immune cell subpopulations by flow cytometry

For the evaluation of immune cell subpopulations blood samples were drawn from all participants under standardized conditions (WHO guideline on drawing blood) and were analyzed by using standard techniques. In details, the peripheral blood immune cell subsets analysis was performed by flow cytometry in a whole-blood assay using 100 μl of whole blood, which was conducted within 8 h from blood sample withdrawal. Ethylenediaminetetraacetic acid (EDTA) blood-collecting tubes were used for the collection of 2ml of whole-blood samples from patients. The following conjugated monoclonal antibodies were used for analysis:

- CD45(BD)
- CD14(BD)
- CD16(BD)
- CD4(BD)
- CD8(BD)
- CD56(BD)
- CD3(BD)
- CD19(BD)
- CD25(BD)
- Fox-P3(eBioscienceTM)

Immune cells subtypes were analyzed using flow cytometry (FACSCalibur) and Cell Quest and FACSDiva software (BD Biosciences). 100 µl of whole-blood was added to flow cytometry tubes and incubated with 10 µl of the respective monoclonal antibodies for 20 minutes in laboratory conditions of low light intensity and room temperature according to the instructions of the manufacturer. 500 µl of Versalyse (Beckman Coulter) was added and incubated for 10min at room temperature (18–25◦C) protected from light, to lyse red blood cells. Samples were processed immediately for flow cytometry analysis. The data were analyzed using the CellQuest V3.1 software (Becton Dickinson).

Accordingly, the following immune cell subsets were measured:

- CD14++CD16- monocytes percentage and absolute number of cells out of the total monocytes
- CD14++CD16+ monocytes percentage and absolute number of cells out of the total monocytes
- CD14+CD16++ monocytes percentage and absolute number of cells out of the total monocytes
- NK cells (CD3+CD16+56+) absolute values and percentage out of the total lymphocytes
- CD3- CD19+ B lymphocytes absolute values and percentage out of the total lymphocytes
- CD3+ CD4+ T cells absolute values and percentage out of the total lymphocytes
- CD3+CD8+ T cells absolute values and percentage out of the total lymphocytes
- Tregs (CD4+CD25+ FoxP3+) absolute values and percentage out of the total lymphocytes

The peripheral blood immune cell subsets CD14++CD16-, CD14++CD16+ and CD14+CD16++ absolute values and percentages out of total monocytes and NK cells (CD3+CD16+56+), CD3-CD19+ B lymphocytes, CD3+ CD4+ T cells, CD3+CD8+ T cells and Tregs (CD4+CD25+ FoxP3+) absolute values and percentages out of total lymphocytes were measured by flow cytometry at baseline (T0) and after 24 months (T1) in CKD patients and KTRs. Delta (Δ) of immune cells subtypes was defined as their respective difference between T1 and T0. Regarding patients with type 2 CRS-, the peripheral blood immune cell subsets CD14++CD16-, CD14++CD16+ and CD14+CD16++ absolute values and percentages out of total monocytes and NK cells (CD3+CD16+56+), CD3-CD19+ B lymphocytes, CD3+ CD4+ T cells, CD3+CD8+ T cells and Tregs (CD4+CD25+ FoxP3+) absolute values and percentages out

of total lymphocytes were measured by flow cytometry only at a single time point during the baseline evaluation.

Figure 7 and Figure 8 represent flow cytometry analysis results from a patient with CKD and a patient with type 2 CRS respectively.

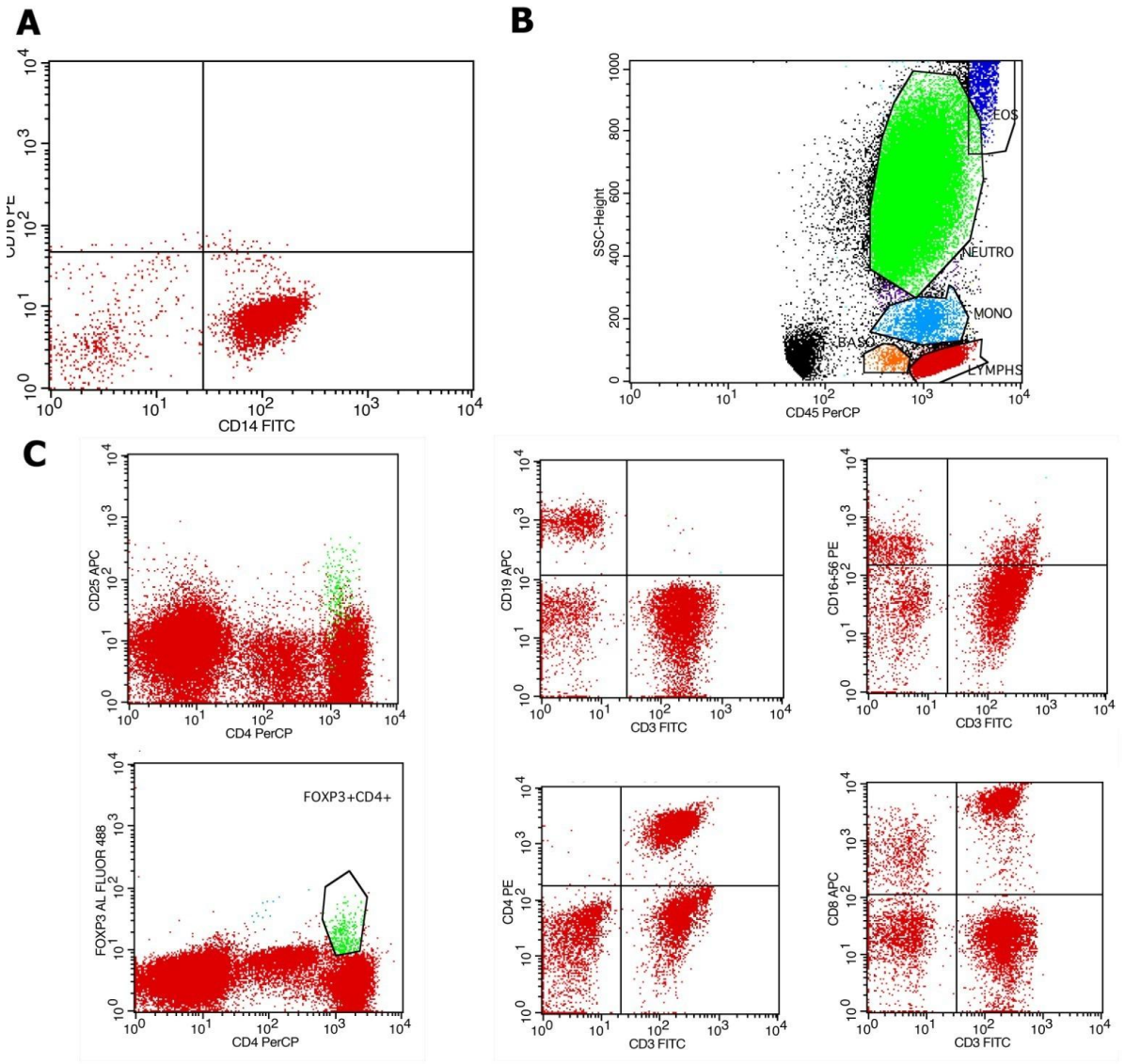

Figure 7. Flow cytometric analysis of a patient with CKD. (A) Representative dot plots depicting monocyte subsets (green color) according to surface expression of CD14 and CD16 in CD14++CD16-, CD14++C16+, and CD14+CD16++ subpopulations. (B) Representative dot plots depicting lymphocyte gating (red colour) with B-lymphocytes, T-lymphocytes, and natural killer (NK) cells defined as CD16+CD56+ cells, CD4+ T cells, and CD8+ T cells. (C). Representative dot plots depicting T regulatory cells (Tregs) defined as CD4+ FoxP3+ CD25high positive cells (green colour).

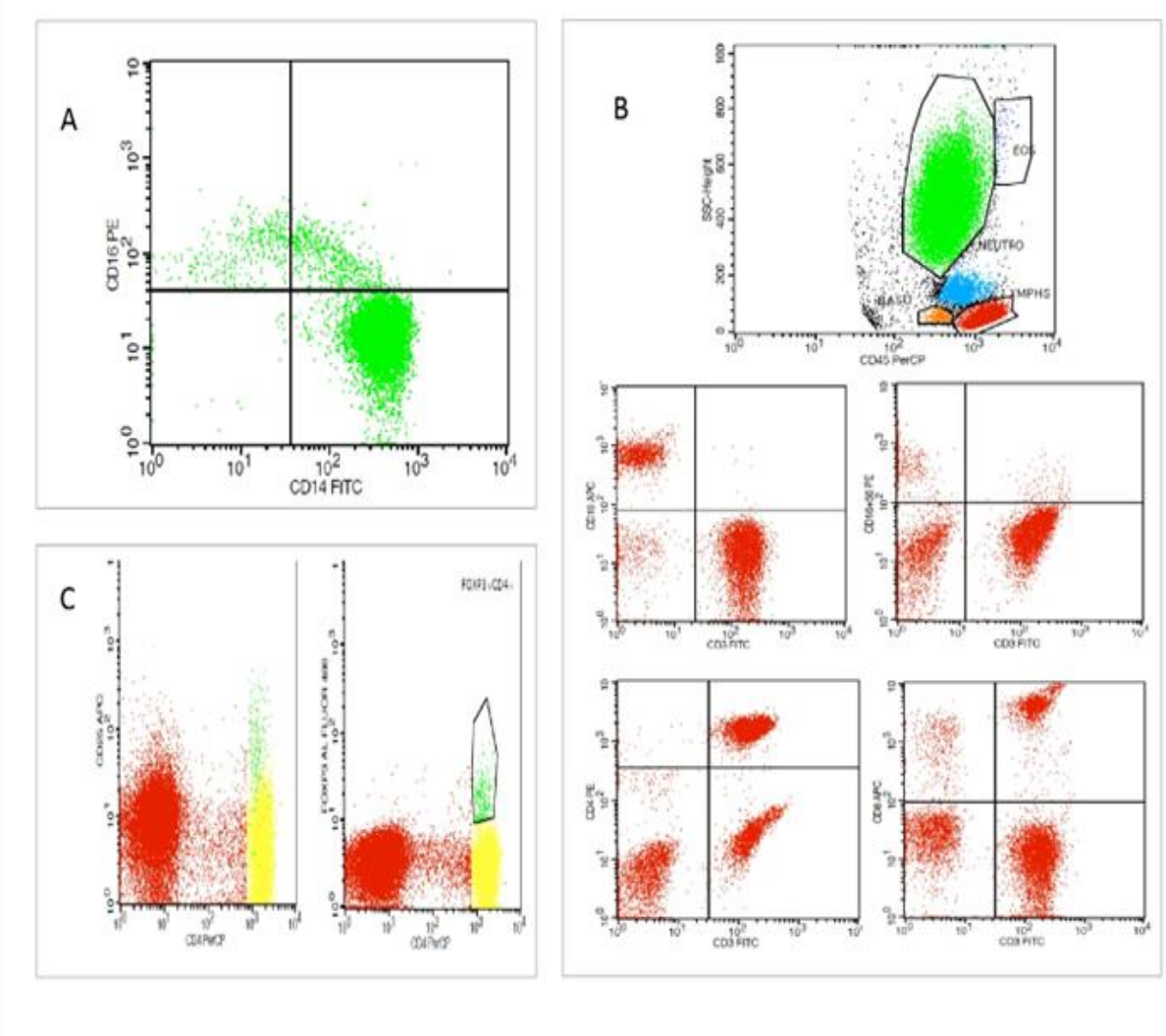

Figure 8. Flow cytometric analysis of a type 2 CRS patient. (A) Representative dot plots depicting monocyte subsets, differentiated according to their surface expression pattern of CD14 and CD16 in CD14++CD16–, CD14++C16+ and CD14+CD16+ subpopulations. (B) Representative dot plots depicting lymphocyte gating with B-lymphocytes, and T lymphocytes, natural killer (NK) cells defined as CD16+CD56+ cells, CD4+ T cells, CD8+ T cells. (C) Representative dot plots depicting regulatory T cells (Tregs) defined as CD4+ FoxP3+ CD25high positive cells.

7.3 The echocardiographic study

The echocardiographic evaluation was performed by a single operator using a Vivid 7 ultrasound machine (GE Vingmed ultrasound AS) in all patients. Standard echocardiographic views were used and acquired images and video loops were stored digitally in high analysis. A single observer blinded of the patients' identity performed offline analysis using EchoPac (version 113 - GE Vingmed ultrasound AS). The echocardiographic studies were performed within 1 month from immune cell subset analysis. Initially, a basic echocardiogram was

performed, and classical systolic and diastolic indices of ventricular function were obtained according to the European Society of Cardiology and European Association of Cardiovascular Imaging guidelines (37).

The following standard parameters were measured:

- coronary flow reserve (CFR)
- left atrial volume index (LAVI)
- left ventricle mass index (LVMI)
- relative wall thickness (RWT)
- ejection fraction (EF)
- tricuspid annular plane systolic excursion (TAPSE)
- mitral annular plane systolic excursion (MAPSE) septal
- MAPSE lateral
- early to late diastolic transmitral wave ratio (E/A)
- E’, early diastolic tissue wave velocity (E')
- E/E'
- medial wall systolic velocity (Sm)
- lateral wall systolic velocity (Sl)

In addition, a two-dimensional STE analysis was performed in both parasternal and apical views (at frame rates 60-90Hz). The endocardial left ventricular borders were manually traced (region of interest). Two-dimensional STE analysis included assessment of:

- global longitudinal strain (GLS)
- global radial (GRS)
- circumferential strain (GCS)
- left ventricular TWIST (calculated as the difference between apical and basal left ventricular rotation as it was assessed from equivalent short-axis views)
- UNTWIST rate (measured as the peak negative time derivative of twist during diastole)

Following the baseline echocardiographic evaluation, infusion of dipyridamole for 6 minutes (0.84mg/kg) was performed. A new echocardiographic assessment (focused mainly on left ventricular systolic and diastolic function indices) was performed. At the end of the dipyridamole infusion, 125-250mg of aminophylline was administered to the patient, to counteract any dipyridamole negative effect. Finally, the differences (Δ) between the values of measured echocardiographic parameters post and prior to dipyridamole infusion were calculated.

With regard to patients with type 2 CRS, echocardiographic data from ultrasounds performed by a skilled operator within 1 month from immune cell subset analysis were recorded including parameters for estimating ventricular function and morphology and for cardiac chamber quantification.

7.4 Clinical and laboratory assessment

Anthropometric and clinical data were recorded at baseline by patients' medical records, including comorbidities such as the presence of DM, arterial hypertension and medications. Specifically, regarding KTRs, dialysis vintage as well as the time from transplantation and the immunosuppressive regimen were recorded. Specifically, with regard to patients with CRS type 2 the presence of coronary artery disease (CAD), peripheral artery disease (PAD) and atrial fibrillation were recorded. Common biochemical parameters were measured in accordance with the standard methods applied in the hospital laboratory simultaneously with flow cytometry analysis. Complete blood counts and classical inflammatory markers including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were measured. Furthermore, serum levels of glucose, uric acid, total protein, albumin, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, calcium, phosphorus, intact parathyroid hormone (iPTH), and ferritin were determined. Urinary protein to creatinine ratio (UPCR) measurement assessment was performed in morning spot urine samples. Whole blood levels of calcineurin inhibitors (CNIs) - cyclosporine and tacrolimus)- were measured in KTRs using high-performance liquid chromatography (HPLC). NT-pro-BNP levels and high sensitivity troponin I (hsTnI) as respective markers of HF severity and subclinical myocardial damage were measured in patients with CRS type II.

Clinical and laboratory parameters were recorded at T0 and T1 in CKD patients and KTRs. Delta (Δ) eGFR (CKD-EPI) and the Δ spot urine protein to creatinine ratio (ΔUPCR) were defined as their respective differences between T1 and T0. Likewise, Delta (Δ) of laboratory indices was defined as their respective difference between T1 and T0.

7.5 Statistical analysis

Descriptive statistics are reported as mean ± standard deviation in normally distributed continuous variables, median and interquartile range (IQR) in skewed continuous variables. Dichotomous parameters are presented as frequency (percentage). Normal distribution of all continuous variables was tested with the parametric Shapiro-Wilk normality test. Differences between groups were determined by independent samples t-test or non-parametric Mann Whitney test, in normally and skewed continuous variables, respectively and the chi-square followed by a Fisher’s exact test for categorical variables (frequency distributions). Univariate correlation analyses were made by assessing the Pearson (R) or the Spearman (Rho) coefficients, as indicated. Linear regression model analysis was used to adjust for confounders in cell subtype differences among various patient subgroups. Multivariate association analysis was performed using stepwise linear regression analysis models that included all variables with a univariate association at the level of p value <0.1. P values were always two-sided and a value of $p<0.05$ was considered significant. Logistic regression analysis was used to identify predictors of mortality among

various cell subtypes in CRS patients. Accordingly, CRS patients were categorized as ≥median value or < median value based on cell subtypes with significant associations in logistic regression analysis. Kaplan-Meier curves were then generated and compared by a Log-Rank test for each of the immune cell subsets of interest. All endpoint analyses were conducted on a time-to-first event basis. The SPSS v23.0 software was applied to analyze all data, and the significance level was set at 0.05 in all cases.

8. Results

8.1 Characteristics of the patient cohorts

Table 6 summarizes the main baseline characteristics of the CKD and KTRs patient cohorts.

There were included 44 patients with CKD and 38 KTRs in the final study analysis. The mean age of KTRs was 53±9 years, whereas patients with CKD had a mean age of 63±11 years, respectively ($p<0.001$) whereas most patients were males in both groups. The cause of primary kidney disease was chronic glomerulonephritis in 7 patients, secondary focal segmental glomerulosclerosis (FSGS) in 9 patients, tubulo-interstitial kidney disease in 4 patients, hypertensive nephropathy in 5 patients, diabetic kidney disease in 7 patients and unknown in 10 patients. As for the presence of diabetes mellitus, 21% of KTRs and 39% of CKD patients were diabetics with no significant differences detected between the two patient groups. Additionally, the majority of patients in both groups had arterial hypertension. With regard to indices of renal function, KTRs displayed significantly higher median eGFR compared to CKD patients [(55 (IQR, 48-72) versus 24 (IQR, 15-41) mL/min/1.73 m2, respectively ($p<0.001$)]. Likewise, the median UPCR was significantly lower in KTRs (0.16 g protein/g creatinine (IQR, (0.09-0.56)) versus 1.31 (IQR, (0.23-2.61)) g protein/g creatinine ($p< 0.001$), respectively.

In specific, the majority of KTRs were on a triple immunosuppressive regimen including corticosteroids, a CNI and an antimetabolite (mycophenolate mofetil or mycophenolic acid) whereas only 12% of the KTRs were on a steroid free regimen. With regard to CNIs, 60% of KTRs were under treatment with tacrolimus whereas 40% received cyclosporine as part of their immunosuppressive regimen. Finally, no significant differences were observed between the two groups with regards to treatment with statins, ACE inhibitors or b-blockers.

There were included 39 patients with CRS 2 in the final study analysis who were compared to 23 patients with CKD matched for gender and eGFR and who were selected from the CKD cohort of our study. Table 6 summarizes the main baseline characteristics of the CRS 2 patient cohort as well as comparisons between CRS 2 patients and matched CKD patients. Mean age of patients with type 2 CRS was 72±10 years whereas patients with CKD had a mean age of 66±10 years respectively ($p=0.01$). As for the presence of diabetes mellitus, no significant differences were detected between the two patient groups. With

regard to indices of renal function, mean eGFR of patients with CRS and CKD patients was 37±14 and 33±16ml/min/1.73m2 respectively (p=0.28) whereas median urinary protein to creatinine ratio (UPCR) was 0.19 gr protein/gr creatinine (IQR, 0.10-0.52) versus 1.03 (IQR, 0.17-2.09) gr protein/gr creatinine (p=0.02) respectively. CRS patients displayed lower levels of total cholesterol (147±40 mg/dl vs 184±41 mg/dl, p=0.001), LDL cholesterol (84±35 vs 110±44, p=0.01) and triglycerides as compared to patients with CKD, whereas no significant differences were found between the use of statins. In specific, within the CRS patient group, 29 patients (74.3%) had ischemic cardiomyopathy in the setting of coronary artery disease (CAD) whereas 5 patients had dilated cardiomyopathy (12.8%). In addition, 13 patients (33%) with CRS had peripheral artery disease (PAD) and atrial fibrillation was present in 26 patients (66%). Left ventricular ejection fraction was less than 30% in 17 patients (44.7%) whereas with regard to NYHA class, 8 patients (21.1%) had NYHA class I, 14 patients (38.1%) had NYHA class II and 16 patients (41%) had NYHA class III HF respectively.

Table 6. Main clinical characteristics in all patients, in CKD patients and in kidney transplant recipients (KTRs). Statistically significant differences between subgroups are highlighted in bold.

	All Patients (N = 82)	**CKD Patients (N = 44)**	**KTRs (N = 38)**	***p*-Value ***
Age (years)	58 ± 11	63 ± 11	53 ± 9	**<0.001**
Males, N (%)		28 (64)	27 (71)	
DM, N (%)	26 (32)	17 (39)	9 (24)	0.147
Arterial Hypertension, N (%)	71 (86)	39 (88)	32 (84)	0.558
Transplantation vintage	-	-	77.5 (58–111)	-
eGFR (mL/min/1.73 m^2)	42 (20–57)	24 (15–41)	55 (48–72)	**<0.001**
UPCR (g protein/g creatinine)	0.32 (0.13–1.92)	1.31 (0.23–2.61)	0.16 (0.09–0.56)	**<0.001**
Hemoglobin (g/dL)	12.9 ± 2.0	12.4 ± 1.9	13.4 ± 2.0	**0.028**
Uric acid (mg/dL)	7.1 ± 1.7	7.4 ± 1.8	6.8 ± 1.5	0.123
ESR (mm/hour)	24 (13–34)	30 (21–42)	15 (12–26)	**0.001**
CRP (mg/L)	3 (2–6)	3 (2–6)	3.5 (3–7)	0.442

Glucose (mg/dL)	101 (91–115)	100 (89–114)	101 (93–118)	0.429
Albumin (g/dL)	4.2 (4–4.5)	4.2 (3.8–4.4)	4.2 (4–4.5)	0.253
Total proteins (g/dL)	7.0 ± 0.6	7.1 ± 0.6	7.0 ± 0.5	0.614
Total cholesterol (mg/dL)	187 ± 37	183 ± 43	190 ± 28	0.339
Triglycerides (mg/dL)	144 (113–192)	150 (113–200)	142 (113–166)	0.468
LDL cholesterol (mg/dL)	109 ± 35	108 ± 41	109 ± 26	0.835
HDL cholesterol (mg/dL)	46 (40–55)	43 (36–50)	50 (44–62)	**0.001**
Ferritin (ng/mL)	65 (40–108)	79 (48–112)	57 (31–104)	0.200
Calcium (mg/dL)	9.5 (9.1–9.7)	9.3 (8.8–9.6)	9.7 (9.4–10.1)	**<0.001**
Phosphorus (mg/dL)	3.4 (2.8–4.1)	3.9 (3.3–4.6)	2.9 (2.7–3.5)	**<0.001**
iPTH (pg/mL)	121 (85–226)	156 (88–294)	109 (74–169)	**0.048**
Cyclosporine N (%)	-	-	15 (40)	-
Tacrolimus N (%)	-	-	23 (60)	-
Statins N (%)	57 (70)	29 (66)	28 (74)	0.464
ACEI/ARB N (%)	48 (58)	25 (57)	23 (60)	0.656
B-blockers N (%)	49 (60)	23 (52)	26 (68)	0.115

Values are expressed as the mean (±SD) or median (IQR 25–75th percentiles). ACEI/ARB, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; iPTH, intact parathyroid hormone; N, number; UPCR, urinary protein to creatinine ratio. * *p* refers to *t* test significance for normal distribution variables, to the Mann–Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

Table 7. Main clinical characteristics in all patients, in patients with type 2 CRS and in matched CKD patients. Statistically significant differences between subgroups are highlighted in bold.

	All patients (N=63)	CRS patients (N=39)	CKD patients (N=24)	p-value
Age (years)		72±10	66±10	**0.01**
Diabetes Mellitus N, %	31 (47.7)	22 (56.4)	9 (37.5)	0.15
eGFR ml/min/1.73m^2	35±15	37±14	33±16	0.18
UPCR (gr protein/gr creatinine)	0.39 (0.12- 1.13)	0.19 (0.10-0.52)	1.03 (0.17-2.09)	**0.02**
Hemoglobin (mg/dl)	12.3(11.0-14.4)	11.8 (11.0-14.4)	13 (11.1-14.6)	0.78
Uric Acid (mg/dl)	7.0 (5.9- 8.4)	6.9 (5.5-7.8)	7.9 (6.05-8.9)	0.17
ESR (mm/hour)	33±18	33±19	32±15	0.87
CRP (mg/L)	4 (2-8)	4 (2-8)	4 (3-8)	0.92
Glucose (mg/dl)	107 (93-137)	120 (98-160)	99 (91-113)	**0.007**
Albumin (gr/dl)	4 (3.7-4.4)	3.9 (3.7-4.3)	4.2 (3.7-4.5)	0.26
Total Proteins (gr/dl)	7.0 (6.4-7.6)	6.9 (6.3-7.5)	7.2 (6.7-7.6)	0.17
Total cholesterol (mg/dl)	161±44	147±40	184±41	**0.001**
Triglycerides (mg/dl)	125 (94-178)	104 (77-155)	150 (118-216)	**0.006**
LDL cholesterol (mg/dl)	94±40	84±35	110±44	**0.01**
HDL cholesterol (mg/dl)	38±10	37±11	40±10	0.29
Ferritin (ng/ml)	76 (43-114)	63 (36-115)	88 (58-112)	0.31
Calcium (mg/dl)	9.4 (9.0-9.7)	9.4 (9.1-9.7)	9.4 (8.7-9.6)	0.16
Phosphorus (mg/dl)	3.7±0.7	3.7±0.6	3.7 ±0.8	0.83
iPTH (pg/ml)	130 (86-235)	134 (92-176)	128 (62-281)	0.55
hsTNI (ng/ml)	/	25.3 (16.4- 42.4)	/	/
NT-proBNP (pg/ml)	/	324 (184-797)	/	/
Statins N, %	49 (79.0)	32 (84.2)	17 (70.8)	0.22
ACEI/ARB N, %	30 (48.4)	14 (36.8)	16 (66.7)	**0.02**
B-blockers N, %	44 (71)	34 (89.5)	10 (41.7)	**0.000**

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). ACEI/ARB, angiotensin converting enzyme inhibitors/angiotensin receptor blockers; CKD, chronic kidney disease; CRP, C-reactive protein; CRS, cardiorenal syndrome; ESR, erythrocyte sedimentation rate; hs-TNI, high sensitivity troponin I; N, number; NT-proBNP, N-terminal pro hormone BNP; iPTH, intact parathyroid hormone.

8.2 Differences in the profile of immune cells subsets expression between the patient cohorts

The number and percentages of immune cells subsets in CKD patients, the number and the percentages of immune cells subsets in normal healthy controls as well as their respective differences are depicted in Table 8. Accordingly, a significantly lower percentage of the classical CD14++CD16- monocytes were observed in CKD patients [81.7 (IQR ,75.9–85.6)%] compared to healthy control subjects [87.7 (IQR, 85.7-92.5)%, p=0.009]. Likewise, the number of the classical CD14++CD16+ monocytes was lower in CKD patients as compared to healthy controls, however it did not reach statistical significance. Notably, both the number and the percentage of the pro-inflammatory, intermediate CD14++CD16+ monocytes were higher in CKD patients [32 (IQR, 24-53)/μL and 8.2 (IQR, 5.9-11.3)%] compared to healthy controls [16 (IQR, 13-18)/μL and 3.7 (IQR, 2.54-4.41)%], p=0.002 for both. Similarly, the number of the non-classical CD14+CD16++ monocytes were higher in CKD patients [25 (IQR, 19-36)/μL] compared to healthy controls [19 (IQR, 10-21)/μL], p=0.044. Finally, with regard to lymphocytes subsets, CKD patients displayed both a lower number and a lower percentage of B lymphocytes [94 (IQR, 61-161)/μL and 5.6 (IQR, 3.7-7.9)%] compared to healthy control subjects [224 (IQR, 171-261)/μL and 10.3 (IQR, 8.5-11.3)%], p= 0.001 and p=0.009 respectively.

Table 8. Immune cell subpopulations in CKD patients and healthy control subjects. Statistically significant differences between subgroups are highlighted in bold.

	CKD patients (N=44)	**Controls (N=10)**	**p-value**
WBC (N)	7045 (5745–8925)	7270 (6460-8570)	0.962
Monocytes (N)	400 (300–600)	450 (400-500)	0.981
Monocytes (%)	6.4 (5.3–7.4)	6.2 (6.0-702)	0.696
CD14++CD16- (N)	366 (258–438)	396 ±77	0.368
CD14++CD16- (%)	81.7 (75.9–85.6)	87.7 (85.7-92.5)	**0.009**
CD14++CD16+ (N)	35 (24–53)	16 (13-18)	**0.002**
CD14++CD16+ (%)	8.2 (5.9–11.3)	3.7 (2.5-4.4)	**0.002**
CD14+CD16++ (N)	25 (19–36)	19 (10-21)	**0.044**
CD14+CD16++ (%)	5.8 (4.3–8.2)	4.1 (2.1-4.8)	0.052
Lymphocytes (N)	1790 (1585–2405)	2188 ± 493	0.145
Lymphocytes (%)	26.4 ± 7.5	30.1 (27.1-34.3)	0.097
T-lymphocytes (N)	1376 (1114–1796)	1672 ± 387	0.237

T-lymphocytes (%)	76.7 ± 9.6	76.6 ±8.3	0.932
B-lymphocytes (N)	94 (61–161)	224 (171-261)	**0.001**
B-lymphocytes (%)	5.6 (3.7–7.9)	10.3 (8.5-11.3)	**0.009**
NK cells (N)	304 (178–370)	269 (240-305)	0.636
NK cells (%)	16.5 (11.3–19.3)	12.1 (8.6-17.6)	0.286
CD4+ T-Cells (N)	830 (595–1101)	1038 (768-1101)	0.303
CD4+ T-cells (%)	45.2 ± 10.2	46.9 ±6.7	0.636
CD8+ T-cells (N)	567 (411–781)	636 (408-724)	0.991
CD8+ T-cells (%)	32.1 (25.0–37.3)	24.9 (22.2-31.3)	0.201
Tregs (%)	1.75 (1.13–2.44)	1.9 (1.7-3.4)	0.325
T Regs (N)	33 (19–48)	57 (38-68)	0.112

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; NK, natural killer; No, number; Tregs, T regulatory cells. *p refers to t test significance for normal distribution variables, to Mann-Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

The number and percentages of immune cells subsets in KTRs as well as their differences to immune cells subsets in healthy controls are depicted in Table 9. Accordingly, KTRs displayed a higher number of total monocytes compared to healthy controls [600 (IQR, 400-800)/μL vs 450 (IQR, 400-500)/μL, p=0.028]. Additionally, the number of the proinflammatory intermediate CD14++CD16+ monocytes was higher in KTRs compared to healthy controls [25 (IQR, 16-45)/μL vs 16 (IQR, 13-18)/μL,p=0.016]. With regard to lymphocytes subsets, the percentage of total lymphocytes was lower in KTRs compared to healthy controls [24.7 ±8.5% vs 30.1 (IQR, 27.1-34.3)%, p=0.02]. In line with the above, both the number and the percentage of Tregs were lower in KTRs compared to healthy individuals [19 (13-28)/μL vs 57 (38-68/μL and 0.93 (0.63-1.71)% vs 1.9 (1.7-3.4)%, p=0.002 and p<0.001 respectively]. Finally, KTRs displayed a lower number as well as a lower percentage of B-lymphocytes compared to healthy controls [88 (33-140)/μL vs 224 (171-261/μL and 4.3 (1.9-6.7)% vs 10.3 (8.5-11.3)%, p<0.001 and p=0.002 respectively].

Table 9. Immune cell subpopulations in kidney transplant recipients and healthy control subjects. Statistically significant differences between subgroups are highlighted in bold.

	KTRs (N=39)	**Controls (N=10)**	**p-value**

WBC (N)	7710 (6920–10790)	7270 (6460-8570)	0.245
Monocytes (N)	600 (400–800)	450 (400-500)	**0.028**
Monocytes (%)	7.1 (5.9–8.7)	6.2 (6.0-702)	0.291
CD14++CD16- (N)	479 (354–599)	396 ±77	0.091
CD14++CD16- (%)	87.1 (83.6–90.1)	87.7 (85.7-92.5)	0.465
CD14++CD16+ (N)	25 (16–45)	16 (13-18)	**0.016**
CD14++CD16+ (%)	4.6 (2.8–7.3)	3.67 (2.54-4.41)	0.214
CD14+CD16++ (N)	18 (13–28)	19 (10-21)	0.636
CD14+CD16++ (%)	3.2 (1.9–5.4)	4.1 (2.1-4.8)	0.570
Lymphocytes (N)	2000 (1450–2710)	2188 ± 493	0.525
Lymphocytes (%)	24.7 ± 8.5	30.1 (27.1-34.3)	**0.02**
T-lymphocytes (N)	1732 (1156–2228)	1672 ± 387	0.957
T-lymphocytes (%)	81.4 ± 8.3	76.6 ±8.3	0.124
B-lymphocytes (N)	88 (33–140)	224 (171-261)	**<0.001**
B-lymphocytes (%)	4.3 (1.9–6.7)	10.3 (8.5-11.3)	**0.002**
NK cells (N)	257 (150–324)	269 (240-305)	0.636
NK cells (%)	13.2 (7.9–18.8)	12.1 (8.6-17.6)	0.914
CD4+ T-Cells (N)	835 (610–1299)	1038 (768-1101)	0.725
CD4+ T-cells (%)	47.4 ± 9.6	46.9 ±6.7	0.883
CD8+ T-cells (N)	612 (448–896)	636 (408-724)	0.552
CD8+ T-cells (%)	33.1 (28.4–37.1)	24.9 (22.2-31.3)	0.152
Tregs (%)	19 (13–28)	57 (38-68)	**0.002**
T Regs (N)	0.93 (0.63–1.71)	1.9 (1.7-3.4)	**<0.001**

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; NK, natural killer; No, number; Tregs, T regulatory cells. *p refers to t test significance for normal distribution variables, to Mann-Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

The differences between the peripheral blood levels of immune cell subpopulations between CKD patients and KTRs are shown in Table 10 and Figure 9. KTRs displayed both a higher number and percentage of classical CD14++CD16− monocytes [479 IQR (354–599)/μL and 87.1 (IQR, 83.6–90.1)%] compared to their CKD counterparts [366 (IQR, 258–438)/μL and 81.7 (IQR, 75.9–85.6)%, p = 0.001 and <0.001, respectively]. On the other hand, the

percentage of intermediate CD14++CD16+ monocytes was lower in KTRs [4.6 (IQR, 2.8–7.3)%] compared to CKD patients [8.2 (IQR, 5.9–11.3)%, p < 0.001]. Similarly, the number and percentage of the non-classical CD14+CD16++ monocytes were lower in KTRs [18 (IQR 13–28)/μL and 3.2 (IQR, 1.9–5.4)%] compared to their CKD counterparts [25 (IQR, 19–36)/μL and 5.8 (IQR, 4.3–8.2)%, *p* = 0.001 and <0.001, respectively]. With regard to lymphocyte subpopulations, KTRs displayed a higher percentage of T-lymphocytes (81.4 ±8.3%) and a lower percentage of B-lymphocytes [(4.3 (IQR, 1.9–6.7)%] compared to CKD patients [76.7 ± 8.3%, *p* = 0.02 and 5.6 (IQR, 3.7–7.9)%, *p* = 0.04, respectively]. Finally, KTRs had lower number and percentage of Tregs [19 (IQR, 13–28)/μL and 0.93 (IQR, 0.63–1.71)%, respectively] compared to CKD patients [33 (IQR, 19–48)/μL and 1.75 (IQR, 1.13–2.44)%, *p* = 0.002 and *p*< 0.001, respectively]. Following adjustment for confounders including age, eGFR and UPCR, the differences in immune cell subsets between the two groups remained statistically significant for the percentage of classical monocytes (*p* =0.02), both the number and percentage of non-classical monocytes (*p* < 0.001), the percentage of T-lymphocytes and B lymphocytes (*p* = 0.03 and *p* = 0.003, respectively) as well as the Tregs number (*p* = 0.008).

Table 10. Immune cell subpopulations in all patients, in kidney transplant recipients (KTRs) and in CKD patients. Statistically significant differences between subgroups are highlighted in bold.

	All Patients (N = 82)	**CKD Patients (N = 44)**	**KTRs (N = 38)**	***p*-Value**
WBC (N)	7485 (6080–9260)	7045 (5745–8925)	7710 (6920–10790)	**0.023**
Monocytes (N)	500 (400–600)	400 (300–600)	600 (400–800)	**0.001**
Monocytes (%)	6.7 (5.4–7.9)	6.4 (5.3–7.4)	7.1 (5.9–8.7)	**0.033**
CD14++CD16− (N)	415 (318–522)	366 (258–438)	479 (354–599)	**0.001**
CD14++CD16− (%)	83.7 (79.2–88.2)	81.7 (75.9–85.6)	87.1 (83.6–90.1)	**<0.001**
CD14++CD16+ (N)	31 (18–48)	35 (24–53)	25 (16–45)	0.095
CD14++CD16+ (%)	6.5 (3.6–9.2)	8.2 (5.9–11.3)	4.6 (2.8–7.3)	**<0.001**

CD14+CD16++ (N)	22 (15–32)	25 (19–36)	18 (13–28)	**0.012**
CD14+CD16++ (%)	4.6 (3.1–6.9)	5.8 (4.3–8.2)	3.2 (1.9–5.4)	**<0.001**
Lymphocytes (N)	1845 (1520–2560)	1790 (1585–2405)	2000 (1450–2710)	0.451
Lymphocytes (%)	25.6 ± 8.0	26.4 ± 7.5	24.7 ± 8.5	0.335
T-lymphocytes (N)	1474 (1125–2053)	1376 (1114–1796)	1732 (1156–2228)	0.173
T-lymphocytes (%)	79.0 ± 9.2	76.7 ± 9.6	81.4 ± 8.3	**0.026**
B-lymphocytes (N)	91 (48–157)	94 (61–161)	88 (33–140)	0.196
B-lymphocytes (%)	4.8 (3.2–7.4)	5.6 (3.7–7.9)	4.3 (1.9–6.7)	**0.042**
NK cells (N)	276 (170–358)	304 (178–370)	257 (150–324)	0.201
NK cells (%)	14.4 (9.7–18.1)	16.5 (11.3–19.3)	13.2 (7.9–18.8)	0.056
CD4+ T-cells (N)	830 (608–1187)	830 (595–1101)	835 (610–1299)	0.395
CD4+ T-cells (%)	46.2 ± 9.9	45.2 ± 10.2	47.4 ± 9.6	0.321
CD8+ T-cells (N)	582 (447–838)	567 (411–781)	612 (448–896)	0.370
CD8+ T-cells (%)	32.7 (26.5–37.1)	32.1 (25.0–37.3)	33.1 (28.4–37.1)	0.491
Tregs (N)	23 (15–39)	33 (19–48)	19 (13–28)	**0.002**
T Regs (%)	1.47 (0.81–2.02)	1.75 (1.13–2.44)	0.93 (0.63–1.71)	**<0.001**

Values are expressed as the mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; NK, natural killer; N, number per µL; Tregs, T regulatory cells. * *p* refers to t test significance for normal distribution variables, to the Mann–Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

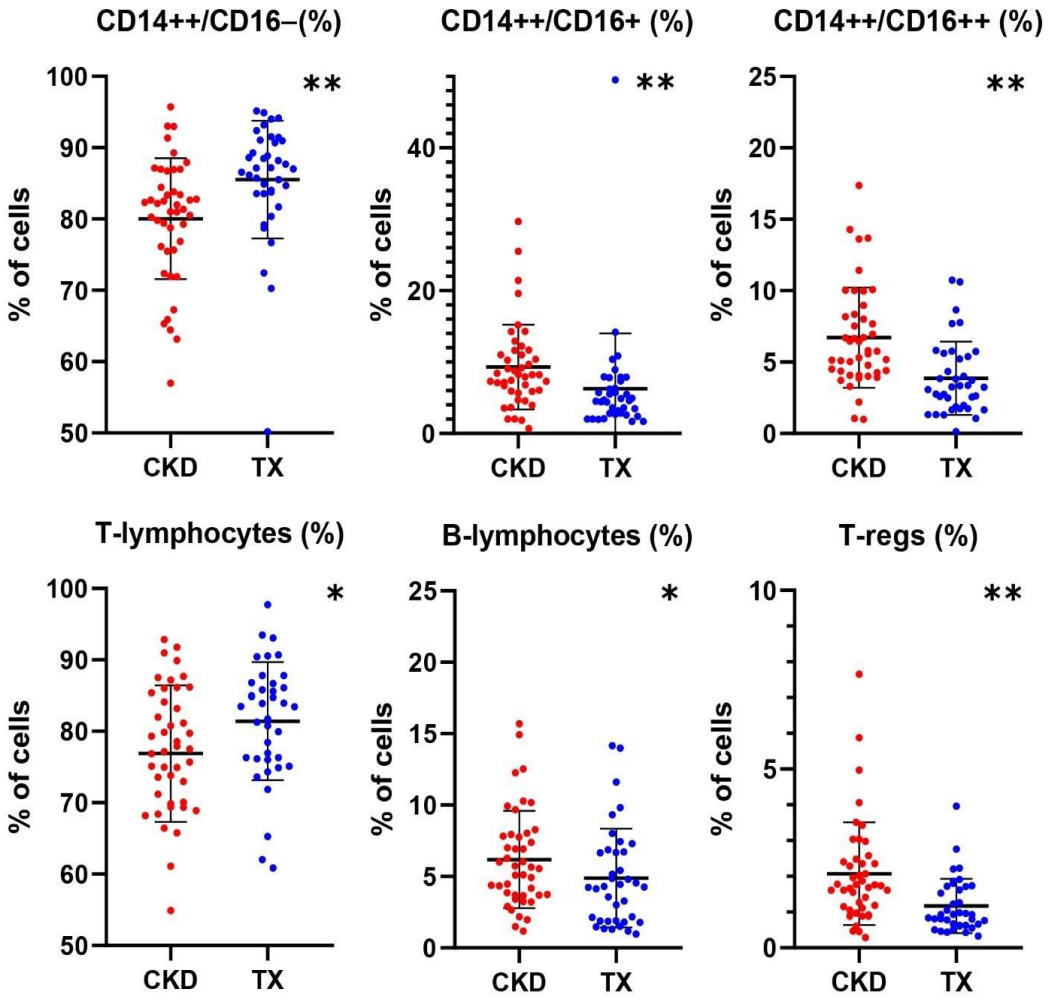

Figure 9. Immune cell subpopulations count in CKD patients and KTRs. * $p < 0.05$, ** $p < 0.01$. Values are expressed as the means or medians. The number and percentages of immune cells subsets in patients with type 2 CRS as well as their differences with immune cells subsets in healthy controls are depicted in **Table 11**. Accordingly, patients with type 2 CRS displayed both an increased number and increased percentage of the pro-inflammatory intermediate CD14++CD16+ monocytes [41 (IQR, 24-78)/μL and 8 (IQR, 5.6-12)%] compared to healthy controls [16 (IQR, 13-18)/μL and 3.67 (IQR, 2.54-4.41)%] (p<0.001 and p=0.001). With regard to lymphocytes subsets, both the number and the percentage of total lymphocytes was lower in patients with type 2 CRS (1557 ±691/μL and 18.7 ±8.3%) compared to healthy control subjects [2188 ±493/μL and 30.1 (IQR, 27.1-34.3)%] (p=0.016 and p<0.001). Similarly, the number of T lymphocytes was lower in patients with type 2 CRS (1227 ±510/μL) compared to healthy controls (1672 ±387/μL)(p=0.025). In line with the above, both the number and the percentage of B lymphocytes were lower in patients with type 2 CRS [68 (IQR, 31-104)/μL and 4.2 (IQR, 2.2-9.0)%] compared to healthy controls [224 (IQR, 171-261)/μL and 10.3 (IQR, 8.5-11.3)%,p<0.001 and p=0.014 respectively]. Notably, patients with type 2 CRS exhibited lower counts of NK cells (148 (IQR, 103-258)/μL) compared to healthy controls (269 (IQR, 240-305)/μL), p=0.015.

Table 11. Immune cell subpopulations in patients with type 2 CRS and healthy control subjects. Statistically significant differences between subgroups are highlighted in bold.

	CRS patients (N=39)	**Controls (N=10)**	**p-value**
WBC (N)	8360 (IQR 6730-9940)	7270 (6460-8570)	0.211
Monocytes (N)	600 (IQR 400-700)	450 (400-500)	0.148
Monocytes (%)	6.5 (IQR 5.4-8.1)	6.2 (6.0-702)	0.899
CD14++CD16- (N)	450±184	396 ±77	0.241
CD14++CD16- (%)	80.6±10	87.7 (85.7-92.5)	0.024
CD14++CD16+ (N)	41 (IQR 24-78)	16 (13-18)	**<0.001**
CD14++CD16+ (%)	8 (IQR 5.6-12.0)	3.67 (2.54-4.41)	**0.001**
CD14+CD16++ (N)	22 (IQR 12-36)	19 (10-21)	0.193
CD14+CD16++ (%)	4.2 (IQR 2.7-6.6)	4.1 (2.1-4.8)	0.579
Lymphocytes (N)	1557±691	2188 ± 493	**0.016**
Lymphocytes (%)	18.7±8.3	30.1 (27.1-34.3)	**<0.001**
T-lymphocytes (N)	1227±510	1672 ± 387	**0.025**
T-lymphocytes (%)	81.7±8.7	76.6 ±8.3	0.120
B-lymphocytes (N)	68 (IQR 31-104)	224 (171-261)	**<0.001**
B-lymphocytes (%)	4.2 (IQR 2.2-9.0)	10.3 (8.5-11.3)	**0.014**
NK cells (N)	148 (IQR 103-258)	269 (240-305)	**0.015**
NK cells (%)	10.7 (IQR 7.1-16.6)	12.1 (8.6-17.6)	0.472
CD4+ T-Cells (N)	732±308	1038 (768-1101)	0.057
CD4+ T-cells (%)	48.6±10.4	46.9 ±6.7	0.583
CD8+ T-cells (N)	411 (IQR 224-720)	636 (408-724)	0.261
CD8+ T-cells (%)	28.5 (IQR 23.3-38.0)	24.9 (22.2-31.3)	0.503
Tregs (%)	2.7 (IQR 2.0-3.9)	1.9 (1.7-3.4)	0.202
T Regs (N)	36 (IQR 24-49)	57 (38-68)	0.250

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; NK, natural killer; No, number; Tregs, T regulatory cells. *p refers to t test significance for normal distribution variables, to Mann-Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

The differences between the peripheral blood levels of immune cells subpopulations between patients with type II CRS and CKD patients are depicted in Table 12 and Figure 10. CRS patients displayed increased levels of pro-inflammatory, intermediate CD14++CD16+ monocytes [41 (IQR, 24-78)/μL] compared to their CKD counterparts [35 (IQR, 18-43)/μL] (p=0.04). A higher Tregs percentage was found in CRS patients [2.7% (IQR, 2.0%-3.9%)] compared to CKD patients [2.0 % (IQR, 1.6%-2.6%)] (p=0.03). Lower mean levels of lymphocytes were observed in CRS patients (1557±691/μL) compared to the CKD cohort (1920±545/μL) (p=0.04). Finally, CRS patients displayed lower NK cell counts [148 (IQR, 103-258)/μL] compared to CKD patients [324 (IQR, 179-368)/μL] (p=0.001). Furthermore, we determined whether the differences regarding the expression of immune cells between patients with type 2 CRS and their CKD counterparts remained significant after adjusting for other significant correlates of immune cells subsets in patients with type 2 CRS and their matched CKD controls. Accordingly, apart from group, significant positive correlations were found between intermediate CD14++CD16+ monocytes and the inflammatory markers ESR (r=0.294, p=0.02), CRP (r=0.331, p=0.008) and ferritin (r=0.293, p=0.02). The Tregs percentage correlated negative with serum triglyceride levels (r =-0.399, p=0.001). Total lymphocytes count correlated with serum albumin (r=0.389, p=0.04) and eGFR (r=0.002, p=0.04). Finally, NK cell number correlated positively with serum albumin (r=0.388, p=0.002). Notably, age and UPCR, parameters which differ significantly between the patients with type 2 CRS and the respective matched CKD patients, did not correlate with immune cells subsets in all patients or in each sub-group (that is patients with type 2 CRS and matched CKD cohort) separately. Following univariate regression analysis, the differences in immune cells subsets between patients with type 2 CRS and matched CKD patients remained statistically significant for the CD14++CD16+ monocytes (p=0.01), total lymphocytes (p=0.04) and NK cells (p=0.002), whereas the difference in the Tregs percentage between the two groups was lost following adjustment for triglycerides levels.

Table 12. Immune cell subpopulations in all patients, in patients with type 2 CRS and in matched CKD patients. Statistically significant differences between subgroups are highlighted in bold.

	All patients (N=63)	**CRS patients (N=39)**	**CKD patients (N=24)**	**p-value**
WBC (N)	7730 (IQR 6224-9495)	8360 (IQR 6730-9940)	7330 (IQR 6070-8830)	0.15
Monocytes (N)	500 (IQR 400-650)	600 (IQR 400-700)	500 (IQR 400-600)	0.06
Monocytes (%)	6.5 (5.4-7.9)	6.5 (IQR 5.4-8.1)	6.6 (IQR 5.3-7.8)	0.64
CD14++CD16- (N)	427±167	450±184	391±132	0.14
CD14++CD16-	81.4±8.9	80.6±10	82.6±6.9	0.35

(%)				
CD14++CD16+ (N)	38 (IQR 22-62)	41 (IQR 24-78)	35 (IQR 18-43)	**0.04**
CD14++CD16+ (%)	7.4 (IQR 5.4-11.2)	8 (IQR 5.6-12.0)	7.3 (IQR 4.7-9.6)	0.30
CD14+CD16++ (N)	25 (IQR 14-35)	22 (IQR 12-36)	25 (IQR 19-32)	0.80
CD14+CD16++ (%)	4.6 (IQR 3.0-6.7)	4.2 (IQR 2.7-6.6)	5.1 (IQR 4.0-6.7)	0.14
Lymphocytes (N)	1699±658	1557±691	1920±545	**0.03**
Lymphocytes (%)	21.3±8.7	18.7±8.3	25.3±8.0	**0.002**
T-lymphocytes (N)	1320±500	1227±510	1465±455	0.06
T-lymphocytes (%)	79.6±9.7	81.7±8.7	76.3±10.3	**0.03**
B-lymphocytes (N)	75 (IQR 37-140)	68 (IQR 31-104)	87 (IQR 58-163)	0.08
B-lymphocytes (%)	4.7 (2.9-8.3)	4.2 (IQR 2.2-9.0)	5.1 (IQR 3.4-7.9)	0.57
NK cells (N)	182 (124-328)	148 (IQR 103-258)	324 (IQR 179-368)	**0.001**
NK cells (%)	1.7 (IQR 8.2-18.3)	10.7 (IQR 7.1-16.6)	16.5 (IQR 11.2-19.6)	**0.01**
CD4+ T-Cells (N)	787±312	732±308	873±304	0.08
CD4+ T-cells (%)	47.5±10.6	48.6±10.4	45.7 ±10.9	0.30
CD8+ T-cells (N)	508 (IQR 353-750)	411 (IQR 224-720)	585 (IQR 447-786)	0.14
CD8+ T-cells (%)	29.9 (IQR 23.5-37.9)	28.5 (IQR 23.3-38.0)	31.5 (IQR 24.4-36.8)	0.73
Tregs (%)	2.4 (IQR 1.7-3.3)	2.7 (IQR 2.0-3.9)	2.0 (IQR 1.6-2.6)	**0.03**
T Regs (N)	37 (IQR 25-51)	36 (IQR 24-49)	40 (IQR 26-61)	0.94

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; NK, natural killer; No, number; Tregs, T regulatory cells. *p refers to t test significance for normal distribution variables, to Mann-Whitney test significance for non-parametric variables, or to chi square test significance for categorical variables.

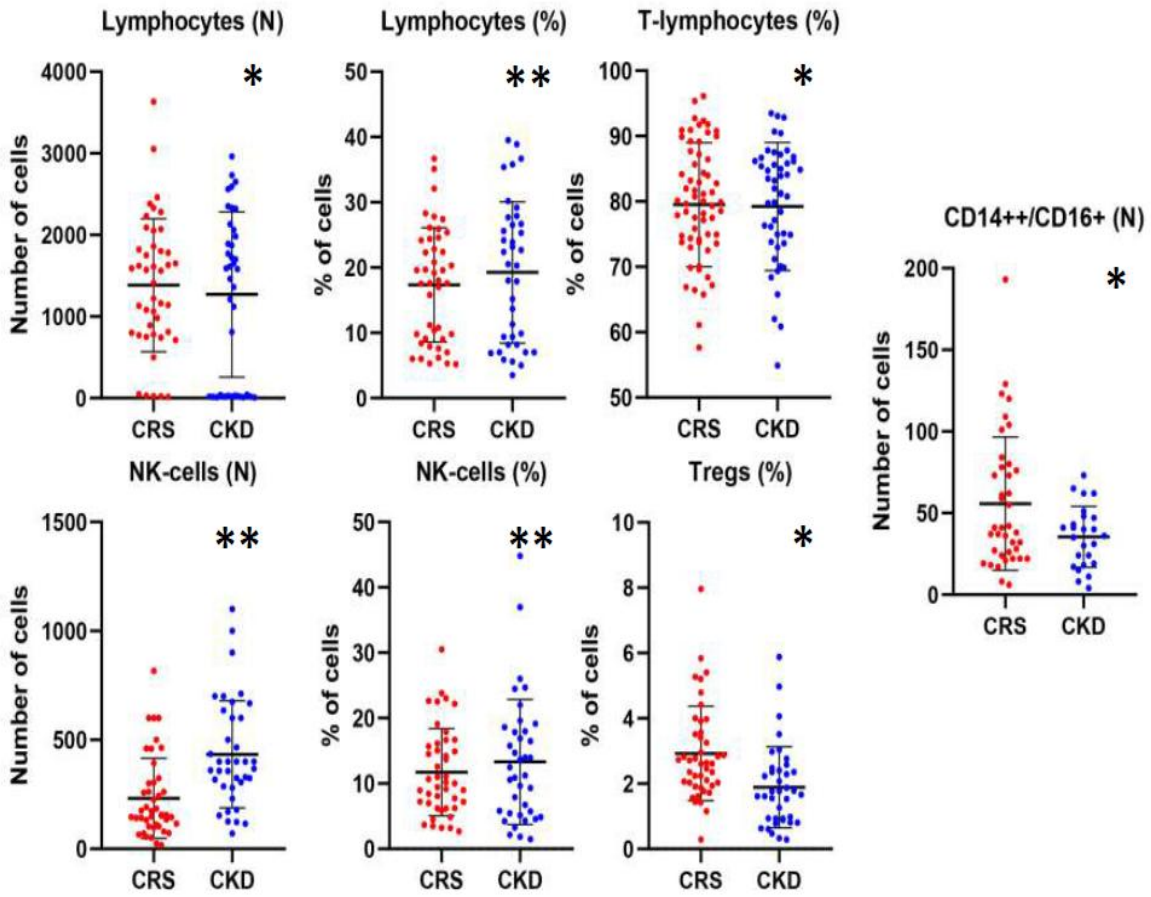

Figure 10. Immune cell subpopulations count in patients with type 2 CRS and respective matched CKD patients. * $p < 0.05$, ** $p < 0.01$. Values are expressed as the means or medians.

8.3. Correlations of Immune Cell Subsets with Clinical and Laboratory Parameters in CKD patients, KTRs and patients with type 2 CRS

Univariate associations of various immune cell subtype levels with other clinical and laboratory parameters in the CKD and KTRs patient subgroups are shown in **Table 13** and **Table 14** respectively. Regarding kidney function markers, eGFR (CKD-EPI) was directly associated with total lymphocytes count (r = 0.388, $p = 0.009$), T-cell counts (r = 0.328, $p = 0.03$) and CD4+ T-cells (r = 0.495, $p = 0.001$) in CKD patients (Table 13). In line with the above, a positive correlation was found between the eGFR, and total lymphocytes counts (r= 0.359, $p = 0.027$), T-cell counts (r = 0.376, $p = 0.02$) and CD8+ T-cell (r = 0.362, $p = 0.02$) in KTRs (Table 14). Furthermore, UPCR was inversely correlated with the percentage of total lymphocytes (r = −0.439, $p = 0.003$) as well as with NK cell counts (r = −0.302, $p = 0.04$) in CKD patients and with B-lymphocytes counts (r = −0.405, $p = 0.01$) in KTRs, respectively (Table 13 and Table 14). Regarding CKD-MBD markers, serum phosphorus levels were

directly correlated to intermediate CD14++CD16+ monocytes count both in CKD patients (r = 0.436, p = 0.003) and in KTRs (r = 0.333, *p* = 0.04 (Table 13 and Table 14).

Table 13. Associations of immune cells subpopulations in CKD patients with markers of kidney function, inflammatory markers and indices of CKD-MBD.

Immune cell subsets	**eGFR**	**UPCR**	**Cr**	**Urea**	**Hb**	**Ca**	**Pi**	**PTH**	**CRP**	**Ferritin**
CD14++CD16+ (N)	/	/	/	/	/	/	r = 0.436, *p =0.003*	/	/	/
CD14++CD16+ (%)	/	/	/	/	/	/	r = 0.410, *P=0.006*	/	/	/
CD14+CD16++ (N)	/	/	/	r = 0.298, *p = 0.005*	/	/	/	/	/	/
NK cells (N)	/	r = -0.302, *p = 0.04*	/	/	r = 0.319, *p = 0.03*	r = 0.329, *p = 0.03*	/	r = -0.327, *p = 0.03*	/	r =0.307, *p = 0.04*
Lymphocytes (N)	r = 0.388, *p = 0.009*	/	r = -0.408, *p= 0.006*	r = -0.374, *p = 0.01*	r = 0.469, *p = 0.001*	r = 0.378, *p= 0.01*	/	/	/	/
Lymphocytes (%)	r = 0.395, *p = 0.008*	r = -0.439, *p = 0.003*	r = -0.475, *p = 0.001*	r = -0.408, *p = 0.006*	r = 0.370, p = *0.013*	r = 0.478, *p = 0.008*	/	/	/	/
T-lymphocytes (N)	r = 0.328, *p = 0.03*	/	r = -0.361, *p = 0.02*	/	r = 0.437, *p = 0.003*	/	/	/	r = -0.344, *p = 0.02*	/
CD4+ T-cells (N)	r = 0.495, *p = 0.001*	/	r = -0.505, *p = 0.000*	r =-0.381, *p = 0.01*	r = 0.522, *p =0.000*	r = 0.371, *p = 0.01*	/	r = -0.324, *p = 0.03*	/	/
CD8+ T cells (N)	/	/	/	r = -0.345, *p = 0.02*	/	/	/	/	/	r = -0.318, *p = 0.04*

Correlations were assessed by Spearman's or Pearson's rank tests. Only correlations reaching statistical significance are presented. Ca, calcium; Cr, creatinine; eGFR, estimated glomerular filtration rate; iPTH, intact parathyroid hormone; Pi, phosphorus; UPCR, urine protein to creatinine ratio.

Table 14. Associations of immune cells subpopulations in KTRs with markers of kidney function, inflammatory markers, indices of CKD-MBD and blood levels of CNIs.

Immune cell subsets	**eGFR**	**UPCR**	**Cr**	**Urea**	**Hb**	**Glucose**	**Ca**	**Pi**	**PTH**	**CRP**	**ESR**	**CyAC0**	**CyAC2**	**Tac**
CD14++ CD16- (%)	/	/	/	/	r=-0.37, *p=0.05*	r=-0.33, *p=0.04*	r=-0.39, *p=0.014*	/	/	/	/	/	/	/
CD14++ CD16- (N)	/	/	/	/	/	/	/	/	r= -0.328, *p=0.04*	/	/	/	/	/
CD14++ CD16+ (%)	/	/	/	/	/	/	/	r=0.33, *p=0.04*	/	/	/	/	/	
CD14+CD16++ (N)	/	/	/	/	/	/	r= 0.388, p=*0.01*	/	/	/	/	/	/	/
CD14+CD16++(%)	/	/	/	/	/	/	r=0.38, *p=0.01*	/	/	/	/	/	/	/
NK cells (N)	/	/	/	/	/	/	r = 0.356, *p = 0.028*	/	/	/	r = 0.395, *p = 0.01*	/	/	
NK cells (%)	/	/	/	/	/	/	/	/	/	/	r =0.393, *p = 0.01*	/	/	/
Lymphocytes (N)	r=0.359 *p=0.02*	/	/	/	/	/	/	/	/	/	/	r=-0.594, *p=0.02*	/	/
Lymphocytes (%)	r=0.359 *p=0.02*	/	/	/	/	/	/	/	/	/	/	r=-0.594, *p=0.*	/	/

												02		
T-lymphocytes (N)	r=0.376 *p=0.02*	/	/	/	/	/	/	/	/	/	/	r= -0.550, *p= 0.04*	/	r = 0.439, *p = 0.03*
T-lymphocytes (%)	/	/	/	/	/	/	/	/	/	/	/	/	r = 0.609, *p= 0.02*	0.419, *p = 0.047*
CD4+ T-cells (N)	/	/	/	/	/	/	/	/	r = -0.340, *p = 0.03*	/	/	/	/	/
CD4+ T-cells (%)	/	/	/	/	/	/	/	/	/	/	/	/	/	r = 0.708, p = 0.000
CD8+ T cells (N)	r = 0.362, *p= 0.02*	/	/	/	r = 0.358, *p= 0.02*	/	/	/	/	/	/	/	/	/
B-lymphocytes (N)	/	r= -0.335, *p = 0.039*	r = -0.369, *p= 0.02*	r = -0.426, *p = 0.008*	/	/	/	r = -0.430, *p= 0.007*	/	/	/	r = 0.686, *p = 0.007*	/	/
B-lymphocytes (%)	/	r = -0.405, *p = 0.01*	/	r = -0.349, *p = 0.03*	/	/	/	r = -0.411, *p = 0.01*	/	/	/	/	/	/
T regs (%)	/	/	/	/	/	/	/	/	/	/	/	/	/	r=0.504, *p=0.01*

Only correlations reaching statistical significance are presented. Ca, calcium; Cr, creatinine; C0, trough blood levels; C2, blood levels 2 h post-dose; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; iPTH, intact parathyroid hormone; Pi, phosphorus; UPCR, urine protein to creatinine ratio.

Univariate associations of various immune cell subtype levels with other clinical and laboratory parameters in patients with type 2 CRS are shown in Table 15. Specifically, in patients with CRS, distinct monocyte subpopulations were found to be associated with inflammatory markers. Thus, we found a positive correlation between monocytes number with ESR (r=0.485, p<0.001) and CRP (r=0.402, p=0.001). Likewise, both the number and percentage of intermediate CD14++CD16+ monocytes correlated positively with CRP [(r=0.476, p=0.002) and (r= 0.319, p=0.04) respectively]. Additionally, the number of the classical CD14++CD16- monocytes as well as the intermediate CD14++CD16+ monocytes correlated with ESR [(r=0.353, p=0.03 and (r=0.378, p=0.02) respectively]. On the other hand, serum hemoglobin levels displayed a negative association with both classical CD14++CD16- and non-classical CD14+CD16++ monocytes count [(r=-0.332, p=0.004) and (r= -0.385, p=0.01), respectively]. Regarding indices of kidney function, a positive correlation was found between eGFR and total lymphocytes (r=0.427, p=0.007), T- cells (r=0.425, p=0.007) as well as CD4+ T cells counts (r=0.439, p=0.005) whereas the CD4+/CD8+ ratio displayed a negative correlation with UPCR (r=0.401, p=0.02). Significant reverse associations were detected between serum levels of total cholesterol and LDL cholesterol with B-lymphocytes counts [(r=-0.336 p=0.03) and (r= -0.388, p=0.01), respectively] and percentage of B-lymphocytes [(r=-0.470, p=0.003) and (r= -0.441, p=0.0005), respectively]. Additionally, both CD8+ lymphocytes number and percentage correlated positively with HDL cholesterol [(r=0.318, p=0.04) and (r=0.333, p=0.04), respectively]. Finally, the negative association that was detected between Tregs and serum triglycerides in the whole study cohort, was confirmed within the CRS patient group as well (r=-0.377, p=0.03).

Regarding cardiac indices, a positive association was found between NT terminal pro-BNP levels and CD14++CD16- monocytes (r=0.565, p=0.02), whereas hsTnI levels correlated negatively with the percentage of total lymphocytes (r=0.575, p=0.006).

We further determined differences regarding expression of immune cell subpopulations with respect to left ventricular ejection fraction (LVEF), to the CRS etiological background as well as to clinical features of CRS patients. Accordingly, the number and percentage of nonclassical CD14+CD16++ monocytes were higher in CRS patients with LVEF less than 30% compared to patients with LVEF above 30% [33 (IQR, 18-37)/μL versus 13 (IQR, 10-29)/μL (p=0.02) and 4.5% (IQR, 3.4%-7.2%) versus 2.7% (IQR, 1.9%-5.4%) (p=0.03) respectively]. With regard to CRS etiological background, patients with dilated cardiomyopathy compared to patients with ischemic CVD displayed increased counts of intermediate CD14++CD16+ monocytes [75 (IQR, 41-104)/μL versus 36 (IQR, 22-61)/μL (p=0.01)] and non-classical CD14+CD16++ monocytes [37 (IQR, 35-49)/μL versus 21 (IQR, 12-32)/μL (p=0.02)]. Finally, NK cells and Tregs levels were lower in patients with atrial fibrillation compared to those without [133 (IQR, 79-173)/μL versus 260 (IQR, 151-314)/μL (p=0.01)] and [32 (IQR, 21-43)/μL vs 47 (IQR, 34-85)/μL (p=0.006)] respectively.

Table 15. Associations of immune cells subpopulations in patients with type 2 CRS with hemoglobin, inflammatory markers, eGFR and serum lipids levels.

Immune cell subsets	eGFR	Hb	Chol	LDL Chol	HDL Chol	Triglycerides	CRP	ESR
Monocytes (N)	/	/	/	/	/	/	r=0.402 *p=0.001*	r=0.485 p≤=0.001
CD14++CD16- (N)	/	r=-0.332, p=0.004	/	/	/	/	/	r=0.353, *p=0.030*
CD14++CD16+ (N)	/	/	/	/	/	/	r=0.476 *p=0.002*	r=0.378, *p=0.020*
CD14++CD16+ (%)	/	/	/	/	/	/	r=0.319, p=0.040	/
CD14+CD16++ (N)	/	r=-0.385, p=0.01	/	/	/	/	/	/
Lymphocytes (N)	r=0.427 *p=0.007*	/	/	/	/	/	/	/
T-lymphocytes (N)	r=0.425 *p=0.007*	/	/	/	/	/	/	/
CD4+ T-cells (N)	r=0.439 *p=0.005*	/	/	/	/	/	/	/
CD8+ T cells (N)	/	/	/	/	r=0.318, *p=0.04*	/	/	/
CD8+ T cells (%)	/	/	/	/	r=0.333, *p=0.04*	/	/	/
B-lymphocytes (N)	/	/	r=-0.336, *p=0.03*	r=-0.388, p=0.01	/	/	/	/
B-lymphocytes (%)	/	/	r=-0.470 *p=0.003*	r=-0.441, p=0.0005	/	/	/	/
T regs (%)	/	/	/	/	/	r=-0.377, *p=0.03*	/	/

Correlations were assessed by Spearman's or Pearson rank tests. Only correlations reaching statistical significance are presented. Chol. Cholesterol; CRP, C-reactive protein; CRS, cardiorenal syndrome; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate, Hb, hemoglobin.

8.4 Correlations of immune cells with classical and novel indices of left ventricular function in patients with CKD

In this sub-chapter, correlations of immune cells with classical and novel indices of left ventricular function in patients with CKD will be presented. Independent correlations with immune cells are depicted in tables, while correlations losing significance at multivariate analysis are reported only as text. Moreover, interesting important correlations of immune cells with other indices will be presented in the text only.
The final analysis included 38 patients with CKD, following exclusion of 7 patients due to poor echocardiographic acoustic window. Results of the echocardiographic measurements of CKD patients at baseline and following dipyridamole infusion as well as their respective differences are presented in Table 16.

Table 16. Echocardiographic parameters at baseline and following dipyridamole infusion in patients with CKD.

	Baseline	**Post dipyridamole**	**Δ**
CFR		2.59 (2.34-3.46)	
LAVI	32.6±10.2	NA	NA
LVMI	112.3 ±36.5	*NA*	*NA*
RWT	0.46 (0.42-0.54)	*NA*	*NA*
EF	68±7	71±8	3±9
TAPSE	2.5 ±0.43	2.5±0.4	0.05 ±0.44
MAPSEsep	1.3 (1.2-1.6)	1.49±0.30	-0.26±0.35
MAPSElat	1.7±0.3	1.79± 0.33	0.43±0.32
E/A	0.80 (0.65-97)	0.78 (0.68-0.96)	-0.05 (-0.13-0.09)
E'	0.09±0.01	0.10±0.02	0.012 ±0.020
E/E'	8.4 ±2.8	8.0 (7.2-10.9)	0.1 (-1.3 - 1.5)
Sm	0.08 (0.06-0.09)	0.09 (0.07-0.1)	0.01 ±0.02
Sl	0.09 (0.08-0.11)	0.10 (0.09-0.13)	0.02 (0.00-0.02)
St	0.13 (0.11-0.16)	0.15 (0.13-0.17)	0.016 ±0.03
GLS	-20.3 ±3.1	-22.3 ±3.1	-1.9±4.1
GRS	27.9 ±15.3	27.6 (13.3-44.5)	0.0 (-11.0-15.7)

GCS	-25.9 (-30.6 - -21.0)	-29.2±8.7	-3.0±7.8
TWIST	9.1 ±4.3	9.6 (4.7-11.6)	-1.1 (-2.7 - 3.2)
UNTWIST	-77.6 ±34.1	-87.4±45.5	-10.5±40.2

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CFR, coronary flow reserve; E', early diastolic tissue wave velocity; E/A, early to late diastolic transmitral wave ratio; GCS, global circumferential strain; GLS, global longitudinal strain; GRS, global radial strain; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVMI, left ventricle mass index; MAPSE, mitral annular plane systolic excursion; RWT, relative wall thickness; Sm, medial wall systolic velocity; Sl, lateral wall systolic velocity; TAPSE, tricuspid annular plane systolic excursion; Δ, difference between values of echocardiographic parameters post and prior to dipyridamole infusion.

Correlations at univariate followed by multivariate regression analyses of classical ventricular function indices at baseline as well as the differences between values following and prior to dipyridamole infusion with immune cells subpopulations, clinical characteristics and laboratory parameters in CKD patients are depicted in Table 17. Table 18 presents the correlations at univariate followed by multivariate regression analyses of classical ventricular function indices following dipyridamole infusion with immune cells subpopulations, clinical characteristics and laboratory parameters in CKD patients. Relative wall thickness, in univariate analysis was positively correlated with the WBC number (r = 0.41, p=0.013), both the number and percentage of monocytes (r = 0.599, p=0.000, and r= 0.503, p = 0.002, respectively) as well as with both the number and percentage of CD14++ monocytes (r = 0.638, p=0.000, and r = 0.355, p=0.034, respectively) (Table 17). On the other hand, a negative correlation was found between RWT and the percentage of CD14+CD16++ monocytes (r = -0.5, p=0.001) as well as the percentage of total lymphocytes (r= -0.317, p=0.06) and B lymphocytes (r = -0.363, p=0.03) (Table 17). Finally, a positive association was found with the presence of arterial hypertension (r = 0.484, p=0.003) and a negative correlation with serum albumin (r = -0.441, p=0.007), serum proteins (p 0.023, r = -0.383), HDL (r = -0.356, p=0.033) and serum calcium (r = -0.338, p=0.044) (Table 17). Multivariate analysis revealed that left ventricular RWT was independently correlated with the classical CD14++ monocytes count (β=0.447, p=0.004) and the percentage of B lymphocytes (β=-0.328, p=0.03) (Table 17). Left ventricular EF at baseline was negatively associated with both the number and percentage of total lymphocytes (r= -0.345, p=0.04 and r= -0.353 p=0.03 respectively), the number of T cells (r=-0.364, p=0.023) as well as with the number of CD4+ lymphocytes (r=- 0.451, p=0.006), only the CD4+ T-cell count remained an independent correlate of left ventricular EF (β=-0.431, p=0.009) (Table 17). EF post dipyridamole infusion was negatively associated with the number of CD14++CD16+ monocytes (r = -0.377, p 0.024) (Table 18). At stepwise multiple regression analysis, only the number of CD14++CD16+ monocytes remained significantly associated with EF post dipyridamole infusion Table 18). Finally, the difference in EF

between post and pre dipyridamole infusion values correlated only with the presence of DM (r= -0.471, p 0.004).

No significant correlations were found for MAPSE septal at baseline whereas MAPSE septal following dipyridamole infusion was associated with the number of WBC (r= -0.420, p=0.011), the number of monocytes (r = -0.364, p=0.029), both the number and percentage of CD14++CD16+ monocytes (r= -0.565, p=0.000 and r = -0.392, p=0.018, respectively) as well with serum phosphorus levels (r = -0.414, p 0.012). UPCR levels failed to reach statistical significance (p 0.075, r = -0.301) (Table 18). At multiple regression analysis only the number of CD14++CD16+ monocytes and UPCR remained significant correlates of MAPSE septal post dipyridamole infusion (Table 18). The difference in MAPSE septal between values post and prior to dipyridamole infusion was negatively correlated with CD14++CD16+ monocytes number (r = -0.468, p=0.004) as well as with serum urea (r = -0.333, p=0.048), UPCR (r = -0.399, p=0.016) and with the presence of arterial hypertension (r = -0.363, p=0.03) whereas a positive correlation was found with serum HDL values (r= 0.340, p=0.043) (Table 17). Finally, the ΔMAPSE septal was independently correlated with the number of CD14++CD16+ monocytes (β=-0.359, p=0.007) and HDL levels (β=0.431, p=0.006) (Table 17).

Regarding other interesting correlations detected, the CFR was negatively associated with the number of WBC (r= -0.334, p=0.046) and CRP (p 0.024, r = -0.376), however at stepwise multiple regression analysis, all correlations were lost. Correlates of LVMI included arterial hypertension (r = 0.383, p=0.021), UPCR (r= 0.421, p = 0.01) and PTH (r =0.37, p=0.02), whereas a negative correlation was found with the percentage of total lymphocytes (r= -0.397, p=0.02), eGFR (r = -0.386, p=0.02), as well as HDL level (r= -0.415, p=0.01). However, in line with our findings in KTRs, at stepwise multiple regression analysis, only UPCR (β=0.447, p=0.004) and arterial hypertension (β=0.447, p=0.004) remained significant correlates of LVMI. Interestingly, although both the number and percentage of CD14++CD16+ monocytes showed an inverse association with TAPSE at baseline (r =-0.36, p=0.03 and r =-0.335, p=0.04 respectively) together with phosphorus (r=- 0.474, p=0.004,) and serum creatinine levels (r=-0.338, p=0.04), following multivariate regression analysis only phosphorus remained an independent correlate of TAPSE (β=-0.506, p=0.02). TAPSE post dipyridamole infusion was negatively associated with the percentage of NK cells (p 0.037, r= -0.35) as well as with serum ferritin (p 0.011, r = -0.424), serum phosphorus (p 0.017, r = -0.397) and finally serum urea (p 0.027, r= -0.368) whereas the correlation to serum creatinine just failed to reach statistical significance (p 0.066, r= -0.310). At stepwise multiple regression analysis, only phosphorus remained significantly associated with TAPSE prior to and post dipyridamole infusion. The difference in TAPSE between values post and prior to dipyridamole infusion did not show any significant correlations with any of the immune cell subsets. No significant associations were found for MAPSE lateral at baseline, following dipyridamole infusion or for the difference in MAPSE lateral post and prior to dipyridamole infusion. No significant associations with regard to immune cells or other parameters were found for E/A ratio at baseline or for the difference in E/A ratio between

values post and prior to dipyridamole infusion, except for a correlation with correlated with the presence of DM II and only borderline with the presence of arterial hypertension (r = 0.321, p=0.064). E/A ratio following dipyridamole infusion was associated with the number of CD8+ lymphocytes (r = 0.341, p=0.049) as well as serum calcium levels (r = -0.36, p=0.049) and inversely associated with serum ferritin (r= -0.346, p=0.049) and serum urea (r = -0.36, p=0.033). However, all associations were lost a stepwise multiple regression analysis.
No significant associations were found between Sm and immune cells at baseline, whereas Sm post dipyridamole infusion was associated both with the number and percentage of CD8 lymphocytes (r = 0.378, p=0.023, and r = 0.342, p=0.041, respectively). However, no correlation remained significant following stepwise multiple regression analysis.
Likewise, no significant associations were found between Sl and immune cells at baseline. Sl post dipyridamole infusion as associated with male gender and with CD8+ lymphocytes (r= 0.343, p=0.041); however, at stepwise multiple regression analysis, the correlation for CD8+ lymphocytes was lost. Although the difference in Sl post and prior to dipyridamole infusion displayed an only borderline association with the number of NK cells (r = 0.332, p=0.051) as well as with serum albumin (r = 0.429, p=0.01), all correlations were lost at stepwise multiple regression analysis. E/E' ratio at baseline correlated with serum cholesterol (r = -0.356, p 0.036) and only borderline with the percentage of T cells (r = -0.321, p=0.06), however both correlations were lost at stepwise multiple regression analysis. No significant associations between E/E' ratio following dipyridamole infusion and immune cells were observed.

Table 17. Univariate and multivariate correlates of classical indices of left ventricular function in CKD patients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**Anova R^2 P value**	**β**	**P value**
RWT			0.338, p=0.001		
CD14++ monocytes No	*0.638*	*0.000*		*0.447*	***0.004***
**CD14++ monocytes %*	*0.355*	*0.034*			
B lymphocytes %	*-0.363*	*0.03*		*-0.328*	***0.03***

WBC	0.41	0.013		-0.92	0.67
Monocytes No	0.559	0.000		-0.02	0.997
CD14+CD16++ monocytes %	-0.53	0.001		-0.240	0.181
Lymphocytes %	-0.317	0.06		-0.151	0.309
Arterial hypertension	0.484	0.003		0.241	0.109
Albumin	-0.441	0.007		-0.176	0.298
Proteins	-0.383	0.02		-0.174	0.259
HDL	-0.356	0.03		-0.176	0.258
Calcium	-0.338	0.04		*-0.150*	*0.320*
LVEF			0.186, p=0.009		
CD4+ T cells No	*-0.451*	*0.006*		*-0.431*	***0.009***
T cells No	-0.364	0.03		0.012	0.969
*Lymphocytes No	-0.345	0.04			
Lymphocytes %	-0.353	0.035		-0.171	0.377
Hemoglobin	-0.317	0.06		-0.191	0.26
ΔMAPSE septal			0.360, p=0.001		
CD14++CD16+ monocytes No	*-0.468*	*0.004*		*-0.405*	***0.007***
Urea	-0.333	0.048		-0.263	0.074
UPCR	-0.399	0.016		-0.155	0.333
Arterial hypertension	-0.363	0.03		-0.276	0.07
HDL	*0.340*	*0.043*		*0.413*	***0.006***

LDL, low density lipoprotein; LVEF, left ventricle ejection fraction; MAPSE, mitral annular plane systolic excursion; N, number; RWT, relative wall thickness; UPCR, urine protein to creatinine ratio; Δ, difference between values of echocardiographic parameters post and prior to dipyridamole infusion.

Table 18. Univariate and multivariate correlates of classical indices of left ventricular function following dipyridamole infusion in CKD patients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**ANOVA R2 P value**	**β**	**P value**
LVEF (post)					
CD14++CD16+ No	*-0.377*	*0.024*			
MAPSE septal (post)			0.373, p=0.000		
WBC	-0.420	0.011		-0.244	0.107
Monocytes No	-0.364	0.029		-0.199	0.180
CD14++CD16+ monocytes No	-0.565	0.000		*-0.372*	**0.013**
CD14++CD16+ monocytes %	-0.392	0.018			
Phosphorus	-0.414	0.012		-0.11	0.949
UPCR	-0.301	0.075		*-0.402*	**0.008**

LVEF, left ventricle ejection fraction; MAPSE, mitral annular plane systolic excursion; N, number; UPCR, urine protein creatinine ratio, WBC, white blood cells

Correlations at univariate followed by multivariate regression analyses of novel ventricular function indices as well as the differences between the values following dipyridamole infusion and baseline values with immune cells subpopulations, the clinical characteristics and laboratory parameters in CKD patients are depicted in Table 19 and in Figure 11 whereas Table 20 presents correlations at univariate followed by multivariate

regression analyses of novel ventricular function indices following dipyridamole infusion with immune cells subpopulations, clinical characteristics and laboratory parameters in CKD patients.

GLS at baseline was only associated with the ESR (r= -0.377, p =0.026,) whereas GLS post dipyridamole infusion was associated with WBC count (r = 0.382, p=0.021), the number of monocytes (r = 0.502, p=0.002) as well as the number of CD14++ monocytes (r = 0.428, p=0.009) (Table 20). A negative correlation was found between GLS post dipyridamole infusion and serum albumin (r = -0.373, p=0.025) (Table 20). At stepwise multiple regression analysis, the number of CD14++ monocytes remained significant correlates of GLS post dipyridamole infusion (Table 20).

Significant correlates of GRS included both the number and percentage of CD14++CD16+ monocytes (r = 0.042, p=0.01, and r = 0.352, p=0.04) (Table 19). No significant associations were found for GRS following dipyridamole infusion and immune cell subsets. Furthermore, although the ΔGRS was associated with the CD14++CD16+ monocytes number (r =0.331, p=0.006), apart from hemoglobin (r = 0.441, p=0.01), ferritin (r = -0.447, p=0.01) and urea (r = -0.408, p=0.02), this correlation was lost at stepwise regression analysis.
With regard to left ventricular TWIST, the percentage and the number of CD8+ T-cells were the sole independent correlate both at baseline and following dipyridamole infusion (β=0.405, p=0.02 and β=0.359, p=0.037 respectively) (Table 19 and Table 20). Thus, TWIST post dipyridamole infusion correlated with the number of CD8+ T cells (r = 0.379, p=0.027) as well as serum cholesterol (r = 0.348, p 0.043) and LDL levels (r = 0.438, p=0.010) (table 20). A negative association was found between TWIST post dipyridamole infusion and CRP (r= -0.389, p=0.023) whereas an association of marginal significance was found for the percentage of NK cells (r = -0.337, p= 0.051) (Table 20).

Likewise, an independent correlation was found between left ventricular UNTWIST with the number of CD8+ T cells (β=-0.363, p=0.03) (Table 19). Accordingly, UNTWIST at baseline was negatively associated with age (r = -0.389, p=0.025) and the number of CD8+ T cells (r = -0.371, p=0.033) which was the only variable remaining significantly associated with UNTWIST at baseline (Table 19). UNTWIST post dipyridamole infusion was positively associated with the percentage of NK cells (r = 0.352 p=0.041) and CRP (r = 0.410, p=0.016). A negative association was found between UNTWIST post dipyridamole infusion and serum cholesterol (r = -0.418, p=0.014) and LD (r = -0.399, p 0.020). However, at stepwise multiple regression analysis, all associations were lost with regard to UNTWIST following dipyridamole infusion.

Table 19. Univariate and multivariate correlates of novel indices of left ventricular function in CKD patients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**Anova R2 P value**	**β**	**P value**

GRS					
CD14++CD16+ monocytes %	*0.351*	*0.041*			
CD14++CD16+ monocytes No	*0.042*	*0.015*			
TWIST			0.164, p=0.021		
CD8+ T cells %	*0.309*	*0.08*		*0.405*	***0.021***
Glucose	0.398	0.028		-0.191	0.26
Triglycerides	-0.348	0.051		-0.278	0.1
UNTWIST			0.135, p=0.03		
CD8+ T cells %	*-0.371*	*0.033*		-0.367	**0.03**
CD8+ T cells No	-0.416	0.016			
Age	-0.389	0.025		-0.287	0.08

GRS, global radial strain, No, number

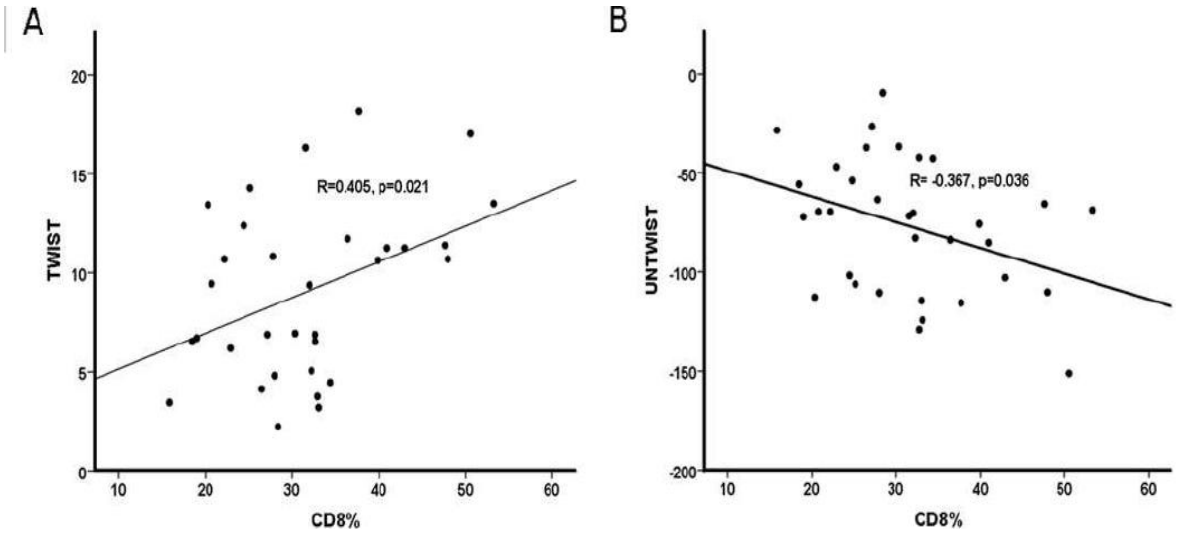

Figure 11. Associations between myocardial strain echocardiographic indices of cardiac function with immune cells subsets in patients with CKD. A. Higher baseline left ventricular TWIST (better) is positively associated with the percentage of CD8+ T-cells in CKD patients B.

More negative baseline left ventricular UNTWIST (better) is inversely associated with the percentage of CD8+ T-cells in CKD patients.

Table 20. Univariate and multivariate correlates of novel indices of left ventricular function following dipyridamole infusion in CKD patients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**ANOVA R2 P value**	**β**	**P value**
GLS post			0.327, p=0.002		0.694
Monocytes No	0.502	0.002		-0.161	0.856
CD14++CD16- monocytes No	0.428	0.009		*0.320*	**0.036**
Albumin	-0.373	0.025		-0.156	0.354
TWIST post			0.129, p=0.037		
CD8+ T cells (No)	0.379	0.027		**0.359**	**0.037**
NK cells %	-0.337	0.051		-0.258	0.135
CRP	-0.389	0.023		-0.245	0.143
Cholesterol	0.348	0.043		0.258	0.120

CRP, serum reactive protein; GLS, global longitudinal strain, No, number

8.5 Correlations of immune cells with classical and novel indices of left ventricular function in kidney transplant recipients.

In this sub-chapter, correlations of immune cells with classical and novel indices of left ventricular function in KTRs will be presented. Independent correlations with immune cells are depicted in tables, while correlations losing significance at multivariate analysis are reported only as text. Moreover, interesting important correlations of immune cells with other indices will be presented in the text only. Following exclusion of 7 KTRs due to poor echocardiographic acoustic window, the analysis included 31 KTRs. Results of the

echocardiographic measurements in KTRs at baseline and following dipyridamole administration as well as their respective differences are presented in Table 21.

Table 21. Echocardiographic parameters at baseline and following dipyridamole infusion in kidney transplant recipients.

	Baseline	**Post dipyridamole**	**Δ**
CFR		2.68±0.80	
LAVI	32.2±10.6	*NA*	*NA*
LVMI	99.09 (84.3-134.6)	*NA*	*NA*
RWT	0.46 (0.38-0.51)	*NA*	*NA*
EF	65±7	75±8	10±7
TAPSE	2.2 (1.9-2.4)	2.4±0.5	0.14 ±0.46
MAPSEsep	1.32±0.29	1.47±0.22	0.14±0.24
MAPSElat	1.6±0.3	1.6± 0.2	0.029±0.27
E/A	0.91 ±0.24	0.82 (0.73-1.08)	-0.05 (-0.19 - 0.16)
E'	0.088±0.018	0.113±0.027	0.023 (0.010-0.040)
E/E'	8.8 (7.6-9.6)	7.5 (6.8-9.1)	-1.2 (-2.7 - 0.7)
Sm	0.08 (0.07-0.09)	0.10 (0.08-0.12)	0.02 (0-0.04)
Sl	0.09 (0.08-0.1)	0.11 (0.09-0.12)	0.017 (±0.024)
St	0.14 (0.12-0.16)		
GLS	-21.1 (-21.9 - -18.1)	-22.8 ±4.2	-2.5±3.3
GRS	21.9 (13.2-37.4)	20.6 (14.9-30.6)	-1.84 ±21.4
GCS	-28.7 ±7.0	-30.1±7.6	-1.73±9.2
TWIST	6.2 (3.4-9.5)	9.1 (5.7-13.3)	3.2±6.7
UNTWIST	-55.0 (-88.9 - -34.4)	-116±50	-51.4±48

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CFR, coronary flow reserve; E’, early diastolic tissue wave velocity; E/A, early to late diastolic transmitral wave ratio; GCS, global circumferential strain; GLS, global longitudinal strain; GRS, global radial strain; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVMI, left ventricle mass index; MAPSE, mitral annular plane systolic excursion; RWT, relative wall thickness; Sm, medial wall systolic velocity; Sl, lateral wall systolic velocity; TAPSE, tricuspid

annular plane systolic excursion; Δ, difference between values of echocardiographic parameters post and prior to dipyridamole infusion.

Significant correlations at univariate followed by multivariate stepwise regression analyses of classical echocardiographic indices at baseline as well as differences in values following and prior to dipyridamole infusion with immune cells subpopulations, clinical characteristics and laboratory parameters are depicted in Table 22 whereas Table 23 presents significant correlations at univariate followed by multivariate stepwise regression analyses of classical echocardiographic indices following dipyridamole infusion with immune cells subpopulations, clinical characteristics and laboratory parameters in KTRs.
EF at baseline was positively correlated with female gender (p 0.01, r= 0.456) and negatively associated with the percentage of Tregs (p 0.033, r= -0.384) (Table 22). EF following dipyridamole administration was associated with female gender (r = 0.402, p=0.025), the percentage of NK cells (r = 0.416, p = 0.02), whereas a negative correlation was found with the percentage of monocytes levels (r = -0.380, p=0.035), both the number and percentage of T cells (r = -0.384, p=0.033 and r = -0.416, p=0.001 respectively), as well as the number of CD4+ lymphocytes (r= -0.358, p=0.0.048) and Tregs (r = -0.429, p = 0.016) (Table 23). Following multiple regression analysis, gender and the levels of Tregs remained significantly associated with EF both prior to and following dipyridamole administration (Table 22 and Table 23). With regard to EF increase following dipyridamole administration, a positive association was observed between serum albumin levels (r = 0.447, p= 0.012) and serum LDL levels (r= 0.381, p 0.034), whereas a negative correlation was observed with the percentage of CD14++CD16- cells (r= - 0.447, p 0.012), both the absolute number and percentage of lymphocytes (r = -0.476, p=0.007 and r = - 0.378, p=0.036 respectively), the percentage of T cells (r= -0.475, p=0.007), the number of CD4+ T cells (r= -0.483, p=0.006), as well as the number of CD8+ T cells (r= -0.371, p=0.004) (Table 22). The number of CD4+ lymphocytes (β=-0.378, p=0.02) and serum albumin levels (β=0.353, p=0.03) were significant independent correlates of the ΔEF at stepwise multiple regression analysis (Table 22).
The tricuspid annular plane systolic excursion was associated with the percentage of CD8+ T cells (r= 0.494, p=0.005) and serum glucose levels (r= 0.404, p=0.024) and inversely associated with the number of CD4+ T cells (r= -0.456, p=0.01) (Table 22). With regard to TAPSE following dipyridamole infusion, it was directly associated with CD8+ T cells levels (r = 0.404, p=0.033) whereas it was negatively correlated with the presence of arterial hypertension (r = -0.515, p 0.005) (Table 23). Similar to TAPSE at baseline**,** CD8+ T cells remained significantly associated with TAPSE following stepwise multiple regression analysis, together with arterial hypertension (Table 23). Overall, the tricuspid annular plane systolic excursion (TAPSE) was associated both at baseline and following dipyridamole infusion with CD8+ T cells (β=0.559, p=0.00 and β=0.450, p=0.004 respectively), whereas arterial hypertension was independently associated with TAPSE post dipyridamole infusion (β=-0.51, p=0.001) (Table 22 and Table 23). On the other hand, no significant associations were observed between immune cells subsets and the difference in TAPSE following

dipyridamole infusion, which correlated only with the presence of arterial hypertension (r= -0.012, p 0.012).

With regard to MAPSE, serum HDL levels (r=0.417, p=0.017) and phosphorus levels (r = 0.369, p=0.041) displayed a direct positive association whereas the CD4+ T cells counts just failed to show a statistically significant relationship at univariate analysis (r—0.303, p=0.098) (Table 22). Nevertheless, following stepwise regression analysis, CD4+ T-cell counts were independent correlates of septal MAPSE at baseline (β=-0.359, p=0.01) (Table 22). Furthermore, MAPSE septal following dipyridamole infusion was significantly associated with ESR (r=0.382, p=0.034), whereas a negative association was found with the percentage of CD4+ T cells (r = -0.468, p=0.008), both the percentage and number of Tregs (r = -0.417, p=0.02 and r = -0.449, p=0.01) as well the serum Tacrolimus levels (r = -0.518, p=0.023) (Table 23). However, at stepwise multiple regression analysis only the percentage of CD4+ T cells remained significantly associated with MAPSE septal post dipyridamole infusion (β=-0.463, p=0.04) (Table 23). The Sm at baseline was negatively associated with serum glucose (p 0.000, r= -0.600) and it displayed no association with immune cells, whereas the Sm post dipyridamole infusion was directly associated to the percentage of CD14+CD16++ monocytes percentage (r=0.562, p 0.004) as well as to the percentage of NK cells (r= 0.460, p 0.024) but it was negatively associated with the percentage of the CD14++CD16- monocytes (r= -0.424, p=0.039) (Table 23). At stepwise multiple regression analysis, the Sm following dipyridamole infusion remained significantly associated with the NK cells and CD14++ monocytes (Table 23). Finally, the difference between Sm following and pre dipyridamole infusion was associated with the percentage of CD14+CD16++ monocytes (r= 0.643, p=0.01) and inversely correlated with the percentage of CD14++CD16- monocytes (r= -0.489, p=0.015) (Table 22). At stepwise multiple regression analysis, only the percentage of CD14++CD16- monocytes remained significantly associated with Sm difference between post and pre dipyridamole infusion values (β=-0.516, p=0.01) (Table 22).

No significant association between baseline Sl and immune cells were observed. Sl following dipyridamole infusion was significantly associated with the both the number and percentage of NK cells (r = 0.527, p=0.008, and r= 0.645, p=0.001 respectively) whereas it was inversely associated with the percentage of T cells (r = -0.571, p=0.004) and uric acid levels (r= -0.494, p=0.014) (Table 23). At stepwise multiple regression analysis, only the percentage of NK cells remained significantly associated to Sl post dipyridamole infusion (Table 23). Finally, regarding the difference in Sl between levels post and pre dipyridamole infusion, it was positively associated with CD14+CD16++ number and percentage (r=0.484, p=0.017 and r= 0.446, p=0.029, respectively) as well as with serum calcium levels (r=0.451, p=0.027) and inversely associated with the percentage of CD14++CD16- monocytes (r= -0.747, p=0.000) (Table 22). At stepwise multiple regression analysis only the association of Sl with CD14++CD16- monocytes remained significant (β=-0.707, p=0.000) (Table 22).
The E/A values were negatively associated with age (r= -0.466, p=0.008), and the number of NK (r= -0.359, p=0.047) as well as with hemoglobin levels (r = -0.429, p=0.016) whereas a positive association was found with transplantation vintage (r = 0.596, p=0.000) (Table 22).

At stepwise multiple regression analysis, an independent correlation with age (β=-0.481, p=0.004) and NK cells count (β=-0.387, p=0.02) was confirmed for E/A values (Table 22). On the other hand, E/A post dipyridamole infusion displayed a positive association with the percentage of Tregs (r = 0.397, p=0.033,) and a negative correlation with CD8+ T cells number (r= -0.502, p=0.006) and hemoglobin levels (r= -0.460, p=0.012) (Table 23). At stepwise multiple regression analysis, only the levels of CD8+ counts remained significantly associated with E/A post dipyridamole infusion (Table 23). The difference in E/A before and after dipyridamole infusion was associated with Cyclosporine C0 levels (r =0.685, p= 0.029,) and it was inversely associated with the percentage of CD8+ T cells (r= -0.419, p=0.024), ESR (r=-0.386, p=0.039) and serum triglycerides (r= -0.386, p=0.019). However, all significant associations were lost at stepwise multiple regression analysis.

Left atrial volume index (LAVI) was inversely correlated with total monocytes counts (r=-0.517, p=0.003) and positively associated with the presence of DM (r=0.420, p=0.02), however only the presence of diabetes mellitus (DM) remained independently associated with LAVI. No significant correlates for LVMI were found among immune cells subsets, whereas among the rest correlates only UPCR remained independently correlated to LVMI (r=0.425, p=0.01). Finally, the percentage of total lymphocytes (r = -0.442, p 0.013), serum albumin (r = - 0.046, p=0.008), serum proteins (r = -0.392, p=0.029) and serum HDL levels (r = -0.555, p=0.001) were negatively correlated with the difference in MAPSE septal between pre and post dipyridamole infusion whereas transplantation vintage displayed a positive association (r = 0.366, p 0.043). However, at stepwise multiple regression analysis all significant associations were lost. On the other hand, no independent correlates were found for lateral MAPSE among immune cell subsets. The E/E'ratio at baseline was inversely associated with the percentage of total lymphocytes and CD4+ lymphocytes (r= -0.390, p=0.03 and r= -0.456, p=0.010, respectively) as well as the number of Tregs (r= -0.419, p=0.019). Furthermore, a negative correlation was found between E/E'ratio and serum Tacrolimus levels (r= - 0.557, p=0.013), which was the only parameter at stepwise multiple regression analysis, only the association with Tacrolimus remained significant. E/E' ration after dipyridamole infusion was associated with gender female (r= 0.491, p=0.02) as well as inversely associated with the percentage of CD14+CD16++ monocytes (r= -0.492, p=0.02). However, following stepwise multiple regression analysis all significant associations were lost. No significant associations were observed regarding the difference in E/E' ratio after and prior to dipyridamole infusion with immune cells subsets.

Table 22. Univariate and multivariate correlates of classical indices of left ventricular function at baseline and their respective differences between values post and prior to dipyridamole infusion in kidney transplant recipients. Only correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis	**Multivariate analysis**

	R/Rho	P VALUE	Anova R^2 P value	β	P VALUE
LVEF			0.357, p=0.002		
Tregs %	*-0.384*	*0.03*		*-0.341*	***0.03***
Female gender	*0.456*	*0.01*		*0.454*	***0.006***
ΔLVEF			0.319, p=0.005		
Albumin	*0.447*	*0.012*		*0.353*	***0.03***
LDL	0.381	0.034			
CD14++CD16 - %	-0.447	0.03		-0.174	0.179
T cells %	-0.475	0.007		0.95	0.557
CD4+ T cells No	*-0.483*	*0.006*		***-0.378***	***0.02***
CD8+ T cells No	-0.371	0.004		-0.262	0.140
TAPSE			0.288, p=0.001		
CD8+ T cells %	*0.494*	*0.005*		*0.559*	***0.001***
CD4+ T cells No	-0.456	0.001		-0.043	0.805
Glucose	0.404	0.024		0.221	0.161
MAPSE septal			0.483, p=0.000		
Hypertension	-0.364	0.044		-0.13	0.414
HDL	*0.417*	*0.017*		*0.474*	***0.002***
Phosphorus	*0.369*	*0.041*		*0.376*	***0.01***
Albumin	0.331	0.069		0.87	0.582
CD4+ T cells %	*-0.303*	*0.098*		*-0.359*	***0.01***
ΔSm			0.266, p=0.01		
CD14+CD16++ monocytes	0.643	0.01		0.151	0.576
CD14++CD16 - monocytes %	*-0.489*	*0.01*		*-0.516*	***0.010***

ΔSl			0.500, p=0.000		
Age	0.439	0.03		0.137	0.405
CD14++CD16 - monocytes %	*-0.747*	*0.000*		*-0.707*	***0.000***
C14++CD16+ monocytes %	0.484	0.02		0.104	0.519
C14+CD16++ monocytes %	0.466	0.03		-0.068	0.762
Calcium	0.451	0.03		0.200	0.217
E/A			0.350, p=0.02		
Age	*-0.466*	*0.008*		*-0.481*	***0.004***
Transplant vintage	0.596	0.000		0.211	0.179
NK cells No	*-0.359*	*0.047*		*-0.387*	***0.02***
Hemoglobin	-0.429	0.016		-0.140	0.435

E/A, early to late diastolic transmitral wave ratio; LDL, low density lipoprotein; LVEF, left ventricle ejection fraction; MAPSE, mitral annular plane systolic excursion; No, number; Sm, medial wall systolic velocity; Sl, lateral wall systolic velocity; TAPSE, tricuspid annular plane systolic excursion; Δ, difference between values of echocardiographic parameters post and prior to dipyridamole infusion.

Table 23. Univariate and multivariate correlates of classical indices of left ventricular function following dipyridamole infusion in kidney transplant recipients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P**	**R^2 P value**	**β**	**P value**
LVEF post			0.683 P=0.01		
Monocytes %	-0.380	0.035		-0.089	0.723
T cells No	-0.384	0.033			
T cells %	-0.416	0.001		-0.299	0.153
CD4+ T cells No	-0.358	0.048		0.053	0.854
NK cells %	0.416	0.02		0.351	0.076
Tregs %	-0.429	0.016		*-0.481*	**0.046**

Female gender	0.402	0.025		*0.580*	**0.021**
TAPSE post			0510, p=0.000		
CD8+ T cells No	0.404	0.033		*0.450*	**0.004**
Arterial hypertension	-0.515	0.005		*-0.521*	**0.001**
MAPSE septal post			0.214 P=0.046		
ESR	0.382	0.034		0.110	0.663
CD4+ T-cells %	- 0.468	0.008		*-0.463*	**0.046**
Tregs No	-0.449	0.01		-0.073	0.746
Tregs %	-0.417	0.02			
Tacrolimus	-0.518	0.023		-0.149	0.609
E/A post			0.211, P=0.012		
Tregs %	0.397	0.033		0.107	0.601
CD8+ T cells	-0.502	0.006		*-0.459*	**0.012**
Hemoglobin	-0.460	0.012		-0.140	0.444
Sm post			0.440, p=0.002		
CD14+CD16++ monocytes	0.562	0.004		-0.184	0.451
NK%	0.460	0.024		*0.499*	**0.007**
CD14++CD16-%	-0.424	0.039		**-0.347**	**0.05**
Sl post			0.385, p=0.001		
NK No	0.527	0.008			
NK %	0.645	0.001		*0.621*	**0.001**
T-lymphocytes %	-0.571	0.004		-0.209	0.733
Uric acid	-0.494	0.014		-0.252	0.163

E/A, early to late diastolic transmitral wave ratio; ESR, erythrocyte sedimentation rate; LVEF, left ventricle ejection fraction; MAPSE, mitral annular plane systolic excursion; No, number; Sm, medial wall systolic velocity; Sl, lateral wall systolic velocity; TAPSE, tricuspid annular plane systolic excursion.

Correlations at univariate followed by multivariate stepwise regression analyses of novel echocardiographic indices at baseline and the differences between their values following and prior to dipyridamole infusion with immune cells subpopulations, with clinical characteristics and laboratory parameters in KTRs are depicted in Table 24 and in Figure 12 whereas Table 25 presents the correlations at univariate followed by multivariate stepwise regression analyses of novel echocardiographic indices following dipyridamole infusion with immune cells subpopulations, with clinical characteristics and laboratory parameters in KTRs.

The GLS was associated with male gender (r= -0.433, p=0.017) and directly correlated with LDL levels (r=0.384, p=0.036) whereas it was inversely associated with the number of NK cells (r= -0.447, p=0.013) (Table 24). At stepwise multiple regression analysis, only gender and NK cell number (β=-0.362, p=0.01) remained significantly associated with GLS (Table 24). GLS following dipyridamole infusion, was significantly associated with the percentage of monocytes (r= 0.362, p 0.046) and inversely associated with the percentage of NK cells (r= -0.365, p=0.044), with NK cell percentage (β=-0.517, p=0.004) retaining significance at stepwise multiple regression analysis (Table 25). The difference in GLS between post and pre dipyridamole infusion values were positively associated with the percentage of CD14++CD16- monocytes (r= 0.455, p=0.012) as well as the number and percentage of CD4+ T cells (r = 0.386, p=0.035 and r= 0.386, p=0.034 respectively) whereas it was negatively associated with the percentage of CD14++CD16+ monocytes (r = -0.374, p=0.042) (Table 24). Finally, at stepwise multiple regression analysis, independent correlates of the ΔGLS included the CD14++CD16+ monocytes (β=-0.423, p=0.009) and CD4+ T cells (β=0.403, p=0.01) whereas the positive association of CD14++ monocytes (p = 0.012, r= 0.455) was subsequently lost (Table 24).

Left ventricular TWIST at baseline was negatively associated with monocytes counts (r= -0.412, p=0.024) and positively with the percentage of CD14++CD16+ monocytes (r = 0.442, p=0.015) (Table 24). Independent correlates of left ventricular TWIST at baseline were total monocytes counts (β=-0.335, p=0.04) and the percentage of CD14++CD16+ monocytes (β=0.416, p=0.01) (Table 24). TWIST post dipyridamole infusion correlated with the percentage of lymphocytes (r= 0.396, p=0.03) and age (r= 0.421, p=0.021), however at stepwise multiple regression analysis only the percentage of lymphocytes remained significant (Table 25). Finally, at stepwise multiple regression analysis no significant associations were found for the difference between TWIST levels post and pre dipyridamole infusion.

UNTWIST was inversely associated with the percentage of CD14++CD16+ monocytes (r = -0.400, p=0.029) which remained significant at stepwise multiple regression analysis among other covariates (β=-0.742, p=0.09) (Table 24). UNTWIST post was positively associated with the percentage of monocytes (r= 0.523, p=0.03) and the presence of hypertension (r= 0.366, p=0.047) and negatively associated with the percentage of NK cells (r= -0.392, p=0.032) (Table 25). At stepwise multiple regression analysis only the

CD14++CD16+ monocytes (β=-0.412, p=0.02) emerged as a significantly correlate of UNTWIST post dipyridamole infusion, even though at univariate analysis they failed to reach statistical significance (r=-0.310, p=0.096) (Table 25). Finally, regarding the difference of UNTWIST between pre and post dipyridamole infusion, a positive association was found with the percentage of monocytes (r= 0.419, p=0.024), however at multivariate multiple regression analysis, the significance was lost.

No significant associations were found between GCS and immune cell subsets. GCS was associated with male gender, serum hemoglobin (r= 0.451, p=0.011) serum albumin (r= 0.423, p=0.018), serum cholesterol (0.015, r= 0.431), serum calcium levels (r= 0.609, p=0.000) as well as inversely associated with serum urea (r= -0.404, p=0.024), serum creatinine (r= -0.416, p= 0.02), positively with eGFR levels (r= 0.582, p=0.001) and negatively with UPCR (r= -0.477, p=0.007). At stepwise multiple regression analysis, only eGFR remained significantly associated with GCS. No significant associations were observed with GCS post dipyridamole infusion with immune cell subsets. Regarding difference of GCS between values following and prior to dipyridamole infusion, it was positively associated with the percentage of CD14++ monocytes (r= 0.372, p=0.043) and trough cyclosporine levels whereas it was inversely associated with age (r= -0.379, p=0.039), serum protein levels (r= -0.396, p=0.03) and serum calcium levels (r= -0.443, p=0.014). However, following stepwise multiple regression analysis, all associations lost significance except for serum cyclosporine levels.

Table 24. Univariate and multivariate correlates of novel indices of left ventricular function in kidney transplant recipients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**ANOVA R^2 P Value**	**β**	**P value**
GLS			0.271, p=0.01		
Gender male	*-0.443*	*0.017*		*-0.434*	***0.01***
NK cells number	*-0.447*	*0.01*		*-0.362*	***0.03***
LDL	0.384	0.036		0.180	0.311
ΔGLS			0.271, p=0.01		
Hypertension	*0.315*	*0.09*		0.364	**0.022**
CD14++CD	0.455	0.012		0.248	0.156

16-monocytes %					
CD14++CD 16+ monocytes %	*- 0.374*	*0.042*		*-0.423*	***0.009***
CD4+ T cells %	0.386	0.035		*0.403*	***0.01***
CD4+ T cells No	*0.386*	*0.034*			
Tregs %	0.324	0.081		0.274	0.107
Albumin	-0.320	0.085		-0.260	0.097
TWIST			0.435, p=0.002		
Monocytes No	*-0.412*	*0.024*		*-0.335*	***0.04***
CD14++CD 16+ monocytes %	*0.442*	*0.015*		*0.416*	***0.01***
Lymphocytes No	-0.343	0.064		-0.03	0.874
Lymphocytes %	-0.33	0.075			
UNTWIST			0.550, p=0.009		
DM	*0.326*	*0.079*		0.113	0.647
CD14++CD 16+ monocytes %	*-0.400*	*0.029*		*-0.742*	***0.009***
Ferritin	-0.344	0.063		-0.360	0.116
CyA C0	-0.545	0.083		-0.198	0.436

C0, trough blood levels; DM, diabetes mellitus; GCS, global circumferential strain; GLS, global longitudinal strain; LDL, low density lipoprotein; No, number; Δ, difference between values of echocardiographic parameters post and prior to dipyridamole infusion.

Table 25. Univariate and multivariate correlates of novel indices of left ventricular function following dipyridamole infusion in Tx patients. Only independent correlations of echocardiographic indices with immune cells subsets maintaining significance at stepwise regression analysis are depicted.

Univariate analysis			**Multivariate analysis**		
	R/Rho	**P value**	**R2** **P value**	**β**	**P value**
GLS post			0.127, p=0.049		
Monocytes %	0.362	0.046		0.265	0.136
NK cells %	-0.365	0.044		*-0.357*	**0.049**
TWISTpost			0.176, p=0.021		
Age	0.421	0.021		0.311	0.078
Lymphocytes %	0.396	0.03		*0.419*	**0.021**
UNTWIST post			0.170, p=0.024		
Monocytes	0.523	0.03			
CD14++CD16+ monocytes %	-0.310	0.096		*-0.412*	**0.024**
NK cells %	-0.392	0.032		-0.273	0.215
Arterial hypertension	0.366	0.047		0.229	0.215

GCS, global circumferential strain; GLS, global longitudinal strain.

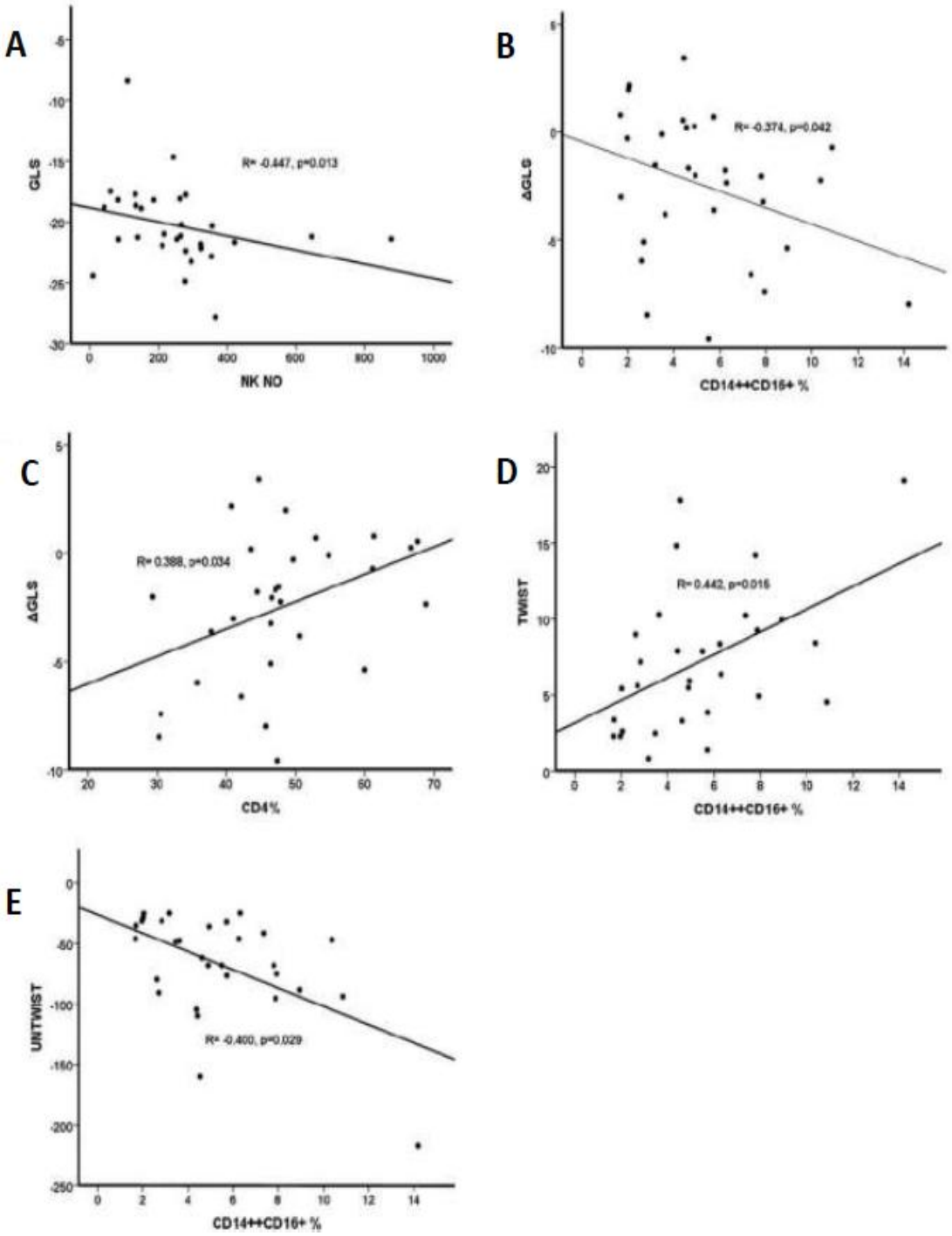

Figure 12. Associations between myocardial strain echocardiographic indices of cardiac function with immune cells subsets in kidney transplant recipients. A. More negative (better) baseline GLS is inversely associated with NK cells in kidney transplant recipients. B. DIPSE-induced improvement in GLS is associated with a higher percentage of CD14++CD16+ monocytes in kidney transplant recipients. C. DIPSE-induced improvement in GLS is associated with a lower percentage of CD4+ T-cells in kidney transplant recipients. D. Higher baseline left ventricular TWIST (better) is positively associated with the percentage of CD14++CD16+ monocytes in kidney transplant recipients. E. More negative baseline left

ventricular UNTWIST (better) is negatively associated with the percentage of CD14++CD16+ monocytes in kidney transplant recipients.

8.6 Survival analyses in CRS patients categorized by circulating immune cell subsets expression

During a mean follow-up of 29.8 ± 3.4 months, 23 patients out of 39 patients with CRS type 2 (59%) reached the study endpoint with no patients being lost to follow-up. At binary logistic regression analysis, immune cells subpopulations that predicted all cause and cardiovascular death included total lymphocytes counts (OR 0.85 per 100 cells/μL increase; 95% CI 0.75-0.97; p=0.01), T-cells number (OR 0.82 per 100 cells/μL increase; 95% CI 0.70-0.96; p=0.01), CD4+ T-lymphocytes number (OR 0.66 per 100 cells/μL increase; 95% CI 0.50-0.87; p=0.004), CD8+ T-lymphocytes counts below their median value cut-off of 410/μL (OR 4.67; 95% CI 1.14-19.07; p=0.03), Tregs counts below their median value cut-off of 35/μL (OR 6.63; 95% CI 1.36-23.27; p=0.01) and CD14++CD16+ monocytes counts above their median cut-off value of 40/μL (OR 4.13; 95% CI 1.06-16.1; p=0.04). In a multivariate model including all six immune cell subsets, only the CD4+ T-lymphocytes remained independent predictors of mortality (OR 0.66; 95% CI 0.50-0.87; p=0.004). In contrast, no such associations were found for age, eGFR, UPCR, hsTnI, BNP, as well as the rest clinical or laboratory indices.

Subsequently, Kaplan-Meier survival curves for patient with CRS type 2 according to the levels of immune cells subpopulations (i.e. below vs above median value) are shown in Figure 13 (CD14++CD16+ monocytes), Figure 14 (total lymphocytes), Figure 15 (T lymphocytes), Figure 16 (CD4+ T cells), Figure 17 (CD8+ T cells) and Figure 18 (Tregs). Decreased levels of lymphocytes, T-lymphocytes, CD4+ T-cells, CD8+ T cells and Tregs were associated with mortality at a mean follow-up of 30 months ($p<0.05$ for all log-rank test). Increased levels of the pro-inflammatory, intermediate CD14++CD16+ monocytes counts showed a non-significant trend for increased mortality (p=0.093).

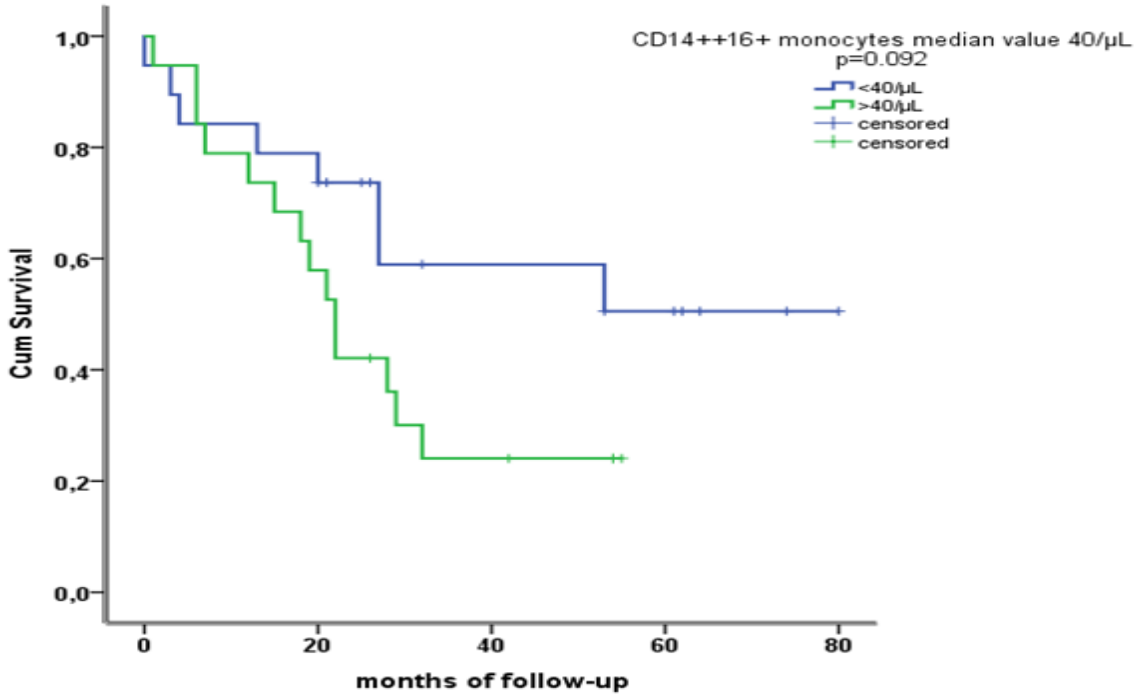

Figure 13. Kaplan-Meier curves of endpoint-free patients with CD14++CD16+ monocytes number expression below or above median value derived cut-offs.

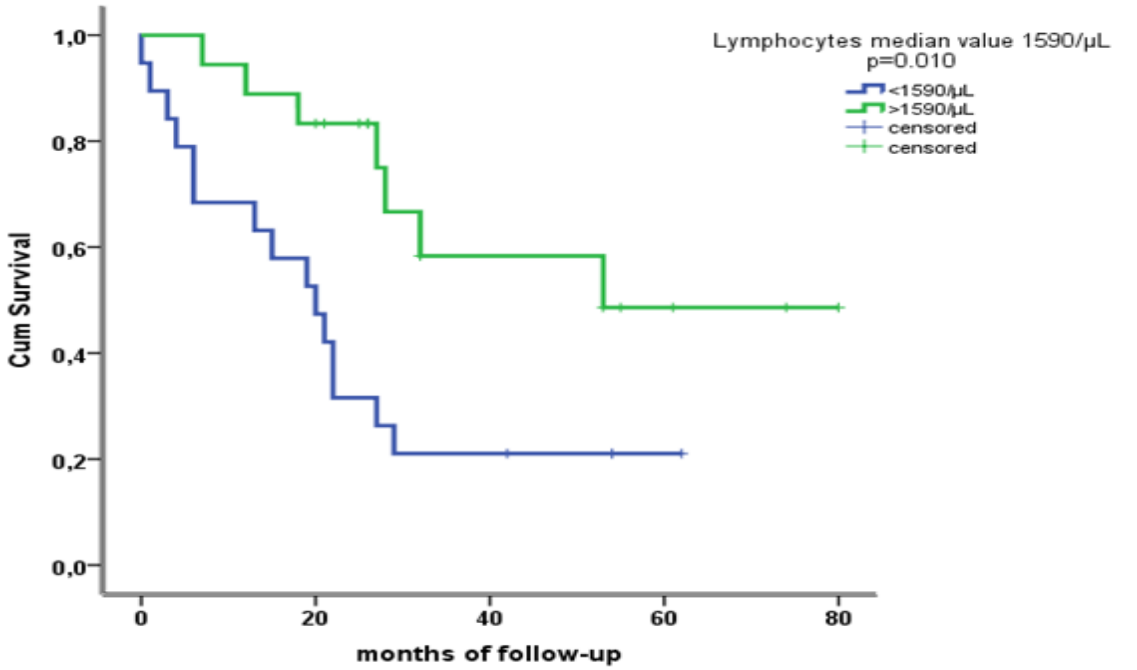

Figure 14. Kaplan-Meier curves of endpoint-free patients with total lymphocytes number expression below or above median value derived cut-offs.

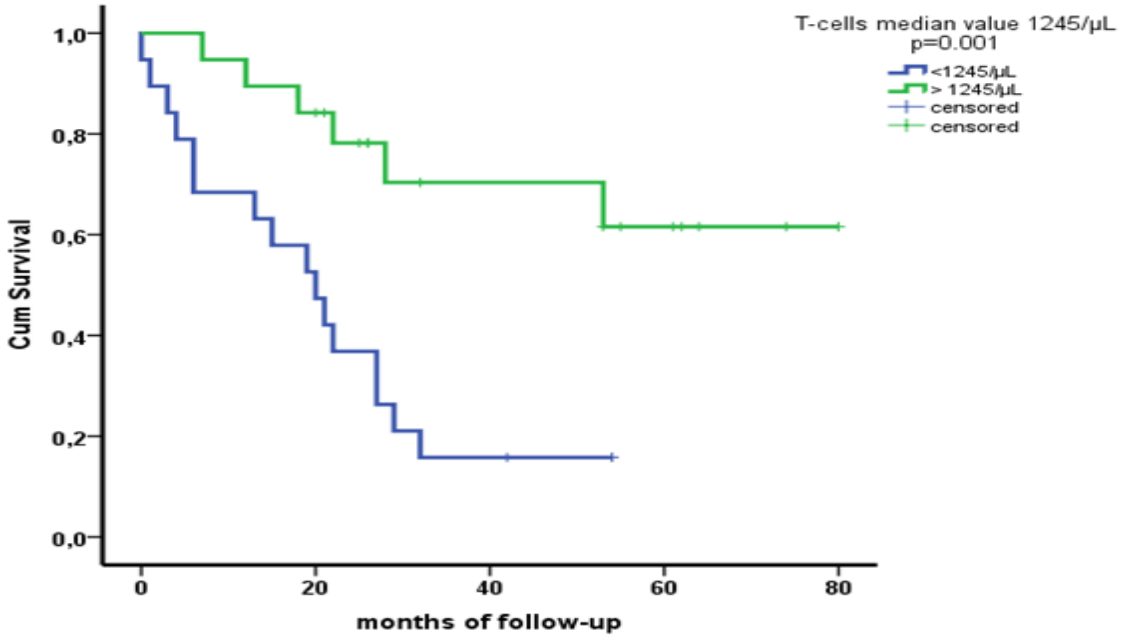

Figure 15. Kaplan-Meier curves of endpoint-free patients with T lymphocytes number expression below or above median value derived cut-offs.

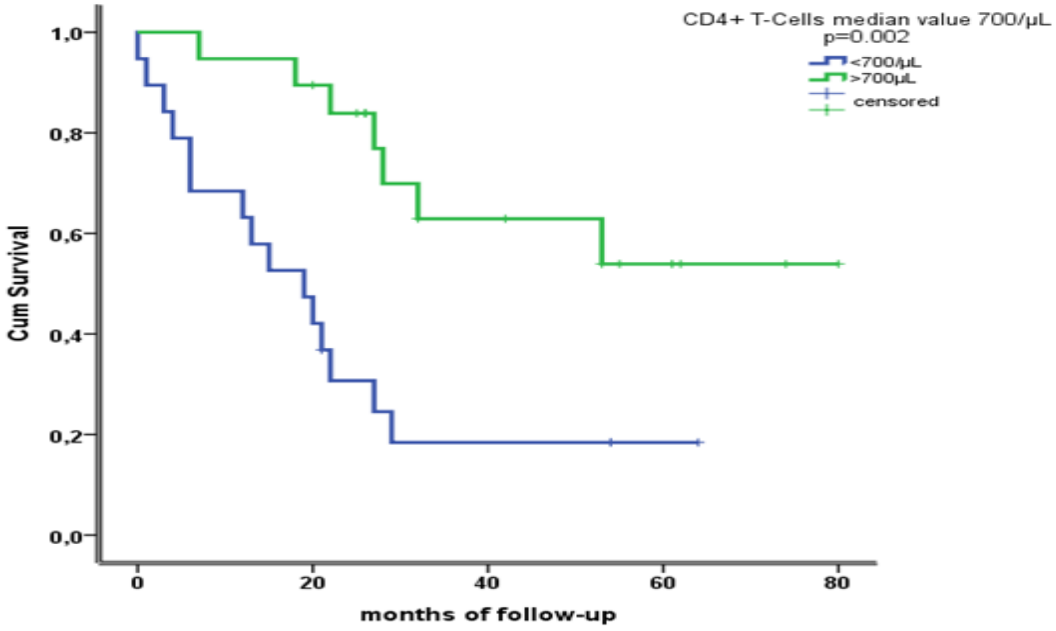

Figure 16. Kaplan-Meier curves of endpoint-free patients with CD4+ T cells number expression below or above median value derived cut-offs.

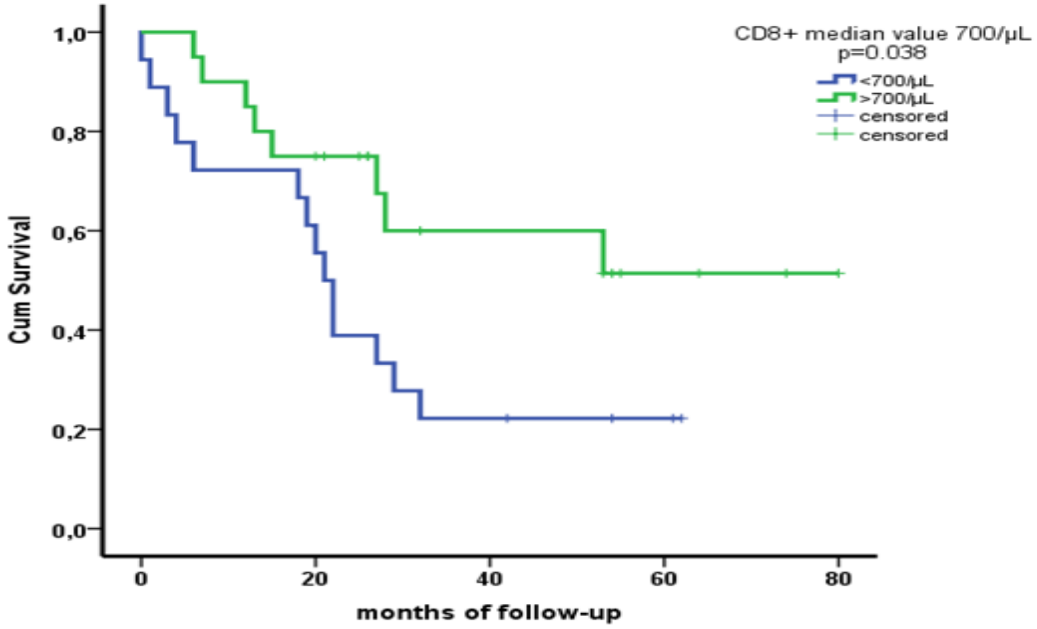

Figure 17. Kaplan-Meier curves of endpoint-free patients with CD8+ T cells number expression below or above median value derived cut-offs.

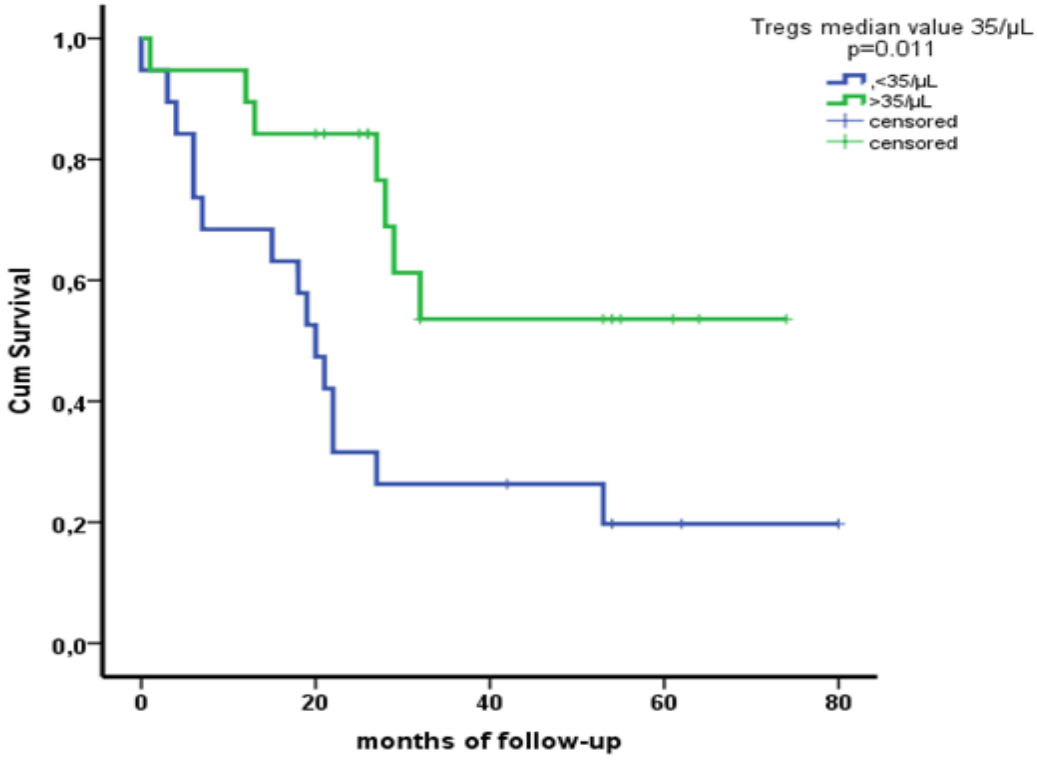

Figure 18. Kaplan-Meier curves of endpoint-free patients with Tregs number expression below or above median value derived cut-offs.

8.7 Longitudinal analysis of immune cell phenotypes in the circulation of CKD patients and clinical correlations.

Out of 44 CKD patients that were recruited at baseline, 21 CKD patients (mean age 60 ±12 years, 50% males) were included in the final longitudinal analysis. No significant differences were observed at follow-up with regard to eGFR, UPCR, or the inflammatory markers (Table 26). Differences in immune cells subsets between To and T1 are presented in Table 27. With regard to immune cell subsets, a significant increase was observed between T0 and T1 in the percentage of the non-classical CD14+CD16++ monocytes (4.7 ±1.9% at T0 vs 7.9 ±4.3%, p=0.044 at T1). Likewise, a significant increase was observed in the number of B lymphocytes (87 ±39 μL at T0 vs 107 ±53/ μL, p=0.047 at T1). Finally, a drop in the percentage of CD4+ T cells was observed (49.3 ±11.5% at T0 vs 45.1 ±12.3 at T1, p=0.037). The rest immune cells subsets did not show any significant differences between T0 and T1. No significant associations were observed between the ΔeGFR with any of the immune cell subsets or the laboratory indices. Likewise, no significant associations were observed between the ΔUPCR with any of the immune cell subsets or the laboratory indices.

Table 26. Main laboratory parameters in To and T1 in CKD patients

Immune cells	**T0**	**T1**	**p-value**
eGFR (ml/min/1.73m^2)	26 ±11	25 ±12	0.502
UPCR (g protein/g creatinine)	0.91 ±0.95	2.3 ±2.77	0.182
Hemoglobin (g/dl)	12.5 ±1.4	12.6 ±1.1	0.857
Uric Acid (mg/dl)	7.9 ±2.0	72.2 ±1.6	0.388
ESR (mm/h)	29 ±19	39 ±25	0.091
CRP (mg/L)	3.1 ±1.9	2.6 ±1.4	0.487
Albumin (g/dl)	4.2 ±0.2	4.3 ±0.4	0.598

Table 27. Immune cell subsets at T0 and T1 in CKD patients.

Immune cells	**T0**	**T1**	**p-value**
Monocytes (N)	462 ±168	450 (400-550)	0.748

Monocytes (%)	7.3 ±1.2	6.8 ±1.4	0.306
CD14++CD16- (N)	379 ±134	382 ±82	0.941
CD14++CD16- (%)	82.2 ±6.1	80.1 ±4.9	0.414
CD14++CD16+ (N)	39 ±19	37 ±16	0.886
CD14++CD16+ (%)	8.8 ±3.5	7.9 ±3.1	0.578
CD14+CD16++ (N)	22 ±11	37 ±19	0.104
CD14+CD16++ (%)	4.7 ±1.9	7.9 ±4.3	**0.044**
Lymphocytes (N)	1691 ±515	1870 ±681	0.471
Lymphocytes (%)	27.3 ±5.4	25.9 ±7.2	0.528
T-lymphocytes (N)	1391 ±431	1415 ±474	0.900
T-lymphocytes (%)	81. 9 ±4.7	76.5 ±6.1	0.092
B-lymphocytes (N)	87 ±39	107 ±53	**0.047**
B-lymphocytes (%)	5.2 ±1.9	5.9 ±2.7	0.257
NK cells (N)	219 ±108	345 ±236	0.142
NK cells (%)	12.7 ±4.4	13.3 ±6.9	0.117
CD4+ T-Cells (N)	831 ±234	816 ±324	0.869
CD4+ T-cells (%)	49.3 ±11.5	45.1 ±12.3	**0.037**
CD8+ T-cells (N)	532 ±309	574 ±305	0.675
CD8+ T-cells (%)	30.6 ±10.2	30.2 ±9.3	0.776
Tregs (N)	30 ±23	28 ±11	0.706
T Regs (%)	1.6 ±0.8	1.6 ±0.5	0.766

8.8 Longitudinal analysis of immune cell phenotypes in the circulation of kidney transplant recipients and clinical correlations.

There were included 35 KTRs (mean age 53 ±9.28 years, 71% males, mean transplant vintage 96 ±66 months, 63% on Tacrolimus and 37% on Cyclosporine) out of 38 KTRs in the final analysis. The main laboratory parameters at T0 and T1 and their respective differences are presented in Table 28. Mean eGFR declined from 58 ±17 at T0 to 53 ±18ml/min/1.73 m^2 at T1 (p=0.004). No significant changes were observed between T0 and T1 in median UPCR [0.16 (IRQ, 0.09-0.56) at T0 and 0.16 (IQR, 0.10-0.70) g protein/g creatine at T1, p=0.489], in median CRP [4.0 (IQR, 3-7) at T0 and 5 (IQR, 2.5-7.5) mg/L) at T1, p= 0.919], in mean ESR (20±14 at T0 and 22 ±19 mm/hour at T1, p=0.381) or other parameters, including CNIs blood

levels. The absolute numbers and the percentages of immune cell subsets at T0 and T1 and their respective differences are presented in Table 29. Significant differences were observed between T0 and T1 in monocytes number (653 ±244 and 538 ±197/μL respectively, p=0.001), monocytes percentage (7.6 ±2.9 and 6.6 ±2.2% respectively, p=0.006) as well as in the number of classical CD14++CD16- monocytes (534 ±225 and 452 ±185/μL respectively, p=0.04). The rest immune cells subsets did not show any significant differences between T0 and T1. The significant correlates of ΔeGFR and ΔUPCR are presented in Table 30. Accordingly, ΔeGFR was correlated with the T0 percentage of monocytes (r =0.359, p= 0.037), the T0 number and T0 percentage of CD14++CD16+ monocytes (r= 0.502, p = 0.003 and r =0.438, p= 0.008 respectively). On the other hand, a borderline inverse correlation was observed between ΔeGFR and ΔCD14++CD16+ monocytes number (r =-0.339, p= 0.05). Additional correlates of ΔeGFR included serum albumin at T0 (rho=0.395, p=0.021) and Δuric acid (r =0.567, p<0.001). At stepwise linear regression analysis, CD14++CD16+ monocytes (β=0.338, p=0.04) and Δuric acid (β=0.477, p=0.006) remained independent significant correlates of ΔeGFR. ΔUPCR was significantly correlated with the percentage of B-lymphocytes (rho=0.385, p=0.027) and CD4+ T-cells (r=0.352, p=0.044) at T0, and inversely correlated with the T0 percentage of T-lymphocytes (r=-0.402, p=0.02) and CD8+ T cells (r =-0.603, p<0.001) as well as with ΔHemoglobin (r =-0.385, p=0.027). At stepwise linear regression analysis, only the CD8+ T cells percentage at T0 remained independently correlated to ΔUPCR (β=–0.379, p=0.03).

Table 28. Main laboratory parameters in T0 and T1 in kidney transplant recipients

	To	**T1**	**p-value**
eGFR (ml/min/1.73m^2)	58 ±17	53 ±18	**0.004**
UPCR (g protein/g creatinine)	0.16 (0.09-0.56)	0.16 (0.10-0.70)	0.489
Hemoglobin (g/dl)	13.5±1.8	13.2±2.0	0.182
Uric Acid (mg/dl)	6.8±1.5	7.1±1.8	0.425
ESR (mm/hour)	20±14	22±19	0.381
CRP (mg/L)	4.0 (3-7)	5 (2.5-7.5)	0.919
Albumin (g/dl)	4.2±0.38	4.8±0.55	0.330
Cyclosporine Co (ng/ml)	114±32	129±30	0.259
Tacrolimus C0 (ng/ml)	6.8± 2.3	7.0±2.2	0.744

Values are expressed as the mean (±SD) or median (IQR 25–75th percentiles). CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; UPCR, urinary protein to creatinine ratio. * *p* refers to *t* test significance for normal distribution variables, to the Mann–Whitney test significance for non-parametric variables

Table 29. Immune cell subsets at T0 and T1 in kidney transplant recipients.

Immune cells	**T0**	**T1**	**p-value**
Monocytes (N)	653 ±244	538 ±197	**0.001**
Monocytes (%)	7.6 ±2.9	6.6 ±2.2	**0.006**
CD14++CD16- (N)	534 ±225	452 ±185	**0.044**
CD14++CD16- (%)	85.5 ±8.5	83.5 ±11	0.419
CD14++CD16+ (N)	25 (16-45)	25 (20-42)	0.871
CD14++CD16+ (%)	4.6 (2.8-7.3)	5.4 (4.0-8.4)	0.403
CD14+CD16++ (N)	18 (13-28)	17 (10-25)	0.518
CD14+CD16++ (%)	3.2 (1.9-5.4)	3.3 (2.3-4.8)	0.781
Lymphocytes (N)	2083 ±817	1897±819	0.06
Lymphocytes (%)	24.3 ±8.4	23.2 ±7.9	0.428
T-lymphocytes (N)	1718 ±716	1578 ± 713	0.132
T-lymphocytes (%)	81 ±8.2	81.3 ±7.7	0.774
B-lymphocytes (N)	88 (33-140)	69 (24-151)	0.800
B-lymphocytes (%)	4.3 1.9-6.7)	4.2 (1.9-5.8)	0.967
NK cells (N)	274 ±176	234 ±130	0.068
NK cells (%)	13.8 ±8.0	13.6 ±7.7	0.879
CD4+ T-Cells (N)	965 ±455	917 ±468	0.366
CD4+ T-cells (%)	46.7 ±9.4	46.6 ±11	0.967
CD8+ T-cells (N)	713 ±356	642 ±332	0.060
CD8+ T-cells (%)	34.0 ±9.3	34.1 ±10.8	0.948
Tregs (N)	22± 14	20 ±16	0.626
T Regs (%)	0.93 (0.63-1.71)	0.88 (0.62-1.48)	0.913

Values are expressed in mean (±SD) or median (IQR 25–75th percentiles). CD, cluster of differentiation; N, number per microliter; NK, natural killer; No, number; Tregs, T regulatory cells.

Table 30. Univariate correlates of ΔeGFR and ΔUPCR in kidney transplant recipients.

ΔeGFR	ΔUPCR
Monocytes % (T0)	**B-lymphocytes % (T0)**
r=0.359, p=0.037	*r =0.385, p = 0.027*
CD14++CD16+ N (T0)	**CD4+ T-cells% (T0)**
r=0.502, p=0.003	*r=0.352, p=0.044*
CD14++CD16+ % (T0)	**T-lymphocytes % (T0)**
r=0.438, p=0.008	*r=-0.402, p=0.02*
ΔCD14++CD14+	**CD8+ T cells % (T0)**
r=-0.339, p=0.05	*r=-0.603, p<0.001*
serum Albumin (T0)	**ΔHemoglobin**
r=0.395, p=0.021	r=0.385, p=0.027
Δuric acid	
r= 0.567, p<0.001	

Correlations were assessed by Spearman's or Pearson rank tests. Only correlations reaching statistical significance are presented. eGFR, estimated glomerular filtration rate; UPCR, urine protein to creatinine ratio; Δ, difference between values of eGFR and UPCR between T1 and T0 respectively.

9. Discussion and conclusion

9.1 Discussion

There are substantial gaps in our understanding and addressing the unique mechanisms underlying the systemic impact of CKD on various organ systems both prior to and following organ transplantation. Thus, the identifications of novel, specific biological traits and clinical parameters which characterize CKD patients and KTRs would be of great significance and up to date. Accordingly, the timing and the inceptive pathogenic immune mechanisms underlying the subclinical cardiovascular damage in CKD remain to be clarified. To the best of our knowledge, this exploratory study is the first to indicate independent correlations between cellular components of the innate and acquired immune system and in

specific classical CD14++ monocytes, intermediate CD14++CD16+ monocytes, CD4+ T-cells, CD8+ T-cells and NK cells with the novel strain related echocardiographic indices of subclinical myocardial dysfunction in CKD patients and KTRs without established CVD. With regard to patients with CRS, to the best of our knowledge, this is the first report in the literature examining the profile of immune cell subtypes, including the CD14++CD16+ proinflammatory monocyte subpopulation, NK cells and lymphocytes subpopulations in patients with type 2 CRS as well as comparing the profile of immune cell subtypes in type 2 CRS and patients with CKD but without established CVD. In addition, the potential associations of immune cells with clinical parameters, biomarkers and outcomes have not been studied in these patients with type 2 CRS until now.
In the discussion, the most important results of the study will be presented. An attempt will be made to interpret them based on existing experimental and clinical knowledge and our findings will be compared with results from other relevant studies in CKD patients, if these are available, or with other high-risk populations for CVD.

9.1.1 Monocytes subsets correlate with subclinical indices of LV function in CKD patients and kidney transplant recipients before the establishment of overt CVD and bear prognostic implications in type 2 CRS

Monocytes subsets appear to be involved in the inflammatory pathways underlying the extracellular matrix deposition and cardiomyocyte hypertrophy as occur in diastolic dysfunction and HFpEF. Even though there is very little evidence available at present, it is plausible that the promotion of inflammation by monocytes and macrophages is directly associated with the development of myocardial fibrosis in HFpEF. Thus, increased monocyte number leads to an increase in collagen deposition and conversion of cardiac fibroblasts to myofibroblasts. In vitro studies of human cardiac myofibroblasts co-cultured with peripheral blood monocytes isolated from healthy human donors, showed that direct cell-cell interaction between monocytes and cardiac myofibroblasts promotes TGF-β release and subsequently local matrix remodeling (741). Clinical evidence regarding the role of blood monocytes in myocardial remodeling is scarce. A study of asymptomatic subjects with data available on the size of the common carotid artery and circulating total WBC counts showed that higher monocytes counts were independently correlated with an increased RWT and LVMI (742). Moreover, higher monocytes counts as well as a larger common carotid artery diameter were the strongest predictive factors for the development of HF and occurrence of all-cause death in the same cohort (742). A study including patients with arterial hypertension, LV diastolic dysfunction and HFpEF showed that the percentage of peripheral blood monocytes was more markedly increased in patients with LV diastolic dysfunction and HFpEF compared to hypertensive subjects (743).

In CKD patients, higher peripheral blood monocyte counts have been associated with adverse kidney outcomes. Accordingly, in a large observational cohort study of more than 1.5 million United States veterans followed for nearly ten years, a graded association was found between monocyte count and risk of development of CKD, risk of CKD progression

and development of kidney failure (744). Still, in the setting of CKD, the link and the prognostic role of peripheral blood monocytes to adverse LV myocardial remodeling remains obscure at present. Experimental models of impaired kidney function have shown expansion of cardiac macrophages, both through the proliferation of local subsets and via the influx and subsequent polarization of circulating monocytes whereas on the other hand monocytopenia appears to prevent the increase in resident macrophages and myocardial remodeling (503). Our data are in accordance with evidence from non-CKD cohorts as we found a positive correlation of monocytes counts with RWT in CKD patients. Furthermore, increased monocytes counts were associated with impaired novel, subclinical strain and deformation related indices of LV function, including GLS, TWIST and UNTWIST in CKD patients and in KTRs.

However, acknowledging the fact that the role of monocytes in CVD is complex and that distinct subsets of monocytes have been identified, which possess diverse properties with potentially detrimental or alternatively beneficial effects on myocardial remodeling, that however have not yet been determined, we further examined the association of monocytes subsets with classical and novel indices of subclinical myocardial dysfunction in CKD patients and KTRs. Taking into consideration models of hypertensive cardiomyopathy in order to draw potential similarities, a progressive decrease in the classical monocytes with a simultaneous increase in the percentage of CD16+ monocytes has been associated with increasing hypertension severity in hypertensive subjects (745). Thus, exposure of human monocytes to endothelial cells submitted to mechanical stretch promotes the differentiation of monocytes into the CD14++CD16+ intermediate and proinflammatory subtype. Similarly, according to findings from the MESA study, increments in the classical CD14++CD16- monocytes are associated with declining systolic blood pressure levels (515). Accordingly, a one standard deviation elevation in classical monocytes was associated with a decrease in the level of systolic BP by a 2.01 mmHg (95% CI 0.79–3.24) (515).

On the other hand, in our CKD patients, we found a direct association of RWT, a measure of left ventricular concentricity broadly used as an index of LVH, with CD14++CD16- monocytes count (β = *0.447*, p=0.004) whereas the correlation of this parameter with arterial hypertension was lost at multivariate analysis. Our finding is hypothesis generating, allowing us to speculate that diverse immune mechanisms may be implicated in the pathogenesis of LV hypertrophy in the setting of arterial hypertension and CKD respectively. Furthermore, our results are in line with evidence from another model of concentric ventricular hypertrophy due to pressure overload as occurs with aortic stenosis, which have shown an increased number of the classical $CD14^{++}$CD16- monocytes in patients with severe aortic valve stenosis (746). Finally, we should take into account the available evidence from experimental studies indicating that it is the classical CD14++CD16-monocytes subtype that enters the myocardium and polarizes to become the pro-fibrotic macrophage subset, which in turn activates fibroblasts to synthesize more collagen and fibronectin, subsequently leading to augmented myocardial stiffness and eventually causing LVH and diastolic dysfunction (747, 748). Thus, the specificities of immune cell dysfunction in CKD should be

taken into consideration and future studies are required to clarify the possible implication of CD14++CD16- monocytes in uremic cardiomyopathy.

Furthermore, we found in our study that the classical CD14++CD16- monocytes count was inversely associated with improvements in GLS following the administration of dipyridamole infusion both in CKD patients (β=0.320, p=0.036) and in KTRs (r= 0.455, p=0.012), albeit in the latter group the significance of the correlation was lost at multivariate analysis. This finding was further reinforced by the inverse association of the CD14++CD16- monocytes with systolic wall motion indices, such as with improvements in Sm and Sl following dipyridamole infusion (β=-0.516, p=0.01 and β=-0.707, p=0.000) respectively) and with LVEF (r= - 0.447, p 0.012) following dipyridamole administration, albeit the significance of the latter was lost at multivariate analysis, in KTRs. According to evidence from experimental models of ischemic myocardial dysfunction, the $Ly6C^{high}$ monocytes, the murine equivalents to human classical CD14++CD16- monocytes, are the first cells to be recruited during the initial proinflammatory phase of AMI, whereas later during the proliferation phase, $Ly6C^{low}$ monocytes, equivalents to human CD16+ monocytes become the predominant cells regulating fibroblast function and angiogenesis (452, 477).

On the other hand, data from clinical studies are few and heterogenous in terms of the aims and the populations involved. Classical CD14++CD16- monocytes are the first to be activated and accumulate following occurrence of MI and reach the highest levels nearly two and a half days after infarct onset, a chronological pattern that parallels the evolution of the monocyte subsets in response to injury (749). In patients with AMI, peak levels of the classical CD14++CD16- monocytes have been inversely associated with the magnitude of myocardium salvaged as well as with the recovery of left ventricular function (453). Of note, a decrease in classical monocytes counts has been observed following cardiac resynchronization therapy in the setting of HF, indicating a potential role of these cells in the myocardial remodeling process (750). Even though the pathogenesis of ischemic cardiomyopathy might bear significant differences compared to uremic cardiomyopathy and though mere associations are not an equivalent of causality, the inverse correlation of the classical CD14++CD16- monocytes with classical indices of LV systolic function as well as their association with impaired LV strain in the absence of established CVD, as we observed in our patients, support a potential link to the involvement of classical monocytes in the initial stages of the development of myocardial dysfunction in CKD and should prompt further investigation in this area.

Overall, most available studies concur that the distribution of the monocyte subsets, shifts in CVD with respect to the cardiovascular phenotype as well as depending on temporal circumstances (749). However, the etiology and the patterns of this shift in the setting of uremic cardiomyopathy still need to be elucidated. Other studies conducted in patients with HFpEF have found increased levels of all the monocytes subtypes, including the classical, intermediate and non-classical monocytes (741, 743). These findings along with ours underscore the relevance of CD14++CD16− monocytes in the myocardial responses during diverse clinical settings. Nevertheless, further investigation is required to

determine whether the classical monocytes play a direct role from the early stages in myocardial injury and the adverse remodeling associated with kidney disease as well as to clarify any potential singularities of the related inflammatory pathways in comparison to other disease states such as ischemic cardiomyopathy.

An interesting finding of our study is the trend of independent correlations between the intermediate CD14++CD16+ monocytes with strain related LV myocardial performance indices, both in CKD patients and in KTRs. Thus, the CD14++CD16+ monocytes were the sole parameter associated with higher baseline GRS values in CKD patients ($r= 0.351$, $p=0.04$) whereas in KTRs they were directly associated with better LV Twist ($\beta =0.416$, $p=0.015$) and Untwist parameters ($\beta =-0.742$, $p=0.009$) as well as with improved GLS following dipyridamole infusion ($\beta=-0.423$, $p=0.009$). Previous studies have shown elevated counts of the intermediate CD14++CD16+ monocytes in patients with both acute and stable chronic HF. Additionally, direct associations have been observed between these the intermediate CD14++CD16+ monocytes and the severity of HF, the number of hospitalizations due to HF decompensation as well as HF related mortality (493). Similarly, the CD14++CD16+ monocytes are increased in patients with atrial fibrillations and are considered to promote the fibrotic remodeling of the atria through increased expression of TGFβ by these cells (523, 751). However, recent studies aiming to shed light on the mechanisms of various monocytes subsets recruitment in the dysfunctional myocardium and their involvement in myocardial remodeling, hint to potentially protective features of the intermediate monocytes as well (492, 524). Notably, a less marked decrease of the CD14++CD16+ monocytes levels at the first month following AMI has been associated with a better LVEF after six months (524). Findings from a recent study also involving patients with AMI, indicate a direct relationship between augmented collateral vessel formation and a higher percentage of the CD14++CD16+ monocytes in the circulation, which subsequently translated into beneficial effects regarding infarct healing and LV remodeling in these patients (752). About our results, a potential explanation, though speculative, would be that a compensatory augmentation in strain related myocardial performance indices is promoted in the setting of the microinflammatory milieu of CKD, which would subsequently lead to myocardial damage and remodeling. Accordingly, relying on the sole available data so far, which comes from MI models and aiming to find common ground between these findings and our results, we might make an assumption that the accumulation of the intermediate monocytes in the setting of myocardial tissue injury is required during the early stages of wound healing, however, the maladaptive persistence of the intermediate CD14++CD16+ monocytes beyond the initial repair process could lead to long-term inflammation-related deleterious effects in the myocardium (748). Notably, accumulating evidence indicates that the inflammatory response induced by the innate immune system can be physiological in certain circumstances and results in the promotion and the accentuation of cytoprotective responses that allow the heart to adapt to stress (748). So as to spark further controversy, we might further take into consideration the fact that the intermediate CD14++CD16+ monocytes produce among other mediators, the anti-inflammatory IL-10, which has been

found to exert beneficial effects promoting tissue repair and the resolution of inflammation following acute myocardial injury, thus preventing the development myocardial dysfunction in the setting of AMI. In contrast, IL-10 has been associated with adverse effects in the chronic setting as it appears to promote myocardial fibrosis and diastolic dysfunction in HFpEF (748, 753). Another anti-inflammatory cytokine, IL-4 which is mainly secreted by resident cardiac macrophages has been as well associated with profibrotic actions by stimulating collagen synthesis in cardiac fibroblasts through activation of STAT6 (754). Thus, in the TAC model, neutralization of IL-4 leads to improvement of fibrotic changes (754, 755).

In line with the above, the fact that myocardial hypertrophy, triggered by various physiological or pathological stimuli, including hemodynamic stimuli, metabolic ones or infarction, remains above all an initial adaptive compensatory response of the heart, to injury, should be underscored (754). Accordingly, CKD and the related uremic environment might as well represent a specific injurious process to the myocardium, which in this setting undergoes a spectrum of changes from adaptive to maladaptive hypertrophy. Of note, the common pathways underlying the structural foundations of the progression from compensated hypertrophy to decompensated hypertrophy and HF, regardless of the pathophysiological background, remain undetermined (754). However, early activation of the inflammatory response pathways represents a reparative or protective action against the primary injurious stimuli whereas later on and following decompensation and overt HF development, the systemic activation of inflammatory signaling cascades has deleterious effects in the myocardium (754). Consequently, the apprehension of molecular and cellular immune mechanisms set in motion during the early remodeling process is essential so as to reverse it or at least to hinder its progression to overt HF. The seminal work by Levine et al, three decades ago revealed the tight link between HF and inflammation, with proinflammatory cytokines emerging as key factors for the initiation, coordination and perpetuation of the myocardial reaction to injury (756).

Another great example of the pluripotent and multidirectional effects of inflammation is the paradigm of TNFα which among the inflammatory mediators is considered the master regulator of inflammation. Continuous and excessive expression of TNFα has been considered a major culprit which promotes transition from early cardiac remodeling to overt myocardial decompensation (754). Furthermore, TNFα contributes to the adverse cardiac remodeling that occurs in the pressure-overload TAC model, as was demonstrated in TNFα-knockout mice (757). In contrast, strikingly, the results of the multicenter clinical trials conducted in HF patients using medications that inhibit TNFα, such as infliximab, an antibody against TNFα, and etanercept, a soluble recombinant receptor of TNFα, did not show any benefits or even led to HF worsening in these patients (758, 759). The disappointing results of these studies were subsequently translated in the revised cytokine hypothesis which took into consideration the complex impact of inflammation on the myocardium, including the beneficial short-term effects and the deleterious long-term consequences (754, 758, 759).

Actually, a significant body of evidence supports the hypothesis that short-term expression of the pro-inflammatory molecules might be beneficial and play the role of an early warning system (754, 760). Of note, with regard to pressure overload models, clinical data have shown higher myocardial TNFα gene expression in patients with compensated aortic stenosis compared to patients with decompensated stenosis, thus suggesting a potential adaptive role of TNFα during the early cardiac remodeling process and development of compensatory concentric LV hypertrophy (754). Inhibition of the TNFR (TNF receptor) 1 in TNFα overexpressing mice attenuates myocardial hypertrophic remodeling and protects myocardial function whereas on the other hand, inhibition of $TNFR_2$ exacerbates dilation and HF (754). Accordingly, $TNFR_1$ activation exerts pro-inflammatory, pro-hypertrophic effects and subsequently leads to the development of myocardial dysfunction, whereas co-activation of $TNFR_2$ during β adrenergic stimulation in the setting of stress suppresses inflammation thus creating equilibrium with the adverse effects of TNFR1 activation (754, 761, 762). In line with the above, increased expression and activity of NF-κB may exert contrasting effects to the myocardium such as reducing apoptosis on one hand whereas on the other leading to the development of various heart diseases (754). Thus, studies of patients with valvular disease have shown higher NF-κB activity and accentuated fibrosis in those with atrial fibrillation as compared to patients with sinus rhythm whereas in other clinical studies, a loss of function mutation of NF-κB was associated with increased risk of LV dysfunction as well as HF development and progression (763, 764).

The altered Ca^{2+} entry is implicated in the pathological LVH development (765). An alternative Ca^{2+} entry pathway, independent of store-depletion, involves the key participation of the Orai3 molecule (765). CD11b/c inflammatory cells, including monocytes and macrophages in the myocardium appear to regulate store-independent activation of Orai3-calcium influx via a TNFα triggered $TNFR_2$-dependent signaling pathway in the cardiomyocytes, which leads to the development of early, adaptive, augmented myocardial hypertrophy and simultaneously increased resistance to oxidative stress and delayed transition to HF (765). Global depletion of macrophages in arterial hypertension worsens cardiac function but improves fibrosis suggesting dual protective and pathological functions of the macrophage populations (754). CCR2+ monocyte-derived macrophages stimulate fibrosis in hypertension, which in turn reflects the activation of reparative or maladaptive processes. However, in the setting of extensive cardiomyocytes necrosis as occurs with MI, reparative fibrosis is a necessary process of crucial importance for the preservation of the structural integrity of the infarcted ventricle. Recent evidence from a TAC model of pressure overload indicates that early inflammatory cellular infiltration in the myocardium is associated with increased tissue and plasma levels of myeloid-derived growth factor (MYDGF), a proangiogenic factor linked to wound healing, which is secreted by monocytes, macrophages, as well as other immune cells. MYDGF inhibits cardiomyocyte hypertrophy through increased expression of the serine/threonine kinase PIM1 to increase expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (761).

Various pathophysiologic stimuli such as pressure overload, volume overload, metabolic dysregulation, and the uremic environment may cause interstitial and perivascular fibrosis with the participation of immune pathways (754). Notably, CKD is the classical example of premature aging and immune senescence is a classic example of CKD manifestations. Aging is associated with evolution towards a low-grade oxygen environment, where cardiomyocytes release pro-inflammatory cytokines and chemokines, stimulating an immune response. This leads to the increase in cardiac monocyte-derived CCR_2^+ $Ly6C^{high}$ macrophages, the so called "inflammaging", which promotes the development of fibrosis (754). Considering the findings from other studies which are in parallel with ours, the results of our study are thought provoking and merit additional research considering the complex properties of the pro-inflammatory CD14++CD16+ monocytes regarding their potential implication in the pathogenesis of myocardial remodeling in CKD and following kidney transplantation. Overall, the question remains as to whether monocytes subsets and their functions should be considered as causal factors directly involved in the pathophysiological mechanisms of HF development or whether they are simple bystanders displaying pathological alterations in the setting of the HF milieu. Even though the implication of the pro-inflammatory CD14++CD16+ monocytes appears to be more prominent in HFpEF ,whereas the classical CD14++CD16- monocytes are mainly involved in HFrEF, further research is mandatory to clarify the interplay between monocytes subsets, related inflammatory mediators and other cardiac resident cells such as macrophages, fibroblasts, and cardiomyocytes (748). Furthermore, it is of paramount importance to examine the additional effects of comorbidities such as diabetes mellitus and the metabolic syndrome, anemia, arterial hypertension and chronic uremia, on the levels and properties of monocytes subsets so as to untangle the complex and intertwined underlying mechanisms. In particular, with regard to CKD, considering that the proinflammatory milieu is a major feature of the uremic environment, the high burden of comorbidities in this setting, as well as the establishment of subclinical myocardial abnormal function since early during the disease process, the definition of the specific roles of monocytes subsets remains a necessity. As a result, considering the latest evidence on the novel therapeutic regimens targeting inflammatory pathways in order to reduce cardiovascular risk in CKD, future treatments directly aiming monocytes subsets might emerge (748).

Of note, in our study we found increased levels of the pro-inflammatory intermediate CD14++CD16+ monocytes in CRS patients as compared to healthy controls [41 (IQR, 24-78)/μL and 8 (IQR, 5.6-12)% vs 16 (IQR, 13-18)/μL and 3.67 (IQR, 2.54-4.41)%, $p<0.001$ and $p=0.001$] as well as to CKD patients [41 (IQR, 24–78)/μL vs 35 (IQR, 18–43)/μL, $p = 0.04$], albeit no significant differences were detected between other robust markers of inflammation such as CRP or ESR between the CRS and CKD cohorts. Although monocytes are considered an essential component of the inflammatory cascade in HF, relevant evidence until now has been based mainly on studies regarding monocyte-derived cytokines implicated in the pathogenesis of HF such as TNFα and IL-6 (766, 767). There are few and

relatively recently published studies which have found elevated CD14++CD16+ monocyte counts in patients with both acute and stable chronic HF as well as association of these pro-inflammatory monocytes with HF severity and decreased GFR levels (493-495). In patients with HF, proinflammatory CD14++CD16+ exhibit accentuated ACE (CD143) expression (493). Notably, the CD14++CD16+ monocytes, in addition to producing TNFα, IL-1β and IL-10, also produce IL-13, an anti-inflammatory cytokine that may attenuate the adverse remodelling of the failing myocardium (495). Thus, according to our findings, subtle and possibly additive immune mechanisms might further aggravate the pro-inflammatory milieu of CKD in the setting of HF.

Moreover, our results indicate a direct association between the pro-inflammatory CD14++CD16+ monocytes and adverse outcomes in type 2 CRS patients. This is in accordance with evidence from previous studies revealing that higher counts of CD14++CD16+ monocytes are associated with higher incidence of cardiovascular events and death in high atherosclerotic risk populations including non-end stage CKD and haemodialysis patients (460, 464). With regard to the aetiology of HF, we found increased levels of the CD16+ monocytes, that is both CD14++CD16+ [75 (IQR, 41–104)/μL versus 36 (IQR, 22–61)/μL, $p = 0.01$] and CD14+CD16++ monocytes [37 (IQR, 35–49)/μL versus 21 (IQR, 12–32)/μL, $p = 0.02$] in patients with non-atherosclerotic CVD compared to patients with ischemic CVD, however attempts to justify this finding would be speculative at present considering the paucity of relevant data in the literature. Thus, until very recently, the evidence for pathophysiological implication of monocytes in HF stemmed from HFrEF of mixed aetiology (464, 492, 493). Accordingly, patients with ischemic HF display similar levels of classical monocytes, increased levels of intermediate monocytes compared to patients with CAD but without established HF whereas the status of the non-classical monocytes remains controversial (492, 493, 768). Recent findings suggest that raised intermediate monocyte counts are observed in HFpEF as well (769). Regarding the non-classical CD14+CD16++ monocytes, we found an inverse association between them and LVEF in CRS patients.

Finally, it is worth mentioning that among our patients, KTRs compared to the CKD patients, displayed a higher level of the classical CD14++CD16- monocytes [479 IQR (354–599)/μL and 87.1 (IQR, 83.6–90.1)% vs 366 (IQR, 258–438)/μL and 81.7 (IQR, 75.9–85.6)%, $p = 0.001$ and 0.000, respectively] together with a lower level of the pro-inflammatory CD14++CD16+ monocytes [4.6 (IQR, 2.8–7.3)% vs 8.2 (IQR, 5.9–11.3)%, $p < 0.001$] , which is in line with data from previous studies (468, 770). Thus, in CKD patients the intermediate CD144++CD16+ monocyte counts are higher than those in healthy subjects and appear to decrease early after transplantation (463, 468). A partial reduction in the CD16+ monocytes has been reported since early in KTRs, without however making distinctions between the intermediate CD14++CD16+ monocytes and the non-classical CD14+CD16++ monocytes (468). This reduction may be at least partly attributed to the reversal of the uremic environment following kidney transplantation (468). A prospective study of KTRs evaluating the phenotypic patterns of peripheral monocytes by multicolor flow cytometry showed that

the percentage of the classical CD14+CD16− monocytes increased one week following transplantation and despite a slight decline after one month, a further increase was observed one year later (770). On the contrary, the intermediate CD14+CD16+ monocytes decreased immediately after transplantation, they displayed an augmenting trend one month later but further declined one-year post-transplant (770). With regard to the percentages of non-classical CD14+CD16++ monocytes, they decreased substantially during the first week after transplantation remaining at lower levels compared to pre-transplant values and displayed a declining trend by the end of the first year (770). It should be taken into consideration that immunosuppression involving corticosteroids is a significant factor that may potentially influence and might induce a shift in the distribution of monocyte subsets, whereas therapies consisting of mycophenolate mofetil, CNIs and mammalian target of rapamycin inhibitors (mTORI) have not been shown to play a role (285, 468). Monocytopenia caused by steroid treatment has been demonstrated by many studies. In terms of the monocyte subpopulations, it has been shown that chronic low-dose steroids are associated with higher counts of the classical CD14++CD16- monocytes but by lower levels of the CD16+ monocytes (285, 468). Both in vitro and in vivo data suggest that glucocorticoids cause a selective depletion of the CD16+ monocytes by inducing apoptosis of these cells in a caspase-dependent manner (468).

Another notable finding of our study is the positive correlation between the intermediate CD14++CD16+ monocytes and serum phosphorus both in CKD patients (r = 0.410, *p = 0.006*) as well as in KTRs (r = 0.33, *p = 0.04)*. Elevated phosphate levels in the serum have been recognized as a major risk factor for CVD and CVD related mortality both in the general population as well as in CKD patients, however the pathophysiological background of the connections between phosphate and CVD have not been completely understood (771). Accumulating evidence supports a link between disturbances in phosphate homeostasis and inflammation (772). Calcitriol, PTH, and FGF23 have emerged as the main regulators of phosphorus homeostasis, with increased phosphorus levels stimulating PTH secretion, which enhances bone FGF23 formation and the release as well as the synthesis of calcitriol by the kidneys whereas on the other hand, FGF23 suppresses PTH and calcitriol levels (772). Additionally, αKlotho is an essential player in the FGF23 axis, both as a regulator of FGF23 synthesis and as a necessary cofactor binding intact FGF23 and making possible its signaling (772). Particularly, the role of systemic inflammation in the regulation of FGF23 production has gained much attention as stimulation of FGF23 production and secretion by IL-1β, IL-6, and TNF has been shown (772). Impaired phosphorus clearance in the setting of CKD leads to raised levels of FGF23 very early during the course of CKD, paralleled by a fall in αKlotho and calcitriol levels, whereas PTH and phosphorus increase only later during CKD progression (772). A great body of evidence supports a direct link of phosphorus with pro-inflammatory effects and an association of phosphorus levels and inflammatory markers such as CRP, TNF and IL-6 has been found in CKD patients whereas the administration of phosphate binders is associated with reduced levels of these inflammatory markers (772-774). Experimental studies indicate a direct

effect of phosphorus overload on cardiac remodeling, including LVH and fibrosis and altered phosphate metabolism is associated with LVH in CKD patients (772, 775, 776). Treatment of hyperphosphatemia results in reduced serum FGF23 levels together with regression of LVH in hemodialysis patients (772, 775, 776). Similarly, FGF23 appears to promote myocardial hypertrophy and fibrosis through various mechanisms, including activation of mitogen-activated protein kinase (MAPK) signaling and increases in the expression of Early Growth Response 1 (Egr1) (772, 777). Additionally, FGF23 temporarily augments intracellular calcium levels in primary cardiomyocytes and contractile force in ex vivo ventricular muscle strips (772, 777). Accordingly, several CKD animal models investigating the role of FGF23 in LVH have shown that markers of LVH, ANP, BNP and β-myosin heavy chain (*Myh7*) mRNA expression are upregulated in response to FGF23 (772, 777). Furthermore, in adenine diet-induced CKD rats, increased plasma FGF23 concurred with vascular calcification and increased pulse pressure, pulse-wave velocity as well as increased LVMI (772). Yet, the interaction of phosphorus, FGF23 and calcitriol with inflammation remains a subject of ongoing investigation. Accordingly, human PBMCs express αKlotho and FGFR 1, 2, and 4 receptors (772). In addition, activation of TLR1 and TLR2 and subsequent increase in IL-15 and IFNγ in the innate immune response triggers local calcitriol production by monocytes and macrophages by up-regulation of Cyp27b1 mRNA expression (772). Calcitriol subsequently activates the vitamin D receptor in an auto- or paracrine way and up-regulates cathelicidin, a protein with antimicrobial activity (772). On the other hand, reduced calcidiol levels or inhibition of 1-α-hydroxylase or of the vitamin D receptor suppresses the innate immune response (772). Notably, FGF23 inhibits IL-15-dependent stimulation of Cyp27b1 expression and calcitriol production in PBMCs (772). Taking into consideration available evidence supporting a strong association between cardiac hypertrophy and fibrosis with plasma phosphate concentration and improvements in the cardiac remodeling process after correction of hyperphosphatemia as well as the complex involvement of phosphate, FGF23 and Vitamin D in the inflammatory pathways, further investigation of the potential links of the pro-inflammatory CD14++CD16+ monocytes with the CKD-MBD axis would be appropriate.

With regard to the non-classical CD14+CD16++ monocytes, we found higher counts in CKD patients compared to normal controls [25 (IQR, 19-36)/μL vs 19 (IQR, 10-21)/μL, p=0.044] and KTRs [25 (IQR, 19–36)/μL and 5.8 (IQR, 4.3–8.2)% vs 18 (IQR 13–28)/μL and 3.2 (IQR, 1.9–5.4)%, p = 0.001 and 0.000, respectively]. In addition, we found an inverse correlation of the non-classical monocytes with RWT in CKD patients (r= −0.53, p=0.001) as well as a direct association with indices of systolic function in KTRs, i.e. improvement in Sm following dipyridamole infusion (r=0.643, p=0.01), albeit all the aforementioned correlations lost their significance in the multivariate analysis. The CD14+CD16++ monocytes remain the least understood subtype in the literature. However, available data suggest a complex role of non-classical monocytes which produce both anti-inflammatory cytokines such as IL-10 in response to bacterial stimuli as well as pro-inflammatory cytokines in a TLR7-mediated response against viruses and nucleic acids. Furthermore, the non-classical monocytes are

involved in the initiation of the recruitment and activation of other innate immune cells, like NK cells and neutrophils via TNF-α-induced upregulation of E-selectin on the endothelial cells (748, 749). Overall, considering the patrolling function of the non-classical monocytes in the vascular endothelium and the removal by them of cell debris via Fcγ-mediated phagocytosis, the main function of the CD14+CD16++ monocytes, appears to be the maintenance of the vascular integrity (748, 749). Furthermore, the non-classical monocytes participate in the resolution of inflammation, as they appear to differentiate into wound-healing macrophages and they display increased expression of miR-150 and miR-21 (748, 749). Even though there is paucity of data on their tissue-specific effects, CD14+CD16++ monocytes appear to bear a significant protective association with all-cause death in patients with HF (778). Thus, considering that due to the low counts of the intermediate CD14++CD16+ and non-classical CD14+CD16++ monocytes compared to the classical CD14++CD16+ monocytes in the circulation, most studies have evaluated them together as a pool of CD16 positive monocytes, which does not allow for a clear interpretation of their results or the specification of the unique properties and functions of each subset (749). Accordingly, more studies focusing on the functional differences between the intermediate and non-classical monocytes with regard to CKD and CVD are mandatory.

9.1.2 T-lymphocytes subsets display different patterns of association with the subclinical indices of LV function in CKD patients and in kidney transplant recipients

Among the T-cells subpopulations, it appears that the CD4+ T-cells are the dominant immune mediators involved in the remodeling process that occurs in the post-ischemic myocardium and in maladaptive myocardial hypertrophy (633, 634). Accordingly, in ischemic models of HF, CD4+ T cells have been associated with ischemia and reperfusion injury during the early phases following AMI as well as with the development of interstitial myocardial fibrosis during the chronic phase, via the release of fibrosis promoting cytokines such as IL-4 and IL-13 (645). CD4+ T-cells have been implicated in the fibrotic process, LVH and eventually HF in the setting of pressure overload (609, 610, 614, 616). Mice lacking B and T lymphocytes due to depletion of recombination activating gene 2 expression (RAG2KO), a gene that encodes the enzyme necessary for rearranging and recombining the genes for both TCR molecules and immunoglobulins, did not develop cardiac dilation, displayed improved contractile function and attenuated adverse remodeling compared with wild-type mice in the setting of TAC (609). Furthermore, elevated secretion of the T-cell growth factor IL-2 after ex vivo TCR stimulation has been observed in TAC induced HF (609). Mice deficient in CD4+ T-cells submitted to TAC, did not display collagen deposition and cross-linking within the myocardium as well as did not develop ventricular dilation and dysfunction, in contrast to their wild type counterparts which manifested prominent myocardial fibrosis, ventricular dilation and dysfunction (609). Administration of anti-CD3 antibody injections to deplete T cells in CKD mice with CKD led to significant improvements of both myocardial strain and diastolic function as indicated by E/A ratio, isovolumic relaxation time, and myocardial performance index without causing any changes in LVH,

thus supporting the conclusion that reduced T cell number rather than sustained cytokine release mediate the improvement in cardiac function (656). We may further infer from these findings that the diastolic dysfunction in the mouse model of uremic cardiomyopathy occurs independently of LVH development (656). Nonetheless, there is paucity of clinical studies regarding the implications of T-cells subpopulations in CKD related LV remodeling. CD4+ T-cells producing interleukin-17 and INF-γ, are higher in hypertensive patients compared to healthy subjects (654). About CKD, T-cell phenotyping in the setting of kidney dysfunction has revealed an association between the senescent CD4+ T-cells and improving diastolic function (656). Accordingly, clinical data from a small cohort of children with CKD showed that T cell phenotypes correlated with structural and functional echocardiographic indices of myocardial function (656). Increased levels of T cells expressing the activation markers PD-1 and/or CD57 were associated with impaired diastolic function as represented by the E/E' ratio (656). Additionally, the loss of naïve T cells was associated with exacerbation of LVH whereas the accumulation of terminally differentiated effector memory $CCR7^{-}CD45RA^{+}$ $CD4^{+}$ T cells displayed a moderate association with improved diastolic function in the same pediatric cohort, thus allowing us to speculate that $CD4^{+}$ memory T cells represent senescent cells which are functionally unable to affect cardiac function (656). Notably, a reduced CD4+ to CD8+ ratio, as observed in the setting of continuous antigen stimulation or advanced aging, displayed a significant association with worsening diastolic function and with increased left ventricular mass in CKD children (656).

The results of our study pair well with the currently available evidence as presented above. Overall, with regard to T cells, we found an inverse correlation between T cells with LVEF in CKD patients (r= −0.364, p=0.029) as well as with improvements in the LVEF following dipyridamole infusion in KTRS (r= −0.475, p= 0.007). In specific, we found a negative correlation of CD4+ T-cells with LVEF in CKD patients (β= -0.451, p=0.009) as well as with the dipyridamole induced improvement of LVEF in KTRs (β= *-0.378, p*=0.024). Likewise, in KTRs we found a negative correlation of CD4+ T-cells both with MAPSE (β= -0.359, p=0.016), a sensitive marker of early systolic dysfunction and TAPSE (r= -0.456, p=0.001), a marker of right ventricular function, although the latter was lost following multivariate analysis. In accordance with the above, with regard to strain related indices of LV function, CD4+ T-cells were inversely correlated with dipyridamole induced improvements in GLS in KTRs (β= 0.403, p=0.012). Considering that CKD results in the systemic accumulation of proinflammatory T cells that play a causal role in myocardial pathology, future research targeting T cell function is needed for discovering new treatments aiming to attenuate early subclinical myocardial dysfunction in the setting of CKD.

In contrast to CD4+ T-lymphocytes, the currently available evidence, albeit scarce, points to a complex role of the CD8+ T cells in the development and progression of CVD. In experimental arterial hypertension models, wild type mice undergoing adoptive transfer of CD8+ T-cells from hypertensive mice, developed salt sensitive hypertension (649, 650). In specific, IFNγ is considered to largely mediate the interaction of CD8+ T cells with the nephron structures, eventually leading to sodium retention (649, 650). Accordingly, CD8+ T

cells have been shown to augment arterial resistance in a three-dimensional culture model of hypertension as well as display upregulation of gene pathways involving chemotaxis and response to IFNγ in prehypertensive mice (652). Furthermore, an increased proportion of immunosenescent, proinflammatory CD8+ T cells have been found in hypertensive patients (655). On the contrary, with regard to myocardial remodeling, post-myocardial infarction mice lacking functional $CD8^+$ T-cells displayed delayed removal of necrotic debris and defective scar formation, inferring a profibrotic role of CD8+ T- cells in the myocardium (641). Mice lacking mature CD4+ T cells but with normal $CD8^+$ T-cells were protected from LV fibrosis, dilation and contractile dysfunction whereas mice lacking CD8+ T cells developed adverse remodeling and HF in the TAC pressure overload model, thus suggesting that only the CD4+ T-cell subset plays a determining adverse role in cardiac dysfunction (609). We found an independent association of CD8+ T-cells with both LV twist (β= 0.405, p=0.021) and untwist **(**β**=** -0.367, p=0.036) in patients with CKD. Notably, LV torsion as represented by the systolic twist and the diastolic untwist rates, is greater in hypertensive than normotensive individuals, which might be ascribed to a compensatory mechanism in the setting of increased aortic stiffness during the early stages of hypertensive cardiomyopathy (779). Furthermore, augmented left ventricular twist and twist rates have been observed in asymptomatic CKD patients and appear to be compensatory to impairments in GLS and GRS, suggesting a pattern of subendocardial injury, the effects of which are compensated by the epicardial myocardial fibers which translate in increased LV twist (87). Thus, considering the above, the implication of CD8+ T-cells in arterial hypertension and the absence of established CVD in our patients, further investigation is needed to shed light on the pathways linking CD8+ T-cells, arterial hypertension and myocardial remodeling in the setting of CKD before the development of overt myocardial dysfunction.

9.1.3 Lymphocytes subsets are predictive of adverse outcomes in patients with type 2 CRS.

The results of our study showed lower levels of total lymphocytes, T lymphocytes (1227 ±510/μL vs 1672 ±387/μL, p=0.025) and B lymphocytes [68 (IQR, 31-104)/μL and 4.2 (IQR, 2.2-9.0)% vs 224 (IQR, 171-261)/μL and 10.3 (IQR, 8.5-11.3)%, p<0.001 and p=0.014 respectively] in patients with type 2 CRS compared to healthy controls as well as lower levels of total lymphocytes (1557 ± 691/μL vs 1920 ± 545/μL, p = 0.04) in patients with type 2 CRS compared to their CKD counterparts. Strikingly, an inverse association of lymphocytes counts with mortality was observed in CRS patients. Likewise, levels of lymphocytes subpopulations and specifically, T-lymphocytes, CD4+ T cells and CD8+ T-cells were independently associated with survival in our cohort of CRS patients (p < 0.05 for all). Our results pair well with those from previous studies linking reduced lymphocyte count with adverse prognosis in HF patients (780-782). Several past studies conducted in patients with chronic HF have shown that in the stable outpatient setting lymphocyte counts predict survival up to one year following measurement (780-782). The Seattle Heart Failure Model (SHFM) is a calculator of projected survival at baseline and after interventions for patients with HF (783). The score indicates that the percentage of peripheral blood lymphocytes

together with NYHA class, ischemic aetiology of HF, diuretic dose, EF, systolic BP, sodium, haemoglobin, uric acid and cholesterol, each had independent predictive power for predicting death in patients with HF (783). Of note, the renal function was not an independent predictor of adverse outcomes in the SHFM (783). The most accepted lymphopenia mechanism in HF is a state of chronic subclinical stress, inflammation and sympathetic activation, otherwise known as the neuro-immuno-hormonal axis (784, 785). High cortisol levels, stimulation of beta-adrenergic receptor in the lymphocytes due to increased sympathetic tone and elevated cytokines, have all been shown to cause lymphopenia (784, 785). Peripheral congestion may lead to lymphocyte loss and endotoxin transfer, whereas endotoxin may induce apoptosis of specific subtypes of lymphocytes, resulting in lymphocytopenia (784, 785). Yet, the most significant finding of our study was that in the multivariate model including all six immune cell subsets, only the CD4+ T-lymphocytes remained independent predictors of mortality (OR 0.66; 95% CI 0.50–0.87; p = 0.004), further reinforcing the previously presented evidence regarding the deleterious role of the CD4+ T cells in HF.

With regard to indices of kidney function, we showed that eGFR levels in CRS patients were positively associated with total lymphocytes (r = 0.427 p = 0.007), T-lymphocytes (r = 0.425 p = 0.007) as well as CD4+ T-cells (r = 0.439 p = 0.005) counts which is in line with available findings from studies conducted in patients with advanced stages of CKD (340). Furthermore, we detected an inverse association between the ratio of CD4+ T cells to CD8+ T cells with proteinuria in CRS patients (ρ = 0.401, p = 0.02), despite overall low mean levels of proteinuria in patients with CRS compared to CKD patients. Considering that proteinuria is a marker for both progression of CKD and increased cardiovascular morbidity and mortality, larger studies are needed in the future so as to clarify the pathophysiological and prognostic role of these findings (786). Interestingly, we found that lower B-lymphocytes counts were associated with an adverse lipid profile. Recent data indicate that B cells regulate atherosclerotic plaque formation through production of antibodies and cytokines and their effects are subset specific, thus future research will further evaluate their role in atherogenesis (702).

9.1.4 Natural killer cells correlate with indices of subclinical myocardial dysfunction and are differentially expressed in CKD patients and patients with type 2 CRS

Studies on NK cells in HF are very limited. Interestingly, in our study we detected an independent correlation of NK cells both with E/A ratio (β= -0.387, p=0.017) as well as with GLS (β= -0.362, p=0.038) in KTRs only. These results appear controversial at first sight considering that a physiological reduction in E/A ratio is observed with aging whereas the relationship of this index with LV diastolic function is more complex and should be evaluated in combination with other markers, including the E/E' ratio. Accordingly, in a previously published study of patients undergoing peritoneal dialysis, we found that patients with higher NK cell levels had a higher E/E’ ratio (787). In addition, apart from an

increased E/E' in heart ultrasound, our previous study results indicated that increased NK cells were linked to fluid overload in PD patients, determined either as overhydration by lung ultrasound or by BCM measurements (787). We also found a direct correlation between increased NK cell counts and a fast peritoneal transport status in the PD cohort with faster peritoneal transport status in PD patients being associated among others with intraperitoneal and systemic inflammation (787). Altogether, until now available data suggest that fluid overload is significantly and reciprocally associated with systemic micro-inflammation and it is more frequent in fast transporters. Accordingly, we found a direct correlation between both NK cells count and percentage with ESR in our KTRs.

An inverse correlation between NK cells and proteinuria was observed in CKD patients ($r = -0.302$, $p = 0.04$). We should take into consideration that proteinuria and in particular albuminuria is a hallmark of glomerular damage, whereas chronic inflammation is an established mediator between microalbuminuria and development of macrovascular disease. Thus, in the CRIC study participants, the plasma levels of proinflammatory cytokines and positive acute phase proteins were higher in participants with lower levels of kidney function and higher levels of albuminuria (220). A reciprocal relationship has been indicated between inflammatory mediators and albuminuria, with proinflammatory cytokines being pathogenically involved in promoting proteinuria, whereas albuminuria might selectively activate cytokines which induce hepatic albumin and fibrinogen synthesis (220). NK cells are a significant source of the proinflammatory cytokine IFN-γ in the fibrotic kidney and appear to be actively involved in the progression of CKD, regardless of the underlying cause of kidney disease (788). In human models of kidney fibrosis, NK cells located in the renal interstitium express the NK cell receptor NKp46 which is able to recognize stressed cells and in the setting of chronic inflammation, NK cells could exert direct cytotoxic effects on damaged tubular epithelial cells. Furthermore, kidney NK cells have been shown to produce IFN-γ which could promote the production of proinflammatory mediators by kidney parenchymal cells as well as promote the activation of the pro-inflammatory local macrophages, thus perpetuating renal inflammation (220). On the other hand, while NK cells are likely to participate in the development of interstitial kidney fibrosis in CKD in humans, there is no evidence indicating a significant role of NK cells in the setting of glomerular injury. A potential explanation for the inverse correlation detected between NK cells and proteinuria in our CKD patients, might be ascribed to the accumulation of NK cells in the kidney tissue which would be translated into fewer NK cells in the circulation. Additionally, NK cells might exert different functions and selectively express different properties in various CKD stages, including end-stage CKD and kidney transplantation. In conclusion, the multifaceted nature of NK cells and their role in the propagation versus modulation of inflammation remains at present a subject of dispute.

In addition, it should be noted that inflammation itself has been associated both with the induction of NK cell apoptosis and augmented proliferation in the setting of cytokine stimulation.

Results from the MESA study, point out a direct relationship between increments in NK cells with higher average systolic blood pressure levels (515). Accordingly, NK cells produce cytokines such as IFN-γ and IL-17 that have been shown to be related to arterial hypertension (515). Thus, as already previously described IFN-γ knockout mice did not respond to angiotensin II induced hypertension and appeared protected from angiotensin II induced vascular and kidney dysfunction (515, 547). Yet, results from the MESA study did not show an association between other IFN- γ producing adaptive immune cells, such as Th1 lymphocytes with blood pressure (515). On the other hand, we also found that higher NK cell counts were associated with improved LV systolic function as well as strain and deformation indices in KTRs. Experimental data point out a cardioprotective, antifibrotic role of NK cells in the setting of HF, via production of IFNγ, suppression of cardiac myocyte apoptosis and collagen deposition, as well as increases of neovascularization (570, 571). Thus, NK cells appear to be involved in the regulation of the proliferation phase following cardiac injury, during which NK cells prevent the development of cardiac fibrosis by directly limiting collagen formation of cardiac fibroblasts and the accumulation of specific inflammatory and profibrotic cell populations, including eosinophils (789). Furthermore, NK cells interact with cardiac endothelial cells to expand vascularization and angiogenesis following MI (788). Although depletion together with modulation of the phenotypic and cytotoxic characteristics of NK cells may contribute to the immune dysfunction in advanced CKD, the role of these alterations in the pathogenesis of CKD associated CVD remains currently hypothetical and requires further investigation (291). We found lower levels of NK cells in patients with type 2 CRS compared to healthy controls which pair well with findings from studies conducted in patients with HF (568, 569). In addition, our results showed lower levels of NK cells [148 (IQR, 103–258)/μLvs 324 (IQR, 179–368)/μL, $p = 0.001$] in patients with type 2 CRS compared to their CKD counterparts as well as in CRS patients with chronic atrial fibrillation compared to those without [133 (IQR, 79–173)/μL versus 260 (IQR, 151–314)/μL ($p = 0.01$)]. The depleted NK cells levels in HF and advanced CKD have been ascribed to the chronic inflammatory state and upregulation of Il-6 pathways which induces NK cell dysfunction and anergy (569).

There are scarce data regarding the implications of NK cells in patients with CRS. A study aiming to characterize the immune landscape of the kidney throughout AKI-CKD transition in the setting of type 1 CRS showed that innate immune cells are the first to infiltrate the kidney, including neutrophils and NK cells (789). NK cell infiltration immediately preceded mesenchymal cell expansion, suggesting that transient NK cell infiltration induces long-lasting changes in the kidney (790). As a result, further research so as to clarify and specify the role of NK cells within the heterogenous pathological entities included in the CVD and CKD spectrum.

9.1.5 T regulatory cells display altered expression in CKD patients and in kidney transplant recipients

In our study , we found that Tregs had lower counts both in KTRs [19 (13-28)/μL vs 57 (38-68/μL and 0.93 (0.63-1.71)% vs 1.9 (1.7-3.4)% respectively] compared to healthy control subjects, whereas the lower level of Tregs observed in KTRs compared to CKD patients [19 (IQR, 13–28)/μL and 0.93 (IQR, 0.63–1.71)% vs 33 (IQR, 19–48)/μL and 1.75 (IQR, 1.13–2.44)%, $p = 0.002$ and $p< 0.001$, respectively] might potentially be ascribed to the effects of immunosuppression. These findings are in line with other CKD populations such as patients with diabetic nephropathy who have reduced levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in the periphery, which negatively correlate with the UACR (791). Moreover, regarding the role of Tregs in transplantation, the effect of current and novel immunosuppressive agents on Tregs and their interactions with Tregs have been overlooked until now. Accordingly, available immunosuppressive regimens have not evolved with consideration of their effects on Tregs. Having in mind the immunoregulatory and immunotolerance promoting role of the Tregs, novel therapies that do not themselves negatively affect Tregs function in vivo, as occurs with mTOR inhibitors at present, are needed. Finally, we found no significant association between Tregs and subclinical indices of LV dysfunction. Experimental data suggest a protective role of Tregs from the development of arterial hypertension and pressure overload induced myocardial hypertrophy, thus larger clinical studies are required to elucidate potential alterations in the features and behavior of Tregs in CKD and related cardiovascular complications (625). The role of Tregs in CVD and CKD has attracted a great deal of research interest during the last years however available evidence is controversial. We found decreased levels of Tregs in patients with type 2 CRS compared to CKD patients, although the association was lost after correcting for triglycerides levels. Moreover, lower levels of Tregs were observed in patients with CRS and atrial fibrillation compared to patients with siunus rhythm [32 (IQR, 21–43)/μL vs. 47 (IQR, 34–85)/μL, $p = 0.006$]. Initial studies showed depletion of Tregs in patients with HFrEF, however recent data indicate that there might be a change of the Tregs phenotype towards a profibrotic one in the chronic HF setting (682, 695, 700).

9.1.6 Strengths and limitations of the study

Our study provides preliminary but noteworthy evidence regarding the potential associations between a selected panel of immune cells subsets in the circulation with an array of echocardiographic indices of subclinical myocardial dysfunction in CKD patients and KTRs. The main strength of our study is the research question. Thus, the investigation of immune mechanisms involved in the pathogenesis and progression of CVD in CKD and kidney transplantation as well as their prognostic role remain currently an uptodate and trending research topic. Yet, there are very limited data available until now regarding the potential association of immune cell subsets with the development of preclinical myocardial dysfunction in CKD and kidney transplantation as well as regarding their role as cardiovascular risk markers. Considering the evolution of immune therapies in CVD, such as

monoclonal antibodies targeting interleukin signaling pathways for the treatment of atherothrombosis, the elucidation of specific immune mechanisms is of paramount importance for the development of novel targeted agents addressing myocardial dysfunction in high-risk populations such as CKD patients. Another strength of our study is its design in terms of including a cohort of homogenous patient groups. Thus, we included 2 homogenous groups, CKD patients and KTRs respectively, in terms of clinical characteristics and specifically with no established CVD. Additionally, we included a third group, a homogeneous cohort of patients with type 2 CRS in terms of clinical characteristics who were matched by gender and eGFR to a subgroup of patients from the CKD cohort, in order to make adequate comparisons. In addition, the prospective arm of the study with an appropriate follow-up duration and an inclusive combined endpoint allowed us to examine the predictive value of immune cells subsets for hard adverse outcomes.

Nonetheless, there are some limitations that need to be mentioned. First, it was a single center study. Second, the sample size was relatively small, but this fact was counterbalanced partially by the longitudinal design of the study. Third, the observational and cross-sectional nature of the study arm evaluating correlations of immune cells sub-populations with subclinical indices of myocardial dysfunction in CKD patients and KTRs precludes us from drawing conclusions about causality in the associations detected between immune cell subsets and conventional and novel deformation related indices of left ventricular function in our CKD patients and KTRs. Yet, investigation of causality was not included in the study aims and this is an exploratory and preliminary study, first and foremost designed to detect associations in an obscure research field. Notably, the fact that our results are hypothesis generating should be underscored. In line with the above, with regard to patients with type 2 CRS, the key limitations are the small sample size, gender limitations and its observational nature which does not allow us to confirm causality in the associations found between circulating immune cells, clinical variables and patient outcomes. Moreover, we examined the expression of a selected panel of immune cell subsets in the peripheral blood; however the alterations of their functional characteristics and their potential consequences remain to be assessed.

9.2 Conclusions

Immune cell subsets, including classical CD14++CD16 and intermediate CD14++CD16+ monocytes, NK cells and lymphocytes subsets independently correlate with subclinical indices of myocardial dysfunction, including left ventricular strain and torsion-related parameters, in patients with CKD and in KTRs without established CVD. Our findings provide novel insights suggesting a potential role for specific immune cell phenotypes for CKD-related cardiomyopathy, even at a preclinical stage. Taking into consideration that subtle alterations in left ventricular function commence early in CKD, future mechanistic studies are required to shed light on the potential pathophysiological significance of the role of specific immune cell subsets during the initial phases of myocardial remodelling in these

patients. Prospective studies are highly needed to clarify the utility of immune cell populations as potential prognostic markers for development of cardiomyopathy. Finally, development of interventions that modulate the expression and activity of specific immune cell subsets might represent a new target of remarkable therapeutic prospect for uremic cardiomyopathy. In our cohort, patients with CRS-2 exhibit clear alterations of the immune cell subsets profile in the circulation compared to CKD patients of similar kidney function but without established cardiovascular disease. Our findings suggest that distinct immune mechanisms might be involved in the pathogenesis or during the chronic clinical course of CKD in the setting of heart failure as compared to CKD without established CVD. Future research is required to elucidate further and specify the pathophysiological role of immune cell subpopulations as well as evaluate their potential value as markers of prognostic significance.

Summary

“A study of the immune system in patients with chronic kidney disease and kidney transplant recipients – correlations with markers of cardiovascular disease.”

Cardiac remodeling is a hallmark of chronic kidney disease (CKD) manifesting as myocardial fibrosis, left ventricular hypertrophy (LVH), impaired myocardial strain and eventually left ventricular diastolic and systolic dysfunction. Kidney transplantation is associated with significant improvements in left ventricular size and function, as well as regression of LVH, otherwise known as reverse remodeling. Nevertheless, subclinical abnormalities in the biventricular strain may be observed in kidney transplant recipients (KTRs) even when other classical indices of myocardial function such as ejection fraction (EF) are normal. Maladaptive activation of the immune system plays an essential role in the pathogenesis of CKD and cardiovascular disease (CVD). The role of immune system components in the development of myocardial remodeling in CKD and kidney transplantation remains an open question. Likewise, little evidence has been generated until now regarding potential alterations of the cellular components of the immune system in patients with CKD due to heart failure, as occurs in type 2 cardiorenal syndrome (CRS-2).

We therefore conducted a cross-sectional study in a cohort of non-end-stage CKD patients and KTRs without established CVD to investigate for the first time the relation of a selected panel of immune cell subpopulations in the peripheral circulation with classical and novel, strain-related indices of myocardial performance as evaluated by two-dimensional STE. In addition, we investigated the expression of a selected panel of immune cell subsets in the peripheral blood of a cohort of CRS-2 patients and subsequently made comparisons to a group of patients with CKD but without established CVD matched for gender and estimated glomerular filtration rate (eGFR). We further examined the clinical correlations as well as the prognostic value of specific immune cells with respect to overall and cardiovascular mortality in patients with type CRS-2.

There were enrolled 44 consecutive patients with CKD and without established CVD, who were under regular follow-up by our outpatient nephrology clinic and 38 KTRs without established CVD who were under follow-up by the kidney transplant unit of our hospital as

well as 39 stable male patients with CRS-2 under regular follow-up by the outpatient chronic heart failure and CKD clinic of our hospital. After baseline evaluation, patients with CRS-2 were followed until the end of the established observation period, or the study endpoint was reached, which was defined as a combined outcome of all-cause mortality and cardiovascular mortality.

The peripheral blood immune cell subsets analysis was performed by flow cytometry. Accordingly, CD14++CD16-, CD14++CD16+, and CD14+CD16++ percentage and the absolute number of cells out of the total monocytes, as well as NK cells (CD3+CD16+56+), CD3- CD19+ B-lymphocytes, CD3+ CD4+ T cells, CD3+CD8+ T cells, and T regulatory cells (Tregs) (CD4+CD25+ FoxP3+) absolute values, and percentage out of the total lymphocytes were measured.

Anthropometric and clinical data were recorded at baseline by patients' medical records, including comorbidities such as the presence of diabetes mellitus (DM) and medications. In addition, common biochemical parameters were measured at baseline in accordance with standard methods applied in the hospital laboratory. Echocardiographic data from ultrasounds performed by a skilled operator within 1 month from immune cell subset analysis were recorded, including parameters for estimating ventricular function and morphology and for cardiac chamber quantification. Specifically, regarding CKD patients and KTRs, classical and novel strain-related indices of ventricular function were measured by speckle-tracking echocardiography at baseline and following dipyridamole infusion. Two-dimensional STE analysis included assessment of global longitudinal strain (GLS), global radial strain (GRS) and global circumferential strain (GCS). In addition, left ventricular twist and untwist rates were measured. Following the baseline echocardiographic evaluation, dipyridamole was administered, and a new echocardiographic assessment was performed. The differences (Δ) between the values of measured echocardiographic parameters post and prior to dipyridamole infusion were calculated.

Following adjustment for confounders including age, eGFR and UPCR, the differences in immune cell subsets between CKD patients and KTRs remained statistically significant for the percentage of classical monocytes ($p = 0.02$, both the number and percentage of non-classical monocytes ($p < 0.001$), the percentage of T-lymphocytes and B lymphocytes ($p = 0.03$ and $p = 0.003$, respectively) as well as the Tregs number ($p = 0.008$). Following univariate regression analysis, the differences in immune cell subsets between patients with CRS-2 and CKD patients remained statistically significant for CD14++CD16+ monocytes, total lymphocytes, and NK cells ($p < 0.05$ for all) but not for Tregs, after adjustment for various confounders including age. Interestingly, we found increased levels of proinflammatory intermediate CD14++CD16+ monocytes in CRS-2 patients as compared to CKD patients, albeit no significant differences were detected between other robust markers of inflammation, such as CRP or ESR between the two cohorts. Our results showed lower levels of NK cells in patients with CRS-2 compared to their CKD counterparts as well as in CRS-2 patients with chronic atrial fibrillation compared to those without.

In CKD patients, a direct association of RWT, a measure of left ventricular concentricity broadly used as an index of LVH with CD14++CD16− monocytes count ($\beta = 0.447$, $p = 0.004$) was found, whereas the correlation with arterial hypertension was lost at multivariate analysis. Furthermore, in KTR, the classical CD14++CD16− monocytes count was inversely associated with improvements in systolic wall motion indices, such as the medial and lateral wall systolic velocity of the left ventricle (Sl and Sm) ($\beta = -0.516$, $p = 0.01$ and $\beta = -0.707$, $p < 0.001$, respectively).

An interesting finding of the study is the trend of independent correlations between the intermediate CD14++CD16+ monocytes with strain-related left ventricular myocardial performance indices, both in CKD patients and in KTRs. Thus, the CD14++CD16+ monocytes were independently associated with higher baseline GRS values in CKD patients, whereas in KTRs, they were associated with better baseline left ventricular twist (β = 0.416, p = 0.01) and untwist parameters (β = −0.742, p = 0.09). About our results, another potential explanation, though speculative, would be that a compensatory augmentation in strain-related myocardial performance indices is promoted in the setting of the microinflammatory milieu of CKD, which would subsequently lead to myocardial damage and remodeling. The results are thought provoking and merit additional research considering the complex properties of the pro-inflammatory CD14++CD16+ monocytes in the pathogenesis of myocardial remodeling in CKD and following kidney transplantation. It is worth mentioning that among our patients, KTRs displayed a higher classical CD14++CD16− monocytes count together with a lower level of pro-inflammatory CD14++CD16+ monocytes compared to the CKD patients, which is in line with data from previous studies.

A negative correlation was found between CD4+ T-cells with left ventricular EF in CKD patients (β = −0.431, p = 0.009) as well as with the dipyridamole induced improvement of left ventricular EF in KTRs (β = −0.378, p = 0.02). Likewise, in KTRs we found a negative correlation of CD4+ T-cells both with MAPSE (β = −0.463, p = 0.04), a sensitive marker of early systolic dysfunction and TAPSE, a marker of right ventricular function, although the latter was lost following multivariate analysis. In accord with the above, about strain-related indices of left ventricular function, CD4+ T-cells were inversely correlated with dipyridamole induced improvements in GLS in KTRs (β = 0.403, p = 0.01). Further characterization of the CD4+ T-cell responses is needed in order to discern possible pathogenic links to the development of uremic cardiomyopathy, evaluate their role as novel biomarkers of disease and subsequently examine the effects of immunomodulation on the CKD-related myocardial remodeling.

An independent association of CD8+ T-cells with both left ventricular twist and untwist in patients with CKD was found (β = 0.405, p = 0.02 and β = −0.363, p = 0.03 respectively). Considering the differences in the pathogenic models implemented by the above studies as well as the preliminary nature of our results, it remains to be determined whether CD8+ T-cells are simple bystanders or active and independent players in the mechanisms of CKD-related myocardial dysfunction.

Interestingly, we detected an independent correlation of NK cells both with E/A ratio (β = −0.387, p = 0.02) as well as with GLS (β = −0.362, p = 0.01) in KTRs only. These results appear controversial at first sight considering that a physiological reduction in E/A ratio is observed with aging, whereas the relationship of this index with left ventricular diastolic function is more complex and should be evaluated in combination with other markers, including the E/E’ ratio. On the other hand, we also found that higher NK cell counts were associated with improved left ventricular strain in KTRs.

Results of the prospective arm of the study regarding patients with CRS-2 showed that during a median follow-up of 29.8 ±3.4 months, 23 CRS-2 patients (59%) reached the study endpoint with no patients being lost to follow-up. At binary logistic regression analysis, immune cell subpopulations that correlated with all-cause and cardiovascular death included total lymphocyte counts, (OR 0.85 per 100 cells/μL increase; 95% CI 0.75–0.97; p = 0.01), T-cell number (OR 0.82 per 100 cells/μL increase; 95% CI 0.70–0.96; p = 0.01), CD4+ T-lymphocyte number (OR 0.66 per 100 cells/μL increase; 95% CI 0.50–0.87; p =

0.004), CD8+ T-lymphocyte counts below their median value cut-off of 410/μL (OR 4.67; 95% CI 1.14–19.07; p = 0.03), Treg counts below their median value cut-off of 35/μL (OR 6.63; 95% CI 1.36–23.27; p = 0.01), and CD14++CD16+ monocyte counts above their median cut-off value of 40/μL (OR 4.13; 95% CI 1.06–16.1; p = 0.04). In a multivariate model including all six immune cell subsets, only the CD4+ T-lymphocytes remained independent predictors of mortality (OR 0.66; 95% CI 0.50–0.87; p = 0.004). In contrast, no such associations were found for age, eGFR, UPCR, hsTnI, BNP, as well as the rest of the clinical or laboratory indices. Subsequently, Kaplan–Meier survival curves for CRS-2 patients according to the levels of immune cell subpopulations (i.e., below vs. above median value) were generated. Decreased levels of lymphocytes, T-lymphocytes, CD4+ T-cells, CD8+ T-cells, and Tregs were associated with mortality at a median follow-up of 30 months ($p < 0.05$ for all log-rank tests). Increased levels of proinflammatory intermediate CD14++CD16+ monocyte counts showed a trend for increased mortality (p = 0.093).

Overall, this study provides preliminary but noteworthy evidence regarding the independent associations of immune cell subsets, including classical CD14++CD16 and intermediate CD14++CD16+ monocytes, NK cells and lymphocytes subsets independently correlate with subclinical indices of myocardial dysfunction, including left ventricular strain and torsion-related parameters, in patients with CKD and in KTRs without established CVD. Taking into consideration that subtle alterations in left ventricular function commence early in CKD, these findings provide novel insights suggesting a potential role for specific immune cell phenotypes for CKD-related cardiomyopathy. Notably, the CD4+ T-lymphocytes were shown to independently predict fatal cardiovascular events in patients with CRS-2. Furthermore, the study results showed that patients with CRS-2 exhibit alterations of the immune cell subsets profile in the circulation compared to CKD patients of similar kidney function but without established cardiovascular disease, thus indicating that distinct immune mechanisms might be involved in the pathogenesis or during the chronic clinical course of CKD in the setting of heart failure as compared to CKD without established CVD.

Περίληψη

Η δυσπροσάρμοστη ενεργοποίηση του ανοσοποιητικού συστήματος φαίνεται να παίζει ουσιαστικό ρόλο στη παθογένεια της ΧΝΝ και των καρδιαγγειακών παθήσεων. Ο ρόλος των συστατικών του ανοσοποιητικού συστήματος στην παθογένεια του remodeling του μυοκαρδίου στη ΧΝΝ και μετά τη μεταμόσχευση νεφρού, παραμένει αδιευκρίνιστος. Τα δεδομένα σχετικά με τις πιθανές μεταβολές και διαταραχές των κυτταρικών στοιχείων του ανοσοποιητικού συστήματος στους ασθενείς με ΧΝΝ σε έδαφος καρδιακής ανεπάρκειας, δηλαδή με καρδιονεφρικό σύνδρομο τύπου 2 (ΚΝΣ-2) παραμένουν ελάχιστα μέχρι τώρα.

Με βάση τα παραπάνω πραγματοποιήθηκε διαστρωματική μελέτη σε μια κοορτή ασθενών με ΧΝΝ χωρίς εγκατεστημένη καρδιαγγειακή νόσο και παρομοίως σε μια κοορτή ληπτων νεφρικού μοσχεύματος (ΛΝΜ) χωρίς εγκατεστημένη καρδιαγγειακή νόσο, με στόχο την διερεύνηση για πρώτη φορά στη βιβλιογραφία των πιθανών συσχετίσεων ενός επιλεγμένου πάνελ υπό-πληθυσμών ανοσοκυττάρων στο περιφερικό αίμα, με κλασσικούς και νεότερους δείκτες λειτουργίας του μυοκαρδίου, συμπεριλαμβάνοντας τους δείκτες παραμόρφωσης της αριστερής κοιλίας, μέσω δισδιάστατης ηχοκαρδιογραφίας «speckle tracking”. Επιπλέον, εξετάστηκαν οι πιθανές διαφορές στην έκφραση των ανοσοκυττάρων μεταξύ των ασθενών με ΧΝΝ και των ΛΝΜ. Διερευνήθηκε η έκφραση του επιλεγμένου

πάνελ των ανοσοκυττάρων στο περιφερικό αίμα σε μια κοορτή ασθενών με ΚΝΣ-2 καθώς και έγιναν συγκρίσεις με μια ομάδα ασθενών με ΧΝΝ, χωρίς εγκατεστημένη καρδιαγγειακή νόσο, εξομοιωμένων ως προς το φύλο και το ρυθμό σπειραματικής διήθησης (eGFR). Επιπλέον, διερευνήθηκαν οι πιθανές κλινικό-εργαστηριακές συσχετίσεις των ανοσοκυττάρων στις ομάδες των ασθενών. Το προοπτικό σκέλος της μελέτης είχε ως κύριο στόχο τη διερεύνηση για πρώτη φορά στη βιβλιογραφία της προγνωστικής άξιας των υπότυπων των ανοσοκυττάρων με καταληκτικό συνδυαστικό σημείο την ολική ή καρδιαγγειακή θνητότητα στους ασθενείς με ΚΝΣ-2. Επιπλέον, πραγματοποιήθηκε προοπτική ανάλυση των υποτύπων των ανοσοκυττάρων στο περιφερικό αίμα των ασθενών με ΧΝΝ και των ΛΝΜ σε δυο χρόνους (αρχική ανάλυση- Τ0 και μετά από 24 μήνες – Τ1) ενώ διερευνήθηκαν και πιθανές κλινικές συσχετίσεις.

Στη μελέτη συμπεριλήφθησαν 44 ασθενείς με ΧΝΝ και χωρίς εγκατεστημένη καρδιαγγειακή νόσο, 38 ΛΝΜ χωρίς εγκατεστημένη καρδιαγγειακή νόσο, 39 ασθενείς με ΚΝΣ-2 καθως και 10 υγιή άτομα. Η ανάλυση των υπότυπων των ανοσοκυττάρων στο περιφερικό αίμα διενεργήθηκε με κυτταρομετρία ροής. Μετρήθηκαν το ποσοστό των CD14++CD16-, CD14++CD16+, and CD14+CD16++ μονοκυττάρων επί των ολικών μονοκυττάρων καθώς και ο απόλυτος αριθμός των υπότυπων των μονοκυττάρων. Επίσης, μετρήθηκαν το ποσοστό των NK κυττάρων (CD3+CD16+56+), CD3- CD19+ Β-λεμφοκυττάρων, CD3+ CD4+ Τ λεμφοκυττάρων, CD3+CD8+ Τ λεμφοκυττάρων, and Τ ρυθμιστικών κυττάρων (Tregs) (CD4+CD25+ FoxP3+) επί των ολικών λεμφοκυττάρων καθώς και ο απόλυτος αριθμός τους.

Τα ανθρωπομετρικά και τα κλινικά δεδομένα καταγράφηκαν από τους κλινικούς φακέλους των ασθενών, συμπεριλαμβανομένων των συνοσηροτήτων και της φαρμακευτικής αγωγής ενώ συγχρόνως μετρήθηκαν κλασσικοί εργαστηριακοί δείκτες. Τα υπερηχοκαρδιογραφικά δεδομένα, αντλήθηκαν από υπερήχους καρδιάς που διενεργήθηκαν εντός ενός μήνα από τη μέτρηση των υπότυπων των ανοσοκυττάρων. Ειδικά, όσον αφορά τους ασθενείς με ΧΝΝ και τους ΛΝΜ, μετρήθηκαν οι κλασσικοί και οι νεότεροι δείκτες παραμόρφωσης της αριστερής κοιλίας, συμπεριλαμβανομένων των επιμήκες (GLS), ακτινικό (GRS) και κυκλοτερές (GCS) strain, twist και untwist, με «speckle-tracking» υπερηχοκαρδιογραφήμα στην αρχική φάση και μετά τη χορήγηση διπυριδαμόλης (DIPSE). Επίσης, υπολογίστηκαν οι διαφορές (Δ) μεταξύ των τιμών των υπερηχοκαρδιογραφικών παραμέτρων μετά και πριν τη χορήγηση διπυριδαμόλης.

Τα αποτελέσματα της μελέτης ως προς τις διαφορές των υπότυπων των ανοσοκυττάρων μεταξύ των υπό-ομάδων ανέδειξαν μετά από προσαρμογή για συγχυτικούς παράγοντες όπως ηλικία, eGFR και πρωτεινουρία (UPCR), πως οι ΛΝΜ είχαν υψηλότερο ποσοστό κλασσικών μονοκυττάρων (p=0.02) και Τ-λεμφοκυτταρων (p=0.03) καθώς και χαμηλότερο αριθμό και ποσοστό των CD4+CD16++ μη-κλασσικών μονοκυττάρων (p<0.001), των Β-λεμφοκυττάρων (p = 0.003) και των Tregs (p=0.008). Όσον αφορά τις διαφορές μεταξύ των ασθενών με ΚΝΣ-2 και των ασθενών με ΧΝΝ, μετά από μονομεταβλητή ανάλυση παλινδρόμησης και προσαρμογή για διάφορους συγχυτικούς παράγοντες, συμπεριλαμβάνοντας την ηλικία, φάνηκε πως οι ασθενείς με ΚΝΣ-2 είχαν υψηλοτέρα επίπεδα των προ-φλεγμονωδών CD14++CD16+ μονοκυττάρων καθώς και χαμηλότερα επίπεδα ολικών λεμφοκυττάρων και ΝΚ κυττάρων (p < 0.05 για όλα τα κύτταρα). Αξιοσημείωτο εύρημα είναι τα αυξημένα επίπεδα των προ-φλεγμονωδών ενδιάμεσων μονοκυττάρων στους ασθενείς με ΚΝΣ-2 σε σύγκριση με τους ασθενείς με ΧΝΝ, ενώ δεν διαπιστώθηκαν σημαντικές διαφορές ως προς τους υπόλοιπους κλασσικούς δείκτες φλεγμονής, όπως η CRP ή η ταχύτητα καθίζησης ερυθρών (ΤΚΕ) μεταξύ των δυο

υπό-ομάδων. Επίσης τα αποτελέσματα ανέδειξαν χαμηλότερα επίπεδα των ΝΚ κυττάρων σε ασθενείς με ΚΝΣ-2 και με συνυπάρχουσα χρόνια κολπική μαρμαρυγή συγκριτικά με τους ασθενείς με ΚΝΣ-2 που δεν είχαν κολπική μαρμαρυγή.

Όσον αφορά τις συσχετίσεις μεταξύ των υπότυπων των ανοσοκυττάρων με τους κλασσικούς και τους νεότερους δείκτες δυσλειτουργίας της αριστερής κοιλίας, στους ασθενείς με ΧΝΝ παρατηρήθηκε μια απευθείας ανεξάρτητη συσχέτιση του σχετικού πάχους της αριστερής κοιλίας (relative wall thickness – RWT), ένας δείκτης εκτίμησης του βαθμού συγκεντρικής υπερτροφίας της αριστερής κοιλίας, με τα κλασσικά CD14++CD16− μονοκύτταρα (β = 0.447, p = 0.004), ενώ η συσχέτιση με την αρτηριακή υπέρταση χάθηκε κατά την πολυπαραγοντική ανάλυση. Επίσης, στους ΛΝΜ, ο αριθμός των κλασσικών CD14++CD16− μονοκυττάρων ανέδειξε αντίστροφη συσχέτιση με τη βελτίωση των δεικτών συστολικής κινητικότητας των τοιχωμάτων της αριστερής κοιλίας, όπως οι medial and lateral wall systolic velocity (Sl and Sm), μετά τη χορήγηση διπυριδαμόλης, (β = −0.516, p = 0.01 and β = −0.707, p < 0.001, αντίστοιχα). Ένα άλλο ενδιαφέρον εύρημα της μελέτης αποτελεί η ανεύρεση ενός trend ανεξάρτητων συσχετίσεων μεταξύ των ενδιάμεσων, προ-φλεγμονωδών CD14++CD16+ μονοκυττάρων με τους δείκτες του μυοκαρδιακού strain στους ασθενείς με ΧΝΝ και στους ΛΝΜ. Συγκεκριμένα, τα CD14++CD16+ μονοκύτταρα ανέδειξαν ανεξάρτητη συσχέτιση με υψηλότερες τιμές του GRS στους ασθενείς με ΧΝΝ, ενώ στους ΛΝΜ συσχετίστηκαν άμεσα με καλύτερους δείκτες twist (β = 0.416, p = 0.01) και untwist (β = −0.742, p = 0.09). Μια πιθανή εξήγηση για τα αποτελέσματα της μελέτης, αν και υποθετική, θα ήταν πως η αντισταθμιστική αύξηση στους strain δείκτες επίδοσης του μυοκαρδίου, προωθείται σε έδαφος της χρόνιας προ-φλεγμονώδους εξεργασίας της ΧΝΝ, η οποία στη συνέχεια ωστόσο, θα οδηγούσε σε δεύτερο χρόνο σε βλάβη και remodeling του μυοκαρδίου. Τα αποτελέσματα αυτά διεγείρουν ερωτήματα ως προς τις πολύπλοκες ιδιότητες των προ-φλεγμονωδών CD14++CD16+ μονοκυττάρων και τη συμμετοχή τους στη παθογένεια του remodeling του μυοκαρδίου στη ΧΝΝ καθώς και μετά τη μεταμόσχευση νεφρού.

Σχετικά με τους υπό-πληθυσμούς των λεμφοκυττάρων, αρνητική συσχέτιση ανευρέθηκε μεταξύ των CD4+ Τ-λεμφοκυττάρων και του κλάσματος εξωθήσεως της αριστερής κοιλίας (EF) (β = −0.431, p = 0.009) στους ασθενείς με ΧΝΝ, όπως και επίσης και με βελτιωμένες τιμές του EF μετά τη χορήγηση διπυριδαμόλης στους ΛΝΜ (β = −0.378, p = 0.02). Παρομοίως, στους ΛΝΜ, ανευρέθηκε αρνητική συσχέτιση των CD4+ Τ-λεμφοκυττάρων με τη παράμετρο MAPSE (β = −0.463, p = 0.04), έναν ευαίσθητο δείκτη πρώιμης συστολικής δυσλειτουργίας της αριστερής κοιλίας και τη παράμετρο TAPSE, δείκτης λειτουργίας της δεξιάς κοιλίας, αν και η συσχέτιση με την τελευταία παράμετρο χάθηκε κατά την πολυπαραγοντική ανάλυση. Σε συμφωνία με τα παραπάνω, όσον αφορά τους δείκτες strain της αριστερής κοιλίας στους ΛΝΜ, τα CD4+ Τ-λεμφοκύτταρα, ανέδειξαν αρνητική συσχέτιση με βελτιωμένες τιμές του GLS μετά τη χορήγηση διπυριδαμόλης (β = 0.403, p = 0.01). Δεδομένου των ανωτέρων ευρημάτων, απαιτείται περαιτέρω χαρακτηρισμός των απαντήσεων των CD4+ Τ-λεμφοκυττάρων ώστε να διευκρινιστούν πιθανές παθοφυσιολογικές συσχετίσεις με την ουραιμική μυοκαρδιοπάθεια, να αναλυθεί ο ρόλος τους ως πιθανοί βιοδείκτες της νόσου και στη συνέχεια να εκτιμηθεί η θεραπευτική επίδραση της ανοσοτροποποίησης στο remodeling του μυοκαρδίου στη ΧΝΝ. Όσον αφορά τα CD8+ Τ-λεμφοκύτταρα, ανευρέθηκαν ανεξάρτητες συσχετίσεις με το twist και το untwist της αριστερής κοιλίας στους ασθενείς με ΧΝΝ (β = 0.405, p = 0.02 and β = −0.363, p = 0.03 αντίστοιχα).

Τα αποτελέσματα της προοπτικής μελέτης των ασθενών με ΚΝΣ-2 ανέδειξαν πως κατά τη διάρκεια μέσης παρακολούθησης 29.8 ±3.4 μηνών, 23 ασθενείς με ΚΝΣ-2 (59%) εκδήλωσαν το συνδυαστικό καταληκτικό σημείο της μελέτης. Κατά την ανάλυση λογιστικής παλινδρόμησης, οι υπό-πληθυσμοί των ανοσοκυττάρων που συσχετίστηκαν με την ολική ή καρδιαγγειακή θνητότητα ήταν τα ολικά λεμφοκύτταρα (OR 0.85 ανά 100 κύτταρα/μL αύξηση; 95% CI 0.75–0.97; p = 0.01), τα Τ-λεμφοκύτταρα (OR 0.82 ανά 100 κύτταρα/μL αύξηση; 95% CI 0.70–0.96; p = 0.01), τα CD4+ Τ-λεμφοκύτταρα (OR 0.66 ανά 100 κυτταρα/μL αύξηση; 95% CI 0.50–0.87; p = 0.004), τα CD8+ Τ-λεμφοκυττάρα χαμηλότερα του επιπέδου cut-off της διάμεσης τιμής των 410/μL (OR 4.67; 95% CI 1.14–19.07; p = 0.03), τα Tregs χαμηλοτερα του επιπέδου cut-off της διάμεσης τιμής των 35/μL (OR 6.63; 95% CI 1.36–23.27; p = 0.01) καθώς και τα CD14++CD16+ μονοκύτταρα άνω της διάμεσης τιμής cut-off των 40/μL (OR 4.13; 95% CI 1.06–16.1; p = 0.04). Το πολυπαραγοντικό μοντέλο ανάλυσης που συμπεριέλαβε και τους έξι υπό-πληθυσμούς των ανοσοκυττάρων, ανέδειξε μόνο τα CD4+ Τ-λεμφοκύτταρα να παραμένον ως ανεξάρτητοι προγνωστικοί παράγοντες της θνησιμότητας (OR 0.66; 95% CI 0.50–0.87; p = 0.004). Σε αντίθεση με τα παραπάνω, δεν ανευρέθηκαν συσχετισμοί μεταξύ της ηλικίας, του eGFR, του UPCR, της hsTnI, του BNP ή των υπολοίπων κλινικών και εργαστηριακών δεικτών με την ολική και την καρδιαγγειακή θνησιμότητα. Επίσης, για τους ασθενείς με ΚΝΣ-2 διενεργήθηκε ανάλυση επιβίωσης (καμπύλες Kaplan-Meier) ανάλογα με τα επίπεδα των υπό-πληθυσμών των ανοσοκυττάρων (ανώτερα η κατώτερα της διάμεσης τιμής τους). Μειωμένα επίπεδα των ολικών λεμφοκυττάρων, των Τ-λεμφοκυττάρων, των CD4+ Τ-λεμφοκυττάρων, των CD8+ Τ-λεμφοκυττάρων και των Tregs συσχετίστηκαν με τη θνησιμότητα κατά τη μέση παρακολούθηση των 30 μηνών ($p < 0.05$ για όλα τα log-rank τεστ). Από την άλλη, αυξημένα επίπεδα των προφλεγμονωδών ενδιάμεσων CD14++CD16+ μονοκυττάρων ανέδειξαν μια τάση για αυξημένη θνησιμότητα (p = 0.093).

Η προοπτική παρακολουθηση των υποτύπων των ανοσοκυττάρων στο περιφερικό αίμα ΛΝΜ ανέδειξε κατά την ανάλυση γραμμικής παλινδρόμησης, ανεξάρτητη συσχέτιση των CD14++CD16+ μονοκυττάρων με μεταβολές του eGFR (β=0.338, p=0.04) και αντίστροφη συσχέτιση των CD8+ Τ-λεμφοκυττάρων με μεταβολές της πρωτενουρίας (β=–0.379, p=0.03).

Συμπερασματικά, τα αποτέλεσμα της μελέτης προβάλλουν προκαταρκτικές ενδείξεις σχετικά με τις ανεξάρτητες συσχετίσεις μεταξύ των υπό-πληθυσμών των ανοσοκυττάρων, όπως τα κλασσικά CD14++CD16- μονοκύτταρα και τα ενδιάμεσα μονοκύτταρα, τα ΝΚ κύτταρα καθώς και τους υπότυπους των λεμφοκυττάρων, με τους υποκλινικούς δείκτες δυσλειτουργίας του μυοκαρδίου συμπεριλαμβάνοντας τους δείκτες παραμόρφωσης της αριστερής κοιλίας, σε ασθενείς με ΧΝΝ και ΛΝΜ χωρίς εγκατεστημένη καρδιαγγειακή νόσο. Λαμβάνοντας υπόψη πως ανεπαίσθητες μεταβολές της καρδιακής λειτουργίας εγκαθίστανται πρώιμα κατά την εξέλιξη της ΧΝΝ, τα αποτελέσματα αυτής της μελέτης παρέχουν νέα δεδομένα τα οποία εισηγούνται πιθανό ρολό συγκεκριμένων ανοσοκυτταρικών φαινότυπων στη μυοκαρδιοπάθεια της ΧΝΝ. Επιπλέον, τα αποτελέσματα της μελέτης ανέδειξαν διαφορές ως προς την έκφραση των υπό-πληθυσμών των ανοσοκυττάρων μεταξύ των ασθενών με ΚΝΣ-2 και των ασθενών με ΧΝΝ χωρίς εγκατεστημένη καρδιαγγειακή νόσο, υποδεικνύοντας έτσι την εμπλοκή διαφορετικών ανοσολογικών μηχανισμών στην παθογένεια και στην εξέλιξη της ΧΝΝ σε έδαφος της καρδιακής ανεπάρκειας. Εν τέλει, η μελέτη ανέδειξε για πρώτη φορά στη βιβλιογραφία το ρόλο συγκεκριμένων υπότυπων των ανοσοκυττάρων ως προγνωστικοί δείκτες έκβασης των ασθενών με ΚΝΣ-2.

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