GFR meets mTOR: value of different methods to measure and estimate GFR & (side) effects of mTOR inhibition in renal transplantation

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The subject of this thesis is twofold: where GFR and mTOR meet. Precise measurement of kidney function is difficult and cumbersome and many, simpler alternatives have been developed to determine GFR. Determination of GFR remains an approximation since the GFR itself is not a static phenomenon. This should be kept in mind when interpreting GFR results. mTOR inhibitors are a new class of immunosuppressive drugs and increasingly used in renal transplantation. They seem to be most promising due to their lack of nephrotoxicity, their potential anti-atherosclerotic effects and their anti-oncogenic effects. However, with the increased use side effects are becoming more clear. Individual tailoring of the immunosuppressive regimen will be the answer to decrease adverse effects and to benefit the most from the advantages of inhibition of the mTOR pathway.
GFR meets mTOR

Value of Different Methods to Measure and Estimate GFR
&
(Side) Effects of mTOR Inhibition In Renal Transplantation

Marije Catharina Baas
GFR meets mTOR
Value of Different Methods to Measure and Estimate GFR &
(Side) Effects of mTOR Inhibition In Renal Transplantation

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 ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
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Marije Catharina Baas
geboren te Hoorn
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Faculteit der Geneeskunde
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Part I

Value of Different Methods to Measure and Estimate GFR
1 General Introduction
Part I
GENERAL INTRODUCTION

The glomerular filtration rate (GFR), the sum of the filtration rate of all functioning nephrons, is used to express kidney function. For many reasons, adequate measurement of renal function is important in patients at risk for or suffering from overt renal failure. Early detection of renal impairment is relevant since it can have major implications for decisions about treatment. Moreover, exact calculation of GFR necessary in the evaluation and follow-up of possible beneficial or harmful interventions. Furthermore, the dose of many drugs has to be adjusted for renal function; overdosing can cause an increase in adverse side effects or can further worsen kidney function. Finally, GFR is an important predictor for all-cause mortality due to especially cardiovascular disease and is thereby a prognostic factor in itself.

The Kidney Disease Outcomes Quality Initiative (K/DOQI) has provided guidelines to optimize the care of patients with chronic kidney disease (CKD), according to a five-stage classification that is based on kidney function. Stage I: GFR $\geq$ 90 ml/min/1.73m$^2$ with signs of kidney damage (defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine test or imaging studies), stage II: 60 - 89ml/min/1.73m$^2$ with signs of kidney damage, stage III: 30 - 59 ml/min/1.73m$^2$, stage IV: 15 - 29 ml/min/1.73m$^2$ and stage V: < 15 ml/min/1.73m$^2$. Each stage of kidney disease asks for a different clinical action plan and is accompanied by its own hazard ratio for mortality and morbidity. Therefore, accurate classification of patients with CKD is important for appropriate treatment and evaluation.

Many attempts have been made to find an ideal method to determine GFR. GFR can be measured or can be estimated. So far, inulin clearance or the use of radioactive markers are considered as the gold standard, but these methods are expensive and cumbersome. Plasma creatinine is the best known and most often used endogenous marker for GFR.

In the next sections, the currently used methods for GFR determination will be reviewed and new methods for GFR measurement and GFR estimation will be discussed.

Methods for GFR measurement

Two approaches to measure GFR can be used. The first one uses a continuous infusion of a tracer. Before the actual measurements are done, a steady plasma level of the tracer is required, enabling the calculation of renal clearance from urine collections. Confounders such as extrarenal clearance and variation in distribution are minimised in this way. The other approach is the single injection method, which measures whole body clearance from the plasma disappearance rate. The main advantage of the latter method is its relative simplicity.
Inulin clearance

Inulin is a starch (fructose polymer) found in the tubers and roots of many plants. Inulin clearance is considered the gold standard, since it has all the characteristics of an ideal marker. Inulin is a low molecular weight solute (5200 D), not bound to plasma proteins and is therefore freely filtered by the glomerulus. No readsoption or secretion takes place in the tubular system. There is only minimal secretion into the bile. The classic method of measuring inulin clearance includes intravenous administration of a priming dose followed by a constant infusion. GFR is calculated as $U \times V/P$, where $U$ = inulin concentration in the urine, $V$ = the urine flow rate per unit of time, determined by bladder catheterization and $P$ = the inulin concentration in plasma.

However, the use of this method has important drawbacks: measurement in laboratories is complex and cumbersome, inulin is not widely available and the use of a bladder catheter for investigation should be avoided.

$^{125}$I-labeled iothalamate/$^{131}$I-labeled hippuran continuous infusion method

This method was first introduced in 1977, enabling simultaneous determination of GFR with $^{125}$I-iodotatamate and effective renal plasma flow (ERPF) with $^{131}$I-hippuran. This makes it possible to calculate the filtration fraction (FF). The administration of $^{131}$I-labeled hippuran makes it possible to correct for incorrect urine collection, making bladder catheterization unnecessary and improving accuracy.

Single bolus injection with $^{51}$-chromium-ethylenediaminetetraacetic acid ($^{51}$Cr-EDTA)

$^{51}$Cr-EDTA is a widely accepted and commonly used method using a single bolus injection with measurement of the plasma disappearance rate of $^{51}$Cr-EDTA. Similar to each injected substance, the plasma concentration curve after a single intravenous injection consists of two phases: an initial rapid decrease representing the distribution of the injected marker in the extra-cellular volume and a second slower decrease representing its clearance. $^{51}$Cr-EDTA is mostly filtered by the glomeruli but extra renal clearance can be as high as 10%. The radiation load is minimal. Other filtration markers used to determine GFR are $^{99}$mTc-diethylenetriaminepenta-acetic acid ($^{99}$mTc-DTPA) or iohexol (a non-ionic, low osmolar radiocontrast medium).

Methods for GFR estimation

GFR estimation by plasma creatinine

Since methods to measure GFR are labour intensive as well as expensive, endogenous markers are used as an alternative to estimate GFR. As mentioned earlier, plasma creatinine is the best known and most commonly used endogenous marker for estimation of GFR, since Popper et al. first described the use of creatinine as a marker for GFR in 1937. Creatinine is a breakdown product of creatinephosphate in muscle tissue. The word ‘creatinine’ (or ‘kreatinin’) is derived from the Greek word ‘kreas’, which means flesh. It is produced at a relatively constant rate, depending on the muscle mass, and filtered in the glomeruli but also actively secreted in the proximal
tubule. Tubular secretion contributes normally to 10 - 40% of renal creatinine removal, but increases when GFR decreases, causing plasma creatinine to remain in the normal range until GFR drops below 60-70 ml/min ('creatinine blind range'). Significant nephron loss can therefore have occurred before a change in creatinine occurs. If GFR decreases below 10 ml/min, extrarenal clearance increases, probably mediated by intestinal microflora\textsuperscript{10-12}. Although plasma creatinine is the most commonly used marker, it has several other limitations. It is influenced by muscle mass and therefore by age, gender and race. Medication, for example prednisolone, can also affect plasma creatinine\textsuperscript{13, 14}. Furthermore, the method used for its determination contributes to variation in the measured value of plasma creatinine. The alkaline picrate (Jaffé) method is influenced by non-creatinine chromogens like glucose, acetoacetate and antibiotics\textsuperscript{15, 16}. Therefore, this method is nowadays more often replaced by an enzymatic determination. But also the enzymatic method can be influenced by exogenous compounds, like dopamine, 5-flucytosine and bilirubin\textsuperscript{17}.

Another factor which is important to interpret plasma creatinine values, is whether the creatinine assay is calibrated to the reference isotope dilution mass spectroscopy (IDMS) creatinine standard. Errors in calibration make little difference in estimating severely decreased GFR (<30 mL/min/1.73 m\textsuperscript{2}), but result in progressively larger differences at higher GFRs\textsuperscript{18}. This factor is especially important when creatinine values of different laboratories are compared.

To estimate GFR, creatinine clearance can be calculated using the following formula: GFR x P = U x V (i.e filtered load), where ‘P’ is the plasma concentration of creatinine, ‘U’ the urine concentration of creatinine and ‘V’ the volume of the urine. This calculation does not correct for tubular secretion. Measurement of creatinine clearance using this method becomes more reliable after the administration of cimetidine, which inhibits tubular secretion\textsuperscript{19-21}.

To overcome some other limitations of creatinine as a marker for GFR, several formulas have been constructed to correct for the influences of weight, age, gender and/or race. The most commonly used formulas (and their advantages and limitations) are listed in the next section.
Creatinine-based formulas

Table 1. Plasma creatinine (Pcr) is expressed in μmol/l, urea in mmol/l, albumin in g/l, weight in kg and height in m.

<table>
<thead>
<tr>
<th>formula</th>
<th>Population in which the formula is developed</th>
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</thead>
<tbody>
<tr>
<td>Cockcroft and Gault&lt;sup&gt;22&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance = ([140- age] x weight)/Pcr</td>
<td></td>
</tr>
<tr>
<td>(male, multiply result by 1.23, female: multiply result by 1.05)</td>
<td>236 Canadian patients (209 male)</td>
</tr>
<tr>
<td>6-variable MDRD&lt;sup&gt;23&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>GFR = 170 x (Pcr ÷ 88.4)&lt;sup&gt;-0.999&lt;/sup&gt; x age&lt;sup&gt;-0.176&lt;/sup&gt; x (P&lt;sub&gt;urea&lt;/sub&gt; x 2.8)&lt;sup&gt;-0.170&lt;/sup&gt; x (P&lt;sub&gt;albumin&lt;/sub&gt;/10)&lt;sup&gt;0.318&lt;/sup&gt;</td>
<td>1628 patients with CKD</td>
</tr>
<tr>
<td>(female: multiply result by 0.762, black multiply result by 1.180)</td>
<td></td>
</tr>
<tr>
<td>4-variable MDRD&lt;sup&gt;24, 25&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>GFR = 175 x (Pcr ÷ 88.4)&lt;sup&gt;-1.154&lt;/sup&gt; x age&lt;sup&gt;-0.203&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>(when creatinine is measured using a non IDMS traceable method)</td>
<td></td>
</tr>
<tr>
<td>(female: multiply result by 0.742, black multiply result by 1.210)</td>
<td></td>
</tr>
<tr>
<td>CKD-EPI&lt;sup&gt;26&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Female and Pcr ≤ 62 umol/l: GFR = 144&lt;sup&gt;*&lt;/sup&gt; x ((Pcr/ 88.4) / 0.7)&lt;sup&gt;-0.529&lt;/sup&gt; x (0.993)&lt;sup&gt;MP&lt;/sup&gt;</td>
<td>8254 patients with various causes of CKD and healthy kidney donors</td>
</tr>
<tr>
<td>Female and Pcr &gt; 62 umol/l: GFR = 144&lt;sup&gt;*&lt;/sup&gt; x ((Pcr/ 88.4) / 0.7)&lt;sup&gt;-1.209&lt;/sup&gt; x (0.993)&lt;sup&gt;MP&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Male and Pcr ≤ 80 umol/l: GFR = 141&lt;sup&gt;**&lt;/sup&gt; x ((Pcr/ 88.4) / 0.9)&lt;sup&gt;-0.411&lt;/sup&gt; x (0.993)&lt;sup&gt;MP&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Male and Pcr &gt; 80 umol/l: GFR = 141&lt;sup&gt;**&lt;/sup&gt; x ((Pcr/ 88.4) / 0.9)&lt;sup&gt;-1.209&lt;/sup&gt; x (0.993)&lt;sup&gt;MP&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>* use 166 if black</td>
<td></td>
</tr>
<tr>
<td>** use 163 if black</td>
<td></td>
</tr>
<tr>
<td>Nankivell&lt;sup&gt;27&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>GFR = 6.7/Pcr + weight/4 – urea/2 – 100/height&lt;sup&gt;2&lt;/sup&gt;</td>
<td>146 renal transplant patients</td>
</tr>
<tr>
<td>(male: add 35, female: add 25)</td>
<td></td>
</tr>
</tbody>
</table>

All formulas have their specific limitations. The Cockcroft and Gault formula generally overestimates GFR because of tubular secretion of creatinine. Especially in patients with a bodymass index above 30 kg/m<sup>2</sup>, whereas it systematically underestimates GFR in elderly people<sup>28</sup>. The 6-variable MDRD formula is derived from patients in the United States with non-diabetic renal disease, with a mean
GFR of 40 ml/min/1.73m². The included patients were largely Caucasian, but the formula seems also accurate across a wide range of subgroups²⁹. To simplify the 6-variable MDRD formula, the 4-variable (abbreviated) MDRD was constructed in which measurement of urea and albumin are not necessary. There are two formulas of the abbreviated MDRD, depending on the method used for the determination of creatinine (i.e. whether creatinine values are standardised to creatinine reference materials using gold standard techniques or not)²⁴, ²⁵. The MDRD and the abbreviated MDRD underestimate GFR in young women and also overestimate GFR in patients with a body mass index below 18.5 kg/m², since these patients have a very low creatinine, reflecting low muscle mass.

Furthermore, the MDRD formulas are less accurate at GFR > 60 ml/min/1.73m² and systematically underestimate true GFR. Attempts have been made to construct new formulas for healthy persons³⁰-³². Recently, the CKD-EPI equation has been developed to be more accurate at higher GFRs²⁶. The CKD-EPI indeed performs better in the higher ranges of GFR³⁵, ³⁴ leading to higher estimates of kidney function and resulting in less CKD (defined as estimated or measured GFR < 60 ml/min/1.73m²) on a general population based level by reclassification of low-risk individuals³⁵, ³⁶ However, it may lead to an increased incidence of CKD in the elderly (> 70 years) since the formula results in lower GFR estimates in this group compared to the MDRD formula³⁷, ³⁸

The Nankivell formula was specially developed for renal transplant recipients (RTR), using 711 GFR measurements in 146 patients²⁷. Thus many data points were repeated measures in the same patients and not independent samples. Although derived in kidney transplant recipients, the Nankivell equation did not perform well in that same group of patients, and was even less accurate than the MDRD equation³⁹. But when GFR is above 60 ml/min/1.73m², the accuracy and precision of the formula improved and exceeded that of the MDRD and Cockcroft-Gault formula⁴⁰. Recently, it was demonstrated that the CKD-EPI performed better in RTR than the MDRD⁴¹ although all equations to estimate GFR overestimate measured GFR⁴².

So far, none of these formulas seem to be good enough to substitute measured GFR, but they seem acceptable to discriminate patients with chronic kidney disease.

New methods and markers
Since the above mentioned methods and markers for GFR determination have their specific limitations, the search for new ones continues.

A new method to measure GFR is the single injection method of ‘Gd-DTPA’, using gadolinium DTPA (Magnevist®). This method is a non nuclear method and can therefore be used in facilities without a nuclear department. Plasma concentrations of gadolinium are measured using an ELISA technique. The whole body clearance is calculated from the plasma disappearance rate⁴³-⁴⁵. Although recently a number of articles has been published describing the occurrence of nephrogenic systemic fibrosis after exposure to gadolinium in patients with end-stage renal failure⁴⁶, ⁴⁷, this is a rare event occurring in patients receiving gadolinium in the context of an MRI scan.
The incidence of nephrogenic systemic fibrosis appears to depend on the total dose of administered gadolinium. Since GFR measurement with Gd-DTPA uses an amount of gadolinium that is only 5% of the dose used with MRI, the risk of developing nephrogenic systemic fibrosis is seems negligible. So far, GFR measurements using Gd-DTPA have only been performed in dogs and in a small group of patients, including renal transplant patients.

Relatively new endogenous markers are cystatin C and beta-trace protein (βTP). Cystatin C is a low molecular weight protein (13.3 kD) that functions as a cysteine protease inhibitor. It is produced at a constant rate by all nucleated cells and removed from the body by glomerular filtration, thereafter reabsorbed and broken down in the proximal tubule\(^\text{48, 49}\). Cystatin C production is thought not to be influenced by sex, age, bodyweight or muscle mass\(^{50, 51}\) although this is contradicted by some reports\(^{52}\). However, cystatin C is influenced by thyroid function; in overt hypo- and hyperthyroidism, but also in subclinical states\(^{53}\). In hyperthyroidism cystatin C concentrations increase and normalize after euthyroidism is achieved. In hypothyroidism, the opposite occurs\(^{54, 55}\). Whether this reflects a change in GFR is not completely clear; plasma creatinine values rise when hyperthyroidism is corrected and decrease when hypothyroidism is corrected. Treatment with corticosteroids also increases cystatin C levels by increasing the production of cystatin C\(^{56-58}\). Several studies showed that cystatin C is a more sensitive indicator of mild reductions of renal function than creatinine\(^{50, 59-65}\). Moreover cystatin C is not removed during intermittent hemodialysis\(^{66}\) or continuous venovenous hemodiafiltration\(^{67}\) and it has been advocated a marker for residual renal function in dialysis patients\(^{68}\).

### Table 2. Cystatin C (cysC) is expressed in mg/l

<table>
<thead>
<tr>
<th>formula</th>
<th>Population in which the formula is developed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoek(^{62})</td>
<td>GFR = -4.23 + 80.35/cysC, 93 patients with various causes of renal disease</td>
</tr>
<tr>
<td>Larsson(^{71})</td>
<td>GFR = 77.24 x cysC(^{-1.2623}), 100 patients with various causes of renal disease</td>
</tr>
<tr>
<td>Rule(^{69})</td>
<td>Native CKD: GFR = 66.8 x cysC(^{1.30}), 460 healthy subjects, patients with various causes of renal disease and renal transplant patients</td>
</tr>
<tr>
<td></td>
<td>Transplant recipient: GFR = 76.6 x cysC(^{1.16}),</td>
</tr>
</tbody>
</table>

Several formulas have been constructed for cystatin C to express GFR\(^{62, 69-73}\). The formulas are each extracted from and validated in different patient populations and the ones used in the articles in this thesis are summarized in Table 2.

**Creatinine- and cystatin C based formula**

From pooled data from the MDRD study\(^{23}\), the African American Study of Kidney Disease (AASK)\(^{74}\), the Collaborative Study Group (CSG)\(^{75}\) and a clinical population in
Table 3. Creatinine (Pcr) is expressed in µmol/l, cystatin C (cysC) is expressed in mg/l

<table>
<thead>
<tr>
<th>Formula</th>
<th>Population in which the formula is developed</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR = 177.6 x (Pcr/88.4)^-0.65 x cysC^-0.57 x age^-0.20</td>
<td>3418 patients with CKD (pooled data)</td>
</tr>
</tbody>
</table>

Paris\textsuperscript{76} a creatinine and cystatin C combined formula was constructed\textsuperscript{76}. This formula was more accurate than the formulas based on creatinine or cystatin C alone, but this formula requires further testing in various patients groups.

\textbf{Beta-trace protein (bTP)}\textsubscript{77}, also known as lipocalin prostaglandin D2-synthase, is another low molecular weight protein (22-26 kD, depending on the degree of posttranslational glycosylation\textsuperscript{77}). It is mainly produced in the central nervous system (leptomeninges, choroid plexus epithelium and oligodendrocytes) and forms one of the principal proteins of the cerebrospinal fluid (CSF), which contains the highest bTP concentration of all body fluids. Male gonads, the human heart, spleen, bone marrow, thymus also produce bTP and it has been found in serum, urine, ascites and seminal plasma. The concentration of bTP in serum and urine is very low compared to that in CSF\textsuperscript{78-81}. Glycosylation patterns of bTP differ in cerebrospinal fluid, serum and urine. bTP in the brain (bbTP) is smaller, due to truncated oligosaccharide side-chains and absent sialyzation. bbTP is metabolized in the liver and therefore cleared from the circulation; the sialyzed glycoforms are eliminated by glomerular filtration\textsuperscript{77}. Both isoforms of bTP can be distinguished by immunonephelometry. bTP is advocated as an early marker for deterioration of renal function in the ‘creatinine blind range’\textsuperscript{77, 82-84}. bTP might also be of value to estimate residual renal function in patients treated with hemodialysis (HD) or hemodiafiltration (HDF), since HD and HDF do not induce clinical relevant alterations on bTP serum levels. However, HDF reduced bTP levels by more than 20\% in some patients. bTP serum concentrations appeared strongly associated with residual diuresis (i.e. probably Gfr) in these patients\textsuperscript{85}.

Like cystatin C, bTP also appears to be influenced by the use of corticosteroids. In contrast to cystatin C, bTP decreases during the use of prednisolone\textsuperscript{86, 87}. The mechanism underlying the decrease in bTP is not elucidated yet. Down-regulation of bTP synthesis, permeability changes of the blood-brain barrier or increased extrarenal elimination are proposed mechanisms\textsuperscript{77, 87}. A comparison between eGFRs using cystatin C and bTP, showed no benefit of bTP over cystatin C\textsuperscript{87}.

The following GFR-equations have been constructed in adults for estimation of GFR, using bTP plasma-levels (Table 4):
Chapter 1

Patients at Risk

Many renal transplant patients develop renal failure. This can be multi-factorial. In the first months after transplantation acute rejection is the main cause of kidney deterioration. Later on, calcineurin-inhibitor toxicity, chronic rejection, chronic allograft nephropathy and BK-nephropathy become important. Each of these conditions demands a different treatment. Early detection is critical, so that further diagnostic procedures such as a kidney biopsy can be performed without delay.

In the past years renal transplantation with a kidney from a living donor has gained more and more grounds due to organ shortage and excellent results. Nowadays half of the kidney transplantations in the Netherlands are performed with living donors. This has increased the urge to accurately assess renal function in potential donors, ensuring that pre-donation renal function is above a certain minimal standard and minimizing the risk of future renal failure in these donors. Reliable assessment of pre-donation kidney function is therefore necessary to exclude those patients with diminished kidney function.

Renal function in HIV-infected patients can be affected by the HIV-infection itself, manifesting as interstitial nephritis, collapsing FSGS or thrombotic microangiopathy, or by other HIV-related infections, like post-infectious glomerulonephritis. Furthermore, nephrotoxicity is the dose-limiting toxicity associated with certain components of highly active retroviral therapy (HAART), such as cidofovir, adefovir and tenofovir.

Fabry’s disease is an X-chromosomal-linked lysosomal storage disorder. Due to deficient activity of the lysosomal enzyme α-galactosidase A, glycosphingolipids, mainly globotriaosylceramide (Gb-3) or ceramide trihexoside, accumulate in lysosomes of various cell types. The kidney is also affected because of lysosomal storage in endothelial, mesangial, interstitial cells and podocytes. Proteinuria is an early sign of renal involvement. Microvascular endothelial deposits of Gb-3 cause ischaemic injury, global glomerulosclerosis, tubular atrophy and interstitial fibrosis. Renal replacement therapy may already be necessary in the third and fourth decade of life. Renal function is an important predictor of mortality. Since enzyme replacement therapy (ERT) became available for patients with Fabry disease, the course of the disease has altered. Studies have shown that the treatment benefit of ERT is more pronounced in patients with a better renal function at baseline.

Table 4. Beta-trace protein (βTP) is expressed in g/l, urea in mmol/l

<table>
<thead>
<tr>
<th>Formula</th>
<th>Population in which the formula is developed</th>
</tr>
</thead>
<tbody>
<tr>
<td>White^88 [ GFR = 112.108 \times \beta TP^{0.662} \times \text{urea}^{0.280} ] (female: multiply result by 0.88)</td>
<td>163 Renal transplant patients</td>
</tr>
<tr>
<td>Pöge^89 [ GFR = 89.85 \times \beta TP^{0.5541} \times \text{urea}^{0.3018} ]</td>
<td>187 Caucasian renal transplant patients</td>
</tr>
</tbody>
</table>
Breunig et al. showed that a decline of GFR to 71 ml/min already was a risk factor for the development of a clinical end point (death, cardiac and cerebrovascular event, renal failure)\textsuperscript{92} stressing the importance of adequate monitoring of renal function to assess the effect of ERT.

**AIM AND OUTLINE OF PART I OF THIS THESIS**

GFR is measured and estimated in renal transplant recipients, healthy kidney donors, HIV-infected patients and patients with Fabry disease. As mentioned above, these patients are at risk of developing renal failure and normal formulas to estimate GFR may not apply to them because of low muscle mass, interference of medication with the markers for GFR or because their GFR is above 60 ml/min and creatinine will not be able to detect renal deterioration. Chapter 2 discusses the value of formulas using other markers than plasma creatinine, i.e. cystatin C and $\beta$-trace protein, to estimate GFR in Fabry patients treated with enzyme replacement therapy. The new non nuclear method to measure GFR with gadolinium (Gd-DTPA) as an alternative to $^{51}$Cr-EDTA is examined in chapter 3, in which simultaneous GFR measurements with Gd-DTPA and $^{51}$Cr-EDTA are performed in renal transplant recipients, healthy kidney donors, HIV- and Fabry patients.

Since creatinine is cleared from the circulation by dialysis and continuous venovenous hemofiltration (CVVH), it cannot be used as a parameter of renal function in patients who receive this form of renal replacement therapy. However, in some situations, it would be very useful to be able to estimate residual renal function to determine if renal replacement therapy is still necessary. In chapter 4, cystatin C is examined as an alternative marker for residual GFR in critically ill patients treated with CVVH because of acute renal failure.


20. Kemperman FA, Silberbusch J, Slaats EH, Prins AM, Krediet RT, Arisz L. Follow-up of GFR estimated from plasma creatinine after cimetidine administration in...


58. Bjarnadottir M, Grubb A, Olafsson I. Promoter-mediated, dexamethasone-


The Value of Estimated GFR in Comparison to Measured GFR for the Assessment of Renal Function in Adult Patients with Fabry Disease

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ABSTRACT

Background  Renal disease is one of the major complications in Fabry disease, an X linked lysosomal storage disease due to deficiency of the enzyme α-galactosidase A. The aim of our study was to determine the value of creatinine-, cystatin C- and beta-trace- based formulas for the estimation of glomerular filtration rate (eGFR) in Fabry patients. For comparison, the gold standard method $^{125}$I-labelled iothalamate/$^{131}$I-labelled hippuran (mGFR) was used.

Methods  GFR was estimated by using eleven different formulas based on creatinine, cystatin C and beta-trace protein. Accuracy and precision, detection of early decline of renal function and follow-up of renal function by eGFR was compared to mGFR.

Results  136 GFR measurements and plasma samples were available from 36 (20 male) Fabry patients, treated with agalsidase α or β with a median follow-up of 3.1 (range 1.5 – 5.2) years. Median mGFR was 97.3 (15.5-148.6) ml/min/1.73 m$^2$ in males and 84.4 (23.0-131.0) ml/min/1.73 m$^2$ in females at start of follow-up.

Conclusions  Although none of the investigated endogenous markers proved to be an equivalent substitute for mGFR in Fabry patients, the Stevens equation, a creatinine and cystatin C based formula, most closely approximated the mGFR. When a creatinine based formula is preferred, considering that there is no standardized method available for cystatin C, the aMDRD and the recently developed CKD-EPI had the best performance. In male Fabry patients, the aMDRD may overestimate GFR, especially in the higher ranges. In these cases, CKD-EPI may perform better.
INTRODUCTION

Renal disease is one of the major complications in Fabry disease, an X-linked lysosomal storage disease due to deficiency of the enzyme α-galactosidase A. Accumulation of several glycosphingolipids, the main compound being globotriaosylceramide (Gb3), occurs in various cell types. This results in complications of mainly vascular origin, of which progressive renal insufficiency, cardiac hypertrophy and cerebral infarctions are the most severe. Due to the X-linked nature of the disease, males are most severely affected but females can express symptoms as well. In the kidney, extensive storage has been identified in glomerular, tubular, vascular and interstitial cells. Renal involvement can already be present during childhood. Before the era of enzyme replacement, the mean age of onset of clinical nephropathy (i.e. proteinuria or chronic kidney disease (CKD)) has been reported for male Fabry patients to occur at the age of 27 years. Thereafter, the mean rate of GFR decline was 12.2 ml/min/year. Overall, 13 - 23% of male Fabry patients and approximately 3% of female patients developed ESRD. Before decline of renal function, hyperfiltration can be present early in the course of the disease. Enzyme replacement therapy (ERT) is available since 2001 and improvement of cardiac disease and stability of renal disease is better achieved in patients with a preserved renal function. Early detection of renal impairment is therefore important. Unfortunately, an easy and accurate method to assess renal function is lacking. GFR can be measured as the clearance of exogenous or endogenous filtration markers. The use of exogenous marker methods such as inulin clearance or nuclear methods are considered to be the gold standard for the assessment of renal function in Fabry patients. However, these methods are expensive and time consuming. Creatinine is the most commonly used marker. Apart from the fact that creatinine is influenced by muscle mass, age, sex and race, loss of renal function may already have occurred without a significant rise in plasma creatinine. The MDRD and abbreviated MDRD (aMDRD), based on creatinine, are commonly used. A recent study showed that by using the MDRD formula in Fabry patients, GFR is overestimated more specifically in male Fabry patients at the higher end of GFR range. This hampers early detection of a decline in renal function. Recently a new formula, the CKD-EPI has been published, developed to assess GFR more accurately, especially in the higher ranges of GFR. This formula may therefore be of additional value in Fabry disease.

Cystatin C (cysC) is another endogenous marker. It is a low molecular weight protein (13.3 kD), produced at a constant rate by all nucleated cells and is freely filtered by the glomerulus and completely reabsorbed and broken down in the proximal tubule. Its production is not influenced by sex, age, bodyweight or muscle mass, although it can be influenced by thyroid dysfunction and large dosages of glucocorticoids. CysC appears to be a more sensitive marker than creatinine to detect a decline in renal function and is reported to detect changes in GFR below 80 ml/min. To our knowledge, besides one review, only one study has been published investigating cysC in Fabry nephropathy, concluding that cysC is a sensitive and reliable marker. However, a reference method in that study was lacking.
Beta-trace protein (βTP) is also advocated as an early marker for deterioration of renal function in the so-called “creatinine blind range” 29-32. βTP, also known as lipocalin prostaglandin D2-synthase, is a low molecular weight protein (22-26 kD) 29, that is mainly produced in the central nervous system (leptomeninges, choroid plexus epithelium and oligodendrocytes). Furthermore, it is found in serum, urine, ascites and seminal plasma. Although a comparison between eGFRs using cysC and βTP in paediatric renal transplant patients showed no benefit of βTP over cysC 33, so far no studies have investigated the value of βTP as a parameter of renal function in patients with Fabry disease.

The aim of the present study was to determine the value of creatinine-, cysC- and βTP - based formulas for the determination of GFR and the follow-up of renal function in Fabry patients in comparison to a gold standard method, consisting of a continuous infusion of 125I- iothalamate in combination with 131I-hippuran 34-36.

SUBJECTS AND METHODS

Patients
The Academic Medical Center in Amsterdam is a tertiary referral center for lysosomal storage diseases in the Netherlands since 1999.

Currently 100 Fabry adult patients are closely monitored in our outpatient clinic. Yearly, GFR measurement with 125I-labelled iothalamate/ 131I-labelled hippuran is routinely performed in patients on ERT. All Fabry patients in whom 3 or more GFR measurements were available for follow-up (n = 36), were included.

The diagnosis of Fabry disease was made by demonstrating decreased enzyme activity in α-galactosidase A in leukocytes in males as well as genotyping in both males and females. Patients were either treated with agalsidase-α (Replagal, 0.2 mg/kg/ 2 weeks) or algalsidase β (Fabrazyme 0.2 mg/kg/ 2 weeks or 1.0 mg/kg/ 2 weeks). Thirty one patients participated in earlier ERT trials 37, 38. Patients provided informed consent for reanalysis of samples.

Measurements
Plasma samples, frozen at -20 °Celsius, collected during follow-up of treatment were used for this analysis. Creatinine, cysC and βTP measurements were performed in samples from one year after start of treatment due to lack of availability of samples before start of treatment. Samples for measurements of creatinine, cysC and βTP were obtained at the same day. Plasma samples were taken with a median of 1 day (range -83 to + 97 days, with one exception of 175 days) before or after GFR measurement. Urine samples were taken one day before the plasma sample was taken.

Creatinine was measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyser (Roche, Almere, the Netherlands). The creatinine determinations were calibrated according to the IDMS traceable creatinine standard.
CysC was measured in heparinized plasma samples with the N Latex Cystatin C test kit, a particle-enhanced immunonephelometric method, on a BN ProSpec analyser (Siemens, Breda, the Netherlands).

bTP was measured in heparinized plasma samples with the N Latex b-Trace Protein test kit on a BN ProSpec analyser (Siemens, Breda, the Netherlands).

**Estimated GFR (eGFR)**

We chose formulas to estimate GFR which had been constructed in adult patient populations with various causes of renal failure \(^{13, 27, 39-45}\). For bTP, only formulas constructed from data of renal transplant recipients with decreased renal function (mean mGFR < 60 ml/min/1.73 m\(^2\)) were available \(^{42, 45}\). The formulas used to estimate GFR are given in Table 1.

All results are presented in ml/min/1.73m\(^2\). The Cockcroft and Gault formula, 24h urinary creatinine clearance and the eGFR using the Larsson formula were corrected for body surface area (BSA) using the duBois formula \(^{46}\).

**Gold standard GFR measurement**

A method with continuous infusion of 125I-iothalamate and 131I-hippuran was used to determine GFR \(^{34-36}\). With this method, GFR is calculated as the mean urinary clearance of [125I] iothalamate of two 2h periods after a 2h equilibration period. Corrections are made for incomplete urinary collections by using 131I-hippuran and for fluctuations in plasma concentrations. GFR was then corrected for BSA using the duBois formula \(^{46}\).

**Statistical analysis**

Statistical analyses were performed using SPSS16. Demographic data were expressed as median (range). The Wilcoxon signed ranks test was used to compare differences in GFR course over time. For comparison between males and females, we used the Mann-Whitney U test. A p < 0.05 was considered statistically significant. For comparison of the various eGFR methods with mGFR, we used Bland and Altman analysis \(^{47}\). Accuracy was defined as the mean difference between mGFR and eGFR. Limits of agreement were defined as mean difference -2SD and mean difference +2SD and precision as ± 1 SD. To correct for repeated measurements, we used a correction factor proposed by Bland and Altman \(^{48}\). To compare the accuracies of the various formulas we used Student’s t-test.

To investigate whether cysC and/or bTP would be superior to creatinine to detect decreased renal function, receiver operating characteristic (ROC) curves were used. A decreased renal function was defined as a GFR < 90 ml/min/1.73 m\(^2\). MedCalc statistical software was used to compare AUC of the ROC curves.
Table 1. Formulas based on creatinine, beta trace and cystatin C for estimation of GFR.

**Creatinine-based:**
1. MDRD formula\(^{13}\):
   \[
   \text{GFR} = 170 \times \left(\frac{P_{\text{cr}}}{88.4}\right)^{0.997} \times \text{age}^{-0.176} \times \left(\frac{P_{\text{urea}} \times 2.8}{0.81}\right)^{-0.170} \times \left(\frac{P_{\text{album}}}{10}\right)^{0.318}
   \]
   (Female: multiply result by 0.762, if black multiply result by 1.180)
2. Abbreviated MDRD\(^{41}\):
   \[
   \text{GFR} = 175 \times \left(\frac{P_{\text{cr}}}{88.4}\right)^{-1.154} \times \text{age}^{0.203}
   \]
   (Female: multiply result by 0.742, if African-American multiply result by 1.210)
3. CKD-EPI\(^{16}\):
   Female and Pcr ≤ 62 umol/l: GFR = 144 x \((P_{\text{cr}} / 88.4) / 0.7\)x \(0.993)^{\text{age}}\)
   Female and Pcr > 62 umol/l: GFR = 144 x \((P_{\text{cr}} / 88.4) / 0.7\)^{1.209} x \(0.993)^{\text{age}}\)
   Male and Pcr ≤ 80 umol/l: GFR = 141 x \((P_{\text{cr}} / 88.4) / 0.9\)^{0.411} x \(0.993)^{\text{age}}\)
   Male and Pcr > 80 umol/l: GFR = 141 x \((P_{\text{cr}} / 88.4) / 0.9\)^{1.209} x \(0.993)^{\text{age}}\)
* use 166 if black
** use 163 if black
4. Cockcroft and Gault formula\(^{39}\):
   \[
   \text{Creatinine clearance} = \frac{(140 - \text{age}) \times \text{weight (kg)}}{P_{\text{cr}}}
   \]
   (Male: multiply result by 1.23, if female: multiply result by 1.05)
5. 24h\(_{\text{urine}}\) creatinine clearance:
   \[
   \text{creatinine clearance} = \frac{U_{\text{cr}} \times V}{P_{\text{cr}}}
   \]

**Cystatin C-based:**
6. Hoek formula\(^{27}\):
   GFR = -4.23 + 80.35/cysC.
7. Larsson formula\(^{40}\):
   GFR = 77.24 x cysC\(^{-1.263}\)
8. Rule formula\(^{43}\):
   GFR = 66.8 x cysC\(^{-1.30}\)

**Beta-trace protein based:**
9. White formula\(^{45}\):
   GFR = 112.108 x βTP\(^{-0.662}\) x urea\(^{-0.280}\)
   (Female: multiply result by 0.88)
10. Pöge formula\(^{42}\):
    GFR = 89.85 x βTP\(^{-0.5541}\) x urea\(^{-0.3018}\)

**Creatinine- and cystatin C-based:**
11. Stevens formula\(^{44}\):
    GFR = 177.6 x \((P_{\text{cr}} / 88.4)\)^{0.65} x cysC\(^{-0.57}\) x age\(^{0.20}\)
    (Female: multiply result by 0.82, black: multiply result by 1.11)

**RESULTS**

**Patients**
Thirty-six patients (20 (56%) male, 16 female) underwent 136 GFR measurements.
The median number of GFR measurements per patient was 4 (range 3 – 5); in males 4 (range 3-5), in females 3.5 (range 3-5). Median follow-up was 3.1 years (range 1.5–5.2) in males 3.1 (1.8-5.2), in females 2.2 (1.5-4.0) years). At the time of the first renal assessment, 30 patients were treated for one year with enzyme replacement therapies and 6 patients for 2 to 5 years: 10 with agalsidase α at 0.2 mg/kg, 13 with agalsidase β at 0.2 mg/kg and 13 with agalsidase β at 1.0 mg/kg. BMI,
weight and height are given in table 2. Table 3 shows the distribution of patients and number of measurements according to mGFR. Twenty seven and thirty one patients participated in the previous ERT trials\textsuperscript{37,38}.

**Table 2.** Demographics at start of follow-up. Data are expressed as median (range).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BSA</th>
<th>BMI (kg/m²)</th>
<th>GFR (ml/min/1.73 m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>20</td>
<td>(18.7-63.0)</td>
<td>(60.0-97.0)</td>
<td>(164-191)</td>
<td>(1.66-2.23)</td>
<td>(18.8-29.8)</td>
<td>(15.5-148.6)</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>(17.1-72.5)</td>
<td>(58.0-100.0)</td>
<td>(153-178)</td>
<td>(1.58-2.15)</td>
<td>(20.3-35.0)</td>
<td>(23.0-131.0)</td>
</tr>
<tr>
<td>All</td>
<td>36</td>
<td>(17.1-72.5)</td>
<td>(58.0-100.0)</td>
<td>(153-191)</td>
<td>(1.58-2.23)</td>
<td>(18.8-35.0)</td>
<td>(15.5-148.6)</td>
</tr>
</tbody>
</table>

There was a significant decline in GFR between the first and last measurement from 89.0 (15.5-148.6) ml/min/1.73m² towards 83.4 (11.5-146.6) ml/min/1.73m² (p = 0.004). This decline can be completely attributed to the decline in GFR in males (from 97.3 (15.5-148.6) to 79.3 (11.5-146.6) ml/min/1.73m², p = 0.002 in males vs 84.4 (23-131.0) to 88.3 (18.2-133.1) ml/min/1.73m², p = 0.51, in females). The median decline of GFR per year was 1.9 (range – 4.0 to 15.2) ml/min/1.73m² in males and -0.26 (range -7.2 to 16.7) ml/min/1.73m² in females. Six patients (4 males) showed hyperfiltration (> 125 ml/min/1.73m²).

**Table 3.** Distribution of patients and number of measurements according to mGFR.

<table>
<thead>
<tr>
<th>GFR (ml/min/1.73 m²)</th>
<th>All</th>
<th>Male</th>
<th>Female</th>
<th>Number of measurements (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 90</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td>62 (45 %)</td>
</tr>
<tr>
<td>60-89</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>34 (25%)</td>
</tr>
<tr>
<td>30-59</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>24 (18%)</td>
</tr>
<tr>
<td>&lt; 30</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>16 (12%)</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>20</td>
<td>16</td>
<td>136 (100%)</td>
</tr>
</tbody>
</table>

**Receiver operating characteristic (ROC) curve**

To discover the most sensitive and specific non-invasive method for detection of a decline in GFR < 90 ml/min/1.73m², ROC curves were plotted for creatinine, cysC and βTP. The area under the curve (AUC) was largest for CysC (0.877), compared to creatinine (0.839) and βTP (0.791). The cutoff value to detect a GFR < 90 ml/min/1.73m² was 69.5 μmol/L (sens 81%, spec 76%) for creatinine, 0.90 mg/L (sensitivity 75%, specificity 90 %,) for cysC and 1.04 mg/L (sensitivity 68%, specificity, 77%) for βTP.
All the respective formulas performed better than the AUC of plasma creatinine, cysC and βTP alone, all with an AUC ≥ 0.838. The Stevens formula had the highest AUC (0.926), Cockcroft and Gault (0.918), 24h\textsubscript{urine} creatinine clearance (0.916), CKD-EPI (0.894), aMDRD (0.889), Hoek (0.884) and Rule (0.884) had the best AUC to detect a GFR < 90 ml/min/1.73m\(^2\). Figure 1 shows the ROC curves of the best performing formula of each biomarker: in case of creatinine and cysC combined the Stevens formula, in case of creatinine the Cockcroft and Gault formula, for cysC the Hoek formula and for βTP the White formula.

**Figure 1.** ROC-curves of the Stevens formula, Cockcroft and Gault, Hoek formula and the White formula to detect a GFR < 90 ml/min/1.73m\(^2\). AUC Stevens 0.926 (solid line —), AUC Cockcroft and Gault 0.918 (dashed-line ——) and AUC Hoek 0.884 (dotted line ⋯⋯) and AUC White 0.887 (dashed-dotted line —⋯⋯).

**Accuracy and limits of agreement of different methods to estimate the GFR**

Bland and Altman analysis of the MDRD-, aMDRD-, CKD-EPI, 24h\textsubscript{urine} creatinine clearance-, Cockcroft and Gault-, Larsson-, Hoek-, Rule, White- Pöge- and Stevens equations are shown in Figure 2. Table 4 shows the level of performance of each formula to accurately estimate GFR within 10%, 20%, 30% and 50% of the mGFR, respectively.

The Stevens formula approximated mGFR most closely with 41.9% of cases within 10 % range of mGFR and 81.6 % within 30% of mGFR. However, the Rule formula had 87.5% of cases within 30% range of mGFR but only 36.8% of cases within 10% range of mGFR. The aMDRD, the Larsson- and the Hoek formulas showed the best overall accuracy (Figure 2) and were within 30% range of mGFR in 77.9 %, 77.9%
Figure 2. Bland and Altman analysis of the MDRD (A), aMDRD (B), CKD-EPI (C), 24h urine creatinine clearance (D), Cockcroft and Gault (E), Larsson (F), Hoek (G), Rule (H), White (I), Pöge (J) and Stevens (K) versus gold standard (GFR) using isotope GFR ($^{125}$I-iothalamate/$^{131}$I-hippuran) formula. Males are represented as •, females as △.
and 80.1 % of all cases, respectively (Table 4). 24h urine creatinine clearance and Cockcroft and Gault systematically overestimated GFR in both males and females; MDRD, aMDRD, CKD-EPI and Stevens systematically overestimated GFR in males but not in females. CKD-EPI performed better in the higher range as compared to the MDRD and aMDRD. Hoek and Larsson overestimated GFR in females. In males, however, the Hoek, Larsson and Rule underestimated GFR in the higher ranges. The White and Pöge formula underestimated GFR in both males and females. The limits of agreement of all formulas were large, but smallest using the Stevens, CKD-EPI, MDRD and aMDRD formulas.

Subgroup analysis showed that when GFR was < 60 ml/min/1.73m$^2$ (40 measurements), the performance (i.e. accuracy and limits of agreement) of the βTP-based formulas exceeded that of the creatinine- and cysC-based equations. Age did not influence the performance of the formulas. Overall, accuracy and limits of agreement were smaller in females compared to males.

**Follow-up**

Figure 3 shows the follow-up of renal function by the mGFR and the creatinine-based (aMDRD and CKD-EPI), cysC-based (Hoek) and combined formula (Stevens). The latter had the best limits of agreement as shown by the Bland and Altman analysis (figure 2). Only the first three consecutive measurements (i.e. two years of follow-up) are shown, since the number of patients who had more than three sequential GFR
measurements, was too small (number of patients after 3, 4 and 5 years of follow-up was 23, 7 and 2 patients, respectively).

The Hoek formula using cysC did not demonstrate a decline in renal function for males and females combined, where the mGFR, aMDRD, CKD-EPI- and Stevens formula did.

**Figure 3.** Course of renal function at start of follow-up (n = 36, 20 males), 1 year (n = 33, 17 males) and 2 years (n = 33, 17 males) of follow-up, measured by isotope GFR (¹²⁵I-iothalamate/¹³¹I-hippuran) (mGFR) and estimated by the aMDRD-, CKD-EPI, Hoek- and Stevens formula. All (A), males (B) and females (C).
DISCUSSION

In this study, we assessed the value of known formulas for estimation of GFR using plasma creatinine, cystatin C and βTP in patients with Fabry disease. The creatinine/cystatin C combined formula (Stevens) showed the highest AUC to detect a decline in renal function below a GFR 90 ml/min/1.73 m². Moreover, the mGFR values during yearly follow-up of renal function were most closely represented by this formula as well. Together with the aMDRD and CKD-EPI, the Stevens formula had the smallest limits of agreement. Even for these best performing formulas, a small proportion of measurements deviated more than 30% from the gold standard measurement of GFR. The aMDRD and CKD-EPI approached the mGFR nearly as well as the Stevens formula but, like the Stevens formula in general overestimated renal function in males. The CKD-EPI formula was constructed to be more accurate at a higher GFR. Indeed in case of hyperfiltration (n = 14 measurements in 6 patients), the CKD-EPI approximated the mGFR value more closely than the aMDRD (Figure 2). At ranges below < 90 ml/min/1.73 m², aMDRD performed slightly better, but both showing overestimation in males. These results are important, since there is clearly a need for a reliable estimate of early kidney failure in Fabry disease patients, especially males. It is hoped that early installation of ERT will prevent deterioration of kidney function and thus adequate monitoring is mandatory. In this respect the Stevens formula would be the preferred method if measured GFR is not feasible. When a cystatin C assay is not available, among the creatinine based formulas, the CKD-EPI provides the most optimal results in the higher range of GFR as compared to the aMDRD, and is therefore the method of choice. Recently the Stevens formula also showed the best performance in a cohort of autosomal dominant polycystic disease patients compared to the MDRD, Cockcroft and Gault formula and CK-EPI formula.

Further validation studies showed the CKD-EPI was more accurate than the aMDRD in patients with diabetes or a renal transplant. In many studies it has been shown that the MDRD and aMDRD may not be accurate enough in non Fabry patients with normal or near normal renal function. Interestingly, in most patients, the aMDRD results in an underestimation of GFR. Aakre et al found that in Fabry patients there is an overestimation of GFR by using the aMDRD in subjects with only slightly impaired renal function. We have observed the same phenomenon in our study, which indicated that probably in this population the aMDRD behaves differently. The reasons for this are incompletely understood. Aakre et al. postulated that a BMI < 20 mg/kg² in males could contribute to this overestimation. In our study however, most males had a normal BMI at start that did not decrease during follow-up.

For the detection of a decline in renal function over time, the same formulas were most accurate: apart form the gold standard mGFR, the Stevens as well as the aMDRD and CKD-EPI formula showed a decrease of GFR in males over 2 years of follow-up. The CysC-based Hoek-formula could only demonstrate a decrease in the first year of follow-up. One other study investigated the value of cystC (Hoek formula)
in Fabry disease and found that it was an early marker for decline of GFR.\textsuperscript{28} In this study no gold standard (mGFR) was used. However, this finding can be interpreted as supportive of our findings that cysC within a formula can add to the precision of eGFR in Fabry disease. For follow-up, formulas based on cysC alone were not superior to creatinine-based or creatinine/cysC combined formulas, in part due to severe underestimation of GFR in the higher range of renal function (mGFR > 125 ml/min/1.73m\textsuperscript{2}, see figure 3). If these measurements (10 in males and 4 in females) were excluded, the accuracy and limits of agreement of the cysC based formulas would improve substantially. Limitations of the use of CysC as a marker for GFR are well known.\textsuperscript{41, 54} Since corticosteroids and thyroid function are known to affect CysC, we analyzed this in our cohort. None of the patients were on daily corticosteroids which could have influenced CysC levels. Only 4 patients had a slightly raised TSH with normal fT4; we could not detect a correlation with plasma CysC. We did not assess the possible influence of inflammation on CysC in our cohort. Another aspect of CysC which limits its use is the lack of standardization and higher costs compared to creatinine measurement.

\(\beta\)TP is reported as a marker of renal function in the so-called ‘creatinine blind range’.\textsuperscript{32} However, in our study we did not find an advantage of \(\beta\)TP-based formulas over creatinine and/or cysC formulas. \(\beta\)TP-based formulas only resembled mGFR more closely when mGFR was < 60 ml/min/1.73m\textsuperscript{2} (data not shown). Since the mean GFR in the Pöge- and White study was < 60 ml/min/1.73m\textsuperscript{2} (40.1 ± 17 and 59 ± 22 ml/min/1.73m\textsuperscript{2}, respectively), their formula was most probably specifically valuable for this population with already severely impaired renal function. In general, we believe that formulas that are based on cysC or beta trace protein alone severely underestimated GFR in the higher GFR ranges, which make them unsuitable for early detection of renal failure in Fabry patients.

A limitation of our study is that blood samples were not taken on the same day but were collected with a median of 1 day (range from -83 to +97 days, with one exception of 175 days, before or after GFR measurement. Although in theory renal function could have changed during this period of time, only in two female patients, in whom the time between mGFR and the blood sample was 9 weeks, the mGFR had declined slightly. Therefore this is not likely to affect our conclusions.

In summary, although none of the formulas based on cysC, \(\beta\)TP or creatinine proved to be an equivalent substitute for mGFR in Fabry patients, we advise to use the Stevens formula for the estimation of GFR since it showed the best performance over the whole range of mGFR, even when hyperfiltration is present. For creatinine based formulas, the aMDRD or CKD-EPI is advised as both performed nearly as well as the Stevens equation. However, one should be aware of slight overestimation of GFR in males. In patients with a higher range GFR, the most accurate estimation could be obtained using the CKD-EPI.
ACKNOWLEDGEMENTS

We would like to acknowledge Karlijn van Stralen of the Department of Medical Informatics for her advice on the Bland and Altman analysis used in this article. The reagents for the BTP determinations were a gift from Siemens, Breda, the Netherlands.

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comparative trial with agalsidase alfa or beta at a dose of 0.2 mg/kg. PLoS ONE 2007; 2(7):e598.


Gadolinium for the Assessment of Glomerular Filtration Rate?

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Submitted
ABSTRACT

**Background** Accurate measurement of renal function using gold standard techniques remains expensive, time consuming and cumbersome. Therefore, the search for cheaper and easier applicable endogenous and exogenous markers continues. Here we studied the performance of a recently developed method using functional immunoassay technology to determine the blood clearance of the nonradioactive marker gadolinium-DTPA (GFR\textsubscript{Gd-DTPA}) to assess glomerular filtration rate (GFR).

**Methods** GFR\textsubscript{Gd-DTPA} was measured in 33 patients at risk of - or with overt renal failure. The GFR\textsubscript{Gd-DTPA} results were compared to both the GFR determined with \textsuperscript{51}Cr-EDTA (GFR\textsubscript{\textsuperscript{51}Cr-EDTA}) and the estimated GFR using creatinine- and/or cystatin C-based formulas. The various estimated GFR and measured GFR methods were compared using Bland and Altman analysis. Bias was defined as the mean difference between reference GFR and investigated GFR. Limits of agreement were defined as mean difference -2SD and mean difference +2SD and precision as ± 1 SD. Accuracy was defined as the percentage of measurements that fell within 30% of the reference GFR.

**Results** Compared to GFR\textsubscript{\textsuperscript{51}Cr-EDTA}, GFR\textsubscript{Gd-DTPA} showed a bias of 9.3 ml/min/1.73m\textsuperscript{2} and precision 16.7 ml/min/1.73m\textsuperscript{2}. Accuracy of GFR\textsubscript{Gd-DTPA} was low with only 72.7% within 30% of GFR\textsubscript{\textsuperscript{51}Cr-EDTA}. GFR estimated by formulas using creatinine and/or cystatin C correlated better with GFR\textsubscript{\textsuperscript{51}Cr-EDTA} than with GFR\textsubscript{Gd-DTPA}.

**Conclusion** In our study, assessment of GFR\textsubscript{Gd-DTPA} with functional immunoassay technology did not prove to be accurate and precise enough. GFR\textsubscript{Gd-DTPA} therefore seems to be unsuitable to replace GFR measurement with radioisotope based techniques.
INTRODUCTION

In patients at risk of - or with overt renal failure, adequate measurement of glomerular filtration rate (GFR) is warranted and often of utmost importance. In case of deterioration of renal function, treatment of the underlying cause and/or adjustment of medication must be initiated without delay.

Many methods are available to determine GFR. Radioisotopes have been studied as filtration markers because they can be used without continuous intravenous (IV) infusion. Moreover, analysis is relatively simple, compared to assessment of GFR with inulin, which is considered gold standard but cumbersome and time-consuming. Recently, GFR measurement using the MRI contrast agent Magnevist® has been proposed as an alternative nonradioactive method.

Apart from measuring GFR (mGFR), many formulas have been developed to estimate the GFR based on plasma creatinine values in combination with other clinical parameters such as weight, gender and age. One of the first and most frequently used formula was that of Cockcroft and Gault, introduced in 1976. Since then, many more formulas have been constructed to estimate GFR (eGFR) from surrogate markers of kidney function using plasma creatinine and more recently cystatin C levels.

In our hospital, the single bolus injection method with 51-chromium-ethylenediaminetetra-acetic acid (51Cr EDTA) is used as gold standard method to determine GFR. In search of a method to measure GFR without the use of radioisotopes, we tested the above mentioned method using a single injection with Gadolinium-Diethylene triamine penta-acetic acid (Gd-DTPA, Magnevist®) and compared this with mGFR using 51Cr EDTA and with eGFR using formulas containing plasma values of creatinine and/or cystatin C.

METHODS

Patients

Patients seen at the outpatient clinic of the Academic Medical Center were eligible to participate in the study in case of: (1) Otherwise healthy kidney donors, in need of a GFR measurement as part of preoperative evaluation, (2) Renal transplant recipients with stable transplant function, (3) HIV infected patients seen in the outpatient clinic who participated in an earlier GFR trial (Vrouenraets et al, manuscript submitted) and (4) Patients with Fabry disease, an X-linked lysosomal storage disease characterized by gradual deterioration in kidney function, in need of a yearly GFR measurement during treatment with enzyme replacement therapy. The patients had to be at least 18 years old, had to show stable renal function as estimated by a stable plasma creatinine during the previous year and had to give written informed consent to participate. Patients were excluded if their eGFR was < 30 ml/min/1.73m² because of the risk of nephrogenic systemic sclerosis, after administration of gadolinium based...
contrast agents\textsuperscript{16,17}. Other exclusion criteria were pregnancy or a known allergy to either $^{51}$Cr-EDTA or Gd-DTPA.

Institutional review board approval for the study was obtained. The study was conducted in accordance with the declaration of Helsinki.

### Measurement of GFR

Patients were instructed not to drink coffee or tea from the evening before and not to eat or drink protein rich products at the day of measurement, as these substances could interfere with GFR measurements. Diuretics were withheld on the day of GFR measurement. GFR was simultaneously measured using a single bolus injection with $^{51}$Cr-EDTA and a single bolus injection with Gd-DTPA.

GFR measurement using $^{51}$Cr-EDTA was performed according to the guidelines of the British Society of Nuclear Medicine\textsuperscript{25}. After a blood blank (P1) is drawn, a single bolus of 3.7 MBq $^{51}$Cr-EDTA was injected in an ante-cubital vein. The syringe was weighed and radioactivity was measured before and after injection to exactly determine the injected amount of $^{51}$Cr EDTA. Blood samples (P2-4, 10 ml each heparinized) were drawn at 2, 3, and 4 hours after the injection with $^{51}$Cr-EDTA. Samples P2-4 were taken from the contra-lateral ante-cubital vein. The samples were centrifuged and 4 ml of plasma was transferred into counting tubes. These were counted together with two 4 ml standards taken as aliquots from 1 MBq $^{51}$Cr-EDTA diluted to 500 ml. Activities of $^{51}$Cr-EDTA in the different samples were determined in a well-type scintillation counter. $^{51}$Cr-EDTA clearance was calculated using the slope intercept method, assuming a one-compartment model. After correction for overestimation\textsuperscript{18}, GFR was corrected for body surface area using the Haycock formula\textsuperscript{19} (mGFR\textsubscript{$^{51}$Cr-EDTA}).

Functional immunoassay technology (FIT) was used to determine GFR after a single bolus injection with Gd-DTPA (469.01 mg dimegluminegadopentetate/ml, Magnevist®) as described by Reinhardt et al\textsuperscript{6}. The blood clearance method was used to determine GFR. After the blood blank (P1) was drawn, a single bolus of 10 ml/kg $^{51}$Cr-EDTA was injected in the same ante-cubital vein as $^{51}$Cr-EDTA. The syringe containing Gd-DTPA was weighed before and after injection on an exacta P5 Bovenweger scale (Optech). The exact injected amount (in ml) of Gd-DTPA was determined as follows: \( \text{weight syringe before} - \text{weight syringe after} \) (g) ÷ density\textsubscript{Gd-DTPA} (g/ml), where the density of Gd-DTPA is 1.195 g/ml at room temperature. Blood samples (P2-5, 4.5 ml serum) were drawn 1, 2, 3, and 4 hours after injection. Samples P2-5 were taken from the contra-lateral ante-cubital vein. Samples were centrifuged and the serum was stored in the refrigerator at 4 °C until analysis (samples could be stored in the refrigerator for at least 6 months; personal communication, Reinhardt, January 2008).

Blood Gd-DTPA concentrations were determined by ELISA using a FIT-GFR kit of BioPAL, Inc., Worcester, MA. For calculation of GFR, a one-compartment blood clearance method was assumed. The data were fit to a one-exponential decay
function: \( Y = Be - bX \). The function was integrated over the limits zero to infinity to obtain the area-under-the-curve (AUC): \( AUC = \frac{B}{b} \) (mg*min/ml). Next, the GFR value was obtained by dividing the administrated dose by the AUC. GFR was corrected for body surface area using the Haycock formula\(^1\) (mGFR\(_{Gd-DTPA}\)).

**Estimation of GFR (eGFR)**

GFR was estimated using the formulas listed in table 1. Plasma creatinine values were measured by an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyzer (Roche, Almere, the Netherlands). The plasma creatinine determinations were calibrated according to the IDMS traceable creatinine standard. Cystatin C levels were measured in heparinized plasma samples by the N Latex Cystatin C test kit, a particle-enhanced immunonephelometric method, on a BN ProSpec analyzer (Siemens, Breda, the Netherlands).

**Table 1. Formulas used to estimate GFR.**

<table>
<thead>
<tr>
<th>Formula Type</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>re-expressed 4-variable (abbreviated) MDRD</strong>(^4)</td>
<td>GFR = ( 175 \times \frac{Pcr}{88.4}^{1.154} \times \text{age}^{-0.203} )</td>
</tr>
<tr>
<td>(Female: multiply result by 0.742, if African-American multiply result by 1.210)</td>
<td></td>
</tr>
<tr>
<td><strong>CKD-EPI</strong>(^9)</td>
<td>Female and Pcr ( \leq 62 ) umol/l: ( \text{GFR} = 144^* \times \left(\frac{Pcr}{88.4}\right) \times 0.7^{1.3207} \times 0.993^{0.993} \times \text{age}^{-0.203} )</td>
</tr>
<tr>
<td>Female and Pcr ( &gt; 62 ) umol/l: ( \text{GFR} = 144^* \times \left(\frac{Pcr}{88.4}\right) \times 0.7^{1.209} \times 0.993^{0.993} \times \text{age}^{-0.203} )</td>
<td></td>
</tr>
<tr>
<td>Male and Pcr ( \leq 80 ) umol/l: ( \text{GFR} = 141^{**} \times \left(\frac{Pcr}{88.4}\right) \times 0.9^{1.209} \times 0.993^{0.993} \times \text{age}^{-0.203} )</td>
<td></td>
</tr>
<tr>
<td>Male and Pcr ( &gt; 80 ) umol/l: ( \text{GFR} = 141^{**} \times \left(\frac{Pcr}{88.4}\right) \times 0.9^{1.209} \times 0.993^{0.993} \times \text{age}^{-0.203} )</td>
<td></td>
</tr>
<tr>
<td>* use 166 if black</td>
<td></td>
</tr>
<tr>
<td><strong>Hoek formula</strong>(^11)</td>
<td>GFR = -4.23 + 80.35/cysC</td>
</tr>
<tr>
<td><strong>Stevens formula</strong>(^14)</td>
<td>GFR = ( 177.6 \times \left(\frac{Pcr}{88.4}\right)^{-0.65} \times \text{cysC}^{0.57} \times \text{age}^{-0.20} )</td>
</tr>
<tr>
<td>(female: multiply result by 0.82, black: multiply result by 1.11)</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

Statistical analyses were performed using SPSS16. Demographic data were expressed as median (range). For comparison of the various eGFR methods with mGFR, the Bland and Altman analysis was used\(^{10}\). Bias was defined as the mean difference between reference GFR and investigated GFR. Limits of agreement were defined as mean difference -2SD and mean difference +2SD and precision as ± 1 SD. Accuracy was defined as the percentage of measurements that fell within 30% of the reference GFR. Differences in bias and accuracy of the various methods for GFR determination, were tested with the Wilcoxon signed ranks test and McNemar’s test, respectively. A p < 0.05 was considered to indicate a statistically significant difference.
RESULTS

Forty-seven patients were included in this study: 8 candidate kidney donors, 22 renal transplant recipients, 7 HIV-1 infected patients and 10 patients with Fabry disease. The demographic data and the results of the measured GFR with the two methods are displayed in table 2.

Table 2. Demographic data of 47 patients who underwent GFR measurement with $^{51}$Cr EDTA. Data are expressed as median (range).

<table>
<thead>
<tr>
<th></th>
<th>Kidney donors (n = 8)</th>
<th>Renal transplant recipients (n = 22)</th>
<th>HIV patients (n = 7)</th>
<th>Fabry patients (n = 10)</th>
<th>Total (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48.1 (28.4 – 76.1)</td>
<td>58.3 (22.4 – 78.8)</td>
<td>48.8 (33.4 – 68.6)</td>
<td>48.8 (21.0 – 55.6)</td>
<td>52.8 (21.0 – 78.8)</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>2/6</td>
<td>12/10</td>
<td>7/0</td>
<td>6/4</td>
<td>27/20</td>
</tr>
<tr>
<td>GFR$_{51Cr-EDTA}$ (ml/min/1.73m$^2$)</td>
<td>86.7 (77.6-108.7)</td>
<td>48.3 (22.6-86.0)</td>
<td>80.3 (69.8-99.2)</td>
<td>88.5 (42.9-119.0)</td>
<td>69.8 (22.6-119.0)</td>
</tr>
<tr>
<td>GFR$_{Gd-DTPA}$ (ml/min/1.73m$^2$)</td>
<td>76.6 (5.2–105.3)</td>
<td>44.8 (13.7– 69.1)</td>
<td>79.7 (75.9–93.5)</td>
<td>77.0 (28.7–118.1)</td>
<td>51.2 (5.2-118.1)</td>
</tr>
</tbody>
</table>

In all patients GFR was measured with $^{51}$Cr-EDTA. However, only 33 patients could be included in the comparative analysis with Gd-DTPA: in 3 patients GFR Gd-DTPA could not be determined due to omission of blood withdrawal and in 11 patients, due to a slow inclusion rate, blood samples containing Gd-DTPA had been stored longer than 180 days (maximum number of days during which blood samples are allowed to be stored after collection until analysis of this parameter).

Figure 1 shows the Bland and Altman analysis of mGFR$_{51Cr-EDTA}$ versus mGFR$_{Gd-DTPA}$, Figure 2 shows the Bland and Altman analysis of mGFR$_{51Cr-EDTA}$ and mGFR$_{Gd-DTPA}$ versus eGFR.

Table 3 lists the accuracies of mGFR$_{51Cr-EDTA}$ versus mGFR$_{Gd-DTPA}$ and of mGFR$_{51Cr-EDTA}$ and mGFR$_{Gd-DTPA}$ versus eGFR. Compared to $^{51}$Cr-EDTA, Gd-DTPA showed a bias of 9.3 ml/min/1.73m$^2$. However, the limits of agreement were wide (-24.2 to 42.8 ml/min/1.73m$^2$). When the most extreme outlier was excluded from the analysis, bias (limits of agreement) improved to 7.0 (-13.8 to 27.8) ml/min/1.73m$^2$. Accuracy was low, 72.7% was within 30% of mGFR$_{51Cr-EDTA}$. GFR estimated by either creatinine and/or cystatin C corresponded better with mGFR$_{51Cr-EDTA}$ than with mGFR$_{Gd-DTPA}$.

Of the equations used to estimate GFR, GFR estimated with the CKD-EPI formula demonstrated the overall best accuracy and precision in comparison to mGFR$_{51Cr-EDTA}$.
Figure 1. Bland and Altman analysis of measured GFR with $^{51}$Cr-EDTA versus measured GFR with Gd-DTPA (n = 33).

Table 3. Comparison between the accuracy (percentage of measurements within 30% of reference GFR) of GFR measured with $^{51}$Cr-EDTA and Gd-DTPA respectively (n = 33). * McNemar’s test.

<table>
<thead>
<tr>
<th>$^{51}$Cr-EDTA versus</th>
<th>% within 30%</th>
<th>Gd-DTPA versus</th>
<th>% within 30%</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DTPA</td>
<td>72.7</td>
<td>MDRD</td>
<td>39.4</td>
<td>0.001</td>
</tr>
<tr>
<td>MDRD</td>
<td>72.7</td>
<td>CKD-EPI</td>
<td>39.4</td>
<td>0.003</td>
</tr>
<tr>
<td>CKD-EPI</td>
<td>72.7</td>
<td>Hoek</td>
<td>28.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Hoek</td>
<td>56.3</td>
<td>Stevens</td>
<td>21.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Stevens</td>
<td>62.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIT GFR

No adverse events were observed during and after the administration of Gd-DTPA (Magnevist*). After a median (range) follow-up of 523 (118 – 810) days, there were no reports of nephrogenic systemic sclerosis.

After exclusion of the samples containing Gd-DTPA stored beyond 180 days, the mean time to analysis was 47 (5 - 175) days (i.e. storage time). When all samples were combined (included those stored longer than 180 days) there was a significant correlation between storage time and bias ($\text{mGFR}_{^{51}\text{Cr-EDTA}} - \text{mGFR}_{\text{Gd-DTPA}}$, figure 3). This correlation disappeared when the storage time was reduced to a maximum of 120 days.

DISCUSSION

We measured GFR using a recently proposed nonradioactive method with the MRI contrast agent Gd-DTPA (Magnevist*) and used the $^{51}$Cr-EDTA based GFR as a reference. Bias and precision of the Gd-DTPA based method deviated significantly
Figure 2. Bland and Altman analysis of estimated GFR with the creatinine-based CKD-EPI formula (A, n = 47 and D, n = 33), the cystatin C-based Hoek formula (B, n = 46 and E, n = 32) and the creatinine/cystatin C-based Stevens formula (C, n = 46 and F, n = 32) versus measured GFR with 51Cr-EDTA and measured GFR with Gd-DTPA, respectively.

from the 51Cr-EDTA based GFR. Accuracy of mGFR{sub}Gd-DTPA was low with only 72.7% within 30% of mGFR{sub}51Cr-EDTA. GFR measurement with Gd-DTPA versus mGFR{sub}51Cr-EDTA performed similar or worse in comparison to estimated GFR with formulas using creatinine and/or cystatin C versus mGFR{sub}51Cr-EDTA. Furthermore, eGFR correlated better with mGFR{sub}51Cr-EDTA than with mGFR{sub}Gd-DTPA.

Measurement of GFR using Gd-DTPA was recently proposed as an alternative, nonradioactive, method to assess renal function. In a population of 20 patients with a GFR ranging from 10.9 to 102.1 ml/min/1.73m², among whom 12 renal transplant recipients, concentrations of Gd-DTPA were determined using a simple ELISA of blood and urine samples taken 1, 2, 3 and 4 hours following a single bolus injection of Gd-DTPA. Thereafter, GFR was calculated using the blood clearance method,
assuming a one-compartment model or from the classic UV/P method. An excellent performance of this GFR method was found in comparison to gold standard GFR measurement using $^{125}$Iothalamate. Although our study population was comparable, we could not reproduce these results.

Reinhardt et al demonstrated that, in comparison to the blood clearance GFR calculation, the UV/P method showed a (non significant) better accuracy (90 versus 95%, in their study defined as percentage within 20% of reference GFR, and better precision (3.0 versus 6.2 ml/min/1.73m$^2$) even when we excluded the samples analyzed beyond the maximum storage time of 180 days, there was an inverse correlation between bias and storage time of the samples (figure 3), resulting in a significant underestimation of GFR when storage time increased. This correlation only disappeared when storage time was limited to 120 days or less. This effect most probably points to degradation of Gd-DTPA in the samples over time.

Another possibility that could contribute to the differences between our study and the study of Reinhardt, is the fact that we used another radioisotope based method as reference GFR, namely $^{51}$Cr-EDTA instead of $^{125}$Iothalamate. Although not often compared ‘head to head’, measured GFR can vary when different markers are applied.

Next to inuline, radioactive markers are considered gold standard. However, tubular secretion is suggested for $^{125}$Iothalamate and extrarenal clearance for DTPA and $^{51}$Cr-EDTA. There are indications that DTPA clearance is systemically higher
than that of EDTA\textsuperscript{25,26}, although we found underestimation of Gd-DTPA clearance in comparison to that of \textsuperscript{51}Cr-EDTA. Nevertheless, the various formulas to estimate GFR corresponded better with mGFR using \textsuperscript{51}Cr-EDTA than mGFR using Gd-DTPA. This let us to conclude to prefer mGFR\textsubscript{\textsuperscript{51}Cr-EDTA} over mGFR\textsubscript{Gd-DTPA}.

Nephrogenic systemic fibrosis (NSF) is a much feared complication of gadolinium administration in the context of MRI in patients with a reduced GFR (< 30 ml/min/1.73m\textsuperscript{2})\textsuperscript{27,28}. The incidence of NSF depends on the total dose of administered gadolinium; GFR measurement using Gd-DTPA needs a gadolinium dosage that is only 5% of the dose used with MRI. Despite this low dose of gadolinium administration, we excluded patients from the study with an eGFR of less than 30 ml/min/1.73m\textsuperscript{2}. However, two patients turned out to have a mGFR of 22.6 and 28.1 ml/min/1.73m\textsuperscript{2}, both renal transplant recipients. None of the patients developed NSF during a median (range) follow-up of 530 (145 -810) days.

Exact measurement of GFR remains difficult and even gold standard methods can result in different values of renal function. One may even wonder if exact determination is possible and whether an observed decline in measured GFR actually reflects a pathologic decrease in kidney function. For, many physiologic factors can influence GFR for example fluid intake, diet and the circadian rhythm\textsuperscript{29}. Nevertheless, functional immunoassay technology using the nonradioactive MRI contrast agent gadolinium-DTPA, recently proposed as a simple and easy applicable method for the assessment of renal function, appeared in our hands, safe but unsuitable to replace GFR measurement with radioisotope based methods in clinical practice.

**ACKNOWLEDGEMENTS**

We greatly acknowledge J.W.J. de Jong en M. Spaeth for their dedicated help in processing the samples. Furthermore, we thank S.L. Yong for performing the ELISA. Two FIT-GFR kits were kindly provided by BioPAL, Inc.
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Cystatin C in Critically Ill Patients Treated with Continuous Venovenous Hemofiltration

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Haemodialysis International 2006; 10:S11-15
ABSTRACT

Background Assessment of residual renal function in critically ill patients with acute renal failure (ARF) treated with continuous venovenous hemofiltration (CVVH) is difficult. Cystatin C (CysC) is a low molecular weight protein (13.3 kD) removed from the body by glomerular filtration. Its serum concentration has been advocated for assessment of renal function in patients with kidney disease.

Objective To investigate whether the removal of CysC by CVVH is likely to influence its serum concentration.

Patients and Methods Concentrations of CysC were measured in three consecutive samples in 18 patients with oliguric ARF treated with CVVH (2 L/h). Samples were taken from the afferent and efferent blood lines and from the ultrafiltrate line.

Results Concentrations of CysC did not change during the studied time interval. The mean serum concentrations of CysC were 2.25 ± 0.45 mg/l in the afferent and 2.19 ± 0.56 mg/l in the efferent samples (NS); ultrafiltrate concentrations of CysC were 1.01 ± 0.45 mg/l. The sieving coefficient of CysC was 0.52 ± 0.20; clearance of CysC was 17.3 ± 6.6 ml/min; removed quantity of CysC averaged 2.13 mg/h.

Conclusion During CVVH (2 L/h) the removed quantity of CysC is less than 30% of its production and no rapid changes in its serum concentration are observed. Therefore CVVH (2 L/h) is unlikely to influence serum concentrations of CysC significantly which suggests that it can be used to monitor residual renal function during CVVH.
INTRODUCTION
Critically ill patients are at risk of acquiring acute renal failure (ARF). Its incidence is 15-20% of all intensive care admissions of which 4-6% require some form of renal replacement therapy\(^1\). During renal replacement therapy adequate monitoring of renal function is severely hampered because plasma creatinine and urea are removed by the hemofilter. This problem could be overcome by the use of Cystatin C (CysC).

CysC has been advocated as a marker of renal function. It is a low molecular weight protein (13.3 kD), produced at a constant rate by nucleated cells\(^2\) and removed from the body by glomerular filtration\(^3\). CysC production is not influenced by sex, age, bodyweight or muscle mass\(^4,5\) and several studies have shown that CysC is a more sensitive indicator of mild reductions of renal function than creatinine\(^6-11\). Moreover CysC was not removed during intermittent hemodialysis\(^12\) or continuous venovenous hemodiafiltration\(^13\). However, critically ill patients with ARF will often receive continuous venovenous hemofiltration (CVVH). In contrast to hemodialysis, removing substances by diffusion, clearance of solutes with hemofiltration is achieved by convection (ultrafiltration) and adsorption\(^14\). With the use of conventional membranes convection is associated with higher removal of middle and high molecular weight molecules.

The aim of this study was to investigate whether the removal of CysC by CVVH is likely to influence its serum concentration. If not, serum concentrations of CysC can be used as a marker for GFR in patients treated with CVVH.

METHODS
Patients
The present study was conducted as part of a wider study on the effect of CVVH on antimicrobial dosing\(^15\). The study was conducted in a 28 beds multidisciplinary closed format intensive care in a university hospital and was performed in accordance with the guidelines of the local ethics committee. Consecutively admitted critically ill patients who required CVVH for oliguric ARF of any cause, in whom antimicrobial therapy was started to treat a known or suspected infection were included.

Continuous venovenous hemofiltration
Hemofiltration was performed with computer controlled fully automated hemofiltration machines (Diapact, Braun, Melsungen, Germany). Vascular access was obtained by cannulation of the femoral, jugular or subclavian vein using the Seldinger technique and a double lumen catheter (GamCath, Gambro, Hechingen, Germany). A 1.9 m\(^2\) cellulose triacetate hollow fiber membrane (in vitro cut-off 60 kD, ultrafiltration coefficient 37 ml/h/mmHg, sieving coefficient for β2-microglobulin 0.81) was used (CT-190G, Baxter Healthcare Corporation, IL, USA). The extracorporeal circuit was anti-coagulated with heparin (Heparin Leo, Leo Pharma, Ballerup, Denmark). In
Case of severe contraindications for anticoagulation, hemofiltration was performed without anticoagulation. Blood flow rate was 150 ml/min and warmed substitution fluids (SH 19, B-Braun; SB 53-HEP, B-Braun) were administered in predilution mode at a flow rate of 2 L/h.

Samples
Samples were obtained from the afferent (pre-hemofilter) and efferent (post-hemofilter) line of the extracorporeal circuit and from the ultrafiltrate line. They were collected at three different time points, 1.5 to four hours apart, between two successive intravenous gifts of antibiotics. Serum and ultrafiltrate were stored at -80°C.

Assay
CysC was measured with the N Latex Cystatin C test kit, a particle-enhanced immunonephelometric method, on a BN ProSpec analyser (Dade Behring, Leusden, the Netherlands). Normal serum CysC values range from 0.50 to 0.96 mg/l. Urea and creatinine concentrations were measured by standard clinical chemical methods.

Calculations
The prefilter serum concentration was multiplied by the dilution factor to correct for the dilution effects of predilution hemofiltration.

\[
\text{Dilution factor} = \frac{Q_b}{Q_b + Q_{\text{inf}}}
\]

Where \(Q_b\) is the blood flow rate and \(Q_{\text{inf}}\) is the infusion rate of the substitution fluid.

The following equations were used to calculate the sieving coefficient (\(SC\)), CVVH clearance (\(Cl_{\text{CVVH}}\)) and total mass removed in ultrafiltrate (\(M_{\text{uf}}\)):

\[
SC = \frac{2 \times C_{uf}}{C_{aff} + C_{eff}}
\]

\[
Cl_{\text{CVVH}} (\text{mL/min}) = SC \times Q_{uf}
\]

\[
M_{\text{uf}} (\text{mg/h}) = C_{uf} \times Q_{uf}
\]

Where \(C_{uf}\) is the concentration in ultrafiltrate and \(C_{aff}\) and \(C_{eff}\) are the concentrations in the afferent and efferent blood line respectively.

Statistics
Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows version 11 (SPSS, Chicago IL, USA). Results are presented as mean ± SD. To examine changes over time, we used linear mixed models. This analysis studies average changes in subjects, taking into account the association between variables for individual patients at separate time points. To compare afferent and efferent values, we used the paired T-test. Differences at the level of \(P < 0.05\) were considered to be statistically significant.
RESULTS

Eighteen patients were included (Table 1). Per patient three afferent, three efferent and three ultrafiltrate samples were available. In one patient only two afferent samples were available and in two patients only two efferent samples were present. Figure 1 shows the afferent CysC concentrations in the three consecutive samples. Changes over time were not statistically significant for CysC, creatinine and urea. Therefore the mean individual data were used for further analysis and are summarized in Table 2. Figure 2 shows the individual data for CysC. The SC for creatinine averaged 0.9 and that of urea 1.0. The mean SC for CysC was 0.47. When corrected for possible incomplete mixing by using the SC of urea, which should be 1.0 by definition, a value of 0.52 ± 0.20 was found. Likewise, the corrected CysC hemofilter clearance was 17.3 ± 6.6 ml/min (Figure 3).

Serum CysC concentration was 2.21 ± 0.48 mg/l in the 12 patients receiving corticosteroids and 2.34 ± 0.41 mg/l in the others (NS).

Table 1. Patient characteristics (n=18). Values are mean ± SD or number.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD or Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / Female</td>
<td>9 / 9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.0 ± 14.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.2 ± 18.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>170.2 ± 8.4</td>
</tr>
<tr>
<td>Urine production (ml/24 hrs)</td>
<td>84.7 ± 98.8</td>
</tr>
<tr>
<td>APACHE-II score</td>
<td>21.7 ± 6.4</td>
</tr>
<tr>
<td>Admission type</td>
<td></td>
</tr>
<tr>
<td>medical</td>
<td>10</td>
</tr>
<tr>
<td>surgical</td>
<td>8</td>
</tr>
<tr>
<td>Corticoid therapy</td>
<td>12</td>
</tr>
</tbody>
</table>

Figure 1. CysC concentrations in three consecutive afferent samples (horizontal lines represent mean ± SD). No statistically significant differences were observed over the three collection periods (P = 0.334).
Table 2. Concentrations, sieving coefficient, hemofilter clearances and removed quantity of CysC, creatinine and urea. Mean values ± SD.

<table>
<thead>
<tr>
<th></th>
<th>CysC</th>
<th>creatinine</th>
<th>urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afferent concentration</td>
<td>2.25 ± 0.45 mg/L</td>
<td>166 ± 99 μmol/L</td>
<td>13.0 ± 5.6 mmol/L</td>
</tr>
<tr>
<td>Efferent concentration</td>
<td>2.19 ± 0.56 mg/L</td>
<td>154 ± 90 μmol/L</td>
<td>12.4 ± 5.3 mmol/L</td>
</tr>
<tr>
<td>Ultrafiltrate concentration</td>
<td>1.01 ± 0.45 mg/L</td>
<td>160 ± 124 μmol/L</td>
<td>13.5 ± 9.0 mmol/L</td>
</tr>
<tr>
<td>Sieving coefficient</td>
<td>0.47 ± 0.19</td>
<td>0.90 ± 0.45</td>
<td>1.00 ± 0.45</td>
</tr>
<tr>
<td>Clearance</td>
<td>15.8 ± 6.5 ml/min</td>
<td>30.1 ± 14.9 ml/min</td>
<td>33.3 ± 15.0 ml/min</td>
</tr>
<tr>
<td>Removed quantity</td>
<td>2.13 ± 0.95 mg/h</td>
<td>340 ± 263 μmol/h</td>
<td>28.5 ± 19.0 mmol/h</td>
</tr>
</tbody>
</table>

Figure 2. Mean individual CysC concentrations in the afferent and efferent samples in 18 patients.

Figure 3. Mean corrected clearance of CysC by CVVH. Clearance was calculated from the urea-corrected sieving coefficient (horizontal lines represent mean ± SD).

DISCUSSION

In the present study the SC for CysC is 0.52 and the CVVH clearance is 17 ml/min. Adsorptive removal is unlikely because there is no difference in CysC level between the afferent and efferent concentrations. The removed quantity averages 2.13 mg/h. Data from literature show that the generation rate of CysC is 7.44 ± 1.44 mg/h. Consequently CVVH CysC removal is less than 30% of its generation and unlikely to influence serum CysC levels in a clinical significant way. This is confirmed by the absence of rapid changes in the CysC concentrations in individual patients.
Our study is the first study evaluating the removal of CysC during CVVH in critically ill patients with ARF. Balik et al.\textsuperscript{13} studied the effects of continuous venovenous hemodiafiltration (CVVHDF) with polysulphone membranes in critically ill patients and concluded that CysC is not removed during CVVHDF to a significant extent. However, solute removal during hemodialysis is based on diffusion and not convection as in CVVH.

Our study has several limitations. The conclusion is based on the generation rate of CysC as reported in the literature in non critically ill patients\textsuperscript{16}. Several conditions may have an effect on CysC levels, in particular thyroid disease\textsuperscript{17,18} and the use of corticosteroids\textsuperscript{19-21}. It is possible that the generation rate of CysC is influenced by critical illness; however this would affect our conclusion only in case of a reduced generation rate. In our study the difference in CysC levels between the patients with and without corticosteroids is not statistically significant, however the number of patients per group is small. We studied one filter and one ultrafiltration rate in the predilution mode. Membrane material and pore size might affect the SC for CysC. Convective removal of CysC was reported earlier during in vitro hemofiltration\textsuperscript{22}. In that study the SC for CysC was somewhat higher than in our study, most likely because high-cut-off membranes were used (in vitro cut-off = 100 kDa). Moreover, adsorptive removal might be more prominent with other filters, in particular the polyacrylonitrile filter\textsuperscript{23}. In our study, applying an ultrafiltration rate of 2 L/h, the removal of CysC is not likely to affect serum levels. However, during the application of higher ultrafiltration rates, larger quantities of CysC will be removed and this may affect its concentration.

In conclusion, during CVVH (2 L/h) CysC is removed from the circulation; however the removed quantity is less than 30% of its production. Therefore CVVH (2 L/h) is unlikely to influence serum concentrations of CysC which suggests that it can be used to monitor residual renal function during CVVH.

REFERENCES


Part II

(Side) Effects of mTOR Inhibition in Renal Transplantation
General Introduction
Part II
**GENERAL INTRODUCTION**

The discovery of the mammalian target of rapamycin (mTOR) pathway originates from the early 1970's when rapamycin was isolated from a soil sample obtained on Easter Island (Rapa Nui). This macrolide was found to be produced by *Streptomyces hygroscopicus* and to have antifungal properties. In 1994 the mammalian target of rapamycin, a serine/threonine kinase, was indentified and cloned. Rapamycin selectively inhibits mTOR and could be used to elucidate the mTOR pathway. mTOR is ubiquitously present in cells throughout the body; plays an important role in cell signaling and is involved in cell growth and cell proliferation in response to various stimuli, like growth factors, glucose and oxygen.

mTOR consists of two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Either complex is associated with a scaffolding protein: mTORC1 with rapamycin associated protein of mTOR (raptor) and mTORC2 with rapamycin insensitive companion of mTOR (rictor). Activation of mTOR starts with activation of the lipid kinase phosphatidylinositol-3-kinase (PI3K), which in turn activates Akt. Once activated, Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC) thereby activating mTOR by releasing it from the inhibitory effects of TSC. mTORC1 and mTORC2 have discrete functional roles. Activation of mTORC1 results in inhibition of autophagy, cell proliferation and mRNA translation, whereas activation of mTORC2 contributes to regulation of cell polarity, of the actin cytoskeleton and, according to recent findings, to cell-cycle progression, anabolism and cell survival.

Rapamycin acts by forming a complex with FK506–binding protein 12 (FKBP-12) which then binds to - and inhibits mTORC1 by blocking its interaction with raptor. No direct effect of rapamycin has been shown on mTORC2. However, prolonged treatment with rapamycin can inhibit mTORC2 in a subset of tissues and cell lines, possibly by sequestrating the cellular pool of mTOR in a complex with rapamycin-FKBP12 thereby making mTOR unavailable for assembly into mTORC2.

Although discovered as an antifungal agent, rapamycin, by blocking the mTOR pathway, has multiple effects. It has potent immunosuppressive properties by influencing the innate and adaptive immune system. Rapamycin inhibits T-cell proliferation by preventing transition from the G1 to S phase. It impairs dendritic cell differentiation and function, partly by reducing (co-stimulatory) responses to allogenic stimuli and influencing antigen uptake, thereby modulating events that are associated with antigen presentation. Furthermore, it has tolerance inducing properties, for example by increasing the frequency of regulatory T-cells. mTOR inhibitors, first sirolimus/rapamycin (1999) and later everolimus (2001), have proven their efficacy in renal transplantation. They were first introduced in renal transplantation because of their supposed lack of nephrotoxicity, which contrasts to calcineurin inhibitors (CNI) which by their nephrotoxic side effects contribute to long-term graft failure. mTOR inhibitors can be used to withdraw or decrease CNI dose, when calcineurin-inhibitor associated nephropathy is suspected, although...
Figure 1. mTOR pathway signaling (simplified). Activation of mTORC1 starts with activation of the lipid kinase phosphatidylinositol-3-kinase (PI3K) by growth factors, cytokines, co-stimulatory factors or antigen receptors. PI3K, in turn activates Akt. Once activated, Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC) thereby activating mTOR by releasing it from the inhibitory effects of TSC. TSC can also be directly activated by hypoxia, cellular stress or DNA damage or via AMPK, which in turn is activated by low energy levels, detected as high levels of AMP. Activation of mTORC1 leads to inhibition of autophagy, stimulation of cell proliferation and mRNA translation. mTORC2 is involved in regulation of cell polarity and cytoskeleton and most probably in cell survival, cell cycle progression and anabolism. Rapamycin acts by forming a complex with FK506-binding protein 12 (FKBP-12) which then binds and inhibits mTORC1. Prolonged treatment with rapamycin can inhibit mTORC2 possibly by sequestering the cellular pool of mTOR in a complex with rapamycin-FKBP12 thereby making mTOR unavailable for assembly into mTORC2.

a GFR < 40 ml/min and the presence of proteinuria seem to be contra-indications. An early switch to everolimus at 4 months after transplantation improved renal function at one year compared to CNI continuation. Furthermore, mTOR inhibitors may have beneficial effects on the vessel wall and have anti-cancer properties. They have been shown to be effective in the prevention of treatment of cancers occurring more often in the immunocompromised renal transplant recipients, like non-melanoma skin cancers, haematologic malignancies, Kaposi sarcoma and renal carcinoma.

Unfortunately, the use of mTOR inhibitors is associated with a high incidence of adverse effects. Early use of sirolimus or everolimus after transplantation increases the incidence of impaired wound healing, lymphoceles and the incidence of delayed graft function. Amongst others, prolonged treatment is
associated with lymphoedema, dyslipidaemia and microcytic anemia\textsuperscript{36,37}, resembling anemia of chronic disease. Another well known side effect is de novo occurrence of - or an increase in proteinuria\textsuperscript{38}. A more severe complication is mTOR associated pneumonitis\textsuperscript{39}, reported as a rare but potential life threatening condition.

Possible explanations for the high incidence of adverse effects accompanying the use of mTOR inhibitors can be postulated. First, since the mTOR pathway is present in almost all cells of the body, it is not surprising that more systems are affected than only the targeted one. Second, although suppressing the adaptive immune response, inhibition of the mTOR pathway enhances innate immunity\textsuperscript{17,40}. This might explain in part the chronic inflammatory state often associated with the use of mTOR inhibitors.

To further clarify the place of mTOR inhibitors in the immunosuppressive drug treatment after renal transplantation, the MECANO trial was conducted, ‘Mycophenolate sodium versus Everolimus or Cyclosporine with Allograft Nephropathy as Outcome’. From January 2005 to September 2009, three academic hospitals, the Academic Medical Center, Leiden University Medical Center and the University Medical Center in Groningen, participated in this multi-center randomized controlled trial, studying the effects of early withdrawal of the calcineurin-inhibitor cyclosporine A. Preliminary data were recently published\textsuperscript{41}. In short, renal allograft recipients, receiving their first or second kidney transplant, were treated with quadruple immune suppression consisting of prednisolone (P), cyclosporine A (CsA), mycophenolate sodium (MPA) and basiliximab. After 6 months, patients were randomized to one of three immunosuppressive drug regimens: P/CsA, P/MPs and P/everolimus (EVL), provided no signs of rejection were detectable in protocol transplant biopsy. Drug exposure of CsA, MPA and EVL was monitored by calculating the Area Under the Curve (AUC) at pre-fixed time-points. The primary outcome was interstitial graft fibrosis and arteriolar hyalinosis. Secondary outcome was, among others, graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with > 50% panel reactive antibodies. The P/MPs arm was prematurely halted because of an increase in severe acute rejection episodes. Substudies of the MECANO trial provided the data for part II of this thesis.

**AIM AND OUTLINE OF PART II OF THIS THESIS**

Part II will focus on the (side) effects of treatment with everolimus. Pneumonitis, as mentioned above, is one of the potentially most severe complications of mTOR inhibitors. Although several reports have been published on sirolimus-induced pneumonitis, far less is known about everolimus-induced pneumonitis (EIP). **Chapter 6** reports a case-control study in patients of the MECANO cohort treated with everolimus. Incidence, radiologic features and risk factors of EIP are studied in an attempt to elucidate the etiology of this adverse effect.
When mTOR inhibitors were introduced in transplantation, they were used as a calcineurin inhibitor sparing regimen to halt calcineurin associated nephropathy. However an increase in proteinuria was observed after decreasing or withdrawing calcineurin-inhibitors. CNIs have antiproteinuric properties, so it was speculated that the increase of proteinuria resulted from withdrawal of the antiproteinuric properties of CNIs. However, nephrotic range proteinuria was also observed after de novo use of mTOR inhibitors. To clarify this important issue, chapter 7 compares proteinuria and renal biopsy data, light- and electron microscopy, from patients treated with either everolimus or the calcineurin-inhibitor cyclosporine.

A less well known complication of mTOR inhibitor treatment is discussed in chapter 8. It reports a pilot study, initiated after observing an increased incidence of thrombotic events in patients treated with either sirolimus or everolimus, investigating various parameters of hemostasis in patients treated with everolimus or a non-mTOR containing regimen.

REFERENCES


Everolimus Induced Pneumonitis: a Case-Control Study

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* both authors contributed equally to this manuscript

Submitted
ABSTRACT

Background The use of inhibitors of the mammalian target of rapamycin (mTORi) in renal transplantation is associated with many side effects: one of the potentially most severe being interstitial pneumonitis. Although several reports have been published on sirolimus-induced pneumonitis, far less is known about everolimus-induced pneumonitis (EIP). With the present case-control study in renal transplant recipients (RTR), we aimed to assess the incidence of EIP after renal transplantation and to identify risk factors for its development.

Methods This study was retrospectively conducted as a substudy of a multi-center randomized controlled trial. All patients treated with prednisolone/everolimus were included. RTR who developed EIP, were identified as cases. RTR without pulmonary symptoms served as control patients.

Results Thirteen out of 102 patients (12.7%) developed EIP. We did not find any predisposing factors for the development of EIP, especially no correlation with everolimus dose. EIP presented as organizing pneumonia, non-specific interstitial pneumonitis or a combination of both. Median time (range) to the development of EIP after start of everolimus was 162 (38-407) days.

Conclusion EIP is a common side-effect of everolimus in RTR, presenting as organizing pneumonia and/or non-specific interstitial pneumonia. No clear predisposing factors could be identified.
INTRODUCTION

Inhibitors of the mammalian target of rapamycin (mTORi), sirolimus and everolimus, are potent immunosuppressive drugs widely used after organ transplantation. They have been introduced in renal transplantation because of their supposed lack of nephrotoxicity and potential anti-oncogenic and anti-atherosclerotic effects. Unfortunately, the use of mTORi is associated with many side effects like edema, impaired wound healing, mouth ulcers, anemia, proteinuria, development of lymphoceles, hyperlipidemia and hypertriglyceridemia. Also interstitial pneumonitis may complicate treatment with an mTOR inhibitor. There are many reports of sirolimus-induced pneumonitis (SiP). Estimates of the incidence of SiP vary between 5 and 15% in solid organ transplant recipients. Clinical presentation ranges from asymptomatic to respiratory failure, but published reports suggest that SiP generally has a mild course and resolution of symptoms usually occurs after dose reduction or discontinuation of sirolimus. Far less is known on everolimus-induced pneumonitis (EiP) and no systematic case-control studies have been performed in renal transplant recipients (RTR).

The mechanism responsible for pulmonary toxicity by mTORi is not completely understood. Some suggest a dose-dependent risk, but there are also reports of cases with low mTORi trough levels. Apart from the dose of mTORi, other possible risk factors have been identified in patients with non-small cell lung cancer, like smoking and pre-existing pulmonary disease. Other studies found plasma creatinine and glomerular filtration rate (GFR) to be risk factors for development of EiP, indicating that the tolerance to mTORi may be altered in the presence of severe renal insufficiency.

The presence of lymphocytes and eosinophils in broncho-alveolar lavage fluid suggests an immune mediated reaction. It has been hypothesized that sirolimus binds to plasma proteins and that this complex is processed by antigen presenting cells in the lungs with consecutive T-cell recognition and recruitment of inflammatory cells like macrophages. Others suggested that sirolimus exposes cryptic alveolar antigens evoking an ongoing cellular immune response. Histopathological patterns include bronchiolitis obliterans organizing pneumonia, lymphocytic interstitial pneumonia, non-necrotizing granulomatous inflammation and vasculitis which support the immune mediated hypothesis. The mechanisms involved in EiP are speculative due to the lack of detailed studies. However, a recent study suggests a similar immunological mechanism for EiP, although there are also reports of resolution of SiP after conversion to everolimus. In conclusion, ongoing exposure to mTORi may lead to a persistent inflammatory response in the lungs presenting clinically as pneumonitis.

With the present case-control study we aimed to describe the incidence, clinical presentation, radiologic findings and predisposing factors of EiP in RTR.
METHODS

Patients
This study was conducted as part of a larger prospective, multicenter randomized trial studying the effects of withdrawal of cyclosporin A (CsA) from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), CsA, prednisolone (P) and mycophenolate sodium (MPS) early after transplantation. Three university hospitals in the Netherlands participated in this trial from January 2005 until September 2009: the Academic Medical Center in Amsterdam (AMC), the Leiden University Medical Center (LUMC) and the University Medical Center in Groningen (UMCG). Institutional review board approval has been obtained. The study was conducted in accordance with the declaration of Helsinki. Informed consent was obtained from every patient. The details and results of an interim analysis of this trial have previously been published (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl) 21. In short, RTR, receiving their first or second renal transplant, were treated with quadruple immunosuppressive therapy consisting of P, CsA, MPS and basiliximab. After 6 months, RTR were (in the absence of rejection, proven by renal biopsy) randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and P/everolimus. Drug exposure of CsA and everolimus was monitored by calculating Area Under the Curves (AUC) at fixed moments. The target value of the AUC for CsA was 5400 μg*h/l in the first 6 weeks and 3250 μg*h/l thereafter. The target AUC for everolimus was 150 μg*h/l for Fluorescence Polarization Immunoassay (FPIA) and 120 μg*h/l for Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), corresponding to the average 23% overestimation of FPIA 22. The primary outcome was interstitial graft fibrosis and hyalinosis. Secondary outcome was, among others, graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with >50% panel reactive antibodies.

Case definition
For this retrospective sub-study, all RTR who were randomized to treatment with P/everolimus and/or effectively switched to treatment with P/everolimus during the study were included. RTR, who developed symptoms of an EIP, were identified as cases. We used the following criteria for EIP 10: (1) exposure to everolimus before the onset of pulmonary symptoms, (2) exclusion of other pulmonary disease, especially infection, (3) radiographic findings on computed tomography of the chest not compatible with other diagnoses and (4) resolution of pulmonary symptoms after discontinuation of everolimus. When available, histopathological diagnosis consistent with drug-induced lung-toxicity was considered gold standard.

RTR who were treated with P/everolimus, but did not develop pulmonary symptoms, served as control patients. Patients in whom everolimus was discontinued because of pulmonary symptoms which, however, could not be attributed to everolimus with certainty because of the lack of chest CT imaging, were excluded from the analysis. These patients were classified as possible EIP.
The following data were retrospectively collected from medical records: sex, age, race, original renal disease, organ origin (living related or deceased), data on rejection episodes and CMV infection, dialysis mode, history of pulmonary disease, smoking, everolimus AUCs and trough levels. Chest X-rays and (HR) CT of the chest from possible cases were re-analyzed by a radiologist (IB) and pulmonologist (RJ). Pulmonary function tests (when performed) were also recorded. The course of the EIP was analyzed and time to (partial) recovery was noted.

**Radiologic classification**
Imaging findings on chest CT scan were classified into three distinct patterns (a simplified version of the approach by Endo et al 23): 1) multifocal areas of airspace consolidation primarily peripherally localized, compatible with organizing pneumonia (OP), 2) extensive bilateral ground-glass attenuation or airspace consolidation with traction bronchiectasis, compatible with a non-specific interstitial pneumonitis (NSIP), or 3) a combination of OP and NSIP.

**Measurements**
Plasma creatinine was measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyzer (Roche, Almere, the Netherlands). Estimated GFR was calculated using the abbreviated MDRD formula: 
\[ \text{GFR} = 175 \times (\text{Pcr} ÷ 88.4)^{-1.154} \times \text{age}^{0.203} \]
(female: multiply result by 0.742, black: multiply result by 1.210).

AUCs\(_{0-12h}\) for everolimus were calculated from blood samples drawn at T=0, 1, 2, 3, 4, 5 and 6 hours after administration. The everolimus AUCs\(_{0-12h}\) consisted of full AUCs (seven or six time points) and sparsely sampled AUCs (four time points), calculated using linear trapezoidal rule. Everolimus levels were determined by immunoassay (Innofluor® Certican® Assay System) according to manufacturers’ instructions (Seradyn Inc, IN, USA) or by a validated LC-MS/MS method 22. Since there is an average overestimation of 23 \% by FPIA 22, the average AUC\(_{0-12h}\) measured with LC-MS/MS was corrected by this 23 \% to eliminate the differences between both methods.

Pulmonary function (vital capacity [VC] and measurement of the single-breath carbon monoxide diffusion capacity [DLCO]) was measured using standard testing procedures.

**Statistical analysis**
All statistical analyses were performed using SPSS statistical software, version 16.0 (SPSS Inc., Chicago, Illinois, USA). Univariate analysis was performed to identify risk factors associated with EIP. Associations of discrete variables with EIP are expressed in terms of exact odds-ratios with their 95\% confidence interval and analyzed with a chi-square test. Associations of continuous variables were analyzed with a Mann-Whitney U test. A p-value < 0.05 was considered statistically significant. Areas under the curve (AUCs\(_{0-12h}\)) were calculated using the linear trapezoidal rule.
with everolimus trough concentrations used as 12-hour values. AUCs were grouped into three different time periods (range): 1 month (0.2-3.5), six months (4.0-8.1) and 12 (9.4-14.5) months after start of everolimus. If one patient had multiple AUC measurements within one time period, the average AUC was calculated and used in the analysis.

RESULTS

102 RTR were treated with P/everolimus during the study period. We identified 13 cases, corresponding with an incidence of 12.7% (i.e. 13/102). Seven cases were classified as ‘possible cases’ and were excluded from the definite analysis. A detailed description of these patients can be found as supplementary data (supplementary table S1).

Eighty-two RTR who did not develop pulmonary symptoms, served as control patients. Table 1 shows the demographic data of cases and control patients. The characteristics of the 13 patients who developed an EIP are listed in table 2. The median (range) time on everolimus of all patients was 752 (32-1502) days. In the cases, the median time (range) on P/everolimus until confirmation of EIP by CT was 162 (38-407) days. Beyond 407 days, no more EIP occurred (figure 1).

<table>
<thead>
<tr>
<th>Table 1. Univariate analysis of risk factors for EVL-induced pneumonitis among renal transplant recipients (n = 102).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cases (n=13)</strong></td>
</tr>
<tr>
<td>Male gender n (%)</td>
</tr>
<tr>
<td>Recipient age, median (range)</td>
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<td>Caucasian n (%)</td>
</tr>
<tr>
<td>Underlying renal disease</td>
</tr>
<tr>
<td>-vascular</td>
</tr>
<tr>
<td>-immunological</td>
</tr>
<tr>
<td>-urological</td>
</tr>
<tr>
<td>-other</td>
</tr>
<tr>
<td>-eci</td>
</tr>
<tr>
<td>Renal transplant type (living) n (%)</td>
</tr>
<tr>
<td>Smoking</td>
</tr>
<tr>
<td>-yes</td>
</tr>
<tr>
<td>-stopped prior to Tx</td>
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<tr>
<td>-no</td>
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</table>
Table 1. Continued.

<table>
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<tr>
<th></th>
<th>Cases (n=13)</th>
<th>Control patients (n=82)</th>
<th>Odds ratio (CI)</th>
<th>p-value</th>
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<tr>
<td>Pulmonary history n (%)</td>
<td>4 (30.8)</td>
<td>14 (17.1)</td>
<td>0.463 (0.13-1.72)</td>
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</tr>
<tr>
<td>Rejection episode n (%)</td>
<td>1 (7.7)</td>
<td>16 (19.5)</td>
<td>2.909 (0.35-24.04)</td>
<td>0.32</td>
</tr>
<tr>
<td>Time on RRT (months)</td>
<td>48.1 (0-277)</td>
<td>28.8 (0-344)</td>
<td>-</td>
<td>0.23</td>
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<tr>
<td>Dialysis mode n (%)</td>
<td></td>
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<tr>
<td>- pre-emptive</td>
<td>1 (7.7)</td>
<td>13 (15.9)</td>
<td>1.0</td>
<td>0.34</td>
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<td>- HD</td>
<td>7 (53.8)</td>
<td>23 (28.0)</td>
<td>4.0 (0.4-35.8)</td>
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<tr>
<td>- PD</td>
<td>3 (23.1)</td>
<td>31 (37.8)</td>
<td>1.3 (0.1-13.2)</td>
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</tr>
<tr>
<td>- HD &amp; PD</td>
<td>2 (15.4)</td>
<td>15 (18.3)</td>
<td>1.7 (0.1-21.4)</td>
<td></td>
</tr>
<tr>
<td>GFR* (ml/min),</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 6 months after Tx</td>
<td>59.1 (30.8-87.8)</td>
<td>52.4 (17.4-110.2)</td>
<td>-</td>
<td>0.098</td>
</tr>
<tr>
<td>- 9 months after Tx</td>
<td>54.5 (35.8-79.5)</td>
<td>52.8 (20.6-102.8)</td>
<td>-</td>
<td>0.534</td>
</tr>
<tr>
<td>- 12 months after Tx</td>
<td>50.4 (35.5-75.4)</td>
<td>51.2 (11.7-96.8)</td>
<td>-</td>
<td>0.645</td>
</tr>
<tr>
<td>- 18 months after Tx</td>
<td>54.2 (37.0-93.3)</td>
<td>50.1 (14.3-101.6)</td>
<td>-</td>
<td>0.836</td>
</tr>
<tr>
<td>- 24 months after Tx</td>
<td>58.8 (22.6-97.8)</td>
<td>47.0 (10.1-104.6)</td>
<td>-</td>
<td>0.447</td>
</tr>
<tr>
<td>Time on EVL (days)</td>
<td>157.5 (32-485)</td>
<td>864.5 (69-1502)</td>
<td>-</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>AUC EVL 1 month after start (µg*h/l)</td>
<td>173 (65-447)</td>
<td>169.5 (77-439)</td>
<td>-</td>
<td>0.972</td>
</tr>
<tr>
<td>AUC EVL 6 months after start (µg*h/l)</td>
<td>172 (164-238)</td>
<td>171 (98-356)</td>
<td>-</td>
<td>0.403</td>
</tr>
<tr>
<td>AUC EVL 12 months after start (µg*h/l)</td>
<td>237</td>
<td>169 (89-261)</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Trough level EVL 1 month after start (µg/l)</td>
<td>9,2 (3,8-25,4)</td>
<td>9,1 (4,0-28,1)</td>
<td>-</td>
<td>0.982</td>
</tr>
<tr>
<td>Trough level EVL 6 months after start (µg/l)</td>
<td>10,8 (8,0-14,0)</td>
<td>9,4 (2,9-22,0)</td>
<td>-</td>
<td>0.438</td>
</tr>
<tr>
<td>Trough level EVL 12 months after start (µg/l)</td>
<td>14,5</td>
<td>8.9 (4.5-14.7)</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>CMV-infection n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- primary infection</td>
<td>1 (7.7)</td>
<td>7 (8.5)</td>
<td>1.120 (0.13-9.93)</td>
<td>0.92</td>
</tr>
<tr>
<td>- reactivation</td>
<td>3 (23.1)</td>
<td>28 (34.1)</td>
<td>1.728 (0.44-6.79)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

AUC: Area Under the Curve; CI: confidence interval; CMV: cytomegalovirus; EVL: everolimus; GFR: glomerular filtration rate; HD: haemodialysis; NA: not available; PD: peritoneal dialysis; RRT: renal replacement therapy; Tx: transplantation. *GFR estimated by the abbreviated MDRD. Associations of discrete variables with everolimus-associated pneumonitis are expressed in terms of exact odds-ratios with their 95% confidence interval and analyzed with a chi-square test. Associations of continuous variables are analyzed with Mann-Whitney U test.
Table 2. Characteristics of renal transplant recipients with an everolimus-induced pneumonitis (n = 13).

<table>
<thead>
<tr>
<th>patient</th>
<th>age</th>
<th>gender</th>
<th>time on EVL until symptoms (days)</th>
<th>symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>Male</td>
<td>109</td>
<td>dyspnea, coughing</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>Male</td>
<td>206</td>
<td>dyspnea, coughing, fever</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>Male</td>
<td>162</td>
<td>dyspnea</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>Female</td>
<td>279</td>
<td>coughing, fever</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>Female</td>
<td>385</td>
<td>none</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>Male</td>
<td>130</td>
<td>dyspnea, coughing, fever</td>
</tr>
<tr>
<td>7</td>
<td>71</td>
<td>Female</td>
<td>14</td>
<td>dyspnea</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>Male</td>
<td>58</td>
<td>coughing, fever</td>
</tr>
<tr>
<td>9</td>
<td>70</td>
<td>Male</td>
<td>41</td>
<td>dyspnea, coughing, fever</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>Female</td>
<td>106</td>
<td>dyspnea, coughing, fever</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>Male</td>
<td>41</td>
<td>dyspnea, coughing, fever</td>
</tr>
<tr>
<td>12</td>
<td>64</td>
<td>Male</td>
<td>109</td>
<td>coughing, fever</td>
</tr>
<tr>
<td>13</td>
<td>48</td>
<td>Male</td>
<td>7</td>
<td>dyspnea, coughing</td>
</tr>
</tbody>
</table>

AB: antibiotics; OP: organizing pneumonia; CT: computed tomography; EVL: everolimus; NA: not available; NSIP: non-specific interstitial pneumonia; VATS: video assisted thoracoscopic.

4 First AB (ceftriaxone) was given, which did not improve the pulmonary symptoms. Hereafter ceftriaxone was stopped and everolimus was discontinued.

5 First AB (amoxicillin/clavulanic acid) was given which did not improve the pulmonary symptoms and AB was discontinued. After histopathologic prove of organizing pneumonia, everolimus was discontinued and 60 mg prednisolone was started.

10 Everolimus was discontinued and AB (ciprofloxacin and co-trimoxazole) together with 40 mg prednisolone were given. Sputum cultures revealed no bacteria, some candida species. After one day oseltamivir was added and three days later voriconazol. CMV PCR, positive in the broncho-alveolar lavage fluid, was negative in blood. No anti-CMV treatment was given.

11 First AB (doxycycline) was given which did not improve the pulmonary symptoms and AB was discontinued. Then everolimus was discontinued, 30 mg of prednisolone was administered and pulmonary symptoms resolved.

12 AB (cefuroxime) was given due to 10-100 colonies of Escherichia coli in sputum, because of lack of improvement, everolimus was discontinued and pulmonary symptoms resolved.

13 One month before pulmonary CT, patient was admitted with suspected pneumonia. AB were given. BAL cultures remained negative, everolimus was discontinued. Because of continuing pulmonary symptoms, patient was readmitted one month later (while on prednisolone and tacrolimus). CT revealed OP and pulmonary embolism, anticoagulation was started.
Table 2. Characteristics of renal transplant recipients with an everolimus-induced pneumonitis (n = 13).

<table>
<thead>
<tr>
<th>patient age</th>
<th>gender</th>
<th>time on EVL until symptoms (days)</th>
<th>symptoms</th>
<th>radiologic findings on pulmonary CT</th>
<th>Broncho-alveolar lavage</th>
<th>treatment</th>
<th>time to recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 66</td>
<td>Male</td>
<td>109</td>
<td>dyspnea, coughing</td>
<td>OP/NSIP</td>
<td>NA</td>
<td>discontinue EVL</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>2 49</td>
<td>Male</td>
<td>206</td>
<td>dyspnea, coughing, fever</td>
<td>NSIP</td>
<td>NA</td>
<td>discontinue EVL</td>
<td>&lt; 12 months</td>
</tr>
<tr>
<td>3 61</td>
<td>Male</td>
<td>162</td>
<td>dyspnea</td>
<td>NSIP/OP negative</td>
<td>discontinue EVL</td>
<td>&lt; 6 months</td>
<td></td>
</tr>
<tr>
<td>4 48</td>
<td>female</td>
<td>279</td>
<td>coughing, fever</td>
<td>OP</td>
<td>NA 4AB +</td>
<td>discontinue EVL</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>5 32</td>
<td>female</td>
<td>385</td>
<td>none</td>
<td>OP</td>
<td>NA 4AB +</td>
<td>discontinue EVL</td>
<td>unknown</td>
</tr>
<tr>
<td>6 49</td>
<td>Male</td>
<td>130</td>
<td>dyspnea, coughing, fever</td>
<td>OP with GG</td>
<td>negative</td>
<td>discontinue EVL</td>
<td>&lt; 1 month</td>
</tr>
<tr>
<td>7 71</td>
<td>Female</td>
<td>14</td>
<td>dyspnea</td>
<td>OP/NSIP</td>
<td>NA</td>
<td>discontinue EVL</td>
<td>&lt; 12 months</td>
</tr>
<tr>
<td>8 50</td>
<td>Male</td>
<td>58</td>
<td>coughing, fever</td>
<td>OP</td>
<td>negative</td>
<td>discontinue EVL</td>
<td>unknown</td>
</tr>
<tr>
<td>9 70</td>
<td>Male</td>
<td>41</td>
<td>dyspnea, coughing, fever</td>
<td>VATs: OP</td>
<td>negative</td>
<td>9AB + discontinue EVL</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>10 38</td>
<td>Female</td>
<td>106</td>
<td>dyspnea, coughing, fever</td>
<td>OP</td>
<td>CMV positive</td>
<td>10discontinue EVL + AB + corticosteroids</td>
<td>&lt; 1 month</td>
</tr>
<tr>
<td>11 60</td>
<td>Male</td>
<td>41</td>
<td>dyspnea, coughing, fever</td>
<td>OP</td>
<td>negative</td>
<td>11AB + discontinue EVL + corticosteroids</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>12 64</td>
<td>Male</td>
<td>109</td>
<td>coughing, fever</td>
<td>OP/NSIP</td>
<td>negative</td>
<td>12AB + discontinue EVL</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>13 48</td>
<td>Male</td>
<td>7</td>
<td>dyspnea, coughing</td>
<td>OP</td>
<td>negative</td>
<td>13discontinue EVL</td>
<td>unknown</td>
</tr>
</tbody>
</table>

AB: antibiotics; OP: organizing pneumonia; CT: computed tomography; EVL: everolimus; NA: not available; NSIP: non-specific interstitial pneumonia.

AB: antibiotics; OP: organizing pneumonia; CT: computed tomography; EVL: everolimus; NA: not available; NSIP: non-specific interstitial pneumonia; VATs: video assisted thoracoscopy.

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Figure 1. Kaplan Meyer curve demonstrating the time to development of everolimus induced interstitial pneumonitis (EIP) in 102 renal transplant recipients treated with everolimus.
The most common presenting symptoms were dyspnea and cough (10/13 cases). Fever was present in 8/13 cases. One patient was asymptomatic, however 2-deoxy-2-({\textsuperscript{18}}F)fluoro-D-glucose (FDG) positive pulmonary infiltrates were discovered on a PET scan performed because of multiple unexplained bone fractures. A consecutive HRCT scan showed an image compatible with drug-induced pneumonitis.

In all identified cases, the pulmonary CT scan revealed an image of organizing pneumonia (OP), a non-specific interstitial pneumonitis (NSIP) or a combination of the two (figure 2). In one patient, no CT scan could be retrieved, but EIP was confirmed with pulmonary biopsy.

In all cases everolimus was discontinued. In 6/13 cases everolimus was only discontinued when antibiotic therapy did not result in improvement. The absence of any microorganisms in the bronchoalveolar lavage fluid and the failure of empirical antibiotic treatment ruled out infection in these patients. Corticosteroids were administered in three cases. Pulmonary function tests were performed just after the onset of symptoms in 6/13 cases, showing normal to mildly lowered VC 90.2% (range 68–112), normal FEV1 84.8% (70–100) with a decreased diffusion capacity for carbon monoxide (DCLO) in all, 56% (range 38–75).

All patients had a full clinical recovery within one year. No follow-up CT scans have been made.

Risk analysis for EIP development
We could not identify any predisposing factors to EIP, for example a known prior pulmonary history or smoking. Exposition to everolimus, expressed as AUC or trough levels, was similar in cases and control patients (table 1 and figure 3). In cases,
median time between confirmation of EIP by CT scan and most recent AUC was 69 (6-318) days. The (median) AUC of everolimus was 207 (108 -266) μg*h/l. In 6/13 cases a random trough level of everolimus was available 20 (1 – 63) days before a confirmative CT scan with a median (range) of 10.6 (6.6 – 15.2) μg/l. During follow-up, 68.4% and 50% of the AUCs measured in the cases were > 150 and > 200 μg*h/l, respectively, versus 69.0% and 32.2% in the control patients (NS). 73.7% and 38.9% of the trough levels measured in cases versus 69.4% and 23.1% in control patients, respectively, were > 8 and > 12 μg/l.

**Figure 3.** Area Under the Curve of everolimus, 1 month (A), 6 months (B) and 12 months (C) and trough level of everolimus 1 month (D), 6 months (E) and 12 months (F) after switch to everolimus in renal transplant recipients who developed an everolimus-induced pneumonitis (cases) and their control patients.
DISCUSSION

Interstitial pneumonitis is a common adverse event complicating the use of everolimus after renal transplantation, with an incidence of 12.7% in our study cohort. No clear predisposing factors were identified in this case-control study. Pulmonary CT scan revealed organizing pneumonia (OP) or non-specific interstitial pneumonitis (NSIP) with or without ground glass opacities (GGO). The course seemed benign with disappearance of symptoms within one year after discontinuation of the drug.

The incidence of EIP (12.7%) reported in our study is higher than previously reported in RTR on mTORi, varying between 4 and 6.8% \(^{24-26}\). The true incidence of EIP in our cohort might even be higher because not in all possible cases pulmonary CT scans were performed (table A, supplementary data). In one patient, excluded from the analysis because of lack of pulmonary CT scan or biopsy, the chest x-ray could be compatible with EIP, which might have increased the incidence to 13.7%. Furthermore, we could have missed EIP in control patients, since EIP can be present on pulmonary CT scan without causing symptoms as demonstrated by White et al, who routinely performed pulmonary CT scans in patients with advanced non-small cell lung cancer treated with everolimus \(^{13}\). We identified one asymptomatic case in our cohort as well.

In patients treated with everolimus for renal cell carcinoma the incidence of EIP has been reported to be around 25% \(^{13, 27}\). This high incidence of EIP has been attributed to higher dosage of everolimus in these patients in combination with a higher detection level of EIP due to routinely performed pulmonary CT scans. However in our study, drug exposure, although relatively high with an AUC around 170 \(\mu g\cdot h/l\) and trough levels around 10 \(\mu g/ml\), did not differ significantly between cases and controls. Remarkably, all patients who developed EIP, did so within 407 days; hereafter no EIP occurred. When reviewing the literature, we found only two cases of EIP occurring beyond 407 days.

Much debate exists on the aetiology of mTOR induced pneumonitis. White et al. \(^{13}\) showed that patients with interstitial lung disease on baseline CT scans, whether focal or diffuse, had a higher incidence of all types of pneumonitis. This may reflect the tendency of patients with underlying lung disease to develop more serious toxicity. Therefore, we hypothesized that previous pulmonary disorders (reported in the medical charts) could be a predisposing factor to the development of EIP in our patient cohort. However, cases and control patients displayed a similar incidence. Furthermore, we found no difference in GFR which has also been suggested as a potential risk factor \(^{14}\).

Therapeutic drug monitoring (TDM) of everolimus is essential due to the narrow therapeutic window in combination with highly variable pharmacokinetics. Moreover, direct toxicity of everolimus in the aetiology of EIP is suggested \(^{8}\). Since systematic everolimus AUCs and trough levels were determined in our study, we were able to accurately assess the exposure to everolimus in the cases and controls. We could not demonstrate a difference in AUCs or trough levels of everolimus in the EIP cases.
versus controls. Since there is currently no pharmacokinetic model available to predict everolimus exposure, TDM based on troughs and determination of trapezoidal AUC is the only option to monitor everolimus exposure. In our study, everolimus levels were measured using either the FPIA or the LC-MS/MS method. Recently we noted that the use of FPIA can lead to clinically relevant differences in everolimus levels and a higher intra-patient variability compared with the use of LC-MS/MS. However, bias was minimized by using average AUCs and trough levels in our analysis and the application of the correction factor as mentioned in Moes et al. 

Comparable exposure to everolimus in cases and controls makes toxicity simply based on higher exposure unlikely. We were not able to confirm the immune mediated hypothesis, due to lack of flowcytometric analysis of BAL fluid. More research on pharmacodynamics and pharmacogenetics of everolimus in relation to toxicity is needed to explain why EIP occurs in certain patients.

Our study confirms the previous findings of EIP presenting as an organizing pneumonia, a non-specific interstitial pneumonia or a combination of both, making CT imaging a valuable tool to discriminate infection from a direct everolimus effect. All patients subjectively recovered within one year, however, long-term outcome is unclear since NSIP is known to potentially result in pulmonary fibrosis.

In conclusion, EIP is a common side-effect of everolimus in RTR presenting as organizing pneumonia and/or non-specific interstitial pneumonia. No clear predisposing factors could be identified. Since we did not find a correlation with exposure between cases and controls, we advise to halt everolimus instead of reducing the dosage following EIP.

ACKNOWLEDGEMENTS

We greatly acknowledge M. van Dijk, UMCG, for her excellent help in collecting the data. Furthermore, we would like to thank G. Nieuwenhuizen, AMC, and S. Hendriksen, LUMC.
Table S1. Characteristics of 7 renal transplant recipients with pulmonary symptoms not surely attributable to everolimus

<table>
<thead>
<tr>
<th>patient</th>
<th>age</th>
<th>gender</th>
<th>time on EVL until symptoms</th>
<th>symptoms</th>
<th>radiologic findings on pulmonary CT</th>
<th>broncho-alveolar lavage</th>
<th>treatment</th>
<th>time to recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>male</td>
<td>221</td>
<td>dyspnea, coughing</td>
<td>Broncho-pneumonia</td>
<td>NA</td>
<td>1AB + discontinue EVL + corticosteroids</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>male</td>
<td>186</td>
<td>dyspnea, coughing, fever</td>
<td>NA</td>
<td>negative</td>
<td>discontinue EVL</td>
<td>&lt; 1 month</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>female</td>
<td>14</td>
<td>itch, dyspnea</td>
<td>NA</td>
<td>NA</td>
<td>discontinue EVL</td>
<td>unknown</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>male</td>
<td>12</td>
<td>coughing, fever</td>
<td>NA</td>
<td>CMV</td>
<td>4discontinue EVL + AB + ganciclovir</td>
<td>&lt; 1 month</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>male</td>
<td>81</td>
<td>dyspnea</td>
<td>NA</td>
<td>NA</td>
<td>5NA</td>
<td>died</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>male</td>
<td>1</td>
<td>dyspnea, coughing</td>
<td>NA</td>
<td>NA</td>
<td>6discontinue EVL</td>
<td>&lt; 12 months</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>male</td>
<td>6</td>
<td>Dyspnoe, coughing, rash</td>
<td>NA</td>
<td>NA</td>
<td>7discontinue EVL</td>
<td>&lt; 3 months</td>
</tr>
</tbody>
</table>

AB: antibiotics; COP: cryptogenic organizing pneumonia; CT: computer tomography; EVL: everolimus; NA: not available.

1 Patient was admitted with suspected pneumonia, AB (erythromycin/amoxicillin) were given. Since no improvement occurred everolimus was discontinued and prednisolone was increased to 30 mg. Cardiac ultrasound revealed a LVEF of 13%, later improving to 35%.

4 Ganciclovir was started intravenously, cefuroxim was given and everolimus was discontinued. Patient recovered.

5 Patient presented with dyspnoe with unknown cause, symptoms resolved spontaneously after 1 week. One month later symptoms re-occurred accompanied by chest pain. Patient died in his sleep one month hereafter.

6 Patient presented with dyspnoe directly after start of everolimus. Chest X-ray revealed cardiomegaly. After one month everolimus was discontinued. Dyspnoe improved. Symptoms completely resolved after a percutaneous coronary angiography with stent placement one year later.

7 Chest X-ray more compatible with COP than another diagnosis.
REFERENCES


Prolonged Treatment with Everolimus Does Not Induce Podocyte Damage and Leaves the Glomerular Basement Membrane Intact

M C. Baas¹, J. Kers², S. Florquin², J. W. de Fijter³, J. J. Homan van der Heide⁴, M. A. van den Bergh Weerman², I. J.M. ten Berge¹, F. J. Bemelman¹.

¹Renal Transplant Unit, Department of Nephrology, Division of Internal Medicine, ²Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands, ³Department of Nephrology, Leiden University Medical Center, the Netherlands; ⁴Renal Transplant Unit, Department of Nephrology, Division of Internal Medicine, Groningen University Hospital, the Netherlands

Submitted
ABSTRACT

**Background** Inhibitors of the mammalian target of rapamycin (mTOR) were introduced as non-nephrotoxic immunosuppressive drugs; but have been associated with variable degrees of proteinuria. The aim of the present study was to compare proteinuria in renal transplant recipients (RTR) on a maintenance regimen with a CNI to those with the mTOR-inhibitor everolimus, and relate the presence of proteinuria to the histopathological findings of the glomerulus in 2-year protocol biopsies.

**Methods** In a single center study, nested in a multi-center randomized controlled trial, we determined eGFR, proteinuria and renal biopsy data (light –and electron microscopy) of RTR who received prednisolone/everolimus (P/EVL) (n=16) and compared them to a similar patient group treated with prednisolone/cyclosporine A (P/CsA) (n=7). All patients were two years after renal transplantation and had been on the above described maintenance immunosuppression for 18 months.

**Results** Renal function at two years after transplantation did not differ between patients who received P/EVL or P/CsA (eGFR 45.5 vs 45.7 ml/min/1.73m²); proteinuria was borderline increased in the P/EVL vs the P/CsA group (0.29 vs 0.14 g/24h, p = 0.06). There were no differences in light microscopy changes according to Banff 2005 classification, nor in electron microscopic findings. We could not demonstrate increased podocyte effacement or changes in the glomerular basement membrane thickness in the P/EVL treated patients.

**Conclusion** Although there was a marginal increase of proteinuria in patients treated with P/EVL, long-term treatment with everolimus left the glomerular basement membrane and the podocytes unaffected upon light and electron microscopy.
INTRODUCTION

With the introduction of calcineurin-inhibitors (CNI, cyclosporine and tacrolimus) one year renal transplant survival has improved significantly. This improvement has mainly been caused by a decrease in the incidence of early acute rejection episodes. Disappointingly, however, long-term renal graft survival has not improved to the same extent and chronic allograft dysfunction remains the dominant cause for late allograft failure. Chronic allograft nephropathy, defined by interstitial fibrosis and tubular atrophy, is, paradoxically, associated with calcineurin-inhibitors. The mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus, are potent immunosuppressant drugs, with the promise of no or only minor nephrotoxic effects, and other beneficial effects such as on the vessel wall and oncogenesis. Several studies, however, have reported proteinuria after the switch from a CNI-based immunosuppressive regimen to sirolimus. Controversy exists whether this increase in proteinuria results primarily from withdrawing the CNI or from the consequence of direct toxicity of sirolimus on podocytes and/or glomerular basal membrane. The latter is at least supported by new onset proteinuria in patients not on a CNI-based regimen and the development of focal glomerular sclerosis following de novo use of sirolimus. On the other hand, beneficial effects have been described in experimental animals as well as in humans. The mechanism of the sirolimus-induced proteinuria is not clearly defined. Sirolimus has been shown to reduce the in vitro expression of the slit-diaphragm proteins nephrin and transient receptor potential cation channel 6 as well as the cytoskeletal adaptor protein Nck. Moreover, sirolimus has been shown to induce microscopic morphological changes in the cytoskeleton of immortalized podocytes. Injury of the slit-diaphragm and the actin cytoskeleton of podocytes as well as interference with the interaction of podocytes with the glomerular basement membrane can result in proteinuria and morphological alterations such as foot process effacement. Remarkably, although multiple reports exist on the incidence of de novo or significantly increased proteinuria during treatment with sirolimus, only few reports have been published on everolimus. Therefore the effects of everolimus on proteinuria are not well clarified. Successful conversion from cyclosporine to everolimus has been described, without the occurrence of nephrotic range proteinuria, although it was accompanied by a slightly increased proteinuria.

A complicating factor in comparing the effects of sirolimus and everolimus may be the difference in drug exposure. Trough levels of sirolimus in the aforementioned studies were between 8–15 ng/ml, whereas the trough levels of everolimus varied between 3–10 ng/ml. Yet, one study in a liver transplant recipient with diabetic nephropathy reported a decrease in sirolimus-induced proteinuria after switch to everolimus, with comparable trough level of sirolimus and everolimus.

Recently, we performed a prospective multicenter randomized trial in renal transplant recipients withdrawing cyclosporin and/ or mycophenolate mofetil from a triple immunosuppressive regimen at 6 months after transplantation.
Patients continued on double therapy consisting of prednisolone and everolimus or prednisolone and cyclosporine. In this study protocol biopsies were performed just prior to randomization and at 2 years after transplantation. Here, we describe a subset of these patients in whom we studied the amount of proteinuria in relation to the changes in the glomerulus upon light and electron microscopy comparing patients treated with either a CNI (cyclosporine A) or the mTOR-inhibitor everolimus.

**METHODS**

**Patients**

This study was a sub-study nested in the larger prospective, multi-center randomized MECANO trial studying the effects of withdrawal of cyclosporine A (CsA) from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), CsA, prednisolone (P) and mycophenolate sodium (MPS) early after transplantation. Three university hospitals in the Netherlands participated in this trial from January 2005 until September 2009: the Academic Medical Center in Amsterdam (AMC), the Leiden University Medical Center and the University Medical Center in Groningen. Institutional review board approval has been obtained. The study was conducted in accordance with the declaration of Helsinki. Informed consent was obtained from every patient. The details and results of an interim analysis of this trial were previously published (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl). In short, renal allograft recipients, receiving their first or second kidney transplant, were treated with quadruple immune suppression consisting of prednisolone, CsA, MPS and basiliximab. After 6 months, patients (in the absence of rejection upon protocol transplant biopsy) were randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and P/everolimus (EVL). Drug exposure of CsA, MPS and EVL was monitored by calculating the Area Under the Curve (AUC) at pre-fixed time-points. The target values of the AUC$_{12}$ for CsA was $5400 \ \mu g \cdot h/l$ in the first 6 weeks and $3250 \ \mu g \cdot h/l$ thereafter. The AUC$_{12}$ target for MPS was $> 40 \ \mu g \cdot h/l$ during time of triple therapy and $70-85 \ \mu g \cdot h/l$ from month 7 on. Target AUC$_{12}$ for EVL was $150 \ \mu g \cdot h/l$. The primary outcome was interstitial graft fibrosis and arteriolar hyalinosis. Secondary outcome was, among others, graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with $> 50\%$ panel reactive antibodies. The P/MPS arm was prematurely halted because of an increase in severe acute rejection episodes.

For this sub-study, all patients of one center (AMC), who underwent a renal biopsy at 2 years after transplantation, who received either P/CsA or P/EVL during the complete study period and of whom material for light and electron microscopy was available were included. We compared renal function, proteinuria, urine sediment abnormalities and renal biopsy data of patients treated with P/EVL to those treated with P/CsA.
**Measurements**

Plasma creatinine was measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyser (Roche, Almere, the Netherlands).

Estimated GFR was calculated using the abbreviated MDRD formula:

\[
GFR = 175 \times \left( \frac{Pcr}{88.4} \right)^{1.154} \times \text{age}^{0.203}, \]  
(female: multiply result by 0.742, black: multiply result by 1.210).

Total urine protein was measured using a turbidimetric (Roche Diagnostics) assay. Proteinuria is reported as total urine protein/24 hours.

AUC\(_{12}\)s for CsA and EVL were calculated from blood samples drawn at C0, 1, 3, 4, 5 and 6 hr after administration. Everolimus levels were determined by immunoassay (InnoFluor\textsuperscript{®} Certican\textsuperscript{®} Assay System) according to manufacturers’ instructions (Seradyn Inc, IN, USA).

**Light microscopy (LM)**

Biopsies were formalin fixed and paraffin embedded. Haematoxylin and Eosin, Periodic Acid Schiff and Jones’ Silver stainings were performed on all biopsies. Six and 24 months post-transplant protocol biopsies were scored according to the Banff 2005 working classification for allograft pathology by two observers in a simultaneous manner (SF and JK). Only biopsies with the minimal biopsy requirements of at least 7 glomeruli and 1 artery were included in this study.

**Electron microscopy (EM)**

After fixation in Karnovsky fixative, the material was post fixed in 1% osmiumtetroxide and block-stained with 1% uranyl acetate. After one-step dehydration in dimethoxypropane the tissue was embedded in epoxyresin LX-112. LM sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (FEI, Europe BV, Eindhoven, the Netherlands).

For this study, 10 capillary loops of one to two glomeruli in each patient were photographed in a random and unbiased fashion with a final magnification of 10500. The thickness of the glomerular basement membrane (GBM) of 10 different capillary loops was measured at their thinnest points (using Universal TEM Imaging Platfom Software (Soft Imaging System)). Median and range were calculated. GBM length was traced and measured in an image processing and analysis program (ImageJ, http://rsb.info.nih.gov/ij/). The number of podocyte foot processes along the GBM was counted by hand. A foot process was defined as any connected epithelial segment butting on the basement membrane, between two neighbouring filtration pores or slits.

Podocyte foot process effacement of each biopsy was expressed as foot processes width, which was calculated as follows: \(\text{FPW} = \frac{\pi}{4} \times (\Sigma \text{GBM length}/\Sigma \text{foot process})\).

Where ‘\(\Sigma\) foot process’ is the total number of foot processes counted along the GBM in all the available pictures from each biopsy. ‘\(\Sigma\) GBM length’ is the total GBM length measured in all the pictures available of each biopsy, and the correction factor
of $\pi/4$ serves to correct for presumed random variation in the angle of section relative to the long axis of the podocyte$^{22}$.

### Statistical analysis

All statistical analyses were performed under non-Gaussian assumption. Differences in patient characteristics, median Banff scores and glomerular basement membrane thickness between P/CsA and P/EVL were calculated with the use of Mann-Whitney-U signed rank tests. A $p < 0.05$ was considered statistically significant. Analyses were performed with the use of SPSS 16.0 (SPSS Inc. Chicago, IL, United States of America).

### RESULTS

#### Clinical parameters

In the total multicenter trial 361 patients were enrolled$^{22}$, 224 underwent randomization. After two years, 74 patients had been treated with P/CsA during the complete study period, 25 with P/MPS and 58 with P/EVL. At that time-point, mean proteinuria did not differ between both groups (P/CsA 0.30 ± 0.32 g/24h, P/MPS 0.38 ± 0.47 g/24h and P/EVL 0.43 ± 0.48 g/24h). However, proteinuria had significantly increased in the P/EVL group since randomization at 6 months (proteinuria at 6 months 0.24 ± 0.17 g/24h, $p < 0.001$), whereas it had remained stable in the P/MPS and P/CsA group. Estimated GFR was significantly better in the P/MPS and P/EVL versus the P/CsA treated patients (64.3 ± 33.2 and 58.7 ± 21.1 vs 49.2 ± ml/min/1.73m$^2$, $p = 0.01$ and 0.03, respectively) (van der Heide, manuscript in preparation).

Table 1 shows the characteristics of the patients included in this substudy, treated with either P/EVL or P/CsA, of whom material for light and electron microscopy was available at 2 years after transplantation. Twenty-three patients were included, all Caucasian. Sixteen received P/EVL and 7 P/EVL. Three biopsy-proven rejection episodes had occurred, all within 6 months after transplantation (i.e. before randomization to either P/EVL (n=1) or P/CsA (n=2). At two years after transplantation, proteinuria was borderline increased in the P/EVL group ($p = 0.06$), as compared to the P/CsA group.

The incidence of diabetes mellitus, either de novo after transplantation or already pre-existent (defined as the need for either oral anti-diabetics or subcutaneous insulin) was comparable between both groups (P/EVL 4/16 (25%), P/CsA 2/7 (29%)).

Time of exposition of the renal transplant to increased glucose levels was also similar in both groups (P/EVL 11.8 (9.0 – 25.4) months vs P/CsA 12.6 (9.1 - 16.0 months).

The median (range) AUC (at the day of kidney biopsy) of CsA (n = 6) was 2882 (2404 – 5852) $\mu$g*h/l and 164 (84 – 247) $\mu$g*h/l of EVL (n = 16). Median (range) trough levels of EVL were 10.4 (3.6-17.1) $\mu$g/l.

#### Light microscopy

Of the 23 patients, 16 of the 16 patients in the P/EVL group and 6 of the 7 patients in the P/CsA group had an adequate 6 months protocol biopsy. None of the Banff
Table 1. Characteristics of 23 renal transplant recipients 2 years after transplantation, treated with either prednisolone/everolimus (P/EVL) or prednisolone/cyclosporine (P/CsA). Data are expressed as median (range), unless otherwise reported. ns = not significant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P/EVL (n=16)</th>
<th>P/CsA (n=7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females</td>
<td>10/6</td>
<td>6/1</td>
<td>ns</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.2 (24.2 – 69.3)</td>
<td>44.0 (23.2 – 69.9)</td>
<td>ns</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>45.5 (20.4-102.8)</td>
<td>46.2 (38.7 – 57.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Underlying renal disease</td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>- Vascular</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>- Immunological</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>- Urological</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Other</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>- Unknown</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Living/deceased</td>
<td>6/10</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic/diastolic (median/range)</td>
<td>132 (115-150)/(75-88)</td>
<td>140 (112-174)/(80-100)</td>
<td></td>
</tr>
<tr>
<td>Use of ACE/ARB</td>
<td>8/16 (50%)</td>
<td>3/7 (43%)</td>
<td>ns</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>0.29 (0.12 – 0.84)</td>
<td>0.14 (0.08 – 1.04)</td>
<td>0.06</td>
</tr>
<tr>
<td>- 0 – 0.30 g (n)</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>- 0.30 – 1.0 g (n)</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>- &gt; 1.0 g (n)</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>- missing (n)</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Urine sediment abnormalities (n):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Erythrocyturia</td>
<td>10</td>
<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>- Leukocyturia</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Erythrocyturia and leukocyturia</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Parameters were significantly different between P/EV: and P/CsA group at the time of randomization. Table 2 shows the median (range) Banff scores of the 16 patients with an adequate protocol biopsy at 24 months after transplantation (11/16 in the P/EVL group and 5/7 in the P/CsA group). None of the Banff parameters were significantly different between the P/EVL and P/CsA group.

Electron microscopy

Electron microscopy was performed in all patients. In 3/23 patients, no glomerulus was detected in the enclosed material. We measured basement membrane thickness and foot process width in 14/16 P/EVL patients and in 6/7 P/CsA patients (table 3 and figure 1). Basement membrane thickness did not differ between both groups. Podocyte effacement, expressed as foot process width, was similar as well.
**Table 2.** Banff scores at the 2-year post-transplant protocol biopsy, treated with either rednisolone/everolimus (P/EVL) or prednisolone/cyclosporine A (P/CsA). All scores are listed as median (range). ns = not significant

<table>
<thead>
<tr>
<th>Banff parameter</th>
<th>P/EVL (n = 11/16)</th>
<th>P/CsA (n = 5/7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage sclerosed glomeruli</td>
<td>0% (0-18%)</td>
<td>0% (0-14%)</td>
<td>ns</td>
</tr>
<tr>
<td>Tubulitis</td>
<td>0 (0-1)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Interstitial inflammation</td>
<td>0 (0-2)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Glomerulitis</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Arteritis</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Arteriolar hyalinosis</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Mesangial matrix increase</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Allograft glomerulopathy</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Vascular intima thickening</td>
<td>0 (0-3)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Interstitial fibrosis/tubular atrophy</td>
<td>1 (0-3)</td>
<td>0 (0-3)</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Figure 1.** Representative electron microscopy images from patients treated with everolimus. A: Normal GBM (260nm) and normal podocytes. B: Thin GBM: (110nm) and podocyte effacement. C: Thick GBM (480nm). L: capillary lumen, US: urinary space. Bar: 1 μm.

**Table 3.** Glomerular basal membrane thickness and foot process width measured by electron-microscopy in 21/24 renal transplant recipients 2 years after transplantation, treated with either prednisolone/everolimus (P/EVL) or prednisolone/cyclosporine (P/CsA). Data are expressed as median (range). ns = not significant

<table>
<thead>
<tr>
<th></th>
<th>P/EVL (n=14/16)</th>
<th>P/CsA (n=6/7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular basement membrane thickness (nm)</td>
<td>244 (104 – 300)</td>
<td>255 (170 – 276)</td>
<td>ns</td>
</tr>
<tr>
<td>Foot process width (nm/foot process)</td>
<td>546 (421 – 753)</td>
<td>518 (424 - 580)</td>
<td>ns</td>
</tr>
</tbody>
</table>
DISCUSSION

In the total multi-center MECANO trial and in this single center substudy, we found no significant difference in proteinuria 2 years after transplantation in patients treated with prednisolone/everolimus as compared to patients treated with prednisolone/cyclosporine, although there was an increase in proteinuria in patients treated with everolimus 2 years versus 6 months after renal transplantation. However, here we show that this is not accompanied by histopathologic changes as detectable by detailed light and electron microscopic analysis.

From studies performed in animal models, the effect of mTOR inhibitors on glomerular cells appear to be heterogenous and context dependent. For example, in early diabetic nephropathy, characterized by hypertrophy of the glomerulus including the podocytes, mTOR inhibition has been shown to attenuate the progression of diabetic kidney disease by reducing glomerular hypertrophy, mesangial expansion and glomerular basement membrane thickening. On the other hand, mTOR inhibitors have been shown to induce changes in the cytoskeleton and slit-diaphragm. For instance, the expression of nephrin, one of the critical components of the slit-diaphragm was down-regulated. Moreover, mTOR inhibitors reduce VEGF expression, which is relevant for podocyte survival and differentiation. Based on these observations, we hypothesized that mTOR inhibitors could lead to proteinuria by interfering not only with the integrity of the slit-diaphragm but also with the synthesis of the GBM resulting in a thinner membrane. To our knowledge, no previous electron microscopy studies of GBM in protocol biopsies from renal transplant recipients treated with mTOR inhibitors have been reported. Yet, with detailed electron microscopy, we could not demonstrate any difference in the glomeruli between patients treated with EVL or CsA.

No nephrotic range proteinuria occurred in our substudy and since we found no histological differences between the P/CsA and P/EVL group, the observed difference in proteinuria might also be explained by the anti-proteinuric effect of calcineurin inhibitors, caused by a decrease in renal blood flow and stabilization of the actin cytoskeleton through inhibition of synaptopodin degradation. Moreover, we cannot exclude a tubular origin of the proteinuria as previously suggested by Straathof et al. Nevertheless, light microscopy did not reveal any tubular damage. Lastly, mTOR inhibitors could interfere with the expression of cubulin and megalin in the proximal tubular epithelial cells, resulting in decreased tubular receptor-mediated albumin endocytosis.

Multiple reports exist on the association of treatment with sirolimus and an increased incidence of proteinuria. However, only few data are available on possible proteinuric effects of everolimus. This might be explained by the longer use of sirolimus in renal transplantation, differences in actual exposure and/or physico-chemical properties. As already mentioned, the difference in trough levels that were achieved in the several studies may explain a discrepancy in side effects. In our study, we measured a median AUC of 164 μg*h/l, corresponding with trough levels
of 10.4 (3.6-17.1) μg/l, comparable with most of the sirolimus studies. Thus, it has to be elucidated whether everolimus and sirolimus in equimolar concentrations yield similar effects regarding proteinuria. In an *in vitro* model of cultured rat brain cells, everolimus as compared to sirolimus exerted fundamentally different effects on cell metabolism.

In conclusion, prolonged treatment with everolimus did not induce any change in the glomerular basement membrane, in the podocytes or on overall histological examination, neither by light microscopic, nor by electron microscopic analysis. The observed small difference in proteinuria between everolimus and cyclosporine treated patients is most probably explained by the antiproteinuric properties of calcineurin-inhibitors.

### REFERENCES


mTOR Inhibition Enhances the Procoagulant State of Renal Transplant Recipients

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Submitted
ABSTRACT

Background Renal transplant recipients are at increased risk of venous thromboembolic events, which is in part caused by their treatment with maintenance immunosuppressive drugs. Because we observed an increased incidence of venous thrombo-embolic events in renal transplant recipients treated with a mTOR inhibitor (mTORi), we aimed to identify possible prothrombotic mechanisms of this immunosuppressive drug.

Methods In a single center study, nested in a multi-center randomized controlled trial, we measured parameters of endothelial activation, coagulation and fibrinolysis in renal transplant recipients who received the mTORi everolimus (n=16, mTOR group) and compared them to a similar patient group, treated with a calcineurin inhibitor and/or mycophenolate sodium (n=20, non-mTOR group). All patients were at least 6 months following transplantation with a stable transplant function.

Results The use of a mTORi was associated with significantly higher levels of von Willebrand factor, prothrombin fragment 1+2, thrombin-activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 as compared to a non-mTOR based immunosuppressive regimen.

Conclusion Treatment with an mTORi leads to increased endothelial activation, thrombin formation and impaired fibrinolysis. Larger studies with clinically relevant end-points are required to establish the thrombosis risk.
INTRODUCTION

Chronic kidney disease (CKD) is associated with an increased risk of venous thrombo-embolism (VTE), especially in the presence of a nephrotic syndrome\(^1\). The rate of VTE in the first years after kidney transplantation is also increased, mounting up to about 5-9%\(^4,5\). This increase can be explained by several factors. Many renal allograft recipients still suffer from CKD showing a mean estimated creatinine clearance (eGFR) of about 55 ml/minute at 2 years following renal transplantation\(^6\). Furthermore, renal transplant patients are at risk of developing proteinuria, caused in most cases by either recurrence of the original kidney disease or chronic allograft nephropathy.

Another risk factor for thromboembolic disease after renal transplantation is the maintenance immunosuppressive medication. Both steroids and calcineurin inhibitors have prothrombotic properties\(^7,8\). Little is known about the possible prothrombotic side effects of the mTOR-inhibitors (mTORi) sirolimus and everolimus in allograft recipients. Like calcineurin-inhibitors (CNI), they have been associated with thrombotic micro-angiopathy, but the underlying mechanism is uncertain\(^9\). Reduction in (local) production of vascular endothelial growth factor (VEGF) may play a pathogenetic role\(^12\). Also a direct effect on prothrombotic genes encoding for PAI-1\(^13\) and tissue factor (TF), has been observed\(^14\). Recently we performed a multicenter randomized controlled trial (MECANO) studying early cyclosporin withdrawal after six months and comparing maintenance therapy with prednisolone/cyclosporine A (P/CsA) to prednisolone/mycophenolate sodium (P/MPS) or prednisolone/everolimus (P/EVL). Preliminary results have been published\(^15\). Early in this study, we noticed cases of VTE in the everolimus treated patients. This observation, and the known effects on endothelial cells, raised our suspicion of a relationship between mTORi and the occurrence of thrombotic complications.

To study whether indeed treatment with mTOR inhibition leads to procoagulant alterations, we measured parameters of coagulation, fibrinolysis and endothelial activation in renal transplant recipients, participating in the afore mentioned trial, who received the mTOR inhibitor everolimus (EVL) and compared them to patients who were treated with a CNI and/or mycophenolate sodium (MPS).

PATIENTS AND METHODS

Patients

This study was conducted as part of a larger prospective, multicenter randomized trial (MECANO) studying the effects of withdrawal of cyclosporine A (CsA) from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), CsA, prednisolone (P) and mycophenolate sodium (MPS) early after transplantation. Three University Hospitals in the Netherlands participated in this trial from January 2005 until September 2009: the Academic Medical Center in Amsterdam (AMC),
the Leiden University Medical Center and the University Medical Center in Groningen. Institutional review board approval has been obtained. The study was conducted in accordance with the declaration of Helsinki. Informed consent was obtained from every patient. The details and results of an interim analysis of this trial have been published previously (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl). In short, renal allograft recipients, receiving their first or second kidney transplant, were treated with quadruple immune suppression consisting of prednisolone, CsA, MPS and basiliximab. After 6 months, patients were (in the absence of rejection, proven by kidney biopsy) randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and p/everolimus (EVL). Drug exposure of CsA and EVL was monitored by calculating the Area Under the Curve (AUC) at fixed moments. The target values of the AUC for CsA was 5400 μg*h/l in the first 6 weeks and 3250 μg*h/l thereafter. Target AUC for EVL was 150 μg*h/l. The primary outcome was interstitial graft fibrosis and hyalinosis. Secondary outcome was, among others, graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with > 50% panel reactive antibodies. The P/MPS arm was prematurely halted because this form of double therapy resulted in an increase in severe acute rejection episodes.

The present study is a sub study nested in the multicenter trial: renal transplant recipients recruited at one side only (AMC) participated. From February 2008 until December 2009, blood samples were collected from all consecutive patients who were admitted for either a protocol renal biopsy or for protocol drug level monitoring. To study whether treatment with mTOR inhibitors leads to procoagulant alterations, we compared the patients treated with everolimus (mTOR-group) to those who were treated either with CsA or MPS or a combination of the latter two (non-mTOR group). We measured parameters of coagulation, fibrinolysis and endothelial activation. Patients treated with vitamin K antagonists were excluded (n = 1).

**Measurements**

Blood was drawn between 8 and 10 am and anticoagulated with sodium citrate (final concentration, 0.32%). Plasma samples were centrifuged twice at 4°C, 2000 rpm for 20 minutes and frozen at −80°C until assays were performed.

To assess the various contributors to haemostasis: endothelial activation, thrombin/ fibrin formation, anticoagulation and fibrinolysis; the following parameters were measured: von Willebrand factor, vascular endothelial growth factor (VEGF), activated partial thromboplastin time (APTT), prothrombin time (PT), prothrombin fragment F1+2 (F1+2), endogenous thrombin potential (ETP), activated protein C (APC) resistance, protein C, protein S, thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor-1 (PAI-1), plasmin-antiplasmin complexes (PAP) and D-dimer.

Von Willebrand factor antigen (vWF-Ag) was determined with a home-made ELISA using antibodies from DAKO (Glostrup, Denmark), reference values (rv)
50-150%. VEGF was measured by ELISA (R&D systems), rv: <115 pg/ml. PT and APTT were performed on an automated coagulation analyzer (Behring Coagulation System) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany), rv 25.0 – 38.0 and 10.7-12.9 sec, respectively. The plasma concentrations of F1+2 were measured by ELISA (Siemens), rv 53 – 271 pmol/l.

The Calibrated Automated Thrombogram® assays the generation of thrombin in clotting plasma using a micro titer plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinoscope® software (Thrombinoscope BV, Maastricht, The Netherlands). The assay was carried out as described by Hemker et al.16 and the Thrombinoscope® manual.

Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin®, Siemens), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored using the Fluoroscan Ascent fluorometer (ThermoLabsystems, Helsinki, Finland), and the etP was calculated using the Thrombinoscope® software (Thrombinoscope BV). Reference values for ETP were 1155-2606 nM.min.

Resistance to activated protein C was determined by testing the effect of activated protein C on the endogenous thrombin potential (ETP) with the CAT assay. The sensitivity to APC (Enzym Research Laboratories) of each plasma sample was determined in both the presence and absence of ~4 nM APC (final concentration). The APC concentrations used were adjusted to maintain a residual thrombin generation activity of approximately 10% in normal pooled plasma. Normal pooled plasma was run in parallel on each plate. The normalized ratio (APCsr) was determined by dividing the APCsr of an individual by the APCsr of the pooled plasma. A normalized APC sensitivity ratio >1.0 reflects an APC resistant phenotype. Reference value was <1.6.

Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden), rv 70 -120%. Total protein S antigen was assayed by ELISA using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Reference values were 63-137% and 25-130%, respectively. TAFI activity levels were determined with a chromogenic assay (Pefakit TAFI, Pentapharm LTD, Basel, Switzerland) by converting TAFI into its active form and subsequently measuring the carboxypeptidase activity. Measurements were run on a BCS coagulation analyzer (Siemens), rv 64 – 125%. PAI-1 antigen was assayed by ELISA (Innotest PAI-1, Hyphen BioMed, Andrésy, France), rv: < 100 ng/ml. PAP complexes were determined by ELISA (DRG, Marburg, Germany), rv 47 – 563. D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-Dimer, Siemens), rv: < 1.00 mg/l fibrinogen activity units (FEU).

Plasma creatinine levels were measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyzer (Roche, Almere,
The Netherlands). Estimated GFR (eGFR) was calculated using the abbreviated MDRD formula:

\[
GFR = 175 \times \left(\frac{Pcr}{88.4}\right)^{-1.154} \times \text{age}^{-0.203}, \quad \text{(female: multiply result by 0.742, black: multiply result by 1.210)}.
\]

**Statistical analysis**

Statistical analysis was performed using SPSS 16. Data are expressed as median (interquartile range) since not all parameters were normally distributed and numbers were relatively small. For comparison between groups in the substudy, we used Mann–Whitney U test, Chi-square test or Fisher–Freeman–Halton (using StatXact-3), when appropriate. We used the Spearman correlation coefficient to correlate parameters of coagulation with the AUC of CsA, EVL and MPS. Multivariable linear regression analysis was performed to assess the independent influence of various parameters (age, gender, days after transplantation and type of immunosuppression) on the contributors to coagulation. The normality assumption for the linear model was assessed by inspection of the residuals. Since for F1+2 and PAI-1 violations were observed, these variables were analyzed on a transformed scale using \(1/F1+2\) AND \(10\log(PAI-1)\). A p-value ≤ 0.05 was considered statistically significant.

**RESULTS**

We studied 2 differently treated patient groups after renal transplantation (non-mTOR versus mTOR). Patient characteristics are shown in table 1. Forty-two blood samples were collected. After exclusion of 6 blood samples (3 due to clotting, 2 because of the use of oral anticoagulants and 1 because of a too small plasma volume), 36 samples (from 36 patients) were available for analysis. There were twenty patients in the non-mTOR group and 16 in the mTOR group. Of the 20 patients in the non-mTOR group, 11 were treated with triple therapy i.e. prednisolone/cyclosporine/mycophenolate sodium (P/CsA/MPS), 4 were treated with P/CsA, 4 with P/MPS and one was treated with P/tacrolimus. All patients in the mTOR group were on P/everolimus (EVL).

In 29/36 patients a protocol kidney biopsy was performed just following blood withdrawal (20 in the non-mTOR group and 9 in the mTOR group). In 2 cases (both in the non-mTOR group), in the protocol biopsy, a borderline acute cellular rejection was diagnosed, for which no additional treatment was given. They were not randomized to double therapy but continued triple therapy.

The demographic characteristics were similar between both groups, except for the time interval from transplantation to blood withdrawal: this was increased in the patients in the mTOR group as compared to patients in the non-mTOR group (median 754 days versus 570 days). None of the patients developed VTE during the study.
Table 2 shows an overview of the parameters of coagulation. When certain parameters could not be determined due to lack of material, the number of analyzed patients is reported. Figure 1 demonstrates the most important findings. vWF concentrations were elevated in both patient groups and the majority of patients had an abnormal D-dimer result: 24/35 (69%) > 0.5 mg/l and 9/35 (26%) > 1.0 mg/l. CRP did not differ between both groups: median (interquartile range) non-mTOR group 1.2 (0.55 – 3.8) mg/l versus 2.2 (0.55 – 3.0) mg/l in the mTOR group (p = 0.67).

vWF, VEGF, F1+2, protein C, TAFI and PAI-1 were significantly higher in the mTOR treated patients as compared to the non-mTOR group. Multivariable linear regression analysis, including age, gender, days after transplantation and type of immunosuppression, showed that vWF and F1+2 were significantly associated with age and type of immunosuppression, VEGF with gender and immunosuppression. Protein C, TAFI and PAI-1 were affected by type of immunosuppression only.

The median (range) AUC of CsA (n=13) was 3302 (1693 – 5852) µg*h/l, the median (range) AUC of EVL (n=13) was 188 (123 – 229) µg*h/l. We could not demonstrate a significant correlation between the AUCs of EVL and CsA and the level of the assessed parameters of coagulation, fibrinolysis and endothelial activation.

Table 1. Patient characteristics of 36 renal transplant recipients treated with either prednisolone/cyclosporine/mycophenolate sodium (non-mTOR group) or prednisolone/everolimus (mTOR group). Data are expressed as median (interquartile range). M/F = male/female. eGFR = estimated GFR.
Table 2. Parameters of coagulation in renal transplant recipients treated with either prednisolone/cyclosporine/mycophenolate sodium (non-mTOR group) or prednisolone/everolimus (mTOR group). Data are expressed as median (interquartile range). NS = not significant. vWF = von Willebrand factor, VEGF = vascular endothelial growth factor, APTT = activated partial thromboplastin time, PT = prothrombin time, F1+2 = prothrombin fragment F1+2, ETP = endogenous thrombin potential, APC = activated protein C, TAFI = thrombin-activatable fibrinolysis inhibitor, PAI-1 = plasminogen activator inhibitor-1, PAP complexes = plasmin-antiplasmin complexes.

<table>
<thead>
<tr>
<th></th>
<th>non-mTOR (n= 20)</th>
<th>mTOR (n = 16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>192 (175 – 252)</td>
<td>220 (192 – 266)</td>
<td>NS</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>178 (129 – 243)</td>
<td>315 (252 – 361)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>32.4 (20.6 – 38.2)</td>
<td>39.6 (29.2 – 58.4)</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>32.4 (30.9 – 38.0)</td>
<td>33.1 (30.6 – 36.6)</td>
<td>NS</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>11.9 (11.6 – 12.9)</td>
<td>12.0 (11.7 – 12.7)</td>
<td>NS</td>
</tr>
<tr>
<td>F1 + 2 (pmol/l)</td>
<td>356 (307 – 463)</td>
<td>556 (383 – 791)</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>ETP (nM.min)</td>
<td>1399 (1271 – 1583)</td>
<td>1477 (1346 – 1632)</td>
<td>NS</td>
</tr>
<tr>
<td>APC</td>
<td>0.58 (0.00 – 1.07)</td>
<td>0.65 (0.00 – 1.90)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>128 (109 – 163)</td>
<td>173 (152 – 196)</td>
<td>p = 0.009</td>
</tr>
<tr>
<td>Protein S (total) (%)</td>
<td>120 (106 – 125)</td>
<td>131 (111 – 159)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein S (free) (%)</td>
<td>109 (95 – 123)</td>
<td>109 (94 – 121)</td>
<td>NS</td>
</tr>
<tr>
<td>TAFI (%)</td>
<td>102 (90 – 120)</td>
<td>122 (102 – 134)</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>61 (30 – 90)</td>
<td>100 (48 – 149)</td>
<td>p = 0.05</td>
</tr>
<tr>
<td>PAP complexes (µg/l)</td>
<td>488 (365 – 736)</td>
<td>657 (435 – 752)</td>
<td>NS</td>
</tr>
<tr>
<td>D-dimer (mg/l)</td>
<td>0.78 (0.32 – 1.19)</td>
<td>0.76 (0.48 – 0.94)</td>
<td>NS</td>
</tr>
</tbody>
</table>

DISCUSSION

In this pilot study in 36 renal transplant recipients we aimed to find an underlying mechanism for the increased incidence in thrombotic events in patients using mTOR inhibitors. We here demonstrate that von Willebrand factor concentration is elevated in all RTRs as is the F1+2 level, pointing to increased coagulation potential and activation of coagulation following renal transplantation. Indeed, the use of
mTOR inhibitors even further increased vWF levels as compared to a non-mTOR based immunsuppressive regimen. Furthermore, we observed higher F1+2, TAFI and PAI-1 concentrations in the patients on a mTORi. This indicates that treatment with mTORi (i.e. everolimus) leads to increased endothelial activation, thrombin formation and impaired fibrinolysis compared to treatment with a non-mTORi (i.e. CNI and/or MPS).
In our study the high levels of vWF in both groups, and especially in the mTOR group, are striking. Previous studies in renal transplant recipients treated with prednisolone, cyclosporine and/or mycophenolate mofetil, reported vWF values of around 200%, corresponding with our values in the non-mTOR group\textsuperscript{17,19}. To the best of our knowledge no studies on vWF levels in renal transplant recipients treated with mTOR inhibitors have been reported so far. The higher levels of vWF in the EVL group are compatible with either increased endothelial activation or increased release from activated platelets. The latter also applies to the higher levels of VEGF in the EVL group. VEGF has been shown to be crucial to preserve the integrity of the endothelium\textsuperscript{20}. In contrast to our findings, previous studies demonstrated that mTOR inhibitors decrease VEGF levels locally and systemically\textsuperscript{12,21}, possibly mediated through hypoxia inducible factor 1\textalpha (HIF1\textalpha). HIF1\textalpha is regulated via the mTOR pathway: mTOR inhibitors decrease the production of HIF1\textalpha and thereby VEGF\textsuperscript{22}. However, the fact that we found elevated levels of VEGF might be explained by VEGF release from platelets, reflecting an ex vivo phenomenon caused by activation of platelets by blood collection using a tourniquet (Niers et al, unpublished data). The higher concentrations in the mTOR treated group found by us could therefore point to increased platelet activatability in these patients. Increased levels of F1 + 2 in the mTOR group indicate enhanced in vivo thrombin formation leading to fibrin generation and platelet activation. In addition, increased thrombin generation leads to augmented activation of TAFI which impairs fibrinolysis. Both TAFI and PAI-1, another inhibitor of fibrinolysis were increased in the mTOR compared to the non-mTOR treated patients.

The only significant difference between the treatment group was the time after transplantation. Previous studies have demonstrated a decrease in levels of prothrombotic markers and an improvement of endothelial injury markers after transplantation as compared to before or early after transplantation\textsuperscript{23-25}. However, in our study, time after transplantation in patients in the mTOR group was even longer as compared to the non-mTOR treated group.

A possible explanation for the increased procoagulant state in the mTOR-group could be up-regulation of tissue factor (TF) due to the inhibition of the mTOR pathway by everolimus\textsuperscript{14,26}. TF production is known to be stimulated via the VEGF/MAP kinase pathway and inhibited by the PI3K/Akt/mTOR pathway. Inhibition of the mTOR pathway by everolimus thus may lead to up-regulation of TF expression, by removing this additional negative feedback loop, which in turn might lead to activation of coagulation\textsuperscript{26}.

The main limitation of this substudy of the MECANO trial is the relatively small number of patients. None of the patients in our substudy developed a VTE during the study period. However, our initial observation of thrombosis in everolimus treated patients was recently confirmed after completion of the final analysis of the MECANO trial van der Heide, manuscript in preparation). Seven out of 96 (7.3%) everolimus treated patients developed a VTE (5 deep venous thrombosis
and 2 pulmonary embolism) versus 1/39 (2.6%) (DVT) and 0/89 (0%) in the P/MPS and P/CsA treated patients, respectively, p = 0.02 (Fisher-Freeman-Haltman test). This increased incidence of VTE is in line with a recently published study\textsuperscript{27}. In this study significant more VTEs occurred in lung transplant recipients treated with a combination of prednisolone, tacrolimus and sirolimus than in those treated with prednisolone, tacrolimus and azathioprine (15/87 (17.2%) versus 3/94 (3.2%), p < 0.01). Moreover, several case reports have been published about striking arterial or venous thromboembolic events in solid organ transplant recipients treated with an mTORi \textsuperscript{28-30}. Unexpectedly, VTE is not described as an adverse event in many randomized controlled trials studying mTORi in renal transplantation\textsuperscript{31, 32} or for the treatment of renal cell carcinoma\textsuperscript{33, 34}. According to the published incidence of VTE in RTR or in patients with a renal cell carcinoma, one would expect, even without the use of mTORi, an incidence of at least 5% in those trials\textsuperscript{4, 5, 35, 36}. However, venous thrombosis as an adverse event is often underreported in clinical studies.

In conclusion, we found an increased procoagulant state in renal transplant recipients treated with a mTORi (everolimus) compared to those treated with a non-mTOR (i.e. CNI and/or MPS) based immunosuppressive regimen. Increased endothelial activation, thrombin formation and impaired fibrinolysis seem to be responsible, although we could not exclude a possible effect of platelet activation. This suggests an increased risk of thrombotic events in renal transplant recipients treated with mTOR inhibitors. Although more studies with hard end-points are necessary to establish the clinical risk, in the meantime one should be cautious in prescribing mTOR inhibitors to patients with a history of venous or arterial thrombosis.

**ACKNOWLEDGEMENTS**

We greatly acknowledge G. Nieuwenhuizen for her excellent support in collecting the data.

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Part III

Appendices
9 Summary
PART I

Exact assessment of renal function, expressed as glomerular filtration rate (GFR), remains an important subject of research even 75 years after the discovery of creatinine as an endogenous marker to estimate renal function. Since plasma creatinine concentration is influenced by other parameters than kidney function alone, for example age, gender, race and diet, multiple formulas have been developed to overcome these factors. The most known and commonly used formulas are the Cockcroft-Gault - and MDRD formula. Furthermore, other endogenous markers have been identified, like cystatin C and beta-trace protein, possibly representing a better reflection of GFR than plasma creatinine concentration.

In chapter 1, the performance of these various endogenous markers in patients at risk or with overt renal failure, is discussed in comparison to established gold standard methods for the assessment of GFR, like $^{51}$Cr-EDTA and $^{125}$I-iothalamate/$^{131}$I-hippuran. The advantages and disadvantages of formulas based on these endogenous markers are described. Also a newly developed, still experimental method using the MRI contrast agent gadolinium-DTPA, is introduced.

Adult patients with Fabry disease, a lysosomal storage disease due to alpha-galactosidase deficiency, underwent yearly GFR measurement with iothalamate/hippuran to monitor the effect of enzyme replacement therapy. In chapter 2, these data were used to compare the value of formulas based on either plasma creatinine, cystatin C, beta-trace protein or a combination, with the gold standard GFR measurement. Bias, but especially precision and accuracy deviated considerably from gold standard. Furthermore, the creatinine-based formulas overestimated GFR in male Fabry patients, possibly reflecting decreased muscle mass in these patients compared to the normal population. Cystatin C and beta-trace protein alone did not prove to be better markers to estimate GFR. Although, we concluded that GFR estimated by the tested formulas could not replace gold standard GFR when more precise knowledge of renal function is required, the creatinine and cystatin C combined Stevens formula performed best, closely followed by the CKD-EPI and MDRD.

Chapter 3 discusses a new exogenous nonradioactive marker as a gold standard method to assess GFR, gadolinium-DTPA (Gd-DTPA, Magnevist®). We measured renal function in renal transplant recipients, candidate kidney donors, HIV infected patients and patients with Fabry disease. Unfortunately, GFR measurement using Gd-DTPA had an unacceptable bias, precision and accuracy when compared to an established radioactive gold standard method with $^{51}$Cr-EDTA and did not outperform estimated GFR with creatinine and/or cystatin C. Therefore we do not consider this method suitable to be used in the clinic.

Cystatin C is explored in chapter 4 as a marker of residual renal function in critically ill patients treated with continuous venovenous hemofiltration (CVVH). Since creatinine is removed from the blood during haemofiltration, it can not be used to determine residual renal function. However, the low molecular weight protein
Cystatin C, with a molecular weight of 13.3 kD, appeared to be a good candidate for this task. We measured pre- and post filter concentrations of cystatin C and found that the removed quantity averaged 2.13 mg/h, corresponding with less than 30% of its production. We therefore conclude that cystatin C could be representative of residual renal function.

Precise determination of GFR remains extremely difficult and the obtained kidney function can differ depending on the used method. Furthermore, additional physiologic factors like diet, fluid intake or circadian rhythm, can affect GFR not necessarily reflecting an improvement or pathologic decline of GFR. In general, exact measurement of GFR should be reserved for specific situations for example the measurement of GFR in candidate kidney donors or in research settings. Most often, it is sufficient to know whether kidney function is above or below 60 ml/min, for instance if medication has to be adapted. Creatinine-based formulas like the MDRD and recently developed CKD-EPI appear to be precise enough when GFR is between 10 and 60 ml/min. However, one should be careful in interpreting estimated GFR > 60 ml/min, since creatinine is an unreliable marker in this range. The course of estimated GFR in time is the most crucial to monitor, and action should be undertaken when significant decline is noticed.

Cystatin C could have a place in situations when creatinine is notoriously unreliable, for example in patients with extremely low muscle mass which is the case in anorexia nervosa and muscle diseases. Moreover, in the rare case of urine leakage in the peritoneal cavity and hereafter reabsorption of urinary creatinine, plasma creatinine is falsely elevated and cystatin C could be of value since it is broken down in the proximal tubule and does therefore not appear in the urine. There seems no additional value of beta-trace protein.
PART II

Inhibitors of the mammalian target of rapamycin (mTOR) were introduced in renal transplantation because of their supposed lack of nephrotoxicity, possible anticancer effects and their beneficial effects on the vessel wall. Recently a multicenter randomized controlled trial, the MECANO trial, was performed studying the effects of withdrawal of cyclosporine from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), cyclosporine, prednisolone and mycophenolate sodium early after renal transplantation. After 6 months, renal transplant recipients were (in the absence of rejection, proven by renal biopsy) randomized to one of three immunosuppressive regimens: prednisolone/ cyclosporine, prednisolone/mycophenolate sodium and prednisolone/everolimus. The prednisolone/mycophenolate arm was prematurely halted due to an increased incidence of acute rejection. From January 2005 until September 2009, the Academic Medical Center, University Medical Center Groningen and the Leiden University Medical Center participated. The studies mentioned below represent substudies of this trial.

Chapter 5 gives an overview of the mTOR pathway and the general effects of mTOR inhibition. Since the mTOR pathway is ubiquitously present in the body it is not surprising that many side effects accompany its use.

One of the most severe complications caused by mTOR inhibitors is pneumonitis, a possible life threatening condition. Chapter 6 describes a case-control study performed in renal transplant recipients treated with the mTOR inhibitor everolimus, reporting the incidence, radiologic features and risk factors of everolimus-induced pneumonitis (EIP). EIP appeared to occur relatively often with an incidence of 13%, presenting as organizing pneumonia and/or non-specific interstitial pneumonitis, the latter carrying the risk of becoming a chronic condition. Unfortunately no risk factors could be identified, especially no correlation with everolimus dose. This lead to the recommendation to withdraw everolimus completely instead of lowering the dose when EIP is suspected.

Another, much debated side effect is the often observed de novo occurrence of or increase in proteinuria after start of an mTOR inhibitor. This issue is addressed in chapter 7, comparing proteinuria in renal transplant recipients treated with prednisolone/everolimus to those treated with prednisolone/cyclosporine and relating this to renal biopsy data analyzed with conventional light microscopy as well as electron microscopy. We found a slight increase in non-nephrotic range proteinuria in the everolimus treated patients, not accompanied by a decrease in renal function. Moreover, we found no abnormalities upon light- and electron microscopy, especially no signs of podocyte damage. The increase in proteinuria in the everolimus treated patients compared to the cyclosporine treated patients might be explained by the antiproteinuric properties of the latter.

Although chronic kidney disease and the use of immunosuppressive drugs are known to increase the risk of venous-thrombo-embolic events, the observed number of unexpected thrombo-embolic events in patients treated with the mTOR inhibitors
sirolimus or everolimus was remarkable. This was the reason to conduct the pilot study reported in chapter 8. Here we compared various parameters of coagulation in everolimus treated patients (mTOR group) to those treated with cyclosporine and/or mycophelate (non mTOR group). We found that in the mTOR group as well as in the non mTOR group, von Willebrand factor prothrombin fragment 1+2 were elevated, pointing to increased coagulation potential and activation of coagulation following renal transplantation. The use of mTOR inhibitors even further increased vWF levels as compared to a non-mTOR based immunosuppressive regimen. Furthermore, we observed higher F1+2, TAFI and PAI-1 concentrations in the patients treated with an mTOR inhibitor. This indicates that treatment with an mTOR inhibitor leads to increased endothelial activation, thrombin formation and impaired fibrinolysis compared to treatment with a non-mTOR inhibitor. At completion of this pilot study, the overall results of the MECANO study were analyzed and demonstrated an increased incidence of thromboembolic complications in the everolimus - compared to the cyclosporine and mycophenolate treated patients. This suggests caution in prescribing mTOR inhibitors to patients with a history of venous or arterial thrombosis.

The drop-out rate due to side effects of mTOR inhibitors in patients treated with these drugs in study context is high, sometimes mounting 50%. Side effects complicating its use vary from mild diarrhea to life threatening pneumonitis. Considering the high incidence of adverse effects, first line treatment with mTOR inhibitors in renal transplantation is not a matter of course. Nevertheless, in selected patient groups mTOR inhibitors can have a clear place. Calcineurin-inhibitor induced nephrotoxicity is a common cause of graft failure; switch to a calcineurin-inhibitor free regimen with an mTOR inhibitor can lead to stabilization or slowing down of decline in GFR. Furthermore, an increasing amount of data suggest that mTOR inhibitors have beneficial effects on the incidence of non-melanoma skin cancer and are therefore preferred as immunosuppressive drug in these patients. Moreover, it is probably effective in lymphoma and already registered for the treatment of renal cell carcinoma, both more prevalent in renal transplant recipients. If prescribed, adequate monitoring of adverse events is important. Based on the studies mentioned in Part II, next to standard measurements of kidney and liver function, differential count and cholesterol, I would recommend to monitor also pulmonary function by function tests before start and 3 monthly hereafter in the first two years. If the vital capacity or CO diffusion declines, a HRCT should be performed to exclude EIP. If proteinuria occurs or increases during treatment with an mTOR inhibitor, renal biopsy should be performed to be able to make an exact diagnosis. If mTOR inhibitors are prescribed to patients with a history of venous or arterial thrombosis, anticoagulation should be considered pending further studies.
Nederlandse Samenvatting
DEEL I

Nauwkeurige bepaling van de nierfunctie, uitgedrukt als glomerulaire filtratie snelheid (GFR, normaal 100 -120 ml/minuut), blijft een belangrijk onderwerp voor onderzoek. 75 jaar geleden was er een belangrijke doorbraak in het onderzoek naar nierfunctie bepaling met de ontdekking van kreatinine, een lichaamseigen stof in het bloed dat gebruikt kan worden om de nierfunctie te schatten. Kreatinine is afkomstig uit de spier en wordt daarom helaas ook door andere factoren beïnvloed dan door nierfunctie alleen, zoals bijvoorbeeld leeftijd, geslacht en ras: allen factoren die een effect op de spiermassa hebben. Daarom zijn er op kreatinine gebaseerde formules ontwikkeld die deze factoren proberen te corrigeren. De meest bekende en de meest gebruikte zijn de Cockcroft – Gault, MDRD formule en de recent ontwikkelde CKD-EPI formule. Ook zijn er in het bloed andere lichaamseigen stoffen ontdekt die mogelijk een betere weerspiegeling geven van de nierfunctie dan kreatinine, zoals bijvoorbeeld cystatine C en beta-trace protein.

In deel I van dit proefschrift, ingeleid in hoofdstuk 1, wordt de waarde van deze verschillende lichaamseigen stoffen besproken om de nierfunctie te schatten van patiënten die een hoog risico lopen op nierschade of reeds nierfalen hebben. Voor- en nadelen van de verschillende formules worden besproken. De waarde van de formules wordt afgezet tegen methodes waarmee de nierfunctie heel precies gemeten kan worden met behulp van licht radioactieve stofjes zoals $^{51}$Cr-EDTA en $^{125}$I-iothalamate/$^{131}$I-hippuran, de zogenaamde ‘gouden standaard’ methoden. Recent is er een nieuwe, nog experimentele, methode gevonden, waarmee het MRI contrast middel, gadolinium-DTPA, gebruikt wordt om nierfunctie te meten. Deze methode wordt ook besproken.

De ziekte van Fabry is een ziekte waarbij nierschade kan optreden door stapeling van een ziekmakende stof door gebrek aan een enzym dat dit stofje afbreekt. Behandeling met het ontbrekende enzym kan nierfunctie verslechtering tegen gaan. Patiënten met de ziekte van Fabry ondergaan jaarlijks een precieze nierfunctie meting met $^{125}$I-iothalamate/$^{131}$I-hippuran, om het effect van deze behandeling te beoordelen. Omdat precieze meting van de nierfunctie veel werk is en tijd kost (bijna een halve dag in het ziekenhuis), wordt er gezocht naar alternatieven, bijvoorbeeld in de vorm van op kreatinine-, cystatine C- of beta-trace protein gebaseerde formules, die slechts een simpele bloedafname inhouden.

In hoofdstuk 2 wordt gekeken of formules de ‘gouden standaard’ methode kunnen vervangen. De gegevens van de nierfunctie meting met $^{125}$I-iothalamate/$^{131}$I-hippuran in Fabry patiënten worden gebruikt om de waarde te bepalen van de formules om de nierfunctie te schatten, door deze met elkaar te vergelijken. Helaas bleek dat de formules de nierfunctie niet precies genoeg konden bepalen om de gouden standaard methode te vervangen. De nierfunctie werd overschat in mannelijke Fabry patiënten als op kreatinine gebaseerde formules werden gebruikt. Formules waarin alleen cystatine C of beta-traceprotein werden gebruikt, waren niet beter dan die waarin het alom vertrouwde kreatinine werd gebruikt. De formule waarin zowel kreatinine
als cystatine C werd gebruikt, de Stevens formule, bleek het beste te presteren. Als men toch de nierfunctie wil schatten in Fabry patiënten is het advies om de Stevens formule te gebruiken of de alleen op kreatinine gebaseerde CKD-EPI of MDRD formule.

**Hoofdstuk 3** bespreek de nieuwe, ‘niet radioactieve’ methode voor nierfunctie meting met behulp van het MRI contrast middel, gadolinium-DTPA (Gd-DTPA, Magnevist®) als alternatief voor de ‘radioactieve’ methode met $^{51}$Cr-EDTA. Wij hebben de nierfunctie gemeten van niertransplantatie patiënten, kandidaat nierdonoren, HIV geïnfecteerde patiënten en van patiënten met de ziekte van Fabry met zowel $^{51}$Cr-EDTA (een bekende gouden standaard methode) als met Gd-DTPA. De nieuwe methode bleek echter niet zo goed als $^{51}$Cr-EDTA. De methode was zelfs niet beter dan de op kreatinine en/of op cystatine C gebaseerde formules waarmee de nierfunctie geschat wordt. Daarom achten wij deze methode niet geschikt om de bestaande gouden standaard methodes te vervangen.

Cystatine C wordt in **hoofdstuk 4** onderzocht als marker voor restnierfunctie van patiënten die op de intensive care worden behandeld met een bepaalde vorm van dialyse, namelijk continue venoveneuze hemofiltratie (CVVH). Er bestaat tot op heden geen goede manier, anders dan de urineproductie, om de restnierfunctie tijdens behandeling met CVVH te bepalen. Omdat kreatinine kunstmatig uit het bloed wordt verwijderd tijdens hemofiltratie, kan het niet gebruikt worden als maat voor restnierfunctie. Omdat cystatine C een stuk groter is, wordt het minder makkelijk uit bloed gefilterd en zou daarom een goede kandidaat kunnen zijn voor deze taak. Wij tonen aan dat de hoeveelheid cystatine C, verwijderd tijdens CVVH, minder is dan 30% van de productie en dat de concentratie van cystatine C in het bloed niet snel verandert. Cystatine C zou dus gebruikt kunnen worden om de restnierfunctie tijdens CVVH te monitoren.

Precieze bepaling van de nierfunctie blijft zeer moeilijk en de gemeten nierfunctie kan verschillen afhankelijk van welke methode werd gebruikt om deze te bepalen. Daar komt bij dat normale fysiologische factoren zoals dieet, vocht inname en dag/nachtritme ook de nierfunctie kunnen beïnvloeden, wat niet perse een blijvende verbetering of een verslechtering van de nierfunctie hoeft te betekenen. Precieze nierfunctie meting moet gereserveerd worden voor specifieke situaties zoals wanneer er twijfel bestaat over de nierfunctie van kandidaat nierdonoren of in het kader van onderzoek. Over het algemeen zal het voldoende zijn te weten of een nierfunctie beter of slechter is dan 60 ml/minuut, bijvoorbeeld wanneer medicatie moet worden aangepast aan de nierfunctie. De op kreatinine gebaseerde formules zoals de MDRD en de recent ontwikkelde CKD-EPI lijken een adequate schatting van de nierfunctie te kunnen geven als deze tussen de 10 en 60 ml/minuut ligt. Boven de 60 ml/minuut moet men voorzichtig zijn met het interpreteren van de geschatte nierfunctie, omdat kreatinine in dit gebied notoir onbetrouwbaar is. Mijns inziens is het allerbelangrijkst het verloop van de nierfunctie in de tijd te volgen en in actie te komen als de nierfunctie belangrijk achteruit gaat.
Cystatine C zou gebruikt kunnen worden in enkele specifieke situaties waarin kreatinine niet gebruikt kan worden. Bijvoorbeeld bij patiënten met een zeer geringe spiermassa in het kader van anorexia nervosa of een ernstige spierziekte; of in de zeldzame situatie van urine lekkage in de buikholte waarbij de afvalstof kreatinine uit de urine weer via het buikvlies in het bloed wordt opgenomen en de nierfunctie vals verlaagd lijkt. Cystatine C wordt niet in de urine uitgescheiden maar al in de nier afgebroken en kan dus op dat moment een betere weerspiegeling van de nierfunctie geven. Naar mijn mening heeft beta-trace protein geen aanvullende waarde in bepaling van de nierfunctie.

**DEEL II**

In de cellen van ons lichaam worden processen gereguleerd via een aantal paden. Het ‘mTOR pad’ is zo’n belangrijke route. Remmers van ‘the mammalian target of rapamyin’ (mTOR), zoals ‘sirolimus’ en ‘everolimus’, zijn sinds eind jaren negentig als afweeronderdrukkende medicijnen in het kader van niertransplantatie in gebruik genomen. Ze hebben een aantal gunstige eigenschappen omdat ze naast het onderdrukken van de afweer ook beschermende effecten tegen kanker hebben; aderverkalking tegen kunnen gaan en, in tegenstelling tot de veel gebruikte afweeronderdrukkende ‘calcineurine-remmers’ niet schadelijk zijn voor de nier.


**Hoofdstuk 5** geeft een overzicht van het mTOR-pad en beschrijft de algemene effecten die optreden wanneer dit pad geremd wordt door middel van mTOR-remmers. Omdat het mTOR-pad bijna in alle cellen van ons lichaam aanwezig is, is het niet verrassend dat er vele bijwerkingen kunnen optreden bij remming hiervan.

Eén van de potentieel gevaarlijkste complicaties die op kunnen treden bij het gebruik van mTOR-remmers is het ontstaan van een ‘steriele’ longontsteking. Hiermee wordt een ontstekingsreactie in de long bedoeld die niet veroorzaakt wordt door bacteriën of virussen. **Hoofdstuk 6** beschrijft een ‘case-control’ studie,
waarin de karakteristieken van patiënten die behandeld werden met de mTOR-remmer ‘everolimus’ en die zo’n steriele longontsteking kregen, worden vergeleken met de karakteristieken van patiënten die ook everolimus gebruikten, maar die geen longontsteking kregen. De steriele longontsteking wordt in dit geval een ‘everolimus-geïnduceerde pneumonie’ (EIP) genoemd. De case-controle studie was bedoeld om inzicht te krijgen in vóórkomen van EIP, hoe EIP zich presenteert op een gedetailleerde röntgenfoto (CT-scan) en wat de risicofactoren zijn om EIP te ontwikkelen. EIP bleek vaak voor te komen: 13% van de patiënten die everolimus gebruikten, ontwikkelden ook deze steriele longontsteking. De CT-scan toonde het typische beeld van ‘een organiserende pneumonie’ en/of een ‘niet-specifieke interstitiële pneumonitis’. Deze laatste longaandoening kan ontstaan in een chronische ontsteking waarbij de long uiteindelijk verbindweefselt. Wij konden helaas geen risicofactoren vaststellen, vooral vonden wij geen relatie met de hoogte van de dosering van everolimus.

Daarom adviseren wij everolimus te staken in plaats van de dosis te verlagen, als het vermoeden bestaat op een EIP.

Een andere, veel besproken bijwerking van mTOR-remmers is het ontstaan of de verergering van eiwitverlies in de urine door lekkage uit de nier na het starten van dit medicijn. De oorzaak hiervan is nog niet geheel duidelijk. Dit onderwerp wordt besproken in hoofdstuk 7, waarbij eiwitverlies in de urine van niertransplantatie patiënten die behandeld werden met prednisolon/everolimus, wordt vergeleken met het eiwitverlies in de urine van patiënten die behandeld worden met prednisolon/cyclosporine. Dit eiwitverlies relateerden wij aan de uitslag van nierbiopten (dit zijn kleine stukjes weefsel afgenomen uit de nier). De nierbiopten werden bekeken met behulp van standaard microscopisch onderzoek, maar ook met behulp van ‘electronen-microscopisch onderzoek’ waarbij de nier wordt bekeken met een vergroting van 10.500 maal. De nier bestaat uit honderdduizenden afzonderlijke nierfiltertjes, de glomerulus, en met behulp van electronenmicroscopie kunnen de aparte onderdelen van deze nierfiltertjes zeer nauwkeurig bekeken worden. Een van de cellen die een belangrijke rol speelt bij evenwel verlies uit de nier, is de ‘podocyt’, deze cel is er erg belangrijk in de vorming van het nierfilter. In deze substudie vonden wij een minimale toename in eiwitverlies via de nier in patiënten die everolimus gebruikten in vergelijking met de patiënten die cyclosporine gebruikten. De nierfunctie van deze patiënten verschilde niet. Ook was er geen verschil in het microscopisch onderzoek van de nierbiopten van deze patiënten, met name geen tekenen van schade aan de ‘podocyt’.

Hoewel een chronische nierziekte en het gebruik van afweeronderdrukkende medicijnen beiden het risico op trombose (het spontaan vormen van een bloedstolsel) verhogen, leek er opmerkelijk meer trombose voor te komen bij patiënten die behandeld werden met de mTOR-remmers sirolimus en everolimus. Dit was reden om het onderzoek te starten wat in hoofdstuk 8 besproken wordt. In deze studie worden verschillende parameters van stolling gemeten bij patiënten behandeld met everolimus (mTOR groep) en vergeleken met die van patiënten behandeld met
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cyclosporine en/of mycofenolaat (non mTOR groep). Wij vonden dat 2 parameters, namelijk von Willebrand factor en prothrombine fragment 1+2 duidelijk verhoogd waren in zowel de mTOR groep als de non mTOR groep. Dit wijst op een toegenomen neiging tot stolling en ook daadwerkelijk activatie van stolling na niertransplantatie. In de mTOR groep bleek von Willebrand factor echter nog belangrijk hoger dan in de non-mTOR groep, dit gold ook voor protrombine fragment 1 + 2, TAFI en PAI-1, passend bij activatie van het endotheel (het cellaagje dat de bloedvaat wand bekleeft), daadwerkelijke bloedstolsel vorming en verminderde afbraak van dit stolsel in de met everolimus behandelde patiënten. Uit de uiteindelijk resultaten van de gehele MECANO studie bleek inderdaad dat er significant meer trombose voorkwam in de met everolimus behandelde patiënten in vergelijking met de cyclosporine en mycofenolaat behandelde patiënten. Dit suggereert terughoudendheid in het voorschrijven van mTOR remmers aan patiënten met een voorgeschiedenis van trombose.

Het percentage patiënten dat in studieverband wordt behandeld met een mTOR remmer en vanwege bijwerkingen moet stoppen met dit middel is groot, tot wel 50%. De bijwerkingen kunnen variëren van milde diarree tot een levensbedreigende steriele longontsteking. Gezien de grote kans op bijwerkingen, is het niet vanzelfsprekend om mTOR remmers voor te schrijven als standaard behandeling na niertransplantatie. Desalniettemin kunnen mTOR remmers zeer geschikt zijn in geselecteerde groepen patiënten. Schade aan de nier, veroorzaakt door calcineurine remmers is een belangrijke oorzaak van niertransplantatiefalen. Het omzetten naar een behandeling zonder calcineurine remmers en met een mTOR remmer, kan leiden tot stabilisatie of vermindering van de achteruitgang in nierfunctie. Huidkanker is een veel voorkomende complicatie van chronisch afweeronderdrukkende medicatie. mTOR remmers verkleinen de kans op het ontstaan van huidkanker en kunnen dus gebruikt worden als afweeronderdrukkend middel in niertransplantatie patiënten met deze vorm van kanker. Daar komt bij dat mTOR remmers effectief zijn gebleken in de behandeling van lymfklierkanker en nierkanker, beiden meer voorkomend in transplantatie patiënten.

Indien mTOR remmers worden voorgeschreven is adequate monitoring van de bijwerkingen belangrijk. Gebaseerd op de studies in deel II van dit proefschrift, zou ik willen adviseren, naast de standaard bloed controle van nier- en leverfunctie, bloedbeeld en cholesterol, om ook de longfunctie te vervolgen. De longfunctie moet worden gemeten voor de start van behandeling en daarna 3 maandelijks in de eerste 2 jaar na start van de mTOR remmer. Als de longcapaciteit vermindert of de gaswisseling afneemt, moet een CT van de longen worden gemaakt om een mTOR geinduceerde steriele longontsteking uit te sluiten. Als belangrijk eivit verlies in de urine ontstaat of verergerd tijdens behandeling met een mTOR remmer, moet een nierbiopt worden verricht om een exacte diagnose te stellen. Als mTOR remmers moeten worden voorgeschreven aan patiënten met een voorgeschiedenis van trombose, moet vooralsnog behandeling met antistolling worden overwogen.
Dankwoord
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Voilà, klaar! Enige reflectie..

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Hou van jullie.
CURRICULUM VITAE


LIST OF PUBLICATIONS


M.C. Baas, J.C. Wetssteyn, T.J. van Gool. Patterns of imported malaria at the Academic Medical Center, Amsterdam, the Netherlands. Travel Med. 2006 Jan-Feb;13(1):2-7