KDU Journal of Multidisciplinary Studies (KJMS)
Volume 6. Issue 2 Nov 2024
DOI: https://doi.org/10.4038/kjms.v6i2.131

KJMS 2024 VOL.6 (2): 79-87

CROSS-SECTIONAL STUDY ON HEMATOLOGICAL VARIATION ASSOCIATED WITH ADVANCE CHRONIC KIDNEY DISEASE (CKD) IN A CKDu ENDEMIC AREA IN SRI LANKA

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ABSTRACT

A cross-sectional study was undertaken to investigate the etiology and disease progression in terms of CKD (chronic kidney disease)-initiation risk factors (IRF) and hematology in Nachchaduwa, a CKDu (CKD of unknown etiology)-endemic area in North Central Province of Sri Lanka. Whole blood, spot urine and risk factor history were obtained following informed consent from all medically confirmed CKD/CKDu cases in the area. CKDu was substantiated when medically accepted IRFs did not exist prior to CKD diagnosis. The disease progression stage (G1-G5) was established by kidney dysfunction markers, eGFR (MDRD, and CKD-EPI-sCr) and UACR using serum creatinine (sCr), urine creatinine (uCr) and urine (uAlb) measured in the study. Hematology variables were compared between early (G2-G3b) and advance (G4-G5) stages to reveal changes over disease progression and assessed for linear associations to disease progression in terms of kidney dysfunction markers and renal outcomes. Multivariate dendrogram and principal component analyses were utilized to study inter-variable relations. The results showed that advance disease (G4-G5) in Nachchaduwa had significantly declined RBC counts and RDW-CV, amid unchanged MCV, MCH, MCHC, hematocrit and total hemoglobin suggesting predominantly normocytic anemia. RBC depletion was further evident in its positive associations to eGFRs, and negative association to sCr ($p<0.05$, rho stronger than ±0.45). Multivariate analyses revealed that RDW-CV was following the decreasing eGFRs and uCr over disease progression. Results showed the potential utility of erythrocyte count and RDW-CV as markers of advance disease in CKDu endemic areas of Sri Lanka. IRF distribution in Nachchaduwa pointed to a minor fraction of unknown etiology among total CKD, and the major IRFs that led to chronic renal failure did not include Diabetes Mellitus.

KEYWORDS: *Red Blood Cells, Red Cell Distribution Width, Estimated Glomerular Filtration Rate, Chronic Kidney Disease, Initiation Risk Factors*

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1. INTRODUCTION

The chronic kidney disease of unknown etiology (CKDu) of Sri Lanka was first reported by Abesekara et al, (1996) in late 1990s. The disease is prevalent in a distinct climatic area of the island, known as the dry zone. CKDu is often described as epidemic since a high prevalence exceeding 30,000 reported cases were in the north central province of the dry zone alone (Ranasinghe et al, 2019). With vague distinction in practice, non-endemic CKD and CKDu coexist in the area and share the same pathology, symptoms and medical management. The difference is that etiology of CKDu cannot be described by medically accepted CKD initiation risk factors (IRFs). CKDu is presumed to be causally linked to a hitherto unidentified IRF restricted to the dry zone. Such notion may explain the geographical distribution of the high prevalence disease which is often described as endemic to the region. In this context, further characterization of the development of the dry zone disease and its risk factor history deserve attention.

Hematology variations symptomatic to the chronic renal failure in the CKDu endemic area may provide benchmarks of the disease progression. In peripheral blood of the CKD/CKDu affected in the area, significant decreases reported in total red cell, lymphocyte, platelet, and monocyte counts and increases red cell indices and basophils over disease progression (Gunawickrama et al, 2022). The knowledge gap remains notable with scarcity of further data on hematology trends in the endemic disease. The authors attempted to characterize the disease in the area by following blood cell counts and indices over the entire length of the disease progression, in concert with initiation risk factor history. Nachchaduwa is a typical area of CKDu endemicity in the North Central Province, and it shared the same climate, demography and socioeconomics with the rest of the CKDu endemic area of the island. The study involved all CKD/ CKDu affected people in the village. The participants were field farmers or their family members who relied on irrigated water from a nearby reservoir, for vocational rice cultivation.

2. METHODOLOGY

The households of individuals who had already been medically diagnosed with chronic kidney disease were visited in Nachchaduwa area (8°14'38.0"N+80°27'37.0"E) of the North Central Province in Sri Lanka. The chronic renal failure was verified from the medical records in possession of the affected as the village community provided locations of such households. Following informed consent and on volunteer basis, each subject answered a questionnaire on their own history of CKD initiation risk factors (IRF) and demography. Subsequently at a center, the same participants provided a whole blood (3-4mL), and spot urine samples.

Subject Interview

IRF were followed as put forward by the National Kidney Foundation of USA (Levey et al, 2005). IRF existed prior to initial diagnosis with CKD was gathered individually by interview and from medical records where available. Data were collected by a medical practitioner.

Samples, Initial Processing and Analyses

Blood cell counts and RBC-indices including total RBC, hematocrit, total hemoglobin, MCV, MCH, MCHC, red cell distribution width-coefficient of variation (RDW-CV) total WBC, and the counts of lymphocytes, platelets, neutrophils, eosinophils, basophils, and monocytes were obtained from EDTA-whole blood with an automated hematology analyzer (HumaCount 5 L, Wiesbaden) on the same day. Samples were transported on ice to a commercial medical laboratory for the purpose. At the center, fraction of whole blood was allowed to clot before serum was separated by centrifugation (room temperature, 3000 *g* for 10 min) for determination of serum creatinine (sCr; mg/dL) by colorimetry. Urine albumin (uAb; mg/L) and urine creatinine (uCR; mg/dL) were determined by immunoturbidometry (Bakker, 1988) and Jaffe method (Jaffe, 1886) respectively. Serum and urine samples were delivered on dry-ice and biochemical determinations were made with a Mindray BS-200 clinical chemistry analyzer (Shenzhen) at a commercial laboratory in compliance with the QC guidelines and using reagents of the manufacturer.

The kidney dysfunction markers, estimated glomerular filtration rate (eGFR in mL/min/1.73 m^2) using sCr by the equations, chronic kidney disease epidemiology collaboration (CKD-EPI), and by abbreviated modification of diet in renal disease (MDRD) (Inker et al, 2012; Levey et al, 2003) were calculated. Urine albumin to creatinine ratio (UACR, mg/g) was determined using urine albumin (mg/L) and urine creatinine (mg/dL). All counts and determinations were made for each subject of the study.

Subject Sorting

Individual eGFR< 60 in the study (CKD-EPI; sCr equation) and prior medical diagnosis were considered confirmatory of CKD/CKDu. Participants with higher eGFR were considered to be CKD/CKDu when medical diagnosis was available. This was followed by sorting into CKD stages from G1 to G5 as outlined elsewhere (National Kidney Foundation, 2002). Stages G4 and G5 (ESRD) were considered to be the advance disease. CKDu was presumed when CKD initiation risk factors were absent in own medical history prior to the chronic renal failure (Levey et al, 2005). G1 subjects were not identified in the study. Notably, recruited participants (n=23) were vocational paddy farmers or their family members in total.

Statistical Analyses

The erythropoietin administered and the dialyzed were not recruited. Univariate analyses were performed following $\log_{10}$ transformation of data for approximation of normality. Variables were compared between early (G2-G3b) and advance disease (G4-G5) stages by student's t-test to reveal differences ($p<0.05$) over disease development. Pearson's correlation analysis was conducted to identify linear associations of blood cell counts and indices with kidney dysfunction markers (eGFRs, and UACR) and other renal outcomes of the study (sCr, uCr, and uAlb). An association was considered when correlation coefficient (r) was stronger than ±0.4 and at $p<0.05$. Multivariate cluster dendrogram involving complete linkage and correlation coefficient distance was developed, and principal component analysis (PCA) by correlation-based matrix was performed to visualize nonlinear relations among selected variables. Hematology variables measured in the study, which showed a Pearson's r stronger than ±0.2 with any of eGFRs, UACR, sCr, uCr, or uAlb were selected for multivariate analyses. Weight and body mass index (BMI) were included as well on their symptomatic relation to CKD progression. All analyses were conducted in Minitab 17 software program. The study was conducted under ethical approval (RP/2017/03) from the ethical review committee at Faculty of Medicine, General Sir John Kotelawala Defence University.

3. RESULTS

CKD progression in to advance stages (G4-G5) significantly decreased ($p<0.05$) red blood cell count (RBC; $10^6/\mu L$) and red cell distribution width-coefficient of variation (RDW-CV; %) as compared to initial G2-G3b stages of the disease (**Figure 1**). Linear correlation analyses showed that total RBC counts from stages G2 to G5 were in a positive association with eGFR by both CKD-EPI; sCr and MDRD ($r=0.5$, $p<0.05$), and in a negative association ($r= -0.4$, $p<0.05$) with sCr (**Figure 2**). Results collectively suggest anemic tendency towards advance stages. However, total hemoglobin and RBC-indices such as MCV, MCH, MCHC were not associated ($p>0.05$) to progressive kidney dysfunction. Disease progression over stages G2-G5 resulted in increasing total WBC and lymphocyte counts (**Figure 3**).

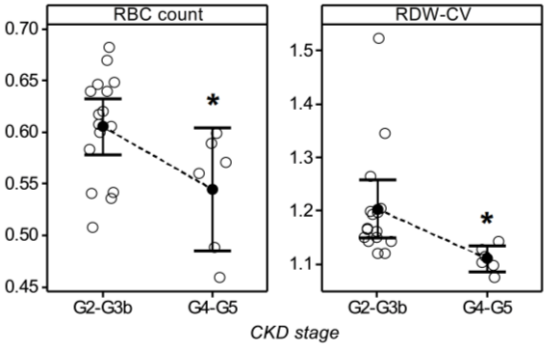

Figure 1 Variation of red blood cell count ($10^6/\mu L$) and red cell distribution width - coefficient of variation (%) as CKD/CKDu progresses into advance stages in Nachchaduwa

Log_{10} transformed data were compared between CKD initial (n=16) and advance stages (n=6), and plotted on linear scale as mean, 95% confidence interval for the mean, and individual values.

* $p<0.05$ (student's t-test)

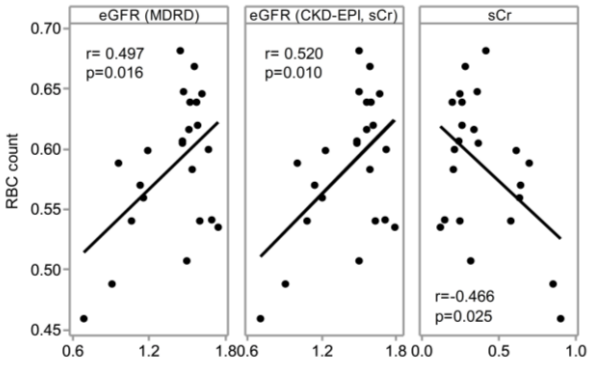

Figure 2 RBC associations with progressive kidney dysfunction in Nachchaduwa

Log_{10} transformed data of RBC count (10^6/µL) and serum creatinine (mg/dL), and eGFR (mL/min/1.73m^2) were tested by Pearson's correlation, and plotted in linear axes (n=22).

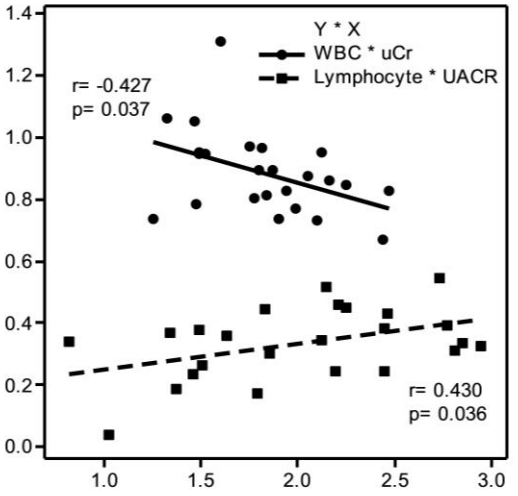

Figure 3 Leukocyte associations with progressive kidney dysfunction in Nachchaduwa

Pearson correlations of WBC count (10^3/µL) with urine creatinine (mg/dL), and lymphocyte count (10^3/µL) with urine albumin to creatinine ratio (mg/g) are shown. Data were log_{10} transformed before analyses and plotted in linear scale (n=22).

RDW-CV showed a close relation with progressive kidney dysfunction (eGFRs) as indicated in both dendrogram (similarity; 69.2%, **Figure 4A**), and PCA (**Figure 4B**). Tendencies of the platelet, neutrophil and total WBC counts to follow sCr were evident as well.

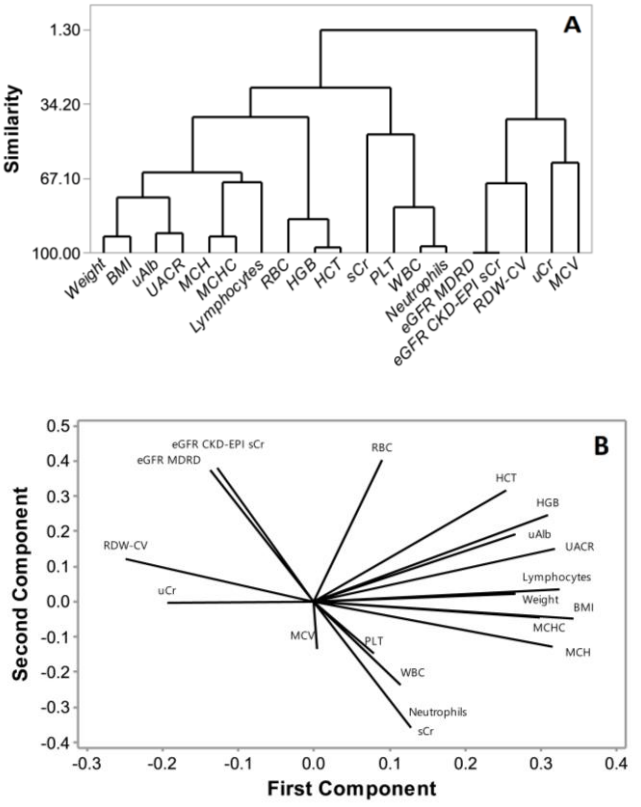

Figure 4 Red cell distribution width was related to eGFR during progressive kidney dysfunction in Nachchaduwa (n=23); A) cluster dendrogram, and B) loading plot of principal component analysis

Most of the CKD/CKDu patients in the study reveled one or more IRF that existed prior to initial diagnosis (**Table 1**). Hypertension remained the most predominant by percentage (60.9%) within the study population, as family history of nephropathies (56.5%) was a major IRF as well. Dyslipidemia (39.1%), systemic infections (malaria mostly of multiple episodes; 26.1%) and urological disorders (21.7%) such as urinary stones and recurrent urinary tract infections were notable.

Table 1 CKD Initiation Risk Factor (IRF) History of the participants of Nachchaduwa

IRF	% from total CKD
Diabetes mellitus	8.69
Hypertension	60.9
Autoimmune diseases	0
Cardiovascular diseases	0
Systemic infections; malaria	26.1
Systemic infections; leptospirosis	4.3
Dyslipidemia	39.1
Acute kidney injury	0
Urological disorders/ urinary stones/ urinary track obstructions/ recurrent urinary tract infection	21.73
Family history of CKD/ nephropathy	56.5
Drug toxicity	4.3
Snake bites	4.3

n=23 with many reporting more than one IRF

Less than 10% the participants in the study reported diabetes mellitus, the systemic infection; leptospirosis, drug toxicity, or snake bites as suspected IRF prior to diagnosis with the chronic renal failure. Minority of CKD subjects did not confirm prior presence of IRF suggesting that the fraction of unknown etiology (CKDu) among total CKD could be about 8% in the Nachchaduwa which is considered to be within CKDu endemic zone of the country.

4. DISCUSSION

Prior medical records of diagnosis and management were used to verify the chronic renal failure. The disease was further verified before recruitment using individual kidney dysfunction marker levels, eGFR (<90 mL/min/1.73 m^2) and UACR (>30 mg/g) measured in the present study. It resulted in recruitment of twenty-four CKD/ CKDu cases from inhabitants in Nachchaduwa village into a study group irrespective of non-proteinuria. All of them participated in the study. Moderate sample size of the verified chronic renal failure cases remains a limitation. However, data analyses essentially reproduced what has been reported from CKDu endemic North Central and Uva provinces of the country and elsewhere in relation to hematology and risk factor profiles pertaining to the chronic renal failure, in terms of the onset of anemia (Dmitrieva et al, 2013; Gunawickrama et al, 2022) and hypertension as a more dominant IRF than diabetes mellitus (Gunawickrama et al, 2020) in the endemic area. A true control of renal health is difficult to be constituted from CKDu endemic areas of the country. For that reason, data were compared between early (G2-G3b) and advance (G4-G5) stages of the chronic kidney disease and variations with the disease progression were followed accordingly.

PCA and cluster dendrogram were essentially used to reveal inter-variable relations among hematology, kidney dysfunction, and renal outcomes. The stringency was maintained by including only the variables that had a linear association (*rho*) stronger than ±0.2 with the disease progression.

Significantly declined ($p<0.05$) RBC count and RDW-CV in advance G4-G5 CKD stages as compared to early G2-G3b stages (**Figure 1**) could be considered as indicative of CKD associated anemia. RDW-CV indicates erythrocyte size variation, and its decrease mostly suggests hampered RBC production. Declining of the RBC count in advance stages (**Figure 2**) points to an increasing incidence of anemia in the study group as the chronic renal disease progresses, and as reported before (Dmitrieva et al, 2013). In participants, MCV, MCH and MCHC as well as hematocrit and total hemoglobin were neither changed ($p>0.05$) between early and advance stages nor correlated to disease progression, in terms of eGFR, UACR, sCr, or uCr. Further, the MCV remained within its normal range, 80-100fL (Bessman and Johnson, 1975) in 86% of the participants. Solak et al (2013) reported an unchanged MCV across stages G1-G5 involving 309 CKD patients. In such context, the results may collectively be considered as suggesting that renal malfunction induced anemia in Nachchaduwa could predominantly be normocytic.

Anemia is multifactorial in CKD (Portolés et al, 2021). Solak et al (2014) reported, anemia may emerge as early as stage G1 (>40% total CKD cases) and grow further in incidence as the disease progresses to end-stage renal disease (G5; ESRD) notably with a fraction showing low RDW-CV (<14%). It suggests

other routes to anemia in CKD as well. Normocytosis was shown to be the most common morphologic form in anemia in CKD (Mohammed and Mahmood, 2022) and its proportion increases as the disease progresses (Dmitrieva et al, 2013; Gluba-Brzózka et al, 2020). RDW-CV drop at G4-G5 of CKD/CKDu in the present study was notable. Low RDW occurs in CKD and other chronic illnesses (Hsieh et al, 2016; Tonelli et al, 2019) as certain authors report an increase as CKD progresses (Lu et al, 2017; Yonemoto et al, 2018; Roumeliotis et al, 2020). In a correlation study with anemic CKD patients, Emans et al (2011) reported that low RDW associated weak erythropoiesis and dwindling reticulocyte fraction. In such context, decreased RDW could mean hampered erythropoisis in Nachchaduwa study participants of chronic renal failure.

Increasing WBC and lymphocyte counts towards advance CKD stages in relation to uCr and UACR variations (**Figure 3)** and the relation of WBC and neutrophils to sCr (**Figures 4A, 4B)** could perhaps be linked to oxidative stress and chronic inflammation which understandably drive initial CKD towards ESRD (Oberg et al, 2004; Tucker et al, 2015).

The health, genetic, environmental, demographic or other factors that could be causal to initial renal damage of the chronic kidney failure are envisaged as CKD initiation risk factors (IRF). The geographical distribution of high-prevalence CKD that is commonly described as CKDu in dry zone of Sri Lanka is presumed by the present authors, to be linked to one or more hitherto unidentified risk factors limited to the same area. On the other hand, such proposition could explain the geographical delimitation of the disease as well. CKD is cosmopolitan and initiated by established IRF (Levey et al, 2005). The authors believe investigation into the individual risk factor history prior to chronic renal impairment may reveal obscure IRFs operating in the area. Literature on IRFs that could explain endemic CKDu in the dry zone of Sri Lanka appears to be scarce with exceptions that linked the disease to genetic predisposition (Wanigasuriya, 2012), agrochemical usage & paddy farming (Wimalawansa, 2014), metal exposure (Gunawickrama et al, 2022) and hydrogeochemistry & drinking-water (Chandrajith, 2011). Search for unconventional IRF operating in the area is also the key to reveal etiology relations of CKDu, establish its frequency among total CKD in the dry zone, and for disease migration. Nachchaduwa CKD/CKDu population had only a minority (8%) whose disease could not be traced back to a conventional IRF hence CKDu. Present study points to the uncontrolled hypertension as the major conventional IRF in Nachchaduwa village (**Table 1**). The results are consistent with previous report (Gunawickrama et al, 2020) where hypertension was reported as a major IRF in both CKDu endemic Padaviya (31% total CKD) and Girandurukotte/ Madawachchiya (38% total CKD). Diabetes Mellitus has been reviewed as the dominant cause of CKD initiation internationally (Kazancioglu, 2013). In Sri Lanka dry zone however, IRFs such as hypertension, and systemic infections (multiple episodes of Malaria and Leptospirosis in particular), family history of nephropathy could be the leading causes of CKD.

5. CONCLUSION

The limited statistical power was a constraint in the present study. However, entire CKD/CKDu population was studied in Nachchaduwa which is endemic to CKDu and data were in general agreement with prior reports. The participants were comparable in demography so that confounders were minimal supporting results and conclusions. Results pointed to a predominantly normocytic anemia. Red cell count and RDW-CV followed kidney dysfunction and eGFR. It is suggested that fraction of unknown etiology among total CKD could be a clear minority in the area, and the major IRFs that led to chronic renal failure did not include Diabetes Mellitus.

6. ACKNOWLEDGEMENTS

The study was supported (100%) by the research grant, RPHS/2016/CKDu/04 from National Science Foundation of Sri Lanka. Assistance from the village community and the buddhist temple in Nachchaduwa is duly appreciated.

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