Original article

Macro CK2 accumulation in tenofovir-treated HIV patients is facilitated by CK oligomer stabilization but is not predictive for pathology

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Background: Ubiquitous mitochondrial creatine kinase (uMtCK) accumulates as macroenzyme creatine kinase type 2 (macro CK2) in the serum of HIV-infected patients under a tenofovir disoproxil fumarate (TDF)-containing antiretroviral regimen. The genesis and clinical significance of this finding is unclear.

Methods: A prospective observational 5-year follow-up study was performed on those patients in which macro CK2 appearance was initially described ('TDF switch study' cohort). In addition, tenofovir (TFV), its prodrug TDF and its active, intracellular derivative TFV diphosphate (TDP) were tested *in vitro* for their effects on different key properties of uMtCK to clarify possible interactions of uMtCK with TFV compounds.

Results: In just under 5 years of continuous TDF treatment, only 4/12 (33%) patients remained macro CK2-positive, whereas 8/12 (66%) originally positive patients were macro CK2-negative at the end of follow-up. Prospective clinical follow-up data indicate that macro CK2 appearance under TDF is not associated with significant cell damage or occurrence of malignancies. A trend towards grade 1 hypophosphataemia suggests subclinical proximal tubular dysfunction in macro-CK2-positive patients, although it was not associated with a significant decrease in estimated glomerular filtration rate. *In vitro*, TFV, TDF and TDP did not interfere with uMtCK enzyme activity as competitive inhibitors or pseudo-substrates, but TFV and TDF stabilized the native uMtCK octameric structure in dilute solutions.

Conclusions: Appearance of octameric uMtCK as macro CK2 in the serum of TDF-treated patients is suggested to result from a combination of low-level mitochondrial damage caused by subclinical renal tubular dysfunction together with possible compensatory uMtCK overexpression and a putative concomitant stabilization of uMtCK octamers by higher levels of TFV in proximal tubules.

Introduction

The macroenzyme creatine kinase type 2 (macro CK2) is a high molecular weight serum species of the enzyme creatine kinase (CK). Macro CK2 is predominantly detected in the sera of severely ill patients, particularly with malignant tumours [1], but also in HIV-infected patients treated with tenofovir disoproxil fumarate (TDF) [2]. The association of macro CK2 with malignancy is likely caused by direct release of large amounts of mitochondrial CK (MtCK) from malignant or necrotic cells.

Macro CK2 in TDF-treated patients consists of predominantly octameric complexes of MtCK, in particular the ubiquitous (uMtCK) and not the sarcomeric isoenzyme (sMtCK) [2]. The uMtCK isoform is abundantly expressed in the brain, and at somewhat lower levels in the aorta, intestine, kidney, placenta or uterus, whereas sMtCK is exclusively expressed in skeletal and heart muscle [3,4]. Generally, MtCK is most abundant in cells with oxidative metabolism and high or fluctuating energy requirements, such as myocytes, neurons or many epithelial cells like renal epithelial cells [5,6].

CKs are key players in cellular energy homeostasis within a complex signalling network and have been

involved in numerous pathologies [4,7]. They catalyse the reversible generation of phosphocreatine (PCr) and ADP from creatine and ATP, with the 'high-energy' compound PCr serving as a cellular energy buffer and transportation mechanism [4,6]. In healthy cells, MtCK isoenzymes are restricted to cristae and the intermembrane space of mitochondria where they form mainly octameric complexes, which can dissociate into dimers, for example, at low concentration or under oxidative stress [8–11]. As a result of natural cell turnover, MtCK also occurs at some negligible and hardly detectable level in the blood of probably every patient. There, kinase octamers undergo rapid dissociation into dimers that can be eliminated by regular clearance. Thus, accumulation of uMtCK as macro CK2 in the sera of HIV-1-infected patients under TDF could be highly relevant, not only as an unexplained diagnostic finding but also as the pathological correlate of cellular or subcellular mitochondrial damage provoked by the drug.

Tenofovir (TFV) is an acyclic nucleoside phosphonate that is administered orally in the form of the cell-permeable prodrug TDF. Once taken up into the cell, TFV is transformed into TFV monophosphate by adenylate kinase (AK) and finally into the mainly bioactive compound TFV diphosphate (TDP) by different enzymes, including nucleoside diphosphate kinase (NDPK) and cytosolic CK [12]. Phosphorylated TFV derivatives have been identified as competitive inhibitors (with respect to dATP) and substrates of HIV-1 reverse transcriptase, but, in comparison to most of the other nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), TDP is a rather weak inhibitor and substrate for mitochondrial DNA polymerase-γ *in vitro* [13]. TDF is the only nucleotide analogue reverse transcriptase inhibitor approved by the FDA and is available also in a fixed-dose combination with emtricitabine and efavirenz (Atripla®) [14] or rilpivirine (Complera® or Eviplera®).

The clinical significance of macro CK2, in particular whether it reflects mitochondrial damage or predicts nephrotoxicity and proximal tubular dysfunction [15–17], remains to be clarified. Persistent appearance of macro CK2 would require continuous cellular release of uMtCK and stabilization of its octameric state, which is labile in dilute solution. Here, we systematically analysed the clinical long-term consequences of macro CK2 appearance under TDF and studied the effects of the different forms of TFV on uMtCK function *in vitro*. Our findings indicate that macro CK2 appearance is not associated with significant damage to cells, malignancy or severe tubular damage to the kidney, leading to renal impairment. TFV and its related compounds did not negatively affect uMtCK structure and function but rather had a slightly protective and stabilizing effect, which could partially explain the accumulation of octameric uMtCK as macro CK2 in the serum.

Methods

Patients/study population

Detailed clinical characteristics of the 'TDF switch study' cohort were recently described [2,18]. The 'TDF switch study' cohort consisted of 60 patients who were treated successfully (viral load <50 copies/ml), including with stavudine and/or didanosine. In 2003, the patients were 2:1 randomized to have the stavudine or didanosine exchanged for TDF or to continue on the previous regimen. A total of 41 patients changed their treatment to TDF. In 20 of the 41 (48%) TDF-treated patients, macro CK2 appeared in sera after TDF initiation. Since 2003, 40 of these TDF-treated patients were prospectively followed in 3-month intervals over 5 years in order to systematically analyse potential differences in laboratory and clinical findings between initially macro-CK2-positive and -negative patients. At each visit a history was taken and an examination conducted, and the $CD4^+$ T-cell count (BD TriTest; Becton Dickinson and Company, Heidelberg, Germany) and HIV RNA level (VERSANT HIV-1 RNA 3.0 assay; Bayer Health Care, Leverkusen, Germany) were measured. All patients denied iatrogenic muscle injury or performing vigorous exercise and did not complain of myalgia. Routine laboratory workup included activity of CK, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), as well as serum creatinine, lactate and phosphate. The estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD)2 formula [19] and the Cockcroft–Gault formula [20]. At the end of the observation period, all eligible patients were tested for macro CK activity. Electrophoresis was performed using a semi-automated Hydrasys agarose gel electrophoretic apparatus (Sebia GMBH, Fulda, Germany), as described [2]. The study was approved by the local Institutional Review Boards, and written informed consent was obtained for all participants.

Production of recombinant uMtCK and TDP

Recombinant human uMtCK was produced as described [21] with some modifications [22]. Purification was carried out at 4°C with an Aekta Explorer HPLC (GE Healthcare, Vélizy, France). Bacterial extract was first purified by Blue Sepharose chromatography (GE Healthcare), then CK-active fractions were pooled and the buffer exchanged by three cycles of concentration/dilution in an ultrafiltration device (Vivaspin, Sartorius, Göttingen, Germany; 10 kDa cutoff) to 20 mM MES pH 6.2, 1 mM ADP and 1 mM β-mercaptoethanol (β-ME). The concentrate was applied to a Resource S cation exchange column (1 ml; GE Healthcare) and uMtCK eluted with a linear NaCl gradient in MES buffer at approximately 100 mM salt. Fractions containing pure uMtCK, as

judged by Coomassie-stained SDS-PAGE, were pooled. Protein concentrations were determined by Bradford-based microassays (Biorad, Marnes-la-Coquette, France) and bovine serum albumin as standard.

TDP was synthesized with a coupled enzymatic reaction using AK (rabbit muscle; Sigma–Aldrich, St Louis, MO, USA) and recombinant NDPK as modifying enzymes [23]. A reaction mixture of 100 μM TFV (Gilead, Munich, Germany), 1 mM ATP, 0.5 mg/ml AK and NDPK, each in 50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl2, 1 mg/ml bovine serum albumin and 1 mM β-ME was incubated at 4°C for 24 h. AK and NDPK-D was removed by ultrafiltration (Vivaspin; 10 kDa cutoff) and the filtrate applied to a Resource Q anion exchange column (1 ml; GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8 and calibrated with ATP, ADP and TFV. Elution by a linear NaCl gradient at 1 ml/min revealed a new peak at 260 nm absorption containing TDP.

Determination of catalytic properties and oligomeric state of MtCK

Enzymatic activity of MtCK was determined by a photometric coupled enzyme assay for the forward (PCr production) and reverse reactions (ATP production), as described [21] with some modifications [22]. In short, initial rate constants were measured with a matrix of CK substrate concentrations: 0.5, 2, 5 and 10 mM creatine (Cr); 0.2, 0.8, 2 and 4 mM MgATP; 0.2, 0.5, 2 and 20 mM PCr; and 0.05, 0.1, 0.4 and 2 mM MgADP. Preliminary experiments revealed that coupling enzymes were not affected by TFV, TDF or TDP. Alternatively, MtCK activity was determined with an electrochemical assay (pH stat; Radiometer Copenhagen, Villeurbane, France) [24]. This method can reliably detect low CK activities because it is not altered by effects on coupling enzymes or 'parasite reactions' caused by contaminating enzymes. Proton consumption in the CK reverse reaction was measured at 25°C with 0.3–1.0 μg uMtCK and 0.02, 0.04, 0.1, 0.2, 0.4, 1, 2 and 4 mM ADP, and 0.8 and 10 mM PCr. Apparent K_m constants were calculated from double reciprocal plots of initial velocity data.

Quantification of the two oligomeric states of uMtCK dimers and octamers was carried out by calibrated gel permeation chromatography, as established and described in detail earlier [11,25,26]. In short, a stock solution at 3 mg/ml uMtCK (entirely octameric) was diluted into 100 mM triethanolamine buffer pH 8 containing 2 mM β-ME to reach 700 μg/ml or 70 μg/ml enzyme and incubated at 4°C for 24 h or 48 h, respectively, with or without 500 μM TFV or TDF. The uMtCK oligomers were then separated and identified by HPLC, using 25 μl aliquots injected onto a Superose 12 gel permeation column (GE Healthcare) in GF buffer (50 mM Na-phosphate, 2 mM β-ME, 0.2 mM EDTA and 150 mM NaCl, pH 7.0) run at 0.7 ml/min and 4°C. The column was calibrated for Stoke's radii [27] and for uMtCK oligomers using pure recombinant enzyme [11,27].

Statistical analyses

The results obtained were analysed using SPSS for windows software version 12.0 (SPSS Inc., Chicago, IL, USA). Results are presented as mean ±SEM, percentages and tables. Comparison was done with the Friedman test for continuous variables or χ^2 test for categorical variables, and *P*-values <0.05 were considered statistically significant. Results of *in vitro* experiments are presented as mean ±SD, and comparison was performed by Student's T-test.

Results

Macro CK2 can persist over years in sera of TDF-treated patients but always disappears after TDF discontinuation

Follow-up of 'TDF switch study' patients and results for macro CK testing are summarized in Figure 1. Of the initial 20 macro-CK2-positive patients, 16 were eligible for testing 5 years after the start of a TDF-containing regimen. During follow-up, TDF was continued in 12/16 (75%) and stopped in 4/16 (25%) patients. The attending physicians' motives for TDF discontinuation were virological failure in two patients, improved convenience of the antiretroviral treatment regimen in one patient and suspected, but not secured, TFV-associated eGFR decline in one patient. Under continuous TDF treatment, a significant portion of patients (4/12; 33%) remained macro-CK2-positive.

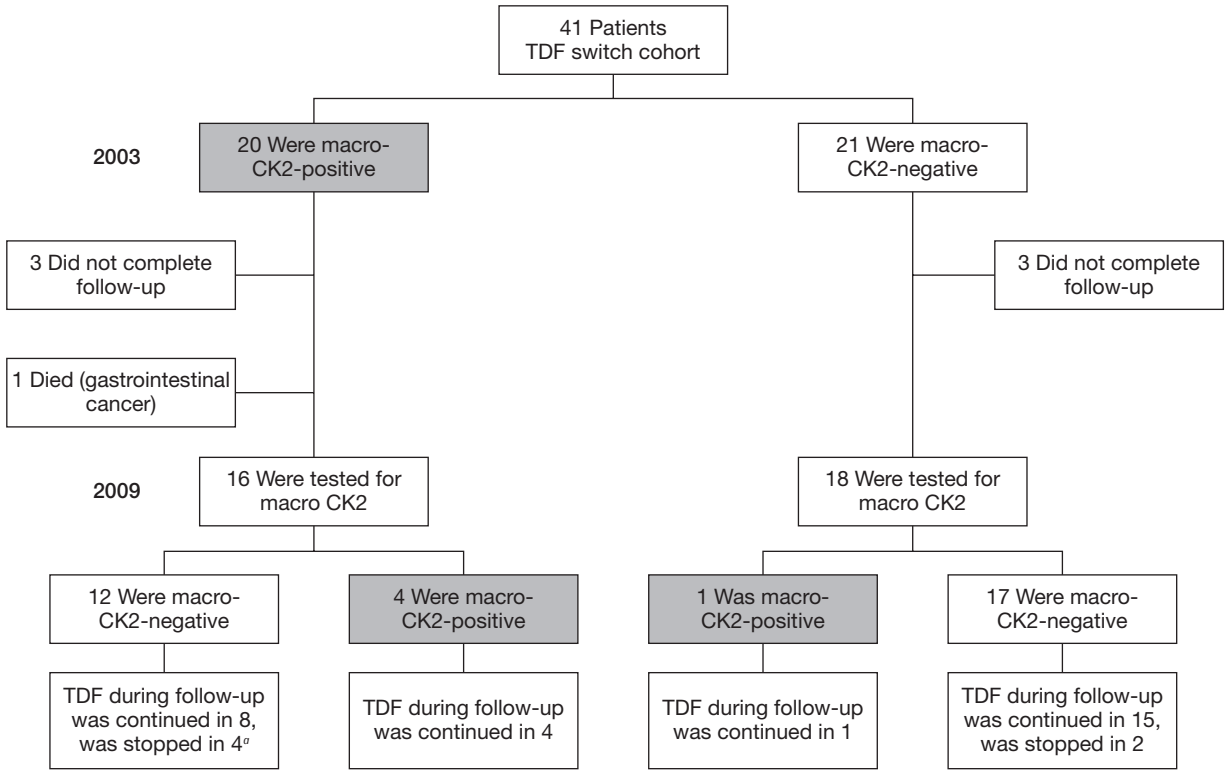
In those patients who developed macro CK2 the occurrence was at an average of 100 days after initiation of treatment [2]. However, in one initially (in 2003) macro-CK2-negative patient, macro CK2 was detected after 5 years of uninterrupted TDF intake. Of note, macro-CK2 appearance was restricted to patients with a continuous TDF treatment, irrespective of whether tenofovir was given in its individual formation (Viread®), in combination with emtricitabine (Truvada®) or as efavirenz/emtricitabine/TFV disoproxil fumarate single-tablet regimen (Atripla®). After TDF discontinuation (6/34 patients tested; 18%), macro CK2 was undetectable in all of the patients.

Macro CK2 appearance is not associated with cell damage or malignancy but indicates a trend towards hypophosphataemia

Prospective clinical and laboratory baseline, as well as 5-year follow-up data of initially macro-CK2-positive and macro-CK2-negative patients, are compared in Table 1. A slight increase in absolute CK values within the reference limits, a non-significant trend towards

lower lactate levels and stable (for initially macro-CK2-negative), or even decreasing (for initially macro-CK2-positive), LDH, AST and ALT values within the reference range were consistent findings. Despite a trend towards grade 1 hypophosphataemia (defined as 2–2.5 mg/dl) in macro-CK2-positive patients, there was no occurrence of clinically significant hyper- or hypophosphataemia in our cohort. These data indicate that macro CK2 is

Figure 1. Study enrolment and results for macro CK2 testing after a 5-year follow-up



Grey boxes indicate macro-CK2-positive patients. [a]Reasons for tenofovir disoproxil fumarate (TDF) discontinuation in initially macroenzyme-creatine kinase type 2 (macro-CK2)-positive patients were virological failure in two patients, improved convenience of the antiretroviral treatment regimen in one patient and estimated glomerular filtration rate decline in one patient.

Table 1. 'TDF switch study' cohort 5-year follow-up data

	Macro-CK2-positive patients				Macro-CK2-negative patients			
Parameter	Trend	Year 1	Year 5	*P*-value	Trend	Year 1	Year 5	*P*-value
CK<180 U/l[a]	↑	151	165	0.674 (ns)	↑	156	177	0.05 (ns)
AST<40 U/l[a]	↓	54	39	0.220 (ns)	↔	28	28	0.303 (ns)
ALT<45 U/l[a]	↓	50	42	0.289 (ns)	↔	28	28	0.381 (ns)
LDH<250 U/l[a]	↓	173	162	0.138 (ns)	↔	165	167	0.374 (ns)
Phosphate 2.5–4.8 mg/dl[a]	↓	2.57	2.45	0.794 (ns)	↑	2.74	2.95	0.463 (ns)
Lactate 0.5–2.2 mg/dl[a]	↓	2.05	1.80	0.594 (ns)	↓	1.92	1.53	0.699 (ns)
S-creatinine 0.5–1.2 mg/dl[a]	↑	0.85	0.90	0.185 (ns)	↑	0.85	0.89	<0.001
eGFR, ml/min[a,b]	↓	96	91	0.423 (ns)	↓	113	104	<0.0001
eGFR, ml/min/1.73 m^2[a,c]	↓	93	86	0.094 (ns)	↓	102	96	0.001
CD4$^+$ T-cell count, cells/ml	↑	412	487	<0.0001	↑	553	627	<0.005

[a]Trend of laboratory parameters in a 5-year follow-up period, mean values after 1 and 5 years and level of significance analysed by the Friedman test are presented. [b]Cockroft–Gault 1. [c]Modification of diet in renal disease 2. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; eGRF, estimated glomerular filtration rate; LDH, lactate dehydrogenase; macro CK2, macroenzyme creatine kinase type 2; ns, not significant; TDF, tenofovir disoproxil fumarate.

not associated with significant damage or breakdown of cells that is measurable by standard laboratory analysis.

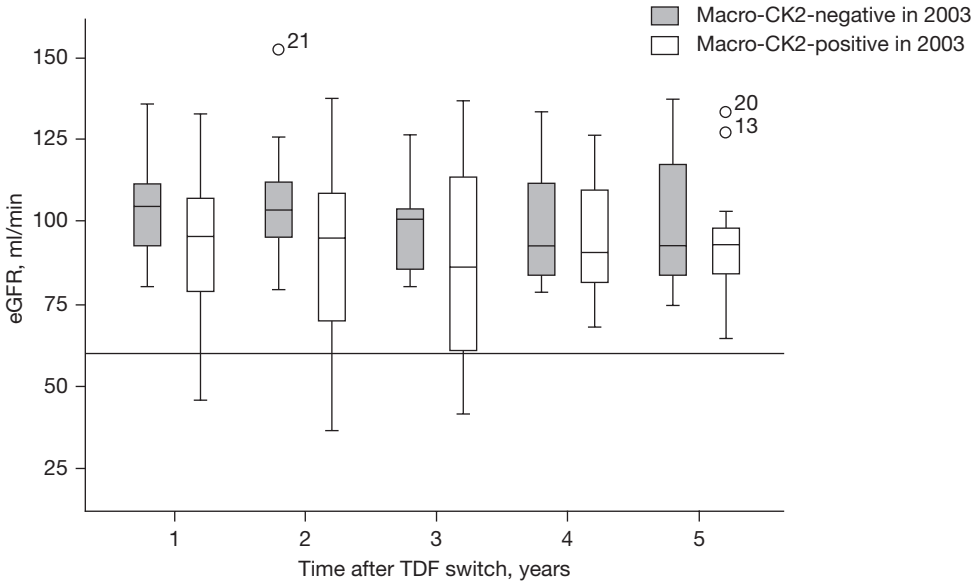
Literature data suggest association of macro CK2 with disseminated malignancies [28,29]. Indeed, in the macro-CK2-positive cohort, one patient was diagnosed with adenocarcinoma of the colon in 2003, and died shortly after as a consequence of metastatic disease; this patient was, therefore, excluded from follow-up. In the macro-CK2-negative cohort, in 2003, a male patient presented with a relapse of Hodgkin's disease after a remission period of 4 years [2]. This patient was successfully treated and was followed without a detectable macro CK2 until August 2006 but did not complete follow-up. In all 34 patients completing the prospective observational study in 2009, including those four patients that remained macro-CK2-positive over 5 years, we observed neither AIDS-defining nor non-AIDS-defining malignancies in follow-up, indicating that appearance of uMtCK in TDF-treated patients is not caused by active carcinoma.

TDF treatment is associated with a favourable immunological and virological response but a decrease in eGFR, which is independent of macro CK2 status

A multivariate logistic regression analysis at baseline showed no association of viral load or CD4+ T-cell count with macro CK2 appearance [2]. Re-evaluation between initially macro-CK2-positive and macro-CK2-negative patients after 5 years demonstrated no virological failure (defined as two consecutive plasma HIV RNA levels of >1,000 copies/ml) and, despite a lower initial CD4+ T-cell count at baseline in macro-CK2-positive patients, a significant CD4+ T-cell count reconstitution in both patient groups (Table 1).

In the initial study evaluation, differences between macro-CK2-positive or -negative patients in eGFR, determined by the MDRD2 formula, nearly reached statistical significance. In addition, macro-CK2-positive patients showed a significant elevation of serum β2-microglobulin levels, suggesting mild proximal tubular damage [2]. As chronic tubular dysfunction could lead to a gradual decrease in eGFR, we compared creatinine values and eGFR between initially macro-CK2-positive and -negative patients over a 5-year period. During follow-up, we found no case of acute renal failure or Fanconi syndrome in the cohort. In TDF-treated patients, an increase in mean serum creatinine within the reference limit and a decrease in eGFR over time, irrespectively of the equation formulas used, was a prominent finding. Using the Cockraft–Gault formula, mean eGFR in macro-CK2-positive patients decreased from 96 to 91 ml/min

Figure 2. eGFR decreased in macro-CK2-positive and macro-CK2-negative patients after TDF switch



Using the Cockroft–Gault formula, mean estimated glomerular filtration rate (eGFR) in macroenzyme creatine kinase type 2 (macro CK2)-positive patients decreased from 96 to 91 ml/min (P=0.423) and from 113 to 104 ml/min (P<0.0001) in macro-CK2-negative patients. Application of the modification of diet in renal disease 2 formula yielded similar results. Significant reduction in eGFR between years 1 and 5 was observed only in the group of macro-CK2-negative patients. Straight line indicates an eGFR of 60 ml/min. The circles and numbers represent outliers with the corresponding patient code after anonymization. TDF, tenofovir disoproxil fumarate.

(P=0.423) and from 113 to 104 ml/min (P<0.0001) in macro CK2-negative patients, respectively (Figure 2). Application of the MDRD2 formula yielded similar results (Table 1). Of particular note, the observed trends reached significance only in macro-CK2-negative patients.

TFV, TDF and TDP do not affect the catalytic parameters of uMtCK

Our prospective clinical data argue against the hypothesis of macro CK2 genesis being associated with malignancies or massive cell damage in general. Thus, TFV compounds may not only target proximal tubular cells in the kidney but, also, affect uMtCK complexes directly. We, therefore, analysed TFV, TDF and TDP for their direct *in vitro* effects on structure and function of recombinant human uMtCK. Obviously, the relevant compounds *in vivo* are TFV in serum (TDF being rapidly converted into TFV) and TFV and TDP for intracellular effects. In these and following *in vitro* experiments, we used supraclinical concentrations of these drugs (up to 500 μM) to account for accumulation of TFV and TDP in proximal tubular cells up to the higher micromolar range [30,31].

Catalytic parameters were first determined by a very sensitive electrochemical assay. Although specific activity of uMtCK with its natural substrates PCr and ADP was 105 U mg^{-1}, no enzyme activity was detectable with PCr and TFV or TDF. As reported for cytosolic CK [12], only analogues with a diphosphate moiety as in TFV monophosphate (the phosphonate group plus the added phosphate) may serve as a CK substrate. We then analysed whether TFV, TDF or TDP can interact with the uMtCK active site to competitively inhibit the uMtCK reaction by using spectrophotometric (Figures 3 and 4) or electrochemical assays (data not shown). Dose–response curves in the range of 5–500 μM drug and preincubation times for up to 24 h did not affect maximal uMtCK enzymatic activity in either forward or reverse reactions (data not shown). We, therefore, continued to use 500 μM drug for a detailed kinetic analysis of the two-substrate reaction. There were no systematic effects of TFV and TDF on enzyme kinetics (Figure 3), independently of the assay method applied. Neither was there systematic departure from linearity in Lineweaver–Burk plots, so the drugs did not affect classical Michaelis–Menten kinetics. Calculated apparent K_m values confirmed no statistically significant difference between controls and TFV- or TDF-treated samples (Table 2). The uMtCK activities in the presence of 500 μM TDP, determined with a more limited set of MtCK substrate concentrations, again did not reveal significant differences (Figure 4). This also indicates that, in contrast to TFV monophosphate and cytosolic CK [12], TDP does not significantly participate in the uMtCK forward reaction as a phosphate donor.

TFV and TDF stabilize the octameric form of uMtCK

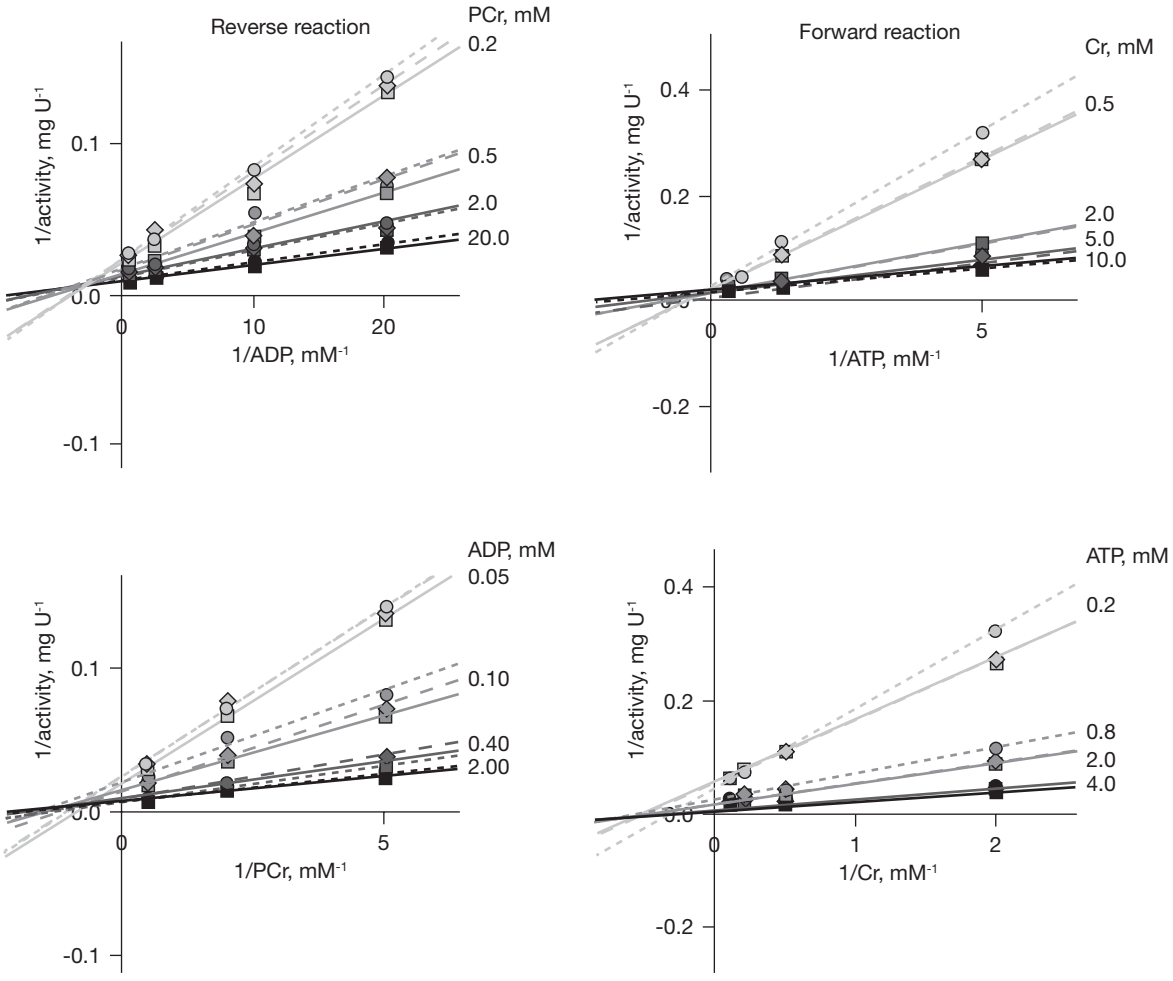
In TDF-treated patients, macro CK2 mainly consists of uMtCK octamers, the native oligomeric form in mitochondria. Such octamers are labile and readily dissociate into dimers upon dilution to concentrations below 0.5 mg/ml [11]. However, this seems not to be the case in patients' sera. We, therefore, analysed the effect of 500 μM TFV or TDF on the oligomeric state of recombinant octameric uMtCK *in vitro* by gel permeation chromatography under two conditions: a rather stabilizing (high-protein concentration, 24 h incubation), and a rather destabilizing (low-protein concentration, 48 h incubation) [11] (Figure 5). Although the drugs had no major effect under stabilizing conditions, where few dimerizations occurred, this was different under destabilizing conditions. Controls without drug addition showed a substantially reduced octamer content of only 61%, but this octamer loss was less pronounced in presence of TFV or TDF, preserving 65% or 70% of octamers, respectively (Table 3). This indicates a significant stabilizing interaction of the two drugs with the uMtCK octamer, similar to non-productive substrate–enzyme complexes [4]. Because no concomitant effects on enzyme kinetics have been observed, this binding may not occur in the active site or is of too low affinity to significantly affect enzyme kinetics.

Discussion

Here, we performed a prospective observational 5-year follow-up study on those HIV-infected TDF-treated patients, in which macro CK2 appearance was initially described and identified as octameric uMtCK ('TDF switch study' cohort) [2]. Our findings indicate that macro CK2 can persist over years under TDF treatment but always disappears after TDF discontinuation. The data exclude macro CK2 as a marker for imminent nephrotoxicity and significant cell damage. They rather suggest that TDF-induced macro CK2 occurs due to combined cellular and molecular events: subclinical renal tubular dysfunction together with possible compensatory uMtCK overexpression and a putative concomitant stabilization of uMtCK octamers by higher levels of TFV in proximal tubules.

TFV nephrotoxicity is typically characterized by a failure of proximal tubular cells, with glomerular renal function being decreased or even preserved [16,32]. TFV is eliminated by a combination of glomerular filtration and tubular secretion. Approximately 30% of the drug is actively transported into the proximal tubule by organic anion transporter-1 in the basolateral membrane and then secreted into the urinary space by apical efflux transporters [17]. Dysfunction of these transporters leads to accumulation of the drug in proximal tubule epithelia. This strongly affects

Figure 3. TFV and TDF do not affect uMtCK enzyme kinetics

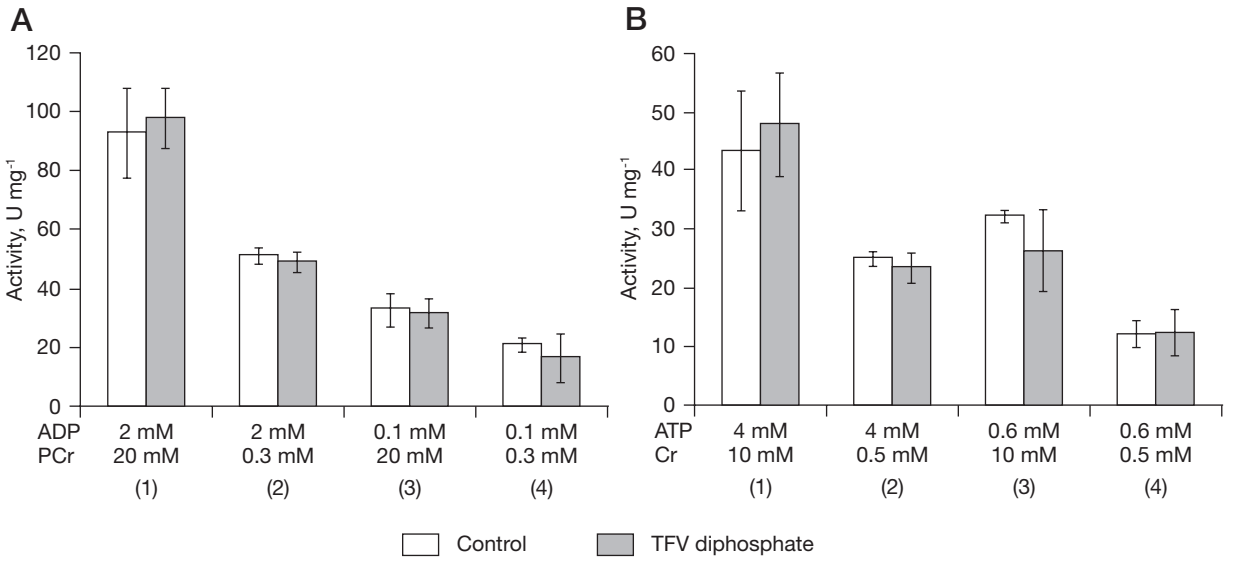


Substrate concentration, mM				Drug added		
ADP	ATP	PCr	Cr	No	TFV	TDF
0.05	0.2	0.2	0.5			
0.10	0.8	0.5	2.0			
0.40	2.0	2.0	5.0			
2.00	4.0	20.0	10.0			

Double reciprocal Lineweaver–Burk plots of ubiquitous mitochondrial creatine kinase (uMtCK) enzyme kinetics in reverse (ADP and phosphocreatine [PCr] as substrates) or forward reaction (ATP and creatine [Cr] as substrates), determined in a spectrophotometric assay without (full line) or directly after adding (dashed lines) 500 μM tenofovir (TFV) or 500 μM tenofovir disoproxil fumarate (TDF; dotted lines), using four different substrate concentrations (see legend and *Methods*). Data are presented as mean (n=3), and calculated K_m values are given in Table 2.

mitochondrial and cellular structure, including prominent swelling of tubules, enlarged mitochondria, and disruption of cristae architecture [15], possibly due to inhibition of mitochondrial DNA (mtDNA) replication [33]. In addition, it should be noted that TFV is a fairly weak inhibitor of, and substrate for, mtDNA polymerase-γ compared with many other NRTIs [34]. Because proximal tubular cells are highly oxidative with a very large number of mitochondria, TFV and/or TDP accumulation may impair their bioenergetics and

Figure 4. TDP has no significant effect on uMtCK enzyme activity



TDP does not affect ubiquitous mitochondrial creatine kinase (uMtCK) enzyme activity in **(A)** the reverse or **(B)** the forward reaction. The ubiquitous mitochondrial creatine kinase enzyme kinetics were determined in a spectrophotometric assay without or directly after adding 500 μM tenofovir diphosphate. Substrate concentrations (from left to right) were chosen to be (1) close to saturation of the reaction, (2,3) close to saturating for one and close to K_m for the other and (4) close to their respective K_m's for both. Data are presented as mean ±SD (n=4). Cr, creatine; PCr, phosphocreatine; TFV, tenofovir.

general functioning, thus TFV nephrotoxicity has been described as 'renal tubular mitochondriopathy' [35]. It is a limitation of our study that renal biopsies were not routinely obtained to systematically compare ultrastructural features and mtDNA in proximal tubules. However, given the substantial uMtCK expression in renal epithelia [36], low-level mitochondrial and cellular damage may be sufficient for substantial uMtCK to leak into the blood stream.

Appearance of macro CK2 in our earlier study was related to lower eGFR as compared with macro-CK2-negative patients [2], suggesting macro CK2 as an indicator of imminent TDF nephrotoxicity. During follow-up, eGFR, calculated by using the MDRD2 and the Cockcroft–Gault formula, decreased in both groups, reaching statistical significance only in macro-CK2-negative patients, irrespective of the equation formula used. Underlying causes for this eGFR decrease remain unclear. The reduced glomerular function in our cohort was neither significantly associated with trough plasma TDF concentration, older age, diabetes, hypertension or concomitant therapy, for example, with a protease inhibitor [37]. Of note, TDF was discontinued during follow-up in only one patient as a result of a suggested, but not secured, TDF-associated eGFR decrease, excluding a systemic selection bias. Thus, TDF exposure appears to cause a modest, gradual decrease in eGFR, usually without significantly impairing glomerular function.

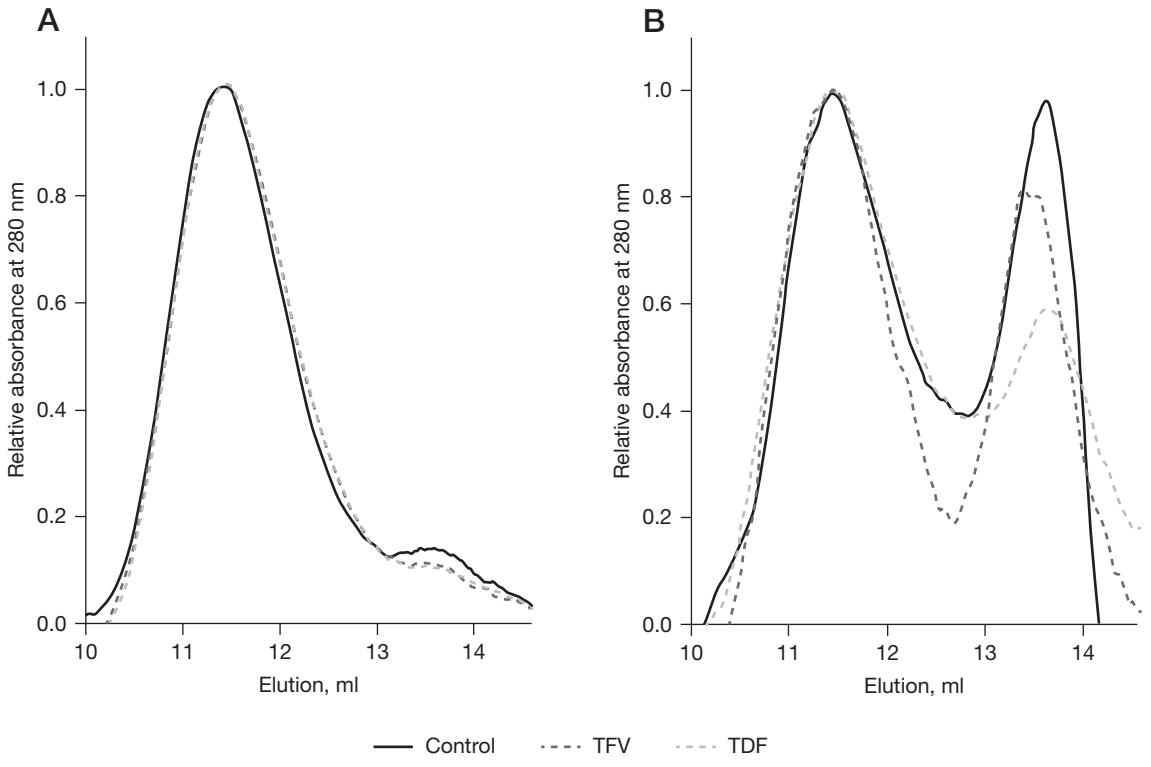
Table 2. Apparent K_m constants of the uMtCK reaction in presence or absence of TFV or TDF

Substrate	Control[a]	TDF[a]	TFV[a]
Forward reaction			
MgATP	661 ±180	494 ±120	543 ±145
Cr	542 ±87	604 ±79	621 ±158
Reverse reaction			
MgADP	121 ±25	130 ±31	138 ±20
	100 ±26[b]	–	100 ±1[b]
PCr	341 ±63	349 ±54	332 ±37

Data are given as mean ±SD for three independent experiments. [a]Data represented as K_m (μM). Data obtained by a spectrophotometric assay or [b]the pH-stat assay. Cr, creatine; PCr, phosphocreatine; TDF, tenofovir disoproxil fumarate; TFV, tenofovir; uMtCK, ubiquitous mitochondrial creatine kinase.

However, some limitations may apply to this conclusion. First, the effect of long-term TDF use on renal function has not been fully evaluated. A recent meta-analysis revealed a small but significant loss of kidney function during TDF treatment [38]. In another non-randomized large cohort, increasing exposure to TDF was associated with a higher incidence of chronic kidney disease [39]. Second, eGFR is determined by measurements of serum creatinine, which is filtered by the glomerulus but also secreted across the proximal tubule. Decreasing eGFR, rather than being indicative of glomerular dysfunction, may, thus, be caused by inhibited tubular creatinine

Figure 5. TFV and TDF stabilize the octameric state of ubiquitous mitochondrial creatine kinase *in vitro*



Typical ubiquitous mitochondrial creatine kinase (uMtCK) elution profiles from Superose 12 gel permeation chromatography separating octamers (eluting at 11.4 ml) from dimers (eluting at 13.8 ml). **(A)** 700 μg/ml or **(B)** 70 μg/ml human uMtCK have been incubated for (A) 24 h or (B) 48 h with 500 μM tenofovir (TFV) or 500 μM tenofovir disoproxil fumarate (TDF). Elution profiles were normalized to the octamer peak to facilitate comparison. Calculated octamer and dimer content is given in Table 3.

Table 3. Octamer and dimer content of diluted uMtCK in the presence or absence of TFV or TDF

Condition	Octamer, %	Dimer, %
700 μg/ml uMtCK		
Control	89.7 ±0.4	10.3 ±0.1
TFV	91.4 ±0.6[a]	8.6 ±0.0[b]
TDF	91.5 ±0.6[b]	8.5 ±0.1[b]
70 μg/ml uMtCK		
Control	61.7 ±1.6	38.3 ±2.0
TFV	65.2 ±1.4[a]	34.8 ±1.6[a]
TDF	70.0 ±1.2[b]	30.0 ±1.3[b]

Incubation of 700 μg/ml ubiquitous mitochondrial creatine kinase (uMtCK) for 24 h or of 70 μg/ml uMtCK for 48 h with buffer alone or an additional 500 μM tenofovir (TFV) or tenofovir disoproxil fumarate (TDF). Data are given as mean ±SD for three experiments. [a]$P<0.05$ and [b]$P<0.01$ as compared with control.

excretion [40]. Finally, although the Cockroft–Gault formula appears to be the best reflection of real glomerular filtration rate (GFR) in patients with preserved renal function and suppressed HIV-infection [41], due to a lack of data, it is currently unclear whether other equations, for example the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula or cystatin c, are more appropriate methods for monitoring GFR in patients on HAART [42].

Direct toxic TDF effects in tubular epithelial cells could explain phosphaturia, proximal tubular acidosis, polyuria and a reduced urinary concentrating ability observed without changes in GFR [43].

Hypophosphataemia is relatively common in HIV-infected patients [44–46]. Based on our study design, we were not able to analyse urine samples at baseline and during follow-up, but, presuming that redistribution of phosphate from the extracellular fluid into cells and intestinal absorption of phosphate are unchanged, we used phosphate levels as an indirect marker of increased urinary phosphate excretion. We found a trend towards grade 1 hypophosphataemia in macro CK2-positive, but not in macro CK2-negative, patients, without documented cases of severe hypophosphataemia (≤1.9 mg/dl). This finding excludes clinically significant proximal tubular dysfunction with renal phosphate wasting, resulting in prolonged

hypophosphataemia. Our conclusion is consistent with a recent prospective study on 424 HIV-infected patients on HAART, in which the use of TDF was not statistically involved in tubular damage [47].

Our prospective clinical data suggest that continuous administration of TDF and related subclinical dysfunction of proximal tubular epithelial cells with low-level mitochondrial damage are both necessary for macro CK2 to accumulate in serum without, however, being sufficient to fully explain the observed macro CK2 levels. It is yet another limitation that we are not able to define the definite time point when initially macro-CK2-positive patients become electrophoretically macro-CK2-negative. This is based on our study design that schedules macro CK2 testing after a 5-year follow-up and the used electrophoresis assay for macro CK2 detection, which seems to have minor sensitivity compared with a recently developed immunoinhibition technique using MtCK-inhibiting antibodies [48]. As a result of the latter fact, it is also possible that some electrophoretically macro-CK2-negative patients still have substantial levels of uMtCK in the blood stream under TDF treatment, which just escape our assay.

An additional factor for high macro CK2 levels could be compensatory uMtCK overexpression as it occurs under bioenergetic stress induced by mitochondrial dysfunction in different pathologies [4]. In mitochondrial myopathies, octameric sMtCK generates intramitochondrial inclusions in the form of crystalline sheets [49,50]. Inflammatory or infectious muscle disease, for example, HIV-associated polymyositis were excluded in our cohort. In some metastatic malignancies uMtCK is overexpressed [51–53]. Its continuous and massive release into the bloodstream as macro CK2 is associated with poor prognosis [1,28,29,52]. No such relationship was observed in our study, indicating that mechanisms for uMtCK accumulation in TDF-treated patients differ from those in cancer.

Another factor in the genesis of macro CK2 could be direct interference of TFV and its derivatives with uMtCK. As we showed, TDF, TFV and TDP do not significantly interfere with uMtCK enzymatic activity, but TFV, and mainly TDF, clearly stabilize the uMtCK octameric structure in dilute solutions. In contrast to exclusively dimeric cytosolic CK isoenzymes, MtCKs occur in dynamic octamer/dimer equilibrium [4,11,54,55]. The octameric form is largely predominant *in vivo*, but readily dissociates into dimers in a concentration-, pH- and temperature-dependant manner [4,11] or as a result of oxidative stress [54,55]. No other uMtCK oligomers occur *in vivo*, and inactive monomers only form under denaturating conditions [26,56]. The molecular structure of human uMtCK reveals that octamers are stabilized by hydrophobic interfaces and additional polar interactions between the N-termini of neighbouring dimers [25]. Although functional analysis revealed that they are more stable than those of sMtCK, they also readily dimerize below concentrations of 0.5 mg/ml [11]. It is known that induction of the 'closed' MtCK conformation by CK substrates or substrate analogues can stabilize the octameric structure [4]. TDF and TFV may act in a similar way, although it remains unclear whether they can directly attach to the nucleotide binding site of uMtCK as they do not affect the enzymatic reaction.

Stabilizing complexes of uMtCK octamers with TFV (and possibly also TDP) would already form in proximal tubular cells, where these drugs accumulate to higher micromolar concentrations [30,31]. More stable uMtCK octamers in the mitochondria of these cells would favour the known anti-apoptotic function of the enzyme [4,6,57]. TFV could, thus, have a direct protective effect in proximal tubular cells, which are highly susceptible to apoptosis. Furthermore, upon cellular damage, leading to uMtCK leakage into the bloodstream, such TFV-stabilized octameric complexes would be significantly protected from rapid dissociation into dimers. Under these conditions, uMtCK is diluted to concentrations much below the 0.07 mg/ml used in our assays (estimated from [2]), which represents a known and efficient dimerization trigger [11]. TFV (and TDF) present in the bloodstream, albeit at much lower concentrations, could maintain stabilized octameric complexes and slow octamer–dimer transition. Although TDF is instable in serum, some TDF may rapidly enter into complexes with octameric uMtCK and stabilize both TDF and uMtCK. Given the large size of the cuboidal octamers (edge length >100 Å [25,58]), these could not be eliminated by renal clearance as is the case with uMtCK dimers. As a consequence, octameric uMtCK would accumulate as macro CK2 in serum.

In conclusion, prospective data exclude the hypotheses that macro CK2 appearance in TDF-treated patients is a predictor of TDF-mediated GFR decrease or clinically relevant tubulopathy, a danger signal for imminent malignancy or an indicator of significant cellular damage. They rather argue for combined molecular and cellular events to be responsible for macro CK2 appearance: a continuous low-level stress on proximal tubules of TDF-treated patients would lead, in some patients, to minimal release of octameric uMtCK into the bloodstream, possibly because they also show compensatory uMtCK overexpression. In the blood, TFV-stabilized octameric uMtCK would escape renal clearance and accumulate as macro CK2 in serum.

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Disclosure statement

The authors declare no competing interests.

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