Vincent Lim

PROJECT TITLE: The Prognostic Value of Cystatin C and Urinary NGAL in Patients With the Cardiorenal Syndrome

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SUMMARY:

Congestive heart failure (CHF) is a common disease and leads to numerous deaths in Canada annually. Part of the reason is due to the high prevalence of renal dysfunction in this patient population, a comorbidity that acts as an independent risk factor for the progression of cardiovascular disease and therefore dramatically increases morbidity and mortality. The phenomenon linking the interaction between these two vital organ systems is called the cardiorenal syndrome. Further understanding of the pathophysiology as well as the ability to predict the development of cardiorenal syndrome in patients with CHF can aid clinicians in guiding therapy towards prevention, and optimizing patient management. To further the understanding of this disease, as well as risk stratify patients with CHF it is important to look at novel biomarkers like serum cystatin C and urinary neutrophil gelatinase-associated lipocalin (NGAL). Cystatin C has been shown to be a robust measure of renal function as it is not subjected to many of the limitations that exist for creatinine. NGAL is a marker of renal tubular injury and levels in both urine and serum are quickly increased when the renal tubules are damaged. By looking at the predictive value and pattern of both of these biomarkers in the development and progression of renal dysfunction in ambulatory CHF patients, we can identify individuals with greater risks of adverse events as well as further our understanding in the development of the cardiorenal syndrome.

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Student's Signature **Supervisors' Signatures**

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Introduction

Congestive heart failure (CHF) is a major cause of morbidity and mortality worldwide, with a prevalence of over 500 000 in Canada alone. It is estimated 50 000 patients are diagnosed with CHF each year in Canada, with its incidence rising due to the aging population. One-year mortality after diagnosis has been reported to be between 25 to 40% (1). In the setting of concomitant renal dysfunction, mortality rises substantially (2).

Cardiac and renal dysfunction occurring simultaneously is termed the cardiorenal syndrome (CRS). Often viewed as renal insufficiency occurring as a direct result of heart failure, it has recently been recognized CRS is in fact more complex, and also encompasses the deleterious effects renal dysfunction has on the cardiovascular system. Ronco and colleagues have attempted to clarify this constellation of disorders with a classification system, which take into account primary organ dysfunction, and chronology of the disease (3) (see Table 1).

There is a high prevalence of renal dysfunction in patients hospitalized with acute, decompensated heart failure, and renal insufficiency has been shown in multiple, observational cohort studies to be an independent marker of mortality and re-hospitalization (1, 4-8). However, there is a relative paucity of evidence regarding stable ambulatory CHF patients with known renal disease, and the impact of gradual deterioration in renal function in worsening clinical outcomes.

The Candesartan in Heart Failure: Assessment of Reduction in Morbidity and Mortality (CHARM) investigators showed an association between chronic kidney disease (CKD) and an increased risk of death and hospitalization from heart failure in an ambulatory cohort of 2680 CHF patients with left ventricular ejection fraction (LVEF) $\leq$40% (9). Unfortunately, similar to many other prospective trials, patients with severe renal impairment (creatinine >265 μmol) were excluded. Similarly Mahon et al. showed an association between worsening renal function, CHF, and functional status (10). To date, the majority of studies linking renal dysfunction in the CHF population with outcomes are retrospective in nature.

The pathophysiology of CRS has yet to be fully elucidated. For several decades type II CRS has been mechanistically rationalized by the "Low-Flow Hypothesis" (11). It was postulated renal dysfunction occurred in the setting of heart failure as a result of a reduced cardiac output which in turn led to reduced renal blood flow, declining glomerular filtration rate (GFR), and an activation of the renin-angiotensin-aldosterone system (RAAS). This is thought to be further exacerbated by sodium and water retention, sympathetic nervous system activation, and inflammatory cytokine production (see Figure 1). While this may seem to be a reasonable mechanism in patients with systolic dysfunction, and indeed novel studies using ^{125}I-Iothalamate and ^{131}I-Hippuran clearances to measure renal blood flow seem to support the low-flow hypotheses, it cannot account for declining renal function in CHF patients with preserved ejection fractions, who by definition, are not in a low-flow state (12, 13).

The ESCAPE trial has shown elevated right atrial pressure, but not decreased LVEF or arterial blood flow, correlated with an elevated serum creatinine in patients with acute

decompensated heart failure (14). This suggests venous congestion may also be responsible for progressive renal dysfunction. This may be a result of diminished transrenal perfusion pressure gradient, which reduces renal blood flow, causing hypoxic damage, and reduced GFR (15). This hypothesis is consistent with clinical signs of congestion, including elevated JVP, peripheral edema, orthopnea, and ascites, which are all associated with renal impairment and mortality in the heart failure population (16-18).

CRS is difficult to treat due to a lack of evidence based guidelines for therapy, and an inability to predict its development before renal function is grossly abnormal, and essentially irreversible. Serum creatinine measurements, used to calculate eGFR, is the most traditional marker of renal function. However, creatinine has many known limitations and its interpretation must take into account muscle mass, diet, sex, age, and ethnicity (19). Serum creatinine may overestimate renal function and is insensitive to mild to moderate renal insufficiency, tending to rise only when at least 50% of nephrons are no longer functioning (20, 21).

The eGFR, as calculated by the Modification of Diet in Renal Disease (MDRD) equation attempts to adjust for the limitations of using creatinine alone by taking into account factors like age, sex, and ethnicity. However, the MDRD is largely based on muscle mass and an ideal estimate of body water and can be inaccurate, especially in the CHF patient population (22, 23). Further, the MDRD equation has been shown to overestimate renal function in patients with a GFR>60 mL/min per $1.73m^2$, thus ineffective for patients with only mild kidney dysfunction (20, 24).

Cystatin C is a cysteine proteinase inhibitor produced by all nucleated cells in the body. It is produced at a constant rate, freely filtered by the kidneys, not secreted or reabsorbed, and not metabolized outside of the renal tubules. Unlike creatinine, cystatin C is not affected by age, gender, diet, ethnicity, and body composition. In essence, it appears to be a more ideal marker of renal function than serum creatinine (19). Serum measurements of cystatin C alone have been shown to perform nearly as well as the MDRD equation in patients with GFR <60 mL/min per $1.73m^2$. Cystatin C appears to be more sensitive than creatinine and the MDRD equation, with the ability to detect mild renal dysfunction. Nevertheless, its elevation is only seen after renal dysfunction has already occurred because like creatinine, it is a measure of renal function, and not injury (20, 25).

A promising new biomarker for the detection of kidney injury is neutrophil gelatinase-associated lipocalin (NGAL). Part of the lipocalin superfamily, it functions in innate immunity. However, in vitro studies have also shown that NGAL has a role in the development of the kidney and can promote proliferation and differentiation of kidney cells, even in adults (26, 27). Originally found to be released by neutrophils when activated during times of a bacterial infection, studies in mice models have shown that NGAL is also produced by other tissues, and its production dramatically upregulated in the setting of tissue injury. Notably, in renal tubules, NGAL production is rapidly increased and released in the setting of ischemia or exposure to nephrotoxic agents, including high dose cisplatin (28, 29). Further studies of NGAL in humans have suggested increased serum and urine levels are a strong predictor of kidney injury in patients undergoing procedures associated with relatively high rates of acute kidney injury (AKI), including cardiac surgery and catheterization. Unlike creatinine, which is

only elevated about 1-3 days after kidney injury, NGAL levels in both the serum and urine can be found to be elevated as soon as 2 hours after injury (26, 30).

As NGAL is elevated with renal tubular injury, it follows that subtle changes in urinary NGAL may be predictive of impending deteriorations in renal function. Combining NGAL with a robust and sensitive marker of fluctuations in GFR, cystatin C, may allow for early detection of renal injury and subsequent preventative measures.

Objectives

In this study, we set out to determine changes in urinary NGAL and serum cystatin C and their impact on the outcomes of death and hospitalization in a prospective cohort of ambulatory CHF patients.

Methods

Participants

An observational cohort of adult patients (>18 years old) being followed in a CHF clinic at a tertiary academic centre in Winnipeg, Manitoba was prospectively studied. Ethics approval was obtained from the local Research Ethics Board.

For the purpose of the study, CHF was defined as an LVEF ≤40% as measured by imaging (echocardiography, MUGA, MRI, MIBI). CKD was defined as estimated glomerular filtration rate (eGFR) ≤60 mL/min per 1.73m^2 on two consecutive measurements calculated using the MDRD equation.

Patient recruitment began September 1, 2008 and the recruitment period lasted until September 2010. All study patients were screened for eligibility and provided written consent. Only patients who were >18 years old, had LVEF ≤40% and willing to consent were included in the study. Patients were also screened by urinalysis for active urine sediment (RBC casts, dysmorphic RBCs) and excluded if this or active glomerulonephritis was found. These patients were then subdivided into two study populations: patients with congestive heart failure (CHF group), and patients with congestive heart failure and chronic kidney disease (CHF-CKD group).

All study patients were followed every 3 months for 2 years. Clinical data, laboratory data, plasma and urine samples were obtained according to a predetermined schedule. Adverse events occurring in between follow-up appointments were recorded. All patients' serum and urine samples were stored at -80°C for subsequent biomarker analysis. For the purpose of the study, patients were considered to have met endpoints when death, heart transplantation, hemodialysis, or peritoneal dialysis occurred.

Assay

Enzyme-linked immunosorbent assays (ELISA) were performed to measure the concentrations of cystatin C and NGAL in serum and urine respectively. All ELISA kits came from Quantikine®.

Prior to performing assays, all specimens were stored at -80°C. Specimens were first thawed and appropriately diluted before incubating at 4°C in a 96 well polystyrene plate coated with a mouse monoclonal antibody against cystatin C or NGAL depending on the assay performed (3 hours incubation for cystatin C and 2 hours for NGAL). All specimens were plated in duplicates. Following the incubation, the plates were thoroughly washed to remove excess substrate and incubated at 4°C with a monoclonal antibody conjugated to horseradish peroxidase with specificity to either cystatin C or NGAL depending on the assay performed (1 hour incubation for cystatin C and 2 hours for NGAL). Plates were thoroughly washed again to remove excess antibody and incubated with a substrate containing hydrogen peroxide and tetramethylbenzidine for 30 minutes at room temperature while protected from light. Sulfuric acid was added as a stopping solution. Plates were read in an ELISA reader at 450 nm and wavelength correction at 540 nm. All cystatin C and NGAL concentrations were calculated with computer software based on a standard curve with a four parameter logistic curve-fit. If specimens were diluted, the appropriate dilution factor was multiplied to attain the concentration of cystatin C and NGAL in serum and urine respectively.

Statistical Analysis

Differences in baseline characteristics were determined by student's t-test or the chi-squared test as appropriate. To determine the prognostic utility of serum cystatin C and urinary NGAL in its ability to predict mortality, all-cause hospitalizations, and CHF related hospitalizations; urinary NGAL and serum cystatin C at baseline entry into the study were included in a COX proportional hazards model to determine their association with outcomes. The models were adjusted for variables including age, diabetes, patient history of myocardial infarction (MI), history of smoking, and diastolic blood pressure. These variables were chosen based on clinical importance and their association with long-term survival in patients with CHF. To determine the test characteristics of cystatin C, creatinine, and eGFR using the MDRD equation, at baseline, receiver operator curves were created and the area under the curve (AUC) calculated. P values <0.05 were considered statistically significant and all analyses were conducted using PASW version 18.

Results

A total of 138 patients were enrolled into the study, 88 belonging to the CHF group and 50 belonging to the CHF-CKD. During the study period, 14% of patients died, 56.5% experienced a hospitalization with 33% being CHF-related. There were 115 males and 23 females with the majority of patients being Caucasian (93.6%) (see Table 2). No differences in sex or ethnicity were seen between the two groups. The most common cause of cardiomyopathy in the patients studied was ischemic cardiomyopathy. The mean age of the patient population was 62.6±11.3 and there was a significant age difference between the CHF-CKD group (68.6±8.3) and the CHF group (60±11.2) with a $p<0.0001$. As expected, in the CHF-CKD group, creatinine (161.8±62.6 μmol/L), urea (16.4±1.2 mmol/L), and cystatin C (1704±616 mg/L) were all significantly higher than the creatinine (83.0±14.8 μmol/L), urea (9.2±2.2 mmol/L), cystatin C (1060±291 mg/L) in the CHF group (p values of<0.001, 0.05 and <0.001 respectively). The two groups

showed no difference in proportion of patients with COPD, coronary artery disease, previous CABG, hypertension, hyperlipidemia, and atrial fibrillation. However, the CHF-CKD group had significantly more diabetics, previous or current cigarette smokers, and patients with previous MIs. Diastolic blood pressure but not systolic blood pressure was also significantly lower in the CHF-CKD group. Concentrations of urinary NGAL and serum cystatin C at different time points are displayed in Table 3.

A baseline measure of cystatin C was associated with mortality and CHF-related hospitalization. Unadjusted Cystatin C was associated with death (HR 1.002 per 1 mg/L increase in cystatin C [95% CI, 1.001 to 1.002]; $p<0.0001$) and this remained consistent after adjustment for age, diabetes, smoking, history of MI, and diastolic blood pressure (HR 1.001 per 1 mg/L increase in cystatin C [95% CI, 1.000 to 1.002]; $p=0.012$). Baseline cystatin C was not associated with all-cause hospitalizations. Cystatin C was associated with CHF-related hospitalization (HR 1.001 per 1 mg/L increase in cystatin C [95% CI, 1.001 to 1.002]; $p<0.0001$) however not after multivariate adjustment (HR 1.001 per 1 mg/L increase in cystatin C [95% CI, 1.000 to 1.002]; $p=0.056$). Baseline urinary NGAL measures were not associated with outcomes.

To investigate the test characteristics of cystatin C compared to serum creatinine and eGFR by the MDRD equation, receiver operator curves and the AUC were determined (see Figures 2-7). Cystatin C (AUC 0.833 [95% CI, 0.75 to 0.92]) was found to be superior to both creatinine (AUC 0.818 [95% CI, 0.702 to 0.934]) and eGFR (AUC 0.816 [95% CI, 0.697 to 0.936]) for predicting mortality. Cystatin C (AUC 0.739 [95% CI, 0.641 to 0.837]) was also found to be superior to both creatinine (AUC 0.592 [95% CI, 0.461 to 0.723]) and eGFR (AUC 0.588 [95% CI, 0.458 to 0.718]) for predicting CHF-related hospitalizations. Results for time-varying analyses of both NGAL and cystatin C up to one-year follow up are currently pending.

Discussion

Cystatin C was shown to be associated with CHF hospitalizations and deaths. This association with mortality held after adjusting for age, diabetes, smoking history, patient history of MI, and diastolic blood pressure. This demonstrates renal dysfunction is an important prognostic factor in ambulatory patients with CHF. This association has been demonstrated previously, however only in older adults over the age of 65 (31). Our population involved patients as young as 28. Therefore, we can extend the association to a more generalized adult CHF population.

Cystatin C was superior to both creatinine and eGFR in its ability to predict CHF-related hospitalizations and mortality. This is likely because cystatin C is a more robust marker of renal function and not affected by muscle mass, diet, age, sex, and ethnicity. When comparing receiver operator curves, the eGFR calculated using the MDRD equation performed least well for both outcomes. Perhaps this is because the MDRD equation was derived for patients with CKD and performs best in patients with GFR <60 mL/min per $1.73m^2$, and is not accurate at more mild levels of renal dysfunction. The majority of our study patients (63.8%) were in our CHF cohort, where inclusion criteria included LVEF

≤40% and eGFR >60 mL/min per 1.73m^2. In addition, the MDRD equation is based on an ideal amount of body water, which in the CHF population is not accurate as these patients generally carry excess intra and extravascular fluid. Time-varying analysis examining levels of cystatin C over one-year follow up is currently in progress.

Urinary NGAL, the marker of renal tubular injury, did not appear to have any association with hospitalizations or mortality. Most studies in the past have centered on NGAL being predictive of renal dysfunction and adverse events in the setting of acute kidney injury (AKI). Our study has shown the predictive value of NGAL in the acute setting does not necessarily translate into the chronic setting with ambulatory CHF patients. This may be due to the fact that in the development of type II CRS, the mechanism of renal injury does not directly involve the renal tubules, but other components like the renal interstitium. In addition, NGAL levels may also appear to be lower due to decreased renal function with less tubules being present to secrete this marker. Further analyses are currently being performed to adjust for renal function to see if the GFR had an influence on the findings. Also, time-varying analysis looking at levels of NGAL over one-year follow up is currently in progress.

Our results demonstrate patients with type II CRS are at higher risk of hospitalizations and death, which may be a result of the higher rate of comorbidities in this group. Significant medical issues seen more often in the CHF-CKD study group included diabetes and smoking. Acute coronary syndrome occurred more frequently in the CHF-CKD group, which may be in part due to their higher rate of smoking and diabetes, known risk factors for cardiovascular disease. However it is difficult to determine whether increased cardiac events resulted in progression of renal insufficiency, or vice versa, however it is likely the two organ dysfunctions potentiated each other.

The CHF-CKD group had significantly lower diastolic blood pressures and wider pulse pressures when compared to the CHF group, likely secondary to hardening of vessels due to calcific changes. Arteriosclerosis is a known sequelae of chronic renal dysfunction, and is more common in the elderly, and diabetics, all of which contribute significantly to cardiac morbidity in this population. NYHA functional class was worse in the CHF-CKD group, as most patients in this group were NYHA classes III and IV, while the majority in the CHF group were NYHA class II. This seems to correlate with the higher rate of death seen in the CHF-CKD group, as there has been a demonstrated association between higher NYHA functional class and patient mortality (10, 32).

Limitations

Our study did have limitations. At baseline recruitment of study patients, it was not feasible to order new evaluations of heart functions (echocardiography, MUGA or MIBI). Therefore, baseline LVEFs were based on most recent measurement available prior to enrolment. Individuals with LVEF evaluations >40% during the study period were said to have “normalized LVEFs” and subsequently removed. Secondly, evaluation of renal function at baseline was measured using creatinine and the MDRD equation. As per the results of this study, these methods of evaluating GFR may be inaccurate in the CHF patient population. Our study was also limited by certain patients’ erratic compliance

with follow up appointments, which unfortunately resulted in censure of their data in preliminary data analysis. The assays of urinary NGAL and serum cystatin C were also done manually using ELISA kits from Quantikine®. Despite caution being taken, human error cannot be ruled out. In attempt to account for this, all samples were plated in duplicate and repeated if differences between duplicates were significant. Lastly, because patients attended follow up appointments based on a predetermined schedule, adverse events usually occurred between appointments. Interesting results may have been found if levels of urinary NGAL and serum cystatin C were measured immediately prior, as well as during patient hospitalizations.

Conclusion

In this prospective study of 138 ambulatory CHF patients, we have demonstrated cystatin C's association with CHF-related hospitalizations and death. This study also showed that cystatin C was superior to creatinine and eGFR (determined from the MDRD equation) in predicting CHF-related hospitalizations and mortality. This is evidence that cystatin C can be useful in risk stratification of ambulatory CHF patients. The lack of association with outcome displayed by urinary NGAL is evidence that it may be of little value in risk stratification and prognostication of ambulatory CHF patients. All of our results helped further the understanding of these novel biomarkers, as well as the development of type II CRS.

Table 1. **Subtypes of Cardiorenal Syndrome**

Type	Name	Description
1	Acute cardiorenal syndrome	Acute cardiac dysfunction → Renal dysfunction
2	Chronic cardiorenal syndrome	CHF → Renal dysfunction
3	Acute reno-cardiac syndrome	Acute Kidney Injury → Cardiac dysfunction
4	Chronic reno-cardiac syndrome	CKD → Cardiac dysfunction
5	Secondary	Cardiorenal syndrome due to sepsis, diabetes, amyloidosis, vasculitis, etc.

Classification system for CRS by Ronco and colleagues. This classification system takes into account primary organ dysfunction, and chronology of the disease.
Adapted from Ronco et al. (3)

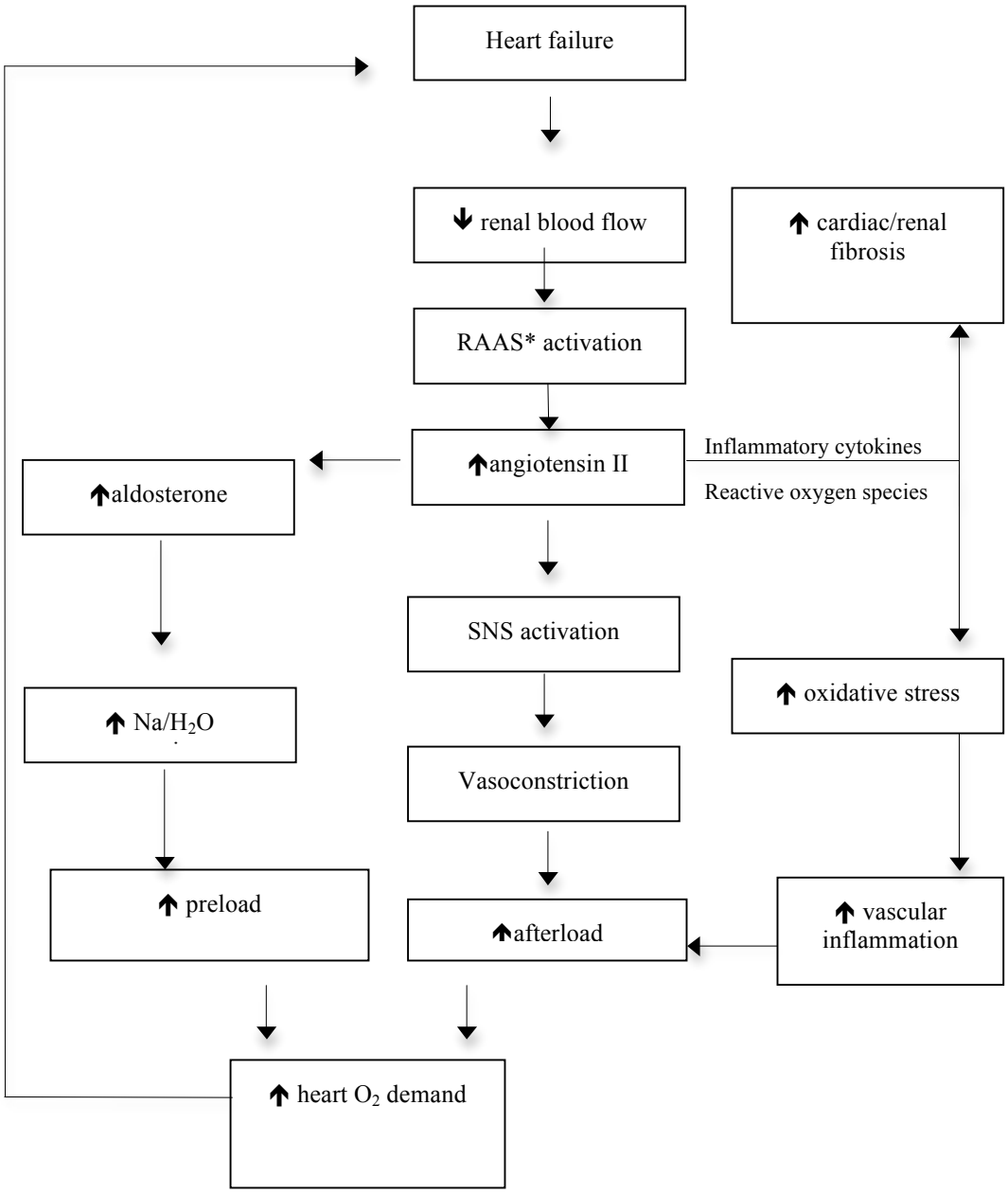
Table 3. **Urinary NGAL and Serum Cystatin C at Different Time Points**

Biomarker	Total Mean	CHF-CKD	CHF	P value
logNGAL baseline (mg/L)	0.89±0.64	0.96±0.67	0.85±0.62	0.3
logNGAL 6 months (mg/L)	0.83±0.72	0.93±0.79	0.79±0.75	0.3
logNGAL 12 months (mg/L)	0.81±0.61	0.91±0.71	0.77±0.57	0.3
Cystatin C baseline (mg/L)	1286±531	1704±616	1060±291	<0.0001
Cystatin C 6 months (mg/L)	1396±584	1851±731	1166±299	<0.0001
Cystatin C 12 months (mg/L)	1214±560	1688±723	1017±313	<0.0001

Table 2. **Baseline Characteristics of Study Sample**

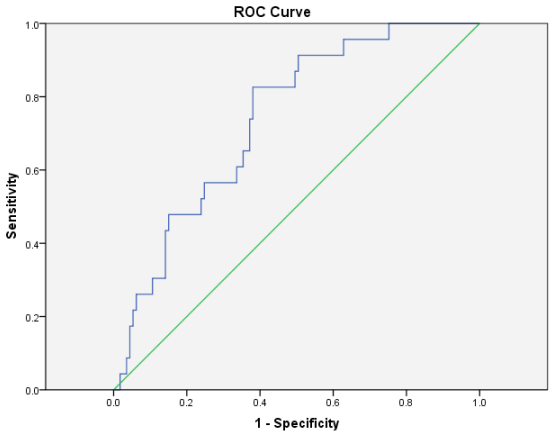
Characteristic	Total (n=138)	CHF-CKD (n=50)	CHF (n=88)	P value
Age	62.6±11.3	68.6±8.3	60.0±11.2	<0.0001
Sex (%)				
Female	17	16	17	1.0
Male	83	84	83	
Race (%)				
Caucasian	93.6	90	95.4	0.1
Aboriginal	3.5	2	4.5	
Black	1.4	4	0	
Hispanic	0.7	2	0	
Other	0.8	2	0.1	
Co-morbidities (%)				
COPD	12.3	8	14.8	0.3
Coronary artery disease	53	56	46.6	0.4
CABG	29	30	28.4	0.7
Acute coronary syndrome	43.7	62	33.3	0.001
Hypertension	68.8	72	67	0.7
Hyperlipidemia	60.0	56.2	61.3	0.7
Diabetes	39.1	52	32	0.03
Smoker	56.9	62	48	0.03
Atrial fibrillation	40.0	50	34	0.3
New York Heart Association Functional Class (%)				
NYHA 1	19.1	18	19.3	0.07
NYHA 2	45.4	34	52.3	
NYHA 3/4	35.5	48	28.4	
Measurements				
Systolic blood pressure (mm Hg)	115.9±19.3	113.2±21.3	117.9±18.2	0.2
Diastolic blood pressure (mm Hg)	69.4±11.3	64.8±10.2	71.9±11.3	<0.0001
Heart rate (bpm)	68.5±11.7	65.6±9.5	70.0±12.3	0.05
Creatinine (μmol/L)	110.8±55.4	161.8±62.6	83.0±14.8	<0.0001
Urea (mmol/L)	11.8±17.5	16.4±1.2	9.2±2.2	0.005
Albumin to creatinine ratio	11.1±49.2	20.4±11.6	6.1±1.7	0.2

***Figure 1*. Neurohormonal Activation in Heart Failure**



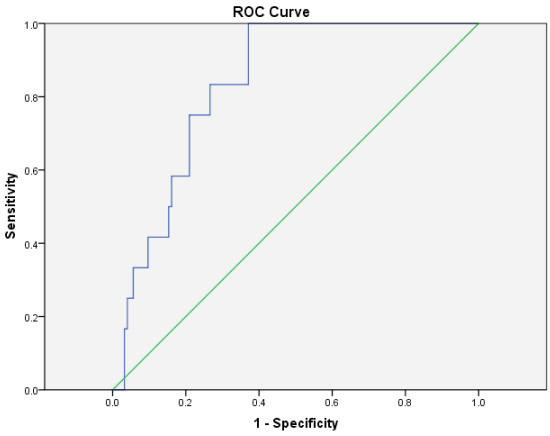
*RAAS = renin-angiotensin aldosterone system
Adapted from Shah et al. (11)

Figure 2. **ROC Cystatin C Predicting CHF Hospitalizations**



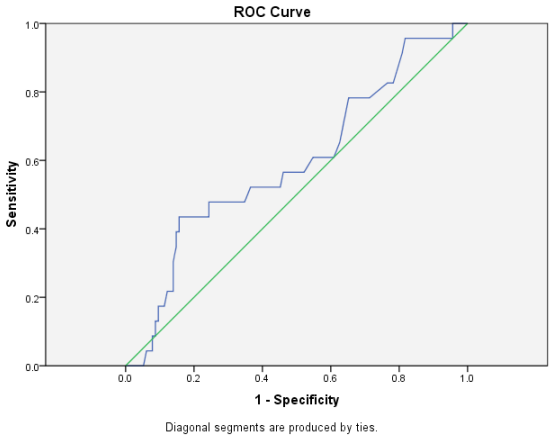
AUC 0.739 [95% CI, 0.641 to 0.837]

Figure 3. **ROC Cystatin C Predicting Mortality**



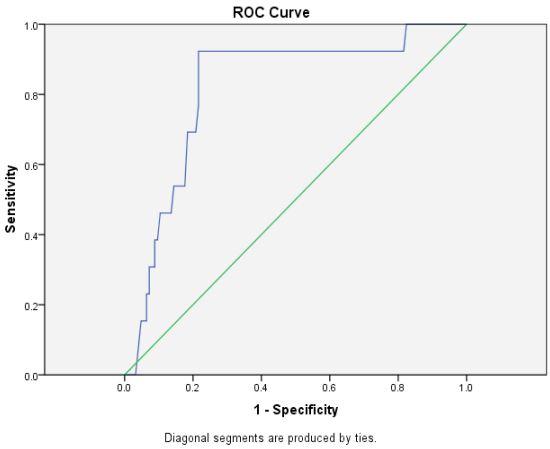
AUC 0.833 [95% CI, 0.75 to 0.92]

Figure 4. **ROC Creatinine Predicting CHF Hospitalizations**



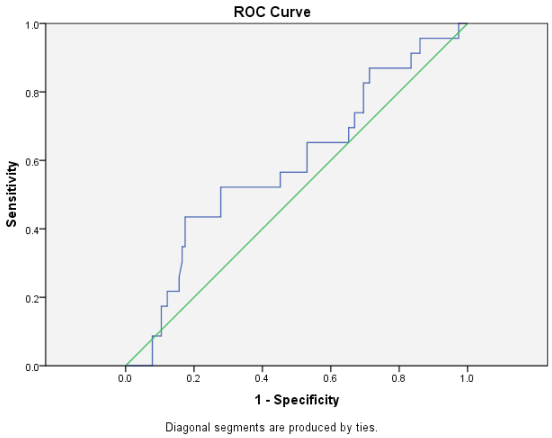
AUC 0.592 [95% CI, 0.461 to 0.723]

Figure 5. **ROC Creatinine Predicting Mortality**



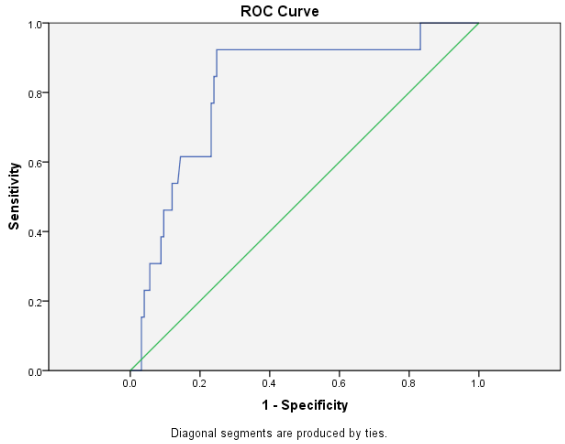
AUC 0.818 [95% CI, 0.702 to 0.934]

***Figure 6.*ROC eGFR Predicting CHF Hospitalizations**



AUC 0.588 [95% CI, 0.458 to 0.718]

***Figure 7.*ROC eGFR Predicting Mortality**



AUC 0.816 [95% CI, 0.697 to 0.936]

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Student's Signature

Supervisors' Signatures