Cathepsin L and Dynamin- Biomarkers of Proteinuric Renal Disease

Mpoti Seboka

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment for the degree of Master of Medicine in Internal Medicine

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Declaration

I, Mpoti Seboka declare that this MMed research report is my own work. It is being submitted for degree of Masters of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

Signature: M. Seboka

Date: 12 April 2016

Dedication

My family, my number one cheer leaders. Thank you for always believing in me. I am because you are.

To God be all the glory.

My supervisors for their patience and guidance.

Publications and Presentations arising from the dissertation

Cathepsin L and Dynamin –Biomarkers of Proteinuric renal disease?

School of Clinical Medicine Research Day 30 September 2015.

ABSTRACT

Dynamin and Cathepsin L: Biomarkers of Proteinuric Kidney Disease?

Background

Chronic kidney disease (CKD) is a major public health problem. It is important to be able to identify those individuals at high risk of CKD progression in order to implement strategies to delay progression to end stage renal disease. Hence, early more sensitive biomarkers are required. Recently, promising new biomarkers have been identified for monitoring CKD progression.

Objectives

- To determine whether Dynamin and Cathepsin L can be used as biomarkers for proteinuric chronic kidney disease (CKD).
- To compare the levels of Dynamin and Cathepsin L in serum and urine of participants with proteinuric kidney disease to those of normal controls.
- To determine if the levels of Cathepsin L and Dynamin correlates with the degree of proteinuria.

Methods

A prospective study of 37 patients with proteinuric kidney disease versus a healthy control group of 40 individuals, where the serum and urine levels of Cathepsin L and Dynamin were determined using an Enzyme Linked immunosorbent assay and the levels compared between the two groups.

Data Analysis

The sample size was determined from previous similar studies, with assistance of a statistician. Sample size was calculated by comparing the means of the groups where the average value for sample 1= 1.0 (standard deviation=0.5); average value sample 2= 1.5 (standard deviation=0.5; alpha= 5% and beta= 20%. A sample size of 20 was initially selected for the kidney disease group and 20 for the Control group (to give a 1:1 ratio). The numbers were there after doubled to increase sample size in order to improve the statistics.

An independent sample t-test was used to assess whether the mean serum Dynamin, urine Dynamin, serum Cathepsin L and urine Cathepsin L differed for the control group compared with kidney disease group. Pearson's correlation analysis was used to measure the strength of the relationship between variables. Statistical significance was $p<0.05$.

Results

There was a significant increase in the level of urine Cathepsin L in the renal disease group 10.44±11.47 pg/ml compared with the control group 2.91±2.88 pg/ml; p= 0.000. There was no difference in the levels of serum Cathepsin L between the renal disease and the control groups (p= 0.23). There were no significant differences in the levels of Dynamin in the serum and urine of patients with proteinuric renal disease and controls (p-values 0.11 and 0.13 respectively).

Although serum Cathepsin L (r = -0.22, p-value = 0.19), urine Cathepsin (r = -0.07, p-value = 0.68), and urine Dynamin (r = -0.04, p-value = 0.83) are negatively related to the degree of proteinuria, the correlation is not significant; all the p-values were greater than 0.05. Serum Dynamin (r = 0.12, p-value = 0.49) had a positive correlation to the degree of proteinuria but

the correlation was not significant at the 5% significance level. Thus, there is no correlation between Cathepsin L and Dynamin levels with the degree of proteinuria.

Discussion

Podocyte dysfunction is a key element in understanding the progression of CKD resulting in proteinuria. In this study, levels of Cathepsin L and Dynamin were determined in participants with proteinuric renal disease and compared with healthy controls. Cathepsin L levels were elevated in the urine of the renal disease group, in keeping with the notion that Cathepsin L proteolysis plays a critical role in the various forms of proteinuria. There was negative correlation between the levels of proteinuria and Dynamin in the serum; however the correlation was not significant statistically.

Conclusion

Cathepsin L could potentially serve as a biomarker of proteinuric kidney disease.

Acknowledgements

To my two supervisors Prof S Naicker and Dr R Duarte. The research team in renal unit; Therese Dix-Peek, Alex Kasembeli, Lerato Mpye and Caroline Dickens, thank you ever so much for all the assistance. Your patience with me and all the encouragement will forever be treasured.

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Abbreviations

AngII –Angiotensin II

CKD-Chronic Kidney Disease

DNM1-Dynamin 1

ELISA- Enzyme linked immunosorbent assay

ESRD- End Stage Renal Disease

ET1- Endothelin

FP-Foot Processes

FSGS-Focal Segmental Glomerulosclerosis

GBM-Glomerular Basement Membrane

GFR-Glomerular Filtration Rate

HIV-Human Immunodeficiency Virus

HIVAN-HIV Associated Nephropathy

HRP-Horseradish Peroxide

MCD-Minimal Change

MPGN-Membranoproliferative Glomerulonephritis

NKF-KDOQI-National Kidney Foundation-Kidney Disease Outcomes Quality Initiative

SD- Slit Diaphragm

TRPC6-Transient Receptor Potential Cation Channel 6

Preface

Kidney disease is still a major problem in South Africa and worldwide. Early detection can aid in optimal management and hence delay requirement of renal replacement therapy and prevention of premature death.

Understanding the pathobiology of the kidney can assist in discovery of biomarkers for proteinuric kidney disease.

This research report is a prospective of 37 patients with proteinuric kidney disease versus a control group of 40 individuals where serum and urine levels of Dynamin and Cathepsin L levels will be compared between the two groups.

Chapter 1- Introduction and literature review

1.1 Background

Chronic kidney disease (CKD) has a significant impact on morbidity, mortality and high medical expenditure in South Africa and globally. It is therefore essential to detect it early in order to institute optimal management and prevent premature death. This will aid in preventing or delaying the requirement for renal replacement therapy. CKD may be present in more than 10% of the adult population in high risk groups. [Nicholas et al. 2005]. Risk factors include socio-demographic, genetic predisposition or the presence of diseases which can initiate and propagate kidney disease. [Nicholas et al. 2005]. In South African adults, hypertension (60-65%) or type 2 diabetes (another 20-25%) are the most common aetiologies of kidney failure [NATIONAL KIDNEY FOUNDATION SOUTH AFRICA]. The increased incidence of hypertension in the South African black population has led to kidney failure being four times more frequent than in other ethnic groups [NATIONAL KIDNEY FOUNDATION SOUTH AFRICA]. Glomerulonephritis was responsible for end-stage renal failure (ESRD) in 52.1% of hypertension and in 45.6% of patients on dialysis in South Africa [Naicker 2003]. It is recognized that the secondary pathological pathway leading to ESRD is a common pathway to all CKD and may inevitably progress to ESRD due to the repeated cycle of nephron destruction by progressive glomerulosclerosis and tubulointerstitial fibrosis [Zandi –Nejad et al. 2004].

The National Kidney Foundation - Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) defines CKD as : "*The presence of markers of kidney damage for ≥3 months, as defined by structural or functional abnormalities of the kidney with or without decreased glomerular filtration rate (GFR), that can lead to decreased GFR, manifest by either*

pathological abnormalities or other markers of kidney damage, including abnormalities in the composition of blood or urine, or abnormalities in imaging tests OR The presence of GFR <60 mL/min/1.73 m2 for ≥3 months, with or without other signs of kidney damage as described above" [Koppel et al. 2011].

1.2 Proteinuria

Proteinuria is the presence of an excess of proteins in the urine. Several hundred million people worldwide, including South Africa, are affected by proteinuria. It serves as a prognostic indicator and sign of kidney disease and is the second most important parameter in the clinical evaluation of kidney function [Madias 2009]. In healthy individuals not more than 150 mg/day protein is excreted and consists predominantly of filtered plasma proteins and tubular Tamm-Horsfall proteins. Therefore, excretion of more than 150mg of protein is pathological and indicates increased permeability of the glomerular basement [Mundel 2010].

There are various forms and different levels of severity for proteinuria. Types of proteinuria include the following: (i) Transient proteinuria, characterised by normal renal function, with no significant underlying renal disease. The proteinuria disappears upon repeat testing. (ii) Orthostatic proteinuria more common in tall, thin adolescents including younger adults of less than 30 years where renal function is normal and proteinuria is said to be less than 1g/day. (iii) Persistent proteinuria: where albumin excretion is usually less than 500 mg/day; not indicative of progressive underlying renal disease, while persistent proteinuria more than 500mg/day, is more likely to be due to glomerular disease [Springberg et al. 1982].

Proteinuria does not merely reflect glomerular injury; it is also harmful. Intracellular signals are activated by increased glomerular permeability to proteins. This includes endothelin (ET-1 vasoactive mediator), growth factors and inflammatory mediators. In the interstitium, these

substances will lead to recruitment of local inflammatory cells. In addition, there will be a release of cytokines/chemokines and growth factors and the production of extracellular matrix collagen and fibronectin that are responsible for interstitial fibrosis [Benigni 2009]. According to this, proteinuria is both a biomarker of kidney injury and a mediator of progressive kidney injury.

1.3 Podocytes

The kidney filters metabolic waste but, the passage of larger molecules e.g. albumin is prevented by filtration across the glomerular capillary wall [Gagliardini et al. 2010]. Therefore, defects in the glomerular capillary wall result in increased permeability to albumin and other plasma proteins causing proteinuria. The podocytes of the kidney and their foot processes function is to prevent urinary protein loss and maintain the ultrafiltration barrier [Gagliardini et al. 2010]. In proteinuria, podocyte membrane extensions reduce in number and size. The podocytes consist of foot process, major processes and a cell body, and they have a complex cellular organisation [Gagliardini et al. 2010]. To better appreciate the biology of glomerular podocytes, understanding the structural and functional anatomy of the glomerular capillary wall is important.

The filtrate must pass through the glomerular capillary wall, which consists of three layers

(i) A fenestrated capillary endothelium-coated with a layer of polyanionic glycosaminoglycans and glycoproteins.(ii) A glomerular basement membrane (GBM), containing heparan sulphate and other anionic glycosaminoglycans and podocytes (or epithelial cells), which are attached to the GBM by discrete foot processes (FP). (iii)The slit diaphragm (SD) is a thin membrane that enclose the pores between the foot processes (slit

pores). Therefore SD functions as a modified adherens junction and may also be permeated by anatomical pores [Gagliardini et al. 2010].

Proteinuria results if there is any interference in any of the components of the glomerular capillary wall [Singh et al. 2007]. The adhesion molecules, such as alpha3beta1 integrin complex and dystroglycan (which are present on the basal membrane of foot processes), attach the podocyte to the GBM [Gagliardini et al. 2010]. Slit diaphragms join the interdigitating foot processes of adjacent podocytes and bridge the intervening filtration slits. The following proteins have been found to comprise the slit diaphragm [Tryggvason et al. 2006]: Nephrin, Neph1 and Neph2, FAT1 and FAT 2, Podocin, Transient receptor potential cation channel 6 (TRPC6), Tight junction proteins, including junctional adhesion molecule A, occludin and cingulin [Fukasawa et al. 2009].

Mutations of some of the genes encoding slit diaphragm proteins causes rearrangement of the actin cytoskeleton and this results in foot process effacement, hence proteinuria [Jones et al. 2009]. (Fig 1) below shows the architecture of normal podocytes and compares this to that where effacement has occurred leading to proteinuria

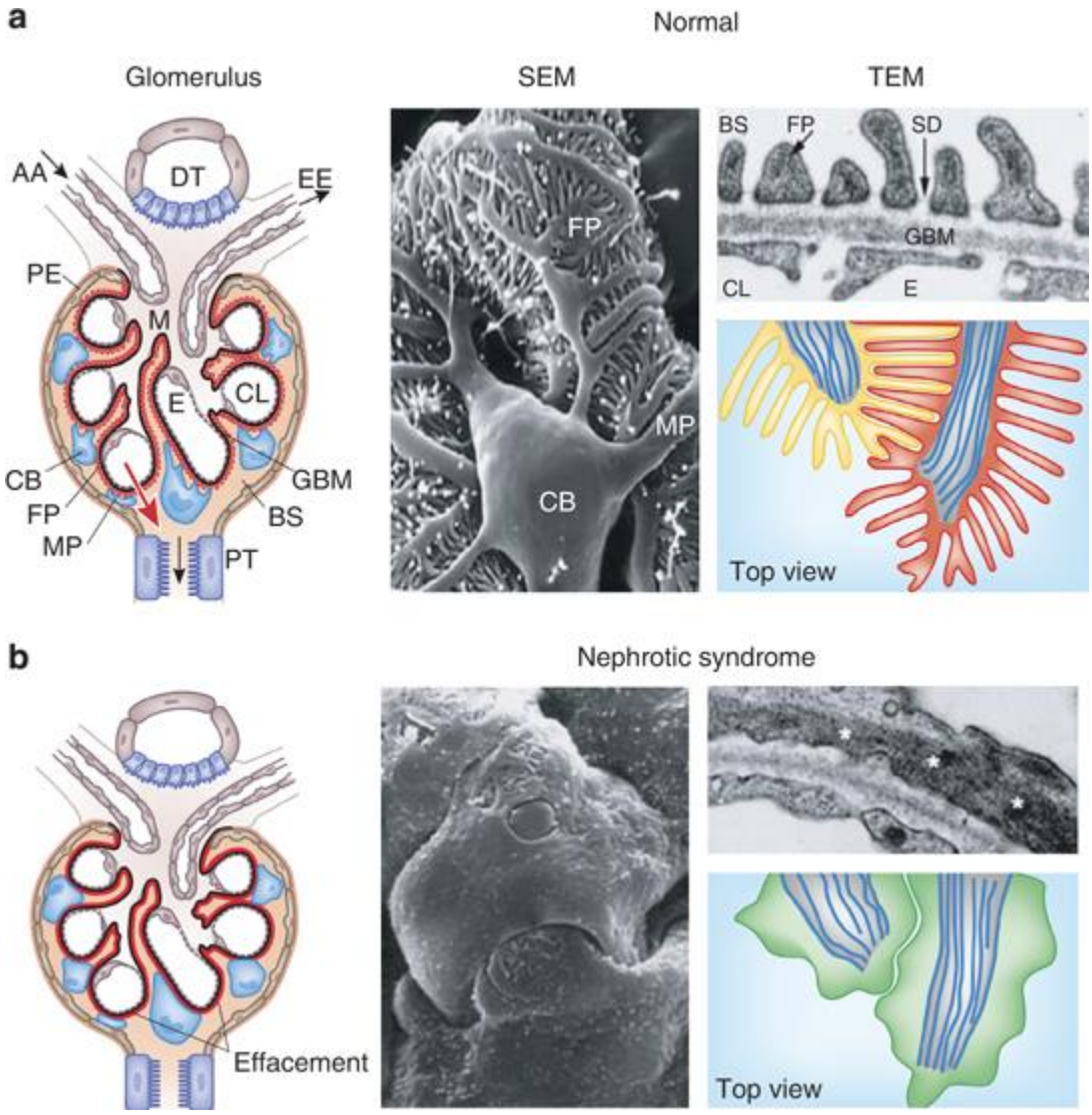

Fig1: a) Architecture of normal podocytes b) Podocytes in nephrotic syndrome

GBM giving support to glomerulus capillary tuft. Capillary lumen (CL) and mesangial cells are embraced by glomerular endothelial cells (E). Podocyte foot process (FP) cover the outer aspect of GBM. Podocyte cell bodies (CB) and major processes (MT) floats in primary urine in the Bowman's space (BS). The plasma ultra-filtrate passes through fenestrated glomerular capillary endothelium, the GBM, and the filtration slits between neighbouring podocyte foot process. AA afferent arteriole, DT distal tubule, EE efferent arteriole. SEM, scanning electron microscopy, TEM, transmission electron microscopy

b) Effacement of the podocytes FP lose their normal interdigitating pattern

Proteinuria: an enzymatic disease of the podocyte? [Mundel and Reiser. 2010]

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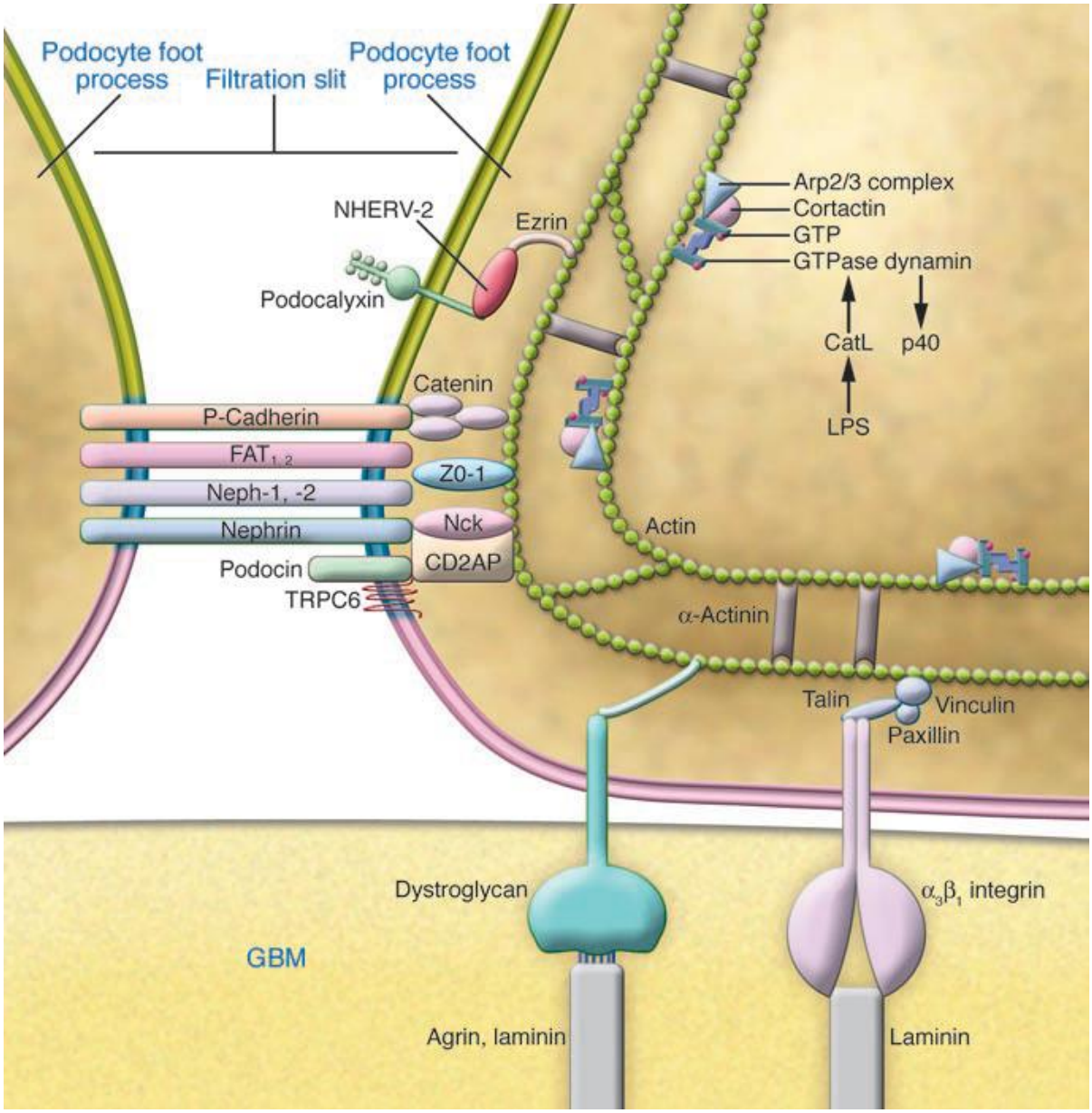

Fig 2: Podocyte slit diaphragm and proteins involved in the slit diaphragm [Ronco 2007].

The cytoskeleton and components of the podocytes are represented. Dynamin cleavage by Cathepsin L, interferes with the normal function of Dynamin and induces cytoskeleton reorganization, and foot process effacement leading to proteinuria.

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In glomerular diseases, including membranous glomerulopathy, minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS) and diabetic nephropathy the podocytes can be injured. Characteristic changes are disruption of the actin cytoskeleton which results in FP effacement and slit diaphragm (SD) disruption. Fig 2 [Mundel et al. 2010].

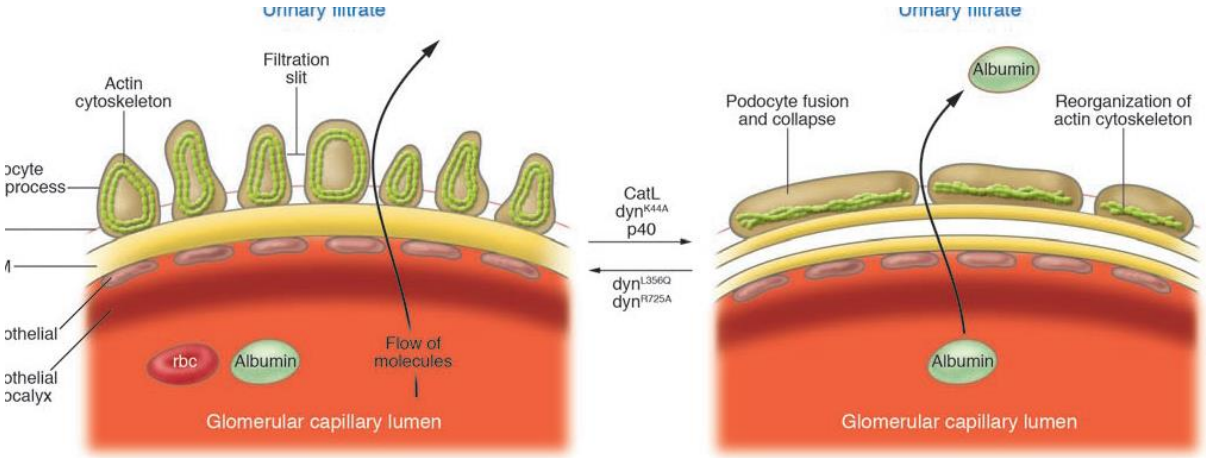

Fig 3: In healthy individuals there is no podocyte effacement and passage of macromolecules is prohibited. [Ronco P. 2007]

The glomerular filtration barrier. (Left) Normal filtration barrier restricts the passage of macromolecules. (Right) Cleavage of Dynamin by Cathepsin L leads to podocyte effacement and hence passage of macromolecules.

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Interference with any components of FP will lead to changes of the actin cytoskeleton to a dense network with FP effacement from the normal parallel contractile bundles structure. There are also changes in slit diaphragm function and structure. The interference of GBM or podocyte–GBM interaction or the dysfunction of podocyte actin cytoskeleton leads to modulation of the negatively charged podocytes surface and activation of Cathepsin L-mediated proteolysis (Figs 2, 3 and 4). [Mundel 2010]

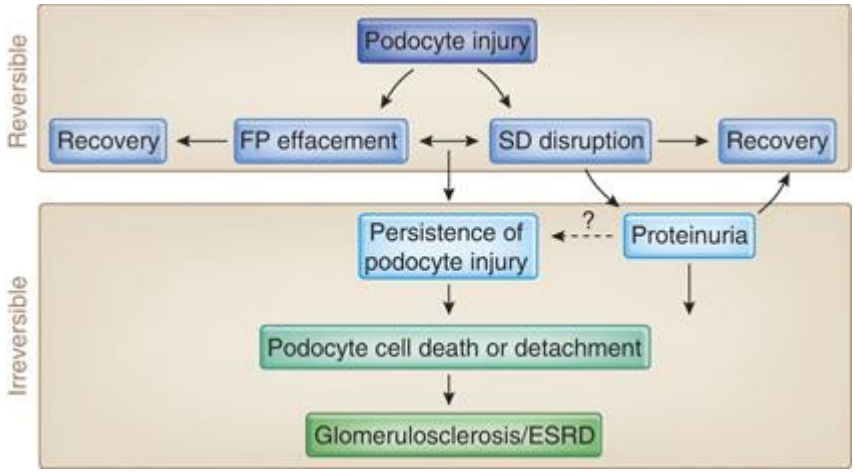

Fig 4: Consequence of podocyte injury [Mundel and Reiser 2010]

Glomerular injury by different glomerular disease can be reversible or irreversible. In irreversible glomerular injury there is cell death and disruption of podocyte cytoskeleton and leads to end stage renal disease (ESRD). FP, foot process. SD, slit diaphragm.

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1.4 Dynamin

According to mouse genetic and cell biologic studies, many proteins control the plasticity of the podocyte actin cytoskeleton [Sever et al. 2007]. These proteins include Dynamin, which

is a substrate for Cathepsin L. Dynamins belong to a super family of GTP-binding proteins that are involved in a diversity of membrane trafficking based processes [McNiven 2000]. Dynamins function as mechano-chemical scaffolding that hydrolyse GTP to constrict and alter biological membranes and recruit many different signalling, cytoskeletal and membrane coat proteins [Praefcke et al. 2004]. Dynamins are also essential for the development of clathrin-coated vesicles at the plasma membrane during endocytosis. They are also essential for the control of actin dynamics in some cell types. In other studies, Dynamin was showed to have a role in mediating microtubule sliding [Reems 2008]. Dynamins are involved in a variety of cellular processes. This includes the development of organelles involved in cell motility, membrane vesiculation from the plasma membrane and trans–Golgi network (e.g. endocytosis and secretion) and also in cytokinesis [Reiser et al. 2010].

The first Dynamin protein was isolated in 1989. Three members of the Dynamin family have been described. Dynamin 1 (DNM1) is neuronal–specific isoform, Dynamin 2 (DNM2) is ubiquitously expressed, and Dynamin 3 (DNM3) localizes in testes, brain, and lungs. The proteins encoded by these genes share the same domain organization and an overall 80% homology but have distinct expression patterns [Ferguson et al. 2012].

1.5 Cathepsin L

The name Cathepsin L alludes to its "catheptic activity" which means to boil down or digest, originate from the Greek word Kathépsein. Cathepsin L was initially discovered in the gastric juice during the 1920s. [Reiser et al. 2010]*'' It is a lysosomal cysteine protease/ enzyme that plays a major role in intracellular protein catabolism. Cathepsin L is potent in degrading collagen, laminin, elastin as well as alpha 1 protease inhibitor and other structural proteins of basement membranes"* [Q1A94 Cathepsin L Elisa kit protocol]. Cathepsins are present in most organisms including all animals. There are several members of this family and are

characterised by their structure, function, mechanism, and which proteins they cleave. Low pH in lysosomes is essential for Cathepsins to become activated [Nicholas et al. 2005]. Cathepsin L mRNA is found mostly in the kidney glomeruli, and is therefore characterized as a glomerular-specific transcript [Sever 2007]. In the podocytes, the cytosolic Cathepsin L degrades the GTPase Dynamin and Synaptopodin; the actin-binding protein. This results in the disruption of actin cytoskeleton hence FP effacement and subsequent proteinuria [Sever 2007]. Foot process effacement can be prevented by the protecting of the target proteins from Cathepsin L proteolysis or by inhibition of Cathepsin L activity (figure 5).

Dynamin mutants, which contain mutated Cathepsin L cleavage sites, are not prone to proteinuria and can arrest and also reverse podocyte foot process effacement. [Nicholas 2005]. These observations propose that Dynamin is important in preserving the ultrafiltration barrier in glomeruli, possibly by modulation the actin cytoskeleton and by Cathepsin L "switching off" the active, GTP-bound form of Dynamin. This suggests that Dynamin is a key for podocyte structure in healthy kidneys. In support of this concept, expression of dominant-negative Dynamin in mice podocytes caused severe proteinuria [Schell et al. 2012].

Levels of Cathepsin-L were found to be increased in the micro-dissected glomeruli of the biopsy of a murine model of minimal change disease- characterised by reversible podocyte foot process effacement. However, this increase was to a lesser extent compared to that of patients with membranous nephropathy, diabetic nephropathy and focal segmental glomerulosclerosis. The expression of a Cathepsin-L-degraded Dynamin fragment causes foot process effacement and proteinuria, whereas the expression of Cathepsin-L-resistant Dynamin mutants decreases proteinuria [Sever 2007].

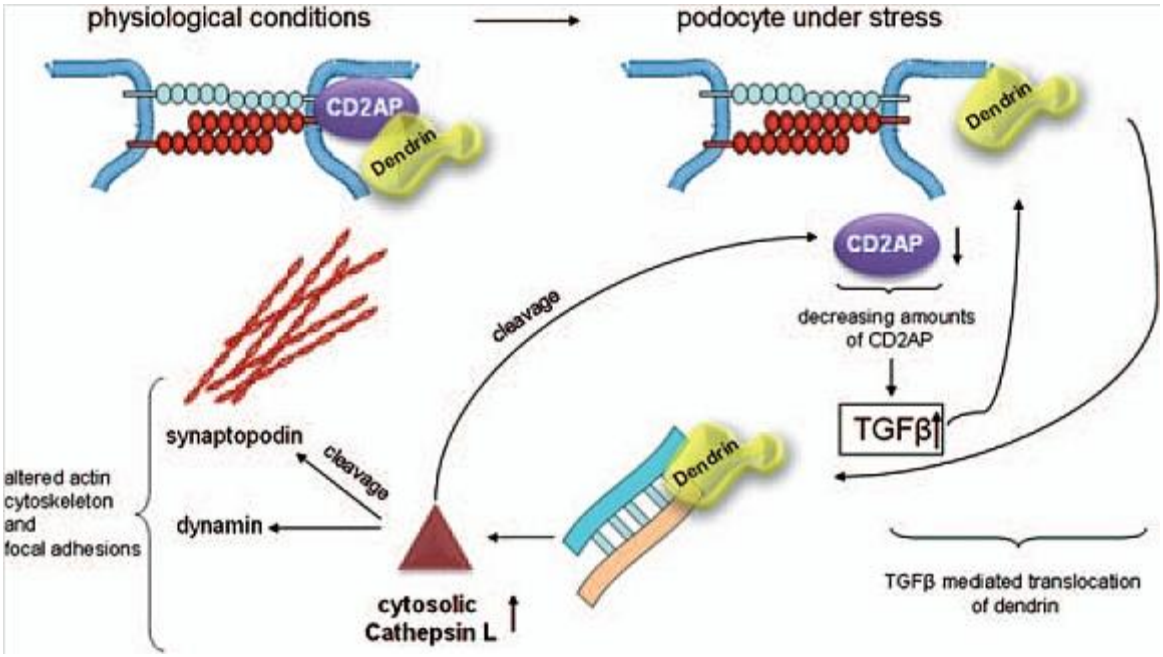

Fig 5: Left: physiological normal podocyte. On the right: podocyte under stress with Cathepsin L cleaving its substrates Dynamin and Synaptopodin leading to effacement [Schell C *et al.* 2012].

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A better understanding of podocyte pathobiology may pave the way for developing a cure for kidney diseases. To date, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II (AngII) receptor blockers have been used as the most effective anti-proteinuric drugs. They are also beneficial in haemodynamic effects and they may be involved in inhibiting AngII-induced actin cytoskeleton reorganization [Macconi 2006]. Further investigation is still required to determine whether this effect also involves Cathepsin L inhibition, or active GTPase Dynamin stabilization. Use of Dynamin mutants and Cathepsin L inhibitors seems to be on the horizon for management of proteinuric diseases, however, further investigation

must be explored. Literature further shows that cysteine protease inhibitors reduce proteinuria in rats possibly by inhibiting proteolysis of the glomerular basement membrane [Reiser 2010]. Inhibition of cytosolic Cathepsin L and its substrate Dynamin, provide promising starting point for the development of selective, anti-proteinuric, and podocyte protective drugs.

Even though albuminuria and proteinuria are used for non-invasive assessment of kidney diseases, they are however non-specific [Fukuda et al. 2011]. As mentioned above, evidence strongly suggests podocyte depletion from glomeruli results to glomerulosclerosis. Podocytes and their products can be detected in urine [Fukuda et al. 2011]. This potentially can be a non-invasive method to monitor the well-being of the podocytes. It suggests the prospect that biological markers of podocyte stress, disorganisation, or loss could be used together with proteinuria to more reliably monitor and detect progression and response to treatment.

While measures of kidney function are still used to observe kidney diseases they are however insensitive as the kidney has considerable reserve function such that more than 50 % of nephrons have to become non-functional before kidney function becomes measurably abnormal [Fukuda et al. 2011]. The screening strategy that relies on functional measurements will, therefore, be insensitive and will tend to identify late stage disease. Podocyte injury and depletion are hence a driver for most forms of glomerular disease. Therefore, this might indicate that podocytes and their products could be utilised as biomarkers of proteinuric kidney disease. Cathepsin L seems to be a promising biomarker in diagnosing of renal dysfunction [Huang et al. 2012]. Hence, the objective of my study is to determine the role of Dynamin and Cathepsin L as potential biomarkers of proteinuric kidney disease.

1.6 Hypothesis

Serum and urine levels of Dynamin and Cathepsin L are increased in patients with proteinuric kidney disease compared to those without proteinuria.

1.6 Objectives

1. To determine the serum and urine levels of Cathepsin L and Dynamin in patients with proteinuric renal disease and normal cohorts using enzyme linked immunosorbent assays (ELISA)

2. To compare the levels of Cathepsin L and Dynamin in patients with proteinuric renal disease to those of normal controls.

3. To determine if the level of Cathepsin L and Dynamin correlates with degree of proteinuria.

Chapter 2-Material and methods

2.1 Ethics

Informed consent was obtained from all the participants. This study was approved by the University of the Witwatersrand Human Research Ethics Committee (Medical). Ethics clearance number M120967.

2.2 Sample collection and preparation

Human samples: 77 participants were recruited and divided into two groups. The first group comprised patients attending the Renal Clinic at Charlotte Maxeke Johannesburg Academic Hospital. This group was made up of patients known with proteinuric renal disease, mostly focal segmental glomerular sclerosis (FSGS) and HIV associated nephropathy (HIVAN). The second group was of 40 normal healthy controls with no background medical history, no proteinuria on dipsticks, and normal urinalysis with normal blood pressures.

Exclusion criteria

- Patients with diabetes mellitus
- Patients with malignancy
- Age<18 >60 years
- Patients with systemic lupus erythematosis or rheumatoid arthritis

Blood and urine samples were collected from each participant. Urine samples were spun at 1000xg for 20 minutes to remove particulate matter and aliquots stored at -80C. For the serum preparation, blood was collected and allowed to clot for 30 minutes before being centrifuged for 10 minutes at 1000xg. The serum was removed and aliquoted into cryotubes for storage at -80C.

2.3 Cathepsin L and Dynamin ELISA

A Sandwich ELISA (enzyme linked immunosorbent assays) was used to determine the serum and urine levels of Cathepsin L and Dynamin and the procedures were carried out according to the manufacturer's instructions (eBiosciences- and Cusabio Biotech Co Human Dynamin 2 Elisa kit, respectively). The protocols are based on the same principle whereby the amount of antigen (Cathepsin L and Dynamin) is measured between two layers of antibodies namely, the capture and detection antibody. In this method, microtitre plates are coated with capture antibody. Following a blocking step, the urine and serum samples were added to each well in duplicate and following an incubation step, the plates were washed. A detection antibody was then added to each well. This antibody recognizes a second epitope of the target protein. After incubation and washing steps, a detection step was carried out. Both assays utilize the Horseradish peroxidase (HRP) enzyme for a colorimetric detection of signal. The substrate for HRP is hydrogen peroxide. Hydrogen peroxide cleavage is coupled to the oxidation of hydrogen donor which causes a colour change.

The measurement of each signal was carried out using an ELISA plate reader (set at a primary wavelength of 450nm). The concentrations of Cathepsin L and Dynamin in each sample were interpolated from a standard curve prepared from readings obtained from duplicate sets of standards included in the assay.

2.4 Data Analysis

The sample size was determined from previous similar studies, with assistance of a statistician.

Sample size was calculated by comparing the means of the groups where the average value for sample 1= 1.0 (standard deviation=0.5); average value sample 2= 1.5 (standard deviation=0.5; alpha= 5% and beta= 20%A sample size of 20 was selected for the kidney disease group and 20 for the Control group (to give a 1:1 ratio). The numbers were there after doubled in order to improve the statistical significance.

The levels of Cathepsin L and Dynamin were compared between the two groups using STATA 12.0 Wilcoxon non-parametric test. This was done due to the skewed distribution. This is an independent sample t-test to assess whether the mean Serum Dynamin, Urine Dynamin, Serum Cathepsin L and Urine Cathepsin L differs for the control group against the disease group. Pearson's correlation analysis was used to measure the strength of the relationship between the variables. Statistical significance was considered as p-value <0.05.

Chapter 3-Results

3.1 Demographic data

Participants were divided into two groups: controls and those with proteinuria.

- **Control group-** 40 health care workers and medical students with no apparent background medical history.

  Normal urinalysis was present in all controls. 60% of the control group was females and 40% males. The age group ranged between 20 to 40 years.

- **Proteinuria group**- comprised 37 patients attending the renal and HIV clinics with proteinuria on dipsticks and abnormal renal function (urea > 7.5 creatinine > 100 mmol/L). 51% of proteinuric group were females and 49% males.

3.2 Aetiology of chronic kidney disease

HIV associated nephropathy (HIVAN) n=22 (59%)

Focal segmental glomerulosclerosis (FSGS) n=10 (27%)

Membranoproliferative glomerulonephritis n=5 (14%)

3.3 Levels of Cathepsin L and Dynamin in patients with proteinuric renal disease compared to normal control

In the kidney disease group, the mean value for Cathepsin L in serum was 5.39±4.31 pg/ml while in the control group was 8.17±13.41 pg/ml showing no statistical significance. For

Dynamin the mean was 396.21±678.15 pg/ml for the kidney disease group while in the control group mean 206.66±174.24 pg/ml. The mean value of Cathepsin L in the urine of disease group was 10.44±11.47pg/ml while that of control group mean was 2.91±2.88 pg/ml with P-value of 0.000….hence statistically significant. The mean value for Dynamin in urine for the kidney disease group was 375.33±338.63pg/ml and for the control group 523.41±492.49pg/ml.

The results are shown in Table 1 below.

Table 1: Comparison of the levels of Cathepsin L and Dynamin in patients with proteinuric renal disease to those of normal controls

	Kidney Disease (n=37)		**Controls (n=40)**		**P-Value**
	Mean (pg/ml)	**Standard Deviation (pg/ml)**	**Mean (pg/ml)**	**Standard Deviation (pg/ml)**	
Dynamin (Serum)	396.2061	678.1511	206.6587	174.2392	0.107
Dynamin (Urine)	375.3332	338.6343	523.4087	492.4917	0.131
Cathepsin L (Serum)	5.3986	4.3180	8.1734	13.4144	0.233
Cathepsin L(Urine)	10.4364	11.4709	2.9086	2.8773	**0.000**

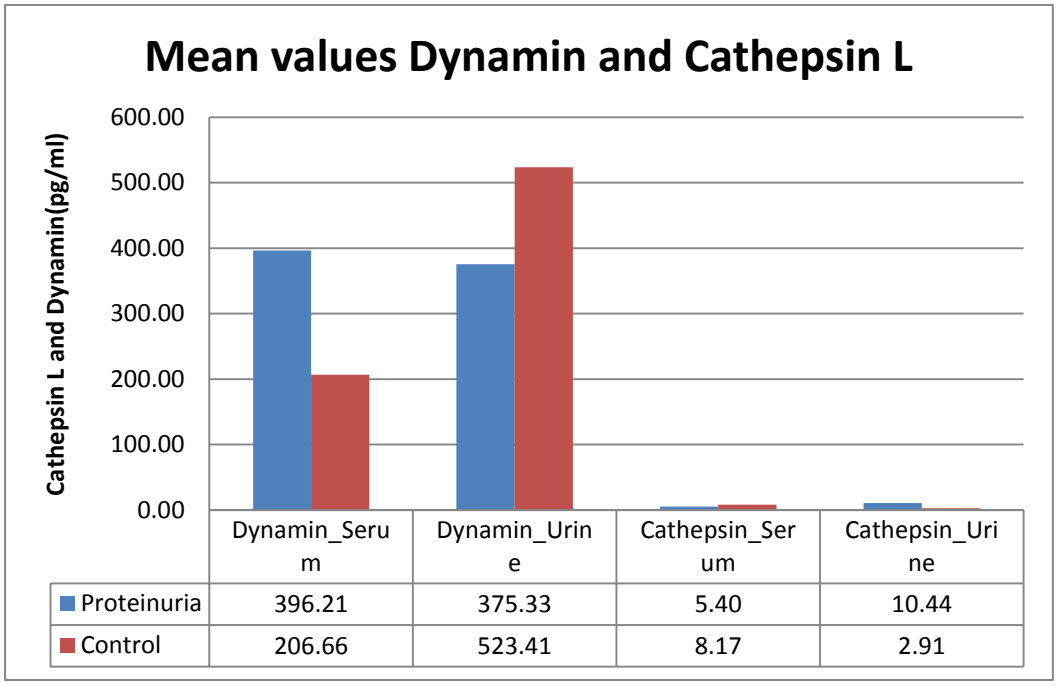

Fig 6: Dynamin and Cathepsin L in serum and urine of disease vs. control group

It is noted that there are no significant differences in the levels of Dynamin in serum and urine of patients with proteinuric renal disease and healthy controls (p-values 0.107 and 0.131 respectively).

The spread of the analyte measurements are shown in the boxplots (Figs 7-10) below.

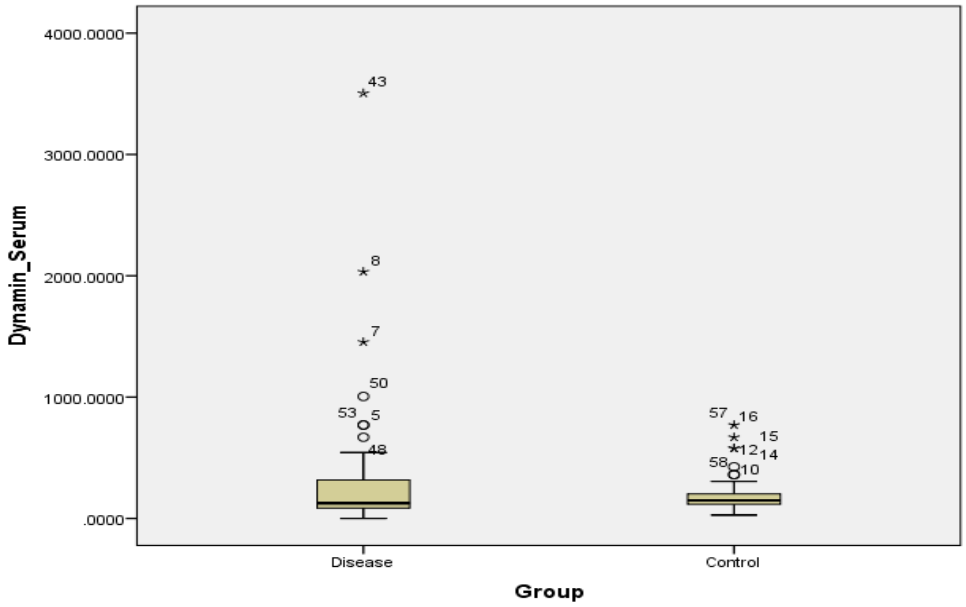

Fig 7: Box plot showing the spread of Dynamin concentration (pg/ml) in serum of the kidney disease group vs the control group

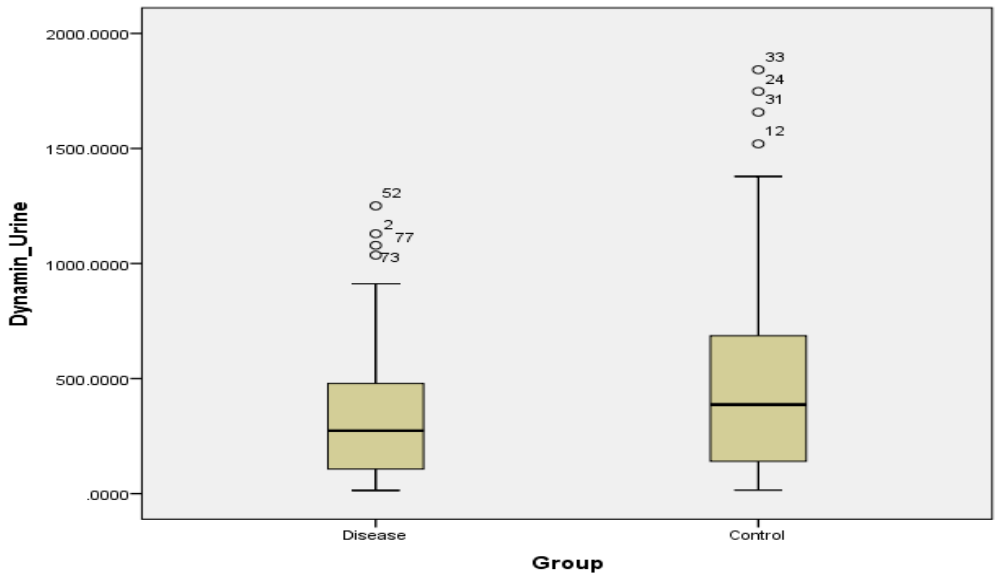

Fig 8: Box plot showing spread of Dynamin levels (pg/ml) in the urine of kidney disease group vs the control group

The results also revealed that there is no significant difference in the level of serum Cathepsin L between the kidney disease and the control groups, the p-value of the independent samples t-test was 0.233.

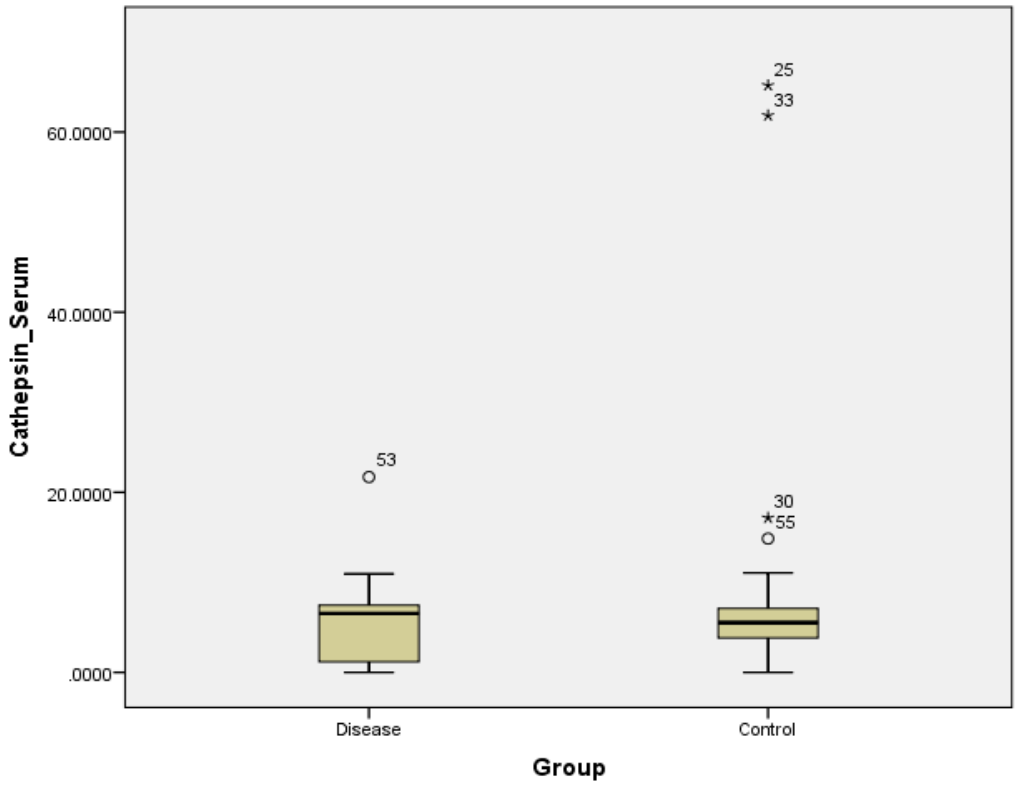

Fig 9: Box plot showing spread of Cathepsin L (pg/ml) in the serum of kidney disease vs control group

The results showed that there is a significant increase in the level of urine Cathepsin L between the disease group (mean = 10.4364) and the control group (mean = 2.9086); p-value= 0.000. The differences are illustrated graphically below;

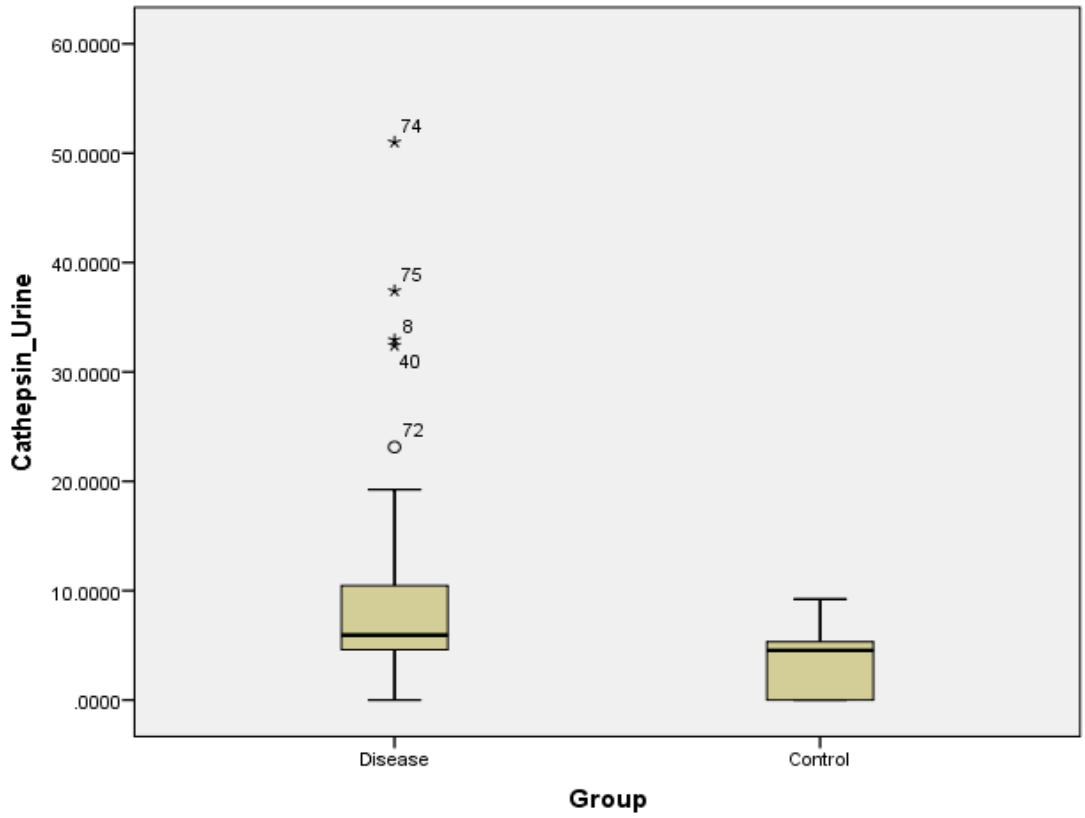

Fig 10: Box plot showing spread of Cathepsin L levels (pg/ml) in the urine of the kidney disease vs control group

3.4 Correlation between Cathepsin L and Dynamin with the degree of proteinuria

Table 2: Correlation between Cathepsin L and Dynamin with the degree of proteinuria

	n	Pearson Correlation with proteinuria	P-value
Cathepsin (Serum)	37	-0.222	0.186
Cathepsin (Urine)	37	-0.070	0.682
Dynamin (Serum)	37	0.116	0.493
Dynamin (Urine)	37	-0.036	0.834

Although serum Cathepsin L (r = -0.222, p-value = 0.186), Urine Cathepsin L (r = -0.070, p-value = 0.682), p-value = 0.665), and urine Dynamin (r = -0.036, p-value = 0.834) are negatively related to the degree of proteinuria, the correlation is not significant, all the p-values were greater than 0.05; table 2.

Serum Dynamin (r = 0.116, p-value = 0.493) had a positive correlation to the degree of proteinuria but the correlation was not significant at 5% significance level (the p-value was greater than 0.05). Thus, there is no correlation between Cathepsin L and Dynamin levels with the degree of proteinuria.

3.5 Association between the level of proteinuria and patient's gender

Table 3: Comparison of the level of proteinuria between male and female patients

	Female (n=19)		Male (n=18)		P-Value
	Mean	Standard Deviation	Mean	Standard Deviation	
PCR(g/mmol)	0.9642	0.2443	0.4498	0.230	0.000

PCR-protein creatinine ratio

The results revealed that the level of proteinuria (as measured by urine PCR) is significantly higher among female patients (mean value = 0.9642) compared to a mean of 0.4498 among male patients; table 3. The means are significantly different since the p-value of the independent samples t-test was 0.000. The differences are also shown graphically in the box plot below (fig 11);

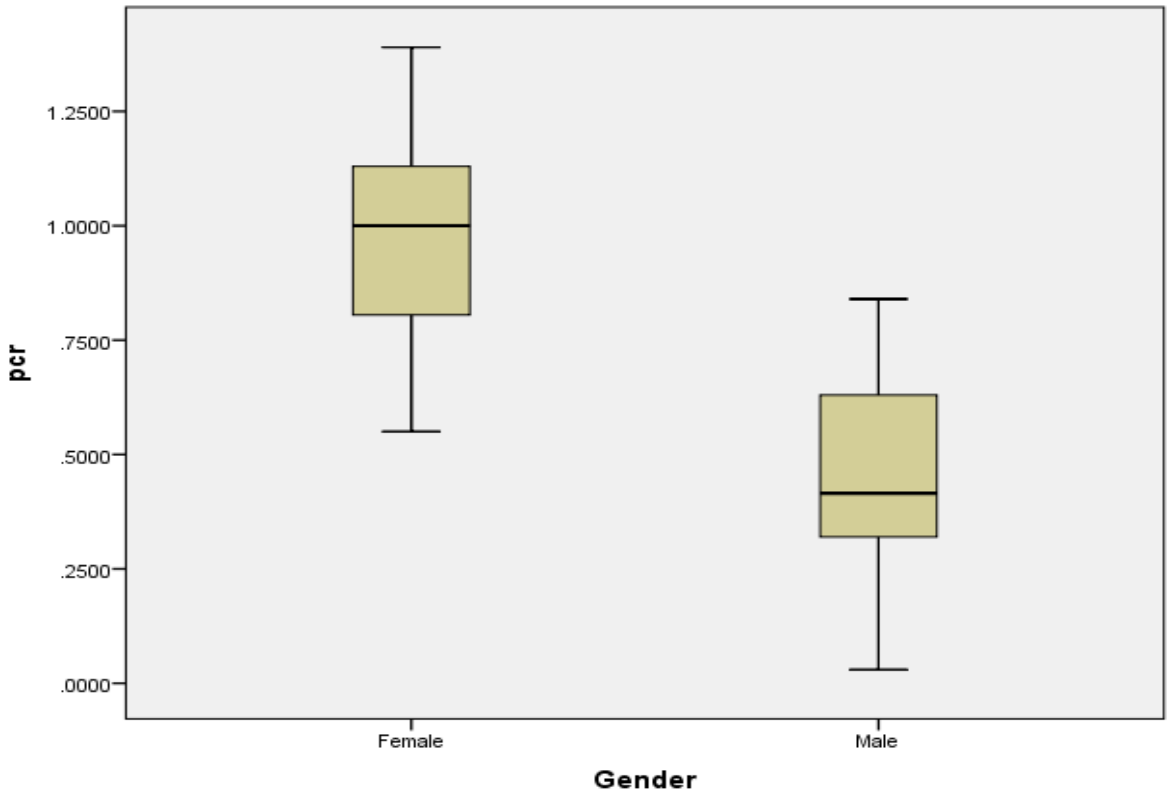

Fig 11: Box plot comparing proteinuria (g/mmol) in females vs. males

It can be clearly seen that the levels of proteinuria is much higher among female patients. This, however is not in keeping with literature which postulates that the same reason that men are at risk for cardiovascular disease, also predispose them to risk of renal disease. However, our results could be due to sampling strategy.

Chapter 4-Discussion

The aim of this study was to determine whether Cathepsin L and Dynamin could be used as biomarkers of proteinuric renal disease. We also wished to determine if the levels of Cathepsin L and Dynamin correlated with the degree of proteinuria. Dynamin and Cathepsin L have been proposed to be promising biomarkers for proteinuric kidney disease [Sever, 2007]. Some biomarkers have been reviewed and have shown promising results but further validation is required. Literature shows podocyte loss as a key determinant of the progression of CKD and this is attributed to one of their specialised proteins, Dynamin – mechano-chemical scaffolding that can hydrolyze GTP to constrict and deform biological membranes and recruit many different signaling, cytoskeletal and membrane coat proteins [Praefcke et al, 2004]. Cathepsin L is responsible for podocyte foot process effacement by cleaving Dynamin in the cytoplasm. This results in reorganization of the podocyte actin cytoskeleton and subsequent proteinuria; this does imply that Dynamin is essential for podocyte morphology in healthy kidneys.

The key finding in our study was that levels of Cathepsin L were elevated in the urine of the proteinuric group compared to the control group(p-value =0.00).This finding is similar to that of Sever, which showed that hydrolysis of Dynamin by a cytoplasmic Cathepsin L leads to kidney podocyte effacement and subsequently, proteinuria in the mouse models. Increased levels of glomerular Cathepsin L mRNA in microdissected glomeruli in some human proteinuric diseases were observed [Sever, 2007]. In another study, using the Cathepsin L inhibitor E-64, in a rat glomerulonephritis model, there was a reduction of proteinuria. This was attributed to inhibition of secreted Cathepsin L during matrix remodelling [Mundel, 2010]. The clinical relevance of this report is that Cathepsin L

expression is increased in proteinuric kidney disease. This, also, supports the concept that Cathepsin L plays an essential role in the occurrence of different forms of proteinuria [Reiser, 2010]. In other studies, Cathepsin S was found in the proximal tubular cells and podocytes of proteinuric failing kidneys [Huang, 2012]. Cathepsin B and L activity was found to be decreased in the tubular cells of the kidneys from patients with early stage diabetic and hypertensive nephropathy [Huang, 2012], suggesting that the role of Cathepsins in various kidney diseases may differ among the various Cathepsin isoforms and stages of CKD [Huang, 2012].

While the spectrum of function for Cathepsin L is very broad, two of its substrates Dynamin and Synaptopodin have been delineated in podocytes [Mundel, 2010]. They are both essential for the functional F-actin structure in normal podocyte FPs. Enzymatic processing of these substrates by Cathepsin L will lead to FP effacement [Mundel, 2010]. Chandel et al. 2013 reported that podocyte Cathepsin L expression diminished Dynamin expression. Therefore, one can hypothesise that lack of Cathepsin L would increase podocyte Dynamin expression. Also, genetic and histological studies have shown that Dynamin reduction was Cathepsin L-dependant, suggesting that the cytoplasmic Cathepsin L targets Dynamin [Sever, 2007]. However, the results of our study did not show any significant decrease in the levels of Dynamin in the proteinuric group compared with the control group.

With Dynamin being Cathepsin L-dependant, we expected levels of Dynamin to be reduced in the proteinuric group. However this was not observed and whether this was due to the fact that Dynamin is ubiquitously expressed requires further studies. Cytoplasmic Cathepsin L seems to be specific, and its substrate is GTP-bound Dynamin. This implies that only a

portion of Dynamin can be hydrolysed *in vivo* [Sever, 2007]. Sever further showed that "*Cathepsin L reduced endogenous Dynamin by only 30%, possibly maintaining Dynamins role in endocytosis*." In another study Soda et al., argued that Dynamin complete destruction is not necessarily required for kidney failure.

Dynamin levels were expected to be higher in the control group than the proteinuric group; as stated earlier, Dynamin plays a vital role in the scaffolding of the podocyte slit diaphragm. Similarly, Cathepsin L levels were expected to be elevated in the proteinuric group. As has been postulated in the literature, Cathepsin L has a role in the development of proteinuria. In support of this, further studies suggested that the antiproteinuric activity of cyclosporine A results from direct protection of the podocyte cytoskeleton from Cathepsin L mediated proteolysis [Soda, 2012]. This explains how Cyclosporine A induces remission of proteinuria in some proteinuric diseases. In our study, the significant finding was elevated Cathepsin L levels in the urine of the proteinuric group. However, there was no significant increase in the serum levels of Cathepsin L, suggesting the possibility of a local paracrine effect of Cathepsin L in the kidney.

In our data, too many outliers were observed in both the control group and kidney disease group (Figs.7-10). This may have impacted on our results, resulting in lack of statistical significance; we therefore suggest an optimal screening of the control group. In a study by Bauer et al., Cathepsin L was revealed as a potential sex-specific biomarker. Using rat models, they reported urinary Cathepsin L to be more elevated in male rats with proteinuria compared to females. Our study showed a similar trend; however it was not statistically significant. However, from our data, males were not exactly matched to females in terms of

numbers. Interestingly there were more females (51%) than males, yet Cathepsin L was found to be elevated in males. Further studies are needed to confirm whether Cathepsin L can be used as a gender specific biomarker. In one study by Verhagen et al., they proposed that men were at greater risk for renal injury as oestrogens had protective effects and androgens predisposed to proteinuria. The exact mechanism is unknown, however the vasodilatory effects of oestrogens were postulated [Verhagen and Marjan 2000].

If Cathepsin L is implicated in proteinuric kidney disease, one may expect a correlation between the levels of Cathepsin L and the amount of proteinuria; however, this was not the case in our study. There was no correlation between either Cathepsin L or Dynamin with the degree of proteinuria. For many years, proteinuria has been used as a diagnostic and monitoring tool for kidney diseases. But, as mentioned earlier, it relies on the functional properties of the kidneys, and hence becomes abnormal at a very late stage. It is therefore not a very specific biomarker as it has to be interpreted in the context of the patient's body habitus as it relies on muscle mass, neurologic conditions and the clinical picture as a whole [Fukuda 2011]. Proteinuria remains a valuable tool for monitoring kidney injury and the response to treatment within a disease spectrum. Podocyte loss is a key determinant of the progression of kidney disease [Fukuda 2011].This raises the possibility that measuring podocyte products in urine could be a powerful tool to aid clinical decision-making, analogous to troponin in cardiac injury [Fukuda 2011]. Podocyte products in urine (including podocyte-specific proteins or mRNAs carried by cells, exosomes, or urosomes) in combination with proteinuria could provide additional information that would improve clinical decision-making [Fukuda 2011].

Limitations

The patients in the two groups were not ideally matched. Therefore with further studies, ideal matching of patients e.g. age groups and ethnicity is recommended.

Screening of the control group was also not ideal. With further studies, the control group should be fully screened; e.g. the control group could be living renal transplant donors who have been shown to have normal renal function prior to uninephrectomy.

Chapter 5 - Conclusion

With CKD, being a significant health problem due to its morbidity and mortality, it has become quite clear that we need better biomarkers that can identify the problem early in disease progression. Different studies have shown that serum creatinine, GFR, and proteinuria are insensitive as they reflect late functional changes, and not early structural alterations in the kidney that would identify subtle damage. Therefore reliance on these may result in an extensive time lapse where successful interventions could be applied.

In our study we showed increased levels of Cathepsin L in the urine of participants with proteinuric kidney disease compared to the control group. This is in agreement with earlier studies done, which showed that Dynamin reduction was Cathepsin L- dependent suggesting that cytoplasmic Cathepsin L targets Dynamin.This raises the possibility that the presence, or level of podocyte products in the urine and serum could be useful as a biomarker for CKD. Hence Cathepsin L is a possible biomarker for proteinuric kidney disease. However, further review and deeper understanding is still needed as both histologic and genetic studies implicate podocytes as targets for CKD therapy.

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Appendix1

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Mpoti Seboka

CLEARANCE CERTIFICATE	**M120967**
PROJECT	Cathepsin I and Dynamin-Biomakers of Proteinuric Kidney Disease
INVESTIGATORS	Dr Mpoti Seboka.
DEPARTMENT	Department of Internal Medicine
DATE CONSIDERED	28/09/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 02/11/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof S Naicker

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 2

INFORMATION LEAFLET AND INFORMED CONSENT

STUDENT NUMBER: 0102409Y

TITLE OF RESEARCH: **CATHEPSIN L AND DYNAMIN- BIOMAKERS OF PROTEINURIC RENAL DISEASE?**

INVESTIGATOR: Dr MPOTI SEBOKA

INTERNAL MEDICINE REGISTRAR

UNIVERSITY OF WITSWATERSRAND

CONTACT DETAILS email mpseboka@yahoo.com

Cell 0828166508

Hi, I am Dr Mpoti Seboka a registrar in the department of internal medicine. I am currently doing a research for my MMED (Masters of Medicine). In this research I will be measuring enzymes Cathepsin L and Dynamin in urine samples and from blood serum, comparing their levels in patients with kidney disease and those without kidney disease (controls).

Kidney disease is a worldwide problem and very expensive to treat. A sign of kidney disease is passing protein in urine due to damaged kidney cells. Recent studies suggest that Cathepsin L and Dynamin are involved in the process of losing protein in urine. Hence these enzymes can be target for medication that treats the protein loss, and can help in reducing this worldwide problem.

You are invited to consider taking part in this research study. Your participation is entirely voluntary. If you agree to participate in the study, I will first ask you questions concerning your health, such as whether you have any diseases like hypertension, diabetes or whether you are on any other treatment. If you agree, a medical examination will be done on you and your medical records reviewed. Volunteers with no medical records, about 5mls of blood will be taken from their forearms, this can inflict minimal discomfort or pain and there is minimal risk of bleeding from the side. You will also be asked to give a urine sample which will be examined for proteins and for the enzymes Cathepsin L and Dynamin. Refusal to participate will involve no penalty or lose of benefits which you are otherwise entitled to.

Your participation in this study will contribute to medical knowledge that may help patients who have chronic kidney disease. You will not be paid to participate in this study. Funds will be sought for payment of all study procedures and expenses that you may incur as a direct result of this study.

The clinical study protocol will be submitted to the University of Witwatersrand Human Research Ethics Committee for approval. If at any point require information regarding your rights as a research participant you are free to contact the ethics committee. Please be informed you are free to ask me any questions for your clarification.

Thanking you in advance for participating in this study.

Dr Mpoti Seboka

Appendix 3

Cathepsin L levels

Micro well strips were washed with a wash buffer and not allowed to dry.

100µl of sample diluent were added in duplicate to the blank wells and 50µl to the sample wells.

50µl of each sample was added in duplicate to the sample wells and 50µl of Biotin-Conjugate was added to each well.

Samples were covered with adhesive film and incubated at room temperature for 2 hours.

Micro wells were then washed and 100µl of Streptavidin-HRP was added to each well including the blank wells.

Micro wells were then covered with adhesive film and incubated at room temperature for 1 hour.

After washing 3 times 100µl of TMB substrate solution was added to each well and incubated at room temperature for 10 minutes.

The stop solution added and absorbance of each micro well read at 450nm as primary wavelength.

Appendix 4

INFORMED CONSENT:

- I hereby confirm that Dr Seboka has informed me about the nature, conduct, benefits and risks of this clinical study.
- I have received, read and understood the participant information leaflet regarding the clinical study.
- I am aware that the results of the study including personal demographics will be anonymously processed into a study report.
- I am aware that at any stage without prejudice I may withdraw my consent to participate in the study.
- I have had sufficient opportunity to ask questions and therefore declare that I am prepared to in the study.

PARTICIPANT:

Printed Name Signature / Mark or Thumbprint Date and Time

I, Dr Seboka herewith confirm that the above participant had been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR:

Dr Mpoti Seboka

Printed Name	Signature / Mark or Thumbprint	Date and Time

WITNESS 1:

Printed Name	Signature	Date and Time

WITNESS 2:

Printed Name	Signature	Date and Time

CATHESPIN L AND DYNAMIN - BIOMARKERS IN PROTEINURIC KIDNEY DISEASE?		
Study Number:		
Investigator: Dr Mpoti Seboka		
Participant Number:		
Group:		
Enrolment Date:		
	Date Done	**Results**
Age		
Sex		
Race		
Blood Pressure		
Weight		
Height		
BMI		
HIV		
HBV		
HCV		

TB		
other infection		
other illness		
Urea		
Creatinine		
Estimated GFR		
Proteinuria		
Protein: Creatinine Ratio		
ARV's		
ACEI		
ARB		
Steroids		

Data Sheet for control and participants with proteinuric kidney disease.

Sa+A1: K68mple	Dynamin_ Serum pg/ml	Dynamin_ Urine pg/ml	Cathepsin _Serumpg /ml	Cathepsin_ Urine pg/ml	Group	Sample type	race	gender	age	pcr
Sample 01	15,54	440,96	6,79	7,71	1	Disease		f		
Sample 02	20,94	47,62	7,34	5,82	1	Disease		m		0,84
Sample 03	5,76	248,69	6,55	19,25	1	Disease		m		0,65
Sample 04	60,20	152,75	6,98	5,69	1	Disease		m		0,43
Sample 05	60,20	1129,26	7,34	6,31	1	Disease		f		1,26
Sample 06	213,69	180,80	6,85	5,94	1	Disease		f		0,55
Sample 07	27,79	88,39	8,02	10,46	1	Disease		f		1,09
Sample 08	145,08	440,96	5,88	4,72	1	Disease		m		0,27
Sample 09	668,99	384,97	5,39	16,38	1	Disease		m		0,25
Sample 10	95,24	180,80	5,33	4,47	1	Disease		m		0,84
Sample 11	1005,63	645,51	9,60	4,60	1	Disease		m		
Sample 12	769,95	106,84	7,47	5,57	1	Disease		f		
Sample 13	118,08	570,88	8,75	7,83	1	Disease		f		
Sample 14	27,79	152,75	6,98	5,94	1	Disease		m		
Sample 15	118,08	1250,51	6,85	5,76	1	Disease		m		
Sample 16	769,95	72,54	21,69	5,08	1	Disease		m		
Sample 17	1454,10	13,89	7,59	17,36	1	Disease		f		
Sample 18	305,50	23,54	6,24	7,10	1	Disease		m		0,03
Sample 19	2033,24	248,69	7,77	32,93	1	Disease		f		
Sample 20	95,24	645,51	7,47	5,08	1	Disease		f		0,77
Sample 21	361,60	502,81	5,94	4,66	2	Control		f		
Sample 22	176,77	384,97	7,04	4,45	2	Control		f		
Sample 23	578,37	1520,11	6,55	5,82	2	Control		f		
Sample 24	305,50	128,20	14,85	4,78	2	Control		m		
Sample 25	145,08	59,04	5,27	4,66	2	Control		m		
Sample 26	118,08	384,97	7,16	4,78	2	Control		f		

Sample 27	769,95	152,75	6,37	5,02	2	Control		m		
Sample 28	361,60	128,20	5,21	5,94	2	Control		m		
Sample 29	425,34	334,50	5,39	7,83	2	Control		f		
Sample 30	578,37	502,81	6,24	4,66	2	Control		f		
Sample 31	213,69	128,20	7,71	5,39	2	Control		m		
Sample 32	668,99	180,80	4,84	4,72	2	Control		f		
Sample 33	95,24	645,51	4,60	5,02	2	Control		f		
Sample 34	47,07	289,18	6,24	5,51	2	Control		m		
Sample 35	118,08	180,80	8,50	4,72	2	Control		f		
Sample 36	27,79	289,18	5,14	5,82	2	Control		f		
Sample 37	76,09	440,96	5,45	5,45	2	Control		f		
Sample 38	27,79	570,88	5,33	5,27	2	Control		f		
Sample 39	118,08	645,51	5,33	6,31	2	Control		f		
Sample 40	76,09	727,05	6,61	5,39	2	Control		f		
Sample 41	140,07	791,05	0,00	0,00	2	Control		m		
Sample 42	91,94	164,15	0,00	0,00	2	Control		m		
Sample 43	117,64	1747,98	7,52	0,00	2	Control		f		
Sample 44	181,81	388,51	65,19	0,00	2	Control		f		
Sample 45	153,56	471,20	0,00	9,22	2	Control		m		
Sample 46	117,09	213,33	0,00	0,00	2	Control		f		
Sample 47	123,78	892,18	6,39	0,96	2	Control		m		
Sample 48	51,67	565,38	3,07	0,00	2	Control		f		
Sample 49	188,75	114,86	7,25	0,00	2	Control		f		
Sample 50	146,74	798,06	5,61	0,00	2	Control		m		
Sample 51	265,39	537,76	6,83	0,00	2	Control		f		
Sample 52	184,57	109,33	0,00	0,00	2	Control		m		
Sample 53	152,31	71,44	0,00	0,00	2	Control		m		
Sample 54	147,97	36,31	17,16	0,00	2	Control		f		
Sample 55	143,08	1657,62	11,04	0,00	2	Control		f		
Sample	85,56	867,89	0,00	0,00	2	Control		m		

56										
Sample 57	190,16	15,35	5,27	0,00	2	Control		m		
Sample 58	167,69	1378,66	0,00	0,00	2	Control		f		
Sample 59	148,59	1842,36	61,84	0,00	2	Control		f		
Sample 60	178,39	76,49	0,00	0,00	2	Control		m		
Sample 61	88,48	223,91	0,00	0,00	1	Disease		m		0,37
Sample 62	212,76	16,13	0,00	9,90	1	Disease		f		1,12
Sample 63	52,90	77,17	10,93	9,96	1	Disease		f		0,63
Sample 64	0,20	323,20	0,00	23,13	1	Disease		m		0,38
Sample 65	275,96	582,15	0,00	0,00	1	Disease		f		1,14
Sample 66	245,90	1078,95	0,00	7,08	1	Disease		m		0,49
Sample 67	201,63	446,58	5,10	9,66	1	Disease		f		0,71
Sample 68	101,07	328,97	1,17	0,00	1	Disease		f		0,58
Sample 69	122,09	348,62	3,54	3,59	1	Disease		f		1,39
Sample 70	516,09	478,52	0,00	51,01	1	Disease		m		0,4
Sample 71	85,56	453,47	6,69	37,42	1	Disease		m		0,63
Sample 72	126,05	74,12	5,10	32,42	1	Disease		f		0,84
Sample 73	139,48	46,23	0,00	2,59	1	Disease		f		1,24
Sample 74	116,54	273,90	0,00	0,00	1	Disease		f		0,84
Sample 75	3504,21	161,26	0,00	12,11	1	Disease		f		1,04
Sample 76	316,30	911,74	7,79	0,00	1	Disease		m		0,56
Sample 77	543,41	1036,55	2,55	3,28	1	Disease		M		0,44

Theresa Kaiser Sep 14 (3 days ago)

to me

Dr. Seboka,

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Pierre Ronco

Published August 1, 2007

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Sincerely,
Theresa Kaiser

If you have any questions, please feel free to contact us.

Sincerely,

Theresa Kaiser

Prof. Dr. Tobias Huber Sep 12 (5 days ago)

to me

Sure, no problem.

However, you should cite the source.

Best,

tobias

Theresa Kaiser <staff@the-jci.org> Jun 8

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Theresa Kaiser