The Association of Mitochondrial Content with Prevalent and Incident Type 2 Diabetes

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Context: It has been shown that mitochondrial DNA (mtDNA) content is associated with type 2 diabetes (T2D) and related traits. However, empirical data, often based on small samples, did not confirm this observation in all studies. Therefore, the role of mtDNA content in T2D remains elusive.

Objective: In this study, we assessed the heritability of mtDNA content in buccal cells and analyzed the association of mtDNA content in blood with prevalent and incident T2D.

Design and Setting: mtDNA content from cells from buccal and blood samples was assessed using a real-time PCR-based assay. Heritability of mtDNA content was estimated in 391 twins from the Netherlands Twin Register. The association with prevalent T2D was tested in a case control study from The Netherlands (n = 329). Incident T2D was analyzed using prospective samples from Finland (n = 444) and The Netherlands (n = 238).

Main Outcome Measures: We measured the heritability of mtDNA content and the association of mtDNA content in blood with prevalent and incident T2D.

Results: A heritability of mtDNA content of 35% (19–48%) was estimated in the twin families. We did not observe evidence of an association between mtDNA content and prevalent or incident T2D and related traits. Furthermore, we observed a decline in mtDNA content with increasing age that was male specific ($P = 0.001$).

Conclusion: In this study, we show that mtDNA content has a heritability of 35% in Dutch twins. There is no association between mtDNA content in blood and prevalent or incident T2D and related traits in our study samples. **(*J Clin Endocrinol Metab* 95: 1909–1915, 2010)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2009-1775 Received August 20, 2009. Accepted January 13, 2010.
First Published Online February 11, 2010
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Abbreviations: A, Additive effects; BMI, body mass index; C, shared environmental influences; CI, confidence interval; DZ, dizygotic; E, unique environmental influences; *HBB*, beta-globin gene; IFG, impaired fasting glucose; IGT, impaired glucose tolerant; mtDNA, mitochondrial DNA; MZ, monozygotic; NGT, normal glucose tolerant; OGTT, oral glucose tolerance test.

Mitochondria contribute to multiple cellular processes such as ATP synthesis, β-oxidation of fatty acids, and apoptosis. They have their own circular genome, of approximately 16 kb, which exists in multiple copies per mitochondrion. Mitochondrial DNA (mtDNA) encodes for two rRNAs, all mitochondrial tRNAs, and 13 subunits of the respiratory chain. These 13 subunits are synthesized by the mitochondrial protein synthesis machinery inside the mitochondrial matrix. Because the complete pool of mitochondrial proteins consists of approximately 1500 proteins, only a fraction of them is encoded and synthesized inside the mitochondrion. Quantities of mtDNA, the so-called mtDNA content, vary between individuals and tissues within an individual. In muscle, dynamic adaptation is observed in relation to physical exercise (1, 2). Mutations in the human *POLG* gene and knockdown of Tfam in mouse models, two of the most important regulators of mtDNA content, result in mtDNA depletion, showing that nuclear factors are involved in the regulation of mtDNA content (3, 4). Furthermore, it has previously been shown in humans that the mtDNA content in blood cells is partially heritable (5, 6). In this study, we readdress the heritability of mtDNA content using buccal swabs from mono- and dizygotic twins and their siblings.

Evidence is accumulating that mtDNA content is associated with type 2 diabetes. However, there is debate about whether mitochondrial dysfunction is primary or secondary to type 2 diabetes. HIV-infected patients treated with highly active antiretroviral therapy containing nucleoside analogs show a 30–50% decreased mtDNA content, redistribution of body fat, insulin resistance, and an increased risk for development of type 2 diabetes, favoring the hypothesis that mitochondrial dysfunction is a cause rather than a consequence of development of type 2 diabetes (7–9).

It has been shown that low mtDNA content precedes type 2 diabetes (10). However, in a sample of 141 nondiabetic adult offspring who had one parent with type 2 diabetes, no difference in mtDNA content in blood was observed compared with controls (11). Other studies showed evidence that mtDNA content is associated with the pattern of triglyceride storage, insulin secretion, insulin sensitivity, and glucose metabolism (12–15). Furthermore, mtDNA content is decreased in Goto-Kakizaki rat, which is a genetic model for type 2 diabetes (16).

Because the association of mtDNA content with type 2 diabetes is not confirmed in all studies, the role of mtDNA content in the onset of type 2 diabetes remains not fully understood. The aim of the current study was to elucidate this important issue. We assessed the heritability of mtDNA content in participants from The Netherlands Twin Register while taking into consideration several lifestyle variables. Next, we assessed the association of mtDNA content with prevalent and incident cases of type 2 diabetes in a Dutch case control study and in selected samples from two prospective studies from Finland and The Netherlands.

Subjects and Methods

Sample selection

Heritability

For estimation of the heritability, participants were recruited from The Netherlands Twin Register (17, 18). The sample consisted of 391 twins and 12 of their siblings from 212 families as part of a study on depression and anxiety (19) and smoking behavior (20). DNA was extracted from buccal cells. Assessment of zygosity was based on DNA polymorphisms. There were 183 complete twin pairs [38 monozygotic (MZ) male, 23 dizygotic (DZ) male, 60 MZ female, 45 DZ female, and 17 DZ pairs of opposite sex] and 25 incomplete pairs (five MZ male, three DZ male, 12 MZ female, and five DZ female). The sibling group consisted of five males and seven females.

Information on body mass index (BMI), smoking, and exercise was obtained from longitudinal surveys and from data collected at the time of DNA sampling.

Case control study of prevalent type 2 diabetes

For the case control study, we selected 175 normal glucose tolerant (NGT) Caucasian participants from the Dutch New Hoorn Study (NHS). A total of 154 prevalent type 2 diabetes patients from NHS and the Diabetes Care System West-Friesland (DCS) were matched for age (21). NHS is a population-based study from the city of Hoorn, The Netherlands, which aims to examine potential determinants for glucose intolerance and related disorders. Glucose tolerance was assessed by oral glucose tolerance test (OGTT) following World Health Organization (WHO) 1999 criteria (22). DCS aims to improve diabetes care by coordinating the diabetes care involving all caregivers and providing education of patients to improve patient empowerment. Patients are referred to the DCS by their physicians and are from the same geographical region as the NHS. DNA was extracted from whole blood.

Prospective study of incident type 2 diabetes

To prospectively analyze the association between mtDNA content and incident cases of type 2 diabetes, we selected samples from two prospective studies. The first was the Botnia study (designated prospective 1), which aims at the identification of genes increasing susceptibility to type 2 diabetes (23). This family study includes 2770 participants, which were healthy at baseline, originating from the Botnia region in Finland. Glucose tolerance at baseline and after follow-up was tested using OGTT following WHO 1999 criteria (22). DNA was extracted from whole blood drawn at baseline. DNA from 133 participants who converted to type 2 diabetes (converters) was available. A total of 311 nonconverters were matched based on age and BMI at baseline and on gender. We allowed the inclusion of impaired glucose tolerant (IGT) and impaired fasting glucose (IFG) participants because

these covered a large proportion of the converter group. The converter group included 55 NGT, 37 IGT, 20 IFG, and 24 IGT+IFG participants at baseline. The nonconverter group included 245 NGT, 45 IGT, 26 IFG, and 15 IGT+IFG participants at baseline. Family history (first-degree relatives) of type 2 diabetes was present in 83.8% of the converter group and 67.2% of the nonconverter group.

The second prospective study was the Dutch Rotterdam study (n = 7983; designated prospective 2), which is a population-based study aiming to investigate determinants of disease occurrence and progression in the elderly (24). DNA was extracted from buffy coat, obtained from blood drawn at baseline. Diabetic state at baseline was assessed using a random glucose tolerance test, which was shown to have a good correlation with OGTT (25). Glucose tolerance after follow-up was assessed using OGTT following WHO 1999 criteria (22). From this study, 113 converters were available. We matched 125 nonconverters based on age and BMI at baseline and on gender. Entry of IGT participants at baseline was allowed (n = 20 in the converter group and n = 1 in the nonconverter group).

All studies were approved by the appropriate medical ethical committees and were in accordance with the principles of the Declaration of Helsinki. Study samples are summarized in Table 1.

Measurement of mtDNA content

mtDNA content was assessed using a modification of the quantitative real-time PCR-based method we described previously (26). In short, the relative amount of mtDNA was quantified by comparison with a nuclear target, which was the beta-globin gene (*HBB*). A fragment of mtDNA was amplified between nucleotide positions 3780 and 3842, which is located in *MT-ND1* on the heavy strand transcript (primer sequences available on request). Quantitative real-time PCR was performed using the Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA). Absolute QPCR SYBR Green ROX mix was used (Thermo Fisher Scientific Inc., Waltham, MA). For quantification, reference curves that were serial dilutions of a standard DNA were used. A ratio between mtDNA and *HBB* was calculated (mtDNA/*HBB*), which is here used as the mtDNA content. Each sample was measured at least in triplicate, and mean values were calculated. Specificity of amplification was tested by dissociation curves and water controls. Approximately 50% of samples were repeated and showed a high concordance in all study samples with initial results ($r^2 \geq 0.8$).

mtDNA content is assessed in several tissues (buccal cells, whole blood, and buffy coats). It is known that DNA extraction from these different samples can result in variance in mtDNA content. Therefore, differences in mtDNA content between the independent study samples is observed (27, 28).

Statistical analysis

Heritability study

Twin studies make use of the genetic relatedness of twins and their family members. MZ twins are genetically identical, whereas DZ twins share on average 50% of their segregating genes, like other siblings (29). Twin studies compare the correlation of a phenotype, such as mtDNA content, within MZ twin pairs with the resemblance within DZ twin and sibling pairs. If the correlation in MZ twins is about twice as large as the correlation in DZ twins, familial resemblance is explained by additive genetic effects (A). When the DZ correlation is more than half the MZ correlation, there is evidence for environmental effects shared by twins and siblings from the same family (C). Differences within MZ twin pairs are due to unique environmental influences (E), which also include measurement error. The observed variance thus can be decomposed into variance components A, C, and E (30).

Statistical analyses were performed using structural equation modeling as implemented in the software package Mx (31). The raw data full information maximum likelihood approach in Mx was used to fit different models to the data (for example, ACE and AE models). Testing of submodels was done by means of likelihood-ratio tests, by subtracting the negative log likelihood (−2LL) for the more restricted model (*e.g.* AE) from the −2LL for the more general model (*e.g.* ACE). This yields a statistic that is distributed as χ^2 with degrees of freedom (*df*) equal to the difference in the number of parameters in the two models. We first fitted a saturated model to the data in which means, variances, and correlations were estimated as well-fixed effects of age, sex, BMI, and physical activity on mtDNA content.

Case control study of prevalent type 2 diabetes

The association of mtDNA content with prevalent type 2 diabetes in the case control study was analyzed with logistic regression with adjustment for age, gender, and BMI. mtDNA content in each cohort was adjusted for age, gender, and BMI with general linear models. Continuous traits were analyzed with linear regression with adjustment for age, BMI, and gender (where appropriate) only in NGT participants. SPSS software (SPSS Inc., Chicago, IL) was used for these statistical analyses.

Prospective study of incident type 2 diabetes

Because the prospective 1 sample (Botnia study) is a family-based study, we adjusted for family relation. To do so, we made use of general estimating equations with logistic regression, which takes into account family relatedness when calculating the SE values. Conversion to type 2 diabetes was adjusted for age, BMI, and glucose tolerance at baseline and gender and family history of diabetes. Estimated average mtDNA content was cal-

TABLE 1. Characteristics of study samples

		Dutch case/control		Prospective 1		Prospective 2	
	Twins/heritability	Cases	Controls	Converters	Nonconverters	Converters	Nonconverters
n (% males)	403 (38)	154 (49)	175 (49)	133 (50)	311 (45)	113 (60)	125 (50)
Age (yr)	39 (12)	55 (4)	52 (6)	53 (12)	53 (9)	66 (6)	65 (5)
BMI (kg/m^2)	24.0 (3.7)	31.1 (5.8)	25.8 (3.5)	28.8 (4.3)	26.8 (2.9)	28.5 (3.5)	28.4 (2.7)

Data are expressed as number (percentage of males) or mean (SD).

culated with generalized estimated equations with linear regression and with similar adjustments as mentioned above.

The prospective 2 sample (Rotterdam study) is a sample of the population of Rotterdam, The Netherlands. We used standard logistic regression with adjustment for baseline measurement of age, BMI, and glucose tolerance. Estimated average mtDNA content and continuous traits were analyzed with a general linear model, using adjustment for age, BMI, and glucose tolerance at baseline and for gender.

Power calculation

Before the study, power calculations were performed using Lenth's power and sample-size calculator (http://www.stat.uiowa.edu/~rlenth/Power/index.html; web site accessed on May 20, 2008). We assumed a population SD of 30%. For the association with prevalent or incident type 2 diabetes, a sample size of 160 cases and controls is needed for 80% power to detect a difference of at least 10% ($\alpha = 0.05$).

Results

Heritability

Regression of gender, age, exercise, and BMI on mtDNA content indicated that mean mtDNA content was lower in subjects who do not exercise, are women, are older, or have a higher BMI. These four covariates thus explain a significant part (4%) of the variance in mtDNA content. Genetic modeling with these covariates yielded twin correlations of 0.34 for MZ and 0.19 for DZ/sib pairs. The corrected mean mtDNA was 1.28 with an SD of 0.02. Comparing an ACE model ($-2LL = 1996.76$) with an AE model ($-2LL = 1996.77$) indicated that C (shared environmental effects) could be dropped without loss of fit. Finally, fitting a model (E model) that specified no familial (genetic) variance showed a significantly worse fit ($-2LL = 2014.24$; χ^2 of 17.49 with 1 *df* when compared with AE model; $P = 2 \times 10^{-5}$), showing that genetic factors do have a significant effect on mitochondrial content. Heritability under the best model (AE) was estimated at 35% [95% confidence interval (CI), 19–48%].

Prevalent type 2 diabetes and mtDNA content

mtDNA content was assessed in a case control study ascertained from the Dutch NHS and DCS, originating from the same geographic location. Estimated mean mtDNA content in prevalent cases of type 2 diabetes and controls was 0.30 (0.28–0.31) and 0.29 (0.28–0.30), respectively ($P = 0.51$) (Table 2). Also, after logistic regression with adjustment for possible confounders (BMI, age, and gender), we could not detect an effect on type 2 diabetes susceptibility ($P = 0.56$). Furthermore, we could not find any evidence for a correlation between mtDNA content and other relevant variables (Supplemental Tables 1 and 2, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

TABLE 2. Association of mtDNA content with (future) type 2 diabetes

Study	mtDNA content		*P* value
	Case	Control	
Case/control[a]	0.30 (0.28–0.31)	0.29 (0.28–0.30)	0.51
Prospective 1[b]	0.38 (0.36–0.40)	0.40 (0.39–0.42)	0.08
Prospective 2[c]	0.55 (0.50–0.59)	0.51(0.46–0.56)	0.18

Estimated mtDNA content is shown as the ratio between nuclear DNA and mtDNA with 95% CI and *P* values.

[a] Adjusted for age, BMI, and gender using general linear model.

[b] Adjusted for age, gender, family history of type 2 diabetes, pedigree clustering, BMI, and glucose tolerance at baseline using generalized estimated equations.

[c] Adjusted for age, gender, BMI, and glucose tolerance at baseline using general linear model.

Incident type 2 diabetes and mtDNA content

mtDNA content was first assessed in the prospective 1 sample. Estimated mean mtDNA content in converters and nonconverters was 0.38 (0.36–0.40) and 0.40 (0.39–0.42), respectively ($P = 0.08$) (Table 2). Using logistic regression with adjustment for possible confounders (age, gender, BMI, glucose tolerance at baseline, and family clustering), we also observed a weak regression toward decreased mtDNA content with increased type 2 diabetes risk, but this did not reach statistical significance ($P = 0.10$). No associations between mtDNA content and OGTT-derived variables were observed.

Next, mtDNA content was assessed in the prospective 2 sample. Estimated mean mtDNA content in converters and nonconverters was 0.55 (0.50–0.59) and 0.51 (0.46–0.56), respectively ($P = 0.18$) (Table 2). Logistic regression with adjustment for possible confounders gave a similar result ($P = 0.17$).

All studies showed a trend toward decreasing mtDNA content with increasing age (Supplemental Table 1). However, for most studies, this did not reach statistical significance. Therefore, we pooled all data to increase power and repeated the analysis. mtDNA content of each independent study was normalized using the average mtDNA content to make the data comparable. To avoid heterogeneity, we excluded the twin data for this analysis because this was the only study for which DNA was extracted from buccal swabs. Using generalized estimating equations with correction for family relatedness and study sample, we observed a statistical significant decrease in mtDNA content with increasing age [B = −0.003/yr (−0.005 to −0.0007); $P = 0.009$]. Correction for BMI did not influence the data. However, the association seems to

be gender specific. No effect of age on mtDNA content was observed in females ($P = 0.53$), whereas a strong association was observed in males [B = −0.005/yr (−0.008 to −0.002); $P = 0.001$]. Furthermore, BMI showed a trend toward association with mtDNA content [B = −0.81 (−1.65 to 0.03); $P = 0.06$] in the pooled data set. After correction for age and gender, the association is borderline significant [B = −0.92 (−1.78 to −0.07); $P = 0.04$]. Exercise data for the pooled sample were only available from the case control and prospective 1 sample and did not influence this association (data not shown). We also observed different mtDNA content between males and females. Females showed an approximately 5% higher mtDNA content ($P = 0.007$). Correction for age and BMI revealed an estimated mtDNA content in males *vs.* females of 0.96 (0.93 to 0.98) and 1.01 (0.98 to 1.04), respectively ($P = 0.007$; Supplemental Table 2). The pooled sample was not corrected for exercise because assessment of exercise was performed with different questionnaires.

Discussion

Heritability

In this study, we showed that mtDNA content has a heritability of 35% (95% CI, 19–48%) in buccal cells. This partially confirms results from other groups who analyzed mtDNA content in blood and reported heritability between 33 and 65% (5, 6). However, the reported heritability of 65% is higher than our finding. This might be caused by a different ethnicity of participants or different types of tissue used.

In our study, mtDNA content in buccal cells is not affected by smoking behavior, so this could not bias our results. However, two other studies did show modest effects of smoking on mtDNA content in buccal cells and whole blood (6, 32).

Prevalent type 2 diabetes and mtDNA content

Inconsistency in literature is observed concerning the association of mtDNA content with type 2 diabetes (10, 11). In our study, we could not detect a relation between mtDNA content in blood and prevalent type 2 diabetes in a case control setup. *Post hoc* power calculations showed that we had greater than 95% power to detect an effect comparable to the results of Lee *et al.* (10) (mtDNA content 35% lower in cases of type 2 diabetes) and 80% power to detect a difference of at least 10%. Therefore, we conclude that mtDNA content in blood is not associated with type 2 diabetes in our sample selection.

Incident type 2 diabetes and mtDNA content

To analyze the relation between mtDNA content and incident cases of type 2 diabetes, we analyzed two prospective studies, but we could not detect a consistent association with incident type 2 diabetes. A previous study had suggested a 25% decrease in converters (10). We had greater than 95% power to detect a similar effect in both prospective 1 and prospective 2 (Botnia and Rotterdam) studies. This indicates that mtDNA content in blood is not a good predictor of future type 2 diabetes. Although the prospective 1 study shows a trend toward decreased risk for type 2 diabetes with increased mtDNA content, the prospective 2 study shows an opposite effect. This might be caused by the differences between the studies. For instance, participants from the prospective 1 sample are younger than those of the prospective 2 sample. Furthermore, there are more IGT and IFG subjects in the nonconverter group of the prospective 1 sample, compared with the prospective 2 sample. However, analyzing only NGT subjects does not change the outcome.

We conclude that mtDNA content in blood is not associated with incident type 2 diabetes, which argues against previous observations where low mtDNA content in blood was associated with type 2 diabetes, triglyceride storage, glucose homeostasis, insulin sensitivity, and insulin secretion (10, 12–15). A major difference between our and previous studies is the difference in ethnicity. All positive findings arose in Asian participants from Korea, whereas no associations were observed in Caucasians (10–14). Our data indicate that mtDNA content in blood is not useful as a predictor of type 2 diabetes in Caucasians. We cannot rule out any effects of mtDNA content in other tissues like muscle, pancreas, and adipose tissue on type 2 diabetes and related traits like insulin resistance and secretion. Muscle seems a highly relevant tissue to examine mitochondrial function because associations with mitochondrial activity and insulin resistance are observed (33, 34), and it has already been reported that mtDNA content decreases with aging in muscle, but also in pancreas, liver, and blood (5, 35–37). It seems that the decline in mtDNA content in muscle is faster than in blood. This may be related to the high level of oxidative metabolism in these tissues. Furthermore, it is speculated that the faster proliferative rate of hematopoietic stem cells provides them with the opportunity to remove damaged mtDNA and perhaps a better maintenance of mtDNA content (38, 39).

In our pooled dataset, we observed a negative correlation between mtDNA content and age that was male specific. To our knowledge, we are the first to show that the decline in mtDNA content might be male specific. The observed gender effect on mtDNA content, also observed by Xing *et al.* (6), is probably caused by this gender-specific correlation between mtDNA content and aging. One might speculate that overall mitochondrial fitness is better retained in females, which might explain the observed dif-

ference in life span between males and females. However, this hypothesis needs further investigation. The association between mtDNA content and BMI was very weak and was not observed by others (10–15). Therefore, it is likely that this is not a true association.

In summary, we have confirmed the heritability of mtDNA content, but could not find evidence for an association of mtDNA content in blood with prevalent or incident type 2 diabetes. The observed decline in mtDNA content in males might suggest enhanced mitochondrial fitness in elderly women. However, this needs further study in more suitable cohorts and experimental settings.

Acknowledgments

The authors acknowledge all participants for their cooperation.

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This project was funded by The Netherlands Organization for Scientific Research, the ZonMW RIDE program, and the Dutch Diabetes Research Foundation. The Netherlands Twin Register study was supported by the Database Twin Register (NWO 575-25-006); Spinozapremie (NWO/SPI 56-464-14192); Centre Neurogenetics/Cognition Research-VU, Center for Medical Systems Biology (NWO Genomics); and the Twin-Family Database for behavior genetics and genomics (NWO 480-04-004). The Botnia study was funded by grants from the Sigrid Juselius and Folkhälsan Research foundations. Work at the Lund University Diabetes Centre was funded by a Linné grant from the Swedish Research Council.

Disclosure Summary: The authors have no conflict of interest to declare.

References

1. **Calvo S, Jain M, Xie X, Sheth SA, Chang B, Goldberger OA, Spinazzola A, Zeviani M, Carr SA, Mootha VK** 2006 Systematic identification of human mitochondrial disease genes through integrative genomics. Nat Genet 38:576–582
2. **Falkenberg M, Larsson NG, Gustafsson CM** 2007 DNA replication and transcription in mammalian mitochondria. Annu Rev Biochem 76:679–699
3. **Naviaux RK, Nyhan WL, Barshop BA, Poulton J, Markusic D, Karpinski NC, Haas RH** 1999 Mitochondrial DNA polymerase γ deficiency and mtDNA depletion in a child with Alpers' syndrome. Ann Neurol 45:54–58
4. **Silva JP, Köhler M, Graff C, Oldfors A, Magnuson MA, Berggren PO, Larsson NG** 2000 Impaired insulin secretion and β-cell loss in tissue-specific knockout mice with mitochondrial diabetes. Nat Genet 26:336–340
5. **Curran JE, Johnson MP, Dyer TD, Göring HH, Kent JW, Charlesworth JC, Borg AJ, Jowett JB, Cole SA, MacCluer JW, Kissebah AH, Moses EK, Blangero J** 2007 Genetic determinants of mitochondrial content. Hum Mol Genet 16:1504–1514
6. **Xing J, Chen M, Wood CG, Lin J, Spitz MR, Ma J, Amos CI, Shields PG, Benowitz NL, Gu J, de Andrade M, Swan GE, Wu X** 2008 Mitochondrial DNA content: its genetic heritability and association with renal cell carcinoma. J Natl Cancer Inst 100:1104–1112
7. **Brinkman K, Smeitink JA, Romijn JA, Reiss P** 1999 Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. Lancet 354:1112–1115
8. **Maassen JA, 't Hart LM, Ouwens DM** 2007 Lessons that can be learned from patients with diabetogenic mutations in mitochondrial DNA: implications for common type 2 diabetes. Curr Opin Clin Nutr Metab Care 10:693–697
9. **Blümer RM, van Vonderen MG, Sutinen J, Hassink E, Ackermans M, van Agtmael MA, Yki-Jarvinen H, Danner SA, Reiss P, Sauerwein HP** 2008 Zidovudine/lamivudine contributes to insulin resistance within 3 months of starting combination antiretroviral therapy. AIDS 22:227–236
10. **Lee HK, Song JH, Shin CS, Park DJ, Park KS, Lee KU, Koh CS** 1998 Decreased mitochondrial DNA content in peripheral blood precedes the development of non-insulin-dependent diabetes mellitus. Diabetes Res Clin Pract 42:161–167
11. **Singh R, Hattersley AT, Harries LW** 2007 Reduced peripheral blood mitochondrial DNA content is not a risk factor for type 2 diabetes. Diabet Med 24:784–787
12. **Park KS, Song JH, Lee KU, Choi CS, Koh JJ, Shin CS, Lee HK** 1999 Peripheral blood mitochondrial DNA content correlates with lipid oxidation rate during euglycemic clamps in healthy young men. Diabetes Res Clin Pract 46:149–154
13. **Park KS, Lee KU, Song JH, Choi CS, Shin CS, Park DJ, Kim SK, Koh JJ, Lee HK** 2001 Peripheral blood mitochondrial DNA content is inversely correlated with insulin secretion during hyperglycemic clamp studies in healthy young men. Diabetes Res Clin Pract 52: 97–102
14. **Song J, Oh JY, Sung YA, Pak YK, Park KS, Lee HK** 2001 Peripheral blood mitochondrial DNA content is related to insulin sensitivity in offspring of type 2 diabetic patients. Diabetes Care 24:865–869
15. **Weng SW, Lin TK, Liou CW, Chen SD, Wei YH, Lee HC, Chen IY, Hsieh CJ, Wang PW** 2009 Peripheral blood mitochondrial DNA content and dysregulation of glucose metabolism. Diabetes Res Clin Pract 83:94–99
16. **Serradas P, Giroix MH, Saulnier C, Gangnerau MN, Borg LA, Welsh M, Portha B, Welsh N** 1995 Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes. Endocrinology 136:5623–5631
17. **Boomsma DI, Vink JM, van Beijsterveldt TC, de Geus EJ, Beem AL, Mulder EJ, Derks EM, Riese H, Willemsen GA, Bartels M, van den Berg M, Kupper NH, Polderman TJ, Posthuma D, Rietveld MJ, Stubbe JH, Knol LI, Stroet T, van Baal GC** 2002 Netherlands Twin Register: a focus on longitudinal research. Twin Res 5:401–406
18. **Boomsma DI, de Geus EJ, Vink JM, Stubbe JH, Distel MA, Hottenga JJ, Posthuma D, van Beijsterveldt TC, Hudziak JJ, Bartels M, Willemsen G** 2006 Netherlands Twin Register: from twins to twin families. Twin Res Hum Genet 9:849–857
19. **Boomsma DI, Beem AL, van den Berg M, Dolan CV, Koopmans JR, Vink JM, de Geus EJ, Slagboom PE** 2000 Netherlands twin family study of anxious depression (NETSAD). Twin Res 3:323–334
20. **Vink JM, Beem AL, Posthuma D, Neale MC, Willemsen G, Kendler KS, Slagboom PE, Boomsma DI** 2004 Linkage analysis of smoking initiation and quantity in Dutch sibling pairs. Pharmacogenomics J 4:274–282
21. **Reiling E, van Vliet-Ostaptchouk JV, van 't Riet E, van Haeften TW, Arp PA, Hansen T, Kremer D, Groenewoud MJ, van Hove EC, Romijn JA, Smit JW, Nijpels G, Heine RJ, Uitterlinden AG, Pedersen O, Slagboom PE, Maassen JA, Hofker MH, 't Hart LM, Dekker JM** 2009 Genetic association analysis of 13 nuclear-encoded mitochondrial candidate genes with type II diabetes mellitus: the DAMAGE study. Eur J Hum Genet 17:1056–1062
22. **World Health Organization** 1999 Definition, diagnosis and classi-

fication of diabetes mellitus, report of a WHO consultation. Part 1: Diagnosis and classification of diabetes mellitus. Geneva: WHO/NCD/NCS/99.2

23. **Groop L, Forsblom C, Lehtovirta M, Tuomi T, Karanko S, Nissén M, Ehrnström BO, Forsén B, Isomaa B, Snickars B, Taskinen MR** 1996 Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. Diabetes 45:1585–1593
24. **Hofman A, Breteler MM, van Duijn CM, Krestin GP, Pols HA, Stricker BH, Tiemeier H, Uitterlinden AG, Vingerling JR, Witteman JC** 2007 The Rotterdam Study: objectives and design update. Eur J Epidemiol 22:819–829
25. **Stolk RP, Pols HA, Lamberts SW, de Jong PT, Hofman A, Grobbee DE** 1997 Diabetes mellitus, impaired glucose tolerance, and hyperinsulinemia in an elderly population. The Rotterdam Study. Am J Epidemiol 145:24–32
26. **Szuhai K, Ouweland J, Dirks R, Lemaître M, Truffert J, Janssen G, Tanke H, Holme E, Maassen J, Raap A** 2001 Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. Nucleic Acids Res 29:E13
27. **Andreu AL, Martinez R, Marti R, García-Arumí E** 2009 Quantification of mitochondrial DNA copy number: pre-analytical factors. Mitochondrion 9:242–246
28. **Guo W, Jiang L, Bhasin S, Khan SM, Swerdlow RH** 2009 DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. Mitochondrion 9:261–265
29. **Boomsma D, Busjahn A, Peltonen L** 2002 Classical twin studies and beyond. Nat Rev Genet 3:872–882
30. **Neale MC, Cardon L** 1992 Methodology for genetic studies of twins and families. Dordrecht, The Netherlands: Kluwer Academic Publishers
31. **Neale MC, Boker SM, Xie G, Maes HH** 2003 MX: Statistical modelling. 6th ed. Virginia Commonwealth University, Department of Psychiatry
32. **Tan D, Goerlitz DS, Dumitrescu RG, Han D, Seillier-Moiseiwitsch F, Spernak SM, Orden RA, Chen J, Goldman R, Shields PG** 2008 Associations between cigarette smoking and mitochondrial DNA abnormalities in buccal cells. Carcinogenesis 29:1170–1177
33. **Nair KS, Bigelow ML, Asmann YW, Chow LS, Coenen-Schimke JM, Klaus KA, Guo ZK, Sreekumar R, Irving BA** 2008 Asian Indians have enhanced skeletal muscle mitochondrial capacity to produce ATP in association with severe insulin resistance. Diabetes 57:1166–1175
34. **Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI** 2004 Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 350:664–671
35. **Barazzoni R, Short KR, Nair KS** 2000 Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. J Biol Chem 275:3343–3347
36. **Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS** 2005 Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci USA 102:5618–5623
37. **Cree LM, Patel SK, Pyle A, Lynn S, Turnbull DM, Chinnery PF, Walker M** 2008 Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets. Diabetologia 51:1440–1443
38. **'t Hart LM, Jansen JJ, Lemkes HH, de Knijff P, Maassen JA** 1996 Heteroplasmy levels of a mitochondrial gene mutation associated with diabetes mellitus decrease in leucocyte DNA upon aging. Hum Mutat 7:193–197
39. **Rajasimha HK, Chinnery PF, Samuels DC** 2008 Selection against pathogenic mtDNA mutations in a stem cell population leads to the loss of the 3243A→G mutation in blood. Am J Hum Genet 82:333–343