

PLATELETS, A TYPICAL SOURCE OF ERROR IN REAL-TIME PCR QUANTIFICATION OF MITOCHONDRIAL DNA CONTENT IN HUMAN PERIPHERAL BLOOD CELLS

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Abstract: Nucleoside analogues can induce toxic effects on mitochondria by inhibiting the human DNA polymerase- γ . The clinically observed toxicities can range from slightly increased serum lactate levels to potentially severe and fatal lactic acidosis.

A growing interest exists for detection of changes in mitochondrial (mt) DNA content in patients receiving antiretroviral therapy (HAART). Most studies use peripheral blood mononuclear cell (PBMC) fractions to investigate mt DNA content via Real-Time PCR in patients, not accounting platelets falsifying the mitochondrial (mt)DNA:nuclear (n)DNA-ratio. In this study we suggest a procedure to eliminate disturbing platelets totally.

8 healthy controls (G1), 6 therapy-naive HIV-infected patients (G2) and 9 HIV-infected patients under HAART (G3) were examined for mtDNA:nDNA-ratio using Real-Time PCR technology. Different blood collection and/or PBMC isolation strategies were analysed for variances of outcome at examinations of the same blood donor.

Using DNA prepared of whole blood specimens, mtDNA:nDNA-ratios showed no differences in all investigated groups (G1, G2, G3). Comparing mtDNA:nDNA-ratios of platelet-depleted PBMC fractions of G1 with G2 revealed a reduction of 22% ($p = 0.128$) and a steeper reduction of 40% ($p = 0.0036$) comparing specimens of G1 with G3. Scrutinising differently processed specimens within the groups themselves, in G2 whole blood versus platelet-containing PBMC specimens showed a difference in mtDNA:nDNA-ratios of 26% ($p = 0.0406$), whereas a comparison of whole blood versus platelet-free PBMC specimens led to a comparatively more distinct reduction of 35% ($p = 0.0089$). The same effect was seen in G3, where whole blood versus platelet-containing PBMC specimens revealed a reduction of 32% ($p = 0.01$) and whole blood versus platelet-free PBMC specimens showed a 42% ($p = 0.0011$) decrease.

Furthermore analysing each single patient in relation to the different methods, a minor fluctuation margin could be found using platelet-free PBMC specimens for Real-Time PCR. Using platelet-free PBMCs for mtDNA content detection, a correlation of low mtDNA:nDNA-ratios to clinical signs, like elevated lactate

levels or lipodystrophy, could be observed. Light-microscopic evaluation for platelets, comparing platelet-containing PBMC fractions versus platelet-depleted PBMC fractions reinforced the Real-Time PCR results.

Our data demonstrate that the first step of the blood sample collection/preparation is critical for valid illustration of mt DNA content in HIV-infected patients using ultra-sensitive Real-Time PCR technology. The use of serum tubes for blood collection is an easy and low-cost alternative to expensive cell sorting for elimination of disturbing platelets. Using platelet-free PBMC fractions for measurement mt DNA content could be a surrogate marker for clinical signs mediated by HAART.

Key words: Platelets, PBMC, mitochondrial DNA content, mtDNA:nDNA-ratio, Real-Time PCR

INTRODUCTION

The use of nucleoside analogues for the treatment of human immunodeficiency virus (HIV) infection includes a spectrum of compounds, such as zidovudine, didanosine, stavudine and lamivudine. Some of these compounds have been associated with adverse effects that have been ascribed to the induction of mitochondrial (mt) toxicity [7, 14, 15]. The toxic effects of zidovudine on mitochondria were the first to be reported [6]. However, nowadays mt toxicity in nucleoside reverse transcriptase inhibitor (NRTI) treated HIV-patients is a well recognised adverse effect of antiretroviral therapy. Regarding the clinical impact of mt toxicity leading to hyperlactatemia, hepatic steatosis, lipodystrophy or severe lactic acidosis in some cases [4, 10], studies of mt DNA content in patient blood specimens are often performed using the high sensitive Real-Time PCR technique [1-3, 8, 11-13]. Effects of NRTIs on mitochondria are mainly due to their capacity to inhibit DNA polymerase- γ , the enzyme responsible for mtDNA synthesis in cells [9]. Therefore quantifying mitochondrial (mt)DNA:nuclear (n)DNA-ratio in PBMCs of HAART treated patients could elucidate direct proof of drug effects on mitochondria [2].

Platelets play a widely underestimated role in the measurement of mt DNA content in PBMC fractions. They adulterate mt DNA content determined as mtDNA:nDNA-ratio by Real-Time PCR, especially when measured in whole blood specimens. Platelets are con-

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stricted from megakaryocytes and therefore are in lack of a nucleus, but contain numerous mitochondria. In healthy humans normal platelet counts rank between 150 000 to 350 000 platelets per μl blood, having relatively stable values intra-individually. Due to the wide range of platelet counts and their small size, platelets can not be fully disposed of by the common method of Ficoll-preparation. Therefore they potentially attenuate the measurement of drug induced mt-toxic effects in Real-Time PCR, while clinical/physiological signs still exist.

In our study we try to depict the problem of platelets influencing the evaluation of mt DNA content in blood specimens using the highly sensitive Real-Time PCR technique. Therefore three sample collection/preparation methods - namely the use of whole blood derived DNA specimens, the commonly used PBMC Ficoll isolation method, which derived platelet-containing PBMC DNA specimens, and platelet depleted PBMC DNA specimens (-which were obtained from blood collected in serum tubes) - are analysed, in respect to variances at the outcome of mtDNA:nDNA-ratio measurements, as well as for the correlations to the clinical signs.

MATERIAL, METHODS AND STATISTICS

REAGENTS AND MATERIAL

QIAamp® DNA Blood Mini Kit was purchased from QIAGEN (QIAGEN GmbH, Hilden, Germany). FastStart DNA Master Hybridization Probes Kit was obtained from Roche Applied Science (Roche GmbH, Mannheim, Germany). Primers and labelled oligonucleotides for the quantitative Real-Time PCR were synthesized by TIB MOLBIOL (TIB MOLBIOL, Berlin, Germany). Serum tubes were purchased from KABE (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) and EDTA tubes from SARSTEDT (SARSTEDT AG & Co., Nümbrecht, Germany)

PATIENT SAMPLE COLLECTION AND DNA EXTRACTION

15 patients (1 female, 14 male) were recruited from our HIV-patient cohort. Healthy controls (4 female, 4 male) were chosen among healthy volunteer individuals. Study participants were stratified into three different groups: HIV-negative healthy controls (n=8; G1), HAART-naive or therapy interrupting (>6 month) HIV-infected asymptomatic patients (n=6; G2) and HIV-infected patients on HAART (n=9; G3). Patients of G3 were set with a therapy regimen which includes two NRTIs. The mean age in our patient collective was 48 years.

10ml blood specimens of peripheral venous blood were optionally collected in either EDTA tubes or serum tubes and processed immediately.

For the recommended procedure we used serum tubes to collect the patients' blood. The serum tubes were rotated for 5 minutes, residual uncoagulated blood was taken for Ficoll-preparation [5] to receive a platelet-free PBMC fraction. The blood collected in EDTA tubes was immediately overlaid above a Ficoll

separating solution, as described elsewhere [5]. Collected PBMCs of both preparation techniques and 200 μl of whole blood, respectively, were taken for DNA extractions. Total DNA was isolated by QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden Germany) according to the manufacturer's protocol. DNA concentration was measured in a photometer and specimens were stored for later use at -20°C .

For the standard curve, needed as reference control in the Real-Time PCR, 6 blood specimens of a non-HIV-infected male volunteer were collected, cells were prepared, total DNA was extracted (as described above) and pooled.

This study was approved by the local ethics committee. All patients in the study provided written informed consent.

QUANTITATIVE REAL-TIME PCR

Within each DNA extract, the nuclear gene for the human DNA polymerase- γ accessory subunit (ASPOL γ) and the mitochondrial gene human cytochrome-c oxidase subunit I (CCO1) were quantified in separate Real-Time PCR reactions in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany).

For the CCO1 gene, the *CCO1-1for* 5'-TTCGCC GACCGTTGACTATT-3' and *CCO1-1rev* 5'-AAGAT-TATTACAAATGCATGGGC-3' primers were used for the PCR amplification. The 3'-fluorescein labelled *fCCO1-2for* 5'-GCCAGCCAGGCAACCTTCTAGG-(F)3' and 5'-LC Red640 labelled and 3'-phosphate-blocked *rCCO1-2rev* 5'(L)-AACGACCACATCTACA ACGTTATCGTCAC-P-3' oligonucleotides were used as hybridisation probes.

For the nuclear gene ASPOL γ , the *ASPOL-1for* 5'-GAGCTGTTGACGGGAAAGGAG-3' and *ASPOL-1rev* 5'-CAGGAAGAGAATCCCGGCTAAG-3' primers were used for the PCR reaction, and the oligonucleotides 3'-fluorescein labelled *fASPOL-2for* 5'-GAGGCGCTGTTAGAGATCTGTCAAGA-(F)3' and 5'-LC Red640 labelled, 3'-phosphate-blocked *rASPOL-2rev* 5'(L)-GGCATTTCCTAAGTGGAAG CAAGCA-P-3' were used as hybridisation probes. Real-Time PCR reactions were performed in duplicate for each gene and sample with the LightCycler Fast-Start DNA Master Hybridization Probes Kit (Roche Applied Science, Mannheim, Germany) as previously described by Côté et al. [2]. The PCR protocol was slightly modified by adding 6% DMSO to the PCR reaction, to achieve a better amplification in our specimens.

In brief, a PCR reaction contained 2 μM of each primer pair, 0.2 μM of the 3'-fluorescein probe, 0.4 μM of the 5'-LC Red640 probe, 6% DMSO and 10ng total DNA. The PCR reaction volume was 20 μl . For the amplification protocol we initially used 10 minutes denaturation at 95°C , followed by 45 cycles of 0 seconds at 95°C , 10 seconds at 60°C , and 5 seconds at 72°C , with a temperature-transition rate of 20°C per second. A single fluorescence acquisition was performed at the end of each annealing step. A standard curve of 0.1, 1, 10, 100 nuclear-genome equivalents for both, the nuclear (ASPOL γ) and mitochondrial (CCO1) gene quantification was defined and included into each PCR run.

The data were analysed using the second-derivative maximum (SD) of each amplification reaction and related to its respective standard curve. The results of the quantitative PCR were expressed as the ratios of the mean mitochondrial DNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements (mtDNA:nDNA) for a given DNA extract.

The intra-assay of the PCR measurements revealed a mean of 9.25 (SD ±0.56) for ASPOLγ and their replicates a mean of 7.82 (SD ±0.85) for COO1. The intra-assay of EDTA tube derived specimens showed a mean of 10.26 (SD ±0.25) for ASPOLγ and a mean of 8.55 (SD ±0.53) for COO1. Whole blood specimens resulted in a mean of 14.85 (SD ±0.63) for ASPOLγ and a mean of 16.78 (SD ±0.56) for COO1 for their replicates in the intra-assay.

STATISTICS

The statistical analysis was done using the StatView software package (Abacus Concepts Inc., Cary, NC) and was considered significant, if p-values were less than 0.05. The Fisher's PLSD test was used as appropriate to compare the significances of the groups.

RESULTS

In our cross-sectional study 23 individuals were analysed for mt DNA content particularly with regard to a platelet specific influence on the measurement of mtDNA:nDNA-ratios detected by Real-Time PCR. As presented in Figure 1 a clear difference in mtDNA:nDNA-ratios was seen, depending on how peripheral venous blood was collected from the patient. Using DNA of whole blood specimens for determination of mtDNA:nDNA-ratios, in all investigated groups no difference was found (Fig. 1a, filled

bars). Filled bars show mtDNA:nDNA-ratios measured in DNA of whole blood specimens. This DNA was directly prepared from whole blood without prior isolation of PBMCs by Ficoll separation. Patterned bars show mtDNA:nDNA-ratios of platelet-free PBMC DNA fractions derived by the use of serum tubes for blood collection. G1 are healthy non-HIV-infected individuals, G2 are HAART-naive or therapy pause (>6 month) HIV-infected asymptomatic patients and G3 indicates HIV-infected patients receiving an antiretroviral therapy.

Collection of blood into serum tubes, Ficoll preparation for PBMCs and further determination of platelet-free PBMC mt DNA content, revealed a marked reduction of mtDNA:nDNA-ratio reflecting the clinical status of the individuals in the groups. The reduction of mtDNA:nDNA-ratio in G2 might indicate a mt DNA depleting effect of the HI-virus itself (22%; n.s.) [3, 12, 13]. The reduction of mtDNA:nDNA-ratio in G3 (40%; p = 0.0036) (compare patterned bars of G1, G2 and G3 in Fig. 1a), might reflect a possible viral effect added with a mt toxic effect of antiviral therapy.

mtDNA:nDNA-ratios G1: filled bar mean 1.17, SD ± 0.32; patterned bar mean 1.11, SD ± 0.2; mtDNA:nDNA-ratios G2: filled bar mean 1.32, SD ±0.04; patterned bar mean 0.86, SD ± 0.33; mtDNA:nDNA-ratios G3: filled bar mean 1.15, SD ± 0.42; patterned bar mean 0.67, SD ± 0.19 (Fig. 1a).

In addition a comparison of the different blood collection/PBMC isolation methods within the groups themselves was performed (Fig. 1b). Filled bars show mtDNA:nDNA-ratios measured in whole blood specimens. Patterned bars show mtDNA:nDNA-ratios of platelet-free PBMC DNA fractions. Chequered bars depict mtDNA:nDNA-ratio measurements using DNA of platelet-containing PBMCs fractions, isolated from blood specimens collected in EDTA tubes. G2

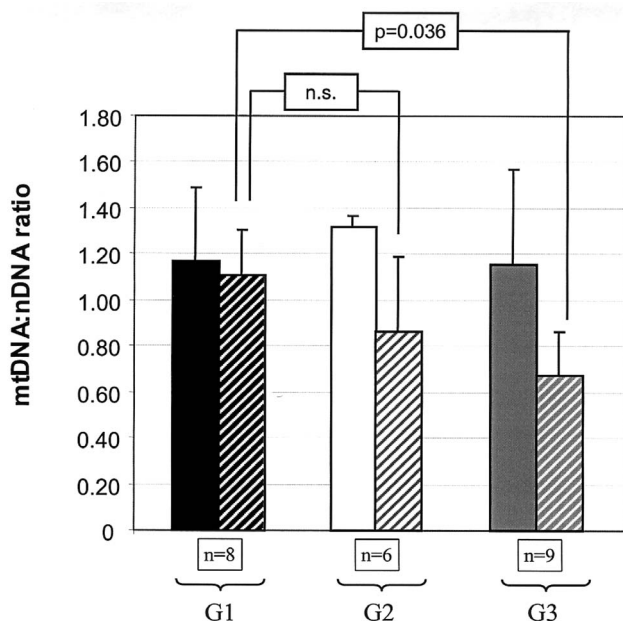


Fig. 1a. Comparison of mtDNA:nDNA-ratios in whole blood specimens versus platelet-free PBMC specimens.

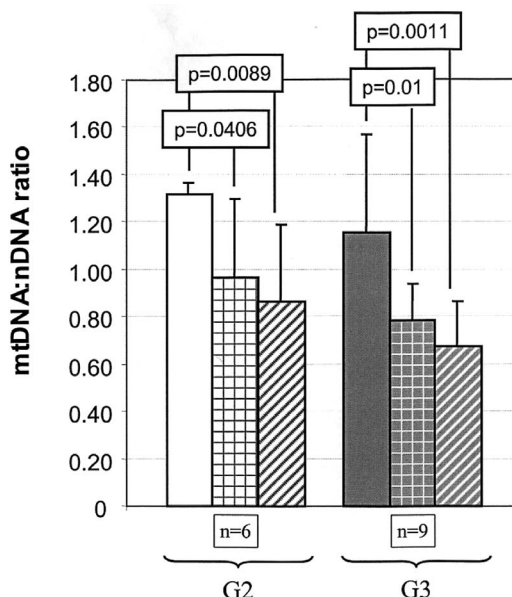


Fig. 1b. Variation of mtDNA:nDNA-ratios in respect of the three different blood collection and preparation methods.

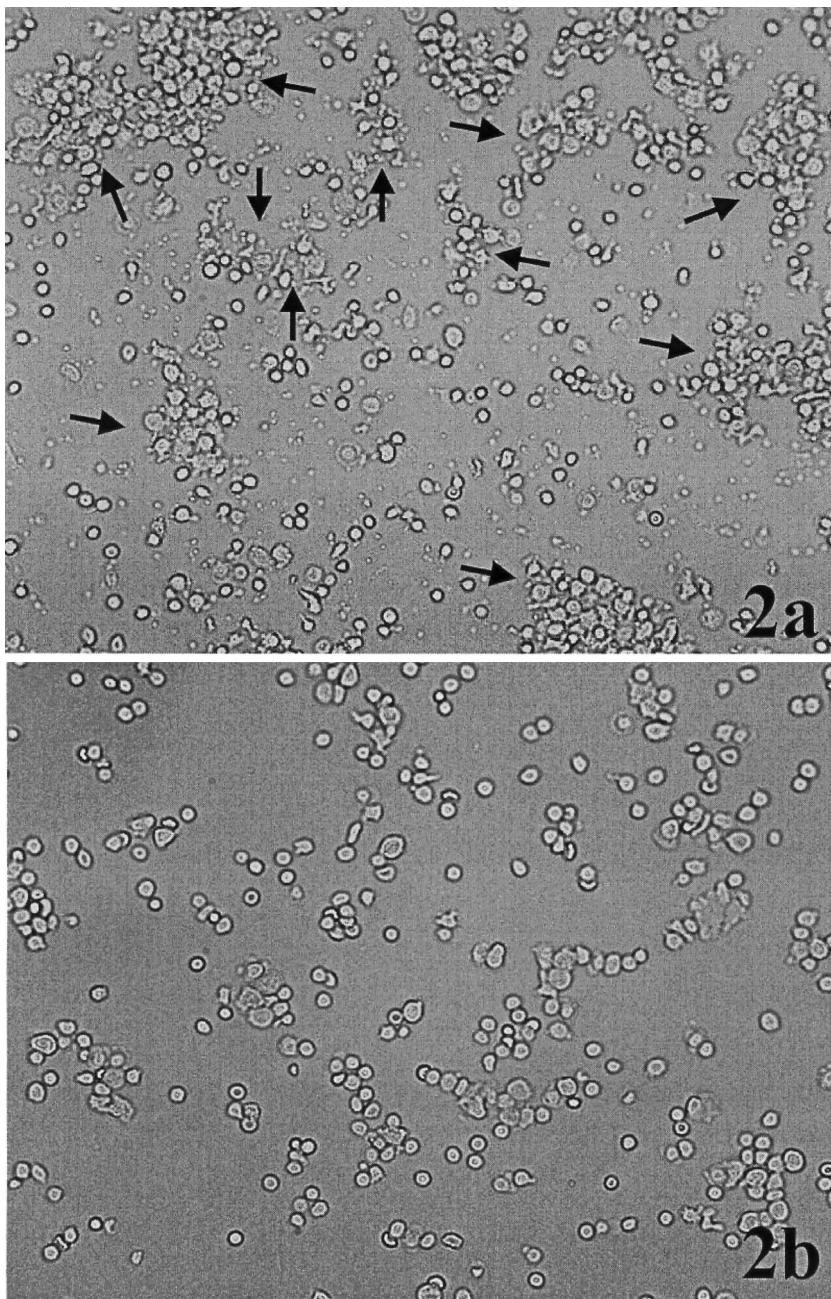


Fig. 2. PBMC fractions derived from EDTA- and serum tubes.

are HAART-naive or therapy pausing (>6 month) HIV-infected asymptomatic patients. G3 indicates HIV-infected patients receiving an antiretroviral therapy.

A significance of $p = 0.0089$ (35%) in mtDNA:nDNA-ratios comparing whole blood specimens versus serum tube collected blood specimens in G2 and of $p = 0.0011$ (42%) in G3 was found. A mtDNA:nDNA-ratio comparison of whole blood specimens versus platelet-containing PBMC specimens, revealed a difference of $p = 0.0406$ (26%) in G2 and $p = 0.01$ (32%) in G3. The reduction in mtDNA:nDNA-ratios comparing platelet-containing PBMC fractions versus platelet-depleted PBMC fractions, seemed to result from the two different blood collection methods.

mtDNA:nDNA-ratios G2: filled bar mean 1.32, SD ± 0.04 ; chequered bar mean 0.97, SD ± 0.33 ; patterned bar mean 0.86, SD ± 0.33 ; mtDNA:nDNA-ratios

mtDNA:nDNA-ratios G3: filled bar mean 1.15, SD ± 0.42 ; chequered bar mean 0.78, SD ± 0.16 ; patterned bar mean 0.67, SD ± 0.19 (Fig. 1b).

To further validate our Real-Time PCR data, we checked Ficoll prepared PBMCs of serum tube and EDTA tube collected blood specimens by visual inspection in the light microscope for presence or absence of platelets. As it is shown in Figure 2b of a PBMC fraction smear, platelets were totally disposed by the use of serum tubes for the collection of blood prior to PBMC isolation (compare Fig. 2a and 2b).

Picture 2a shows a PBMC fraction smear of PBMCs isolated using EDTA tubes for blood collection and the standard Ficoll gradient separation method, followed by two PBS washing steps prior to microscopic inspection. Using this method the PBMC fraction is still contaminated with several platelets. Platelet aggregations are indicated with arrows. Picture 2b illustrates

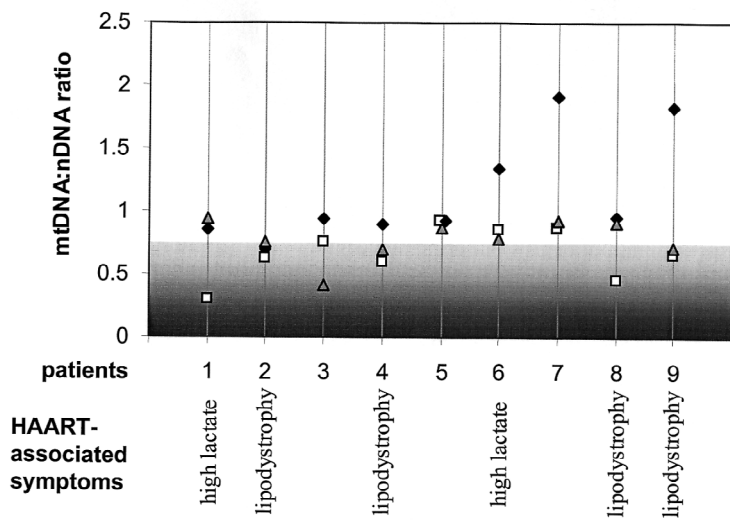


Fig. 3. Comparison of three single mtDNA:nDNA-ratio measurements in each patient of G3.

a PBMC fraction smear of serum tube collected blood and subsequent Ficoll gradient isolated PBMCs, washed two times with PBS. The microscopic pictures clearly demonstrates, that PBMC fractions prepared with serum tube collected blood, generates platelet-free PBMC isolates. Pictures have been taken at a magnification of 20x.

Figure 3 depicts a setting of the measurement for mt DNA content for each patient in G3 using the three different investigated blood collection and PBMC preparation methods, respectively. Patients of G3 were separately analysed in respect to the outcome of mtDNA:nDNA-ratio measurements using each of the different methods. The aim was to scrutinise the applicability and likelihood of each method to identify patients with clinically HAART-associated symptoms.

Using platelet-free PBMC specimens, a minor fluctuation margin was achieved in comparison to the other two methods (shown in Fig. 3). In platelet-free PBMC specimens, a correlation of HAART-associated symptoms, like high lactate levels ($>2\mu\text{mol/ml}$) or lipodystrophy, with low mtDNA:nDNA-ratios in patients 1, 2, 4, 6, 8 and 9 could be found.

Figure 3 shows a diagram of mtDNA:nDNA-ratios depicting the three different blood sample preparation methods for each patient (1 to 9). Ratios derived from whole blood collected in EDTA tubes, directly prepared and measured, without prior isolation of PBMCs by Ficoll separation are indicated with black squares. Ratios of platelet-containing PBMC fractions (EDTA tubes) are indicated with grey triangles. And ratios of platelet-free PBMCs are indicated with white squares. The grey highlighted area indicates critically low mtDNA:nDNA-ratios and therefore a rising risk of HAART-associated symptoms. Patient numbers and HAART-associated symptoms are indicated.

DISCUSSION

To quantify mt DNA depletion in patients, Real-Time PCR technique is widely used [1-4, 7, 8, 10-15]. In this study the mitochondrial DNA concentration in relation to nuclear DNA concentration is determined in blood cells [2]. Peripheral venous blood can be taken

relatively uncomplicated from patients and therefore is a welcome target for study purposes. Thinking of the heterogeneity of blood, we studied the influence of platelets on the ascertainment of mt DNA content by the measurement of mtDNA:nDNA-ratios in Real-Time PCR. As platelets contain high numbers of mitochondria but no nucleus, any determination of mtDNA:nDNA ratio will grossly be perturbed by the presence of thrombocytes, thereby concealing differences caused e.g. by drugs.

In our study three different blood specimen collection and preparation methods were compared with each other. Namely, whole blood specimens collected in EDTA tubes, without a following separation of PBMCs prior to DNA extraction. These specimens contain the whole amount of the patients' individual platelet counts. Therefore using whole blood specimens, platelet counts fully contribute to the measurement of mtDNA:nDNA-ratios.

The second method, we analysed in our study, was the use of peripheral venous blood collected in EDTA tubes, followed by the commonly used Ficoll preparation method for the isolation of PBMCs. The PBMC fraction was washed two times prior to DNA extraction. After the washing steps, the true platelet counts left in the PBMC pellet is a variable which is not known.

The third blood specimen collection method we analysed, was the use of serum tubes for blood collection, followed by Ficoll preparation of PBMCs (see material and methods). After PBMC isolation, PBMC pellets were also washed two times prior to DNA extraction. Using this method for specimen preparation, platelets were fully eliminated from the PBMC fraction.

To take visual inspection of the remaining platelet counts in PBMC pellets after Ficoll preparation, we performed light microscopy of PBMC fraction smears (Fig. 2a and 2b). Performing the common procedure for PBMC isolation, venous blood was collected in EDTA tubes. The whole blood was overlaid over a Ficoll separating solution and centrifuged for 20 minutes [5]. As a result of this centrifugation step, peripheral blood cells were separated in different cell stratifications, depending on their size and specific weight

within the gradient. PBMCs were collected from the specific cell layer. Owing to the small size of platelets, these particles are distributed in all different compartments of the Ficoll gradient and therefore can also be found in the PBMC layer. We found, that it was not possible to receive platelet-free PBMCs using EDTA tube collected blood specimens, even if several washing steps of the PBMC cell pellets were appended (Fig. 2a). The use of serum tubes for blood collection provides platelet-free PBMC fractions. This method is an easy and low-cost alternative to magnetic cell sorting to eliminate contamination of platelets [1]. Serum tubes contain small plastic particles, that activate platelets and insures them to aggregate. Using serum tubes, it is necessary to process the specimens immediately. A rotation time of 5 minutes of the blood filled serum tubes was efficient at our recommended application. The rotation time should not be expanded to prevent PBMCs of being fully incorporated into the prolonged aggregation process. After this platelet cleaning step, Ficoll preparation of the uncoagulated residual blood was carried out and a platelet-free PBMC fraction could be obtained (Fig. 2b).

To further analyse the extent of the platelet specific influence on the measurement of mt DNA content in Real-Time PCR quantification, 8 healthy controls (G1), 6 therapy-naive HIV-infected asymptomatic patients (G2) and 9 HIV-infected patients under HAART, with HAART-associated symptoms (G3) were examined for mtDNA:nDNA-ratios. Figure 1a depicts the differences in measurements of mt DNA content, depending on the used blood collection or specimens preparation method. Using DNA of whole blood specimens for determination of mtDNA: nDNA-ratios, no difference could be found in all investigated groups (G1-G3) (Fig. 1a, filled bars). This might reflect the problem of valid measurement of mt DNA content in whole blood specimens. Separation of PBMCs via Ficoll gradient revealed a detectable difference between all groups at different extent. Collection of blood, especially into serum tubes followed by Ficoll preparation for PBMCs, resulted in the most conspicuous difference in mt DNA content (Fig. 1b). This observation was found to be congruent to the visible impressions at light microscopic examination, that showed the total elimination of platelets, using serum tubes for blood collection (compare Fig. 2a and 2b).

Figure 3 shows each of the mtDNA:nDNA-ratios obtained with the investigated methods of every single patient in G3. The different mt DNA content values were set in relation to the clinic of the HIV-infected patient under HAART. This reflects the applicability of each method to identify patients with HAART-associated symptoms, e.g. high lactate levels ($>2\mu\text{mol/ml}$) or lipodystrophy. As it is plot in the diagram, collection of blood into EDTA tubes, followed by the common method of PBMC isolation is sufficient to detect mt DNA depletion in most cases, but in contrast to our recommended method of blood collection in serum tubes, the correlation of HAART-associated symptoms is less unerring (Fig. 3, compare patient 1, 2, 4, 6, 8 and 9 to 3,5 and 7).

Studies with high patient numbers are needed to establish the critical range of low mtDNA:nDNA-ratios

correlating with HAART-associated symptoms and the best cut off mtDNA:nDNA-ratio value. At this time in our study it is only possible to set an area of critically low mtDNA:nDNA-ratios arbitrarily, correlating to HAART-associated signs and symptoms in our patients. Nevertheless, according to our results, we believe the collection of blood in serum tubes and measurement of platelet-free PBMCs for mt content is a promising tool for predictive ascertainment of HAART-associated symptoms in patients.

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