

**UNIVERSIDADE DE LISBOA**

**FACULDADE DE MEDICINA**



**Strategies for early detection of renal injury in HIV-infected  
patients: the new troponin**

Sara Sofia dos Santos Brilha

**Mestrado em Doenças Infecciosas Emergentes**

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The present dissertation was approved by the Scientific Committee of the Faculdade de Medicina da Universidade de Lisboa on the 15<sup>th</sup> of March, 2011.

*“(...) All our science, measured against reality, is primitive and childlike and yet it is the most precious thing we have.”*

*Albert Einstein (1879-1955)*

## Abstract

**Background:** HIV-infected patients have a known increased risk of kidney disease. For that reason, a biomarker that enables reliable detection of early and mild kidney dysfunction would be advantageous. Cystatin C is considered to be a better marker of kidney function than creatinine.

**Objectives:** This study aimed to evaluate if serum cystatin C was a better marker than creatinine in a HIV-infected population.

**Material and Methods:** This was an observational study of HIV-infected patients that attend the Infectious Diseases Department, at the Hospital Santa Maria, Lisbon. Patients with known kidney disease or HIV-2 infection were excluded. Clinical and demographic data was recorded and serum creatinine and cystatin C levels were determined.

**Results:** A total of 242 patients were included. Cystatin C is an independent factor of age, gender, ethnicity and body mass index. Of the 17 patients with elevated levels of cystatin C but with an estimated creatinine clearance within normal range, 11 were on antiretroviral therapy with tenofovir and/or atazanavir. Fourteen patients were smokers and 10 patients had hepatitis C virus co-infection, which are known causes of inflammation.

In a matched control group of 27 patients, with cystatin C within normal range, the majority of patients also were on an antiretroviral regimen with tenofovir and/or atazanavir. However, none of them had hepatitis C virus co-infection. Moreover, a

statistical association was found between high levels of cystatin C and alanine transaminase.

Differences between biomarkers' levels and patients on combinations with tenofovir, ritonavir boosted atazanavir or both were also studied. Although it was not found any statistically significant difference in creatinine and estimated creatinine clearance, with cystatin C, patients that were on atazanavir or tenofovir and had a higher level of cystatin C, compared with patients that never were on these drugs.

**Conclusion:** Cystatin C levels may be increased in inflammatory conditions. Therefore, in HIV-infected patients, where chronic systemic inflammation is present, often associated to other inflammation sources, such as chronic hepatitis C virus co-infection, the use of cystatin C to monitor kidney function may overestimate kidney impairment.

In what concerns to nephrotoxicity, it was found that patients on atazanavir had higher levels of cystatin C. It is important to develop prospective studies, in order to assess the real long-time impact of the more recent antiretroviral drugs on kidney function.

**Keywords:**

cART, creatinine, cystatin C, HIV, kidney injury

## Resumo

**Introdução:** Os doentes infectados por VIH possuem um risco aumentado para o desenvolvimento de doença renal. Por esta razão, seria importante utilizar um marcador que permitisse uma detecção precoce de alterações da função renal. A cistatina C tem sido referida na literatura como um melhor marcador da função renal, comparativamente com a creatinina.

**Objectivos:** O objectivo deste estudo é analisar se a cistatina C sérica constitui um melhor marcador da função renal que a creatinina, para a população de doentes infectados por VIH.

**Material e métodos:** Este é um estudo observacional de doentes infectados por VIH, seguidos no Serviço de Doenças Infecciosas do Hospital de Santa Maria, Lisboa. Os doentes com doença renal documentada e infecção por VIH-2 foram excluídos deste estudo.

Foram recolhidos dados clínicos e demográficos e foram determinados os níveis séricos de creatinina e cistatina C.

**Resultados:** Um total de 242 doentes foi incluído neste estudo. Os níveis de cistatina C mostraram-se independentes da idade, sexo, etnia e índice de massa corporal. Dos 17 doentes com níveis elevados de cistatina C mas com *clearance* da creatinina dentro dos valores de referência, 11 doentes estavam sob terapêutica antirretroviral com tenofovir e/ou atazanavir potenciado com ritonavir. Catorze doentes eram fumadores e 10 estavam co-infectados por vírus da hepatite C, que constitui um factor conhecido de inflamação. Num grupo controlo de 27 doentes,

com cistatina C dentro dos valores normais, a maioria dos doentes também se encontrava sob terapêutica antirretroviral com tenofovir e/ou atazanavir. No entanto, nenhum possuía co-infecção por vírus da hepatite C.

Foram também analisadas diferenças entre os níveis dos marcadores e doentes em combinações com tenofovir, atazanavir potenciado com ritonavir ou com ambos. Embora não tenha sido encontrada nenhuma diferença estatisticamente significativa nos níveis de creatinina e *clearance* da creatinina estimado, com a cistatina C, doentes sob atazanavir ou tenofovir e atazanavir possuíam níveis mais elevados deste marcador, comparativamente com os doentes que nunca estiveram sob estes antirretrovirais.

**Conclusão:** Os níveis de cistatina C podem aumentar com factores inflamatórios. Por esta razão, no caso dos doentes infectados por VIH, onde existe uma inflamação sistémica crónica, muitas vezes associada a outras fontes de inflamação, como a co-infecção por vírus da hepatite C, a utilização exclusiva da cistatina C para a monitorização da função renal pode conduzir a uma sobrestimação de uma lesão renal.

Em termos de nefrotoxicidade, verificou-se que doentes sob atazanavir possuíam níveis mais elevados de cistatina C. É de extrema importância, o desenvolvimento de novos estudos prospectivos, de forma a permitir a análise do impacto a longo prazo destes antirretrovirais mais recentes na função renal.

**Palavras-chave:**

Cistatina C, creatinina, lesão renal, TARVc, VIH



## Acknowledgments

I dedicate the present dissertation to my grandparents, who I will always carry in my heart. Thank you for everything you taught me.

A warm thanks to my beloved family and dearest friends. Thank you for the everlasting support and understanding.

Here I express my deepest gratitude to my mother, who is my reference of strength and conduct. Thank you for always being present and, more than anybody, for always believing in me.

I wish to express my sincere gratitude to my supervisor, Professor Emília Valadas, for all the support and guidance, all the given opportunities and also for the important life lessons during these almost two years at the Laboratório de Diagnóstico Molecular de Doenças Infecciosas, Faculdade de Medicina de Lisboa.

A kind thanks to Professor Thomas Häscheid, from whom I have also learned so much, and helped and guided me during my last year of the undergraduate degree. If I am now so passionate about research in infectious diseases, it is also due to his mentoring.

I would also want to give a special thank to Professor Francisco Antunes, Director of the Infectious Diseases Department, Hospital Santa Maria, for consenting with the realization of this study.

A sincere thanks to Dr. Robert Badura, Nurse Bárbara Silva, Ana Ferrão and Sandra Sousa, from the Clínica Universitária de Doenças Infecciosas e Parasitárias. Without their help and support this work would not be possible.

I also want to thank Dr. Maria João and Dr. António Santos from the Instituto de Formação Avançada, for always being available to help me during this time as a student of the Master in Emergent Infectious Diseases.

Finally I would like to thank ViiV Healthcare for the financial support.

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## **Abbreviations List**

**ACE-** Angiotensin-converting enzyme

**ADQI -** Acute Dialysis and Quality Initiative

**AIDS-** Acquired immunodeficiency syndrome

**ALT-** Alanin transaminase

**AKI-** Acute kidney injury

**AKIN-** Acute Kidney Injury Network

**AKF-** Acute kidney failure

**ART-** Antiretroviral therapy

**ATN-** Acute tubular necrosis

**ATIN-** Acute tubule interstitial nephritis

**ATV/r –** Atazanavir boosted with ritonavir

**cART-** Combination antiretroviral therapy

**CHLN-** Centro Hospitalar Lisboa Norte

**Cr –** Creatinine

**CrCl-** Creatinine clearance

**Cyst C–** Cystatin C

**CKD-** Chronic kidney disease

**CSF-** Cefalorraquidian fluid

**eCrCl –** Estimated creatinine clearance

**eGFR-** Estimated glomerular filtration rate

**ELISA-** Enzyme-linked immunosorbent assay

**ESKD-** End-stage kidney disease

**FABPs** - Fatty acid binding proteins

**GFR**- Glomerular filtration rate

**GN**- Glomerulonephritis

**HBV**- Hepatitis B virus

**HCV**- Hepatitis C virus

**H-FABP**- Heart fatty acid binding protein

**HIV**- Human immunodeficiency virus

**HIVICK**- Human immunodeficiency virus immune complex-mediated kidney disease

**HSM**- Hospital Santa Maria

**HUS**- Haemolytic uremic syndrome

**ICU** – Intensive Care Unit

**IDV**- Indinavir

**IgA**- Immunoglobulin A

**IL-8**– Interleukin 8

**IQR**- Inter-quartile range

**KIM-1**- Kidney injury molecule 1

**L-FABP**- Liver fatty acid binding protein

**MDRD**- Modified Diet in Renal Disease

**NGAL**- Neutrophil gelatinase-associated lipocalin

**NNRTI**- Non nucleoside reverse transcriptase inhibitor

**NRTI**- Nucleoside reverse transcriptase inhibitors

**NSAID**- Nonsteroidal anti-inflammatory drug

**PIs**- Protease inhibitors

**RBCs**- Red blood cells

**RIFLE**– Risk, injury, fail, lost, end-stage kidney disease classification

**RTV**- Ritonavir

**SD**- Standard deviation

**sNGAL**- Serum neutrophil gelatinase-associated lipocalin

**TDF**- Tenofovir disoproxil fumarate

**uNGAL**- Urinary neutrophil gelatinase-associated lipocalin

**UO**- Urinary output

**w/o** - Without

**Introduction**

**Objectives**

**Material and Methods**

**Results**

**Discussion**

**Concluding remarks**

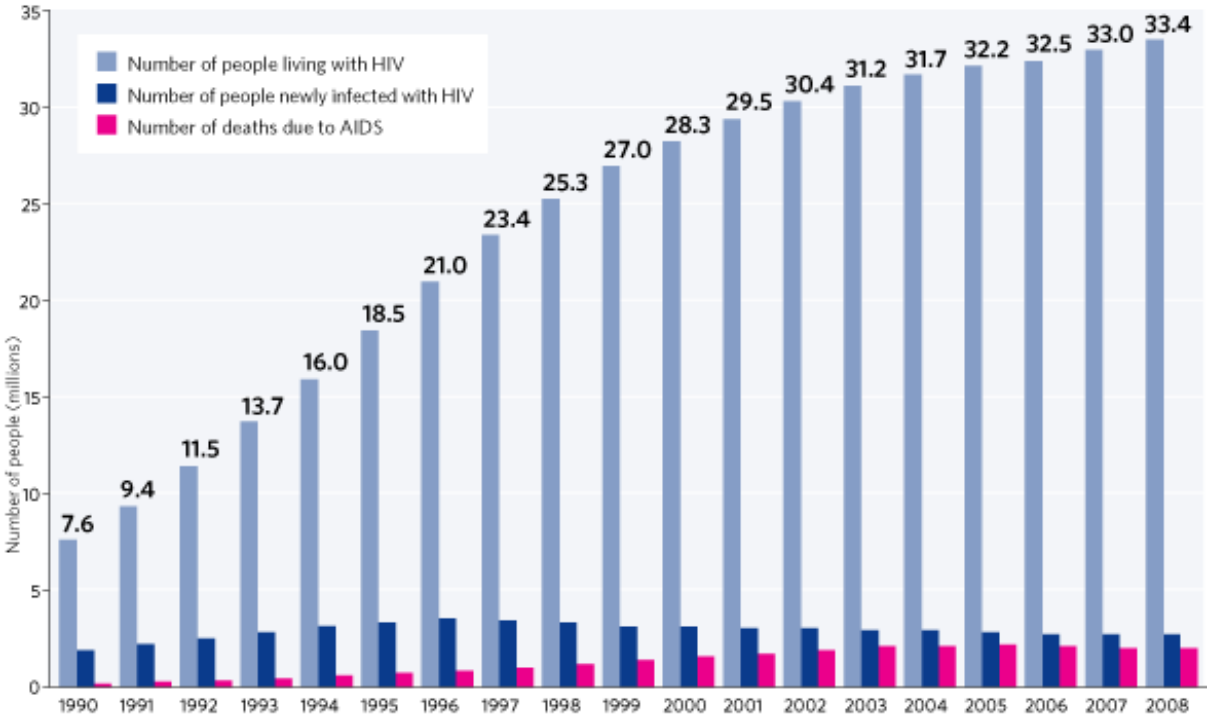
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# Introduction

## 1. Epidemiology of HIV infection

Human immunodeficiency virus (HIV) infection continues to be an important global epidemic, with an estimate of 2.7 million people newly infected and two million deceased people due to AIDS related conditions, in the year of 2008<sup>1</sup>.



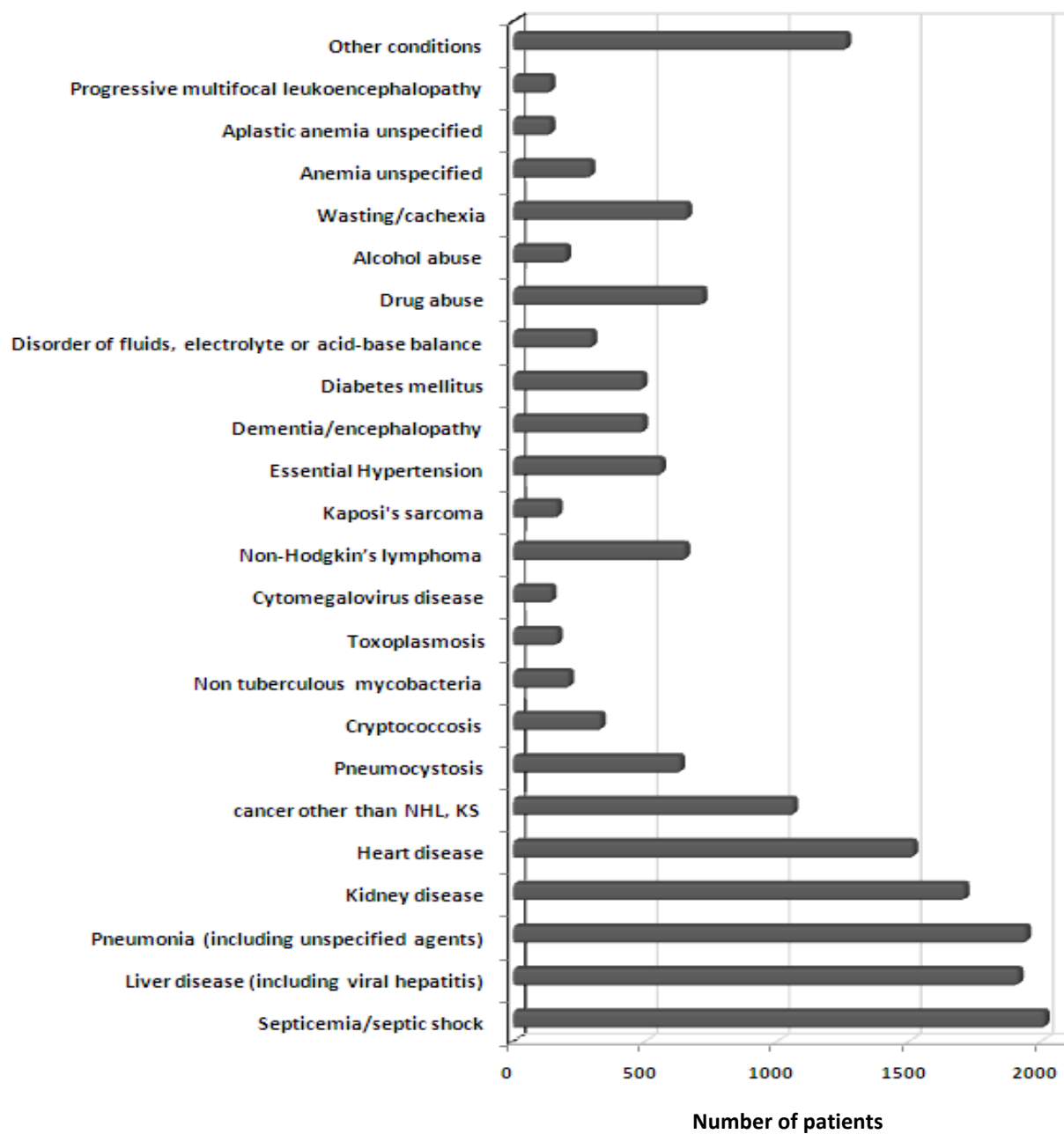
**Figure 1-** General perspective of HIV infection in the world (1990-2008).

Number of people living with HIV, number of people newly infected with HIV and number of AIDS deaths in the world (in Millions), between the years of 1990 and 2008<sup>1</sup>.

Nowadays, the number of HIV infected individuals is higher than in any previous year, as the advent of the combination antiretroviral therapy (cART) had a dramatic impact on morbidity and mortality. In a similar way, the number of AIDS-related deaths has declined by over 10% during the last five years<sup>1</sup>.

Since the introduction of cART in 1996, developed countries are witnessing changes in morbidity and mortality in HIV-infected patients. Opportunistic infections are being replaced by kidney, liver and cardiac diseases which are, in developed countries, the leading causes of death in this population<sup>2</sup> (figure 2). According to the American death certificates, that mentioned HIV infection, in the year 2006, kidney disease were, in fact, the fourth cause of death, only surpassed by pneumonia, liver disease and sepsis<sup>2</sup>.

It is known that a considerable proportion of all HIV-infected patients develop at least transient changes in the kidney function, during the course of HIV infection. Studies have shown that 20-30% of infected individuals have kidney dysfunctions<sup>3,4</sup>, whereas others reported that 15% of these patients develop chronic kidney disease (CKD) or end-stage kidney disease (ESKD)<sup>5</sup>. These patients not only have a risk of HIV-associated kidney disease, but also an increased risk of other diseases, such as thrombotic microangiopathy, associated to the immune system<sup>6,7</sup>, associated with hepatitis virus B or C (HBV/HCV) co-infection<sup>8</sup>, drug induced nephrotoxicity and electrolytic changes. Furthermore, as HIV infection is becoming more frequent in older patient groups, age-associated conditions such as diabetes, hypertension or metabolic syndrome are now more common. These facts led, in 2005, to the publication of guidelines, for Chronic Kidney Disease treatment in HIV-infected patients, by the Infectious Diseases Society of America<sup>4</sup>.



**Figure 2-** Conditions reported in U.S. death certificates that mentioned HIV infection, in 2006.

*Adapted from: Adih et al. JIAPAC 2010 [ahead of print]*

NHL- Non-Hodgkin's lymphoma; KS- Kaposi sarcoma

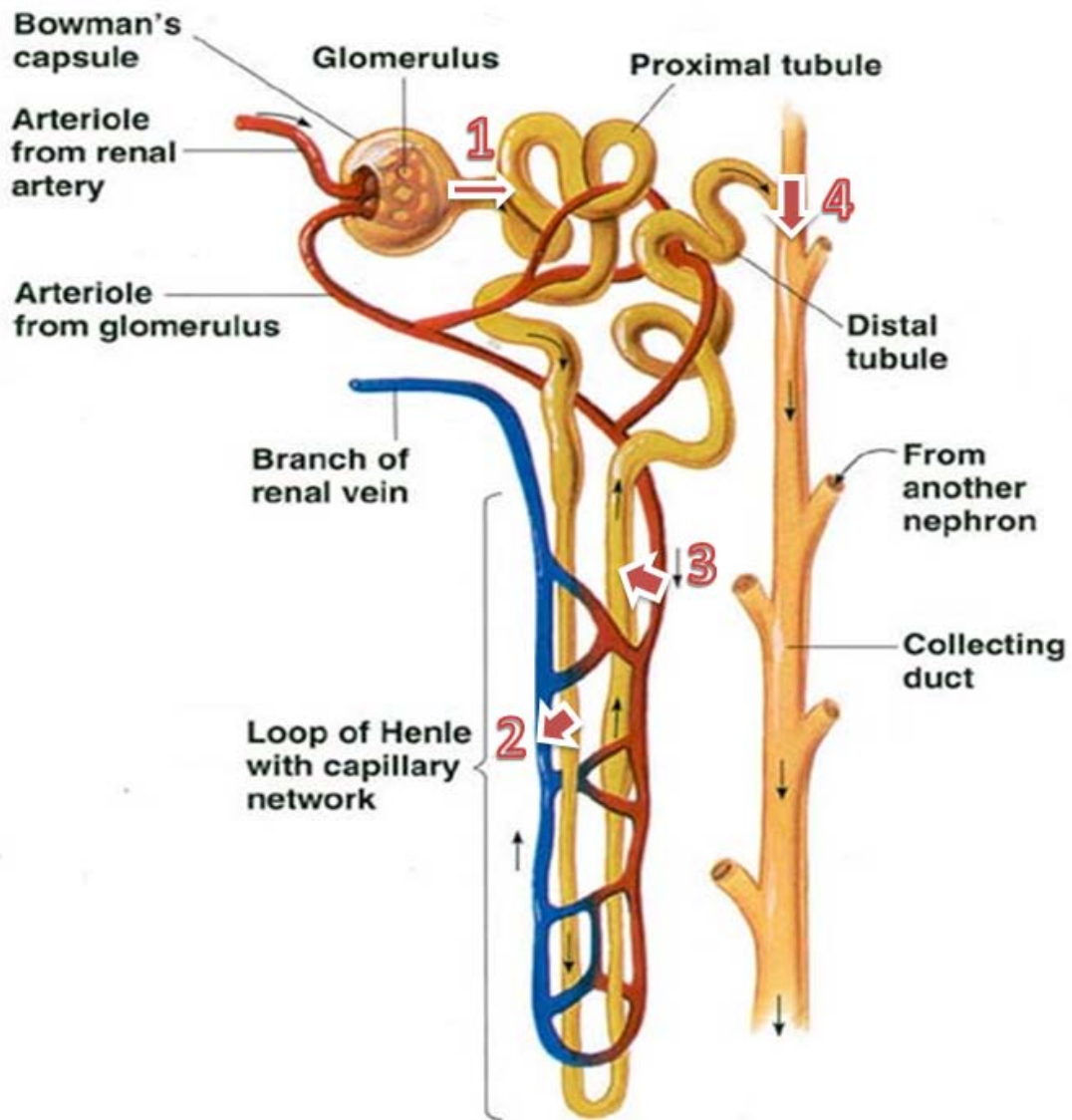
## 2. Mechanism of Kidney Function

The volume of urine excreted per day (around 1.5L) is a result of two opposing processes namely, ultrafiltration and reabsorption. In the first process, 180 L or more fluid per day is filtered by glomerular capillaries<sup>9</sup>. The glomeruli filter the blood, leaving blood cells and large proteins (such as albumin), while other material, such as water, small proteins and waste products are passed through Bowman's capsule, and then into the tubule (figure 3)<sup>10</sup>.

On the reabsorption process, more than 99% of this ultrafiltrate is sent back to the bloodstream<sup>9</sup>. Different parts of the tubules perform different functions in this process. The proximal convoluted tubule processes and reabsorbs the substances that are still necessary (water, electrolytes, glucose, amino acids and minerals) passing them back to the bloodstream. At the same time that this occurs, other substances such as hydrogen ions, ammonia, creatinine and metabolic products of drugs are secreted from the bloodstream directly to the tubule<sup>10</sup>. More water is reabsorbed in the distal tubule, which passes the excess water and waste products to the collecting tubule, which travels through the ureter and into the bladder<sup>10</sup>.

The kidney also secretes hormones namely, erythropoietin, renin, and calcitriol. Erythropoietin stimulates the bone marrow to produce new red blood cells (RBCs). Renin helps regulate blood pressure, and calcitriol is a form of vitamin D and it is important in maintaining bones and the level of calcium in the body<sup>10</sup>.





**Figure 3-** Physiology of a nephron.

Legend: (1)Filtration; (2)Reabsorption; (3) Secretion; (4) Excretion

*Adapted from: [www.cic-caracas.org/departments/science/Topic12.php](http://www.cic-caracas.org/departments/science/Topic12.php)*

Although the kidney is a resilient organ, kidney disorders can occur and be either glomerular, causing problems in the filtration process, or tubular, affecting the reabsorption process, which is often associated with electrolyte imbalances and problems in concentrating urine<sup>10</sup>. The loss of kidney function is usually accompanied

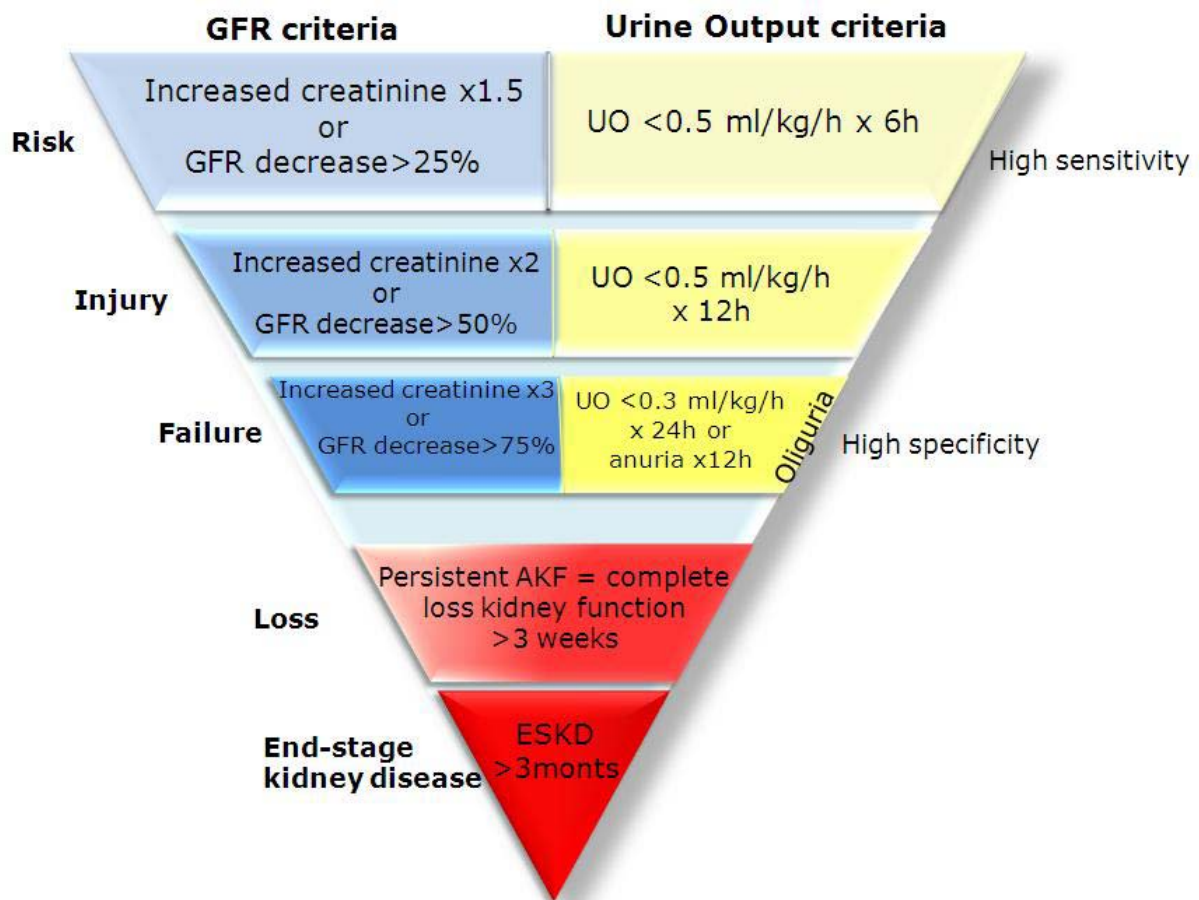
by a progressive distortion of kidney morphology and architecture. At an advanced stage of the disease, usually called kidney failure, the kidney loses the capacity to remove and regulate water and other products and, waste products (causing uremia) and excess water (causing swelling) start to build up<sup>9</sup>. The various kidney diseases are categorized according to whether they are acute and potentially reversible or chronic and generally irreversible, as well as the site of the damage (either glomerular or tubulointerstitial).

### **3. Acute Kidney Injury**

Acute kidney failure (AKF) is a generic term for an abrupt and sustained decrease of kidney function leading to retention of nitrogenous, such as urea and creatinine, and non-nitrogenous waste products<sup>11</sup>.

Despite the advances in treatment strategies and comprehension of the pathogenesis, until recently there was no clear definition of AKF. This definition still remains subjected to controversy and there is a lack of consensus. In fact, there are more than 30 definitions in the literature for AKF. The definitions varied from a 25% increase over baseline serum creatinine to the need for dialysis<sup>12</sup>.

The term acute kidney injury (AKI) has been recently proposed to replace AKF, since an acute decline in kidney function is generally secondary to an injury which leads to functional and structural changes in the kidney<sup>13</sup>. The term AKI is also intended to reflect the entire spectrum of AKF.



**Figure 4 – RIFLE classification scheme.**

In the risk, injury, fail, loss and end-stage kidney disease (RIFLE) classification the criteria (GFR or UO) that lead to the worst possible classification should be used. The Failure class is present even if the increase in serum creatinine is below threefold, as long as the serum creatinine is 4.0 mg per 100 ml or above in the setting of an acute increase of at least 0.5 mg per 100 ml. The shape of the figure indicates that more patients (high sensitivity) will be included in the upper category, including some without actually having kidney failure (less specificity). In contrast, at the bottom, the criteria are strict (high specificity), but some patients will be missed (less sensitivity).

GFR-glomerular filtration rate; UO-urinary output AKF-acute kidney failure; ESKD- End-stage kidney disease.

In order to meet the need of an uniform definition, the Acute Dialysis and Quality Initiative (ADQI) group published, in 2004, a new classification which includes several parameters: the risk, injury, failure, loss and end-stage kidney disease (RIFLE) classification, in which are defined three classes of severity- risk (R), injury (I) and failure (F) classes - and two outcomes – loss (L) and end-stage kidney disease (ESKD)<sup>14</sup>.

The proposed classification of AKI by the RIFLE criteria is shown in figure 4.

Some studies have applied the RIFLE classification to evaluate the occurrence rate and/or outcome of AKI. More recently, this classification has been applied to evaluate clinical characteristics and its predictive ability in intensive care unit (ICU) patients, one of them specifically directed for critically ill HIV-infected patients<sup>15-18</sup>. Those studies reported a good discriminative ability and in a Portuguese study, Lopes *et al.* reported that the RIFLE criteria represent a helpful tool in the prognosis of ICU HIV-infected patients<sup>18</sup>.

In order to further improve the AKI definition, the Acute Kidney Injury Network (AKIN) was created, proposing a modified version of RIFLE classification, known as AKIN criteria<sup>19</sup>. The most important modifications of AKIN criteria include:

- a) the removal of the outcome criteria and the severity criteria are designated as stage 1, 2 and 3;
- b) the broadening of the risk category to include an increase in serum creatinine of at least 0.3 mg/dl, even if it this does not reach the 50% threshold;
- c) the setting of a 48 hour window for the first documentation of any stage;

- d) the categorization of any patient treated with kidney replacement therapy as stage 3 regardless of serum creatinine and urine output.

The AKIN criteria were found to improve detection of AKI sensitivity when compared with the RIFLE classification by *Lopes et al.*<sup>20</sup>. On the other hand, a retrospective study of the Australian and New Zealand Intensive Care Society database did not reveal any significant difference<sup>21</sup>.

AKI has been reported in up to 20% of all hospitalized HIV-infected patients<sup>22</sup> and etiologies of AKI in HIV-infected patients are generally similar to what is seen in non-infected hospitalized patients<sup>23</sup>.

Acute deterioration into kidney failure can be prerenal (hypoperfusion), renal (intrinsic to kidney tissue) or postrenal (obstructive) (table 1).

Prerenal azotemia is characterized by increased levels of nitrogen-containing compounds and in the blood caused by a decrease in blood flow to the kidneys, and therefore by an insufficient filtering of the blood. This condition is the most common cause of AKI in HIV-infected patients<sup>24,25</sup>. This type of hemodynamic kidney failure includes hypovolumetric states, often as a result of gastrointestinal fluid loss, associated with profuse vomiting and/or diarrhea. Disordered kidney regulation of salt and water balance can also contribute to intravascular volume depletion in these patients. Similarly, central or nephrogenic diabetes insipidus in these patients often causes unregulated water loss, which leads to dehydration<sup>26</sup>. Prerenal azotemia can also result from sepsis which develops from a combination of endotoxin-associated systemic vasodilatation, arterial hypotension, capillary leakage and kidney arteriolar vasoconstriction associated with vasopressor drug therapy<sup>26</sup>.

**Table 1.** HIV-related acute kidney diseases.

---

*Acute Kidney Injury*

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<b>Prerenal</b>	Prerenal azotemia	Hypovolemia (diarrhea, vomiting, bleeding, pancreatitis).
<b>Renal</b>	ATN	Tubular necrosis (pentamidine, aminoglycosides, amphotericin B, foscarnet, NSAIDs, contrast based dyes, HUS, microangiopathies, rhabdomyolysis, statins).
	ATIN	Interstitial nephritis (cephalosporin, sulfonamides, quinolones, phenitoin, beta-lactams, allopurinol, protease inhibitors, NSAIDs).
<b>Postrenal</b>	Crystal-induced nephropathy	Internal obstruction (crystal deposition, tumour lysis, blood clots).
	Urinary obstruction	External obstruction (tumour, prostate hyperplasia, urethral obstruction, retroperitoneal fibrosis, drug-associated calculi).

---

ATN- Acute tubular necrosis; ATIN- Acute tubulointerstitial nephritis; NSAIDs- nonsteroidal anti-inflammatory drugs; HUS- haemolytic uremic syndrome.

Acute tubular necrosis (ATN) is the death of tubular cells, when they do not get enough oxygen (ischemic ATN) or when they have been exposed to a toxic drug or molecule (nephrotoxic ATN). This can be due to hypovolemia, sepsis, shock and use of nephrotoxic agents for therapeutic and diagnosis purposes. However, medication-induced ATN is the most common cause of AKI in hospitalized HIV-infected patients

due to the variety of nephrotoxic drugs often required for treatment of opportunistic and nosocomial infections<sup>27</sup>. Commonly implicated drugs include: pentamidine, foscarnet, amphotericin B, aminoglycoside antibiotics, non-steroid anti-inflammatory drugs (NSAIDs). Statin use for hyperlipidemia secondary to cART and concomitant use of gemfibrozil have also been found to cause rhabdomyolysis<sup>26</sup>.

Acute tubulointerstitial nephritis (ATIN) is an inflammation of the tubules that affect the interstitium. ATIN should also be considered as a possible cause of AKI when certain drugs are prescribed for HIV-infected patients. Common implicated drugs include:  $\beta$ -Lactam antibiotics, sulfonamides, quinolones, phenitoin, allopurinol, NSAIDs and indinavir<sup>25</sup>.

The deposition of insoluble crystals in the kidney can also cause AKI in HIV-infected patients. Severe AKI due to crystal-induced nephropathy can develop in the presence of risk factors that increase intra-tubular crystal precipitation. As an example, patients with AIDS-associated lymphoma which produce excessive amounts of uric acid during cell death<sup>24</sup>, that cannot be filtered and secreted by the kidney, which results in precipitation and deposit of uric acid crystals in the tubular lumens of the distal nephron causing intrarenal obstruction<sup>24</sup>. Indinavir, sulfadiazine, foscarnet and acyclovir have been implicated in crystalluria and intrarenal obstruction<sup>28</sup>. Intravascular volume depletion and low urinary flow rates increase the risk for crystal deposition of these substances, as well as uric acid<sup>28</sup>.

Abnormal results on urinalysis usually provide the only evidence of crystal-related kidney injury. Crystalluria, cylindruria, hematuria and proteinuria in varying degrees can be found on urinalysis<sup>24,28</sup>.

Obstruction of the urinary tract is a rare cause of AKI but gains importance in HIV-infected patients. Several unusual causes of obstruction have been described in these patients<sup>23,24</sup>, which are summarised in table 1. The most common cause of obstruction in HIV-infected patients is the development of drug-induced calculi. This has been noted with the antibiotic sulfadiazine and the protease inhibitor indinavir. Symptomatic nephrolithiasis, characterized by classic renal colic with hematuria and dysuria can also occur<sup>28</sup>.

Opportunistic infections and septicaemia are becoming less common causes of kidney injury in cART-treated patients. In opposition, drug toxicity is becoming an increasing cause of both AKI and chronic kidney disease in HIV-infected patients.

#### **4. Chronic Kidney Disease**

Chronic Kidney Disease (CKD) is a syndrome which results from progressive and irreversible destruction of the nephrons, regardless of the cause. It is defined by the maintenance of kidney injury or reduced kidney function for three months or longer (table 2). Proof of chronicity can be also provided by the demonstration of bilateral reduction of kidney size by diagnostic imaging techniques<sup>9</sup>. Other findings consist with long standing kidney disease, such as osteodystrophy, signs of uremia, anemia, hyperphosphatemia or hypercalcemia, however these are not specific. In contrast, the finding of broad casts in urinary sediments is specific for CKD. The wide diameter of



these casts reflects the compensatory function and hypertrophy of surviving nephrons<sup>9</sup>.

Recently, in a study with 1,239 HIV-infected patients, the CKD prevalence was 15.5% <sup>29</sup> and in another study it was reported that HIV-infected patients account for 1-2% of all the end-stage kidney failure population<sup>30</sup>.

In Portugal, the annual incidence of HIV-infected patients on dialysis grew from 0.5%, in 1997, to 0.9% in 2002. Approximately 75% of these patients were receiving treatment in the Lisbon metropolitan area<sup>31</sup>.

**Table 2.** Stages of chronic kidney disease.

Stage	Description	GFR (mL/min/1,73m <sup>2</sup> )
I	Kidney injury with normal or elevated GFR	≥ 90
II	Kidney injury with a mild GFR reduction	60 – 89
III	Moderate GFR reduction	30 -59
IV	Severe GFR reduction	15 – 29
V	Kidney failure	< 15

Chronic kidney disease is defined as kidney injury or GFR<60 mL/min/1.73m<sup>2</sup> for more than three months. Injury is defined as a pathological alteration or existence of injury biomarkers, including changes presented in urinary or imaging exams.

GFR: Glomerular filtration rate.

In cases of CKD in HIV-infected patients, it is important to consider not only infection-associated etiologies, but also factors or comorbidity conditions which are frequent in the general population, such as diabetes *mellitus* or hypertension. In this group of patients, the most frequent cause of CKD is HIV-associated nephropathy (HIVAN).

HIVAN was first reported in 1984 by *Rao et al.*<sup>32</sup>. This author described a focal and segmental glomerulosclerosis in nine patients with AIDS and nephritic syndrome.

Before the cART era, the usual presentation of HIVAN was the onset of asymptomatic proteinuria, followed by a rapid decrease of kidney function and, in a matter of months, end-stage kidney failure<sup>33</sup>. It is considered that HIVAN pathogenesis involves a direct cytotoxic effect, caused by the HIV infection and also by viral gene expression in kidney cells. It seems that a host genetic predisposition for occurrence of HIVAN might also be present<sup>34</sup>. Several studies have shown that HIVAN is predominant and more severe in black individuals. Nowadays in the USA, HIVAN corresponds to the third cause of CKD in the adult black population that initiates dialysis<sup>35</sup>.

Non-HIVAN causes of CKD in these patients include: thrombotic microangiopathy, HIV immune complex-mediated kidney disease (HIVICK), diabetic nephropathy and hypertensive nephropathy (table 3). Thrombotic microangiopathy can occur due to an endothelial cell dysfunction, partially mediated by viral proteins<sup>36,37</sup>. This disease is characterized by five findings with variable expression: fever, neurologic dysfunction, thrombocytopenia, microangiopathic hemolytic anemia and kidney failure with hematuria. Presence of a high level of proteinuria is not frequent, which helps in the differential diagnosis with HIVAN<sup>38, 39</sup>.

**Table 3.** Causes and characteristics of CKD in HIV-infected patients.

<i>Condition</i>	<i>Characteristics</i>
<b>HIV-associated Nephropathy (HIVAN)</b>	Severe proteinuria, large echogenic kidneys, absent of edema, urinalysis often bland.
<b>HIV immune complex disease (HIVICK)</b>	Identical clinical presentation as HIVAN – varying degrees of kidney dysfunction.
Immune complex mediated glomerulonephritis	
Ig A nephritis	Proteinuria, hematuria, mild kidney dysfunction, elevated levels of serum Ig A.
Post-infectious glomerulonephritis	Edema, proteinuria, hematuria, hypertension is frequent.
Membranous nephritis	Sub-epithelial immune deposits (thickened glomerular capillary walls), proteinuria, nephrotic urine sediment.
Membranoproliferative glomerulonephritis	Hematuria with dysmorphic RBCs and casts. Proteinuria (50% of patients have nephrotic proteinuria). Enlarged kidneys and hypercellular.
Mesangial proliferative glomerulonephritis	Edema, hematuria, proteinuria, hyperlipidemia. Increase of mesangial cells.
Fibrillary or immunotactoid glomerulonephritis	Nephrotic proteinuria, microhematuria, Ig G and C3 glomerular deposits.
Lupus-like nephritis	Microhematuria, proteinuria, relatively rapid progression to kidney failure. Large sub-epithelial deposits with a basement membrane reaction.
<b>Interstitial nephritis</b>	Proteinuria, hematuria. Kidney biopsy shows mononuclear and often eosinophilic cellular infiltration of the kidney parenchyma.
<b>Thrombotic microangiopathies</b>	Schistocytes on a blood smear (fragmented RBCs), thrombocytopenia, lactate dehydrogenase level is extremely elevated.
<b>Minimal change glomerulonephritis</b>	Severe proteinuria, oval fat bodies can be observed, edema, hypoalbuminemia, hyperlipidemia, hyponatremia.
<b>Diabetic nephropathy</b>	Persistent proteinuria, microalbuminuria.
<b>Hypertensive nephropathy</b>	Hyaline arteriosclerosis in the kidneys. Nephrons with dilated tubules, often hyaline casts in the lumen.

IgA= immunoglobulin A; RBCs= red blood cells

HIVICK represents a variety of different presentations namely, glomerulonephritis lupus-like, proliferative, membranous, fibrillar, immunotactoid or post-infection<sup>40-43</sup> (table 3). The pathogenesis of the different forms of HIVICK can have common etiologic features, including deposits of immune complexes containing HIV-1 antigens, specific cytokine expression profiles, genetic factors, inflammatory infiltrates, and the development of kidney scarring<sup>30</sup>.

Immunoglobulin A (IgA) nephropathy is characterized by mostly IgA deposition in the glomerular mesangium and, in HIV-infected patients, appears to be the result of immune complexes containing HIV antigens<sup>44</sup>. It is characterized by proteinuria, hematuria, and mild kidney impairment and is less severe than HIVAN and some other variants of HIVICK<sup>45</sup>. Elevated levels of serum IgA, as well as detectable serum IgA immune complexes and rheumatoid factor, may be present<sup>46</sup>.

IgA nephropathy seems to be more frequent in European men, being quite rare in Africa descendents<sup>47</sup>, as in the non-infected population<sup>48</sup>.

Diabetes is one of the leading causes of kidney disease in the general population. In Europe and USA, the prevalence of stage 3 CKD, among people with diabetes is 26.5%<sup>49</sup>. The pathogenesis of diabetic nephropathy is complex. Initially hyperglycaemia, oxidative stress and altered insulin seem to trigger kidney vasodilatation, leading to an increase of the glomerular filtration around 25-50% above the normal range<sup>10</sup>. This causes an increased blood pressure and volume flowing into the glomeruli, which results in glomerular capillary distension. The increased tension and inflammation causes changes to the kidney parenchyma, resulting in glomerular and tubular hypertrophy. This leads to a glomerular capillary compression, resulting in a decrease of glomerular filtration with microalbuminuria

and hypertension<sup>10</sup>. The progressive increase of cellular damage, including kidney podocytes apoptosis, leads to a further decrease on glomerular filtration and the occurrence of severe proteinuria (>3g/day). Without intervention, the patient eventually progresses to ESKD<sup>10</sup>.

Hypertension is another leading cause of CKD. High blood pressure leads to an increase of blood volume that passes through the glomeruli, which creates stress on the glomerular cells, including mesangial and epithelial cells and podocytes<sup>50</sup>. This leads to an overproduction of vasoactive substances that activate the production of inflammatory cytokines and growth factors, which promote cell damage and fibrosis, reducing glomerular filtration<sup>51</sup>. Patients with hypertensive nephropathy usually present severe proteinuria, edema and uremia, which can lead to death, frequently from cerebral hemorrhage<sup>10</sup>.

## **5. Mechanism of HIV infection of kidney cells**

It is still an issue under discussion whether HIV can or cannot directly infect kidney cells. Although viral replication in kidney cells is probably restricted by the lack of CD4 and chemokine co-receptors required for viral entry, it was shown that HIV transfection allows kidney epithelial cells to produce viral particles<sup>52-54</sup>.

The mechanism through which HIV-1 enters kidney cells remains unclear. One possibility is viral transcytosis through lymphocytes to kidney epithelial cells<sup>55</sup>.

Another possibility is CCR5 transfer by microparticles released from peripheral blood mononuclear cells<sup>56</sup>.

The potential role of dendritic cells also needs to be considered. Dendritic cells are involved in binding, dissemination and transfer of HIV to lymphoid and non lymphoid tissues and, for that reason, may also be implicated in HIV infection of kidney cells<sup>55</sup>.

## 5. Combination Antiretroviral Therapy

The first treatment for HIV-infection was available in 1987 and consisted in the nucleoside analog reverse transcriptase inhibitor (NRTI) zidovudine (ZDV, Retrovir®, first manufactured by GlaxoSmithKline). At the time, most HIV-infected patients were dying within months after the diagnosis of AIDS, usually after a sequence of multiple episodes of *Pneumocystis jiroveci* pneumonia<sup>57</sup>.

ZDV was initially developed in 1964 as a candidate for cancer chemotherapy, but was never used before due to its toxicity<sup>57</sup>. In initial clinical trials, ZDV monotherapy was shown to decrease mortality, increase CD4+ T cell counts and lead to fewer opportunistic infections in patients with symptomatic HIV-infection or AIDS<sup>58, 59</sup>. Monotherapy limitations were later demonstrated, as studies showed that the duration of ZDV therapy benefits lasted for just one to two years<sup>60, 61</sup>. However, given the prognosis of a patient diagnosed with AIDS at the time, an apparent extension of a few months of life was regarded as worthwhile. Nevertheless, this opened a window of opportunities for the development of other antiretroviral drugs, with four

additional NRTI drugs licensed for this purpose: didanosine (ddI; Videx<sup>®</sup>, Bristol-Myers Squibb), zalcitabine (ddC; Hivid<sup>®</sup>, Roche), stavudine (d4T; Zerit<sup>®</sup>, Bristol-Myers Squibb) and lamivudine (3TC; Epivir<sup>®</sup>, GlaxoSmithKline).

Combinations of two NRTI constituted the first cART. Compared to ZDV monotherapy, ZDV and ddI<sup>62,63</sup>, ZDV and 3TC<sup>62,63</sup> or ZDV and ddC<sup>64</sup> showed to be more effective in the increase of CD4+ count and decrease of viral load.

Two nonnucleoside reverse transcriptase inhibitors (NNRTI) became available around 1996, which were, delaviridine (Rescriptor<sup>®</sup>, Pfizer), which was never available in Portugal, and nevirapine (NVP, Viramune<sup>®</sup>, Boehringer Ingelheim). With these new antiretroviral drugs, it was possible to treat HIV-infected patients with three drugs. Combination of ZDV, ddI and NVP was found to outperform the efficacy of therapy with two nucleosides<sup>65, 66</sup>.

Protease inhibitors (PIs), the third class of antiretroviral drugs, were on the market by 1995<sup>57</sup>. The introduction of PIs represented one of the biggest breakthroughs in HIV therapy. Highly active antiretroviral therapy (a term now being substituted by cART), that was a combination of a PI and two or three other antiretroviral drugs, help to transform HIV infection into a manageable chronic disease for the majority of patients with access to these drugs<sup>57</sup>.

Nowadays, the situation regarding HIV-infection is completely different from 23 years ago. Today there are more than 20 antiretroviral drugs available and life expectancy of HIV-infected patients is measured in decades. Because of that, today the weighing of antiretroviral drugs' possible benefits of treatment against risk of drug toxicity is extremely important.

## **5.1. cART associated kidney disease**

Studies have confirmed a kidney function improvement in HIV-infected patient, when cART is initiated<sup>67</sup>. However as previously mentioned, other studies have already associated kidney complications with administration of specific antiretroviral drugs<sup>68-95</sup> (table 4).

Indinavir (IDV) (*Crixivan*<sup>®</sup>, Merck) seems to be the only PI with significant urinary excretion. Nephrolithiasis is known as the most important side effect<sup>68</sup> and also pyuria associated with progressive loss of kidney function<sup>69</sup>. Ritonavir (RTV) utilization as IDV booster (IDV/r) elevates the risk of nephrolithiasis<sup>70</sup> and of rhabdomyolysis with possible occurrence of kidney failure<sup>71</sup>.

Risk factors of IDV-associated kidney injury include: low body weight, IDV administration in doses equal or greater than 1000mg, administration twice a day, co-administration with cotrimoxazol<sup>72</sup> and HCV or HBV co-infection<sup>73</sup>.

RTV (Norvir<sup>®</sup>, Abbot Laboratories) has also been associated with AKI<sup>74</sup>. Isolated cases of nephrolithiasis have also been referenced as associated with administration of saquinavir (Invirase<sup>®</sup> and Fortovase<sup>®</sup>, Roche)<sup>75</sup> and nelfinavir (Viracept<sup>®</sup>, ViiV Healthcare)<sup>76</sup>. More recently, there was also a published case of AKI associated with atazanavir (ATV) (Reyataz<sup>®</sup>, Bristol-Myers Squibb) administration<sup>77</sup>.

Regarding NRTIs, dosages might have to be adjusted in patients with a GFR inferior to 60 ml/min/m<sup>3</sup>, with exception of abacavir that does not need dosage adjustments<sup>4</sup>.

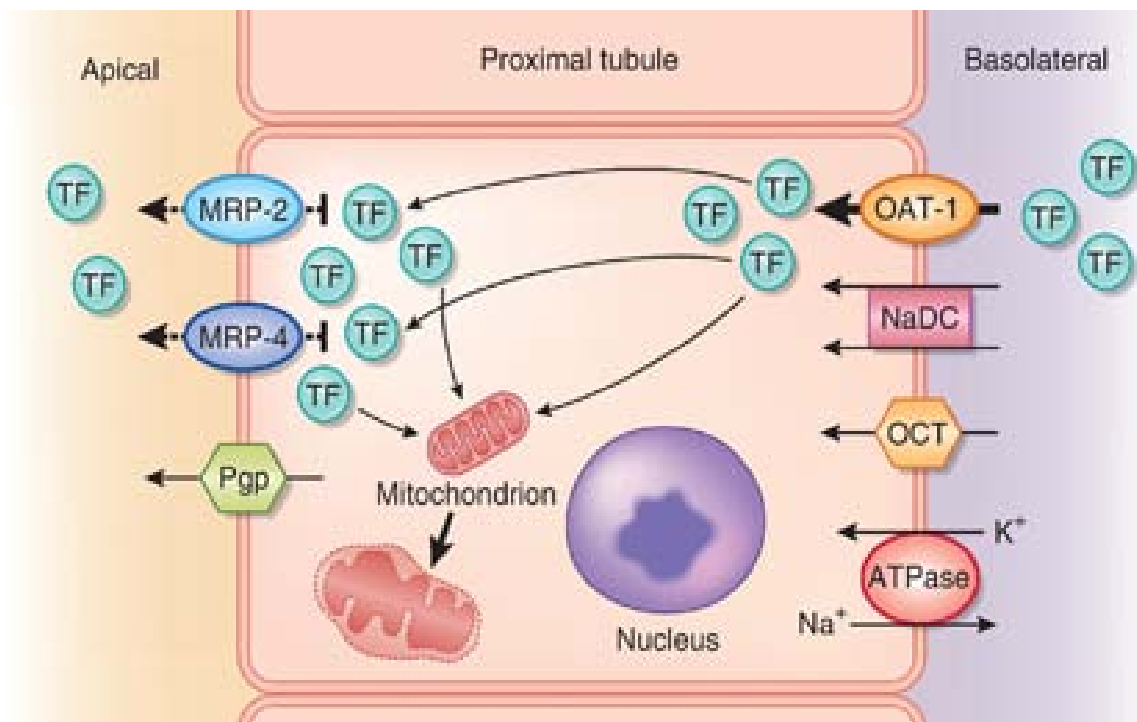
Tenofovir disoproxil fumarate (TDF) (Viread<sup>®</sup>, also present in Truvada<sup>®</sup>, Gilead sciences, and Atripla<sup>®</sup>, Bristol-Myers Squibb & Gilead Sciences) shares its molecular structure with adefovir which utilization is limited by its nephrotoxicity<sup>78</sup>



**Table 4.** Antiretroviral drug associated kidney injury

Antiretroviral drug	Kidney injury	Ref.
<i>Nucleoside reverse transcriptase inhibitors</i>		
Abacavir	AKI (acute interstitial nephritis) in hypersensitivity reaction	[88]
Didanosine	Fanconi's syndrome, AKI, lactic acidosis, nephrogenic diabetes insipidus	[89,90]
Lamivudine	Kidney tubular acidosis and hypophosphataemia	[91]
Stavudine	Kidney tubular acidosis and hypophosphataemia	[91]
Zalcitabine	Not reported	
Zidovudine	Not reported	
<i>Non-nucleoside reverse transcriptase inhibitors</i>		
Nevirapine	Not reported	
Delavirdine	Not reported	
Efavirenz	AKI (multiorgan hypersensitivity reaction)	[94]
Etravirine	AKI	[95]
<i>Nucleotide reverse transcriptase inhibitors</i>		
Tenofovir	Proximal tubulopathy, nephrogenic diabetes insipidus, AKI	[79-83]
<i>Protease inhibitors</i>		
Amprenavir	Not reported	
Indinavir	Intratubular precipitation, nephrolithiasis, kidney colic, AKI and CKD	[70,71]
Lopinavir	Not reported	
Darunavir	AKI, nephrolithiasis (in association with ritonavir)	[96]
Fosamprenavir	Not reported	
Tipranavir	Not reported	
Nelfinavir	Nephrolithiasis	[76]
Saquinavir	AKI (in association with ritonavir)	[75,93]
Ritonavir	AKI, pancreatorenal syndrome, rhabdomyolysis	[74,92,93]
Atazanavir	Nephrolithiasis, AKI (interstitial nephritis)	[77]
<i>Fusion inhibitors</i>		
Enfuvirtide	Membranoproliferative glomerulonephritis in diabetic patients	[97]
<i>CCR5 inhibitors</i>		
Maraviroc	Not reported	
Vicriviroc	Not reported	
<i>Integrase inhibitors</i>		
Raltegravir	Rhabdomyolysis, mild impairment of a pre-existing kidney insufficiency, severe AKI	[98]

AKI – Acute Kidney Injury; CKD- Chronic kidney disease; Ref- Reference



**Figure 5** - Proximal tubular cell transport pathway for tenofovir.

Tenofovir is delivered to the basolateral membrane of proximal tubular cells and is transported into the cell by organic anion transporter-1 (OAT-1). Within the cell, tenofovir is transported via carrier proteins and is subsequently secreted into the kidney tubule by the apical efflux transporters multidrug resistance protein-2 (MRP-2) and MRP-4. Disturbances in this secretory pathway (due to increased OAT-1 activity or decreased MRP efflux transport activity) may lead to increased tenofovir concentrations within the cell, which can cause mitochondrial DNA depletion and dysfunction. Ultimately this can cause proximal tubulopathy (AKI or Fanconi syndrome).

NaDC- sodium–dicarboxylate symporter; OCT- organic cation transporter; OAT-1-organic anion transporter-1; MRP-multidrug resistance protein; Pgp- P-glycoprotein; TF- Tenofovir; AKI- acute kidney injury.

*From: Perazella MA. Kidney Int 2010;78:1060-3.*

Although, initial studies did not show high rates of TDF associated with kidney failure<sup>78</sup> there are already a number of published cases of Fanconi's syndrome post-TDF administration<sup>79-83</sup>. This suggests a possibility of specific risk factors in these patients. On the other hand, in most of these cases, patients were on TDF and RTV, including lopinavir/r, saquinavir/r, amprenavir/r and ATV/r<sup>79-83</sup>. In fact, pharmacokinetics studies demonstrated that an elevation of approximately 32% on TDF exposure occurs, when co-administrated with lopinavir/r and 37% when co-administrated with ATV/r, compared with TDF administration without these PIs<sup>83</sup>.

The observation that around 93% of TDF-associated kidney impairment occurred in individuals on PIs, have led to the idea that RTV mediated inhibition of the multidrug resistance protein 2 (MRP-2) transporter, which mediates TDF excretion into urine in the proximal tubule<sup>84</sup>, may lead to intracellular accumulation of this antiretroviral drug (figure 5). For that reason, the higher TDF concentrations could cause proximal tubule necrosis.

Finally, one must not forget that antiretroviral drugs, besides having a direct toxicity, have been also associated with several metabolic complications that might lead to CKD, including insulin resistance, dislipidemia and hypertension<sup>85-87</sup>.

## **6. Kidney function assessment: traditional methods versus new biomarkers**

Accurate measurement of GFR is costly and time-consuming. Therefore, clinical routine monitoring of kidney function is based on urinalysis, proteinuria and/or albuminuria determination and, most importantly, it is based on serum creatinine (Cr) determination.

Cr is a by-product from the creatinine phosphate break-down of the muscle catabolism and usually is produced at a relatively constant rate. Because there is almost no tubular reabsorption of Cr, if the kidney filtration is affected, Cr blood levels rise. The normal serum Cr level varies with the technique used to measure it. For an adult, the normal range is between 0.6 to 1.2 mg/dl (53 to 106  $\mu\text{mol/L}$ ) by the kinetic or enzymatic method and 0.8 to 1.5 mg/dl (70 to 133  $\mu\text{mol/L}$ ) by the Jaffé reaction<sup>99</sup>.

Cr is considered a reliable biomarker of kidney function. However its levels are influenced by age, gender, diet type, low body mass, and the presence of hepatic disease<sup>100-104</sup>.

To overcome some of these limitations, Cr levels are included in formulas that take into account other factors such as, age and/or weight, in order to obtain an estimated GFR (eGFR) or estimated creatinine clearance (eCrCl).

The two more commonly used formulae are the Cockcroft-Gault and the simplified MDRD (Modified Diet in Renal Disease) equations.

Cockcroft-Gault:

$$Cl_{Cr}(mL/min) = \frac{[140 - age(years)] \times weight(kg) \times [0.85 \text{ if female}]}{72 \times serum \text{ creatinine } (mg/dL)}$$

MDRD:

$$GFR(mL/min/1.73m^2) = 186 \times [serum \text{ creatinine } (mg/dL)]^{-1.154} \times [age(years)]^{-0.203} \\ \times [0.742 \text{ if female}] \times [1.212 \text{ if black}]$$

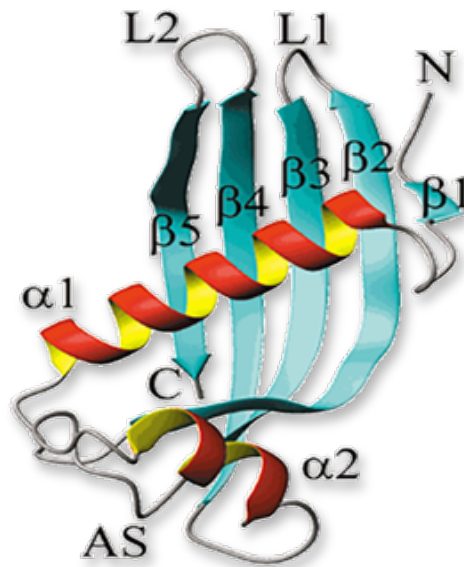
Nevertheless, these formulas are still not validated for HIV-infected patients. As the constant or slow production of Cr makes its rise too small and delayed for early detection of a kidney insult, these formulas also have serum Cr limitations. In addition, serum Cr concentration can remain relatively stable until approximately 50% of kidney function has already been lost<sup>105,106</sup>. In these cases, kidney function changes can be missed if only based on Cr levels.

Therefore, there remains the need for a reliable biomarker of kidney function that allows the detection of mild kidney dysfunction, in order to enable the timely diagnosis and intervention in the early stages of a kidney injury.

## 6.1 Cystatin C

Cystatin C (Cyst C) is a non-glicosilated protein, composed by a single amino acid chain, with a molecular weight of 13.3 kDa (figure 6).

This protein is synthesized in the lisossomes of all nucleated cells<sup>107,108</sup>.



**Figure 6-** Ribbon diagram of human cystatin C structure.

*From: Janowski R et al. Nat Struct Biol 2001,8:316-20*

Cyst C functions like a cysteine protease inhibitor and is present in several biologic fluids, such as serum or cerebrospinal fluid (CSF).

Cyst C is not secreted into the tubules and it is not reabsorbed back into the serum.

Consequently, Cyst C concentration is almost completely dependent of GFR<sup>107, 108</sup>

Studies have demonstrated that Cyst C has a strong negative correlation with GFR<sup>109, 110</sup> and seems to be an earlier<sup>87, 111</sup> and superior marker than Cr, for GFR

estimation<sup>112, 113</sup>. Cyst C also appears to be superior to serum Cr in paediatric<sup>114-117</sup>,

elderly<sup>118</sup> and diabetic patients<sup>119-121</sup>. On the other hand, the concept of Cyst C being

independent of body mass has become controversial over the last years<sup>108, 122</sup> and

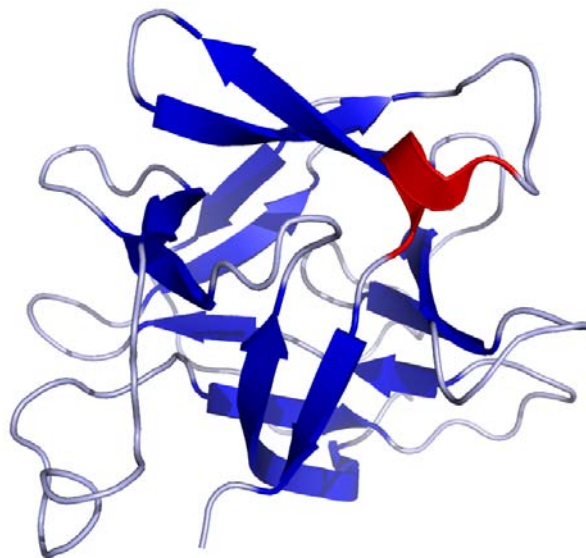
there are still no standardized methods for Cyst C measurement, nor validated

formulas for eGFR<sup>123-125</sup>.

Other factors mentioned as affecting Cyst C production are: thyroid dysfunction, causing a significant decrease in Cyst C levels, as well as high doses of corticosteroids that elevates Cyst C expression<sup>117, 126</sup>. Cyst C serum or plasma levels can be determined by two commercial available, fully automated assays: the particle-enhanced turbidimetric immunoassay and the particle-enhanced nephelometric immunoassay.

## 6.2 Interleukin 18

Interleukin 18 (IL-18) is a pro-inflammatory cytokine, synthesized in the proximal tubules, as an inactive precursor form of 24 kDa, suffering a later cleavage by caspase-1 to originate its active form of 19 kDa<sup>127-129</sup> (figure 7).



**Figure 7** – Ribbon diagram of human Interleukin-18 structure.

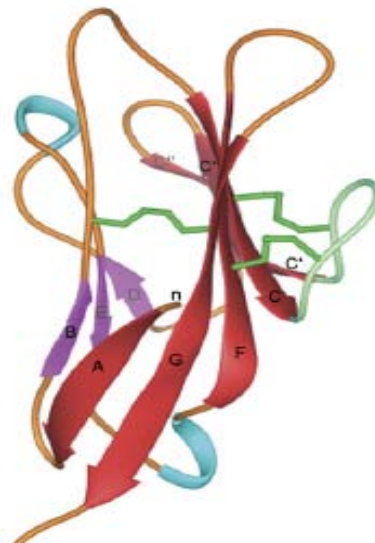
*From: Kato Z, et al. Nat Struct Biol 2003;10:966-71.*

This protein is overexpressed in endogenous inflammatory processes, and seems to have an important role in host defence against several infections<sup>130</sup>.

Pre-clinical trials have indicated that IL-18 mediates acute tubule necrosis in mice<sup>131, 132</sup> and a posterior study reported an IL-18 sensitivity superior to 99% for AKI diagnosis<sup>133</sup>. In another study, it was shown that IL-18 was an earlier marker of AKI in ICU patients, preceding serum Cr elevation in 1-2 days<sup>134</sup>. The elevation of IL-18 occurs approximately 12 hours post kidney injury and can be detected by enzyme-linked immunosorbent assay (ELISA) in urine.

### 6.3 Kidney injury molecule 1

Kidney Injury molecule 1 (KIM-1) is a 100 kDa transmembranar glycoprotein, with an immunoglobulin-like domain and a mucin domain on the extracellular fraction (figure 8)<sup>135</sup>.



**Figure 8** – Ribbon diagram of kidney injury molecule 1 structure.

*From: Santiago C, et al. Immunity 2007;26,299–310.*



The basal expression of this protein in the kidney is very low, in both rodents and humans<sup>136,137</sup>. Nevertheless, in animal models, KIM-1 is the main induced protein in the proximal tubule after a kidney injury, and can be detected in urine. Increase of KIM-1 occurs approximately 12 hours post kidney injury<sup>137</sup>.

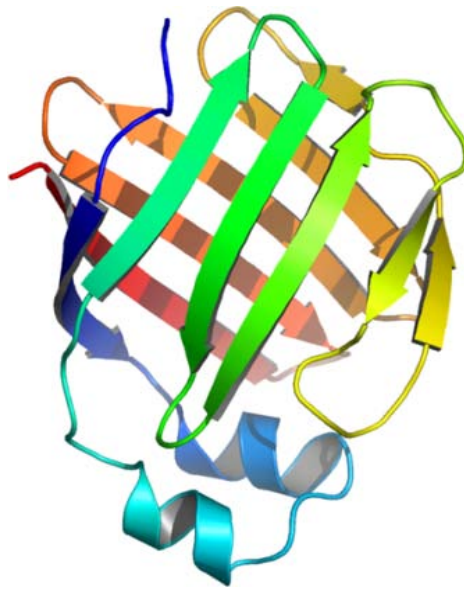
In a recent study performed in mice with different mechanisms of injury in the proximal tubule, using histopathology as the reference method, a good performance of KIM-1 in nephrotoxicity identification was reported. Moreover, KIM-1 was the urinary biomarker with higher sensitivity and specificity from the 21 used in the study<sup>138</sup>. In research, KIM-1 is usually detected by ELISA or microbead. However there are still no commercial methods available.

#### **6.4 Liver fatty acid binding protein**

Fatty acid binding proteins (FABPs) are small cytoplasmatic proteins of 15 kDa which are expressed in all tissues with active metabolism of fatty acids<sup>139</sup>.

FABPs function as chaperons, facilitating free fatty acid chains transport from the cytoplasmatic membrane to oxidation sites such as the mitochondria or peroxissomes<sup>139,140</sup>.

Two types of FABPs were identified in the human kidney: a liver-type FABP (L-FABP) (figure 9), expressed in the proximal tubule and a heart-type FABP (H-FABP), expressed in the distal tubule<sup>141, 142</sup>.



**Figure 9** – Ribbon diagram of liver fatty acid binding protein structure.

*From: Protein Data Bank 2F72*

Pre-clinical and clinical trials reported that urinary L-FABP can be a potential biomarker for several pathologies, such as CKD, IgA nephropathy, contrast nephropathy and diabetic nephropathy<sup>142-145</sup>. Besides that, clinical studies demonstrated that L-FABP has a potential as biomarker for monitoring CKD progression. In another study from Kamijio *et al.* L-FABP increase was shown to be proportional to the kidney function deterioration in non-diabetic patients with CKD<sup>146</sup>.

A preliminary study from Ferguson *et al.* suggested L-FABP as an earlier biomarker of AKI (compared with serum Cr), specifically in the case of aminoglycosides administration<sup>147</sup>.

A different study revealed that L-FABP elevation can be detected approximately four hours after a kidney injury<sup>148</sup>. Still, there seems to be some controversy around L-FABP specificity.

## 6.5 Lipocalin-2 or Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein from the lipocalin family with a molecular weight of 25 kDa. It is composed of eight  $\beta$ -strands assembled in a  $\beta$ -barrel that encloses a calyx. This calyx binds and transports low molecular weight substances<sup>149</sup> (figure 10).

NGAL is expressed by neutrophils and epithelial cells<sup>150</sup>. Distinct NGAL expression levels can be found in different tissues, such as uterus, prostate, lungs, salivary glands, trachea, stomach, colon and kidneys<sup>151</sup>.

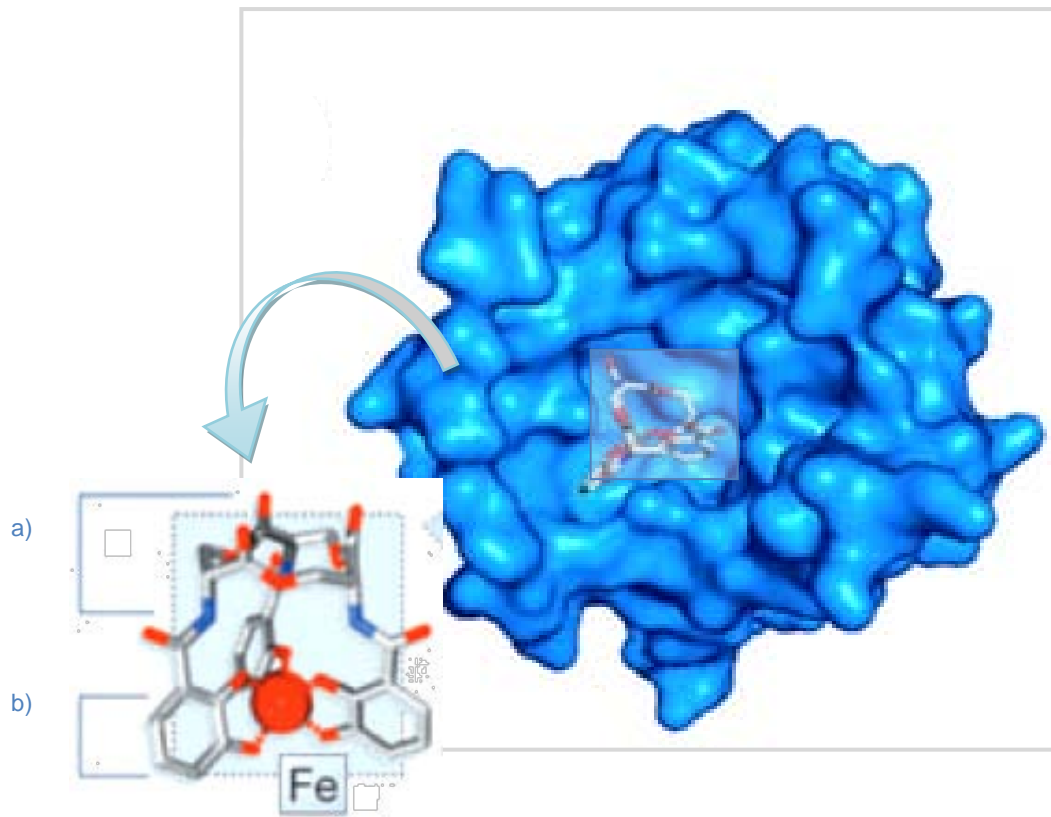
It is known that NGAL binds to siderophores and possibly has an antibacterial action<sup>152-154</sup>. It is thought that this protein can also interact with specific cellular receptors, as the cationic transporter 24p3R, extracellular kinases, hepatocyte growth factors or the gelatinase B enzyme<sup>155-157</sup>.

This protein might also mediate kidney development<sup>158, 159</sup> and act as a cellular growth factor<sup>160-162</sup>.

NGAL is one of the most over expressed proteins in the initial stages of AKI. In the kidney most of this protein is produced in the Loop of Henle and collecting duct<sup>163</sup>.

Studies have shown that NGAL increase can be detected in plasma, serum or urine, just two to six hours after the occurrence of kidney injury<sup>164</sup> and that this protein constitutes a sensitive and specific biomarker of AKI<sup>164-166</sup>. Moreover it was also demonstrated that it has a predictive ability for AKI<sup>167, 168</sup> and CKD severity<sup>169-177</sup>.

In a large prospective study by Wagener *et al.*<sup>178</sup>, it was shown that elevation of urinary NGAL (uNGAL) was correlated with cardio pulmonary bypass time and aortic-clamp time, which are indices of hypoperfusion and are risk factors for AKI<sup>179-181</sup>.



**Figure 10** - NGAL crystal structure with the bacterial enterochelin ligand.

Bacterial enterochelin is composed of: a) a triserine lactone and b) three catecholate groups. These groups, between them, chelate an iron atom or ion.

Enterochelin complex can be buried in the NGAL calyx, between three positively charged amino acids (R81, K125 and K134, where R is arginine and K is lysine).

*Adapted from: Fischbach MA, et al. PNAS 2006;103(44):16502-7*

Several methods have been used for NGAL measurement. Initial studies used Western blot assays<sup>182</sup>, while subsequent studies preferentially used immunoblotting and ELISA assays. There are automated commercial methods available, for instance Triage<sup>®</sup> NGAL (Biosite Incorporated, San Diego, USA) or ARCHITECT<sup>®</sup> (Abbott Diagnostics, Amadora, Portugal).

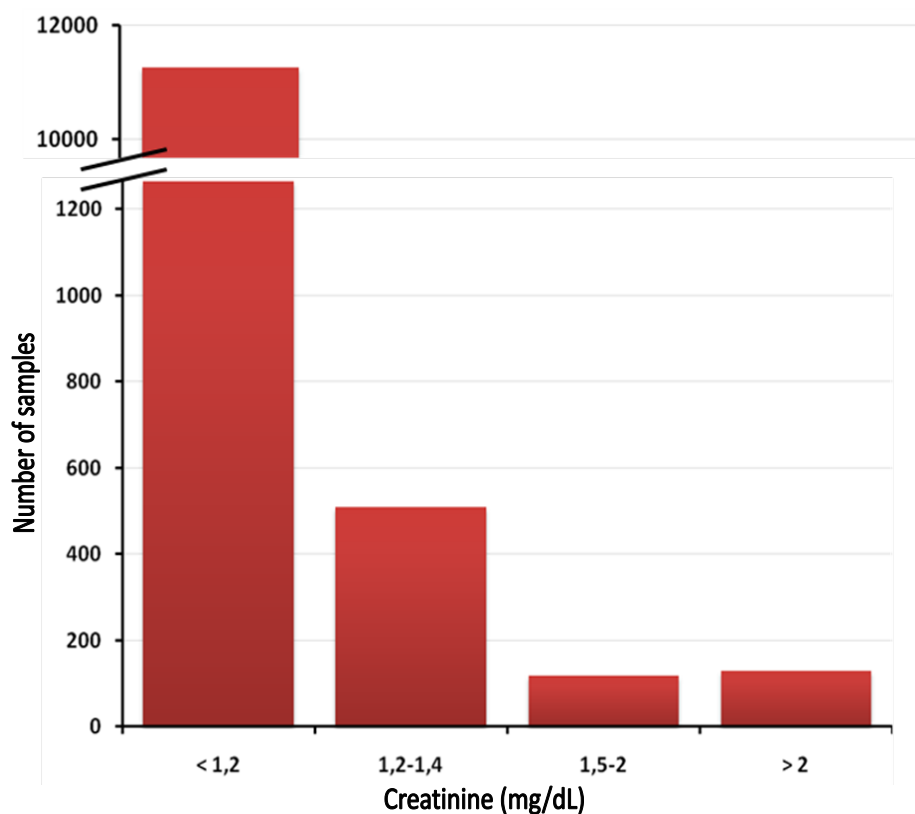
Beside the potential biomarkers mentioned here, there are several others currently in study. However, further studies are still necessary in this field, especially in the case of HIV-infected patients, where studies of these new biomarkers performances are still very limited.

## **7. Kidney dysfunction frequency in HIV patients of Hospital Santa Maria**

Hospital Santa Maria (HSM), Centro Hospitalar Lisboa Norte (CHLN) is one of the largest teaching hospitals in the country. It is a 1,300-bed referral tertiary center and serves a large proportion of the Lisbon population. Clínica Universitária de Doenças Infecciosas (Director: Professor Doutor Franciso Antunes) has a large HIV outpatient clinic. Around 3,000 HIV patients are in a regular follow-up, and most are on cART.

In a previous study at Clínica Universitária de Doenças Infecciosas, during a 20 month period (between January 2007 and August 2008), it was shown that 6% (n=757) of 12,022 samples from 2,761 HIV-infected patients had a creatinine level greater than 1.2 mg/dL (range 1.2-13.5 mg/dL) (unpublished results) (figure 11).

Using the CKD stages guidelines (Guidelines of HIV Medicine Association of the Infectious Diseases Society of America)<sup>4</sup> and eGFR, it was found that 4.1% had moderate GFR reduction (30-59 mL/min/1.73m<sup>2</sup>), 0.6% had severe GFR reduction (GFR 15-29 mL/min/1,73m<sup>2</sup>) and 0.7% had kidney failure (GFR<15mL/min/1,73m<sup>2</sup>) (unpublished results).



**Figure 11-** Samples distribution per creatinine level.

Results refer to 12,022 samples from 2,761 HIV-infected patients.

Early identification of kidney injury is essential to promote initiation/adjustment of cART, suspension of nephrotoxic drugs and control of co-morbid diseases such as diabetes and hypertension. Still, to enable this, sensible (early stage detection) and specific biomarkers of kidney function that can be associated with disease prognosis, are necessary.

In spite of being influenced by several factors, serum Cr maintains a constant basal expression, which makes its rise slowly.

Furthermore, CKD can be present with minimal or no change in Cr, due to its basal reservoir and the raise of Cr tubular secretion. On the other hand, therapeutic interventions that would allow the prevention or treatment of AKI must be introduced in an early stage, before the rise of serum Cr can be detected<sup>181,182</sup>.

Early markers of kidney injury represent the only possibility for an in-time diagnostic and intervention, allowing kidney protection from further injuries and from other risk factors associated with HIV infection.

The HSM study mentioned above led us to study the kidney function obtained by serum Cr in comparison with the one obtained by an apparently promising potential biomarker, serum Cyst C, in HIV-infected patients attending the Infectious Diseases Department of HSM, CHLN, and evaluate whether this marker could be a better alternative than serum Cr for monitoring kidney function in HIV-infected patients undergoing antiretroviral therapy.

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## Study objectives

- I. Cross-sectional observational analysis of kidney function obtained through serum Cr and serum Cyst C determinations:
  - a) Analysis of kidney dysfunction associated risk factors for HIV-infected patients;
  - b) Determination of kidney dysfunction rate using Cr and eCrCl (patients on cART and without treatment);
  - c) Determination of kidney dysfunction rate based on serum Cyst C (same patient group);
  - d) Correlations of Cyst C with Cr, eCrCl and associations with risk factors of kidney injury.
  
- II. Analysis of differences in kidney function between patients undergoing different antiretroviral regimens (with/without TDF, ATV/r).
  - a) Comparison of serum Cyst C, serum Cr and eCrCl levels between patients on cART with TDF, ATV/r and patients on cART that never were on these antiretroviral drugs.

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## Material and Methods

### *Study settings*

Cross-sectional study with HIV-infected patients who currently attend the HIV outpatient clinic, HSM, CHLN.

Inclusion criteria were: (a) HIV-1 infected patients; (b) age over 18 years; (c) capable of having an informed consent.

Exclusion criteria were: (a) HIV-infected patients with known kidney disease; (b) HIV-2 infection.

This study was approved by the Hospital's Ethics Committee and Administration board.

### *Sample size*

The sample size (N) was calculated using the following equation:

$$N \geq [Z^{2*}[P(1-P)]/D^2], \text{ in which}$$

Z-area under a normal curve corresponding to the desired confidence level ( $Z_{0.95}$ );

P-expected frequency of an event in the population

D-maximum difference between the sample mean and the population mean

Assuming a kidney dysfunction frequency (CrCl<60 mL/min) of 5.4% in this population (frequency obtained from the previously mentioned HSM study), the inclusion of at least 218 patients as the sample size, will allow both the determination of kidney dysfunction frequency and associated risk factors with a confidence level of 95% ( $\alpha= 0.05$ ) and a precision of  $\pm 3\%$ .

The sampling method used in this study was the method of convenience sampling.

### ***Study population***

Information on age, gender, ethnicity, country of birth medical history and tobacco and alcohol use was retrieved.

Data on risk factors for HIV, duration and history of antiretroviral therapy were also assessed. The length of HIV infection was considered as the time since the first positive diagnosis.

CD4+ T cell *nadir*, current CD4+ T cell count and HIV RNA levels were also assessed.

HBV co-infection was considered positive when the presence of the hepatitis B surface antigen (HBsAg) was confirmed.

HCV co-infection was considered positive for RNA levels equal or higher than 43 IU/mL (COBAS® Ampliprep/TaqMan® HCV Test, Roche Diagnostics, Amadora, Portugal).

Measurements of alanine transaminase (ALT) and aspartate transaminase (AST) were collected in patients with HCV co-infection.

Height, weight, blood pressure and fasting glucose measurements were also collected.

Hypertension was defined as systolic blood pressure higher than 140 mmHg, and/or diastolic blood pressure greater than 90mmHg and/or use of antihypertensive medication.

Diabetes was defined as fasting glucose levels equal to or higher than 126 mg/dL and/or use of antidiabetic medication.

### ***Kidney function markers measurements***

Serum Cr level was determined using a modified Jaffé method (ADVIA® 2400 Chemistry System, Siemens, Portugal).

CrCl was estimated by the Cockcroft-Gault equation:

$$eCrCl (mL/min) = [140 - age (years)] \times weight (kg) \times [0.85 \text{ if female}] \\ / 72 \times serum Cr (mg/dL)$$

Increased Cr level was defined as a measurement greater than 1.2 mg/dL.

Serum Cyst C was determined with a particle-enhanced immunonephelometric assay (N Latex Cystatin C, Dade Behring, Portugal), according to the manufacturer.

Increased Cyst C was defined as levels greater than 0.96 mg/L.

## ***Statistical analysis***

Statistical analysis was performed using the software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Continuous data were summarized as mean ( $\pm$ SD, standard deviation) and median (IQR, inter-quartile range) for parametric and non-parametric data respectively, while categorical data were represented as proportions. Spearman's and Pearson correlation coefficients were used to evaluate associations between non-parametric and parametric variables.

Pearson's Chi Square ( $\chi^2$ ) and Fisher's exact test were used to compare categorical data, while Student's t-test and Mann-Whitney U test were used to analyse parametric and non-parametric data respectively.

A multiple linear regression was also performed using age, gender, ethnicity, BMI as predictor variables and log transformed cystatin C as the dependent variable.

Differences, associations and regression coefficients between variables were considered significant for a p-value lower than 0.05.

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## Results

### ***Demographic and laboratory description of the sample population***

After the application of inclusion and exclusion criteria, 242 HIV-1 infected patients were enrolled in this study (table 5). Most of them were Portuguese (85%; n=205) and around a seventh of the patients (15%; n=37) were born in foreign countries, mainly Portuguese speaking countries (Angola, Cape Verde, Mozambique, Brazil, Guinea-Bissau), as well as in the United Kingdom, Switzerland and Zaire.

HIV infection was acquired, in most cases, by the sexual route (n=189), followed by intravenous drug use (n=52), and blood transfusion (n=1).

Seventy percent of patients were male (n=169), and 90% were Caucasian (n=218). The median age of the participants was 45 years old (range: 22-83 years; IQR=14). BMI was 24 Kg/m<sup>2</sup> (median; range: 16-33 Kg/m<sup>2</sup>; IQR=5.9). Hypertension was present in 58 patients (24%) and diabetes in 27 patients (11%). Forty nine percent of the patients (n=119) were current smokers and 7% (n=17) had a documented history of alcohol abuse (table 5).

Overall, patients were infected for a median of 14 years (range 1-25 years, IQR=7). Median CD4+ T cell *nadir* was 216/mm<sup>3</sup> (IQR=267), median CD4+ T cell count, at the time of the study, was 686/mm<sup>3</sup> (IQR=509). From the patients on cART, most had viral-RNA levels lower than 40 copies/mL (95%; n=214).



**Table 5.** Demographic and laboratory data of the study population (n=242)

<i>Parameter</i>	
<b>Gender (n, %)</b>	
Male	169 (70%)
Female	73 (30%)
<b>Age (years)</b>	45 (14)
<b>Ethnicity (n, %)</b>	
Caucasian	218 (90%)
Black	24 (10%)
<b>BMI (Kg/m<sup>2</sup>)</b>	24 (6)
<b>Hypertension (n, %)</b>	58 (24%)
<b>Diabetes (n, %)</b>	27 (11%)
<b>Current smoking (n, %)</b>	119 (49%)
<b>Alcohol abuse (n, %)</b>	17 (7%)
<b>Years of infection</b>	14 (7)
<b>Years on cART</b>	12 (6)
<b>On cART (n, %)</b>	225 (93%)
<b>HIV RNA&gt;40copies/mL (n, %)</b>	28 (12%)
Treatment failure	11 (5%)
Treatment <i>naïve</i>	7 (3%)
Treatment interruption	10 (4%)
<b>CD4+ nadir (cell/mm<sup>3</sup>)</b>	216 (267)
<b>CD4+ count (cell/mm<sup>3</sup>)</b>	686 (509)
<b>HBV co-infection (n, %)</b>	5 (2%)
<b>HCV co-infection (n, %)</b>	63 (26%)

Data are presented as mean (standard deviation) and median (inter-quartile range) for parametric and non-parametric data and proportions (n, %) for categorical variables. BMI- body mass index; HBV- hepatitis B virus; HCV- hepatitis C virus; Cr- creatinine; eCrCl- estimated creatinine clearance; Cyst C- cystatin C

Most patients were on antiretroviral treatment (n=225, 93%), of which 5% (n=11) had documented treatment failure. From those patients that were not on cART, seven (3%) were treatment *naïve* patients and 10 (4%) had interrupted treatment (due to patients' own decision, a case of lupus and a case of lipodystrophy).

Among patients on antiretroviral treatment, the median duration of cART was 12 years (range: 5 months-24 years; IQR=6).

**Table 6.** Current cART regimens on 225 HIV-infected patients.

NRTI	N(t)RTI (TDF)	NNRTI	PI/r	II (RAL)	N (%)
X		X			65 (29%)
X	X	X			61 (27%)
X			X		36 (16%)
X	X		X		55 (24%)
X			X	X	2 (1%)
	X		X	X	2 (1%)
		X	X	X	4 (2%)

NRTI- nucleoside reverse transcriptase inhibitors; N(t)RTI- nucleotide reverse transcriptase inhibitor; NNRTI- non-nucleoside reverse transcriptase inhibitor; TDF-tenofovir disoproxil fumarate; ATV- atazanavir; PI/r- ritonavir boosted protease inhibitor; II- integrase inhibitor; RAL- raltegravir.

Regarding current cART regimens on the 225 patients studied, it was possible to see that several antiretroviral combinations were used (table 6). Most patients were on a combination that included two NRTI and one NNRT (n=126). In almost half of those (61/126) TDF was part of the combination. On the group of patients that were on a combination that included PIs (n=99), 52 were on ATV/r. Among the ones that were on TDF, 34 were also on ATV/r.

Regarding HCV and HBV co-infections, 26% (n=63) patients had detectable HCV RNA levels and 2% (n=5) had a positive HBsAg determination. Also, one patient was co-infected with both HCV and HBV. Among patients with HCV co-infection, the HIV infection was acquired through sharing of drug injection materials in most of them (89%; n=56).

### **Kidney function determination based on serum Cr and Cyst C**

Overall, serum Cr level (median) was 0.84 mg/dL (range: 0.3-1.7 mg/dL; IQR=0.29), mean CrCl, estimated by Cockcroft-Gault equation, was 102.5 mL/min (mean,  $\pm$ 30.04) and, for serum Cyst C levels, the median was 0.72 mg/L (range: 0.43-1.42 mg/L; IQR=0.18).

These biomarkers were also compared between patients on antiretroviral therapy (with or without TDF and ATV/r) and *naïve* patients (table 7). Regarding patients without cART, median serum Cr was 0.84 mg/dL (IQR=0.17), mean eCrCl was 100.5 mL/min (SD  $\pm$ 34) and median serum Cyst C was 0.71mg/L (IQR=0.26). Among patients on cART, median serum Cr was 0.85 mg/dL (IQR=0.28), mean eCrCl was 102.7 mL/min (SD  $\pm$ 19) and mean serum Cyst C was 0.72 mg/L (IQR=0.18).

Concerning patients on TDF and ATV/r, simultaneously, only Cyst C levels appeared to be higher for cART with ATV/r and TDF+ATV/r when compared to the overall group of patients on cART (table 7).

**Table 7.** Kidney function according to the type of antiretroviral therapy.

<b>Biomarker</b>	<b>All patients</b>	<b>On cART</b>	<b>On TDF</b>	<b>On ATV/r</b>	<b>On TDF+ATV/r</b>	<b>Not on cART</b>
<b>Cr</b>	0.84(0.29)	0.85(0.28)	0.85(0.22)	0.90(0.25)	0.89(0.25)	0.84(0.17)
<b>eCrCl</b>	102.5(±30)	102.7(±19)	102.9(±29)	102(±18)	95.1(±34)	100.5(±34)
<b>Cyst C</b>	0.72(0.18)	0.72(0.19)	0.73 (0.16)	0.85(0.20)	0.82(0.23)	0.71(0.26)

Data are presented as mean (standard deviation) and median (inter-quartile range) for parametric and non-parametric variables. Cr-creatinine (mg/mL); eCrCl-estimated creatinine clearance (mL/min); Cyst C-cystatin C (mg/L); cART-combination antiretroviral therapy; TDF-tenofovir disoproxil fumarate; ATV/r-ritonavir boosted atazanavir.

**Table 8.** Kidney function according to the duration of HIV infection.

<b>Biomarker</b>	<b>&lt;5 years</b>	<b>5-10 years</b>	<b>11-15 years</b>	<b>16-20 years</b>	<b>&gt;20 years</b>
<b>Cr</b>	0.85(0.23)	0.87(0.21)	0.86(0.20)	0.86(0.18)	0.88(0.21)
<b>eCrCl</b>	117(±42)	103(±34)	104(±31)	99(±23)	100(±31)
<b>Cyst C</b>	0.72(0.17)	0.75(0.16)	0.72(0.20)	0.75(0.21)	0.81(0.15)

Data are presented as mean (standard deviation) and median (inter-quartile range) for parametric and non-parametric variables. Cr-creatinine (mg/mL); eCrCl-estimated creatinine clearance (mL/min); Cyst C-cystatin C (mg/L).

When biomarker levels were analyzed, according to the number of years of HIV infection, a statistically significant difference between the groups was not found ( $p>0.05$ ) (table 8). When analysis was performed according to the number of years on cART, although it was also not found a statistically significant difference between serum Cr and eCrCl levels and years on cART ( $p>0.05$ ), when a comparison was done based on serum Cyst, it was found that patients who were on cART for less than five years had lower levels of Cyst C, comparing to the patients that were on cART for five years or even for longer periods of time ( $p<0.05$ ) (table 9).

**Table 9.** Kidney function according to the number of years on cART.

<b>Biomarker</b>	<b>&lt;5 years</b>	<b>5-10 years</b>	<b>11-15 years</b>	<b>&gt;16 years</b>
<b>Cr</b>	0.85(0.25)	0.85(0.19)	0.85(0.3)	0.90(0.22)
<b>eCrCl</b>	111( $\pm$ 31)	101( $\pm$ 39)	102( $\pm$ 29)	95( $\pm$ 26)
<b>Cyst C</b>	0.69(0.14)	0.72(0.16)	0.73(0.14)	0.77(0.24)

Data are presented as mean (standard deviation) and median (inter-quartile range) for parametric and non-parametric variables. Cr-creatinine (mg/mL); eCrCl-estimated creatinine clearance (mL/min); Cyst C-cystatin C (mg/L).

Biomarker levels were also analyzed according to the CD4+ T cell *nadir* (table 10). Using the same three biomarkers, as before, patients with a CD4+ *nadir* count lower than 200 cells/mm<sup>3</sup> showed a slightly lower kidney function compared to patients that present a CD4+ *nadir* count higher than 200 cell/mm<sup>3</sup>. However, differences found did not reach a significant level ( $p>0.05$ ).

**Table 10.** Kidney function according to CD4+ T cell *nadir*.

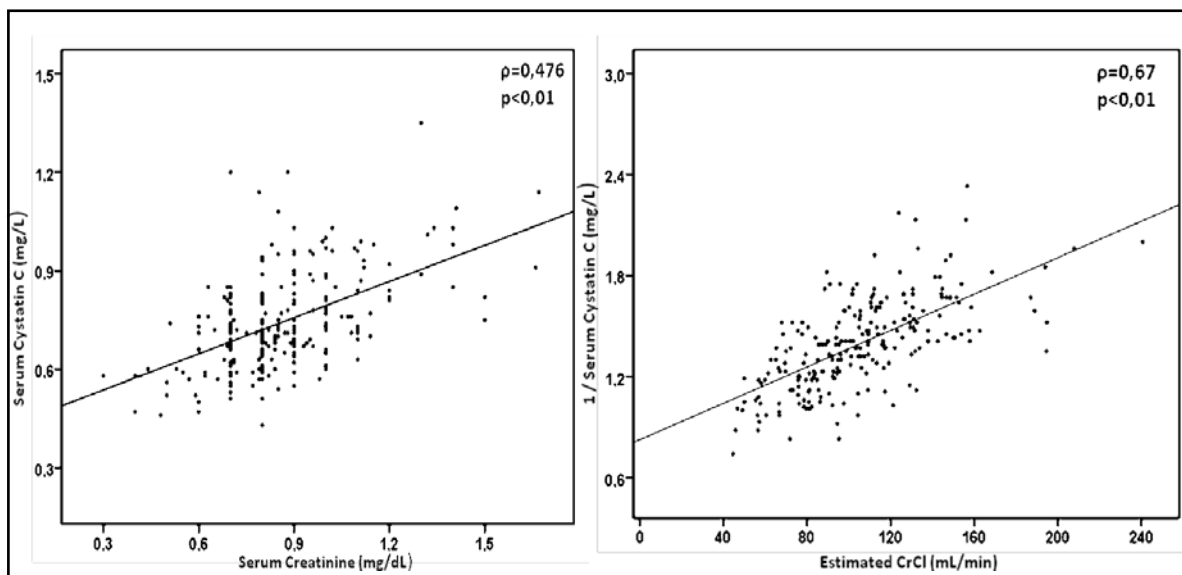
<b>Biomarker</b>	<b>&gt;50 cell/mm<sup>3</sup></b>	<b>51-200 cell/mm<sup>3</sup></b>	<b>201-300 cell/mm<sup>3</sup></b>	<b>301-400 cell/mm<sup>3</sup></b>	<b>&gt;401 cell/mm<sup>3</sup></b>
<b>Cr</b>	0.87(0.27)	0.87(0.2)	0.85(0.29)	0.84(0.11)	0.8(0.25)
<b>eCrCl</b>	97(±29)	102(±34)	104(29)	103(±28)	109(±28)
<b>Cyst C</b>	0.76(0.25)	0.74(0.31)	0.71(0.17)	0.7(0.16)	0.7(0.18)

Data are presented as mean (standard deviation) and median (inter-quartile range) for parametric and non-parametric variables. Cr-creatinine (mg/mL); eCrCl-estimated creatinine clearance (mL/min); Cyst C-cystatin C (mg/L).

Overall, correlations between HIV RNA level and serum Cr was  $\rho=0.082$ , with eCrCl was  $r^2=0.22$  and with serum Cyst C level was  $\rho=0.085$  ( $p>0.05$ ). Concerning CD4+ T cell count, the correlation with serum Cr was  $\rho=-0.021$ , with eCrCl was  $r^2=0.101$  and with serum Cyst C was  $\rho=-0.106$  ( $p>0.05$ ).

### **Evaluation of Cyst C and associations with risk factors for kidney injury**

In order to study the correlation among the overall results of serum Cyst C levels with serum Cr and eCrCl determinations, a Spearman's rank correlation coefficient was applied. It showed a positive correlation of serum Cyst C and serum Cr as well as estimated CrCl ( $p<0.01$ ) (figure 12).



**Figure 12** – Correlation between Cr, Cyst C and estimated CrCl.

Overall Cyst C levels were plotted against Cr and estimated CrCl for each patient. CrCl was estimated using the Cockcroft-Gault equation. Correlations were assessed with Spearman's rank correlation coefficient. Correlations were considered significant for a  $p < 0.01$ .

Cr- creatinine; Cyst C- cystatin C; CrCl- creatinine clearance.

To evaluate the variance of serum Cyst C with age, gender, ethnicity and BMI a multiple linear regression with log transformed Cyst C as the dependent variable was performed (table 11). The variance obtained was low ( $r^2=0.079$ ) and did not reach a significant proportion ( $p > 0.05$ ). In comparison, for the same parameters, the variance with serum Cr ( $r^2=0.523$ ) and with eCrCl ( $r^2=0.178$ ) as the dependent variable, was higher and reached a significant proportion ( $p < 0.05$ ).

Associations among biomarkers and risk factor for kidney injury were also tested. Associations between increased Cyst C levels (greater than 0.96 mg/L) with hypertension, current smoking and HCV co-infection were found to be statistically

significant ( $p < 0.05$ ). In opposition, associations between decreased levels of eCrCl, increased serum Cr (higher than 1.2 mg/mL) and the above mentioned risk factors did not reach significance ( $p > 0.05$ ).

**Table 11.** Multiple linear regression model for age, gender, ethnicity and BMI.

<b>Independent variables</b>	<b>Unstandardized coefficient</b>	<b>Standardized coefficient</b>	<b>p-value</b>
<b>Constant</b>	-0.139	----	0.225
<b>Age</b>	0.001	0.111	0.517
<b>Gender</b>	-0.042	-0.231	0.349
<b>Ethnicity</b>	0.000	0.002	0.994
<b>BMI</b>	0.001	0.024	0.891

$$r^2 = 0.079; p = 0.553$$

Predictor variables are constant, age, gender, ethnicity and BMI and the dependent variable is Log transformed cystatin C.

BMI = body mass index.

### ***Patients with discordant biomarkers determinations***

Using serum Cr determinations, 10 patients (4%) were identified as having kidney dysfunction (Cr levels were higher than 1.2 mg/mL). From these, five patients were black, one had hypertension, one had diabetes, four patients were co-infected with HCV and their ages were between 30-77 years old.



Using eCrCl, 12 patients (5%) were identified as having kidney dysfunction (levels lower than 60 mL/min). When serum Cyst C levels were used, 27 patients (11%) were identified as having kidney dysfunction (higher than 0.96 mg/L) (table 12). Seventeen patients that had normal eCrCl showed increased levels of Cyst C. All these patients were male, six had hypertension, six patients were co-infected with HCV and 14 patients were current smokers.

**Table 12.** Results of eCrCl and cystatin C in 242 HIV-infected patients.

	Normal eCrCl	Reduced eCrCl	Total
Normal cystatin C	213	2	215
High cystatin C	17	10	27
<b>Total</b>	230	12	242

Normal cystatin C is defined by serum levels below or equal to 0.96 mg/L and high cystatin C by serum levels higher than 0.96 mg/L. Normal estimated creatinine clearance (eCrCl) is defined by levels equal to or higher than 60 mL/min and reduced eCrCl by levels below 60 mL/min.

**Table 13.** Comparison between two subgroups of patients: within normal range and elevated cystatin C levels.

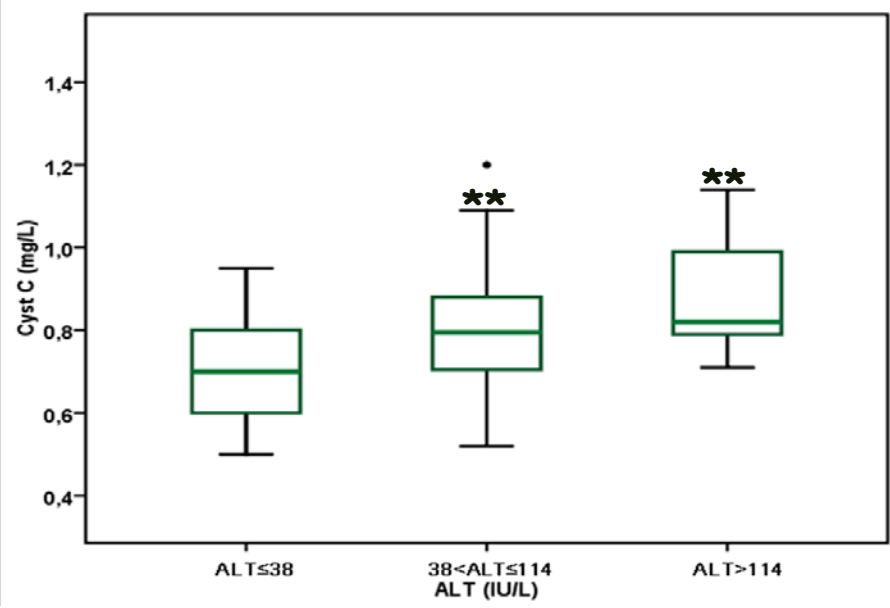
	<b>Cyst C&gt;0,96 mg/L eCrCl&gt;60 mL/min n=17</b>	<b>Cyst C≤0,96 mg/dL eCrCl&gt;60 mL/min n=27</b>	<b>p- value</b>
<b>Age</b>	47±9.9	47.8±5.9	
<b>Male gender</b>	17	27	p>0.05
<b>Years of infection</b>	15.47±3.35	16.23±3.88	
<b>CD4+ T cell count (cells/mm<sup>3</sup>)</b>	762.88±432.9	919.47±293.47	
<b>HIV RNA level (copies/mL)</b>	<40	<40	
<b>HIV non-related factors</b>			
<b>Smoking</b>	14	21	p>0.05
<b>Hypertension</b>	6	3	
<b>Diabetes</b>	0	2	
<b>Antiretroviral therapy</b>			
<b>TDF</b>	4	12	p>0.05
<b>ATV/r</b>	5	4	
<b>TDF+ATV/r</b>	3	1	
<b>w/o TDF, ATV/r</b>	5	9	
<b>HBV co-infection</b>	0	3	p>0.05
<b>HCV co-infection</b>	10	0	p<0.05

Cyst C- cystatin C; eCrCl- estimated creatinine clearance; TDF- tenofovir disoproxil fumarate; ATV/r- atazanavir boosted with ritonavir; ART- antiretroviral therapy; HCV- hepatitis C virus; w/o- without.

Regarding cART on this group (table 12), four patients were on TDF (range 2-5 years of TDF), five patients were on ritonavir boosted atazanavir (ATV/r) (range 1-5 years of ATV/r) and three patients were on cART with both TDF and ATV/r (5 years).

The above mentioned results were further analysed and compared to a matched control group of 27 patients, who had normal Cyst C levels. These two groups were not statistically different in terms of age, time of infection, viral load, or CD4+ T cell count (table 13).

Of note, while ten patients with high Cyst C levels had HCV co-infection, none of the patients in the matched control group did.



**Figure 13-** Box-plot of cystatin C levels in patients with HIV/HCV co-infection, according with the levels of ALT.

ALT levels were considered normal when inferior or equal to 38 IU/L (n=21). The remaining groups correspond to moderate ALT increase (n=31) and an increase over three times the normal upper limit (n=11).

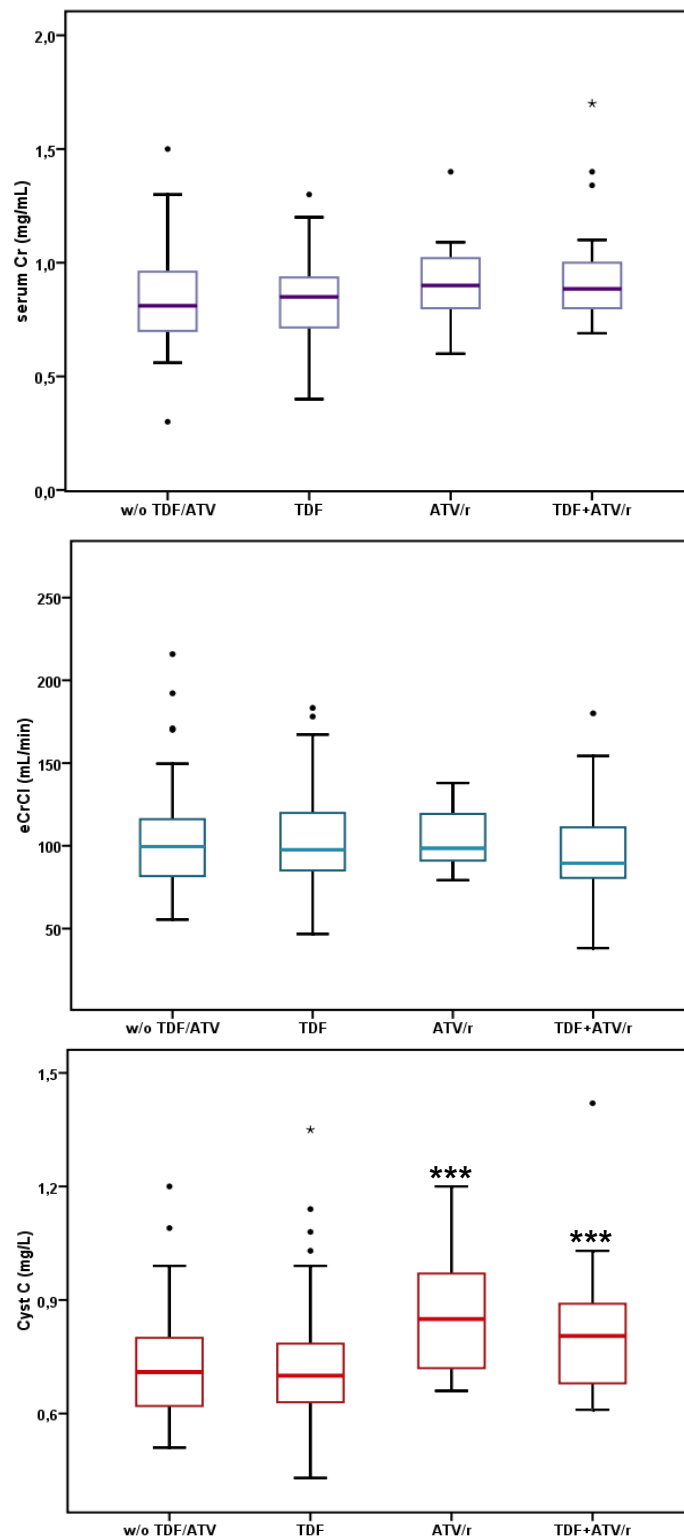
\*\* p-value <0.05; ALT – Alanin transaminase

To test if there was an association between high levels of Cyst C and increased levels of alanin transaminase (ALT) and aspartate transaminase (AST), a Mann-Whitney U test was performed with Cyst C and different levels (within normal range, slightly elevated, and more than threefold the normal range) of ALT and AST, in the patient group co-infected with HIV/HCV (figure 13). It was not found a statistically significant association between increased levels of AST and high level of Cyst C. On the other way, it was found a statistically significant association between increased levels ALT and higher levels of Cyst C compared with the group with  $ALT \leq 38$  ( $p < 0.05$ ).

### **Analysis of kidney function in patients with different antiretroviral regimens**

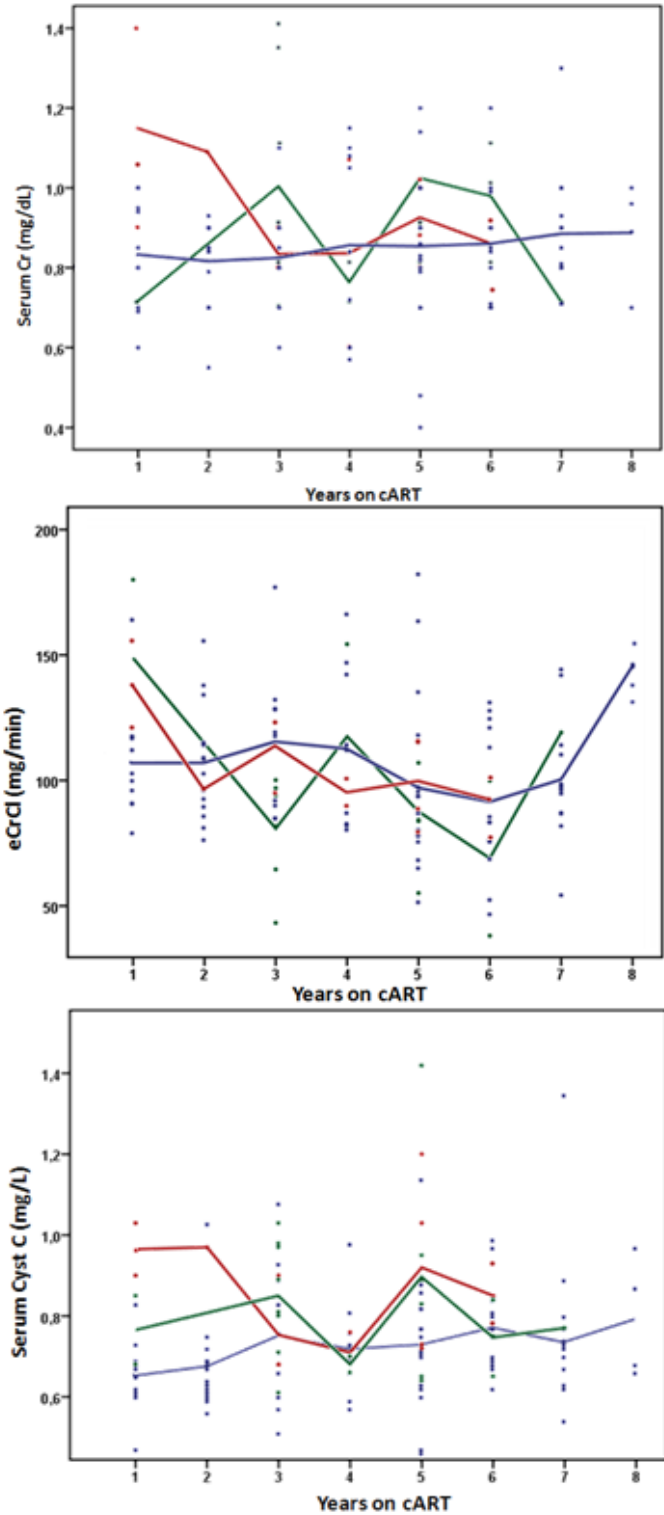
A possible association between high levels of Cyst C, Cr and decreased eCrCl and patients currently on cART with TDF and/or ATV was also studied. Patients currently on TDF, ATV or both drugs were compared to patients who were also on cART but were never on these two antiretroviral drugs.

Statistically significant ( $p < 0.05$ ) associations were found between high levels of Cyst C and cART with ATV/r, as well as cART with TDF and ATV/r (figure 14). However, no statistically significant association was found between these antiretroviral drugs and Cr or eCrCl levels.



**Figure 14-** Box-plot of Cyst C and eCrCl levels per cART group.

w/o TDF/ATV- without tenofovir and atazanavir (n=67); TDF- with tenofovir (n=92); ATV/r- with atazanavir boosted with ritonavir (n=13); TDF+ATV/r- with tenofovir and atazanavir (n=29).Patients with IDV in the past were excluded. Cyst C-cystatin C; eCrCl-estimated creatinine clearance; cART-combination antiretroviral therapy. \*\*\*p<0.05 (group w/o TDF/ATV has reference); •-outliers.



**Figure 16-** Cr, eCrCl and Cyst C determinations per year on cART.

The dots represent individual measurements and the lines represent the mean value of those measurement. The color blue represents patients on tenofovir, red patients on atazanavir and green patients on both antiretroviral drugs. Patients with indinavir in the past were excluded from the analysis. Cr-creatinine; eCrCl-estimated creatinine clearance; Cyst C-cystatin C

Finally, it was studied the kidney function levels of patients on cART with TDF, ATV/r and both antiretroviral drugs (figure 15). Using Cr measurements, patients on ATV/r appear to have higher levels in the first two years on this drug. For patients on TDF, mean Cr levels seemed to remain constant, while for patients on TDF and ATV/r, Cr levels appeared to be higher after the first year on these drugs (figure 16). Estimated CrCl measurements gave similar results to Cr, with a difference on the group of patients on TDF that appeared to have lower mean levels after five years on this antiretroviral drug. Regarding Cyst C measurements, for patients on TDF, it appeared to demonstrate a constant mild increase over the years. For patients on ATV/r, Cyst C levels were higher in the first two years on this drug and also in those who were on ATV/r for five or six years. Concerning the patients on TDF and ATV/r, Cyst C levels demonstrated a variation similar to eCrCl and Cr.

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## Discussion

Since the introduction of effective cART, life expectancy of HIV-infected individuals has increased dramatically<sup>183</sup>. With that, conditions such as kidney, cardiac and hepatic diseases gained importance as causes of morbidity and mortality among this population<sup>2</sup>.

As HIV-infected patients have a known increased risk of kidney disease when compared to the general population<sup>6-8</sup>, a reliable biomarker that enables the detection of mild kidney dysfunction is essential and much needed. Similarly to what was seen with the discovery of troponin I, as an earlier biomarker of myocardial injury, in terms of the impact on in-time decision making and institution of therapy, it is also needed an earlier biomarker of kidney dysfunction, in order to protect the kidney from further injury and prevent the evolution to CKD and end-stage kidney disease.

Previous studies seem to indicate that serum Cr levels are influenced by factors such as body mass, age, gender and ethnicity. The estimation of CrCl or GFR enables the reduction of this effect. However, it is still influenced by limitations associated with Cr determination.

In the present study we tested a potentially alternative biomarker, serum Cyst C against the biomarker most commonly used in clinical practice (serum Cr) in a population of HIV infected patients, followed-up at an Outpatient Clinic, HSM, CHLN. Our sample population was mostly composed by Portuguese, Caucasian males of 45 years old (median), and the majority of patients had acquired the infection through the sexual route (78%) or by intravenous drug use (21%). This sample seem to be representative of the general Portuguese HIV-positive population since, according

with data from the Instituto Nacional de Saúde Dr. Ricardo Jorge, in 2009, 81.4% of the HIV-infected individuals were male, 83.3% had ages between 20-49 years, and HIV-infection was acquired mainly by the sexual route (homo- and heterosexual: 72.7%) and intravenous drug use (23.6%)<sup>184</sup>.

In our study, patients also had a BMI within normal range (normal range: 19-25 Kg/m<sup>2</sup>; median BMI 24 Kg/m<sup>2</sup>), approximately half of the patients were smokers and also 7% had documented history of alcohol abuse. The high rate of smokers was expected, since it is known that a large fraction of HIV-patients smoke cigarettes<sup>185</sup>. Comorbidity conditions that constitute risk factors for kidney disease were present, such as hypertension and diabetes, in 24% and 11% of patients, respectively. According to a study from the Portuguese Cardiology Society, the VALSIM study, the Portuguese hypertension prevalence, for the adult population, was 42.6%<sup>186</sup> which is considerably higher than the levels obtained in our population. This was not expected since our sample population was older than this population. In addition, a study from the Portuguese Diabetes Society, the PREVADILAB study, which included 5,167 adults from different regions of the country, showed that 11.7% had diabetes<sup>187</sup> which is in accordance with our results.

Hypertension and diabetes constitute important risk factors for kidney disease, especially hypertension, since kidney dysfunction can worsen hypertension which, as a consequence, can lead to further kidney damage. The number of patients with hypertension on our study was inferior to the general population numbers. This seems to indicate that this risk factor does not constitute a major cause of kidney disease on this population.

The patients included in our study had a long length of HIV infection (median 14 years) and median CD4+ T cell *nadir* was 216/mm<sup>3</sup>. It is known that HIV infection induces a chronic immune activation and inflammation in patients successfully treated with cART, possibly due to residual HIV replication below the level of detection of standard plasma HIV RNA assays<sup>188</sup>. It also seems to be an association between inflammation and kidney dysfunction. In a pilot study from Gupta *et al.*<sup>189</sup>, HIV infected patients with proteinuria had higher urinary levels of two inflammatory cytokines (MCP-1 and RANTES) when compared to HIV infected patients without proteinuria. With this fact in mind, it was supposed that patients infected for longer would have higher kidney dysfunction rates. However, this was not found in our study. It should be noted that this study could have missed an eventual association since confounding factors are present due to the retrospective characteristics of the study. On the other hand, the mentioned study from Gupta *et al.* did not fully elucidated if the increase on inflammatory biomarkers reflected systemic or intrarenal inflammation. In order to truly determine the association of systemic inflammation and kidney dysfunction it would be necessary to analyze a representative sample of patients with different lengths of untreated infection, which is extremely difficult. Nevertheless, the CD4+ T cell *nadir* can give us some information on this matter, assuming that a lower *nadir* represent longer lengths of infection. In our study, although the three biomarkers seemed to indicate a slightly lower kidney function with patients with a CD4+ *nadir* lower than 200/mm<sup>3</sup>, it was not found a statistically significance difference between the biomarkers levels, which is in accordance with the previous analysis with the length of infection. These results seem to indicate that

the time of exposure to HIV-infection is not the determinant factor on the occurrence of kidney dysfunction.

The median CD4+ T cell was 686/mm<sup>3</sup> which is above the levels generally associated with HIV-related opportunistic infections (CD4+ T cell count lower than 500/mm<sup>3</sup>). Several opportunistic infections, such as, *Pneumocystis jirovecii* or Cytomegalovirus infection, as well as therapies for these infections, can cause kidney dysfunction. However nowadays, with the advent of cART, opportunistic infections do not constitute an important cause of kidney disease of HIV-infected patients in the developed World.

All patients that were on cART (n=225), with the exception of 11 patients with documented treatment failure, had HIV-RNA levels below 40 copies/mL. Since our patients had a long length of HIV infection, they also had a long duration of cART (mean 12 years). The most used combination of antiretroviral drugs was two NRTI and one NNRTI (n=126). This was expected since the two most used combinations are two NRTI plus either one NRTI or one ritonavir boosted PI. Clinicians often choose the NNRTI efavirenz instead of a PI/r, since it was demonstrated that this drug is as effective as the PIs<sup>190</sup> and allow the postponement of PI-associated toxicity.

From the overall regimens, 118 patients were currently on TDF and 53 patients on ATV/r, of which 34 were on a combination of both drugs. Therefore, these patients not only have a potential risk associated with a long duration of infection, but also the risk of nephrotoxicity associated with a long course with some antiretroviral drugs.

Twenty six percent of patients were co-infected with HCV, probably associated with previous sharing of drug injection material, since among these patients almost 90%

acquired HIV-infection by the intravenous drug use route. This percentage is in accordance with European data which estimates that 20-40% of HIV infected patients are co-infected by HCV<sup>191</sup>. Only five patients were co-infected with HBV, mainly due to the fact that the majority of our sample population was Portuguese, where the vaccine against HBV is present on the vaccination plan since 1994<sup>192</sup>.

Concerning the analysis of kidney function through the different strategies, comparisons were always performed between serum Cr, estimated CrCl and serum Cyst C determination. It was not found any statistically significant difference in the biomarkers levels, between patients without treatment and those currently on cART. However, we only had seven treatment *naïve* patients and 10 in treatment interruption in our sample population which limits the statistical analysis. In order to take conclusions, it would be required a similar number of patients in this group. It was also not found any significant difference between the biomarker levels and the duration of infection. While when these levels were analyzed per year on cART, although it was not found any difference with Cr and eCrCl, with serum Cyst C it was found that patients who were on cART for less than five year had lower levels of Cyst C when compared with patients that were on cART for more than five years. These results seem to indicate that Cyst C may have detected mild kidney function changes that the other biomarkers did not detect and, if that so, the length of exposure to antiretroviral drugs may have a more important impact on kidney function than the years exposed to the HIV-infection.

Correlations between the HIV-infection status (HIV-RNA levels and CD4+ T cell count) and the three biomarkers was low (between 0.021 and 0.22;  $\pm 0.7-1.0$  – highly correlated;  $\pm 0.5-0.69$ - moderately correlated;  $0-\pm 0.49$ - poorly correlated) and did not

reach a significant proportion. Although it was already mentioned, in previous studies, that Cyst C had a correlation with HIV-RNA levels and CD4+ T cell count, this was not found in our study. However, it should be noted that our patient group had a long course of antiretroviral therapy, maintaining suppression of viral replication. In one study from Mauss *et al.*<sup>193</sup>, Cyst C levels correlated negatively with CD4+ T cell count and positively with HIV RNA levels in treatment *naïve* patients, while after antiretroviral therapy initiation, serum Cyst C levels decreased. On the other hand, it is possible that high HIV RNA plasma levels might also correspond to an increased HIV replication in kidney cells, resulting in local inflammation and injury with increase of Cyst C levels<sup>194,195</sup>.

Correlations of serum Cyst C with Cr and eCrCl were performed in order to study the congruence between determinations. Cyst C was positive correlated with both but had a higher correlation with eCrCl.

In our study, as in other published studies<sup>113, 114,196</sup> age, gender, ethnicity and BMI, had no significant influence on Cyst C levels contrary in what was found with serum Cr and eCrCl. This fact allows an increase in sensitivity when compared to serum Cr, especially in patients with low BMI, where eGFR overestimation occurs when assessed with Cr. In fact, studies have demonstrated that Cockcroft-Gault and MDRD-based GFR estimations can produce different results in patients with low BMI or during weight changes<sup>197</sup>. This point raises a special concern in HIV-infected patients, where the initiation of antiretroviral therapy might be accompanied of weight gain and therefore, these eGFR alterations may be falsely attributed to new drug regimens.

Kidney dysfunction rates obtained were different, and varied according to the biomarker used. While using serum Cr, 4% (n=10) of patients were identified as having kidney dysfunction, when eCrCl was used, 5% (n=24) were identified. These results are in accordance with the previously mentioned HSM study, where the kidney dysfunction rate, calculated with eCrCl, was 5.4%. Cyst C was the biomarker that showed higher rates of kidney dysfunction with twice as much patients (n=27), then when using eCrCl. Elevated Cyst C levels in normal eGFR settings have been previously described as preclinical kidney disease and have been associated with an increased risk of CKD, cardiovascular events and eventually death<sup>17, 198, 199</sup>. In the present study, 7% of patients (n=17) were identified as having preclinical disease, according to this concept. The majority of these patients (n=11) were in cART with TDF, ATV/r or both antiretroviral drugs (for at least one year), which have been occasionally referenced in the literature as causative agents of tubular dysfunction, Fanconi's syndrome and acute interstitial nephritis<sup>77, 200, 201</sup>. Furthermore, in a recent study with a large cohort of 6,843 patients, increasing exposure to both antiretroviral drugs was associated with a higher incidence of CKD<sup>202</sup>.

On the other hand, in our study, 14 out of 17 patients were current smokers and the majority had HCV co-infection (n=10). Moreover, this study showed that elevated Cyst C levels were associated with current smoking and HCV co-infection besides hypertension and diabetes. In comparison to the overall sample population, these 17 patients were two years older and had one more year of infection (mean) which is not statistically significant. However, the smoking (71%) and hypertension (35%) rates were considerably higher than the overall population rates (49% and 24% respectively). In the matched control group (n=27), with Cyst C levels within normal

range, a major fraction of the patients also had cART with TDF, ATV/r or both. However, none had HCV co-infection. In addition, a statistically significant association was also demonstrated between higher levels of Cyst C and ALT in patients co-infected with HCV.

These facts might indicate that Cyst C could be flagging inflammation rather than kidney dysfunction.

More than two decades ago, Cyst C had already been referenced as a potent regulator in inflammatory processes<sup>203</sup>. Posterior studies have reported increased Cyst C levels in patients with several conditions such as autoimmune diseases<sup>204</sup>, asthma<sup>205</sup> and cigarette smoking<sup>206</sup>. Recently, it has been demonstrated that elevated Cyst C levels are associated with high concentrations of inflammation markers such as, C-reactive protein, tumour necrosis factor- $\alpha$  and Interleukin-6<sup>206, 207</sup>. HCV infection is associated with persistent liver inflammation that can lead to progressive fibrosis. In this process, several enzymes including cathepsins (cysteine proteinases) have been found to be involved<sup>208</sup>.

As cathepsins' activity is increased in the fibrotic liver<sup>209</sup>, Cyst C as a specific inhibitor of these enzymes should also have increased levels. Actually, two previous studies showed that serum Cyst C concentrations were significantly higher in patients with hepatic diseases when compared to the control group. Both studies indicated that Cyst C may be useful for monitoring progression of liver fibrosis<sup>210, 211</sup>.

Regarding the analysis of the kidney function of patients under different cART regimens, although no statistically significant association was found with Cr nor with eCrCl, elevated Cyst C was associated with cART with ATV/r and TDF+ATV/r. In addition, for patients on antiretroviral combinations including either ATV/r, TDF or



TDF+ATV/r, kidney function changes were analyzed per years on these antiretroviral drugs. Using the three biomarkers, patients on ATV/r appear to have lower kidney function in the first two years on this drug, while with TDF, only Cyst C seemed to demonstrate a mild gradual decrease on kidney function during the years. Therefore, while with ATV/r kidney dysfunctions may emerge earlier, with TDF the decrease of kidney function might be gradual and, therefore, be missed by determinations with serum Cr and eCrCl. However, because this is a cross-sectional study, it cannot fully evaluate the long-term impact of antiretroviral drugs. More studies are necessary to assess the true impact of the more recent antiretroviral drugs on kidney function.

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With the increasing life expectancy of HIV-infected patients and the toxicity to which they are subjected due to the various drugs, it is well known the increasing importance of monitoring kidney function in these patients, as AKI and CKD are becoming commoner. Nevertheless, besides serum Cr determination, there are no alternative biomarkers to assess early kidney dysfunction, nor there are validated formulas for GFR estimation in this population.

In the last years, Cyst C has been referenced in several studies as a biomarker that could replace Cr. However, in the present study, Cyst C levels, although not influenced by age, gender, BMI and ethnicity, seemed to be influenced by other factors besides kidney function.

As Cyst C seems to vary with inflammation markers in HIV-infected patients, where a chronic systemic inflammation is present and often associated with other inflammation sources, such as HCV co-infection, this biomarker might lead to an overestimation of kidney impairment. Therefore, caution must be taken when analysing kidney function using Cyst C levels alone.

In this current time of severe cost restrains, another aspect that deserves our attention is the fact that serum Cyst C determination is around seven-fold more expensive than serum Cr determination. Therefore the practical clinical benefits that justify opting for the use of this tool, also need to be further analysed.

One limitation of this study was not having a reference method for GFR determination, such as inulin or  $^{51}\text{Cr}$ -EDTA. Still, the aim of this study was to

compare two non-invasive kidney function biomarkers – the most widely used method in clinical practice (Cr) and the alternative biomarker Cyst C, in the context of HIV infection.

Another limitation was the fact that it was a cross-sectional study. Although it allowed us to recognize some advantages and limitations of Cyst C as an alternative marker of kidney function, still, to definitively acknowledge the ability of Cyst C to detect mild kidney dysfunctions, earlier than Cr, it would be necessary to perform a prospective analysis.

In addition, an initial objective of testing another promising biomarker –NGAL- was not fulfilled in-time for this dissertation, due to time constrains.

In what concerns the nephrotoxicity associated with cART, an association was found between increased levels of Cyst C and cART either with ATV/r or with TDF+ATV/r. In addition, using Cyst C appears that patients on TDF show a mild kidney function decrease over the years. Even though no statistically significant associations were found with increased levels of serum Cr nor with decreased eCrCl, or associations with cART with TDF, we believe that it is extremely important to continue the development of prospective research projects, in order to assess the real long-time impact of these antiretroviral drugs on kidney function. Until then, one should take in consideration the benefits and risks of prescribing these recent antiretroviral drugs, in comparison with those that are available for several years and that clinicians already have the knowledge and experience on the possible complications associated with their long-term use.

Unfortunately, it seems that Cyst C does not completely overcome the limitations associated with serum Cr kidney function determination and does not seem to constitute the final answer to this problematic.

It seems crucial to continue the research in this field, focusing in particular on the development of potential novel biomarkers that could enable an earlier detection of a kidney insult.

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Introduction

Objectives

Material and Methods

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Concluding remarks

Bibliography

**Annexes**

## Annexes

Part of the present work was used to write an original paper that, at the moment of elaboration of this dissertation, was ready to submit to the journal *Clinical Infectious Diseases*.

1

- 1 **Title:** Cystatin C as a marker for kidney disease in HIV infected patients – take a second look
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