The genetics of type 2 diabetes

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Chapter 4

The association of mitochondrial content with prevalent and incident type 2 diabetes

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Abstract

Aims/hypothesis

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. 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.

Methods

Mitochondrial DNA content from cells from buccal and blood samples were 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).

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, which 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.

Introduction

Mitochondria contribute to multiple cellular processes such as ATP synthesis, beta-oxidation of fatty acids and apoptosis. They have their own circular genome, of approximately 16kb which exists in multiple copies per mitochondrion. Mitochondrial DNA (mtDNA) encodes for 2 rRNA's, all mitochondrial tRNA's and 13 subunits of the respiratory chain. These 13 subunits are synthesized by the mitochondrial protein synthesis machinery inside the mitochondrial matrix. Since 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 knock down 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 Anti Retroviral Therapy (HAART) containing nucleoside analogues, show a 30-50% decreased mtDNA content, redistribution of body fat, insulin resistance and an increased risk for development of type 2 diabetes, favouring 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 non-diabetic, adult offspring who had one parent with T2D, no difference in mtDNA content in blood was observed compared to 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).
Since 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 life style 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 form Finland and The Netherlands.

Materials 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 male (MZM), 23 dizygotic male (DZM), 60 monozygotic female (MZF), 45 dizygotic female (DZF) and 17 DZ pairs of opposite sex) and 25 incomplete pairs (5 MZM, 3 DZM, 12 MZF and 5 DZF). The sibling group consisted of 5 males and 7 females.
Information on 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). 154 prevalent type 2 diabetes patients from NHS and the Diabetes Care System West-Friesland

(DCS) were matched for age (21-23). 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 WHO '99 criteria (24). DCS aims to improve diabetes care by coordinating the diabetes care involving all caregivers and providing education of patients in order 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

In order to prospectively analyze the association between mtDNA content and incident cases of type 2 diabetes we selected samples from 2 prospective studies. The first was the Botnia study, here designated prospective 1, which aims at the identification of genes increasing susceptibility to type 2 diabetes (25). 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 '99 criteria (24). DNA was extracted from whole blood, drawn at baseline. DNA from 133 participants who converted to type 2 diabetes (converters) was available. 311 non-converters were matched based on age, and BMI at baseline and gender. We allowed the inclusion of impaired glucose tolerant (IGT) and impaired fasting glucose (IFG) participants since 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 non-converter 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 and 67.2% of the non-converter group.

The second prospective study was the Dutch Rotterdam study (n = 7983), here designated prospective 2, which is a population based study aiming to investigate determinants of disease occurrence and progression in the elderly (26). 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 (27). Glucose tolerance after follow up was

assessed using OGTT following WHO '99 criteria (24). From this study 113 converters were available. We matched 125 non-converters based on age and BMI at baseline and gender. Entry of IGT participants at base line was allowed (n = 20 in the converter group and n = 1 in the non-converter 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.

Table 1. Characteristics of study samples

	Twins / Heritability	Dutch case/control		Prospective 1		Prospective 2	
		Cases	Controls	Converters	Non-converters	Converters	Non-converters
N	403	154	175	133	311	113	125
(%male)	(38)	(49)	(49)	(50)	(45)	(60)	(50)
Age	38.7	55	52	53	53	66	65
(stdev)	(11.7)	(4)	(6)	(12)	(9)	(6)	(5)
BMI	24.0	31.1	25.8	28.8	26.8	28.5	28.4
(stdev)	(3.7)	(5.8)	(3.5)	(4.3)	(2.9)	(3.5)	(2.7)

Mean with SD are shown.
n.a.: not available

Measurement of mitochondrial DNA content

Mitochondrial DNA content was assessed using a modification of the quantitative real time PCR based method we described previously (28). 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 position 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, Applied Biosystems, Foster City, USA). Absolute QPCR SYBR Green ROX mix was used (Thermo Fisher Scientific Inc., Waltham, MA, USA). For quantification reference curves were used, which were serial dilutions of a standard DNA. 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 ($r^2 \geq 0.8$).

Mitochondrial DNA 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 (29;30).

Statistical analysis

Heritability study

Twin studies make use of the genetic relatedness of twins and their family members. MZ twins are genetically identical while DZ twins share on average 50% of their segregating genes, like other siblings (31). 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 (C) effects shared by twins and siblings from the same family. 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 (32).

Statistical analyses were performed using structural equation modeling as implemented in the software package Mx (33). 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 sub models 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. Mitochondrial DNA content in each cohort was adjusted for age, gender and BMI with general linear models. Continuous traits where analyzed with linear regression with adjustment for age, BMI and gender (were appropriate) only in NGT participants. SPSS (SPSS Inc, Chicago, USA) was used for these statistical analyses.

Prospective study of incident type 2 diabetes

Since the prospective 1 sample (Botnia study) is a family based study, we adjusted for family relation. In order to do so, we made use of general estimating equations with logistic regression, which takes into account family relatedness when calculating the standard errors. 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 calculated with generalized estimated equations with linear regression, 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 age, BMI and glucose tolerance at baseline and gender. 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 gender.

Power calculation

Prior to the study, power calculations were performed, using Lenth's power and sample-size calculator (http://www.stat.uiowa.edu/~rlenth/Power/index.html website accessed on May 20 2008). We assumed a population standard deviation 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, in women, who are older and who 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 to AE model; $p = 2 \cdot 10^{-5}$), showing that genetic factors do have a significant effect on mitochondrial content. Heritability under the best model (AE) was estimated at 35% (CI 19%-48%).

Prevalent type 2 diabetes and mitochondrial DNA content

Mitochondrial DNA 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

Table 2. Association of mitochondrial DNA 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 mitochondrial DNA content is shown as the ratio between nDNA and mtDNA with 95% CI and p-values.
a. Adjusted for age, BMI and gender using general linear model.
b. Adjusted for age, BMI, gender, family history of type 2 diabetes, pedigree clustering and glucose tolerance at baseline using generalized estimated equations.
c. Adjusted for age, BMI, gender and glucose tolerance at baseline using general linear model.

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 (supplementary table S1 and S2).

Incident type 2 diabetes and mitochondrial DNA content

Mitochondrial DNA content was first assessed in the prospective 1 sample. Estimated mean mtDNA content in converters and non-converters 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 towards 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 non-converters 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 towards decreasing mtDNA content with increasing age (supplementary table S1). However, for most studies, this did not reach statistical significance. Therefore, we pooled all data in order to increase power and repeated the analysis. Mitochondrial DNA 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, since 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 (-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), while a strong association was observed in males (B = -0.005 / year (-0.008 to -0.002), p = 0.001). Furthermore,

BMI showed a trend towards 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 was 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 a ~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, supplementary table S2). The pooled sample was not corrected for exercise, since assessment of exercise was performed with different questionnaires.

Discussion

Heritability

In this study we showed that mtDNA content has a heritability of 35% (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 behaviour, 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;34).

Prevalent type 2 diabetes and mitochondrial DNA 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 >95% power to detect an effect comparable to the results of Lee H.K. et al (mitochondrial content 35% lower in cases of type 2 diabetes) (10) 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 mitochondrial DNA content

In order 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 >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. While the prospective 1 study shows a trend towards 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 non-converter group of the prospective 1 sample, compared to 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, while no associations were observed in Caucasians (10-14). Our data indicate that mtDNA content in blood is not useful as 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 since associations with mitochondrial activity and insulin resistance are observed (35;36) and it has already been reported that mtDNA content decreases with aging in muscle, but also in pancreas, liver and blood (5;37-39). 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 copynumber (37;38).
In our pooled dataset we observed a negative correlation between mtDNA content and age, which 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 J et al (6), is probably caused by this gender specific correlation between mtDNA content and ageing. One might speculate that overall mitochondrial fitness is better retained in females, which might explain the observed difference 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.

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Supplementary table S1. Regression of mitochondrial DNA content with clinical variables

	Twins / Heritability study (n = 403)	Case / Control study (n = 329)	Prospective 1 study (n = 444)	Prospective 2 study (n = 238)
Age	-0.001 (-0.003 to 0.001) 0.35	-0.002 (-0.004 to 0.0001) 0.06	-0.0008 (-0.002 to 0.0001) 0.09	-0.002 (-0.006 to 0.002) 0.29
BMI	-3.05 (-5.17 to -0.92) 0.005	0.55 (-2.18 to 3.27) 0.69	-0.78 (-3.31 to 1.76) 0.55	-2.26 (-4.57 to 0.04) 0.05
WHR	-0.03 (-0.08 to 0.02) 0.27	0.02 (-0.08 to 0.13) 0.68	0.01 (-0.04 to 0.05) 0.77	0.01 (-0.05 to 0.06) 0.85
0hr gluc	0.49 (0.08 to 0.91) 0.02	0.27 (-0.28 to 0.81) 0.34	0.19 (-0.17 to 0.55) 0.29	n.a.
2hr gluc	n.a.	1.04 (-0.94 to 3.02) 0.30	0.72 (-0.45 to 1.90) 0.23	n.a.

Regression coefficients are shown with 95% CI and p-values.
WHR: Waist Hip Ratio
n.a.: not available
For the case / control study, only NGT participants are included (n = 175)
Regression coefficients, 95% CI and p-values are shown.

supplementary table S2. Gender effect on mitochondrial DNA content in healthy participants

Study	Male		Female
Twins / Heritability	1.01 (0.96 to 1.06)		1.01 (0.97 to 1.06)
p-value		0.98	
Case / Control	0.98 (0.94 to 1.03)		0.98 (0.92 to 1.01)
p-value		0.95	
Prospective 1	0.97 (0.93 to 1.01)		1.02 (0.98 to 1.07)
p-value		0.07	
Prospective 2	0.92 (0.87 to 0.97)		1.04 (0.98 to 1.09)
p-value		0.002	
Pooled	**0.96 (0.93 to 0.98)**		**1.01 (0.98 to 1.04)**
p-value		**0.007**	

Relative mitochondrial DNA content, normalized using the average mtDNA content of every independent study sample are shown with 95% CI and p-values. Netherlands Twin Register is excluded from the pooled sample, since DNA extractions are from buccal cells.
Estimated mean mitochondrial DNA content and 95% CI are calculated using generalized estimating equations with adjustment for age and BMI.

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