Available online at www.sciencedirect.com
SCIENCE DIRECT®
Free Radical Biology & Medicine 38 (2005) 678–683
www.elsevier.com/locate/freeradbiomed

Original Contribution

# Degree of heteroplasmy reflects oxidant damage in a large family with the mitochondrial DNA A8344G mutation

Jeffrey A. Canter[a,*], Alex Eshaghian[b], Joshua Fessel[b], Marshall L. Summar[a], L. Jackson Roberts[b], Jason D. Morrow[b], James E. Sligh[b], Jonathan L. Haines[a]

[a]*Center for Human Genetics Research, Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, 519 Light Hall, Nashville, TN 37232-0700, USA*
[b]*Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232-0700, USA*

Received 30 July 2004; revised 12 November 2004; accepted 12 November 2004
Available online 18 December 2004

**Abstract**

Mitochondria are the source of most oxygen-derived free radicals. Mutations in mitochondrial DNA can impair mitochondrial electron transport resulting in decreased ATP production and increased free radical-induced oxidant injury. The specific mitochondrial DNA mutation A8344G alters the TΨC loop or the mitochondrial tRNA for lysine. We investigated a large five-generational family harboring this mutation to determine whether the degree of heteroplasmy (proportion of mutated mitochondrial genomes) for the mtA8344G mutation correlated with a marker of oxidant damage. We measured $F_2$-isoprostanes because they are specific and reliable markers of oxidant injury formed when free radicals attack esterified arachidonate in cell membranes. Family members with high heteroplasmy (>40%) had significantly higher $F_2$-isoprostane levels (62 ± 39 pg/ml) than those with lower heteroplasmy (33 ± 13 pg/ml, $P < 0.001$). The degree of heteroplasmy for the mtA8344G mutation in this family correlated positively with $F_2$-isoprostane levels ($P = 0.03$). This study highlights the underappreciated role free radicals play in the complex pathophysiology of inherited mitochondrial DNA disorders. The most important novel finding from this family is that some currently asymptomatic individuals with moderate heteroplasmy have evidence of ongoing free-radical mediated oxidant injury.
© 2004 Elsevier Inc. All rights reserved.

*Keywords:* Heteroplasmy; Oxidant injury; $F_2$-isoprostanes; Mitochondrial DNA mutation; mtA8344G

## Introduction

Mitochondrial DNA (mtDNA) encodes for a distinct set of ribosomal RNAs and tRNAs as well as for 13 subunits of the electron transport chain [1]. We report a family that harbors an A–G base transition at nucleotide position 8344 in the mtDNA that alters the TΨC loop of the mitochondrial tRNA for lysine (MTTK gene) [2]. Specific deficiencies in muscle energetics and mitochondrial respiratory complexes I and IV were characterized in a family with maternally inherited myoclonic epilepsy and ragged red fibers (MERRF) even before this phenotype's association with this specific mutation [3]. Since the initial reports of the classic MERRF phenotype, other less clearly defined phenotypes have been associated with the mtA8344G mutation [4–6]. The index case of this family had years of medical evaluations before his progressive muscle wasting and unsteadiness were explained by his high heteroplasmy (proportion of mutated mitochondrial genomes) for the mtA8344G mutation. Mutations in mtDNA are transmitted almost exclusively through the maternal line. Upon learning that the index case's maternal grandmother was one of six sisters, we realized that further investigation of this large family could test the hypothesis that oxidant damage correlates with the degree of heteroplasmy for the mtA8344G mutation.

Under normal physiological conditions, as many as 2% of electrons leak from the mitochondrial electron transport chain and reduce oxygen to superoxide anion [7–9]. Thus, mitochondria are a major source of oxygen-derived free

*Abbreviations:* MERRF, inherited myoclonic epilepsy and ragged red fibers.

* Corresponding author. Fax: +1 615 343 8619.
*E-mail address:* jeff.canter@vanderbilt.edu (J.A. Canter).

0891-5849/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2004.11.031

radicals in the normal physiologic state. When electron transport is impaired, more electrons leak and form superoxide radicals, triggering a cascade of free radicals that indiscriminately damage biological macromolecules [10–12]. Oxidant damage has been implicated in a host of degenerative diseases and is increased in cigarette smokers [13,14]. In this family with a mutation altering the mitochondrial electron transport chain, $F_2$-isoprostanes were measured to determine the extent of oxidant damage. $F_2$-isoprostanes are a specific group of prostaglandin $F_2$-like compounds formed when free radicals attack esterified arachidonate in cell membranes [15]. Measurement of these specific products of lipid peroxidation is emerging as one of the most reliable indices of in vivo oxidant injury [16,17]. Increasing heteroplasmy for the mtA8344G mutation in members of this large, five-generational family correlated positively with plasma $F_2$-isoprostane levels and therefore with oxidant injury.

## Materials and methods

### *Ascertainment/phenotyping*

All participating family members provided informed consent prior to enrollment in this study. This study was limited to adults over the age of 18. Demographic data, including age, sex, height, and weight, were collected. Each participant supplied a medical history including a list of ongoing medical problems, current medications, prior hospitalizations, and past surgeries. A specific symptom survey inquired about muscle weakness, unsteadiness, spontaneous muscle contractions, hearing loss, visual difficulties, seizures, and diabetes. Smoking status, a known potential confounder of $F_2$-isoprostane levels, was reported [13].

### *Genotyping*

Genomic DNA was isolated from 3 ml of whole blood and from buccal epithelium using the Wizard kit (Promega; Madison,WI). PCR in the region of the MTTK gene was carried out using the forward primer CTACCCCCTCT-AGAGCCCAC and the reverse primer GTAGTATT-TAGTTGGGGCATTTCACTGTAAAGCCGTGTTGG as described by Zevani et al. [18]. The reverse primer creates a new *Bgl*I site in the presence of a PCR-amplified A8344G mutation. Products of *Bgl*I (New England Biolabs, Beverly MA) digestion were resolved on 3% agarose gel. The intensities of the wild-type (108 bp) and the mutant (73 bp) were measured using a Hitachi FMBioII (Tokyo, Japan) gel scanner. The raw intensity value for each band was divided by the size of the fragment to obtain a corrected value. Percentage heteroplasmy is presented as the corrected mutant value divided by the sum of the corrected wild-type and mutant values.

### *Plasma sample acquisition/mass spectroscopic analysis of $F_2$-isoprostanes*

Individual blood specimens were centrifuged promptly for 10 min at 14,000 rpm. The separated plasma was then immediately frozen on dry ice to prevent auto-oxidation. No specimens were shipped. All specimens were stored at $-80°C$. No antioxidants were added to the plasma samples prior to storage. Specimens were stored no more than 2 weeks before $F_2$-isoprostane determination. Plasma $F_2$-isoprostanes do not vary diurnally or with meals, so plasma specimens were obtained throughout the day depending on the availability of the subjects. A highly accurate gas chromatographic/negative-ion chemical ionization mass spectroscopic assay was utilized to measure $F_2$-isoprostanes [19].

### *Statistical analysis*

Means of continuous variables measured in two groups were compared using Student's *t* test taking into account unequal variances when necessary. The Pearson product-moment correlation coefficient was used to measure the strength of association between two continuous variables. The level of significance was set at 0.05. All statistical analyses were done utilizing STATA 8.0 (College Station, TX).

## Results

### *Ascertainment/demographics*

Twenty-five of 32 (78%) of adults in this family provided DNA samples for analysis. Of these adults, 23 (92%) had $F_2$-isoprostane levels measured.

Fig. 1 presents the five-generational family pedigree. The mean age of the adult family members was 48 years (range 22–80). Forty-four percent were male. Six (24%) were cigarette smokers. Ten members of Generation III (50%) are deceased. The median age at the time of death was 40 years (range 4 to 71).

### *Symptom survey/selected case reports*

No family members, other than the index case, were previously diagnosed with a mitochondrial disorder. No family member was known to have both myoclonic epilepsy and progressive muscle wasting as would be expected with the classic MERRF phenotype. One deceased member of Generation III (III-7) had a progressive muscle disorder and died at age 54. This person's sister (III-9) had 15% heteroplasmy for mtA8344G detected and transmitted the mutation to all three of her sons (IV-8, 9, 10). Another deceased member of Generation III (III-17) developed seizures at age 31. Systematic survey of family members for symptoms that are related to mitochondrial disorders

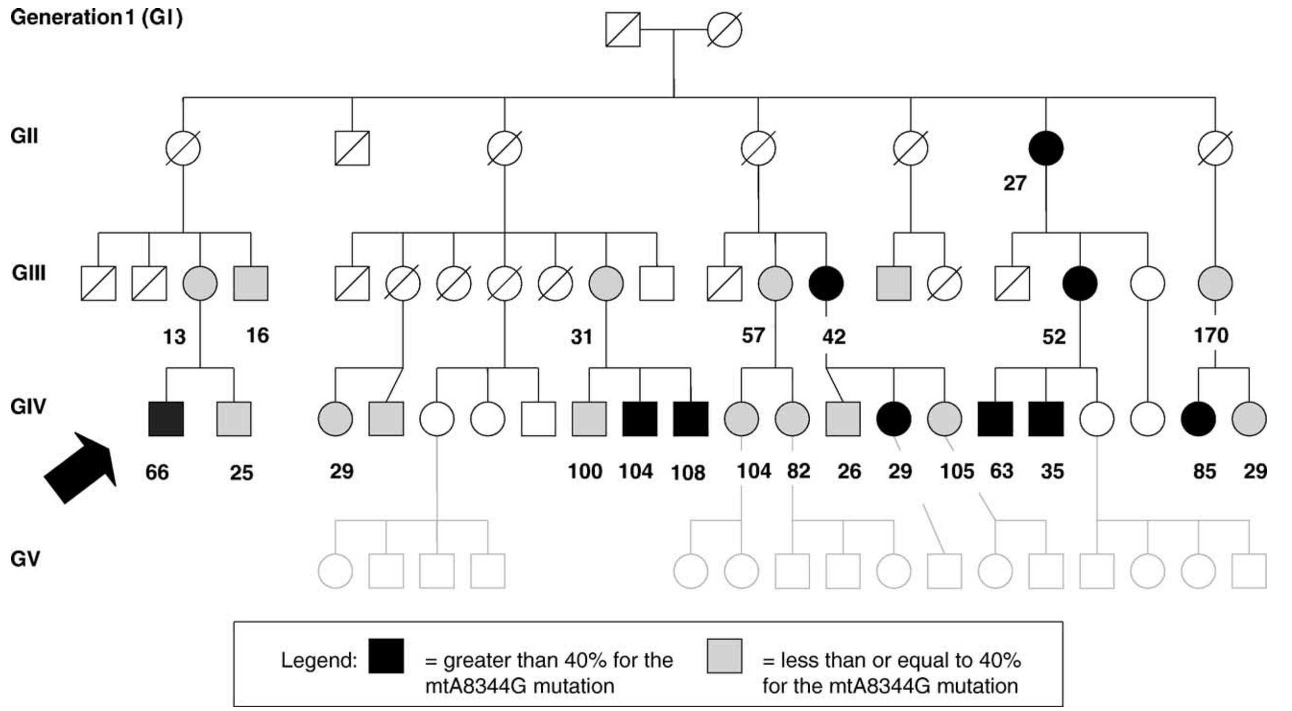


Fig. 1. Pedigree of this family with the mtA8344G mutation highlighting maternal transmission. The legend above shows the symbols used to represent degree of heteroplasmy for this mutation among family members. The $F_2$-isoprostane levels in units of pg/ml for each family member are presented below and slightly to the left of their pedigree symbol. The normal level of $F_2$-isoprostanes is reported as 35 ± 6 pg/ml. All $F_2$-isoprostane values greater than 100 occurred in chronic cigarette smokers, a known confounder of this measure of oxidant injury. The index case has an arrow pointing to his symbol. Children of male family members are not represented in this pedigree. Members of the most recent generation (GV) are all less than 18 years of age and were not ascertained as part of this study. Their symbols are open and light gray in color.

revealed that 7(28%) experienced spontaneous muscle contractions, 6(24%) reported exercise intolerance, 6(24%) reported unsteadiness, and 5 (20%) reported hearing loss. The index case was the only family member taking supplemental vitamin E (800 IU/day).

*Case report 1: index case (IV-1)*

At age 50, the index case noted muscle weakness and increasing fatigue. Over the following 3 years, he had progressive neurologic decline with the development of unsteadiness, profound muscle weakness, spontaneous muscle contractions, and a resting tremor. A brain MRI scan revealed olivopontocerebellar degeneration. Muscle biopsy revealed significant denervation atrophy, but no ragged red fibers. Genotyping utilizing allele-specific oligonucleotide probes revealed that 95% of mitochondrial DNA from the muscle biopsy had the mtA8344G mutation. The patient reported years of occasional myoclonus and limited exercise ability prior to the onset of his illness.

*Case report 2: 39-year-old cigarette smoker with moderate heteroplasmy (IV-10)*

This individual has had numerous medical problems since youth. He has had seizures in the past. He has unsteadiness, spontaneous muscle contractions, and muscle weakness. He had a fatty tumor excised from his neck. He has blood heteroplasmy of 31% and buccal heteroplasmy of 57%. His $F_2$-isoprostane level was 108 pg/ml. (For smokers the range is 85 ± 32 pg/ml [14]).

*Case report 3: asymptomatic young man with moderate heteroplasmy and elevated $F_2$-isoprostane levels (IV-16)*

At age 22, this young man has been very healthy and active. Recently, he has developed hypertension. His degree of heteroplasmy for mtA8344G was 62% in a buccal specimen and 46% in blood. His $F_2$-isoprostane level is 63 pg/ml. His maternal uncle (III-18) developed seizures at the age of 31 and died years later from complications related to a seizure-related fall.

*DNA mutation analysis*

The mtA8344G mutation was detected in either the blood or the buccal specimen of 76% (19/25) family members. The index case had the highest degree of heteroplasmy measured (70% in blood and 69% in buccal specimen). The index case's mother (III-3) had 6% heteroplasmy for mtA8344G detected only on her buccal specimen. Two separate blood specimens from her were negative for the mutation.

*Oxidant damage measurement*

The mean $F_2$-isoprostane level in this family was 55 ± 38 pg/ml. This is significantly higher than published

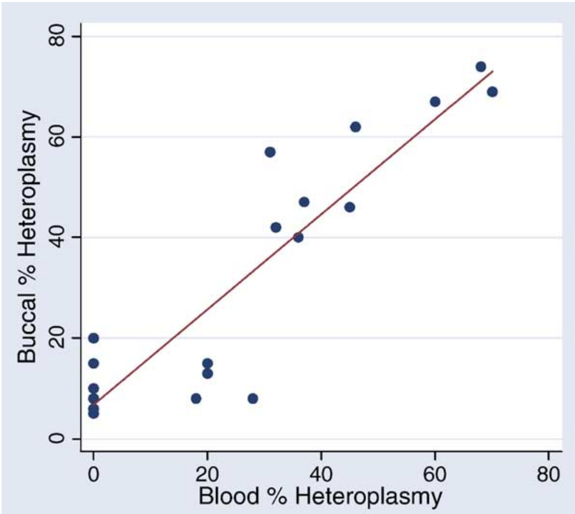


Fig. 2. Scatterplot of blood %heteroplasmy versus buccal %heteroplasmy for the mtA8344G mutation among family members. This scatterplot demonstrates the high degree of correlation between heteroplasmy for the mtA8344G mutation in blood and buccal specimens. The correlation coefficient for this relationship was $r$ = 0.91 ($P$ value < 0.01). While correlated, these values differ somewhat because of tissue heteroplasmy. This variation in the mitochondrial population of different tissues reflects differences in heteroplasmy in progenitor cells as well as local environmental factors effecting mitochondrial proliferation.

normal values, 35 ± 6 pg/ml, using the same assay ($P <$ 0.001) [12]. As Fig. 2 demonstrates, there is a separation in the distribution of degree of buccal heteroplasmy in this family at 40%. Family members with >40% heteroplasmy in mitochondrial DNA had higher $F_2$-isoprostane levels 62 ± 39 pg/ml compared to those with lower heteroplasmy levels 52 ± 38 pg/ml ($P$ = 0.04). The highest isoprostane levels were measured in family members who smoked cigarettes, 102 ± 46 pg/ml ($N$ = 6). In this family all six cigarette smokers also had <40% heteroplasmy for the mtA8344G mutation. Excluding the cigarette smokers because of the known confounding effect smoking has on $F_2$-isoprostane levels, the mean $F_2$-isoprostane levels for family members with less than 40% heteroplasmy was 33 ± 13 vs. 62 ± 39 pg/ml for higher heteroplasmy family members ($P$ < 0.001) [13]. Among nonsmokers, the index case had the highest $F_2$-isoprostane level, despite taking 800 IU/day of vitamin E.

### *Correlation analysis*

Buccal specimen heteroplasmy correlated closely with heteroplasmy measured in mitochondrial DNA extracted from blood, $r = 0.91(P < 0.001)$ (Fig. 2).

Removing the confounding effect of cigarette smoking on patients with lower heteroplasmy revealed a linear correlation between the degree of heteroplasmy in both buccal or blood specimens and measured $F_2$-isoprostane levels. For buccal DNA, the (Pearson) correlation coefficient for increasing degrees of heteroplasmy and increasing $F_2$-isoprostane levels was 0.55 ($P$ = 0.03) (Fig. 3). For blood specimens, the correlation coefficient between these two variables was 0.52 ($P$ = 0.03).

There was no statistically significant correlation between age ($P$ = 0.43) or body mass index ($P$ = 0.31) and $F_2$-isoprostane levels in this family.

## Discussion

This large, five-generational family provides a special opportunity to study the genetic epidemiology of the mtA8344G mutation. Population-based studies have shown that this specific mutation is uncommon [20,21]. However, there are 176 reported disease-causing mitochondrial DNA mutations that are transmitted via the maternal germline [22]. As a group, their prevalence is greater than many other well-known degenerative diseases, such as amytrophic lateral sclerosis [1]. Limiting investigations to well-characterized phenotypes would underestimate the burden of mitochondrial mutations [3]. This index case's diagnosis led to the discovery of 18 other adult family members with this mutation.

Mitochondrial DNA mutations, like mtA8344G in this family, illustrate several unique aspects of mitochondrial genetics. First, transmission of mitochondria and the

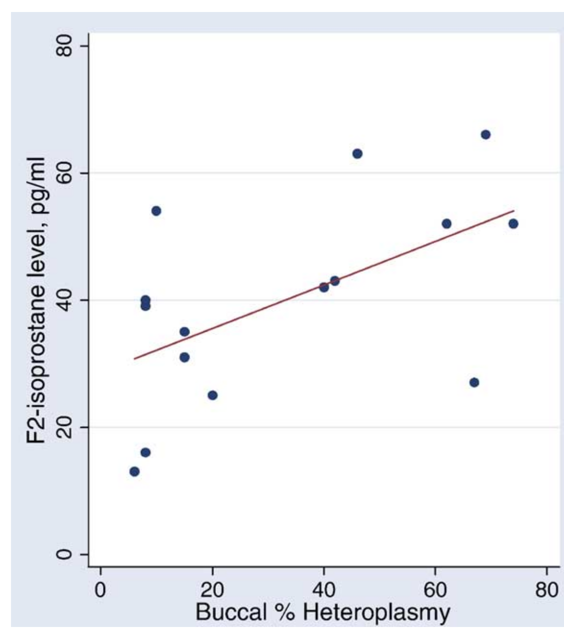


Fig. 3. Scatterplot of $F_2$-isoprostane levels versus buccal %heteroplasmy in nonsmoking members of this family. This scatterplot demonstrates the correlation between the level of $F_2$-isoprostanes, a marker of oxidant injury, and the degree of buccal heteroplasmy for the mtA8344G mutation in this family. Chronic cigarette smoking causes an increase in $F_2$-isoprostane levels. Family members that smoke cigarettes were excluded from this part of the analysis. The correlation coefficient for this relationship is $r$ = 0.55 ($P$ = 0.03). This correlation was similar to that for $F_2$-isoprostanes and blood %heteroplasmy for this mutation, $r$ = 0.52 ($P$ = 0.03).

mtDNA mutations they may carry occurs almost exclusively through the maternal inheritance. The oocyte at fertilization has many thousands of mitochondria while sperm have far fewer which are soon ubiquitin-tagged and destroyed [1]. Next, the proportion of mutated mtDNA genomes can vary between tissues. This heterogeneous distribution is referred to as tissue heteroplasmy. This can arise from the process of replicative segregation where during repeated cell divisions of heteroplasmic cells there can be a shift in the proportion of mutated mtDNA genomes [12]. The two different tissues sampled in this family, blood and buccal samples, did not show a significant variation. However, note the difference in the degree of heteroplasmy in the buccal samples between the index case and his mother (70% vs. 6%). Perhaps the index case's mother has increased tissue heteroplasmy in her ovarian tissue. Stochastic factors could have led to the unequal segregation of mutated mtDNA in the progenitor cells leading to the ova that ultimately became the index case. Finally, mitochondria with mutated mtDNA may have a local selective advantage that leads to proliferation and increased heteroplasmy in a given tissue [12]. Much needs to be discovered regarding the role of free radicals in these mitochondrial processes.

The mtA8344G mutation compromises mitochondrial respiratory chain function and results in decreased ATP production [3]. Cybrid studies reveal that this decline in electron chain transport is independent of nuclear gene involvement. Mutant cybrids containing mitochondria with more that 90% heteroplasmy for the mtA8344G mutation revealed severe impairment of protein synthesis.[23]. Since this mutation is in the gene encoding for the mitochondrial tRNA for lysine, all 13 subunits of the respiratory chain encoded in the mitochondrial genome are affected. Electrons accumulate in the initial steps of the electron transport chain and there interact with oxygen, initiating the production of destructive free radicals.[3,24]. This has been demonstrated in a mouse model where inhibition of oxidative phosphorylation produced markedly increased levels of oxygen-derived free radicals in both muscle and heart tissue [25]. This increase in oxidant injury was accompanied by increased mtDNA damage [25]. Measurement of $F_2$-isoprostanes in this family also reveals free radical-induced oxidant injury. This oxidant damage correlates positively with heteroplasmy for the mtA8344G mutation and suggests again a role for free radicals in the complex pathophysiology of mitochondrial disorders.

Phenotypic variation is a hallmark of mitochondrial disorders and heteroplasmy plays a key role [1]. Oxidant injury is certainly involved in many of the clinical features associated with mitochondrial mutations. Differences in antioxidant defenses may also influence phenotype. The index case was the only family member taking supplemental vitamin E at the time of ascertainment. His dose was subsequently increased to 1600 IU/day and the $F_2$-isoprostane levels decreased. Pro-oxidant factors, like cigarette smoking, may also account for some of this variation in oxidant injury. Cigarette smokers in this family all had moderate heteroplasmy and had higher $F_2$-isoprostane levels than cigarette smokers reported elsewhere [14]. The most novel and challenging discovery in this family was the observation of asymptomatic individuals with moderate heteroplasmy and evidence of ongoing free radical-mediated lipid peroxidation. Future research must focus on what happens to these individuals over time and development of treatments that halt the progression of oxidant injury.

## Acknowledgments

We are greatly indebted to the cooperation and support of family members involved in this study. The authors express appreciation to Asha Kallianpur, M.D., M.P.H., for her insightful review of this manuscript. Supported by NIH Grants GM15431, CA77839, DK48831, and RR000795. J.D.M. is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

## References

[1] DiMauro, S.; Schon, E. A. Mitochondrial respiratory-chain diseases. *N. Engl. J. Med.* **348:**2656–2668; 2003.

[2] Shoffner, J. M.; Lott, M. T.; Lezza, A. M.; Seibel, P.; Ballinger, S. W.; Wallace, D. C. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. *Cell* **61(6):**931–937; 1990.

[3] Wallace, D. C.; Zheng, X.; Lott, M. T.; Shoffner, J. M.; Hodge, J. A.; Kelley, R. I.; Epstein, C. M.; Hopkins, L. C. Familial mitochondrial encephalopathy (merrf): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. *Cell* **55:** 601–610; 1988.

[4] Howell, N.; Kubacka, I.; Smith, R.; Frerman, F.; Parks, J. K.; Parker, W. D. Association of the mitochondrial 8344 MERRF mutation with maternally inherited spinocerebellar degeneration and Leigh disease. *Neurology* **46:**219–222; 1996.

[5] Gamez, J.; Playan, A.; Andreu, A. L.; et al. Familial multiple symmetric lipomatosis associated with the A8344G mutation of mitochondrial DNA. *Neurology* **51:**258–260; 1998.

[6] Austin, S. A.; Vriesendorp, F. J.; Thandroyen, F. T.; Hecht, J. T.; Jones, O. T.; Johns, D. R. Expanding the phenotype of the 8344 transferRNA(Lysine) mitochondrial DNA mutation. *Neurology* **51:** 1447–1450; 1998.

[7] Richter, C.; Park, J. W.; Ames, B. N. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85:**6465–6467; 1988.

[8] Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552:**335–344; 2003.

[9] Halliwell, B., Gutteridge, J. M. C., Eds. *Free radicals in biology and medicine*, third ed. Oxford: Oxford University Press; 1999.

[10] Kang, D.; Hamasaki, N. Mitochondrial oxidative stress and mitochondrial DNA. *Clin. Chem. Lab. Med.* **41(10):**1281–1288; 2003.

[11] Scheffler, I. E. *Mitochondria*. New York: Wiley-Liss; 1999.

[12] Wallace, D. C. Mitochondrial diseases in man and mouse. *Science* **283:**1482–1488; 1999.

[13] Fam, S. S.; Morrow, J. D. The isoprostanes: unique products of arachidonic acid oxidation-a review. *Curr. Med. Chem.* **10:**1241–1253; 2003.

[14] Morrow, J. D.; Frei, B.; Longmire, A. W.; et al. Increase in circulating

products of lipid peroxidation ($F_2$-isoprostanes) in smokers: smoking as a cause of oxidative damage. *N. Engl. J. Med.* **332:**1198–1203; 1995.

[15] Morrow, J. D.; Hill, K. E.; Burk, R. F.; et al. A series of prostaglandin $F_2$-like compounds are produced in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA* **87:** 9383–9397; 1990.

[16] Roberts, L. J.; Morrow, J. D. Measurement of $F_2$-isoprostanes as an index of oxidative stress in vivo. *Free Radic. Biol. Med.* **28:**505–513; 2000.

[17] Morrow, J. D.; Roberts, L. J. The isoprostanes: unique bioactive products of lipid peroxidation. *Prog. Lipid. Res.* **36:**1–21; 1997.

[18] Zevani, M.; Amati, P.; Bresolin, N.; et al. Rapid detection of the A—G(8344) mutation of mtDNA in Italian families with myoclonus epilepsy and ragged-red fibers(MERRF). *Am. J. Hum. Genet.* **48(2):** 203–211; 1991.

[19] Morrow, J. D.; Roberts, L. J. Mass spectrometric quantification of $F_2$-isoprostanes in biological fluids and tissues as a measure of oxidant stress. *Methods Enzymol.* **300:**3–12; 1998.

[20] Remes, A. M.; Karppa, M.; Moilanen, J. S.; et al. Epidemiology of the mitochondrial DNA 8344A>(G mutation for the myoclonus epilepsy and ragged red fibres (MERRF) syndrome. *J. Neurol. Neurosurg. Psychiatry* **74:**1157–1161; 2003.

[21] Chinnery, P. F.; Johnson, M. A.; Wardell, T. M.; et al. The epidemiology of pathogenetic mitochondrial DNA mutations. *Ann. Neurol.* **48:**188–193; 2000.

[22] MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org, 2003.

[23] Enriquez, J. A.; Chomyn, A.; Attardi, G. mtDNA mutation in MERRF syndrome causes defective aminocylation of tRNA(lysine) and premature translation termination. *Nature Genet.* **10:**47–55; 1995.

[24] Wallace, D. C. Mitochondrial defects in neurodegenerative disease. *MRDD Res. Rev.* **7:**158–166; 2001.

[25] Esposito, L. A.; Kokoszka, J.; Waymire, K. Mitochondrial oxidative stress in mice lacking the glutathione peroxidase-1 gene. *Free Radic. Biol. Med.* **28:**754–756; 2000.