

Age influences DNA methylation and gene expression of *COX7A1* in human skeletal muscle

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Abstract

Aims/hypothesis Reduced oxidative capacity of the mitochondria in skeletal muscle has been suggested to contribute to insulin resistance and type 2 diabetes. Moreover, a set

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of genes influencing oxidative phosphorylation (OXPHOS) is downregulated in diabetic muscle. Here we studied whether genetic, epigenetic and non-genetic factors influence a component of the respiratory chain, *COX7A1*, previously shown to be downregulated in skeletal muscle from patients with type 2 diabetes. The specific aims were to: (1) evaluate the impact of genetic (single nucleotide polymorphisms [SNPs]), epigenetic (DNA methylation) and non-genetic (age) factors on the expression of *COX7A1* in human skeletal muscle; and (2) investigate whether common variants in the *COX7A1* gene are associated with increased risk of type 2 diabetes.

Methods *COX7A1* mRNA expression was analysed in muscle biopsies from young ($n=110$) and elderly ($n=86$) non-diabetic twins and related to measures of in vivo metabolism. Genetic variants (three SNPs) from the *COX7A1* locus were genotyped in the twins and in two independent type 2 diabetes case–control cohorts ($n=1466$ and 6380, respectively). DNA methylation of the *COX7A1* promoter was analysed in a subset of twins (ten young, ten elderly) using bisulphite sequencing.

Results While DNA methylation of the *COX7A1* promoter was increased in muscle from elderly compared with young twins ($19.9\pm 8.3\%$ vs $1.8\pm 2.7\%$; $p=0.035$), the opposite was found for *COX7A1* mRNA expression (elderly 1.00 ± 0.05 vs young 1.68 ± 0.06 ; $p=0.0005$). The heritability of *COX7A1* expression was estimated to be 50% in young and 72% in elderly twins. One of the polymorphisms investigated, rs753420, influenced basal *COX7A1* expression in muscle of young ($p=0.0001$) but not of elderly twins. The transcript level of *COX7A1* was associated with increased in vivo glucose uptake and $\dot{V}O_{2\max}$ ($p=0.009$ and $p=0.001$, respectively). We did not observe any genetic association between *COX7A1* polymorphisms and type 2 diabetes after correcting for multiple testing.

Conclusions/interpretation Our results provide further evidence for age as a factor influencing DNA methylation and expression of OXPHOS genes, and thereby in vivo metabolism.

Keywords COX7A1 · DNA methylation · Epigenetic · Gene expression · Genetic · Glucose metabolism · Oxidative phosphorylation · Polymorphism · Skeletal muscle · Type 2 diabetes

Abbreviations

COX	cytochrome c oxidase
GEE	generalised estimating equations
LD	linkage disequilibrium
mtDNA	mitochondrial DNA
OXPHOS	oxidative phosphorylation
SNP	single nucleotide polymorphism
$\dot{V}O_{2\max}$	total body aerobic capacity
WGAS	whole genome association study

Introduction

Type 2 diabetes is characterised by insulin resistance in the liver and peripheral target tissues, hyperglycaemia and impaired pancreatic beta cell function. Reduced oxidative capacity of the mitochondria in skeletal muscle has been found to be associated with insulin resistance in type 2 diabetes [1]. In addition, a number of gene expression profiles have shown reduced levels of genes involved in oxidative phosphorylation (OXPHOS) and reduced levels of their transcriptional regulators, e.g. *PGC-1 α* (also known as *PPARGC1A*) and *PGC-1 β* (also known as *PPARGC1B*), in skeletal muscle from patients with type 2 diabetes [2–5]. Together, these reports propose that defects in the skeletal muscle respiratory chain may contribute to the pathogenesis of type 2 diabetes.

OXPHOS is a process where electrons are passed from NADH and FADH₂ along a series of carrier molecules. In this process protons are pumped across the inner mitochondrial membrane to produce a proton gradient and in a final step this gradient is used to drive the production of ATP from ADP and phosphate. This process occurs in a system that consists of five multiprotein complexes with about 90 known protein subunits. While the majority of these subunits are encoded by the nuclear genome, 13 subunits are encoded by the 16.6 kb mitochondrial genome (mitochondrial DNA [mtDNA]).

COX7A1 is a nuclear encoded OXPHOS gene encoding polypeptide 1 of subunit 7A, a subunit of cytochrome c oxidase (COX), i.e. complex IV in the respiratory chain. COX catalyses the electron transfer from reduced cyto-

chrome c to oxygen, producing H₂O. This reaction is coupled with proton transfer from the matrix to the intermembrane space, thereby contributing to the energy stored in the electrochemical gradient. The catalytic core of COX is composed of mitochondrial encoded proteins whereas nuclear encoded proteins contribute to the structural and regulatory subunits of the complex. *COX7A1* is located on chromosome 19q13.12 within a CpG dense region [6] where tissue-specific DNA methylation has been correlated with gene expression [7]. *COX7A1* is only expressed in skeletal and heart muscle [8], and is significantly downregulated in diabetic muscle [3].

Type 2 diabetes develops as a conspiracy between the genetic background and the environment. Age, reduced physical activity and obesity represent predominant non-genetic factors. Among genetic factors common variants in a number of type 2 diabetes candidate genes, e.g. *PPARG*, *KCNJ11* and *TCF7L2*, have been associated with increased risk of the disease. Additionally, more recent whole genome association studies (WGAS) have not only confirmed the previous associated gene regions, but also identified variants in *FTO*, *CDKN2A/CDKN2B*, *IGF2BP2*, *CDKAL1*, *HHEX*, *WFS1* and *SLC30A8* as being associated with type 2 diabetes [9]. We have previously shown that an age-dependent decrease in skeletal muscle *PGC-1 α* and *PGC-1 β* expression is partially heritable and influenced by the *PGC-1 α* Gly482Ser and *PGC-1 β* Ala203Pro polymorphisms [10, 11]. In addition, insulin-resistant offspring of patients with type 2 diabetes were suggested to have impaired mitochondrial function in skeletal muscle [12]. However, the interaction between genes and environment may be even more complex and could involve epigenetic factors such as DNA methylation and histone modifications in the development of type 2 diabetes. Cytosine residues occurring in CG dinucleotides are targets for DNA methylation in vertebrates and gene expression is often reduced when DNA is methylated in the promoter region. We recently showed that such mechanisms can dramatically change expression of another OXPHOS gene, *NDUFB6*, during a person's life-time [13].

It is well established that mutations in mtDNA can cause diabetes and other metabolic defects [14–16], but less is known about whether common variations in nuclear encoded OXPHOS genes influence the susceptibility to type 2 diabetes.

Our major aim was to investigate the regulation of OXPHOS gene expression in human skeletal muscle and its role in glucose metabolism and type 2 diabetes. For this purpose we chose the *COX7A1* gene, as its expression is decreased in skeletal muscle from patients with type 2 diabetes [3]. Also, the gene resides in a locus on human chromosome 19 showing differential DNA methylation [7]. The specific aims were to: (1) evaluate the impact of genetic (single nucleotide

polymorphisms [SNPs]), epigenetic (DNA methylation) and non-genetic factors (e.g. age) on the expression of *COX7A1* in human skeletal muscle; and (2) investigate whether common variants in the *COX7A1* gene are associated with increased risk of type 2 diabetes.

Methods

Participants

Twins Participants were identified through The Danish Twin Register. Selection criteria for the young and elderly twins have been previously described and their clinical characteristics are described in Table 1 [10, 17, 18].

The Botnia Study We studied 1,466 unrelated individuals from the Botnia Study, 751 of whom have been diagnosed with type 2 diabetes at >35 years of age and 715 non-diabetic controls without first-degree relatives with type 2 diabetes (Table 1). The Botnia Study is a family based study established in 1990 aiming at the identification of type 2 diabetes susceptibility genes [19]. Participants were classified into different stages of glucose tolerance based on an OGTT. Individuals with genetically verified MODY or GAD antibody-positive patients were excluded from the study.

Malmö case-control cohort This included 2,830 Scandinavian type 2 diabetes cases from a local Diabetes Registry [20] and 3,550 unrelated ethnically matched healthy control individuals from the Malmö Diet and Cancer Study [21]

(Table 1). Type 2 diabetes was diagnosed according to WHO criteria [22], with C-peptide concentrations ≥ 0.3 nmol/l, no GAD antibodies and age-at-onset >35 years. The control individuals had fasting blood glucose <5.6 mmol/l and no known family history of type 2 diabetes (Table 1).

All studies were approved by the regional Ethics Committees and conducted according to the Helsinki Declaration.

Clinical examination and muscle biopsies in twins

Twins underwent 2 days of clinical examination including a 2-h euglycaemic-hyperinsulinaemic clamp ($40 \text{ mU m}^{-2} \text{ min}^{-1}$) combined with indirect calorimetry before (basal) and at the end of the clamp as previously described [10, 17, 18]. Rates of glucose disposal, its partitioning into oxidation and non-oxidative glucose metabolism as well as fat oxidation were calculated as previously reported and expressed as $\text{mg (kg lean body mass)}^{-1} \text{ min}^{-1}$ [23]. Muscle biopsies were obtained from the vastus lateralis muscle under local anaesthesia during both basal and insulin-stimulated states. Plasma glucose and insulin concentrations and specific activity of $^3\text{H}_2\text{O}$ were measured as previously described [17, 24, 25].

Analysis of *COX7A1* mRNA levels in skeletal muscle

Total RNA was extracted from muscle biopsies using Tri Reagent kit (Sigma-Aldrich, St Louis, MO, USA). cDNA was synthesised using Superscript II RNase H⁻ Reverse Transcriptase and random hexamer primers (Invitrogen,

Table 1 Clinical characteristics of participants

	Twins		Botnia		Malmö	
	Young	Elderly	Cases	Controls	Cases	Controls
<i>n</i> (male/female)	110 (60/50)	86 (38/48)	751 (399/352)	715 (345/370)	2,830 (1,667/1,163)	3,550 (1,340/2,210)
<i>n</i> (MZ/DZ)	110 (66/44)	86 (42/44)				
Age (years)	28.0±1.9	62.4±2.0*	60.3±9.9	53.7±11.4*	57.9±11.5	57.5±6.0
BMI (kg/m ²)	24.1±3.1	26.1±4.4*	28.9±4.8	25.8±3.7*	29.6±5.5	25.1±3.6*
WHR	0.84±0.08	0.89±0.1*	0.94±0.09	0.87±0.09*		0.84±0.09
LBM (kg)	55.7±11.9	50.2±12.5*	57.1±11.0	54.0±10.4*		
Body fat (%)	22.0±7.0	27.9±9.4*	30.2±8.1	27.8±7.7*		
$\dot{V}\text{O}_{2\text{max}}$ (ml kg ⁻¹ min ⁻¹)	39.6±7.8	26.3±6.9*				
Fasting PG (mmol/l)			9.1±3.2	5.3±0.5*	11.9±4.3	5.4±0.4*
2 h PG (mmol/l)			13.8±5.8	5.2±1.2*		
Fasting serum insulin (pmol/l)			105.7±78.5	48.6±26.5*		46.7±28.1
2 h serum insulin (pmol/l)			461.3±386.2	232.1±179.9*		

Data are expressed as means±SD

In order to adjust for the dependence between MZ and DZ twins, all comparisons of mean differences between age groups were performed using GEE methodology

DZ, dizygotic; LBM, lean body mass; MZ, monozygotic; PG, plasma glucose

* $p < 0.05$

Carlsbad, CA, USA). *COX7A1* mRNA levels were quantified by TaqMan real-time PCR with an ABI 7900 system (Applied Biosystems, Foster City, CA, USA) using gene-specific probes and primer pairs for *COX7A1* covering exon boundary 1–2 (Assays-on-demand, Hs00156989_m1; Applied Biosystems). Transcript quantity was normalised to the mRNA level of cyclophilin A (4326316E; Applied Biosystems). For each probe/primer set, a standard curve was generated, which was confirmed to increase linearly with increasing amounts of cDNA. The mRNA expression data are expressed as mean±SEM.

DNA methylation

Genomic DNA was isolated from muscle biopsies at the same time as RNA using the Tri Reagent kit according to the manufacture's instructions (Sigma-Aldrich). DNA bisulphite modification was accomplished on DNA from ten young and ten elderly twins using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulphite-modified DNA was amplified with primers designed using MethPrimer [26] (forward primer 5'-GGGGAGGAGTTGTATTGTTTT-3' and reverse primer 5'-CTCTATCCAAAACCCCAAAC-3'). A second, semi-nested PCR was performed using the forward primer 5'-TTTGTA AAAATGTATTTTTGGTAT-3'. This amplicon is 238 base pairs and part of the *COX7A1* 5' untranslated mRNA region, 64 bases upstream from the first coding exon (Fig. 1b). It includes 22 possible sites subject to DNA methylation (CpG sites) and is situated in a CpG island, i.e. a region of at least 200 base pairs with a GC content greater than 50% and with observed/expected CpG ratio greater than 60% [27]. The PCR products were cloned into plasmid vectors (pCR 4-TOPO; Invitrogen), further *Escherichia coli* were transformed and DNA from ten colonies of each individual muscle sample were isolated (QIAprep 8 Mini-

prep Kit; Qiagen, Valencia, CA, USA). The individual clones were sequenced and the number of methylated sites was determined using BiQ Analyser [28]. The proportion of methylation for each individual was calculated by dividing the total number of methylated sites in all clones by the total number of possible methylation sites. Of the 20 samples, six were also directly sequenced following bisulphite treatment of the DNA and analysed using ESME (epigenetic sequencing methylation analysis software; Epigenomics, Berlin, Germany) [29] to verify our results.

Genotyping

Genomic DNA was extracted from blood using conventional methods [30]. Native DNA was used for all genotyping except for the controls in the Malmö cohort, where whole genome amplified DNA (DECODE, Reykjavik, Iceland) was used. Genotype data covering the *COX7A1* locus on chr19:41323663–41345611 (10 kb upstream and 10 kb downstream of the *COX7A1* gene transcript) (Fig. 1a) was downloaded from the International HapMap project (www.hapmap.org; last accessed March 2008) [31] for the CEPH (Utah residents with ancestry from northern and western Europe) population. Only three SNPs with minor allele frequency >5% were found: rs11665903, rs753420 and rs7255180; the linkage disequilibrium (LD) structure between these SNPs was analysed using Haploview [32]. The LD relationship between the three SNPs was low ($r^2 < 0.04$) and all the *COX7A1* SNPs were therefore selected for genotyping. rs753420 is located in the 5' mRNA untranslated region, rs11665903 8.4 kb upstream and rs7255180 1.5 kb downstream from the *COX7A1* transcript (Fig. 1a). These SNPs were genotyped using a single based extension method adopted for a MassArray (Sequenom) platform in the Profiling Polygenic Disease Swegene laboratory at Lund University, Malmö, Sweden. Genotyping accuracy was determined by 8% re-genotyping with a concordance rate of 99.8% to 100%. To verify the method, a subset of the samples was also analysed using TaqMan SNP Genotyping Assays on the ABI7900 (Applied Biosystems).

Statistical methods

Generalised estimating equations Because of the strong intrapair correlation of twin data, conventional tests of differences between variable (y) means are not valid. To correct for this dependence, we used generalised estimating equations (GEE) methodology ($y = \alpha + \beta x$) to provide valid standard errors for the β -coefficients [33, 34]. The β -coefficient was estimated from all observations, whereas for calculation of the variance of β , each twin pair was considered as one cluster.

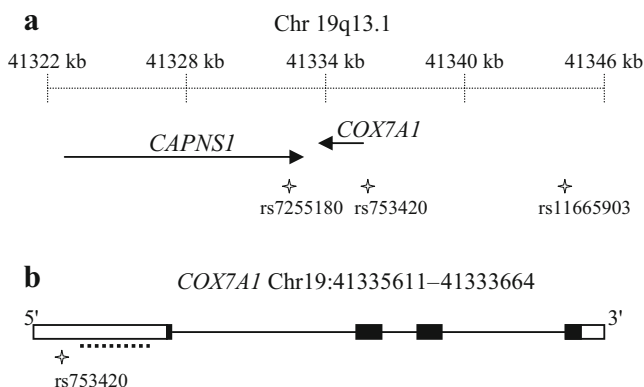


Fig. 1 Location of *COX7A1* on chromosome 19 and the region analysed for SNPs (a). b White boxes illustrate untranslated regions, black boxes represent coding exons, black lines show intron regions and the broken line shows the region analysed for DNA methylation

Linear regression To identify factors independently associated with the response variable, we used backward-elimination multivariate regression analysis with $p > 0.05$ as the defining criterion for exclusion of model terms. Also in this analysis we used GEE methodology to obtain valid tests.

Biometric modelling The total phenotypic variance is the sum of the variance attributable to the effects of both genetic and environmental factors. The degree of genetic and environmental influence of a variable can be estimated using biometric modelling. The models tested included the following parameters: genetic variance due to additive genetic effects (V_A) or dominant genetic effects (V_D) and environmental variance due to an individual environment not shared with co-twin (V_E) or a common environment shared among co-twins (V_C). Biometric modelling was conducted as previously described [35].

Association between genetic variation in the *COX7A1* gene and type 2 diabetes Power to detect association between SNPs in the *COX7A1* gene region and type 2 diabetes in our case–control studies was calculated using the Genetic Power Calculator [36]. With a minor allele frequency of 5%, a type 2 diabetes frequency of 6% and a relative risk of 1.3 at $\alpha = 0.05$, we have 38% power using a dominant model in the Botnia case–control study and a corresponding 93% power in the Malmö case–control study. To determine association of individual SNPs with type 2 diabetes, we performed logistic regression adjusted for BMI, sex and age-at-onset (patients) or age-at-visit (controls), assuming an additive model. Hardy–Weinberg p value, LD and haplotype block structure were calculated using Haploview [32].

DNA methylation We used a robust rank-order test [37] to test whether the total amount of DNA methylation in the analysed *COX7A1* region was increased in elderly compared with young twins. Ten old and ten young twins were analysed for DNA methylation; accordingly results were corrected for inter-twin dependence. Power to detect differences in DNA methylation was calculated to 73.9% ($\alpha = 0.05$) using DSS research statistical power calculator (www.dssresearch.com; Fort Worth, TX, USA).

Results

Heritability of *COX7A1* mRNA expression in skeletal muscle of twins The characteristics of the twins are described in Table 1. The degree of genetic and environmental influence on muscle *COX7A1* mRNA expression in skeletal muscle was estimated by biometric modelling in twins. The

heritability was estimated to be 50% in young and 72% in elderly twins, suggesting that both genetic and environmental factors influence *COX7A1* expression in muscle (Electronic supplementary material [ESM] Table 1).

***COX7A1* mRNA expression in skeletal muscle declines with age** The mRNA level of *COX7A1* was decreased in skeletal muscle from elderly ($n = 68$) compared with young ($n = 88$) twins, both in the basal and insulin-stimulated state (basal 1.00 ± 0.05 vs 1.68 ± 0.06 and clamp 1.04 ± 0.05 vs 1.71 ± 0.06 ; $p = 0.0005$) (Fig. 2). The euglycaemic–hyperinsulinaemic clamp did not influence the mRNA level of *COX7A1* either in young or in elderly twins (Fig. 2).

The level of DNA methylation in the *COX7A1* promoter region increases with age DNA methylation in the *COX7A1* promoter region was analysed in genomic DNA extracted from skeletal muscle of ten elderly and ten young twins. Figure 1b shows the location of the analysed sequence in the proposed *COX7A1* promoter, a sequence within a CpG island including 22 methylation sites. The degree of DNA methylation was higher in elderly than in young twins ($19.9 \pm 8.3\%$ vs $1.8 \pm 2.7\%$, $p = 0.035$; data median \pm SEM). Furthermore, a box-plot illustrating the variation in DNA methylation between individuals suggests that differences in DNA methylation become more pronounced with increasing age (Fig. 3). However, we did not observe any significant correlation between *COX7A1* DNA methylation and mRNA expression ($r = -0.33$, $p = 0.15$).

Genetic variation influences *COX7A1* expression in muscle The heritability estimates demonstrated that both genetic

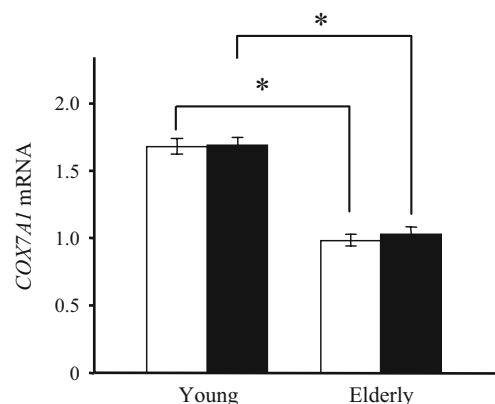


Fig. 2 Effects of age and insulin on human skeletal muscle *COX7A1* mRNA levels in 88 young and 68 elderly twins, before (white bars) and after (black bars) a euglycaemic–hyperinsulinaemic clamp. RNA was analysed for *COX7A1* mRNA expression together with the endogenous control cyclophilin A. The *COX7A1*:cyclophilin A ratio was calculated for each sample and the ratios are presented. Results are expressed as the mean \pm SEM. $*p \leq 0.05$

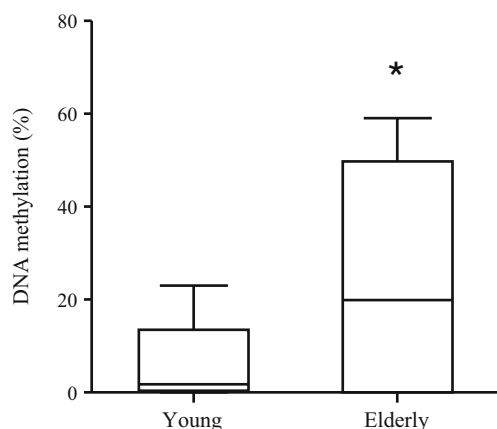


Fig. 3 Age-influenced DNA methylation in the *COX7A1* region in ten elderly compared with ten young twins. The boxes represent the 25th to 75th percentile with median value; upper whiskers show the maximum values. The difference between the two groups was calculated using a robust rank-order test. * $p < 0.05$

and non-genetic factors influence the expression level of *COX7A1* in muscle (ESM Table 1). In order to evaluate the genetic factors influencing *COX7A1* expression, three SNPs were genotyped in the twins and related to *COX7A1* mRNA expression in skeletal muscle. One polymorphism, rs753420, influenced basal *COX7A1* expression in muscle of young (T/T [$n=39$] 1.53 ± 0.08 , T/G [$n=42$] 1.71 ± 0.08 vs G/G [$n=7$] 2.3 ± 0.19 ; $p=0.0001$) but not of elderly twins (Table 2). SNPs rs11665903 and rs7255180 did not significantly influence basal *COX7A1* expression in either of the young or elderly twin groups (Table 2).

***COX7A1* polymorphisms and risk of type 2 diabetes** We also investigated whether common variants in the *COX7A1* gene are associated with increased risk of type 2 diabetes in

two independent case–control cohorts (Table 1). None of the three SNPs showed significant association to type 2 diabetes in the Botnia cohort. In the Malmö case–control cohort, one SNP (rs7255180) showed a nominal association to type 2 diabetes (OR 0.80 [0.66–0.97], $p=0.027$) for an additive model, but this did not stand correction for multiple testing (Table 3). A heterogeneity test excluded significant difference between the Botnia and the Malmö cohorts ($p=0.77$), and to increase power, we pooled our data from the two cohorts. However, even after pooling the two cohorts, we could not detect any genotype association to type 2 diabetes (Table 3).

***PGC-1 α* is associated with *COX7A1* mRNA expression in human skeletal muscle** A multivariate regression analysis was performed to test whether any of the following factors could influence *COX7A1* mRNA levels in skeletal muscle of the twins: *PGC-1 α* mRNA expression in skeletal muscle, age, sex and BMI (ESM Table 2). The final model was reached using backward selection regression. The level of *COX7A1* was positively associated with *PGC-1 α* expression (regression coefficient=0.27; $p=0.003$) and negatively associated with increased age (regression coefficient=−0.52; $p < 0.0001$) and female sex (regression coefficient=−0.21; $p=0.01$; ESM Table 2).

***COX7A1* expression is associated with in vivo glucose uptake and total body aerobic capacity in twins** We also tested whether *COX7A1* mRNA expression in skeletal muscle was associated with insulin-stimulated glucose uptake and total body aerobic capacity ($\dot{V}O_{2\max}$) using multivariate regression analysis including age, sex and BMI as covariates (Table 4). The level of *COX7A1* was positively related with glucose uptake ($p=0.01$) and $\dot{V}O_{2\max}$ ($p=0.001$).

Table 2 Association between SNPs in the *COX7A1* gene region and basal *COX7A1* mRNA expression in skeletal muscle of young and elderly twins

SNP ID	aa	n	aA	n	AA	n	p value ^a	
rs11665903	Young	1.58±0.07	54	1.88±0.1	31	1.49±0.3	3	0.07
	Elderly	1.04±0.07	37	1.10±0.09	23	0.93±0.14	9	0.25
rs753420	Young	1.53±0.08	39	1.71±0.08	42	2.3±0.19	7	0.0001 ^b
	Elderly	1.06±0.06	41	1.04±0.08	25	1.02±0.2	3	0.75
rs7255180	Young	1.71±0.06	74	1.54±0.14	14		0	0.41
	Elderly	1.06±0.05	61	0.96±0.14	8		0	0.55

Results are mean±SEM

a, major allele

^a p values are based on the best ANOVA result and have not been corrected for multiple testing

^b Post hoc test based on a recessive model

Table 3 Association between SNPs in the *COX7A1* gene locus and type 2 diabetes in case and control samples from the Botnia and Malmö cohorts

SNP ID	Major allele	HWP	MAF	Genotype frequency (case/control)			Odds ratio (additive model)	<i>p</i> value ^a
				Case/control	Case/control	aa aA AA		
rs11665903								
Botnia	A	1.0/0.20	0.310/0.279	0.476/0.508	0.428/0.424	0.096/0.068	1.14 (0.95–1.36)	0.15
Malmö	A	0.83/0.29	0.259/0.250	0.550/0.567	0.382/0.368	0.068/0.066	1.03 (0.94–1.14)	0.51
Botnia + Malmö	A	0.82/0.72	0.269/0.255	0.535/0.557	0.391/0.377	0.074/0.066	1.06 (0.98–1.15)	0.16
rs753420								
Botnia	T	0.73/0.85	0.279/0.282	0.524/0.515	0.395/0.401	0.081/0.084	0.98 (0.83–1.17)	0.85
Malmö	T	0.11/0.95	0.257/0.264	0.546/0.541	0.394/0.390	0.060/0.069	0.97 (0.89–1.07)	0.59
Botnia + Malmö	T	0.24/1.0	0.262/0.267	0.541/0.536	0.394/0.392	0.065/0.072	0.99 (0.91–1.08)	0.84
rs7255180								
Botnia	C	0.82/0.32	0.074/0.079	0.855/0.845	0.141/0.153	0.004/0.001	1.03 (0.76–1.39)	0.85
Malmö	C	0.44/0.90	0.046/0.054	0.912/0.895	0.085/0.103	0.003/0.003	0.80 (0.66–0.97)	0.027
Botnia + Malmö	C	0.68/0.20	0.052/0.058	0.900/0.887	0.097/0.111	0.003/0.002	0.89 (0.76–1.04)	0.15

Odds ratios were calculated for additive model using logistic regression adjusted for age, sex and BMI

^a*p* values shown have not been corrected for multiple testing

aa, reference genotype; HWP, Hardy–Weinberg *p* value; MAF, minor allele frequency

Discussion

The key findings of the present study were that age influences both promoter DNA methylation and gene expression of *COX7A1* in human skeletal muscle (Fig. 4). Our results demonstrate how the genome can be modified during life by DNA methylation, which theoretically could influence gene expression and thereby mitochondrial metabolism. It was also shown that the transcript level of this OXPHOS gene was positively associated with in vivo glucose uptake and $\dot{V}O_{2\max}$, although we were unable to

detect any significant association between *COX7A1* polymorphisms and type 2 diabetes.

COX7A1 was chosen as a candidate because it is among the *PGC-1 α* -responsive genes that are involved in OXPHOS and are coordinately downregulated in human diabetes [3]. A close relationship between *COX7A1* and *PGC-1 α* expression was confirmed in the present study. The prevalence of type 2 diabetes increases with age and is increased in individuals with insulin resistance. Oxidative

Table 4 Regulation of insulin-stimulated glucose uptake and $\dot{V}O_{2\max}$ in 110 young and 86 elderly twins

	Regression coefficient	<i>p</i> value
Glucose uptake ^a		
<i>COX7A1</i>	1.1	0.01
Sex	1.3	0.02
BMI	−0.36	<0.0001
$\dot{V}O_{2\max}$ ^b		
<i>COX7A1</i>	3.3	0.001
Age	−9.6	<0.0001
Sex	−6.1	<0.0001
BMI	−0.65	<0.0001

Using regression analysis, we tested whether the following factors correlated with insulin-stimulated glucose uptake and $\dot{V}O_{2\max}$: basal skeletal muscle *COX7A1* expression, age (young coded 0, elderly coded 1), sex (male coded 0, female coded 1) and BMI

^aIn mg (kg lean body mass)^{−1} min^{−1}

^bIn ml kg^{−1} min^{−1}

All final models: *p*<0.05

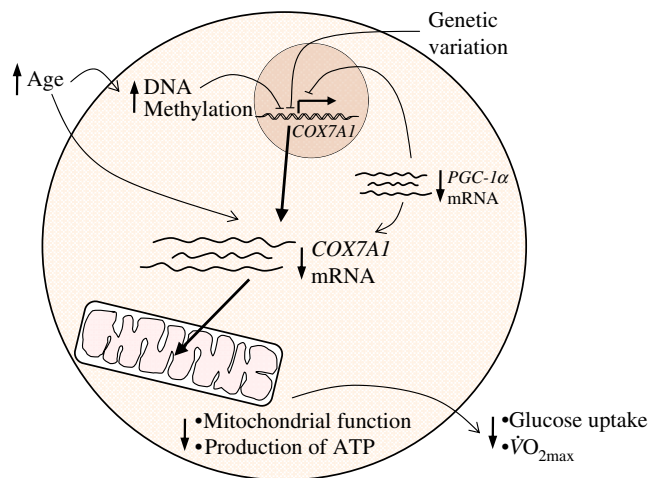


Fig. 4 Proposed mechanisms for regulation of *COX7A1* in human skeletal muscle and its effect on in vivo metabolism. According to our findings, age increases DNA methylation and decreases *COX7A1* mRNA expression. Furthermore, *COX7A1* mRNA expression is associated with genetic variation (SNP rs753420) and *PGC-1 α* mRNA expression. As a result this could affect in vivo metabolism, as *COX7A1* mRNA expression was positively associated with both insulin-stimulated glucose uptake and $\dot{V}O_{2\max}$

capacity in skeletal muscle has been shown to be a good predictor of insulin sensitivity [38]. We analysed the expression level of *COX7A1* in muscle biopsies from young and elderly non-diabetic twins to determine whether the level of this OXPHOS gene declines with age and is related to insulin sensitivity in vivo. The *COX7A1* mRNA level was reduced by 40% in the elderly twins, as compared with the 23% decrease observed in muscle of patients with type 2 diabetes [3]. A decline in *COX7A1* with age may contribute to increased insulin resistance, since elderly twins were more insulin-resistant than younger twins and *COX7A1* expression in muscle was associated with insulin-stimulated glucose uptake. Our results are thus in line with other reports demonstrating a decrease in mitochondrial OXPHOS activity [39] or expression of OXPHOS genes and insulin-stimulated glucose uptake in muscle of elderly individuals [13, 40, 41]. Collectively these studies demonstrate that age has a strong influence on expression of OXPHOS genes in human skeletal muscle.

Expression of *COX7A1* showed clear heritability in both young and elderly twins suggesting that OXPHOS expression is under genetic control. In support of this, the SNP rs753420 was associated with basal *COX7A1* mRNA expression in muscle. However, this did not translate into an increased risk of type 2 diabetes, as there was no significant association between SNPs in and around the *COX7A1* gene and type 2 diabetes. Although the case-control study was fairly well powered for the Malmö cohort, we cannot exclude association with lower odds ratios than 1.3. Recently a meta-analysis based on three type 2 diabetes WGAS was performed [42]. The results include data from 10,128 individuals and ~2.2 million SNPs. None of the three *COX7A1* polymorphisms investigated in our study showed association with type 2 diabetes in this meta-analysis.

It is assumed that DNA methylation of specific sites, particularly in the promoter region, may suppress the expression of the gene [43, 44]. Although the expression of *COX7A1* is tissue-specific, this gene is located in a CpG island, a feature which is commonly seen among housekeeping genes [6]. CpG islands are regions characterised by high C + G and CpG content, found in about 60% of human gene promoters. They are mainly unmethylated, although a subset can show tissue-specific methylation during development [45]. We recently showed increased DNA methylation in another OXPHOS gene, *NDUFB6*, in muscle of elderly compared with that of young twins; this increase in DNA methylation was associated with an age-related decrease in gene expression [13]. We therefore investigated whether this was also true for the *COX7A1* gene region.

Genomic DNA isolated from skeletal muscle was used to analyse DNA methylation in a region of the *COX7A1* gene previously suggested to be important for initiation of transcription [6, 46]. We detected an increased mean methylation rate in elderly compared with young twins. Also, there

was more variation within the group of the elderly twins, suggesting that the variation in DNA methylation between individuals becomes more pronounced with increasing age. Our findings are supported by previous results showing accumulation of epigenetic alterations with ageing [47–49]. Chalaya et al. [7] have previously compared the DNA methylation patterns in selected promoter regions in the *FXYD5–COX7A1* locus in relation to gene expression in human cell lines and tissues (cerebellum, liver, kidney and stomach). Whereas the housekeeping gene *COX6B* (also known as *COX6B1*) showed a clear correlation between the level of DNA methylation and transcription level, others did not.

We could not detect any CpG sites more prone to methylation than others; therefore we calculated the mean methylation level for each individual rather than analysing each specific methylation site. Unlike in our previous study [13], we did not observe a significant correlation ($r=-0.33$) between the level of DNA methylation and gene expression of *COX7A1*. This could be due to low power or the large variation in DNA methylation observed in the elderly, suggesting that non-genetic factors also might influence the age-related decline in *COX7A1* expression. However, the present study, together with our previous data [13, 50], does show that age can alter the level of DNA methylation, which theoretically then could influence gene expression and thereby metabolism. Furthermore, no known polymorphisms in the *COX7A1* gene region affect methylation sites.

In conclusion, we observed an age-related increase in DNA methylation of *COX7A1* as well as a decrease in expression of this gene in skeletal muscle of elderly individuals. Our data also propose a role for *COX7A1* in regulating in vivo glucose uptake and $\dot{V}O_{2\max}$ (Fig. 4).

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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