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New genes and pathomechanisms in mitochondrial disorders unraveled by NGS technologies☆

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ABSTRACT

Next Generation Sequencing (NGS) technologies are revolutionizing the diagnostic screening for rare disease entities, including primary mitochondrial disorders, particularly those caused by nuclear gene defects. NGS approaches are able to identify the causative gene defects in small families and even single individuals, unsuitable for investigation by traditional linkage analysis. These technologies are contributing to fill the gap between mitochondrial disease cases defined on the basis of clinical, neuroimaging and biochemical readouts, which still outnumber by approximately 50% the cases for which a molecular-genetic diagnosis is attained. We have been using a combined, two-step strategy, based on targeted genes panel as a first NGS screening, followed by whole exome sequencing (WES) in still unsolved cases, to analyze a large cohort of subjects, that failed to show mutations in mtDNA and in ad hoc sets of specific nuclear genes, sequenced by the Sanger's method. Not only this approach has allowed us to reach molecular diagnosis in a significant fraction (20%) of these difficult cases, but it has also revealed unexpected and conceptually new findings. These include the possibility of marked variable penetrance of recessive mutations, the identification of large-scale DNA rearrangements, which explain spuriously heterozygous cases, and the association of mutations in known genes with unusual, previously unreported clinical phenotypes. Importantly, WES on selected cases has unraveled the presence of pathogenic mutations in genes encoding non-mitochondrial proteins (e.g. the transcription factor E4F1), an observation that further expands the intricate genetics of mitochondrial disease and suggests a new area of investigation in mitochondrial medicine. This article is part of a Special Issue entitled 'EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2–6, 2016', edited by Prof. Paolo Bernardi.

1. Introduction

Mitochondrial Disorders form a group of rare, genetically and clinically heterogeneous conditions characterized by impairment of the mitochondrial respiratory chain, which is responsible for the synthesis of most of the ATP in the cells through the oxidative phosphorylation (OXPHOS) pathway. Most of the proteins required for the biogenesis, structure and function of mitochondria are encoded by genes contained in the nuclear genome (nuclear DNA, nDNA) but 13 essential subunits of four of the five canonical respiratory-chain complexes are encoded by a physically separated, semi-autonomous, genome, the mitochondrial DNA (mtDNA) [1,2]. Unlike nDNA, which has a diploid organization (i.e. two copies) within the nucleus of somatic cells, and a haploid organization (i.e. one copy) in gametes, mtDNA is present in multiple copies within the mitochondria of each cell. Human mtDNA is a circular, double-stranded molecule, 16,569 base pairs in size, which encodes 37 genes, including 22 tRNAs and 2 rRNAs encoding genes essential for mtDNA-specific translation of the 13 genes encoding as many respiratory chain subunits [3]. Albeit essential for the formation of the mitochondrial membrane potential (ΔP) and ATP production, these polypeptides are outnumbered by far by the >75 respiratory chain subunits encoded by nDNA. Overall, it has been estimated that more than 1000 nuclear genes encode proteins necessary for mitochondrial maintenance and function, including those carrying out mtDNA replication and expression, mitochondrial shape and dynamics (fission and fusion), Coenzyme Q10 (CoQ10) biosynthesis and the complex network controlling respiratory chain formation, activity and turnover [4,5]. In principle, all of these genes should be considered as potential candidates for

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mitochondrial disease, and mutations in approximately 200 of them have already been established to play a causative role. Nevertheless, most of the mitochondrial disease cases, classified on the basis of clinical/biochemical features, still lack a molecular diagnosis [6], so as to make the completion of the molecular dissection a compelling need in the diagnostics, clinical management, and translational research in mitochondrial medicine.

The minimum prevalence of isolated or combined, genetically defined OXPHOS defects is approximately 1:5000 live birth, just considering the most recurrent mutations of mtDNA. This figure is a very conservative one, as it does not take into account the nuclear gene defects associated with mitochondrial disease. A more recent epidemiological survey carried out in Northern UK, which includes some frequent nuclear gene mutations, indicates a more realistic, but still underestimated, figure of 1:4300 live births [6]. Since the mitochondrial respiratory chain is the final common pathway for aerobic metabolism, tissues and organs with high-energy demand based on efficient OXPHOS are preferentially involved in mitochondrial disease [7], notably the central nervous system, the heart and the skeletal muscle. From a medical standpoint, mitochondrial disorders are often suspected on the basis of peculiar clinical presentations, but the diagnostic workup is often complex and difficult, requiring extensive clinical and laboratory evaluation [8]. It can include biochemical investigation (e.g. measurement of respiratory chain complex activity and mitochondrial oxygen consumption), immunohistochemical and histoenzymatic analyses, and evaluation of the amount and integrity of intact respiratory chain complexes and mtDNA in cultured skin fibroblasts or skeletal muscle biopsy; neuroimaging findings; and careful clinical examination and follow-up, with several criteria and evaluation scales suitable for either adults [9] or children [10,11]. Unfortunately, the use of different methods and lack of standardized procedures among different laboratories worldwide, particularly regarding enzymatic analysis, contribute to make the diagnosis even more complicated and uncertain [12]; moreover, not always the affected tissues/organs are available for the analysis and fibroblasts, the most easily obtainable cellular model, may not express any defect. The gold standard for safe diagnosis is clearly based on the identification of the responsible gene. Recent advances in gene screening technologies has become an invaluable tool to rapidly analyze know disease genes and to search for mutations in new candidate genes.

This paper reports our own experience on how the development of Next Generation Sequencing (NGS) approaches has in fact substantially improved the efficiency of mutation discovery and facilitated its translation into routine diagnostics of mitochondrial disease, with remarkable advantages in terms of diagnostic score, and increased value for money and time consumed [13].

NGS includes a series of techniques that enable automation of nucleic acids (RNA and DNA) sequencing, increasing the analysis power from a few hundred base pairs to the entire genome in a single run. NGS became available to the genetics community in 2008–09 when the first NGS machines entered the market [14]. The process required for NGS to implement research or diagnostic procedures comprises a wet laboratory workflow, including the preparation and sequencing of libraries from individual DNA samples. This step is followed by a post-sequencing workflow involving informatics and bioinformatics analyses performed to align the sequences, and call, annotate and filter the variants. This analytical in silico step is crucial to identify and distinguish rare causative mutations from hundreds of frequent or non-pathogenic sequence polymorphisms. Upon completion of this pipeline the selected variants can be either known pathogenic mutations or novel candidate changes. In general, the first option is followed by a review of the literature in order to establish/confirm a causative phenotype–genotype association. As for the second option, the conclusive demonstration of the pathogenic role of a given variant will depend on additional experimental validation. For instance, the detection of mutations in the same gene in unrelated individuals or families with a similar phenotype is a robust and relatively straightforward genetic validation. But when only one case/family is available for genetic analysis, then the pathogenic role of a suspected variant must be confirmed by functional studies focused on the evaluation of its biological effects [13]. In mitochondrial disease, the availability of mutant cells from patients is important to assess the presence of a biochemical defect affecting the OXPHOS system, and as a source of mRNA and proteins for further experimental validations. In addition, cells are essential to carry out complementation assays and evaluate the rescue of the phenotype under expression of the recombinant wild-type cDNA introduced by transfection or viral transduction [15–17]. However, this procedure is applicable only to those conditions in which a specific readout, usually biochemical (e.g. activities of individual respiratory-chain complexes, oxygraphic studies, CoQ measurement) but sometimes also morphological (e.g. structure of the mitochondrial network, and apoptotic nuclei) can be detected in cultured cells (typically fibroblasts, sometimes myoblasts). In other cases, for instance for genes with an ortholog in *Saccharomyces cerevisiae*, mutations can be modeled in a yeast system. A number of biochemical OXPHOS defects, however, are poorly or hardly expressed in cultured cells and involve recent genes (not conserved in yeast). In these cases, a functional complementation approach is difficult, and validation largely relies on the identification of unrelated cases carrying mutations in the same gene and displaying a similar phenotype. For many genes causing mitochondrial disease, the small number of reported cases makes it difficult to safely establish a consistent clinical and neuroimaging nosography. Although some peculiar genotype–phenotype associations have been reported, these observations may be based on an inclusion bias, because once a mutant gene is found in a single or just a few subjects, then the clinical and neuroimaging features are often exploited to identify additional patients. Indeed, thanks to unbiased NGS approaches, very different clinical presentations have recently been reported in patients harboring mutations in the same gene (e.g. *AARS2*, *AIFM1*) [18,19].

In the present study, we analyzed a heterogeneous cohort of 125 patients diagnosed as affected by mitochondrial disease, mostly characterized by early onset. Using a combined NGS approach based on a targeted gene panel and whole exome sequencing (WES), we genetically screened our cohort of mitochondrial disease patients in order to: widen the spectrum of clinical presentations associated with genes already known to cause MD; discover causative mutations in new candidate genes never associated with MD before; evaluate the power of NGS in identifying mutations in patients negative to traditional gene screening based on Sanger sequencing.

2. Materials and methods

2.1. Clinical and biochemical investigation

125 patients (60 males and 65 females; 78 with age of onset ≤1, the mean onset of the remaining ones was 18.6 years) referred to the Neurological Institute "C. Besta" (Milan, Italy) and diagnosed as affected by mitochondrial disease through the integration of clinical, biochemical and morphological investigations were enrolled in this study and coded as NGSP001–NGSP125. Histological analyses and measurements of respiratory chain and pyruvate dehydrogenase (PDH) complex activities were performed according to standard procedures [16,20]. Based on the biochemical profile and mtDNA characterization obtained in either fibroblasts, skeletal muscle, or both, patients were divided into different groups as follows: defects in complex I (n. 5), complex II (n. 18), complex III (n. 15), complex IV (n.21), complex V (n. 5), multiple defects (n. 26), CoQ10 deficiency (n. 3), mtDNA multiple deletions and/or depletion (n. 8) and PDH complex activity (n. 14) (Supplementary Table S1). In 8 subjects the biochemical analysis was not performed because no biological material was available, and in two no biochemical respiratory chain defect was assessed, but they were all included in the study since the clinical and/or neuroimaging features were highly suggestive for a mitochondrial disease. Notably, complex I deficient

patients are underestimated in this cohort because most of them were already analyzed in previous genetic studies [21,22]. The clinical diagnosis is also included in Supplementary Table S1; a detailed clinical description for NGS091 is reported as supplementary material. All patients had previously been screened by traditional Sanger sequencing for mtDNA mutations, and for sets of genes associated with the specific biochemical or molecular (mtDNA) defect and/or clinical presentation, and in no case were mutations found as suggestive for a pathogenic role.

Informed consent for genetic studies was obtained from all investigated subjects in agreement with the Declaration of Helsinki.

2.2. Molecular genetics

Our combined NGS approach was first based on analysis of our entire cohort of 125 individuals by a custom-made targeted mitochondrial panel of 132 genes (Supplementary Table S2). Next, we selected for WES ten samples, which were negative for the genes contained in the panel.

2.2.1. Custom-made targeted gene panel

Genomic DNA was extracted from peripheral blood. We used 250 ng of DNA as template for the construction of a paired-end library, according to the TruSeq Custom Amplicon Library preparation guide (Illumina). Sequencing was performed on an Illumina MiSeq platform. We sequenced a custom panel of genomic regions corresponding to the transcribed sequences of 132 genes (exons and UTR regions), selected to be already associated with mitochondrial disorders or to be candidate genes that take part to the same molecular pathways. The sequencing reads were aligned to the NCBI human reference genome (GRCh37/hg19) using the Burrows–Wheeler Aligner (BWA). Single nucleotide variants (SNVs) and small insertions/deletions (INDELs) calling were performed using the Somatic Variant Caller (Illumina); Variant Studio software (Illumina) was used for variants annotation and filtering. Filtering was carried out by applying a series of steps: low-quality variants were filtered out (Illumina Qscore threshold of 30); variants with a minor allele frequency (MAF) >1% in the 1000 Genomes Project (http://www.1000genomes.org) and Exome Variant Server databases (http://evs.gs.washington.edu) were discharged. Finally, we focused on predicted missense, frame-shift, stop-gain or stop-loss, and splice-site variants. For remained variants in the final list we also checked their frequency in the Exome Aggregation Consortium (ExAC) database.

2.2.2. Whole exome sequencing

WES analysis was conducted using 50 ng of DNA as template for the construction of pair-ended libraries with the Nextera Rapid Exome Capture kit (Illumina), following the manufacturer's instructions. Sequencing was performed on 12 pM libraries with the addition of 1% 12.5 pM PhiX control library on an Illumina MiSeq platform. Libraries were run several times until 12–15 G of data/sample were obtained. Sequences from the FASTAQ files were aligned to the human genome (hg19) by using the BWA aligner. Variants were called by using the GATK HaplotypeCaller, and then filtered by using the Variant Quality Score Recalibration according to the best practices of GATK-2.7 (https://www.broadinstitute.org/gatk/). Variants were annotated by using Annovar. Coverage of the targeted regions was estimated using the GATK DepthOfCoverage. For paired end reads to be included, they needed to have a mapping quality greater than 20 and a base quality greater than 10.

In addition to the standard SNVs and small INDELs annotation and filtering process, the presence of larger insertions/deletions as well as copy number variations (CNVs) was assessed through a deeper analysis of NGS coverage data. Using the cn.MOPS software publicly available as an R package (http://www.bioinf.jku.at/software/cnmops), we analyzed both targeted gene panel sequencing and WES dataset bam files. Using a Bayesian approach, cn.MOPS decomposes variations in the depth of coverage across samples into integer copy numbers and noise by means of its mixture components and Poisson distributions, respectively [23].

Variants identified by NGS were validated by Sanger sequencing. The chromatogram traces were analyzed using the Sequencher software (Gene Codes Corporation). mtDNA content was evaluated by real-time PCR-based quantification (ABI7000 Real-Time PCR System) using specific mtDNA probes and a standard, single-copy autosomal gene (RNaseP) [16]. RNA was extracted from skin fibroblasts and 1 μg was used as template for reverse transcriptase PCR (RT-PCR) to obtain full-length cDNA. Transcripts of candidate genes were amplified through PCR and run by electrophoresis through a 1% agarose gel in order to detect potential splicing alterations. PCR products were also sequenced in order to confirm genomic variants and unmask potential events of nonsense-mediated decay. Real-time quantitative PCR (qPCR) was performed on retro-transcribed cDNA from fibroblasts of patients and controls, using an ABI Prism 7000 apparatus (Applied Biosystems). The amount of specific transcripts was normalized to beta-actin levels (primers available upon request).

3. Results

3.1. Targeted gene panel

Since the majority of our cohort was composed of patients showing an autosomal recessive mode of disease inheritance, we focused our search on homozygous and compound heterozygous variants, although in a smaller set of individuals the mode of inheritance was uncertain or compatible with autosomal dominant or X-linked transmission. In these cases, single heterozygous variants were also considered. In addition, for samples with recessive inheritance but presenting just one heterozygous variant in a likely gene candidate, we performed extended systematic NGS coverage analysis in search of a second allelic variant. This analysis was carried out by looking for: 1) regions not covered or poorly covered by NGS, which were then analyzed by Sanger sequencing; 2) large heterozygous deletions involving entire exons using the cn.MOPS software, followed by cDNA amplification and sequencing to investigate the effect of the two variants on the gene transcript(s); 3) quantitative or qualitative cDNA abnormalities due to variants within intronic or regulative regions not targeted by NGS (Fig. 1).

The targeted gene panel allowed us to analyze both the coding sequences as well as the 5′ and 3′ untranslated regions of 132 genes, for a total of 1078 exons (2565 amplicons). The total amount of the targeted genomic regions is 338 K bp and an average number of 249,625 reads were produced per sample, allowing us to reach an average depth of coverage of 228X per sample. With our pipeline we called an average number of 311 variants per sample (SNVs and INDELs).

After performing variants annotation and filtering steps, we were able to identify the causative mutations of the disease in 19 patients (15.2%), whereas in 27 additional cases we found candidate variants that were considered likely but not conclusively causative (Supplementary Table S1). Of the 19 patients with an ascertained causative mutation, 2 were patients with defective complex I, 2 with defective complex II, 2 with defective complex III, 2 with defective complex IV, 2 with defective PDH complex, 6 with multiple defects, 1 with mtDNA depletion whereas 2 were biochemically undefined (Table 1, Fig. 2).

Of the patients with ascertained or likely recessive inheritance, 7 carried homozygous mutations within the *DLD*, *SDHB*, *ACAD9*, *TTC19*, *LYRM7*, *NDUFV1* and *RNASEH1* genes, whereas 5 carried compound heterozygous mutations within the *TSFM*, *FBXL4*, *RARS2*, *MRPL44*, and *NDUFS3* genes. Two male subjects carried hemizygous mutations, causing amino acid missense substitutions, within the *AIFM1* and *PDHA1* genes, both localized on the X chromosome (Table 1).

The identified variants comprised already known causative mutations [24–27], variants that we have experimentally validated in dedicated studies [17,28,29], or predicted pathogenic variants in genes responsible for mitochondrial disorders with a phenotype overlapping

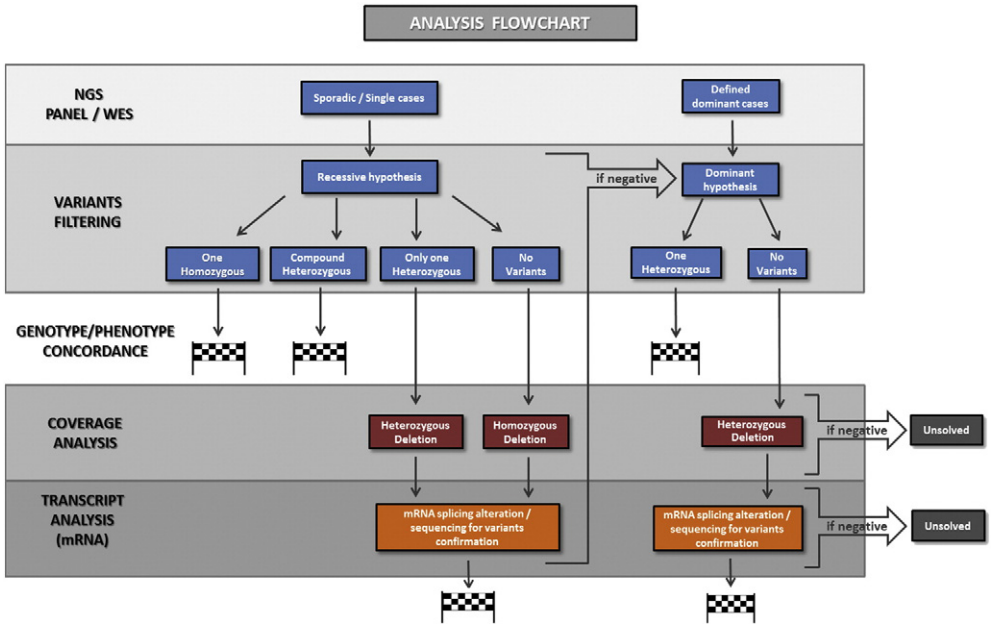

Fig. 1. Flowchart of the genetic analyses. Schematic representation of the different steps of the Next Generation Sequencing (NGS) data analysis used in this study. Checkered flags indicate the achievement of a defined molecular diagnosis. See text for details on each step.

the clinical presentation observed in our mutant subjects. Concordance between genotype and phenotype helped us to achieve definite molecular diagnosis. In order to validate all the variants discovered and their segregation with the disease, we performed Sanger sequencing on the DNA of the probands and of all the available family members. Interestingly, this analysis allowed us to demonstrate reduced penetrance for a homozygous variant identified in the *SDHB* gene in two sisters, the older of whom showed hardly any symptoms, whereas the younger (NGSP124) was affected by severe mitochondrial leukoencephalopathy; this family is described in detail by Ardissone et al. [28].

By performing NGS coverage analysis we were also able to identify the causative mutations in three additional patients who were negative

Table 1
Variants identified by the NGS panel.

Code	Biochemical defect	Gene	Mutations	Exonic deletions	Trait	Notes	ExAC frequency	References
Pathogenic variants								
NGSP36	cI	***NDUFS3***	p.R140P + p.R199W	/	AR	Parents: Heterozygous	<0.01%	
NGSP109	cI	***NDUFV1***	p.R386C homoz	/	AR	Parents: Heterozygous	0.012%	[24]a
NGSP124	cII	***SDHB***	p.D48V homoz	/	AR	Incomplete penetrance	<0.01%	[28]b
NGSP21	cII	***PDHA1***	p.S388P hemiz	/	X		Ø	
NGSP92	cIII	***TTC19***	p.Q261Gfs*8 homoz	/	AR	Parents: Heterozygous	Ø	[29]b
NGSP113	cIII	***LYRM7***	p.D25N homoz	/	AR	Parents: Heterozygous	Ø	[25]a
NGSP90	cIV	***RARS2***	p.M1V + p.S443P	/	AR		0.012%;<0.01%	
NGSP100	cIV	***DARS2***	p.L10P hetero	Ex. 15–16	AR	Mother: p.L10P hetero	Ø	
NGSP70	mtDNA depl	***FBXL4***	p.C547* + p.Q404*	/	AR	Parents: Heterozygous	0.015%;Ø	
NGSP118	Multi	***RNASEH1***	p.V142I homoz	/	AR	3 homozygous siblings	<0.01%	[17]b
NGSP117	Multi	***TSFM***	p.G183S + ss(c.232-3C > G)	/	AR	Altered splicing	Ø;<0.01%	
NGSP35	Multi	***MRPL44***	p.L156R + p.K278*	/	AR		0.028%;Ø	[26]a
NGSP122	Multi	***DNAJC19***		All exons	AR			
NGSP018	Multi	***MTFMT***	p.S209L + p.Q25*	/	AR		0.036%;Ø	[27]a
NGSP38	Multi	***AIFM1***	p.G262S hemiz	/	X	Mother: heterozygous	Ø	[19]b
NGSP33	PDH	***DLD***	p.R482G homoz	/	AR		Ø	
NGSP31	PDH	***PDHA1***	p.R127Q heteroz	/	X	Affected female	Ø	[31]a
NGSP023	nd	***ACAD9***	p.R414C homoz	/	AR	Mother: heterozygous	<0.01%	
NGSP98	Neg	***POLG***	p.R443C heteroz	/	AD	Affected mother and brother: heterozygous	<0.01%	[30]a
Probably non-pathogenic variants								
NGSP60	Multi	*POLG*	p.Y831C heteroz	/		Reported as benign SNP. Unaffected father: heterozygous	0.63%	[35]a
NGSP1	PDH	*POLG*	p.G517V heteroz	/		Reported as benign SNP	0.47%	[34]a
Unlikely causative variants								
NGSP48	Multi	*POLG*	p.R628W heteroz	/		Different phenotype	<0.01%	
NGSP121	nd	*DNA2*	p.A633S heteroz	/		Different phenotype	<0.01%	
NGSP66	cIV	*OPA1*	p.T95M hetero	/		Different phenotype; PREPL deficiency by WES	0.017%	[32]a
NGSP86	cIV	*MFN2*	p.R250Q hetero	/		Different phenotype	0.029%	[33]a

AR: autosomal recessive; AD: autosomal dominant; X: X-linked. Homoz: homozygous; Heteroz: heterozygous. Ø: not reported in the ExAC database.
cI, cII, cIII, cIV: complex I, II, III, IV defect; mtDNA depl mtDNA depletion; multi: multiple RC complex defect; PDH: pyruvate dehydrogenase complex deficiency.
nd: biochemical analysis not done.
[a] Paper reporting the identified mutation.
[b] Paper describing the patient reported in the present study.

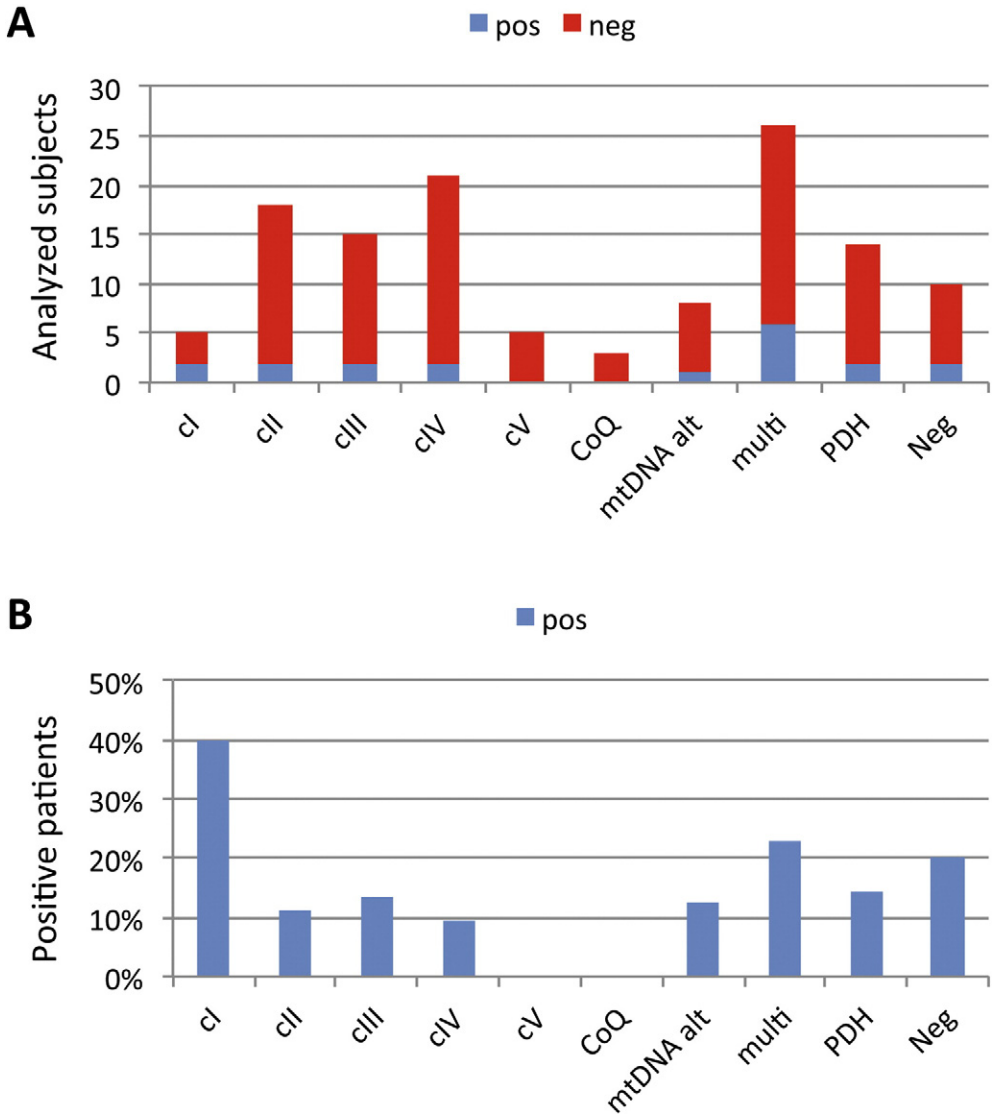

Fig. 2. NGS genes panel analysis. Histograms reporting the patients analyzed by the NGS panel, classified according to their biochemical defect (A) and the percentages of patients with a molecular diagnosis in each class (B). cI, cII, cIII, cIV, cV: complex I, II, III, IV, V defect; CoQ: CoQ10 deficiency; mtDNA alt: mtDNA depletion or deletions; multi: multiple RC complex defect; PDH: pyruvate dehydrogenase complex deficiency; Neg: patients with no biochemical deficiency. Colors indicate subjects with (blue) or without (red) a defined molecular diagnosis.

after the variants-filtering pipeline. In one case (NGSP018) a single heterozygous SNV was identified within the *MTFMT* gene, then coverage analysis revealed a non-covered region within exon 1 of the same gene. Sanger sequencing showed the presence of a second, nonsense heterozygous, allelic mutation in this region. In the second case (NGSP100) we found a heterozygous missense mutation in *DARS2* in a patient with a neuroimaging pattern highly suggestive for *DARS2*-related leukoencephalopathy. Coverage analysis revealed a heterozygous deletion encompassing the last two exons of *DARS2*. Sequencing of the cDNA obtained from patient's fibroblast RNA showed only the presence of the allele with the missense mutation. Accordingly, Western-blot analysis on whole cell lysates of primary patient's fibroblasts revealed a clear reduction of DARS2 protein amount compared with controls. In the third case (NGSP122) no SNV or small INDEL was identified through variants analysis, but coverage analysis allowed us to detect a homozygous large deletion involving the entire *DNAJC19* gene, which was then confirmed by Sanger sequencing.

For cases with a family history clearly supporting dominant inheritance or for sporadic cases with mutations in genes known to be associated with an autosomal dominant trait, we also evaluated genes with heterozygous variants. We identified one subject (NGSP098) with a heterozygous mutation within the *POLG* gene, which was also present in two additional affected subjects of the family and already reported in a patient with myopathy [30]. A second female patient (NGSP031) carried a heterozygous mutation of the X-linked *PDHA1* gene, already described in females with defects of the PDH complex [31]. We also found 6 additional heterozygous missense SNVs within genes previously associated with dominantly inherited mitochondrial disease: three SNVs in *POLG* and one each in *DNA2*, *MFN2* and *OPA1*. However, these findings were not considered conclusive because of either discordance with the phenotype typically associated with mutations in these genes [32,33], uncertain or no pathogenicity of the SNV according to the literature [34,35], or no segregation with the disease (Table 1).

3.2. Whole exome sequencing

From the group of patients that failed to attain conclusive genetic diagnosis after targeted gene panel NGS, we selected 10 patients for WES, based on accuracy of family history and clinical description, parental consanguinity, availability of DNA samples from other family members, suggestive and peculiar phenotypic features and well-established disease inheritance. These patients included 3 cases with defects in

complex IV activity, 2 cases with mitochondrial multiple defects, 3 cases with defect of PDH complex activity, 1 patient with mtDNA depletion and 1 patient with no biochemical diagnosis available, but with an affected sibling and an MRI pattern suggestive for a mitochondrial encephalopathy. WES allowed us to analyze the transcribed regions of the 99.9% of genes present in the human genome, for a total of ~60 Mb. The average number of reads produced per sample was ~59 millions, with an average number of bases with a coverage greater than 20× of 91.6 ± 4.6%. With our pipeline we called an average number of 377 ± 48 variants per sample. Most of the variants were non-synonymous SNVs in the coding regions (359 ± 35.2), including stop gain mutations (7.8 ± 1.5), but a small proportion of INDELs were also detected (14 ± 3.0).

After performing variants annotation and filtering steps, we were able to identify causative mutations in six patients (60%) (Table 2). Four unrelated patients were born from consanguineous parents and carried homozygous mutations in different genes: *PC*, *CYP2U1*, *PREPL* and *E4F1*. In a fifth patient, two allelic heterozygous mutations were identified in a putative assembly factor for complex IV. In a sixth patient, a single heterozygous missense mutation was found in *RANBP2*, inherited from the mother. Notably, only the protein encoded by the *PC* gene, pyruvate carboxylase, and the putative assembly factor specific to complex IV are proteins exclusively and specifically targeted to mitochondria, whereas the other genes encode polypeptides that are localized and operate outside the organelle.

Pyruvate carboxylase is a biotin-dependent mitochondrial enzyme, which converts pyruvate into oxaloacetate in the first step of gluconeogenesis. Pyruvate carboxylase deficiency (OMIM #266150), due to mutations in the *PC* gene, is associated with three clinical forms of different severity and outcome [36–38]. Our patient (NGSP067) displayed a severe condition characterized by psychomotor delay and tetraparesis with leukoencephalopathy, associated with biochemical defects of complexes I and III of the mitochondrial respiratory chain. The presence of the same variant also in the affected sister and a concordant phenotype/genotype association confirmed this homozygous mutation as the cause of the disease. However, the link between *PC* mutation and the respiratory chain deficiency (observed in muscle but not in fibroblasts from NGSP067) is unclear; it could be a secondary effect caused by impairment in pyruvate metabolism.

The two compound heterozygous mutations in a gene encoding a putative mitochondrial assembly factor specific to complex IV were found in a child (NGSP073) with defects in complex IV activity, associated with peripheral neuropathy, hypotonia and a cavitating leukoencephalopathy. According to the literature, no mutations of this gene have been linked to any pathological condition so far. Mutations and function of this protein are currently under study.

As for mutations in non-mitochondrial gene products, in a patient (NGSP065), born from consanguineous parents, presenting with reduced PDH complex activity, hyperlactacidemia, spastic paraparesis and white matter MRI alterations, targeted gene panel NGS showed the presence of a single heterozygous missense variant within the *KARS* gene, encoding the lysyl-tRNA synthetase common to both cytosol and mitochondria. Mutations in this gene are reported to cause Charcot–Marie–Tooth disease, recessive intermediate, B (CMTRIB, OMIM #613641) with an essentially recessive mode of inheritance [39]. This fact and the clear consanguinity of the parents prompted us to deem this variant as non-causative. Conversely, WES analysis revealed a homozygous deletion of 13 nucleotides involving the splicing site of exon 3 in the *CYP2U1* gene, encoding a member of the cytochrome P450 superfamily. *CYP2U1* is mutated in autosomal recessive spastic paraplegia 56, (OMIM #615030). By comparing our case with the clinical presentations described in the literature, an overlapping spectrum of symptoms emerges, strongly suggesting a causative link between our genetic findings and the biochemical and clinical features of the patient. Interestingly, mutations in *CYP2U1* have been reported to alter mitochondrial architecture and bioenergetics [40].

In a patient (NGSP066) born from consanguineous parents, with defective complex IV activity, psychomotor delay, somatic growth retardation and myopathy with neonatal onset, the targeted gene panel NGS screening revealed a heterozygous variant within the *OPA1* gene. This variant was not considered conclusive, because of the marked inconsistencies between the clinical presentation of our patient and the phenotypes reportedly associated with *OPA1* dominant mutations. No homozygous mutations were found by WES but further analysis of raw data revealed a large, completely non-covered DNA region, spanning from exon 6 to exon 14 of the *PREPL* gene, suggesting an intragenic homozygous deletion, This finding was subsequently confirmed by Sanger sequencing. PREPL belongs to the propyl oligopeptidase subfamily of serine peptidases and large deletions involving also this gene have been associated with hypotonia–cystinuria syndrome, also known as the 2p21 deletion syndrome (OMIM #606407) [41]. In addition, Parvari et al. described seven patients affected by cystinuria and mitochondrial disease with a large deletion on chromosome 2p16 involving the *SLC3A1* gene, known to cause isolated cystinuria type 1, the *PP2Cbeta* gene and the *PREPL* gene [42]. Recently, PREPL deficiency has been associated with a congenital myasthenic syndrome with growth hormone deficiency [43], resembling the clinical presentation of our patient. No specific studies have been performed so far to assess any direct effect of *PREPL* defects on mitochondrial function;

Table 2
Patients analyzed by WES.

Code	Biochemical defect	Gene	Mutations	Exonic deletions	Trait	Notes	ExAC frequency	References
Pathogenic variants								
NGSP67	Multi	*PC*	p.N647D homoz	/	AR	Consanguineous parents; 1 homozygous affected sister	Ø	
NGSP73	cIV	"*COA-X*"	compound heteroz	/	AR	Consanguineous parents: heterozygous	Ø	
NGSP65	PDH	*CYP2U1*	c.1283_1288 + 8del	/	AR	Consanguineous parents: heterozygous	Ø	
NGSP66	cIV	*PREPL*	/	Ex. 6–14	AR	Consanguineous parents		
NGSP110	Multi	*RANBP2*	p.T585M	/	AD	"Affected" mother: heterozygous	<0.01%	[46]
Probably pathogenic variants								
NGSP91	PDH	*E4F1*	p.K144Q	/	AR	Parents: heterozygous; 1 homozygous affected brother	<0.01%	
Unsolved WES cases								
NGSP47	mtDNA depl	*TRMT1*	p.N70S + p.A171V	/	AR	1 affected sibling. Variants on the same allele	0.1%; Ø	
NGSP49	Neg	*TENM4*	p.N1799H + p.Q2527K	/	AR	2 affected sibling. Variants on the same allele	0.2%; 0.2%	
NGSP89	PDH	*GNAO1*	p.D134N + p.A165V	/	AR	Different phenotype	Ø	
NGSP116	Multi	*MRS2*	p.R446H homoz	/	AR	Consanguineous parents	0.33%	

AR: autosomal recessive; AD: autosomal dominant. Homoz: homozygous; Heteroz: heterozygous. Ø: not reported in the ExAC database.
"COA-X": gene coding for a putative assembly factor specific to complex IV. cIV: complex IV defect; mtDNA depl: mtDNA depletion; multi: multiple RC complex defect; PDH: pyruvate dehydrogenase complex deficiency. Neg: patient without biochemical deficits.

alternatively, the complex IV deficiency found in our patient could be a secondary finding, due to muscle dysfunction.

In a patient (NGSP091) presenting with reduced PDH complex activity, hypotonia, growth delay, microcephaly, hyperlactacidemia and organic aciduria, brain MRI revealed the presence of symmetric necrotic lesions in the subthalamic nuclei and substantia nigra, suggestive for a diagnosis of Leigh disease (Fig. 3A). A detailed clinical description of this case is reported as supplementary data. WES revealed a homozygous missense variant within the *E4F1* gene (E4F transcription factor 1), affecting a highly conserved amino acid residue (Fig. 3B). The *E4F1* gene variant was homozygous also in an affected sibling whereas the two parents were heterozygous (Fig. 3C). *E4F1* encodes a multifunctional zinc finger protein, which acts as an atypical ubiquitin E3 ligase for p53 [44] and, as a transcriptional factor, regulates the expression of several genes involved in mitochondrial functions and cell-cycle checkpoints [45]. We then tested the transcript levels of some genes regulated by *E4F1* by qPCR. According to Rodier et al. [45], some genes whose expression is clearly activated by E4F1 are: *DNAJC19*, encoding a mitochondrial protein; *DLAT* and *PDPR*, two genes encoding proteins related to the PDH complex; and *CHEK1*, the major component of the CHK1-Dependent Checkpoint of the cell cycle. Interestingly, we observed that all these transcripts were reduced in patient's fibroblasts compared to controls (Fig. 3D): *DNAJC19* was the most affected (46%; $p < 0.001$); *CHEK1*, *DLAT* and *PDPR* showed partial but significant reductions ($p < 0.05$). Given this biological evidence, as well as the rarity and predicted pathogenicity of the *E4F1* variant, we strongly suggest a causative involvement of the mutated *E4F1* gene in the disease.

Finally, in a patient (NGSP110) with combined defects in respiratory chain complexes III and IV and in the PDH complex, presenting with two episodes of acute encephalopathy concomitant with fever, no pathogenic mutations were found by targeted gene panel NGS, and WES failed to identify any candidate among the recessive variants. However, we detected a known heterozygous mutation in the *RANBP2* gene, which encodes Ran-Binding Protein 2, a component of the nuclear pore complex playing a role in nuclear protein trafficking, sumoylation of protein cargoes, intracellular trafficking and energy maintenance [46]. This mutation has already been associated with "Susceptibility to acute, infection-induced encephalopathy 3" (OMIM #608033), a dominantly inherited condition characterized by acute necrotizing encephalopathy following a febrile illness [46–48]. Notably, the mutation in our patient was inherited from his mother, who is now asymptomatic but reported an acute episode of encephalopathy at 6 years of age, requiring Intensive Care Unit admission, during a febrile infection.

4. Discussion

In this study we applied a combined NGS approach based on targeted gene panel sequencing and WES, to analyze 125 patients with a suspect of mitochondrial disease, who had previously been screened with traditional Sanger sequencing for the entire mtDNA and for specific sets of genes known to be associated with the mitochondrial

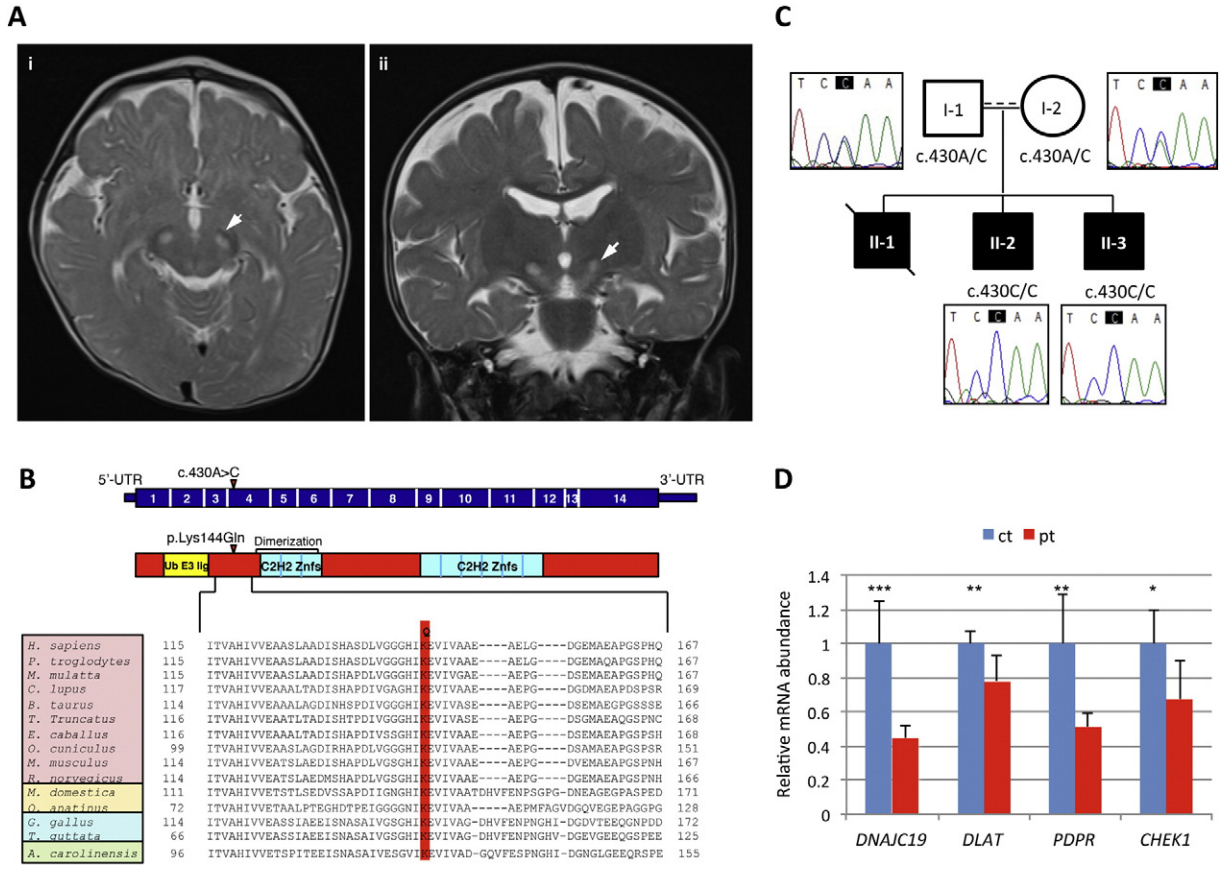

Fig. 3. Clinical and molecular analysis on the *E4F1* mutant patient. (A) Brain MRI: T2 transverse brain sequences showing symmetric lesions in subthalamic nuclei (panel I, arrowhead) and substantia nigra (panel II, arrowhead). (B) Pedigree of the family and electropherograms of the *E4F1* region containing the c.430A > C variant. Black symbols indicate clinically affected subjects. (C) Human E4F1 contains three domains: an ubiquitin E3 ligase-like domain that may mediate ubiquitination of chromatin-associated p53, a domain containing three C2H2 zinc-finger motives required for DNA binding and also involved in protein dimerization and a domain containing six C2H2 zinc-finger motives involved in DNA binding. Phylogenetic alignment of the human protein region containing the substitution found in affected individuals is shown below along with representative protein regions from placental and marsupial mammals, birds and reptiles, where this gene is present. The conserved residue mutated in affected individuals is boxed in red. (D) Transcript levels of *E4F1* target genes in fibroblasts from NGS091 (pt) and controls (ct), measured by qPCR. Data represent the mean ± SD of four experiments. ***: $p < 0.001$, **: $p < 0.01$ and *: $p < 0.05$ by unpaired two-tailed Student's t test.

defect and/or the clinical presentation of each subject. This strategy let us achieve uncontroversial genetic diagnosis in 19 patients (15.2%) by targeted gene panel sequencing and in 6 out of 10 (60%) by WES. Our results demonstrate the power of this combined NGS strategy to achieve genetic diagnosis in several cases, who had failed to be solved by Sanger sequencing based screening of specific genes.

Since the targeted gene panel includes almost all the known genes associated with mitochondrial disease and usually analyzed through Sanger sequencing, which has an average discovery rate of about 10% [49], we estimate a diagnostic success of the NGS panel approach of ≈25%, when used as a first screening strategy. This figure is clearly much higher than the traditional candidate gene approach based on Sanger sequencing.

Importantly, the unbiased, simultaneous analysis of a huge number of genes increases the heuristic value of the investigation, allowing the discovery of new, unexpected genotype/phenotype associations. Although for this scope WES is clearly more powerful, as it enables the discovery of novel mitochondrial disease-linked genes, targeted gene panel NGS is also a valuable tool, as it can widen the spectrum of the clinical presentations associated with known disease-associated genes. For example, the hemizygous mutation in the X-linked *AIFM1* gene, found in one of our male patients, was associated with predominant reduction of complex IV activity, a muscle biopsy already pathological at 13 years of age, with evidence of chronic denervation, ragged-red fibers, and cytochrome c oxidase negative or hyporeactive fibers, and a neuromuscular syndrome of moderate clinical severity [19]. These features markedly differ from previously reported cases of *AIFM1* mutations, which were associated with a spectrum of conditions, from Cowchock syndrome (CMTX4, Charcot–Marie–Tooth disease with deafness and mental retardation, OMIM #310490), caused by a single *AIFM1* founder mutation, to early onset, severe or extremely severe encephalomyopathy, with multiple respiratory-chain defects, and partial mtDNA depletion (Combined Oxidative Phosphorylation deficiency 6, COXPD6, OMIM #300816).

On the other hand, the intrinsically unbiased approach of WES requires a deeper variants analysis, which is more complex than that required for targeted panel NGS, and must be integrated with available clinical, metabolic, neuroimaging and biochemical data. Further functional (experimental) validation is usually necessary to demonstrate the biological consequences of the mutations and their direct causative role in disease. Thus, although the new high-throughput genetic approaches can be applied to large cohorts of poorly characterized patients, the identification of a specific biochemical defect through the collection of suitable biological samples (muscle biopsy, fibroblasts, etc.) remains a very important diagnostic procedure. Not only this is important to direct the filtering process for the selection of the causative variant, but it is also essential for the validation of pathogenic variants through complementation studies, which are typically carried out on biochemically defective cell lines.

In five out of the ten subjects who underwent WES, we were able to identify the causative mutations and in a sixth one we found a strong candidate variant (in *E4F1*). Notably, only in 2 cases the mutant proteins have been demonstrated to exclusively target to mitochondria (PC and a putative complex IV assembly factor). In the remaining cases the responsible genes (*CYP2U1*, *PREPL*, *RANBP2* and *E4F1*) encode proteins not localized into mitochondria, whose functional relation with mitochondrial OXPHOS is unknown, unclear or not exclusive [40,45].

Thus, a fraction of subjects initially classified as mitochondrial-disease patients may indeed carry mutations in genes encoding proteins not targeted to mitochondria, and playing roles not obviously related to mitochondrial physiology, despite the presence of clinical and biochemical features supporting mitochondrial dysfunction. This may well account for a still unknown percentage of genetically unsolved cases classified as mitochondrial disease on the basis of biochemical, clinical or morphological findings. The relatively loose link demonstrated by our WES results between biochemical features and genetic basis of disease is likely due to the central role of mitochondria in a huge number of cellular pathways, although the exact molecular mechanisms of these apparently spurious or possibly indirect associations is far from being clarified [50]. A potentially interesting and conceptually novel finding concerns the mutation in *E4F1*, which, to our knowledge, is the first example of a transcription factor involved in the orchestration of gene expression related to mitochondrial bioenergetics to cause a biochemically defined mitochondrial condition (PDH complex deficiency). Interestingly, a region of E4F1 (aa 30–80) required for its Ub E3 activity presents sequence similarities with IR domains of the SUMO E3 ligase RanBP2, and it has been proposed that E4F1 and RanBP2 E3 domains may originate from a common ancestor [44]. More work is warranted to validate the pathogenic role of the mutation and establish a pathomechanistic link between defective *E4F1* and PDH deficiency. We have obtained evidence strongly suggesting the attractive possibility that the biochemical and clinical features associated with the *E4F1* mutation are the consequence of the direct role of *E4F1* in the expression of gene products, which are part of or play a regulatory role on pyruvate dehydrogenase. Two relevant genes have been shown to contain an E4F1 binding site near the promoter region [45]. The first is *DLAT*, encoding dihydrolipoamide S-acetyltransferase, the E2 component of the PDH complex. Dihydrolipoamide acetyltransferase, accepts acetyl groups formed by the oxidative decarboxylation of pyruvate and transfers them to coenzyme A. Mutations in this gene are also a cause of pyruvate dehydrogenase E2 deficiency (PDHDD, OMIM #245348) which causes primary lactic acidosis in infancy and early childhood. The second gene is *PDPR*, which encodes the pyruvate phosphatase regulatory subunit. PDPR subunit combines with a catalytic subunit, encoded by the *PDP2* gene, to form a pyruvate dehydrogenase phosphatase heterodimer. A second PDH phosphatase is a monomeric enzyme encoded by *PDP1*, which retains both regulatory and catalytic domains. Altogether, these PDH phosphatases are the main activator of the complex, and mutations in *PDP1* are associated with PDH complex deficiency (OMIM #608782) [51,52]. In patient's fibroblasts we found a reduction in the expression of both *DLAT* and *PDPR*; although the decrease is not striking, it is concordant with the results obtained in mouse embryonic fibroblasts knock-out for *E4F1* [45].

5. Conclusions

In summary, NGS strategies offer a wide range of approaches to screen patients with suspected mitochondrial disease, including targeted gene panel sequencing, comprehensive mitochondrial gene panel [53], clinical exome panel (~4.000 clinically relevant genes) and WES. The choice of which technique may be the most suitable for genetic investigation of these disorders depends on several factors, such as availability of funding, space, personnel and bioinformatics expertise.

In our opinion, a two-step approach based on targeted gene panel NGS, followed by WES analysis for patients negative to the former screening, combines high diagnostic score with affordability of costs within an acceptable timeframe. The custom-made targeted panel sequencing allows the screening of large cohorts of patients with relatively easy and manageable interpretation of post sequencing data. WES has been used at a second level, as an unbiased, and more exhaustive approach, to further screen a selected subgroup of patients leftover after targeted panel sequencing. In these selected cases, it was not surprising that the responsible gene would indeed be likely to encode proteins less directly linked to mitochondrial bioenergetics, or for which a specific biological role has not yet been fully elucidated.

Finally, we want to underscore that the identification of a likely candidate gene or mutation is often the beginning of a complex process aimed at validating the pathogenic role of the mutant variant and, in several cases, the very function of novel gene products or proteins previously not considered as mitochondrial. In vitro studies on isolated mutant and wild-type proteins, mutant cell characterization, modeling in yeast or other in vivo systems, including different animal species, are

part of the experimental package available to translational research to contribute to expand medical knowledge on mitochondrial disease and their scientific relevance to understand the complex biology of mitochondria.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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