Quantitative Analysis of Circulating Mitochondrial DNA in Plasma

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Background: Recent studies have demonstrated the existence of circulating mitochondrial DNA in plasma and serum, but the concentrations and physical characteristics of circulating mitochondrial DNA are unknown. The aim of this study was to develop an assay to quantify mitochondrial DNA in the plasma of healthy individuals.

Methods: We adopted a real-time quantitative PCR approach and evaluated the specificity of the assay for detecting mitochondrial DNA with a cell line devoid of mitochondria. The concentrations and physical characteristics of circulating mitochondrial DNA were investigated by experiments conducted in three modules. In module 1, we evaluated the concentrations of mitochondrial DNA in plasma aliquots derived from four blood-processing protocols. In module 2, we investigated the existence of both particle-associated and free forms of mitochondrial DNA in plasma by subjecting plasma to filtration and ultracentrifugation. In module 3, we used filters with different pore sizes to investigate the size characteristics of the particle-associated fraction of circulating mitochondrial DNA.

Results: The mitochondrial DNA-specific, real-time quantitative PCR had a dynamic range of five orders of magnitude and a sensitivity that enabled detection of one copy of mitochondrial DNA in plasma. In module 1, we found significant differences in the amounts of circulating mitochondrial DNA among plasma aliquots processed by different methods. Data from module 2 revealed that a significant fraction of mitochondrial DNA in plasma was filterable or pelletable by ultracentrifugation. Module 3 demonstrated that filters with different pore sizes removed mitochondrial DNA from plasma to different degrees.

Conclusions: Both particle-associated and free mitochondrial DNA are present in plasma, and their respective concentrations are affected by the process used to harvest plasma from whole blood. These results may have implications in the design of future studies on circulating mitochondrial DNA measured in different disease conditions.

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The discovery of circulating nucleic acids in plasma and serum has opened up the possibility of noninvasive diagnosis and monitoring of a wide variety of diseases and conditions (1). Tumor-, fetus-, and donor-derived DNA sequences have been detected in the plasma and serum of cancer patients (2), pregnant women (3), and transplant recipients (4), respectively. Moreover, the detection of tumor- and fetus-associated circulating RNA (6) has provided means for noninvasive gene expression profiling. In addition to the body of work conducted on circulating RNA and DNA of the nuclear genome, there have been several recent studies demonstrating the existence of another species of circulating nucleic acids, i.e., mitochondrial DNA. This was first reported in a study demonstrating the presence of a known mitochondrial DNA mutation in the plasma and serum of patients with type 2 diabetes mellitus (7).

The mitochondrial genome is a circular piece of DNA 16.5 kb in length (8). Up to several thousand copies of this genome can be found in a mammalian cell (9–11). Mutations in the mitochondrial genome are associated with various diseases (8). Maternal inheritance of mutations of mitochondrial DNA is known to cause certain disorders (12). There is also an increasing amount of evidence supporting the association of somatic mitochondrial DNA mutations with aging, degenerative diseases (13), and malignancies (14). Mitochondrial DNA mutations have been identified in tumors of the breast, colon, liver, head and neck, lung, and other tissues (15). Prompted by the promise shown by the use of circulating DNA as a marker

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for noninvasive cancer assessment, several groups have recently investigated and demonstrated the presence of tumor-specific mitochondrial DNA mutations in the plasma and serum of cancer patients (16–19).

To date, most investigations of circulating mitochondrial DNA have been performed in a qualitative manner. The high mitochondrial DNA copy number per cell has been suggested to provide a quantitative advantage for its detection (20, 21), but data on the concentration of mitochondrial DNA in plasma are lacking. Our previous experience on the quantitative analysis of maternal plasma DNA (22) and circulating RNA (23) prompted us to test whether circulating mitochondrial DNA may exist in both free and particle-associated forms and whether its quantification would be affected by the method by which plasma is derived from whole blood. The aim of this study is to investigate the concentrations and physical characteristics of circulating mitochondrial DNA.

**Materials and Methods**

**DEVELOPMENT OF MITOCOHRANDNA-SPECIFIC QUANTITATIVE PCR ASSAY**

**Real-time quantitative PCR.** An assay based on a real-time quantitative PCR format was developed for the quantification of mitochondrial DNA in plasma. The theoretical aspects of real-time quantitative PCR have been described previously (24). Briefly, the PCR process is monitored through the increase in fluorescent signal produced by the enzymatic cleavage of a fluorescent probe. The template DNA concentration is estimated by the number of PCR cycles required for the fluorescent output to reach a defined threshold compared with a series of calibrators with known concentrations.

The primer sequences, Mit 3130F (5′-AGG-ACA-AGA-GAA-ATA-AGG-CC-3′) and Mit 3301R (5′-TAA-GAA-GAG-GAA-TTGT-AAC-CTC-TGA-CTG-TAA-3′), were reported previously by Parfait et al. (25). The TaqMan probe sequence, Mit 3153T (5′-FAM-TTC-ACA-AAG-CGC-CTT-CCC-CCG-TAA-ATG-A-TAMRA-3′), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine) was designed using Primer Express software, Ver. 2.0 (Applied Biosystems). All components of the PCR other than the primers and probes were supplied in the TaqMan PCR Core Reagent Kit (Applied Biosystems). PCR was set up in a reaction volume of 50 μL according to the manufacturer’s instructions. We added 5 μL of plasma or 250 ng of cell line DNA to each reaction mixture, which consisted of 5 μL of 10× buffer A; 300 mM each primer; 50 nM TaqMan probe; 4 mM MgCl₂; 200 nM each of dATP, dCTP, and dGTP; 400 nM dUTP; 1.25 U of AmpliTaq Gold; and 0.5 U of AmpErase uracil N-glyco-sylase. The following thermal profile was used: incubation for 2 min at 50 °C, followed by a first denaturation step of 10 min at 95 °C and 45 cycles of 95 °C for 15 s and 55 °C for 1 min. Real-time quantitative PCR was carried out in an Applied Biosystems 7700 Sequence Detector.

**Assay calibration.** A 172-bp mitochondrial DNA segment between nucleotide positions 3130 and 3301 (GenBank accession no. J01415) was amplified from buffy coat DNA with the primers and conditions described above. The PCR amplicons were cloned into a plasmid vector with use of the TOPO® TA cloning reagent set (Invitrogen) according to the manufacturer’s instructions. The identity of the cloned insert was confirmed by direct sequencing. One confirmed clone was selected, and DNA was extracted and quantified by absorbance measurements and used as a calibrator. The mitochondrial DNA copy number of this calibrator was determined by division of the total DNA concentration by the weight of each plasmid molecule. The plasmid vector was 5300 bp in length; thus, the cloned vector contained 5472 bp. The weight of a cloned plasmid molecule was estimated as follows: (5472 bp × MWt)/A, where MWt denotes the molecular weight of double-stranded DNA (6.6 × 10⁹ g/mole), and A denotes Avogadro’s number (6.02 × 10²³ molecules/mole). Calibrators were prepared by serial dilution of the stock solution and contained 1–100 000 mitochondrial DNA copies/5 μL. During subsequent real-time PCR analysis, a calibration curve was constructed with these calibrators.

**Assay specificity.** The specificity of the quantitative assay for mitochondrial DNA amplification was assessed through the use of a cell line devoid of mitochondria (ρ⁰). The cell line was isolated according to the method described by King and Attardi (26). Ethidium bromide is an inhibitor of mitochondrial DNA replication. A cell line, such as 143B (American Type Culture Collection), that is rich in mitochondria when grown in the presence of low concentrations of ethidium bromide would demonstrate a reduction in mitochondrial number on subculturing. The ρ⁰ cell line was obtained when the subculturing continued until the cells were completely devoid of mitochondria. The mitochondria-negative cells were maintained by supplementation with glucose, pyrimidines, and pyruvate. The mitochondrial specificity of the real-time PCR assay was determined based on whether positive amplification was obtained from the ρ⁰ cell line. The specificity of the assay could also be inferred by demonstration of the expected quantitative difference between the mitochondrial and nuclear DNA concentrations in the 143B cell line before subculturing. A previously described quantitative PCR assay for the β-globin gene (27) was used to estimate the nuclear DNA concentration. DNA was extracted according to the “blood and body fluid protocol” of the QIAamp blood reagent set (Qiagen).

**PLASMA SAMPLE COLLECTION AND DNA EXTRACTION**

Blood samples were collected from healthy volunteers with informed consent into EDTA-containing tubes. The samples were processed within 4 h of blood collection, and plasma was obtained by various means depending on the module of investigation. DNA was extracted from
plasma according to the “blood and body fluid protocol” of the QIAamp blood reagent set (Qiagen). We applied 800 μL of plasma to each column, and the DNA was eluted with 50 μL of deionized water.

**Module 1: Effects of different blood-processing protocols.** We collected 30 mL of blood from each of nine volunteers. Plasma was first prepared by centrifugation at 1600 g for 10 min (Megafuge 1.0R; Heraeus Instruments) and was removed with care to avoid disturbing the underlyinguffy coat layer. The plasma from each individual was then divided into four aliquots, which received either no additional treatment or one of the followings treatments: (a) filtration through a 0.22 μm filter; (b) an additional centrifugation step at 16 000 g for 10 min (Eppendorf Centrifuge 5415D); or (c) centrifugation at 16 000 g for 10 min, followed by filtration through a 0.22 μm filter. The aim of the filtration step using 0.22 μm filters was to remove any residual cells that might persist after centrifugation.

**Module 2: Physical nature of circulating mitochondrial DNA.** To investigate whether plasma contains both free and particle-associated fractions of circulating mitochondrial DNA, we collected 20 mL of blood from each of seven volunteers. Plasma was prepared by centrifugation at 1600 g for 10 min followed by a second centrifugation step at 16 000 g for 10 min. The plasma from each individual was then divided into three aliquots: the first aliquot was filtered through a 0.22 μm filter; the second aliquot was subjected to ultracentrifugation (Optima™, TLX Ultra-centrifuge; Beckman Coulter) at 99 960 g for 2 h; and the third aliquot received no additional treatment.

**Module 3: Size characteristics of the particle-associated fraction of circulating mitochondrial DNA.** We collected 30 mL of blood from each of 14 volunteers and separated the plasma by centrifugation at 1600 g for 10 min followed by an additional 10 min at 16 000 g. The plasma collected from each individual was divided into four aliquots. One aliquot was not subjected to any additional treatments, whereas the remaining three aliquots were filtered through filters with pore sizes of 0.22, 0.45, and 5 μm, respectively.

**Assessment of the characteristics of circulating mitochondrial DNA**
The mitochondrial and β-globin DNA concentrations in all of the plasma aliquots were determined by quantitative PCR. The difference in the mitochondrial DNA concentration among the treatment groups was determined statistically. The physical characteristics of the mitochondrial genome were compared with those of the nuclear genome by comparing the mitochondrial DNA concentration and the corresponding β-globin DNA concentration of the plasma aliquot.

Mitochondrial DNA concentrations in all of the plasma analyses were expressed in copies per milliliter of plasma based on the following calculation:

\[
c = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}
\]

Where \(c\) is the concentration of mitochondrial DNA in plasma (copies/mL); \(Q\) is the quantity (copies) of mitochondrial DNA determined by the sequence detector in a PCR; \(V_{DNA}\) is the total volume of plasma DNA obtained after extraction, typically 50 μL per extraction; \(V_{PCR}\) is the volume of plasma DNA solution used for PCR, typically 5 μL; and \(V_{ext}\) is the volume of plasma extracted, typically 800 μL.

**Statistical analysis**
All statistical analyses were performed using Sigma Stat, Ver. 2.03 (SPSS).

**Results**

**Mitochondrial DNA-specific real-time quantitative PCR**
The linearity of the quantitative assay was assessed with use of the cloned plasmid DNA, which was serially diluted to prepare a series of calibrators with known concentrations. The assay was linear over five orders of magnitude, and its sensitivity allowed the detection of one copy of mitochondrial DNA. A typical calibration curve is displayed in Fig. 1. To assess the specificity of the assay for detecting mitochondrial DNA, we tested successive generations of the 143B cell line. The mitochondrial specificity of the assay was demonstrated by the decreasing mitochondrial DNA concentration over the initial five generations. There was no amplification from the sixth generation, when the cell line was devoid of mitochondria. Fig. 2 shows the mitochondrial DNA concentrations

![Fig. 1. Calibration curve for quantitative analysis of mitochondrial DNA.](image)

The threshold cycle (CT) is the number of PCR cycles required for the fluorescent intensity of the reaction to reach a predefined threshold. The threshold cycle is inversely proportional to the logarithm of the starting concentration of mitochondrial DNA.
of the 143B cell line and the subsequent 12 generations. Before subculturing, the mitochondrial DNA concentration (mean, 30 204 830 copies/250 ng) of the 143B cell line was markedly higher than the β-globin DNA concentration (mean, 5411 copies/250 ng).

**CHARACTERISTICS OF CIRCULATING MITOCHONDRIAL DNA**

**Module 1: Effects of different blood-processing protocols.** The mitochondrial DNA concentrations obtained in plasma derived from the four different treatments are shown in Fig. 3A. The mitochondrial DNA concentration in plasma obtained by a single centrifugation step at 1600g had the strongest signal. The quantitative relationship between the remaining three treatments is further illustrated in Fig. 3B. The mitochondrial DNA concentrations obtained with the different treatments were assessed by the Friedman (repeated-measures ANOVA) test, and there was a statistically significant difference among the corresponding DNA concentrations in the plasma processed by the four respective treatments ($P < 0.001$). The Student–Newman–Keuls test, a pairwise multiple comparison test, was subsequently used to identify pairs of treatments that produced significantly different mitochondrial DNA concentrations. The mitochondrial DNA concentrations in all but one of the paired relationships were significantly different ($P < 0.05$). A significant difference was absent only between the DNA concentrations obtained by the two treatments that involved filtration, one of which was preceded by one-step and the other by two-step centrifugation. The corresponding β-globin DNA concentrations are shown in Fig. 3C. Statistical analysis by the Friedman test revealed no significant difference in the β-globin DNA concentrations among the different groups ($P > 0.05$).

**Module 2: Cell-free or particle-associated nature of circulating mitochondrial DNA.** The relationship between the mitochondrial DNA and β-globin DNA concentrations in plasma obtained by two-step centrifugation, with or without additional filtration (0.22 μm filter) or ultracentrifugation, is illustrated in Fig. 4 (panels A and B, respectively). The DNA concentrations obtained as a result of the three different treatments were compared statistically by the Friedman test. Among the three groups, there was no significant difference for β-globin DNA ($P > 0.05$), but the mitochondrial DNA concentrations were significantly different ($P < 0.001$). The results of the Student–Newman–Keuls test also indicated that there was no significant difference between the filtered and the ultracentrifuged groups but that each of them was significantly different from the group that did not undergo additional filtration or ultracentrifugation.

**Module 3: Size characteristics of the particle-associated fraction of circulating mitochondrial DNA.** The quantitative relationship between the differentially filtered and unfiltered plasma mitochondrial DNA and β-globin DNA concentrations is shown in Fig. 5 (panels A and B, respectively). The Friedman test revealed significant differences in mitochondrial DNA concentrations among the four treatment groups ($P < 0.001$), but not in β-globin DNA ($P > 0.05$). Significance between paired groups was identified by the Student–Newman–Keuls test. The difference was significant ($P < 0.05$) for all but one of the paired combinations. There was no significant difference between the unfiltered plasma and plasma filtered through a 5 μm filter.

**Discussion**

It has long been known that ancient mitochondrial DNA sequences, also termed nuclear pseudogenes, are present in the nuclear genome. The existence of these nuclear pseudogenes may lead to their inadvertent amplification and, thus, the misinterpretation of molecular analyses intended for the study of the mitochondrial genome (28). Recently, Woischnik and Moraes (29) comprehensively searched the human genome database for the number and extent of insertions of mitochondrial sequences into the nuclear genome. Their analysis revealed the presence of 612 nuclear integrations with up to 99% homology with the current mitochondrial DNA. Consequently, in this study, we exercised caution when designing the quantitative assay for detecting circulating mitochondrial DNA. The mitochondrial specificity of the assay was confirmed by the lack of PCR amplification in the ρ0 cell line (Fig. 2) (25). The 5500-fold difference between the mitochondrial DNA and β-globin DNA concentrations of the 143B cell line before subculturing was also a supportive piece of evidence, as this cell line is known to contain up to ~9000 copies of the mitochondrial genome (26). In contrast to previous studies (30, 31) that expressed the mitochondrial DNA concentration as a ratio to nuclear DNA concentrations, we used absolute quantification in this study. The
rationale for this approach was to study cell-free mitochondrial DNA in plasma, as opposed to the previous reports that studied the mitochondrial content of tissues or blood leukocytes.

We have previously studied the factors that affect the quantification of DNA and its physical characteristics in maternal plasma (22) and circulating RNA (23). We noted that different blood-processing protocols would significantly affect the quantification of plasma nucleic acids when the “plasma” fractions contained a heterogeneous population of circulating nucleic acids. The rationale for these observations relates to the presence of both particle-associated and non-particle-associated circulating nucleic acids in plasma. Different centrifugation protocols are effective to various degrees in the removal of particles from the supernatant. Hence, the resulting concentrations of the circulating nucleic acids would vary depending on the protocol used for separation of plasma from whole blood. On the other hand, the quantification of plasma nucleic acids that are predominantly in the free form, for example, fetal DNA in maternal plasma (22), is not affected by variations in the blood-processing protocol.

Consequently, in this study, we investigated the physical characteristics of circulating mitochondrial DNA by the same approach. In module I, we investigated whether quantification of mitochondrial DNA in the plasma of healthy individuals would be affected by the use of different centrifugation and filtration protocols. A significant difference indeed existed. The mitochondrial DNA concentration in the plasma aliquots processed by a single centrifugation step at 1600g was the highest. The most probable explanation is contamination by residual cells that were not effectively removed by the one-step centrifugation but were adequately removed by filtration (see the data for the plasma treated by 1600g centrifugation and filtration in Fig. 3A). Cells may contain up to thousands of copies of the mitochondrial genome (32); hence, the presence of residual cells in plasma would markedly increase the mitochondrial DNA concentration.

On the other hand, a significant difference also existed

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**Fig. 3.** Box plots depicting the effects of four different blood-processing protocols. The upper and lower limits of the boxes and the lines across the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively. ○ indicate outliers. (Statistics are presented in the same format for all of the box plots in this report.) 1600g denotes centrifugation at 1600g; 16000g denotes centrifugation at 16 000g; filtration denotes filtration through a 0.22 μm filter. (A), mitochondrial DNA concentration in plasma obtained by four different processing protocols. (B), mitochondrial DNA concentration of the latter three groups shown in A. (C), β-globin DNA concentration in plasma obtained by four different processing protocols.
among the plasma aliquots processed by the remaining three treatment protocols. Even after blood was processed by two centrifugation steps, the resulting plasma mitochondrial DNA concentration remained significantly higher than the concentrations in the two groups of aliquots that were filtered. Our previous data demonstrated that two centrifugation steps would be effective in producing a supernatant that approximates cell-free plasma (22). The current data therefore suggest the presence of particle-associated mitochondrial DNA in cell-free plasma that was removed more efficiently by filtration. In contrast, nuclear DNA analysis was not affected by the three different treatments. To confirm the presence of both free and particle-associated circulating mitochondrial DNA, we proceeded to module 2.

In module 2, the mitochondrial DNA concentration in plasma prepared by two centrifugation steps was compared with that in plasma processed with additional filtration or ultracentrifugation. Filters with the smallest pore size (0.22 μm) were selected with the aim to concentrate, and therefore to allow the estimation of, mitochondrial DNA in the free form. Ultracentrifugation was performed with the aim to demonstrate that a fraction of the circulating mitochondrial DNA is pelletable. Compared with plasma with no additional treatment, the analysis revealed a significant reduction in the mitochondrial DNA concentration in both the filtered and ultracentrifuged groups. The data support the existence of a significant fraction of particles that contain mitochondrial DNA and are filterable or pelletable by ultracentrifugation.

Fig. 4. Box plots illustrating the effects of filtration and ultracentrifugation on the quantification of mitochondrial DNA (A) and β-globin DNA (B) in plasma.

All of the plasma samples were derived from whole blood by centrifugation at 1600g followed by a second centrifugation step at 16 000g. Untreated denotes the aliquots that received no additional treatment; Filtration denotes plasma aliquots that were processed with additional filtration through a 0.22 μm filter; Ultracentrifugation denotes plasma aliquots that were processed with additional centrifugation in an ultracentrifuge at 99 960g.

Fig. 5. Size characteristics of the particle-associated circulating mitochondrial DNA.

All of the plasma samples were derived from whole blood by centrifugation at 1600g followed by a second centrifugation at 16 000g. NF denotes aliquots that received no additional treatment; 0.22, 0.45, and 5 denote plasma aliquots that were additionally subjected to filtration through filters with a pore size of 0.22, 0.45, and 5 μm, respectively. (A), box plots illustrating the mitochondrial DNA concentration in plasma aliquots derived by the four different treatments. (B), box plots illustrating the β-globin DNA concentration in plasma aliquots derived by the four different treatments.
tion. Despite the process of filtration or ultracentrifugation, a significant amount of mitochondrial DNA was still detectable. These data suggest the coexistence of non-particle-associated and particle-associated mitochondrial DNA in plasma. On the other hand, nuclear DNA was neither filterable nor pelletable by ultracentrifugation; it therefore could be inferred that nuclear DNA circulates predominantly in a free form. This finding confirms our previous results (23).

After confirming the presence of particle-associated mitochondrial DNA in plasma, we studied the size characteristics of these particles. In module 3, we compared the amount of mitochondrial DNA in the particle-containing plasma (prepared by two centrifugation steps) with that in plasma that had undergone additional filtration through filters with different pore sizes: 0.22, 0.45, and 5 \( \mu \)m. Filtration through a 5 \( \mu \)m filter did not produce a significant difference, but filtration through 0.45 or 0.22 \( \mu \)m filters produced a significant decrease in the concentration of mitochondrial DNA in plasma. The data from these two groups were significantly different from the data for the two other groups and also differed between themselves. These data suggest that the mitochondrial DNA-containing particles in plasma exist in different sizes and are possibly of different natures. The present data, however, do not reveal the identity of these particles. Plausible candidates include mitochondria [diameters range from 0.5 to 1 \( \mu \)m, and length from 5 to 10 \( \mu \)m (33)], platelets [each of which contains an average of four copies of the mitochondrial genome (32)], and cellular fragments. In fact, it is our observation (unpublished) that a close correlation exists between the mitochondrial DNA concentration and platelet count in the differentially processed plasma samples. In contrast, the absence of a significant difference in the corresponding concentrations of \( \beta \)-globin gene confirms the lack of "particularity" of circulating nuclear DNA as evidenced by our earlier data.

This study confirms the existence of both particle-associated and non-particle-associated forms of mitochondrial DNA in plasma. Because of their physical differences, in terms of size and density, the proportions of these different forms of circulating mitochondrial DNA would differ when plasma samples are prepared by different protocols. Hence, the mitochondrial DNA concentration in plasma would differ accordingly. At present, the biological significance of the particle-associated or free forms of circulating mitochondrial DNA is unknown. It is also unclear which is the diagnostically or prognostically significant fraction in relation to the study of diseases. For example, as reported recently, tumor-specific circulating mitochondrial DNA is detectable in the circulation of cancer patients (16–19), but it is unknown whether tumors release predominantly particle-associated or free mitochondrial DNA into the circulation. In fact, the relative amounts of the different forms of circulating mitochondrial DNA may vary in different pathologic situations. It has been postulated that the release of circulating nucleic acids is related to cell death, either by apoptosis or necrosis (34). One could further postulate that differing mechanisms of cell death may lead to the release of various forms of circulating mitochondrial DNA.

The implications of the findings of this study include the need to carefully evaluate the protocol that one would adopt for the separation of plasma from whole blood. Different protocols would increase or decrease the fractional concentrations of different forms of circulating mitochondrial DNA and would therefore significantly alter the sensitivity of detecting the disease-related form of circulating mitochondrial DNA. The blood-processing protocol may improve or, on the contrary, be detrimental to the detection of the form of circulating mitochondrial DNA of diagnostic or prognostic interest. It is only when the biological significance of these different forms of circulating mitochondrial DNA is known that recommendations can be made to standardize the procedures used (35). These efforts may enhance the cross-comparability of observations among studies. Furthermore, it is interesting to note the similarities between circulating RNA and mitochondrial DNA (23). Additional studies are needed to characterize the particle-associated fractions of circulating nucleic acids and to determine their origin.

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