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Patient-derived fibroblasts indicate oxidative stress status and may justify antioxidant therapy in OXPHOS disorders

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ABSTRACT

Oxidative phosphorylation disorders are often associated with increased oxidative stress and antioxidant therapy is frequently given as treatment. However, the role of oxidative stress in oxidative phosphorylation disorders or patients is far from clear and consequently the preventive or therapeutic effect of antioxidants is highly anecdotic. Therefore, we performed a systematic study of a panel of oxidative stress parameters (reactive oxygen species levels, damage and defense) in fibroblasts of twelve well-characterized oxidative phosphorylation patients with a defect in the *POLG1* gene, in the mitochondrial DNA-encoded *tRNA-Leu* gene (m.3243A>G or m.3302A>G) and in one of the mitochondrial DNA-encoded NADH dehydrogenase complex I (CI) subunits. All except two cell lines (one POLG1 and one tRNA-Leu) showed increased reactive oxygen species levels compared with controls, but only four (two CI and two tRNA-Leu) cell lines provided evidence for increased oxidative protein damage. The absence of a correlation between reactive oxygen species levels and oxidative protein damage implies differences in damage prevention or correction. This was investigated by gene expression studies, which showed adaptive and compensating changes involving antioxidants and the unfolded protein response, especially in the *POLG1* group. This study indicated that patients display individual responses and that detailed analysis of fibroblasts enables the identification of patients that potentially benefit from antioxidant therapy. Furthermore, the fibroblast model can also be used to search for and test novel, more specific antioxidants or explore ways to stimulate compensatory mechanisms.

1. Introduction

Oxidative phosphorylation (OXPHOS) disorders are the most common group of inherited metabolic disorders characterized by a primary dysfunction of the OXPHOS system. The clinical and biochemical heterogeneity of OXPHOS disorders is partly due to the dual (mitochondrial and nuclear DNA) genetic control of mitochondrial energy production. Other factors that could explain this heterogeneity are only partly resolved, awaiting further insight in the pathophysiological processes. Recently, a number of papers have addressed the issue of oxidative stress in OXPHOS disorders (*e.g.* [1–8]). The electron transport chain in the mitochondria is considered the major source of reactive oxygen species (ROS), which are by-products of the redox reactions necessary to reduce NADH to NAD^+ [9]. At low levels, ROS behave as signaling molecules [10], but increased levels are damaging for DNA, proteins and lipids as well as detrimental for cellular function [11–14]. Therefore, cells are well equipped with antioxidant systems to control ROS levels. Oxidative stress occurs when the balance between pro-oxidants (ROS) and antioxidants is disturbed and antioxidants are no longer able to maintain normal physiological ROS levels.

Increased ROS production has been described in patients with clinically, biochemically [4,15,16] and/or genetically [17–19] diagnosed OXPHOS disorders, in cybrid models [6,8] and in cell lines where the respiratory chain was inhibited by chemicals [20]. The antioxidant status of tissues or cell cultures from OXPHOS patients [1,5,6,16,19,21] was

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investigated with variable and sometimes contradictory results. Only few studies report the total picture of oxidative stress in these patients, including ROS production, antioxidant defense and the eventual oxidative damage [6,19,22]. The goal of this study was to investigate the role of oxidative stress in three different genetically characterized OXPHOS disorders: patients with mutations in the *POLG1* gene, patients with a tRNA leucine (*tRNA-Leu*) mutation in the mtDNA (m.3243A>G and m.3302A>G) causing mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and patients with a mutation in one of the mtDNA encoded complex I subunits. Complex I deficiency and tRNA-Leu mutations have been associated with increased oxidative stress previously [19,23], although not all studies could confirm this [24]. Importantly, different parameters have been characterized to determine oxidative stress, including increased probe oxidation [23] combined with antioxidant expression [24] and increased oxidative damage, or combinations of the three [19]. For *POLG1* mutations, a couple of studies were performed in transgenic mice, but again with conflicting conclusions: transgenic mouse models carrying the D257A mutation in the exonuclease domain of pol γ did not increase oxidative stress [25,26] whereas transgenic mice with cardiac-targeted human mutant Y955C pol γ, affecting the polymerase domain, did [27].

Our study aimed at investigating parameters including ROS levels, ROS detoxification (glutathione levels, antioxidant gene expression) and oxidative damage (protein carbonyls) in patient-derived fibroblasts as a model system. Because the genetic defect might not stress the cells to such an extent that relevant disease-associated changes can be picked up, fibroblasts were deprived of glucose to stimulate energy production through oxidative phosphorylation. The changes due to the genetic defect were corrected for the different culture conditions. The suitability of this model has been demonstrated before [24,28]. This patient-derived model will help to resolve the individual, underlying adaptive mechanisms in each patient, thereby identifying the subgroup of patients that will most likely benefit from antioxidant treatments or will be able to test the efficacy of new targeted candidate antioxidants.

2. Materials and methods

2.1. Patient fibroblast cell lines

Fibroblasts were derived from skin biopsies of five patients with a *POLG1* mutation, four with a tRNA-Leu (m.3243A>G or m.3302A>G) MELAS mutation, three with a mutation in an mtDNA encoded complex I subunit, and three controls (Table 1). Fibroblasts were routinely cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, 0.2 mM uridine (Acros, Geel, BE), penicillin and streptomycin. To stimulate energy production by oxidative phosphorylation, fibroblasts were cultured for 72 h without glucose in the presence of galactose. Galactose medium consisted of DMEM without glucose supplemented with 5.5 mM galactose (Sigma, Zwijndrecht, Netherlands), 20% FBS, 0.2 mM uridine, penicillin and streptomycin [29]. Measurements were performed in primary cell cultures between passage 7 and 20. Except for the quantification of reactive oxygen species, cultures from the same cell line and condition were pooled and the cell pellet was divided for the different assays.

2.2. Quantification of reactive oxygen species

Reactive oxygen species (ROS) levels were measured as described previously [23]. In short, fibroblasts were incubated in HEPES–Tris medium (132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 5.5 mM D-glucose or galactose, pH 7.4), containing 10 μM hydroethidine (HEt; Molecular Probes, Paisley, UK) for 10 min at 37 °C. The reaction was stopped by thorough washing of the cells with PBS. Culture dishes were mounted in an incubation chamber placed on the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, DE) equipped with a Zeiss 40×/1.3 NA fluor objective. The cells were excited at 490 nm using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, DE). Fluorescence emission light was directed by a 525DRLP dichroic mirror filter (Omega) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific, Vianen, NL) with an acquisition time of 100 ms. Hardware was controlled with Metafluor 6.0 software (Universal Imaging Corporation, Downingtown, PA, USA). Processing and analysis of fluorescence images was performed with MetaMorph 6.1 (Universal Imaging Corporation). All cell lines were examined on at least two and if possible up to four different days. In total, at least 150 cells (range 156–568) were analyzed on each day for each cell line and condition.

2.3. Preparation of lysates for glutathione measurements and protein carbonyls

Cell pellets were resuspended in ice-cold extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5) and homogenized with a Teflon pestle. After sonication and 2 freeze-thaw cycles, the suspension was centrifuged for 4 min at 3000 *g* (4°) and

Table 1
Patient characteristics for primary fibroblast cell lines.

Cell line	Sex	Age at biopsy (years)	Passage number	Group[a]	Mutation(s)[b]	OXPHOS complex activity [c]
2862S	F	24	18	POLG	p.467A>T HOM	CI 67, CII 125, CIII 108, CIV 87, CS 93
06E0703	F	16	18	POLG	p.467A>T HOM	CI 54, CII 141, CIII 79, CIV 84, CS 71
00E0741	F	<1	18	POLG	p.227R>P+p.467A>T	CI 34, CII 76,CIII 31, CIV 50, CS 227
3591	M	8	19	POLG	p.305S>R+p.467A>T	CI 50, CII 86, CIII 57, CIV 89, CS 142
05E0536	F	1	19	POLG	p.467A>T+p.957A>P	CI 63, CII 81, CIV 57, CS 150
2400	M	27	15	tRNA-Leu	m.3243A>G (80%)	CI 43, CII 86, CIII 67, CIV 80, CS 125
1933	M	27	11	tRNA-Leu	m.3243A>G (90%)	CI 50, CII 84, CIII 60, CIV 79, CS 117
2830	F	19	8	tRNA-Leu	m.3243A>G (83%)	CI 57, CII 86, CIII 77, CIV 60, CS 100
1330	M	34	12	tRNA-Leu	m.3302A>G (50%)	CI 62, CII 100, CIII 79, CIV 83, CS 108
3765	M	5	10	CI	ND1, m.3890G>A (80%)	CI 34, CII 69, CIII 72, CIV 114, CS 160
2181	M	3	14	CI	ND5, m.13042G>A (86%)	CI 50, CII 90, CIII 59, CIV 70, CS 110
1682	F	4	8	CI	ND5, m.13511A>T (60%)	CI 52, CII 94, CIII 91, CIV 115, CS 105
C0388			19	Control	–	n.a.
C0407			19	Control	–	n.a.
C2244			14	Control	–	n.a.

[a] Patient cell lines are grouped according to their genetic defect: polymerase gamma mutations (POLG), mtDNA tRNA leucine mutation with MELAS phenotype (MELAS) and mtDNA complex I subunit mutation (CI).
[b] Mutations are given at the protein level for POLG and at the mtDNA level for MELAS and CI, the mutation percentage of the mtDNA mutations is given between ( ).
[c] The activities of the different OXPHOS complexes are expressed as the percentage of a control population, normalized to citrate synthase (CS) activity. CI = complex I, CII = complex II, CIII = complex III, CIV = complex IV. n.a. = not applicable.

the supernatant was stored at −70° until further use. Protein concentrations were determined using the Bio-Rad Protein Assay.

2.4. Glutathione measurement

Reduced (GSH) and oxidized (GSSG) glutathione were measured in the cell lysates as described in [30].

2.5. Protein carbonyl detection

To detect oxidative modifications of proteins, the Oxyblot™ Protein Oxidation Detection kit (Millipore, Amsterdam, NL) was used. After derivatization of the carbonyl groups in the cell lysates with di-nitrophenylhydrazone, oxidatively modified proteins were detected by immunoblotting according to the manufacturer's protocol in two replicates. Results were normalized to beta-actin protein levels in different lanes of the same gels.

2.6. Oxidative stress related gene expression

The expression levels of genes involved in oxidative stress and inflammation were measured using the OxyGenes™ microarrays (Probiox SA, Liege, BE) [31]. RNA was isolated from cell pellets with the High Pure RNA Isolation kit (Roche, Woerden, NL). RNA quantity and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was assessed by determining the RNA 28S/18S ratio using the Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). 2 μg of RNA was reverse transcribed into cDNA according to the 3DNA Array 900 protocol (Genisphere, Hatfield, PA, USA). Next, cDNA was hybridized on the OxyGenes™ slides overnight, followed by washing steps and hybridization of Cy™ 3-labeled 3DNA capture reagent according to the manufacturer's procedures (Genisphere). To assess the raw probe signal intensities, slides were scanned using the LS Reloaded laser scanner (Tecan, Männedorf, CH) with gain settings 160, 180 and 200 and analyzed using Array-Pro analyzer software (MediaCybernetics, Bethesda, USA) or scanned using the Agilent High-Resolution Microarray Scanner (Agilent Technologies, Amstelveen, NL) and analyzed using Feature Extraction 10.7 software (Agilent Technologies).

2.7. Data analysis

The data were analyzed using multivariate Gaussian linear regression including a one (protein carbonyls and Oxygenes™) or two level (ROS levels) random effect to take into account the dependence among observations from the same subject (all) and the dependence among the different measurement days (ROS levels). For the ROS level analysis, the medium (glucose and galactose) and the group (control, complex I, tRNA-Leu and POLG) were included during the analysis as well as interactions among these when required. For the protein carbonyl analysis, the reference protein (beta-actin), concentration, day, gel number, medium and group were included. The inference criterion used for comparing the models is their ability to predict the observed data, *i.e.* models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) [32]. First, all cell lines were analyzed together in the patient groups indicated and, next, each cell line was also investigated separately. In each case, the relevant group differences were reported if the model with the smallest AIC contained any combination of one or more groups. For the Oxygenes™ analysis, one of the 30 housekeeping genes, the pooled sample, the background intensity, the scanner used, the medium and group were included during the analysis as well as interactions among these when required. Similarly to the ROS and protein carbonyl analysis, the AIC was used to assess whether there was a group effect. First, each gene was analyzed for all cell lines in the patient groups together and then the analysis was also repeated for all genes but considering each cell line separately. In each analysis, the relevant group differences were reported if the model with the smallest AIC contained any combination of one or more groups. Importantly, the reported fold changes are estimated values by the models, taking into account both the effect of the mutation and the medium. Glutathione data was analyzed using the univariate analysis of variance in the SPSS software package.

3. Results

3.1. Increased ROS levels in most of the patient cell lines

ROS levels, quantified by the oxidation rate of hydroethidine (HEt), were assessed in the control and patient cell lines under glucose and galactose conditions (Table 2). Unlike the fold changes for the patient groups, analysis of individual cell lines and controls did not always estimate a medium effect, *i.e.* a different fold change for the glucose and galactose condition, due to low power. In these cases, the fold changes represent the difference between the specific patient and control fibroblasts regardless of the medium. Except for two cell lines, one *POLG1* mutant (06E0703; p.467A>T homozygote) and one tRNA-Leu mutant (1330; m.3302A>G), all other cell lines showed increased HEt oxidation compared with control cell lines in glucose and galactose medium. HEt oxidation was highest in the CI group, although one of the *POLG1* cell lines (05E0536; p.467A>T + p.957A>P) presented with the highest HEt oxidation rate. Although the main focus was on changes due to the mutation and not the differences between culture conditions, six cell lines showed a larger fold change for HEt oxidation (versus controls) in galactose compared with glucose. Galactose medium also induced a 1.53 fold increase in HEt oxidation in controls (Table 2). Furthermore, an aliquot with a different passage number of cell lines C0388, 2181 and 1682 was thawed at a different time point to check the assay reproducibility. The same results were obtained (results not shown).

3.2. Oxidative damage

The levels of protein carbonyls, an index for oxidative protein damage, were significantly elevated in three patient cell lines cultured with glucose and galactose: tRNA-Leu cell lines 2400 (80% m.3243A>G) and 1933 (90% m.3243A>G) and CI cell line 3765 (80% m.3890G>A). CI cell line 1682 (60% m.13511A>T) only showed increased levels of protein carbonyls compared with controls when cultured with galactose medium (Table 3).

3.3. Stress-related gene expression and antioxidant defense

To examine ROS-related gene expression changes, the transcription levels of stress-related genes were determined using OxyGenes ™ microarrays. Of the 165 genes on the microarray, 38 genes showed more than 20% difference in expression above the background level in at least one of the groups and one of the culture media. Of these 38 genes, 10 were altered due to the genetic defect only, as their expression was not influenced by the culture medium in controls and patients (Table 4). Therefore these were considered specific for the genetic defects and included, amongst others, heat shock protein (*HSPA1A*), oxidative stress related (*SOD1*, *PON2* [33,34], *MT1M* [35,36]) and inflammatory (*ICAM1*, *IL2RG*, *TNFRSF1B*) genes. The expression of the remaining 28 genes was altered due to both the culture medium and genetic defect (Supplementary Table 1). The POLG group showed the highest number of differentially expressed genes compared with controls, both in glucose and galactose medium. The galactose medium induced larger expression differences compared to controls of 17 stress-related genes in the POLG group (Supplementary

Table 1). In contrast, the tRNA-Leu and CI groups showed less differentially expressed genes, both in glucose and galactose (Supplementary Table 1). A closer examination of the gene expression changes of classical antioxidant genes in the individual cell lines (Table 5) revealed an inconsistent picture. On the one hand, genes could behave comparable in multiple cell lines of one or more patient groups in one or both culture conditions (*e.g. GSR* in POLG glucose and galactose, *GPX1* in tRNA-Leu galactose, *SOD1* in tRNA-Leu and CI galactose). Whereas, on the other hand, their expression changed differently, either up or down, in cell lines of the same group in one or both culture conditions (*e.g. GPX1* in POLG glucose and galactose, *SOD1* in tRNA-Leu and CI glucose) (Table 5).

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured to evaluate the oxidative status of the cell lines as indicated by their ratio (Fig. 1). Univariate analysis of variance did not detect any significant difference between the groups or culture conditions. As can be appreciated from Fig. 1, there was a large variation between cell lines of patients within one group, indicating patient-specific effects. Three patient cell lines (tRNA-Leu 1933, CI 3765 and CI 2181) showed a GSH/GSSG ratio below the control range in the glucose situation, indicating a more oxidized state. In addition, in galactose medium all tRNA-Leu cell lines and CI cell line 3765 were in a more oxidized state (lower GSH/GSSG ratio) than the control range (Fig. 1). *POLG1* cell lines seemed to be in a more reduced state than controls, especially in the galactose condition.

3.4. Correlations between different parameters

To investigate correlations between the different parameters (ROS levels, oxidative protein damage, GSH/GSSG ratio and classic antioxidant gene expression) and between these parameters and sex and age, a correlation matrix was created (Table 6). Statistical significant correlations (p value$<$0.05) were only detected between ROS levels and *CAT* expression fold changes (Pearson's r −0.508) and protein carbonyl levels and *GPX1* expression fold changes (Pearson's r 0.454).

Table 2
Statistical analysis of ROS levels as measured by HEt oxidation.

Cell line	Glucose mean (C.I.)	Galactose mean (C.I.)
Comparing patient groups		
Control (3 cell lines)	1.00	**1.53** (1.49–1.57)[a]
POLG	**1.47** (1.12–1.82)	**1.71** (1.34–2.18)
tRNA-Leu	**1.48** (1.14–1.83)	**1.72** (1.35–2.19)
CI	**1.87** (1.47–2.27)	**2.28** (1.73–3.01)
Comparing cell lines		
POLG		
2862S	**1.36** (1.14–1.63)	**1.96** (1.65–2.34)
06E0703	1.12 (0.96–1.30)	
00E0741	**1.18** (1.04–1.33)	
3591	**1.51** (1.35–1.70)	
05E0536	**2.51** (2.13–2.95)	**4.80** (4.08–5.65)
tRNA-Leu		
2400	**1.45** (1.28–1.64)	
1933	**1.30** (1.12–1.51)	**1.92** (1.65–2.23)
2830	**1.74** (1.46–2.08)	**2.52** (2.10–3.03)
1330	1.10 (0.96–1.25)	
CI		
3765	**1.61** (1.37–1.90)	**2.38** (2.01–2.83)
2181	**2.31** (1.98–2.69)	
1682	**1.36** (1.20–1.54)	

HEt oxidation expressed as fold change compared with control in the respective culture medium. Values are estimated fold changes for patients versus controls provided by the model, taking into account both the effect of the mutation and the medium. Values for the patient groups and the individual cell lines were estimated in separate analyses. If the fold change was not significantly different for a cell line between both media, the fold change of the best fitting model was estimated for glucose and galactose together. Values in bold differ significantly from control in the respective medium or glucose and galactose together.

[a] Versus control glucose, only applies for the patient groups comparison.

Table 3
Statistical analysis of oxidative protein damage (protein carbonyls).

Cell line	Glucose mean (C.I.)	Galactose mean (C.I.)
Comparing patient groups		
Control (3 cell lines)	1.00	
POLG (5 cell lines)	1.07 (0.94–1.21)	
tRNA-Leu (4 cell lines)	**1.30** (1.13–1.48)	
CI (3 cell lines)	**1.22** (1.06–1.41)	
Comparing cell lines		
POLG		
2862S	1.07 (0.76–1.50)	
06E0703	0.93 (0.81–1.08)	
00E0741	1.14 (0.96–1.35)	
3591	1.15 (0.92–1.43)	
05E0536	0.92 (0.73–1.66)	
tRNA-Leu		
2400	**1.25** (1.06–1.47)	
1933	**1.56** (1.29–1.89)	
2830	1.26 (0.90–1.77)	
1330	1.14 (0.94–1.39)	
CI		
3765	**1.36** (1.07–1.74)	
2181	1.08 (0.96–1.22)	
1682	1.12 (0.76–1.65)	**2.22** (1.79–2.75)

Protein carbonyls as measured using the Oxyblot assay, expressed as fold change compared with control in the respective culture medium. Values are estimated fold changes for patients versus controls provided by the model, taking into account both the effect of the mutation and the medium. Values for the patient groups and the individual cell lines were estimated in separate analyses. If the fold change was not significantly different for a cell line or group between both media (due to less power compared with the analysis of the groups), the fold change of the best fitting model was estimated for glucose and galactose together. Values in bold differ significantly from control.

4. Discussion

The goal of this study was to characterize different oxidative stress parameters in three groups of patients with a genetically characterized mitochondrial disorder caused by mutations either in the *POLG1* gene or

Table 4
Statistical analysis of stress-related gene expression changes due to disease without medium effect.

Gene	FC CI *vs* control	FC POLG *vs* control	FC tRNA-Leu *vs* control	FC All *vs* control
HSPA1A				3.39 (2.80–4.09)
ICAM1		1.58 (1.33–1.87)		
IGFBP3		1.40 (1.35–1.45)		
IL2RG	0.68 (0.56–0.82)	1.27 (1.03–1.56)	0.68 (0.56–0.82)	
MT1M		2.36 (2.07–2.69)		
PON2				0.58 (0.38–0.89)
SIRT1		2.60 (1.36–4.96)		
SOD1		1.99 (1.71–2.31)		
SOS2		1.38 (1.27–1.50)		
TNFRSF1B	0.67 (0.59–0.76)			

Fold changes of stress-related genes in patients versus controls as measured by the OxyGenes™ microarrays. Fold changes were the same for glucose and galactose medium. Values are estimated fold changes for patient groups versus controls provided by the model, taking into account both the effect of the mutation and the medium. If the fold change was not significantly different for all patient groups, the fold change of the best fitting model was estimated for three groups together and provided in the 'All *vs* control' column. Only significant results are shown. 95% confidence intervals for the fold changes are shown between brackets.

Table 5
Statistical analysis of antioxidant gene expression for each cell line.

Gene	CAT		SOD1		GSR		GPX1	
Cell line	Glu	Gal	Glu	Gal	Glu	Gal	Glu	Gal
POLG								
2862S		1.37	2.35	19.05		2.42	0.77	2.50
06E0703		1.36			1.21			
00E0741	1.29				1.22		1.51	0.58
3591						1.46		
05E0536							0.73	
tRNA-Leu								
2400			14.35	0.64	1.75			0.54
1933								0.67
2830			0.56			2.64		0.42
1330							0.75	0.51
CI								
3765		1.40						
2181			4.11	0.54		1.17		0.79
1682			0.61					1.29

Fold changes are given relative to the average control (glucose or galactose). Values are estimated fold changes for patients versus controls provided by the model, taking into account both the effect of the mutation and the medium. If the fold change was not significantly different for a cell line between both media, the fold change of the best fitting model was estimated for glucose and galactose together. Blank cells indicate no (<10% change) or a non-significant difference compared with controls.

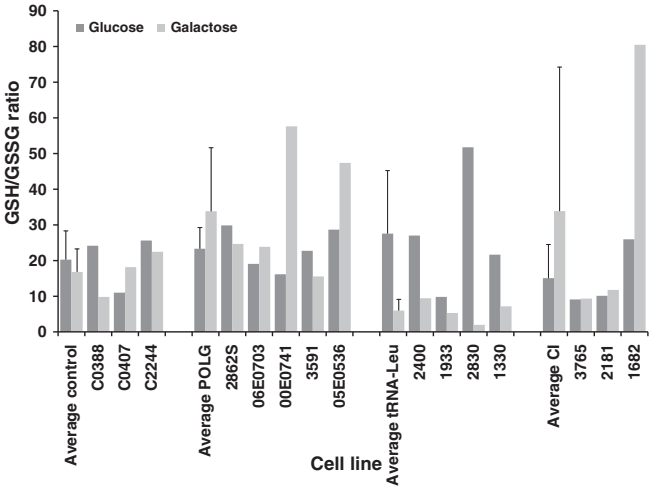

Fig. 1. Ratio of reduced (GSH) to oxidized (GSSG) glutathione levels for each patient group and individual cell line cultured in glucose and galactose.

in mtDNA encoded complex I and tRNA leucine genes. Fibroblast cells predominantly use glycolysis for energy production when cultured with high glucose availability [24,37,38]. Hence, cell lines were forced to derive ATP from OXPHOS using glucose-free galactose medium [39]. An overview of the results is shown in Table 7.

4.1. Increased ROS levels in the majority of primary fibroblasts of OXPHOS patients

ROS levels were increased in most patient-derived cell lines, both when cultured with glucose and galactose medium (Table 7). The moderate increase in ROS levels in control fibroblasts due to the replacement of glucose by galactose might reflect increased basal OXPHOS-related ROS production due to the switch from glycolysis to OXPHOS for energy production and the somewhat more oxidized cellular redox state (2/3 control cell lines, Fig. 1). Our results indicated that ROS levels could already be increased by quite moderate OXPHOS deficiencies (*e.g.* CI activity lower than 70% of the average control activity), as shown for most fibroblasts in this study and previously [23]. The authors have shown before using the same protocol that HEt oxidation occurs with inhibition of complex I and III but not complex V [23,40] and that similar results can be obtained with the MitoSOx Red and CM-H2DCF probes [41]. A drawback is that method cannot distinguish the exact site(s) of HEt oxidation and that the exact mechanism therefore remains speculative [23]. HEt is oxidized by superoxide and non-superoxide free radicals, yielding two different products [42] which are difficult to distinguish using fluorescence microscopy [43,44]. Therefore, we consider the results as 'ROS levels' instead of superoxide. Other proteins that might influence the measured ROS levels are alpha-ketoglutarate dehydrogenase in the tricarboxylic acid cycle [45,46] and NADPH oxidases (NOX) [47], all suggested to be influenced by the redox status of cells [48]. Therefore, genetically defined OXPHOS deficiencies might not only increase ROS levels by electron leakage at the OXPHOS complexes but also by altering the NAD^+/NADH ratio.

4.2. Adaptive processes in fibroblasts of OXPHOS patients

Most cell lines showed increased ROS levels but no increased oxidative protein damage. Stress-induced adaptive gene expression changes, especially in the *POLG1* cell lines, might account for these findings. The existence of such a delicate balance is supported by the presence of oxidative damage in one CI mutant fibroblast cell line in galactose but not glucose medium, associated with higher ROS levels. The increased expression of stress-inducible *HSPA1A, DNAJB1* and *HSF2* in the glucose and/or galactose condition in patient cell lines pointed to a stress condition induced by the genetic defect. The induction of these genes indicates activation of the unfolded protein response to prevent or repair oxidative damage. However, repair processes are highly energy demanding. Therefore, probably also the remaining energy capacity of a defect OXPHOS system plays a role in the faith of cells. Recently, training has been shown to be beneficial for improving oxidative metabolism in muscle [49] and to induce antioxidant-related adaptations, enhancing the ability to cope with oxidative stress [50]. Thus, possibly also the physical activity or training of patients plays a role in how well their antioxidant systems will be able to cope with or adapt to the increased ROS levels caused by their genetic defect. The main specific results for each patient group are being discussed here in more detail.

4.2.1. Patients with mutations in mtDNA encoded complex I subunits

The complex I group presented the highest ROS levels, accompanied with most oxidative protein damage (2/3 cell lines) and only few changes in antioxidant gene expression (Table 7). Interestingly, the CI cell line with the highest ROS levels was the cell line without oxidative damage, indicating sufficient adaptation to or compensation of the defect which was insufficient in the other two cell lines. Increased ROS levels have been detected previously in cells or tissues of patients with a genetically (nuclear [23] or mitochondrial DNA [16]) and/or functionally [4] characterized CI deficiency. The finding is not surprising as CI is one of the major ROS sources in mitochondria [51,52]. Luo et al. observed increased lipid peroxidation in fibroblasts of patients with CI deficiency [53], but this was not detected in another study by Verkaart et al. [15]. The only publication reporting on oxidative protein damage in CI deficiency involved cybrids carrying the 3460, 11778 and 14484 Leber mutations showing increased protein carbonylation [54]. This nicely supports our results. In contrast to the lymphoblast cell lines from biochemically characterized CI deficient patients with predominantly (5/6 cell lines) homoplasmic mtDNA mutations [16], we did not detect consistently increased expression of *SOD*, *CAT*, *GPX* and *GST* in our patients with a genetic defect in mtDNA CI genes [16]. However, in their study, increased expression of the genes in the presence of elevated ROS levels was not correlated with increased expression of the corresponding proteins and they actually measured lower antioxidant activity of these enzymes, except for SOD1. Nevertheless, they concluded, mainly based on

Table 6
Correlation matrix.

	ROS levels	Protein carbonyl level	GSH/GSSG ratio	*CAT* expression FC	*SOD1* expression FC	*GSR* expression FC
ROS levels						
Protein carbonyl level	−0.158					
GSH/GSSG ratio	0.076	0.334				
CAT expression FC	**−0.508**	−0.197	−0.101			
SOD1 expression FC	−0.001	−0.050	0.020	0.227		
GSR expression FC	0.258	−0.170	−0.197	−0.160	0.400	
GPX1 expression FC	0.225	**0.454**	0.401	−0.177	0.327	0.234

Correlation matrix for ROS levels, protein carbonyl levels, the GSH/GSSG ratio and fold changes of classic antioxidant genes. The Pearson correlation values produced by SPSS are shown; significant correlations are depicted in bold ($p<0.05$).

the enzyme data, that ROS levels and alteration of ROS scavenging enzymes were good parameters to assign antioxidant therapy, even without measuring oxidative damage parameters. The absence of antioxidant enzyme activity measurements is a limitation of our study, however, the oxidative damage parameter, which we did examine, is a more appropriate biomarker to estimate the eventual consequences of increased ROS levels in fibroblasts. Antioxidant markers are more a measure of endogenous protection and not of damage.

4.2.2. Patients with mutations in tRNA leucine genes

All three cell lines with the m.3243A>G mutation (80–90% heteroplasmy) experienced increased ROS levels, whereas the cell line with the m.3302A>G mutation (60%) did not (Table 7). In this study with primary patient fibroblast cell lines, the lack of increased ROS levels in the m.3302A>G cell line is likely due to the lower mutation percentage but a different molecular mechanism compared with the m. 3243A>G tRNA-Leu mutation cannot be excluded [55]. The m.3302A>G and m.3243A>G tRNA-Leu mutations have been shown to increase superoxide production in homoplasmic cybrid models with deficiencies of OXPHOS complexes I, III, IV and V [56], whereas in our group complex I deficiency was most prominent, and in muscle of patients with the m.3243A>G mutation [19]. Oxidative protein damage was observed in two m.3243A>G cell lines. Again, the cell line with the highest ROS levels did not show oxidative damage in galactose. For the tRNA-Leu patients, only few studies previously reported on oxidative damage. One study detected increased protein carbonylation in muscle biopsies [19] while two other studies did not observe increased oxidative damage in cybrids carrying the homoplasmic 3243A>G [6,55] or 3302A>G [55] mutations.

Table 7
Summarizing overview of oxidative stress parameters for each cell line.

Cell line	Mutation	OXPHOS complexes	Culture	ROS levels	Oxidative damage	Antioxidant response	Benefit from antioxidant therapy?*
POLG							
2862S	p.467A>T HOM		glu	↑		↑ SOD1 , ↓ GPX1	Follow-up endogenous protection
			gal	↑↑		↑ CAT, ↑↑ SOD1, ↑ GSR, ↑ GPX1	
06E0703	p.467A>T HOM		glu			↑ GSR	No
			gal			↑ CAT, ↑ GSR	
00E0741	p.227R>P p.467A>T	↓	glu	↑		↑ CAT, ↑ GSR, ↑ GPX1	Follow-up endogenous protection
			gal	↑		↓ GPX1	
3591	p.305S>R p.467A>T		glu	↑			Follow-up endogenous protection
			gal	↑		↑ GSR	
05E0536	p.467A>T p.957A>P		glu	↑↑		↓ GPX1	Probably
			gal	↑↑↑		↓ GPX1	
tRNA-Leu							
2400	80% m.3243A>G	↓	glu	↑	↑	↑↑ SOD1, ↑ GSR	Yes
			gal	↑	↑	↓ SOD1, ↓ GPX1	
1933	90% m.3243A>G		glu	↑	↑		Yes
			gal	↑↑	↑	↓ GPX1	
2830	83% m.3243A>G		glu	↑		↓ SOD1	Probably
			gal	↑↑		↓ SOD1, ↑ GSR, ↓ GPX1	
1330	60% m.3302A>G		glu			↓ GPX1	No
			gal			↓ GPX1	
CI							
3765	80% m.3890G>A	↓	glu	↑	↑		Yes
			gal	↑↑	↑	↑ CAT	
2181	86% m.13042G>A		glu	↑↑		↑ SOD1	Probably
			gal	↑↑		↓ SOD1, ↑ GSR, ↓ GPX1	
1682	60% m.13511A>T		glu	↑		↓ SOD1	Yes
			gal	↑	↑	↓ SOD1, ↑ GPX1	

*based on the combination of ROS levels, oxidative damage and antioxidant gene expression in fibroblasts. Cell color code: yellow = no significant change compared with control, red = negative (increased ROS, increased damage, insufficient defense) change compared with control, green = positive (antioxidant response) change compared with control.

There have been no reports on oxidative stress/damage in mutant tRNA-Leu fibroblasts so far.

Our results differ partly from previous studies on antioxidant enzymes in MELAS patients, reporting increased gene expression and activities of SOD, CAT and GPX, although studies are difficult to compare due to differences in cell types, mutation load and analytical approaches [5,6,57]. In one study, the mutation load was considerably lower (<45% heteroplasmy) than in our fibroblasts and myoblast cultures were used [5] and ROS levels, were not determined in these myoblast cultures. Therefore, it is not certain that the gene expression changes resulted from increased ROS production related to the mtDNA mutations. A second study did not quantify their immunohistochemical results in muscle [57], making it difficult to judge and compare the results. Finally, a third study used homoplasmic mutation load and cybrid models [6] instead of primary patient material. Although there is a possible interference of the nuclear cancer background here, the increased expression of antioxidant genes nicely correlates with the lack of oxidative damage. Altogether, all studies point to increased ROS production, whether or not compensated by antioxidant defenses. These studies emphasize the importance of standardization and examining different aspects of oxidative stress to be able to compare the results of different studies, especially if heterogeneous patient populations or different model systems with different adaptive capacities are being used.

4.2.3. Patients with mutations in the POLG1 gene

All except one *POLG1* cell line showed increased ROS levels. *POLG1* cell line 06E0703 has the same mutation as cell line 2862S, while only the latter showed increased ROS levels (Table 7). This may be related to the random effect of pol γ on mtDNA stability and point mutations leading to many, random low level heteroplasmy mutations in contrast to the high heteroplasmic CI and tRNA-Leu mutations. As suggested by the multiple gene expression changes and glutathione results, the *POLG1* cell lines were able to adaptively activate the antioxidant or repair systems associated with increased endogenous ROS levels [58]. No human cells or tissues with *POLG1* mutations have been evaluated for oxidative stress parameters so far. In POLG mutator mice with proofreading deficient D268A polymerase resulting in the random accumulation of mtDNA mutations, no or only mildly increased ROS production and no oxidative damage could be detected in several tissues (liver, heart, muscle) by two groups [25,26]. Contrastingly, a third group examining the same mice, did observe increased oxidative protein damage in the heart which could be attenuated by overexpression of mitochondrial catalase [59]. Additionally, in transgenic mice with cardiac-targeted human mutant Y955C pol γ, affecting the polymerase domain, increased oxidative DNA damage associated with decreased mtDNA copy number was observed [27]. In concordance with the latter publications, the current study implies that ROS levels are increased in most *POLG1* patients and therefore their antioxidant levels and oxidative damage markers should be followed up to supplement them with antioxidants when their endogenous system starts failing.

4.3. Correlations between different oxidative stress parameters

A consistent correlation was observed between classic antioxidant genes and the other oxidative stress parameters. ROS levels were inversely correlated with *CAT* expression and protein carbonyl levels were correlated with *GPX1* gene expression. ROS levels have shown previously to inhibit catalase directly [60], whereas *GPX1* was crucial for protection against protein oxidation in mice [61], both of which might explain the observed correlations. Therefore, the expression of these genes might serve as biological biomarkers, although this remains to be confirmed in a larger patient cohort.

5. Conclusions

In this study using fibroblasts of patients with different genetically characterized OXPHOS disease, it was shown that in the majority ROS was increased and could lead to oxidative damage in different OXPHOS disorders, although not in every patient-derived fibroblast. This has to be determined individually. Whether this also applies to more clinically relevant tissues, like brain and muscle of those patients, is not clear, but is not unlikely given data from literature involving other tissues [19,62,63] and mouse studies [27,64] illustrating that fibroblasts are not the most sensitive system. Therefore, patient-derived fibroblasts can be used to identify patients with increased risk for oxidative damage, who will most likely benefit from boosting their antioxidant defense and energy capacity (training), possibly leading to patient-tailored prevention and antioxidant therapy in the future.

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