Antiviral Therapy 11:1071–1080

Au: title shortened as rather too long for our style - OK?

Macroenzyme creatine kinase (CK) type 2 in HIV-infected patients is significantly associated with TDF and consists of ubiquitous mitochondrial CK

*Holger Schmid[1†], Dieter Mühlbayer[2†], Jörg Röling[1], Thomas Sternfeld[1], Boris Jülg[1], Uwe Schlattner[3], Peter J Nelson[1], Johannes R Bogner[1], Theo Wallimann[3] and Frank D Goebel[1]**

[1]Medizinische Poliklinik, Ludwig Maximilians University Munich, Germany
[2]Institut für Klinische Chemie, Ludwig Maximilians University Munich, Germany
[3]Institut für Zellbiologie, ETH Zurich, Switzerland
[†]These authors contributed equally to this article

*Corresponding author: Tel: +49 89 5160 3550; Fax: +49 89 5160 3593; E-mail: Frank.Goebel@med.uni-muenchen.de

Objective: To evaluate the prevalence and origin of macroenzyme creatine kinase type 2 (Macro CK2) in HIV-1-infected patients on antiretroviral treatment.
Design: CK, CK-MB activity and protein weight, electrophoretic behaviour, glomerular filtration rate (GFR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bone alkaline phosphatase (AP), β2-microglobulin serum levels and proteinuria were analysed in 468 HIV-infected outpatients. Sera with detectable Macro CK2 were further analysed using immunoblotting.
Results: CK-MB isoenzyme activity and mass concentration revealed the presence of Macro CK2 in 32/408 (7.8%) outpatients. Tenofovir DF (TDF) treatment was a prominent common feature in these patients. Prospective examination of sera from 41 patients collected prior to and during TDF exposure showed Macro CK2 in 20/41 (48%) TDF-treated patients and in 0/19 control sera from patients with TDF-free regimens. Macro CK2 was not present prior to TDF exposure. Patients with Macro CK2 showed a significant elevation of serum β2-microglobulin levels. GFR, AST/ALT ratio, bone AP and proteinuria remained unchanged. Electrophoresis and immunoblotting demonstrated that the Macro CK2 in TDF-treated patients consisted of the ubiquitous (uMtCK) and not the sarcomeric type (sMtCK) of mitochondrial CK (MtCK).
Conclusions: Macro CK2 consisting of uMtCK is associated with the use of TDF-containing regimens. Whether the appearance of uMtCK in these patients reflects mitochondrial damage remains to be clarified.

Introduction

Elevation of creatine kinase (CK) in HIV-infected patients is a frequent finding associated with cardiac or muscle disease under highly active antiretroviral treatment (HAART) [1,2]. Macroenzymes are enzymes with high molecular weight resulting in reduced blood clearance. This often leads to an increased serum activity that can confound laboratory tests [3].

Macroenzyme creatine kinase (Macro CK) is an abnormal CK isoenzyme found in approximately 1% of patients tested for CK, and a diagnostic problem in interpretation of elevated total serum CK and CK-MB concentrations. Based on their different molecular weights, CK isoenzyme determination by electrophoresis allows the diagnosis of Macro CK and measurement of the true CK-MB fraction [4].

Two types of Macro CK have been identified: Macro CK type 1 (Macro CK1) is a cytoplasmatic complex formed by one of the CK isoenzymes and immunoglobulins. It has a prevalence rate of 0.4–1.2% and is associated with autoimmune disease [5]. Macro CK type 2 (Macro CK2) represents mitochondrial CK (MtCK) and consists of oligomeric complexes characterized by heat stability, increased molecular mass and high activation energy [6]. MtCKs are usually found exclusively in the mitochondrial compartment of cells attached to the outer surface of the mitochondrial inner membrane and are key players in energy metabolism [7]. Two isoenzymes of MtCK have been defined based on tissue expression. They are designated as sarcomeric (sMtCK) or ubiquitous (uMtCK) MtCK. The appearance of MtCK in the serum is associated with malignant cell proliferation. Macro CK2 is often detected in severely ill patients with malignant tumours with an estimated prevalence rate of 0.5–3.7%, the higher rates being reported from inpatient populations [8,9]. The association of Macro CK2 with malignancy may be linked to the direct release of the enzyme from malignant or necrotic cells. In addition, the compensatory

up-regulation of MtCK gene expression as a response to cellular damage may be a clinical sign of mitochondrial cytopathy and toxicity [10].

Tenofovir disoproxil fumarate (TDF), a tenofovir prodrug, is the first nucleotide analogue reverse transcriptase inhibitor (NRTI) approved for treatment of HIV disease. TDF has a favourable safety profile and shows potent antiviral activity against drug-resistant strains of HIV [11]. Treatment with TDF appears to be free of any major toxicity issues. Studies evaluating the *in vitro* toxicity of TDF on several human cell types have demonstrated low cytotoxicity of TDF as compared with other NRTIs [12]. The most commonly reported laboratory abnormalities seen in clinical studies of TDF treatment include increases in serum CK [13].

An accumulation of macroenzymes in HIV-infected patients has not been reported to date. We present here the results of a study on the detection and prevalence of Macro CK2 in HIV-1-infected antiretrovirally treated outpatients. We demonstrate a significant association of uMtCK appearance and TDF containment of antiretroviral treatment.

Au: please can you clarify this statement?

Patients and methods

Laboratory assays

All laboratory assays were performed in a blinded fashion.

Screening for Macro CK activity

Total CK activity was measured with the N-acetylcysteine-activated method at 37°C in a centrifugal spectrophotometric analyser (Cobas Integra 800 Bio; Roche Analytical Instruments, Penzberg, Germany) using N-acetyl cysteine (NAC)-activated reagents (reagent for CK-NAC; Roche Analytical Instruments) [14]. CK-MB activity was determined by immunoinhibition on the same analyser. Reference limits for total CK activity were <155 U/l for women or <180 U/l for men, and for CK-MB <15 U/l, respectively.

Au: respective to what, do you mean this limit for CK-MB for both men and women?

CK-MB mass was assessed with an electrochemiluminescence immunoassay on an Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). The upper limit of the reference interval for CK-MB mass determined by the manufacturer was less than 5 ng/ml (μg/l).

Patients were classified as 'Macro CK suspect' if the following combination of laboratory findings was present: elevated total CK activity, implausible high CK-MB activity and normal CK-MB mass without evidence of myocardial infarction or skeletal muscle trauma.

To exclude myocardial infarction, cardiac Troponin T levels (reference level <0.01 ng/ml) were measured by electrochemiluminescence (Elecsys 2010; Roche Diagnostics).

β2-Microglobulin (β2-MG; reference range <3.0 mg/l) was measured by a rapid automated particle-enhanced turbidimetric method using an immunoturbidimetric assay kit (Roche Diagnostics) with a Hitachi 917 analyser (Hitachi, Tokyo, Japan). Activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma was determined by routine laboratory testing in compliance with the International Federation of Clinical Chemistry standards with pyridoxalphosphate at 37°C (reference values: AST (37°) <33 U/l for women or <40 U/l for men, ALT (37°) <35 U/l for women or <45 U/l for men). The AST/ALT ratio was calculated as a marker for liver injury [15]. The total activity of serum alkaline phosphatase (AP) and its bone isoenzyme (BAP) was determined with the Hydragel 7 Iso-PAL assay from Sebia GMBH (Fulda, Germany) [reference values: AP ≤80 U/l for women or ≤122 U/l for men, BAP ≤44 U/l (20–74% of AP) for women or ≤73 U/l (23–75% of AP) for men]. The glomerular filtration rate (GFR) was calculated on the basis of the modified Modification of Diet in Renal Disease Study (MDRD) formula ($186.3 \times \text{serum creatinine (mg/dl)}^{-1.154} \times \text{age (years)}^{-0.203} \times 1.210$ if black resp. $\times 0.742$ if female [16].

Au: what does this mean?

Protein excretion by spot urinary protein to creatinine (Crea) ratio (protein excretion mg/g Crea; reference <100.0 mg/g Crea) was determined spectrophotometrically according to the benzethonium chloride method on an Olympus AU 2700 analyser (Olympus Diagnostics GmbH, Hamburg, Germany).

Macro CK2 is believed to represent MtCK, a mostly octameric enzyme actually localized in the cristae and the intermembrane space of mitochondria. Two isoenzymes of MtCK exist (sMtCK and uMtCK) which show a stringent tissue-specific mRNA and protein expression pattern. To study the type of MtCK that corresponds to the Macro CK2 appearance in sera of TDF-treated patients, and to confine thereby the origin of the MtCK, two further experiments were carried out: (i) the electrophoretical pattern of Macro CK2 was compared with that of highly purified, recombinant human sMtCK and uMtCK proteins, and (ii) immunoblots for Macro CK2 with rabbit immune sera against human sMtCK and human uMtCK were examined.

Detection and differentiation of Macro CK

CK macroenzymes electrophoresis was performed using a semi-automated Hydrasys agarose gel electrophoretic apparatus (Sebia GMBH, Fulda, Germany) used according to the manufacturer's protocol. Electrophoretically, Macro CK1 is the so-called anodal Macro CK and usually migrates between CK-MM and CK-MB, whereas Macro CK2 migrates cathodal to CK-MM. Macro CK isoenzymes were diagnosed exclusively on the basis of their electrophoretic behaviour.

Immunoblotting of MtCK

Immunoblotting of MtCK was performed as previously described [17]. Sera from patients with and without

detectable Macro CK2 as well as highly purified, recombinant human sMtCK and uMtCK were separated by standard 12% SDS-PAGE and electrotransferred by semidry blotting (Trans Blot SD; Bio-Rad, Hercules, CA, USA) onto nitrocellulose (HybondECL; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturers' instructions. Loading of the blots was verified by a reversible staining with Ponceau S (0.2% in 0.3% trichloroacetic acid (TCA); Serva, Heidelberg, Germany). After blocking with 4% fat-free milk powder in Tris-buffered saline (TBS) (20 mM Tris-HCl pH 7.5, 150 mM NaCl) each membrane was incubated for 1 h with rabbit immune sera against human sMtCK and human uMtCK (1:1,000 dilution in blocking buffer), washed three times in TBS, incubated 1 h with peroxidase-coupled secondary antibodies (1:3,000 dilution in blocking buffer), either goat anti-rabbit IgG (Nordic, Lausanne, Switzerland) or rabbit anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA, USA), and finally washed three times with TBS. Positive reaction products were identified by enhanced chemiluminescence (ECL; Amersham Biosciences) and autoradiography.

Au: OK?

Statistical analysis

Data are given as mean ±SD. Statistical analysis (univariate and multivariate logistic regression analysis) was performed with SPSS for Windows (Version 12.0; SPSS, Inc., Chicago, IL, USA). Differences between the groups were tested with the Mann–Whitney U-test or Student's *t*-test, as appropriate. Results were considered significant at $P \leq 0.05$.

Results

Study population

The study population consisted of a total of 468 HIV-1-infected outpatients on antiretroviral treatment (female to male ratio 1:4.7) who attended the Infectious Disease Department of the Medizinische Poliklinik, Munich, Germany. The patients' ages ranged from 20 to 71 years with a mean age of 48 ±10 years.

These 468 patients consisted of (i) 408 antiretrovirally treated patients for whom CK isoenzyme analysis was ordered in a 6-month period (July–December 2003), and (ii) an independent/separate cohort of 60 patients ('switch study' cohort) who were not included in the initial retrospective screening.

Retrospective screening for laboratory findings suspicious for presence of macro CK isoenzymes was performed in the 408 patients. Patients with proven Macro CK2 in their sera were followed for 10 ±6 months.

To examine a possible relationship beetween Macro CK2 appearance and specific antiretroviral treatment, sera from an independent cohort of 60 patients (the 'switch study' cohort) were analysed. These 60 patients were on successful treatment regimens (viral load <50 copies/ml) including stavudine (d4T) and/or didanosine (ddI). Patients were randomized 2:1 to have the d4T or ddI exchanged for TDF or to continue on their current regimens. A total of 41 patients switched either d4T or ddI to TDF and the remaining 19 patients served as controls. Of the 41 patients who changed their treatment to TDF, d4T was replaced by TDF in 32 patients and ddI was replaced in nine patients. The switch study included archival sera/urine (baseline/on-treatment) and sera/urine collected prospectively in 3-month intervals with samples available prior to, during, and in some cases after TDF exposure. Samples were also collected from the control group. The switch study was approved by the local ethics committee (Ethikkomission, Ludwig-Maximilians University of Munich, Germany) after receipt of written informed consent.

Au: OK to put CK2 rather then CK here?

Au: OK?

Retrospective screening

Retrospective analysis of total CK activity, CK-MB isoenzyme activity and mass concentration revealed the presence of Macro CK2 in the sera of 32/408 (7.8%) patients. To exclude myocardial infarction, Troponin T levels were assessed and were found to be within the reference limit for each of the patients. Patients with elevated CK and Macro CK2 denied performing vigorous exercise and did not complain of muscle weakness or pain. The clinical details were addressed by a retrospective chart review based on patients' interviews at the outpatient clinic. Rare causes of muscle disease, for example, iatrogenic muscle injury or motor neuron disease were excluded.

Clinical charts of patients with suspected Macro CK2 were characterized with patient's age, sex, race, mode of HIV-1 transmission, known duration of HIV infection, CDC classification, $CD4^+$ T-cell count and HIV-1 RNA load, as well as previous and current HIV medication. A regimen containing TDF was the common finding in 31/32 patients with a Macro CK2 suspicion.

Only one patient with suspected Macro CK2 isoenzyme activity was not under current TDF treatment: a 42-year old male patient who suffered from Kaposi's sarcoma and multicentric Castleman's disease in remission treated with oral etoposide (100 mg/d for 2 days every 2 weeks) and intravenous liposomal doxorubicin (every 3 weeks) [18].

To substantiate our finding that TDF use was significantly associated with appearance of Macro CK2, a multivariate logistic regression analysis including possible confounders [for example, concomitant use of zidovudine (AZT) or d4T or ddI, or a combination of TDF with ddI and/or protease inhibitors (PIs)] was performed. The variables included were (i) the use of

Au: OK to put CK2 rather then CK here?

Au: is this necessary as full confounders are then listed below?

TDF; (ii) concomitant use of antiretroviral compounds [nucleoside reverse transcriptase inhibitors (NRTIs): AZT, lamivudine (3TC), abacavir (ABC), d4T, ddI; non-NRTIs (NNRTIs): nevirapine (NVP), efavirenz (EFV); PIs: fortovase (FTV), saquinavir (SQV), indinavir (IDV), nelfinavir (NFV), lopinavir/ritonavir (LPV/r), atazanavir (ATV), ritonavir (RTV); fusion inhibitor: enfuvirtide]; (iii) the duration of TDF use; (iv) the presence of wasting syndrome; (v) a history of cancer; (vi) the use of other renal toxic drugs (acyclovir, ganciclovir); (vii) an active hepatitis B coinfection; and (viii) an active hepatitis C coinfection. The odds ratio for likelihood of Macro CK2 appearance under TDF is 82.5 (95% confidence interval 10.78–631.82) at a level of significance of $P<0.0001$.

Prospective confirmation of Macro CK2

for five of the 32 patients with evidence of Macro CK2 under TDF treatment, sera were available for prospective detection and differentiation of Macro CK by electrophoresis. Routine laboratory findings for these five patients were as follows: total CK activity 249 ±156 U/l, CK-MB activity 95 ±70 U/l, and CK-MB mass concentration 3.0 ±1.1 ng/ml. Cardiac Troponin T levels were within the reference limit for each patient.

In all five patients, electrophoresis revealed an atypical band migrating cathodal to the CK-MM band, consistent with significant Macro CK2 activity. During a follow-up period of 10 months, one patient died as a consequence of wasting syndrome and in one patient anal cancer was diagnosed.

Prospective and retrospective analysis in an independent TDF-treated ('switch study') cohort

To examine a possible relationship with current TDF treatment, an independent HIV-1-infected cohort of 60 patients was analysed. Archivally and prospectively at 12-week intervals, sera and urine were collected from 41 patients with TDF switch prior to, during, and in one case, after TDF exposure. Control sera from 19 non-switched patients were also investigated. All samples were characterized by electrophoresis.

In 20 of the 41 (48%) TDF-treated patients, a Macro CK2 isoenzyme was detectable at an average of 102 ±69 days after TDF treatment. Macro CK2 was not present before TDF exposure. In a follow-up period of 10 ±6 months, the atypical bands of Macro CK2 persisted in electrophoresis during TDF treatment. Control sera exhibited no Macro CK2. Clinical characteristics and concomitant antiretroviral compounds in the 20 patients with Macro CK2 appearance and the 21 patients without, are summarized in Table 1.

Au: OK? as per 3rd para of "Study population"

All concomitant antiretroviral compounds (NRTIs: AZT, 3TC, ABC, d4T, ddI; NNRTIs: NVP, EFV; PIs: SQV, NFV, LPV/r, RTV) and several other variables

Table 1. Clinical characteristics and concomitant antiretroviral compounds in patients with and without Macro CK2 appearance under TDF-containing antiretroviral treatment

Patient number	Age, years	Gender, f/m	Drug replaced by TDF	Concomitant drugs
Patients with Macro CK2 appearance under TDF treatment (group 1)				
1	52	m	d4T	LPV/r, SQV, 3TC
2	45	f	d4T	NVP, 3TC
3	60	m	d4T	ABC, 3TC
4	59	f	d4T	ddI, NVP
5	54	m	d4T	ABC, EFV
6	52	f	d4T	NFV, 3TC
7	51	m	d4T	NFV, 3TC
8	31	f	d4T	NVP, 3TC
9	36	m	d4T	ABC, 3TC
10	45	f	ddI	NFV, NVP
11	56	m	ddI	EFV, 3TC
12	32	m	ddI	ABC, 3TC
13	44	m	ddI	EFV, 3TC
14	50	m	d4T	ddI, NVP
15	40	f	d4T	ddI, NFV
16	40	f	d4T	ddI, EFV
17	65	m	d4T	ddI, NFV
18	64	m	d4T	ddI, LPV/r
19	43	f	d4T	ddI, NVP
20	43	f	d4T	ddI, EFV
Patients without Macro CK2 appearance under TDF treatment (group 2)				
1	54	m	d4T	ddI, EFV, NFV
2	34	m	d4T	NFV, 3TC
3	37	m	d4T	EFV, NFV, 3TC
4	52	m	d4T	ABC, 3TC
5	46	f	d4T	NVP, 3TC
6	56	m	ddI	LPV/r, 3TC
7	38	f	d4T	ddI, NVP
8	56	m	d4T	RTV, SQV, 3TC
9	37	m	d4T	NFV, 3TC
10	43	m	ddI	AZT, NFV
11	34	m	ddI	ABC, EFV, LPV/r
12	32	m	ddI	ABC, EFV
13	50	m	ddI	EFV, 3TC
14	62	m	d4T	ddI, EFV, NFV
15	44	m	d4T	ddI, IDV, RTV
16	66	m	d4T	ABC, ddI, NVP, LPV
17	44	m	d4T	ddI, NFV
18	52	m	d4T	ddI, LPV/r, RTV
19	54	m	d4T	ddI, RTV
20	54	m	d4T	RTV, 3TC
21	60	m	d4T	EFV, NFV

Clinical characteristics, the drug replaced by tenofovir DF (TDF) and concomitant antiretroviral compounds are listed for patients with macroenzyme creatine kinase 2 (Macro CK2) appearance under TDF treatment and for patients without Macro CK2 appearance under TDF treatment. 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; f, female; IDV indinavir; LPV/r, lopinavir/ritonavir; m, male; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir.

(baseline GFR, viral load detectability, CD4$^+$ T-cell count, body weight) were tested in a multivariate logistic regression analysis for their association with the detection of Macro CK2. Appearance of Macro CK2 was not significantly associated with a specific prior or current HIV medication (that is, concurrent ddI, d4T or LPV/r use) or the other above-mentioned variables.

The association of Macro CK2 with TDF treatment was further supported by an additional observation. In one patient with detected Macro CK2 during TDF treatment, the drug was discontinued 42 days after the start of the treatment because of gastrointestinal intolerance. Three months later (day 98 after first TDF exposure), serum was evaluated for the presence of Macro CK2. Laboratory findings for CK were inconspicuous (total CK activity 85 U/l, CK-MB activity 11 U/l, CK-MB mass concentration <5.0 ng/ml) and electrophoresis showed no atypical bands, indicating disappearance of Macro CK2 activity after discontinuation of TDF treatment.

Evaluation of clinical features and laboratory findings

To help identify additional features of TDF-treated patients with electrophoretically detected Macro CK2 appearance (group 1, *n*=20), and TDF-treated patients without Macro CK2 appearance (group 2, *n*=21), laboratory findings (AST/ALT ratio, estimated GFR, proteinuria and β2-MG levels) from both groups were compared at baseline and at the timepoint of Macro CK2 detection in group 1, or an equivalent timepoint in group 2. A biochemical marker for osteoblastic activity (bone AP) was also determined for both groups and clinical data from a 10-month follow-up period were evaluated for evidence of malignancy. Laboratory and clinical findings of both groups and corresponding levels of significance are summarized in Table 2.

Markers of liver injury (AST/ALT ratio), the GFR as estimated by the simplified MDRD formula, and bone AP (absolute and % values of serum AP) were not significantly different between switch and the appearance of Macro CK2 in group 1, or at an equivalent time point in group 2. It is noteworthy that differences in estimated GFR values at the time of Macro CK2 appearance in group 1, or the equivalent time point in group 2, nearly reached statistical significance *P*=0.051). Based on the spot urine protein excretion referred to urine creatinine we found no significant differences between patients with and those without

Table 2. Laboratory and clinical findings in patients with and without Macro CK2 appearance under TDF-containing antiretroviral treatment

	Patients under TDF-containing antiretroviral treatment		
Laboratory findings	Patients with Macro CK2 appearance (group 1, *n*=20)	Patients without Macro CK2 appearance (group 2, *n*=21)	ρ
AST/ALT ratio			
at baseline	1.19 ±0.67	1.13 ±0.42	0.373
at timepoint of Macro CK2 detection	1.20 ±0.81	1.11 ±0.43	0.335
ρ	0.498	0.441	
GFR			
at baseline	98 ±23	105 ±17	0.266
at timepoint of Macro CK2 detection	91 ±19	104 ±22	0.051
ρ	0.299	0.889	
β2-microglobulin			
at baseline	1.51 ±0.52	1.78 ±0.43	0.138
at timepoint of Macro CK2 detection	2.00 ±0.66	1.77 ±0.45	0.226
ρ	0.039	0.474	
Proteinuria			
at baseline	291.2 ±433.9	153.2 ±180.6	0.129
at timepoint of Macro CK2 detection	168.3 ±157.5	173.3 ±84.5	0.271
ρ	0.146	0.212	
BAP, % of AP/absolute	53.7 ±17.4 / 62.7 ±47.8	41.3 ±16.5 / 49.8 ±31.1	0.138 / 0.312
Malignancy in follow-up, *n* (%)	1/20 (5)	1/21 (4.7)	0.980

Laboratory and clinical findings in patients with and without macroenzyme creatine kinase 2 (Macro CK2) appearance under tenofovir DF (TDF)-containing antiretroviral treatment are summarized. Data are given as mean ±SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAP, alkaline phosphatase bone isoenzyme; GFR, glomerular filtration rate.

AU: rho symbol correct, or should these be P-values

Macro CK2 appearance at any time point during the switch study. However, a slight, but statistically not significant increase in proteinuria after TDF exposure was observed in 10/15 patients with Macro CK2 appearance after a 3-month period of TDF treatment.

Au: "in relation to"?

Patients with detectable Macro CK2 showed a significant increase (P=0.039) in β2-MG levels as compared with the time of TDF switch. However, the induction of β2-MG was slight and within the reference limit (<3.0 mg/l) of the applied laboratory assay. In the follow-up period of 10 ±6 months, the incidence of malignancy was comparable in the two groups. In group 1, a 60-year-old male developed metastatic adenocarcinoma of the colon, and a 56-year-old male patient without detectable Macro CK2 presented with a relapse of Hodgkin's disease after a remission period of 4 years.

Determination of the origin of MtCK

Macro CK2 and uMtCK revealed typical bands migrating to the cathodic site with an identical size, whereas the band for sMtCK was located slightly closer to the CK-MM fraction (Figure 1). This similar electrophoresic pattern of Macro CK2 and uMtCK was confirmed independently for eight patients with Macro CK2 appearance under TDF treatment. Immunoblotting with rabbit immune sera against human sMtCK and human uMtCK showed a band at the expected size of 43 kDa using the anti-human uMtCK antibody (Figure 2). No sMtCK was detected. These results demonstrate that Macro CK2 in the sera of TDF-treated patients consists of the ubiquitous and not the sarcomeric type of MtCK.

Discussion

Au: OK to put CK2 rather then CK here (twice)?

The presence of mitochondrial Macro CK2 is a possible explanation for elevated serum CK in patients on TDF containing HAART without other obvious clinical explanation. This may be the general explanation when the CK-MB isoenzyme is found to make up more than 50% of the total CK activity. The presence of Macro CK2 should always be ruled out by a CK isoenzyme electrophoresis to exclude myocardial damage [19]. Based on electrophoresis and immunoblotting with recombinant human MtCK, we were able to demonstrate that Macro CK2 in patients under TDF consists of the ubiquitous and not the sarcomeric type of MtCK.

Anecdotal reports from inpatient populations have suggested that Macro CK2 can be detected predominantly in severely ill patients of all ages [8]. Macro CK2 appears to be released during: (i) serious cellular damage, for example, severe enough to disrupt mitochondrial membranes, (ii) high rates of cell proliferation and breakdown, or (iii) malignant cell proliferation by a tumour [9].

An accumulation of macroenzymes as a reason for elevated CK levels in HIV-infected antiretrovirally treated patients has not been described to date. The analysis of CK-MB isoenzyme activity, mass concentration and CK isoenzyme electrophoresis of samples from 468 HIV-infected outpatients treated with different antiretrovirals has demonstrated a significant association of Macro CK2 appearance and TDF treatment. The observation that the patients did not present with Macro CK2 prior to TDF exposure and the disappearance of Macro CK2 in one patient after TDF discontinuation substantiates this association.

Since its approval, the tenofovir prodrug TDF has become a widely used component of antiretroviral regimens. It has been well-tolerated in clinical practice to date, without evidence of long-term toxicity, including the mitochondrial toxicity that is often associated with other NRTIs [20]. Moderate elevations in CK, AST and ALT levels have been noted in patients who receive TDF [21]. One case of marked elevation of CK under TDF treatment suggestive of drug interaction has been reported [22].

Detection of Macro CK2 in an inpatient population has suggested an association with various malignant diseases. Occasionally, Macro CK2 disappeared from the peripheral circulation after amelioration of the associated disease [8,23]. However, Macro CK2 has a poor diagnostic sensitivity for neoplastic diseases and lacks prognostic value both in cirrhosis and malignancy. Risk of malignancy in 41 TDF-treated patients did not significantly differ in a follow-up period of 10 ±6 months between patients with, or without, detectable Macro CK2. In contrast to published reports dealing with severely ill inpatient cohorts, Macro CK2 appearance in HIV-infected outpatients has a less clear association with neoplastic diseases. This argues against a direct release of this macroenzyme from malignant cell proliferation in antiretrovirally treated patients.

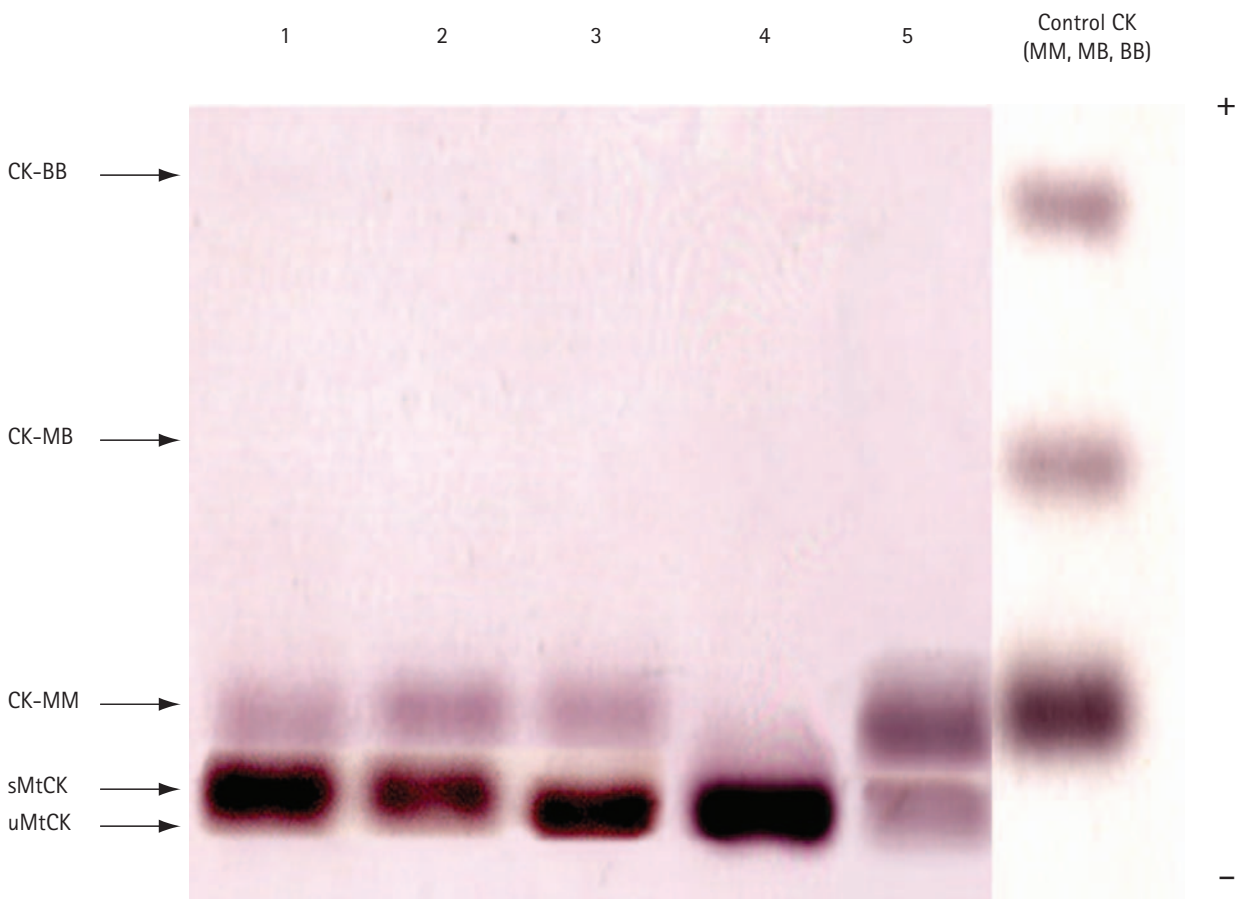
To determine the origin of MtCK in TDF-treated patients, further laboratory investigations were performed. To exclude the possibility that Macro CK2 originated from liver cells, the AST/ALT ratios in our patients were investigated. The utility of the AST/ALT ratio has been used extensively in differentiating several liver diseases and may have prognostic value in severe acute viral hepatitis [24]. In our 41 patients receiving TDF, the mean AST/ALT ratio did not differ between patients with or without detectable Macro CK2, arguing against a serious liver toxicity mediated by TDF leading to significant liver cell necrosis and consequent liver cell regeneration.

Au: OK?

Nephrotoxicity is the dose-limiting toxicity of TDF. A number of different manifestations of kidney disease have been described under TDF treatment including

AU: colour pages charge is £300. Would you therefore prefer this figure to be formatted as black and white?

Figure 1. Electrophoresis of uMTCK and sMTCK and Macro CK2



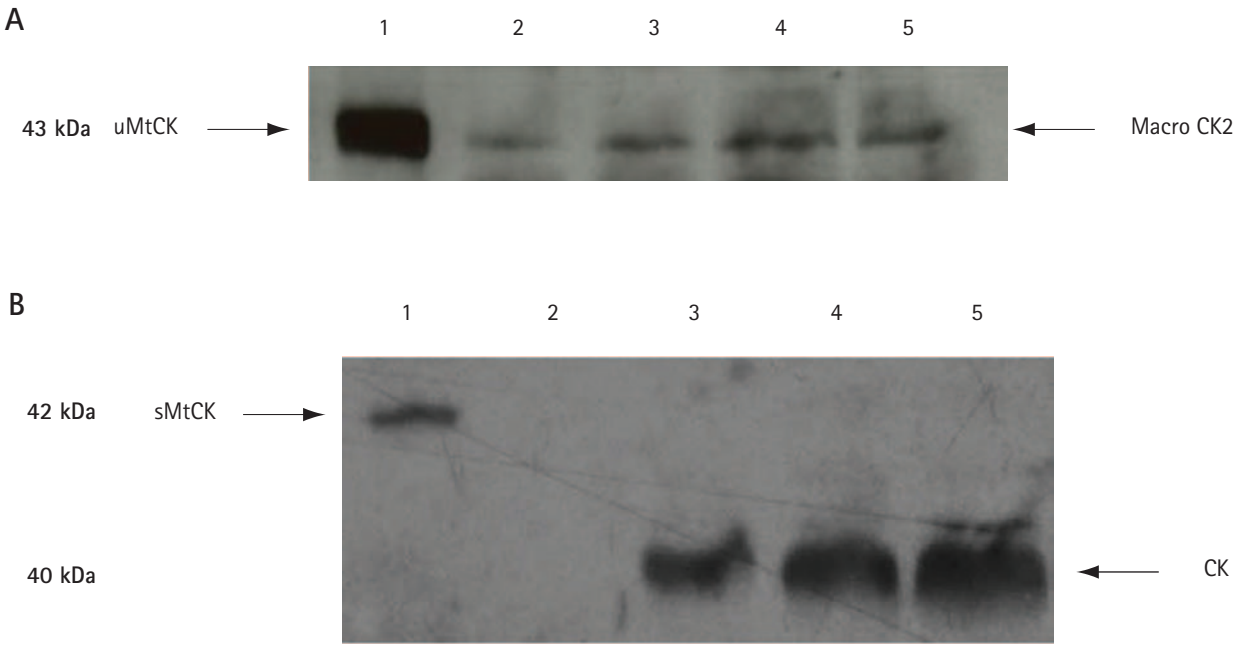
Electrophoretic patterns of recombinant human ubiquitous (uMtCK) and sarcomeric (sMtCK) mitochondrial CK and sera with macroenzyme creatine kinase 2 (Macro CK2) appearance were analysed with a semi-automated Hydrasys agarose gel electrophoretic apparatus (Sebia GMBH) according to the manufacturer's protocol. Macro CK2 (lanes 3 and 5) and recombinant uMtCK (lane 4) revealed typical bands migrating to the cathodic site with an identical size, whereas the band for sMtCK (lanes 1 and 2) was located slightly closer to the CK-MM fraction. The identical electrophoretic behaviour of Macro CK2 and uMtCK was confirmed independently in eight patients with Macro CK2 appearance under TDF treatment. Lanes 1 and 2: recombinant sMtCK, lanes 3 and 5: Macro CK2 from patients under TDF, lane 4: recombinant uMtCK.

Fanconi-like syndrome, diabetes insipidus and acute renal failure [25]. Therefore, patients receiving TDF should be monitored closely for signs of tubulopathy. In addition, antiretroviral therapy with tenofovir is associated with mild renal dysfunction in some cohort analyses [26]. To exclude the possibility that the accumulation of Macro CK2 in TDF-treated patients is a consequence of reduced renal clearance capacity, the GFR using the MDRD formula and proteinuria in spot urine were assessed. The results showed no significant differences between patients with or without Macro CK2 appearance.

Recently, concerns have been raised about TDF-induced bone mineral loss. In animal studies using high-dose TDF, osteomalacia was a frequent adverse event [27]. Furthermore, the development of hypophosphataemic osteomalacia with myopathy in patients with HIV infection receiving TDF have been described [28]. The appearance of MtCK in the sera of TDF-treated patients could be suggestive of an increased activity of osteoblasts. As bone AP is an appropriate biochemical marker for increased osteoblastic activity in hypophosphataemic osteomalacia, we compared this marker in patients with or without Macro CK2. Again, no significant differences were found between the two groups.

β2-MG is a polypeptide chain linked non-covalently to the MHC Class I cell surface antigens present in nearly all cell types [29]. Membrane turnover is the principal source of β2-MG in the blood. β2-MG synthesis is stimulated in various conditions characterized by monoclonal or polyclonal activation and proliferation of lymphoid cells such as malignant tumours, lymphoproliferative B-cell disorders and various chronic

Figure 2. Western blot analysis confirms ubiquitous type of MtCK in sera of TDF-treated patients



(A) Immunoblotting with rabbit immune sera against human ubiquitous mitochondrial creatine kinase (uMtCK) showed a slight band at the expected size of 43 kDa using the anti-human uMtCK antibody in patients with macroenzyme creatine kinase 2 (Macro CK2) appearance under tenofovir DF (TDF) treatment (lanes 2–5). Lane 1 shows positive staining of human recombinant uMtCK. Control sera from HIV-infected antiretrovirally treated patients exhibiting no Macro CK2 remained negative (data not shown). **(B)** Immunoblotting with rabbit immune sera against human sarcomeric mitochondrial creatine kinase (sMtCK) remained negative in patients with Macro CK2 appearance under containing TDF treatment at the expected size of 42 kDa (lanes 3–5). Cross-reactivity of the anti-human sMtCK antibody with cytosolic CK (at 40 kDa) was observed. Lane 1 shows positive staining of human recombinant sMtCK, a blank control (lane 2) remained negative.

inflammatory diseases [30]. Elevated serum levels of β2-MG have been found in systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease and infections [31].

Increased concentrations of β2-MG are predictive of progression of HIV infection to AIDS [32]. It spikes in acute infection, declines, and then rises during the course of HIV infection. Hoffmann and co-workers found that higher β2-MG concentrations predicted a subsequent decline in $CD4^+$ T-cell counts [33].

Patients with detectable Macro CK2 showed a mild, but significant increase of serum β2-MG levels at the point of Macro CK2 appearance in the peripheral blood. As β2-MG is excreted almost exclusively by the kidneys, increased levels are generally found in patients with a decreased GFR [34]. However, the estimated GFR did not significantly differ between TDF-treated patients with or without Macro CK2. We cannot exclude the possibility that the induction of β2-MG levels in patients with Macro CK2 appearance may reflect a direct TDF-dependent cytotoxic effect with subsequent cellular damage and disruption of membranes. Renal tubular epithelial cells may be potential targets for TDF-mediated toxic effects. Acyclic nucleoside phosphonates are efficiently transported from the circulation into tubular cells by the renal basolateral membrane protein hOAT1 [35], leading to high and possibly cytotoxic intracellular drug concentrations.

To further characterize this observation, the type of MtCK leading to Macro CK2 appearance under TDF treatment was determined. Two isoenzymes of MtCK encoded by separate genes have been described. They are designated as uMtCK and sMtCK based on their tissue expression [36]. uMtCK mRNA is abundantly expressed in brain and kidney. Low levels of uMtCK expression can be detected in aorta, intestine, sarcomeric tissue, placenta and, during pregnancy, in uterus [37]. No expression for uMtCK mRNA is detected in liver and lung. In contrast, the sMtCK gene is exclusively expressed in cardiomyocytes, skeletal myocytes of the heart and skeletal muscle, and not in any other organs tested [38,39]. Electrophoresis and immunoblotting have clearly shown that Macro CK2 is present in the sera of TDF-treated patients consisting of the ubiquitous but not the sarcomeric type of MtCK. As the kidney is a major source for uMtCK, this finding suggests that Macro CK2 appearance in sera could reflect a slight renal tubular cytotoxicity of TDF associated with a significant increase of serum β2-MG, but

not leading to a significant renal impairment realized by the MDRD formula.

MtCKs are usually found exclusively in the mitochondrial compartment of cells attached to the outer surface of the mitochondrial inner membrane. MtCKs are responsible for the transfer of high-energy phosphate from mitochondria to the cytosolic carrier, creatine [40]. The compensatory up-regulation of uMtCK gene expression as a response to cellular damage can lead to the accumulation of crystalline MtCK inclusion bodies in mitochondria, which are the clinical hallmarks for mitochondrial cytopathies [41].

Saumoy and co-workers showed that a marked decrease in cellular mitochondrial DNA content was associated with a case of TDF-associated acute renal failure [42]. In a recent study by Cote *et al.*, kidney mitochondrial DNA depletion in HIV-infected patients was associated with concurrent TDF/ddI therapy, but not with TDF use alone [43]. The appearance of Macro CK2 consisting of uMtCK in our cohort was not associated with a ddI-containing regimen. However, kidney ultrastructural mitochondrial abnormalities with a striking distortion of the cristae were seen with TDF use [43]. Mitochondrial damage of tubular epithelial cells, presenting as extremely enlarged mitochondria with loss and disorientation of their cristae, has been proposed as a mechanism for adefovir nephrotoxicity [44]. Taken together, these observations suggest that the appearance of uMtCK in sera may represent mitochondrial damage in tubular epithelial cells.

In conclusion, the finding of Macro CK2 in antiretrovirally treated HIV-infected patients is associated with a TDF-containing regimen. Clinicians should be aware of the possibility of Macro CK2 appearance in TDF-treated patients when results of blood testing lead to suspicion of ischemic disease or muscle injury. In contrast to anecdotal reports from inpatient populations, the appearance of MtCK in sera was not associated with malignancy or severe illness. Based on laboratory findings, we could exclude the possibility that liver cell necrosis, increased osteoblastic activity or decreased renal function measured by the MDRD formula lead to the appearance of Macro CK2 in these patients. Electrophoresis and immunoblotting demonstrated that Macro CK2 consists of the ubiquitous and not the sarcomeric type of MtCK. Whether the appearance of uMtCK reflects a feature of mitochondrial damage in tubular epithelial cells has yet to be clarified.

Acknowledgements

We would like to thank Ilka Edenhofer for expert technical assistance.

This study was supported by a grant from Gilead Sciences, Martinsried, Germany to HS and FDG and grants from the Swiss National Science Foundation (grant 3100-5082.97 to TW and US), Schweizer Krebsliga (to TW and US) and Zentralschweizer Krebsliga (to US and TW).

References

1. Friis-Moller N, Sabin CA, Weber R, *et al*. Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study Group. Combination antiretroviral therapy and the risk of myocardial infarction. *N Engl J Med* 2003; **349:**1993–2003.
2. Johnson RW, Williams FM, Kazi S, Dimachkie MM, Reveille JD. Human immunodeficiency virus-associated polymyositis: a longitudinal study of outcome. *Arthritis Rheum* 2003; **49:**172–178.
3. Remaley AT, Wilding P. Macroenzymes: biochemical characterization, clinical significance, and laboratory detection. *Clin Chem* 1989; **35:**2261–2270.
4. Greco FA. Identifying CK-MB and macro-CK by electrophoresis. *Clin Chem* 1987, **33:**1954–1955.
5. Laureys M, Sion JP, Slabbynck H, *et al*. Macromolecular creatine kinase type 1: a serum marker associated with disease. *Clin Chem* 1991; **37:**430–434.
6. Stein W, Bohner J, Bahlinger M. Analytical patterns and biochemical properties of macro creatine kinase type 2. *Clin Chem* 1985 **12:**1952–1958.
7. Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta* 2006; **1762:**164–180.
8. Lee KN, Csako G, Bernhardt P, Elin RJ. Relevance of macro creatine kinase type 1 and type 2 isoenzymes to laboratory and clinical data. *Clin Chem* 1994; **40:**1278–1283.
9. Stein W, Bohner J, Renn W, Maulbetsch R. Macro creatine kinase type 2: results of a prospective study in hospitalized patients. *Clin Chem* 1985; **31:**1959–1964.
10. Qin W, Khuchua Z, Cheng J, Boero J, Payne RM, Strauss AW. Molecular characterization of the creatine kinases and some historical perspectives. *Mol Cell Biochem* 1998; **184:**153–167.
11. Fung HB, Stone EA, Piacenti FJ. Tenofovir disoproxil fumarate: a nucleotide reverse transcriptase inhibitor for the treatment of HIV infection. *Clin Ther* 2002, **10:**1515–1548.
12. Cihlar T, Birkus G, Greenwalt DE, Hitchcock MJ. Tenofovir exhibits low cytotoxicity in various human cell types: comparison with other nucleoside reverse transcriptase inhibitors. *Antiviral Res* 2002; **54:**37–45.
13. Schooley RT, Ruane P, Myers RA, *et al*. Study 902 Team. Tenofovir DF in antiretroviral-experienced patients: results from a 48-week, randomized, double-blind study. *AIDS* 2002; **16:**1257–1263.
14. Rosalski SB. An improved procedure for serum creatine phosphokinase determination. *J Lab Clin Med* 1967; **69:**696–705.
15. Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology* 1988; **95:**734–739.
16. Levey A, Greene T, Kusek J, Beck G, MDRD Study Group. A simplified equation to predict glomerular filtration rate from serum creatinine. *J Am Soc Nephrol* 2000; **11:**A0828.
17. Schlattner U, Mockli N, Speer O, Werner S, Wallimann T. Creatine kinase and creatine transporter in normal, wounded, and diseased skin. *J Invest Dermatol* 2002; **118:**416–423.
18. Horster S, Jung C, Zietz C, Cohen CD, Siebeck M, Goebel FD. AIDS, multicentric Castleman's disease, and plasmablastic leukemia: report of a long-term survival. *Infection* 2004; **32:**296–298.
19. Galarraga B, Sinclair D, Fahie-Wilson MN, McCrae FC, Hull RG, Ledingham JM. A rare but important cause for a

raised serum creatine kinase concentration: two case reports and a literature review. *Rheumatology (Oxford)* 2003; **42:**186–188.

20. Birkus G, Hitchcock MJM, Cihlar T. Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* 2002, **46:**716–723.
21. Barditch-Crovo P, Deeks SG, Collier A, *et al*. Phase I/II trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. *Antimicrob Agents Chemother* 2001; **45:**2733–2739.
22. Shere-Wolfe KD, Verley JR. Marked elevation of the creatine phosphokinase level in a patient receiving tenofovir. *Clin Infect Dis* 2002; **35:**1137.
23. Galasso PJ, Litin SC, O'Brien JF. The macroenzymes: a clinical review. *Mayo Clin Proc* 1993; **68:**349–354.
24. Giannini E, Risso D, Botta F, *et al*. Validity and clinical utility of the aspartate aminotransferase-alanine aminotransferase ratio in assessing disease severity and prognosis in patients with hepatitis C virus-related chronic liver disease. *Arch Intern Med* 2003; **163:**218–224.
25. Karras A, Lafauri M, Furco A, *et al*. Tenofovir-related-nephrotoxicity in human immunodeficiency virus infected patients three cases of renal failure, Fanconi syndrome, and nephrogenic diabetes insipidus. *Clin Infect Dis* 2003; **36:**1070–1073.
26. Mauss S, Berger F, Schmutz G. Antiretroviral therapy with tenofovir is associated with mild renal dysfunction. *AIDS* 2005; **19:**93–95.
27. Anonymous. Viread (Tenofovir Disoproxil Fumarate) Product Monograph. Foster City, CA, USA: Gilead Sciences; 2001.
28. Parsonage MJ, Wilkins EG, Snowden N, Issa BG, Savage MW. The development of hypophosphataemic osteomalacia with myopathy in two patients with HIV infection receiving tenofovir therapy. *HIV Med* 2005; **6:**341–346.
29. Lawlor DA, Zemmour J, Ennis PD, Parham P. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Annu Rev Immunol* 1990; **8:**23–63.
30. Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F. Analysis of HLA expression in human tumor tissues. *Cancer Immunol Immunother* 2003; **52:**1–9.
31. Miyata T, Jadoul M, Kurokawa K, Van Ypersele de Strihou C. Beta-2 microglobulin in renal disease. *J Am Soc Nephrol* 1998; **9:**1723–1735.
32. Tsoukas CM, Bernard NF. Markers predicting progression of human immunodeficiency virus-related disease. *Clin Microbiol Rev* 1994; **7:**14–28.
33. Hofmann B, Wang YX, Cumberland WG, Detels R, Bozorgmehri M, Fahey JL. Serum beta 2-microglobulin level increases in HIV infection: relation to seroconversion, CD4 T-cell fall and prognosis. *AIDS* 1990; **4:**207–214.
34. Acchiardo S, Kraus AP Jr, Jennings BR. Beta 2-microglobulin levels in patients with renal insufficiency. *Am J Kidney Dis* 1989; **13:**70–74.
35. Cihlar T, Birkus G, Greenwalt DE, Hitchcock MJ. Tenofovir exhibits low cytotoxicity in various human cell types: comparison with other nucleoside reverse transcriptase inhibitors. *Antiviral Res* 2002; **54:**37–45.
36. Schlattner U, Forstner M, Eder M, Stachowiak O, Fritz-Wolf K, Wallimann T. Functional aspects of the X-ray structure of mitochondrial creatine kinase: a molecular physiology approach. *Mol Cell Biochem* 1998; **184:**125–140.
37. Payne RM, Strauss AW. Developmental expression of sarcomeric and ubiquitous mitochondrial creatine kinase is tissue-specific. *Biochim Biophys Acta* 1994; **1219:**33–38.
38. Schlegel J, Wyss M, Schurch U, *et al*. Mitochondrial creatine kinase from cardiac muscle and brain are two distinct isoenzymes but both form octameric molecules. *J Biol Chem* 1988; **263:**16963–16969.
39. Wallimann T, Hemmer W. Creatine kinase in non-muscle tissues and cells. *Mol Cell Biochem* 1994; **133–134:**193–220.
40. Jacobus WE, Lehninger AL. Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. *J Biol Chem* 1973; **248:**4803–4810.
41. DiMauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC. Mitochondrial myopathies. *Ann Neurol* 1985; **17:**521–538.
42. Saumoy M, Vidal F, Peraire J, *et al*. Proximal tubular kidney damage and tenofovir: a role for mitochondrial toxicity? *AIDS* 2004; **18:**1741–1742.
43. Cote HC, Magil AB, Harris M, *et al*. Exploring mitochondrial nephrotoxicity as a potential mechanism of kidney dysfunction among HIV-infected patients on highly active antiretroviral therapy. *Antivir Ther* 2006; **11:**79–86.
44. Tanji N, Tanji K, Kambham N, Markowitz GS, Bell A, D'agati VD. Adefovir nephrotoxicity: possible role of mitochondrial DNA depletion. *Hum Pathol* 2001; **32:**734–740.

Accepted for publication 11 August 2006