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Review

Genetics of mitochondrial diseases: Identifying mutations to help diagnosis

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A R T I C L E   I N F O

A B S T R A C T

Mitochondrial diseases are amongst the most genetically and phenotypically diverse groups of inherited diseases. The vast phenotypic overlap with other disease entities together with the absence of reliable biomarkers act as driving forces for the integration of unbiased methodologies early in the diagnostic algorithm, such as whole exome sequencing (WES) and whole genome sequencing (WGS). Such approaches are used in variant discovery and in combination with high-throughput functional assays such as transcriptomics in simultaneous variant discovery and validation. By capturing all genes, they not only increase the diagnostic rate in heterogenous mitochondrial disease patients, but accelerate novel disease gene discovery, and are valuable in side-stepping the risk of overlooking unexpected or even treatable genetic disease diagnoses.

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1. Introduction

The mitochondria are the so-called "powerhouses of the cell". They are the principal generators of cellular energy as ATP. Our cells house hundreds to thousands of independently proliferating mitochondria, each with 2−10 copies of mitochondrial DNA (mt-DNA) in a network of fusing and budding organelles [1]. Mitochondrial diseases result from disruption to the oxidative phosphorylation (OXPHOS) activity or to integral mitochondrial functions. The responsible mutations harbour in both the circular 16,569 base pair mt-DNA and the nuclear DNA. The mt-DNA encodes 37 genes including 22 tRNAs and two rRNAs essential for mt-DNA-specific translation of the 13 encoded respiratory chain subunits. In the nuclear DNA over 1,000 mitochondrially localising proteins are encoded, translated in the cytoplasm, and translocated to the mitochondria by an elaborate protein import machinery.

Mitochondrial diseases are by far the largest class of inborn errors of metabolism (IEM) [2] with a collective incidence of 1.6 in 5,000 [3]. Organ involvement in the vast majority is multi-systemic with a predilection for the high-energy demanding tissues. These tissues depend on maintaining efficient energetic status and in times of metabolic stress, patients' symptoms characteristically decompensate and regress. Taking mitochondrial respiratory chain complex I

deficiency (MIM:252,010) as an exemplar, this is reflected by a phenotypic spectrum spanning adult-onset isolated eye involvement in Leber's hereditary optic neuropathy (LHON, OMIM:535,000) to devastating infantile onset Leigh syndrome (infantile subacute necrotizing encephalopathy, OMIM:256,000) and remarkably, such a spectrum may by observed in patients with the same mutation [4]. Conversely, each clinical presentation can result from a defect in one of multiple disease genes, such as Leigh syndrome where over 90 genes are implicated (The Leigh Map, https://mseqdr.org/leighmap.php) [5]. Moreover, in mt-DNA encoded disease, mutational heteroplasmy with either a "threshold effect" for disease expression, a correlation between increasing heteroplasmy level and disease severity [6], and conversely, a high heteroplasmic or even homoplasmic burden resulting in a lesser or no disease burden [7] have all been reported. Clinical rating scales, such as the Mitochondrial Disease Criteria (MDC) score, indicate the likelihood of mitochondrial disease. However, they are unable to pinpoint precise genetic diagnoses as phenotypes with high weighting in such scores have equally high locus heterogeneity [8]. Though especially prominent in mitochondrial disease, this phenotypic complexity inflicts the wider genetic field where in up to 90% of patients diagnosed by whole genome sequencing (WGS), the phenotype is found to be a poor predictor of molecular cause [9]. Moreover, there is no single reliable biomarker for mitochondrial disease. Measurement of OXPHOS enzyme activities, along with immunohistochemical and histoenzymatic assays of invasive muscle biopsy, and measurement of lactate, amino acids, and organic acids in blood and urine, have conventionally been at the

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forefront of the diagnostic algorithm [10]. However, given their low specificity and the plethora of genes resulting in a secondary OXPHOS deficiency, they can only bring limited clarity to the molecular cause.

Collectively, these features make the diagnosis of a mitochondrial diseases challenging. Despite the myriad of hurdles, motivation to provide a molecular diagnosis is high. It is invaluable to the patient in tailoring management and anticipatory care, stratifying disease risk, and providing accurate recurrence risk estimates for families. Moreover, it provides an opportunity to individually target treatment, such as replenishing a critical cofactor or restricting a dietary element to ameliorate the disease [11,12].

2. Genetic underpinning of mitochondrial disease

The genetic driven era of mitochondrial disease discovery commenced over 30 years ago with the report of the first mt-DNA encoded disease genes in LHON and Kearns-Sayre syndrome (KSS, OMIM:530,000), shortly followed by the discovery of the first nuclear encoded defect in *PDHA1* [13−15]. Defective OXPHOS is the hallmark, and a wide-array of proteins with a myriad of functional roles converge to influence OXPHOS functionality. In this review, we focus on 338 disease genes, each demonstrated to cause a defect in an enzyme involved in aerobic energy metabolism (OXPHOS, Krebs cycle, pyruvate oxidation, fatty acid oxidation, and ketone bodies) (Fig. 1).

The mitochondrial proteome is under the control of two genomes. Though the mt-DNA is replicated, transcribed, processed, and translated within the mitochondrial itself, almost all of the proteins required are encoded in the nuclear genome. Accordingly, 36 (11%) of the disease genes are mt-DNA encoded and 302 (89%) are encoded in the nuclear DNA. All modes of inheritance are reported and a remarkable 22 genes are inherited by a combination of AR and AD inheritance according to OMIM (https://omim.org) (Fig. 1). The presence of more than one mode of inheritance may be explained by allele heterogeneity with differing consequences of the pathogenic variants, such as loss-of-function, gain-of-function, and dominant negative or variable magnitude of the pathogenic variant effect. *SSBP1* is a recently described example, where missense variants span recessive, dominant, as well as *de novo* dominant inheritance, indicating that simple dosage reduction is not the only molecular mechanism underlying the disease [16].

3. Disease gene discovery

A definitive diagnosis is arguably only possible when genetic definition is reached. Conventionally, by collection of patients with similarity in clinical and biochemical phenotype, candidate gene sequencing with prior linkage analysis or homozygosity mapping and mt-DNA sequencing were utilised. These methods led to the discovery of more than 150 genes over 25 years. An acceleration of disease gene discovery 10 years ago, starting with *ACAD9*, marked the transition into the next generation sequencing (NGS) era (Fig. 2) [17]. Pre-NGS, approximately five disease genes were described per year, monumentally increasing to over 15 thereafter. Though the "low-hanging fruit" may have been picked, we predict many more disease genes await discovery given the vast size of the mitochondrial proteome and increasing use of unbiased WES, WGS, and "multi-omic" methodologies.

4. Genetic analyses over time

4.1. Candidate gene and mt-DNA sequencing

Candidate gene sequencing may be the method of choice in clear clinical syndromes where the majority of cases are explained by a handful of mutations (such as the 95% of LHON cases explained by

mutations in *MT-ND1, -ND4,* and *-ND6*). Beyond these clear circumstances, frequent reassessment is often required, resulting in a protracted diagnostic period for the majority and reaching a diagnosis in just 10% [18].

4.2. Next generation sequencing

NGS encompasses targeted mt-DNA sequencing, panel sequencing, WES, and WGS. In facilitating the identification of patients presenting atypically for their molecular diagnosis, NGS is expanding our understanding of genotype-phenotype relationships in mitochondrial disease, the extent of which are not yet fully appreciated. This has led to an entourage of research articles expanding the clinical phenotype of diseases, most recently in *MT-ATP6* [7].

4.3. Targeted mt-DNA NGS

mt-DNA sequencing screens the entire mt-DNA sequence and allows heteroplasmy level to be measured. While the easily accessible DNA samples such as from blood and urine are convenient measurement starting points, mt-DNA heteroplasmy, replication, and copy number vary from tissue to tissue, presumably to meet local energy demands [19]. Thus, a negative result does not exclude an mt-DNA mutation and sampling of tissues such as the urinary epithelial cells or skeletal muscle is often necessitated. Moreover, some mt-DNA variants, specifically deletions and mt-DNA depletion, are challenging to detect and are detectable only in the high energy demanding tissues from which the clinical phenotype arise. Therefore, even in the era of NGS, tissue biopsies are necessitated in patients when the result of genetic testing is inconclusive. Such examples are progressive external ophthalmoplegia (PEO) and Kearn-Sayre Syndrome where single large-scale mt-DNA deletions are preferentially found in post-mitotic skeletal muscle and are often undetectable in blood [20] and mitochondrial myopathies secondary to muscle specific mt-DNA mutations. These are important exceptions where muscle biopsy is deemed the diagnostic "gold standard". In adults where a diagnosis in the mt-DNA is more likely (75%), it is therefore common practice to sequence the mt-DNA from muscle prior to WES or WGS. While in the paediatric population mt-DNA variants are often detectable in the blood and urine but for definite exclusion further tissues should be analysed [21].

4.4. NGS panel

Panels are targeted towards genes known to be involved in mitochondrial disease and candidate genes predicted to be involved in critical mitochondrial functions. In the literature, panels range from 100 genes implicated in complex I deficiency to more expansive panels targeting the mt-DNA and exons of over 1,000 nuclear genes encoding mitochondrial proteins, the overwhelming majority of which have yet to be assigned a role in mitochondrial disease. These panels achieved a diagnostic rate of 7%−31% [22−27] (Table 1). Today, applying a panel of 300 genes (MitoSure300, Agilent) in a heterogenous cohort of suspected mitochondrial disease patients reaches a diagnostic rate of about 30% (Personal communication). The principal advantages of this focused approach are the high coverage (of over 200x in targeted nuclear, and 12,000x in mt-DNA sites) and relative ease and manageability of interpretation. A major caveat to the panel is its limited shelf-life due to the continual discovery of novel disease genes. Moreover, panels rely on correct *a priori* clinical suspicion as they are unable to appreciate variation beyond the targeted genes, and in cases of phenotypic mimicry the true diagnosis may not be captured.

Of note, "virtual panels" may be applied to WES data to facilitate the analysis of variants in a stepwise manner, focussing firstly on the disease gene panel of interest with the possibility for successive
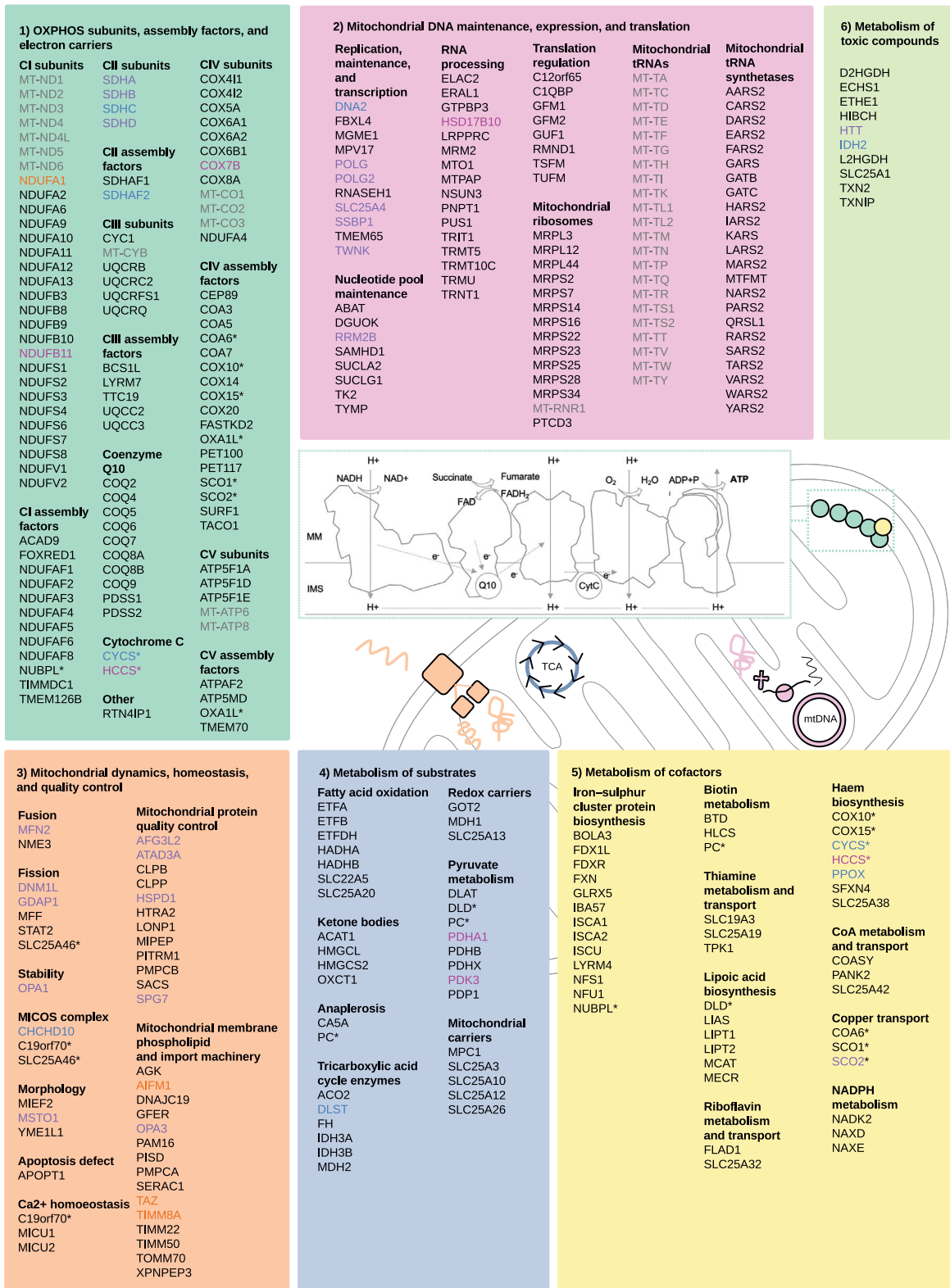
1) OXPHOS subunits, assembly factors, and electron carriers

CI subunits	CII subunits	CIV subunits
MT-ND1	SDHA	COX4I1
MT-ND2	SDHB	COX4I2
MT-ND3	SDHC	COX5A
MT-ND4	SDHD	COX6A1
MT-ND4L		COX6A2
MT-ND5	**CII assembly**	COX6B1
MT-ND6	**factors**	COX7B
NDUFA1	SDHAF1	COX8A
NDUFA2	SDHAF2	MT-CO1
NDUFA6		MT-CO2
NDUFA9	**CIII subunits**	MT-CO3
NDUFA10	CYC1	NDUFA4
NDUFA11	MT-CYB	
NDUFA12	UQCRB	**CIV assembly**
NDUFA13	UQCRC2	**factors**
NDUFB3	UQCRFS1	CEP89
NDUFB8	UQCRQ	COA3
NDUFB9		COA5
NDUFB10	**CIII assembly**	COA6*
NDUFB11	**factors**	COA7
NDUFS1	BCS1L	COX10*
NDUFS2	LYRM7	COX14
NDUFS3	TTC19	COX15*
NDUFS4	UQCC2	COX20
NDUFS6	UQCC3	FASTKD2
NDUFS7		OXA1L*
NDUFS8	**Coenzyme**	PET100
NDUFV1	**Q10**	PET117
NDUFV2	COQ2	SCO1*
	COQ4	SCO2*
CI assembly	COQ5	SURF1
factors	COQ6	TACO1
ACAD9	COQ7	
FOXRED1	COQ8A	**CV subunits**
NDUFAF1	COQ8B	ATP5F1A
NDUFAF2	COQ9	ATP5F1D
NDUFAF3	PDSS1	ATP5F1E
NDUFAF4	PDSS2	MT-ATP6
NDUFAF5		MT-ATP8
NDUFAF6	**Cytochrome C**	
NDUFAF8	CYCS*	**CV assembly**
NUBPL*	HCCS*	**factors**
TIMMDC1		ATPAF2
TMEM126B	**Other**	ATP5MD
	RTN4IP1	OXA1L*
		TMEM70

2) Mitochondrial DNA maintenance, expression, and translation

Replication, maintenance, and transcription	RNA processing	Translation regulation	Mitochondrial tRNAs	Mitochondrial tRNA synthetases
DNA2	ELAC2	C12orf65	MT-TA	AARS2
FBXL4	ERAL1	C1QBP	MT-TC	CARS2
MGME1	GTPBP3	GFM1	MT-TD	DARS2
MPV17	HSD17B10	GFM2	MT-TE	EARS2
POLG	LRPPRC	GUF1	MT-TF	FARS2
POLG2	MRM2	RMND1	MT-TG	GARS
RNASEH1	MTO1	TSFM	MT-TH	GATB
SLC25A4	MTPAP	TUFM	MT-TI	GATC
SSBP1	NSUN3		MT-TK	HARS2
TMEM65	PNPT1	**Mitochondrial**	MT-TL1	IARS2
TWNK	PUS1	**ribosomes**	MT-TL2	KARS
	TRIT1	MRPL3	MT-TM	LARS2
Nucleotide pool	TRMT5	MRPL12	MT-TN	MARS2
maintenance	TRMT10C	MRPL44	MT-TP	MTFMT
ABAT	TRMU	MRPS2	MT-TQ	NARS2
DGUOK	TRNT1	MRPS7	MT-TR	PARS2
RRM2B		MRPS14	MT-TS1	QRSL1
SAMHD1		MRPS16	MT-TS2	RARS2
SUCLA2		MRPS22	MT-TT	SARS2
SUCLG1		MRPS23	MT-TV	TARS2
TK2		MRPS25	MT-TW	VARS2
TYMP		MRPS28	MT-TY	WARS2
		MRPS34		YARS2
		MT-RNR1		
		PTCD3		

6) Metabolism of toxic compounds

D2HGDH
ECHS1
ETHE1
HIBCH
HTT
IDH2
L2HGDH
SLC25A1
TXN2
TXNIP

3) Mitochondrial dynamics, homeostasis, and quality control

Fusion	Mitochondrial protein quality control
MFN2	AFG3L2
NME3	ATAD3A
	CLPB
Fission	CLPP
DNM1L	HSPD1
GDAP1	HTRA2
MFF	LONP1
STAT2	MIPEP
SLC25A46*	PITRM1
	PMPCB
Stability	SACS
OPA1	SPG7

MICOS complex
CHCHD10
C19orf70*
SLC25A46*

Mitochondrial membrane phospholipid and import machinery
AGK
AIFM1
DNAJC19
GFER
OPA3
PAM16
PISD
PMPCA
SERAC1
TAZ
TIMM8A
TIMM22
TIMM50
TOMM70
XPNPEP3

Morphology
MIEF2
MSTO1
YME1L1

Apoptosis defect
APOPT1

Ca2+ homoeostasis
C19orf70*
MICU1
MICU2

4) Metabolism of substrates

Fatty acid oxidation	Redox carriers
ETFA	GOT2
ETFB	MDH1
ETFDH	SLC25A13
HADHA	
HADHB	**Pyruvate**
SLC22A5	**metabolism**
SLC25A20	DLAT
	DLD*
Ketone bodies	PC*
ACAT1	PDHA1
HMGCL	PDHB
HMGCS2	PDHX
OXCT1	PDK3
	PDP1
Anaplerosis	
CA5A	**Mitochondrial**
PC*	**carriers**
	MPC1
Tricarboxylic acid	SLC25A3
cycle enzymes	SLC25A10
ACO2	SLC25A12
DLST	SLC25A26
FH	
IDH3A	
IDH3B	
MDH2	

5) Metabolism of cofactors

Iron–sulphur cluster protein biosynthesis	Biotin metabolism	Haem biosynthesis
BOLA3	BTD	COX10*
FDX1L	HLCS	COX15*
FDXR	PC*	CYCS*
FXN		HCCS*
GLRX5	**Thiamine**	PPOX
IBA57	**metabolism and**	SFXN4
ISCA1	**transport**	SLC25A38
ISCA2	SLC19A3	
ISCU	SLC25A19	**CoA metabolism**
LYRM4	TPK1	**and transport**
NFS1		COASY
NFU1	**Lipoic acid**	PANK2
NUBPL*	**biosynthesis**	SLC25A42
	DLD*	
	LIAS	**Copper transport**
	LIPT1	COA6*
	LIPT2	SCO1*
	MCAT	SCO2*
	MECR	
		NADPH
	Riboflavin	**metabolism**
	metabolism	NADK2
	and transport	NAXD
	FLAD1	NAXE
	SLC25A32	

Inheritance mode Autosomal recessive; Autosomal dominant; Autosomal recessive and autosomal dominant; Maternal; X-linked recessive; X-linked dominant

Fig. 1. Mitochondrial disease genes. Mitochondrial disease genes (338) divided into six subsets according to their functional roles: (1) OXPHOS subunits, assembly factors, and electron carriers (102/338 genes), (2) mitochondrial DNA maintenance, expression, and translation (102/338 genes), (3) mitochondrial dynamics, homoeostasis, and quality control (43/338 genes), (4) metabolism of substrates (40/338 genes), (5) metabolism of cofactors (41/338 genes), and (6) metabolism of toxic compounds (10/338 genes). Numerous genes have dual roles across these categories (indicated by an asterisk). The mode of inheritance is autosomal recessive in 262 genes, maternal in 36, autosomal dominant in 8, X-linked dominant in 6, X-linked recessive in 4, and 22 genes inherited by a combination of AR and AD inheritance.
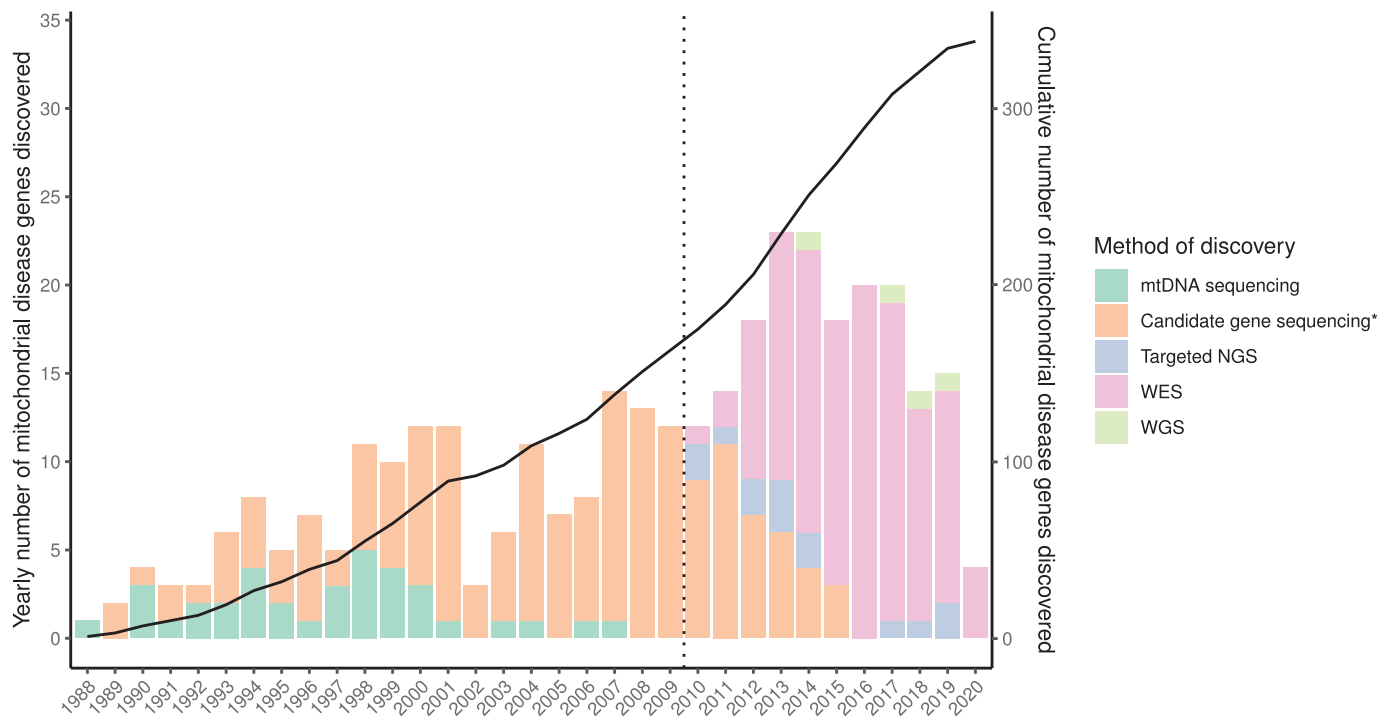
Fig. 2. Methods of disease gene discovery. Demonstration of the shift from mt-DNA and candidate gene sequencing to NGS, marked by the dashed lined in 2010, and reflected by an acceleration in disease gene discovery. Studies marked with an asterisk include a combination of linkage analysis, homozygosity mapping, and candidate gene sequencing.

inclusion of further panels. Virtual panels do however come with the notable disadvantage of lower coverage and blinkering of clinicians to important information beyond genes to which they are restricted, thereby potentially overlooking clearer diagnoses at other loci.

4.5. Whole exome sequencing

WES interrogates the protein coding regions of the genome which harbour approximately 400 protein modifying rare variants [28]. Moreover, the mt-DNA can be analysed from WES data in a holistic approach negating the need for complete mt-DNA sequencing prior or in parallel to WES [29]. Here, the mt-DNA is captured in "off-target" reads with high recall (>95%), precision (>99%), and estimation of heteroplasmy comparable to the "gold standard" targeted mt-DNA

NGS (average difference of <10%). This is especially useful in the paediatric population where the higher frequency of nuclear DNA mutations (75%−90%) deems WES the prudent first-step. It is important to note that WES is typically performed on blood where heteroplasmy can be low, therefore a negative result does not exclude the presence of mt-DNA variants in other tissues.

In total, nine studies have evaluated diagnostic WES in suspected mitochondrial disease, achieving diagnostic yields of 35%−70% [24,30−37] (Table 1), an improvement in mean diagnostic yield from 14% in panel approaches to 47%. The considerably wide variation in diagnostic yield is in the most part a product of the patient selection criteria. Larger cohorts encompass less clearly defined patients representative of everyday clinical practice, in comparison to the small deeply phenotyped, homogenous, and biochemically confirmed

Table 1
The diagnostic yield of next generation sequencing in suspected mitochondrial disease.

	Genes analysed	Publication	Size of cohort	Biochemical confirmation	Age group	mt-DNA analysis	Diagnostic rate	Proportion of mitochondrial diagnoses
Panel	<500	Calvo et al. [22]	60	+	P	Included*	22 (13)	100% (13)
		DaRe et al. [23]	148	+/-	P and A	Included*	9% (13)	46% (6)
		Legati et al. [24]	125	+/-	P and A	Included*	15% (19)	100% (19)
	>500	Calvo et al. [25]	42	+	P	Included*	31% (13)	100% (13)
		Vasta et al. [26]	26	+/-	P	Excluded prior	23% (6)	67% (4)
		Lieber et al. [27]	84	+/-	P and A	Included*	7% (6)	67% (4)
		Panel summary	485				14% (70)	84% (59)
WES	20,000	Haack et al. [30]	10	+	P	Included*	70% (7)	100% (7)
		Taylor et al. [31]	53	+	P	Excluded prior	54% (28)	100% (28)
		Ohtake et al. [32]	104	+	P	Excluded prior	43% (45)	60% (27)
		Wortmann et al. [33]	109	−	P	Excluded prior	39% (42)	50% (21)
		Legati et al. [24]	10	+	P and A	Included*	60% (6)	50% (3)
		Kohda et al. [34]	142	+	P	Included*	35% (49)	86% (42)
		Pronicka et al. [35]	113	−	P	Included*	59% (67)	70% (47)
		Puusepp et al. [36]	28	−	P	Included*	57% (16)	25% (4)
		Theunissen et al. [38]	63	−	P and A	Included*	62% (39)	82% (32)
		WES Summary	632				47% (299)	71% (211)

* Some patient had already undergone mt-DNA screening and were either considered negative or inconclusive (containing variants of uncertain significance) A: adult, P: paediatric.
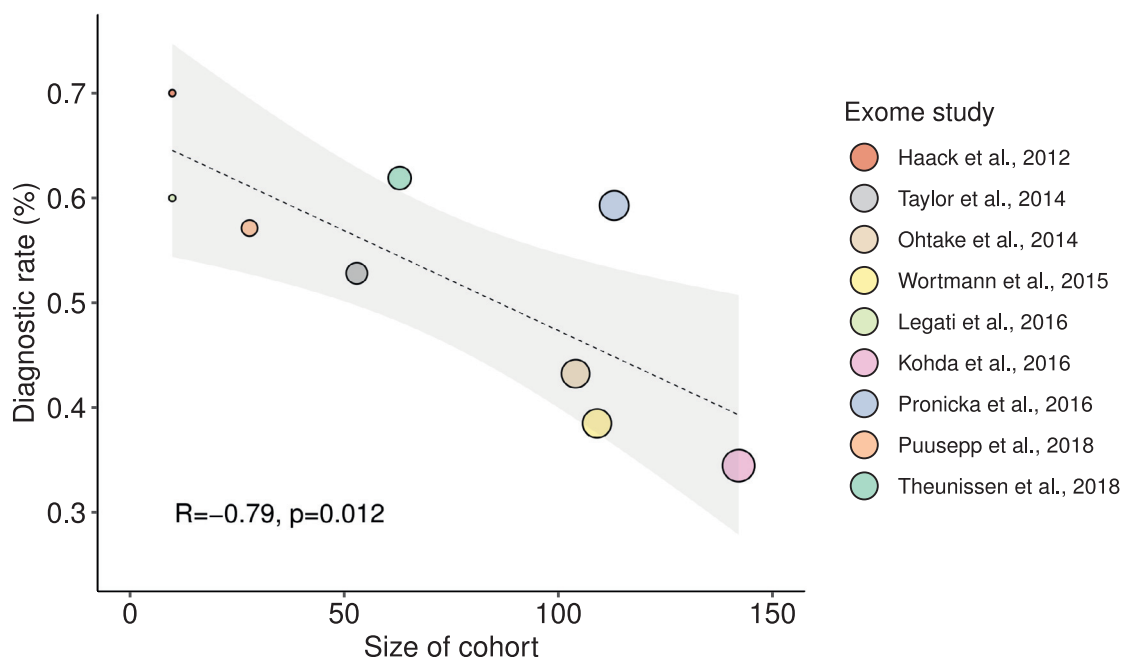
Fig. 3. Diagnostic rate of WES in suspected mitochondrial disease. WES studies of greater cohort size typically encompass more heterogenous cohorts and show a trend towards reduced diagnostic rate. They are, however more reflective of every day clinical practice.

cohorts. Accordingly, a trend of reduced diagnostic rate with increasing study size emerges (Fig. 3) (Table 1). To date, an objective clinical measure of mitochondrial disease likelihood, such as the MDC score, has not been routinely applied. When used with rigour, such scores may help to stratify high-risk patients. It is therefore unsure how biased these studies truly are, and whether there is a clear threshold in MDC score for establishing a mitochondrial molecular diagnosis. Despite this variation, within the rare disease field where the diagnostic rate of WES ranges from 24 to 68%, mitochondrial disease sits at the uppermost end of the spectrum, making them decidedly amendable to WES as a first-tier investigation [38].

Types of genetic variant currently intractable to WES include mosaic variants requiring high sequencing depth, genomic alterations such as small and large deletions/insertions (<50 bp and >50 bp, respectively) especially important in the analysis of the mt-DNA in mitochondrial disease, structural changes such as chromosomal rearrangements (inversions, translocations, deletions, duplications), repeat expansions, and deep intronic and regulatory variants. The inability to delineate the latter changes is overcome in the most part by WGS, with long-read WGS tackling the deletions/insertions.

4.6. Whole genome sequencing

WGS interrogates all regions of the genome. The genome harbours on average 4 million variants, 10,000 of which can alter protein and 40−100 of which have arisen *de novo* in the individual [39,40]. The precise number of disease-causing mutations in the non-coding regions is so far unknown, though evidence suggests that it features as many as 30% of variants impacting gene expression [41]. This is reflected in transcriptomic studies, where almost 50% of disease-causing variants leading to splice aberrations are located in deep intronic regions [42−46]. The relatively limited number of rare disease discoveries made outside of the protein-coding sequence reflects the exponentially more difficult resolution of non-coding variant pathogenicity. In mitochondrial disease, WGS has been instrumental in illuminating three novel disease genes (*COX6A1, TIMMDC1,* and *COQ5*) where the diagnosis relied on the detection of an intronic deletion, a deep intronic variant, and a 3′UTR duplication, respectively. Each resulted in splice aberration, captured by qPCR or

transcriptomics, and subsequently disrupted gene function [42,47,48]. To date, WGS has not been assessed in a systematic way in suspected mitochondrial disease cohorts, it is therefore only possible to infer the benefits to diagnostic rate base on the wider rare disease field where a modest improvement of 2% is seen in comparison to WES (40% vs. 38%, respectively) [38].

4.7. The benefits of WES and WGS

The unbiased approaches of WES and WGS opportune exposure of pathogenic variants in genes beyond those initially suspected by the diagnostic work-up, which is about 30% in published studies (Fig. 4) (Table 1). Moreover, capturing data on all genes regardless of their disease-causing status allows reanalysis of negative cases. In negative WES, from studies totally over 1,000 reanalysed cases, diagnostic gains of up to 10%−20% are demonstrated just 1−3 years after the initial analysis, mostly due to the discovery of novel disease genes [49−52].

4.8. Trio sequencing

WES and WGS are ideally undertaken in trios, by simultaneous analysis of the proband and parents, to strengthen the diagnostic rate with identification of *de novo* variants. A modest 1% increase in diagnostic rate between proband-only and trio sequencing (39% and 40%, respectively) and an increase in *de novo* detection of 2% (47% and 49%, respectively) are reported in the rare disease field to date [38]. Despite these returns, trio sequencing increases concerns of high economic burden and analysis speed. Of the limited studies available for evaluation to date, French and colleagues demonstrate the application of trio WGS in acutely unwell paediatric patients, providing a timely molecular diagnosis (within 2−3 weeks) which influenced clinical management in 65% [9]. Recent studies also provide the first end-to-end cost-effectiveness analyses by prospectively ascertaining patients undergoing parallel WES and conventional diagnostic care. This not only resulted in decreased cost, but in a marked increase in Quality Adjusted Life Years (QALYs), a cost-effectivity which strengthened further still when benefits to first-degree relatives and
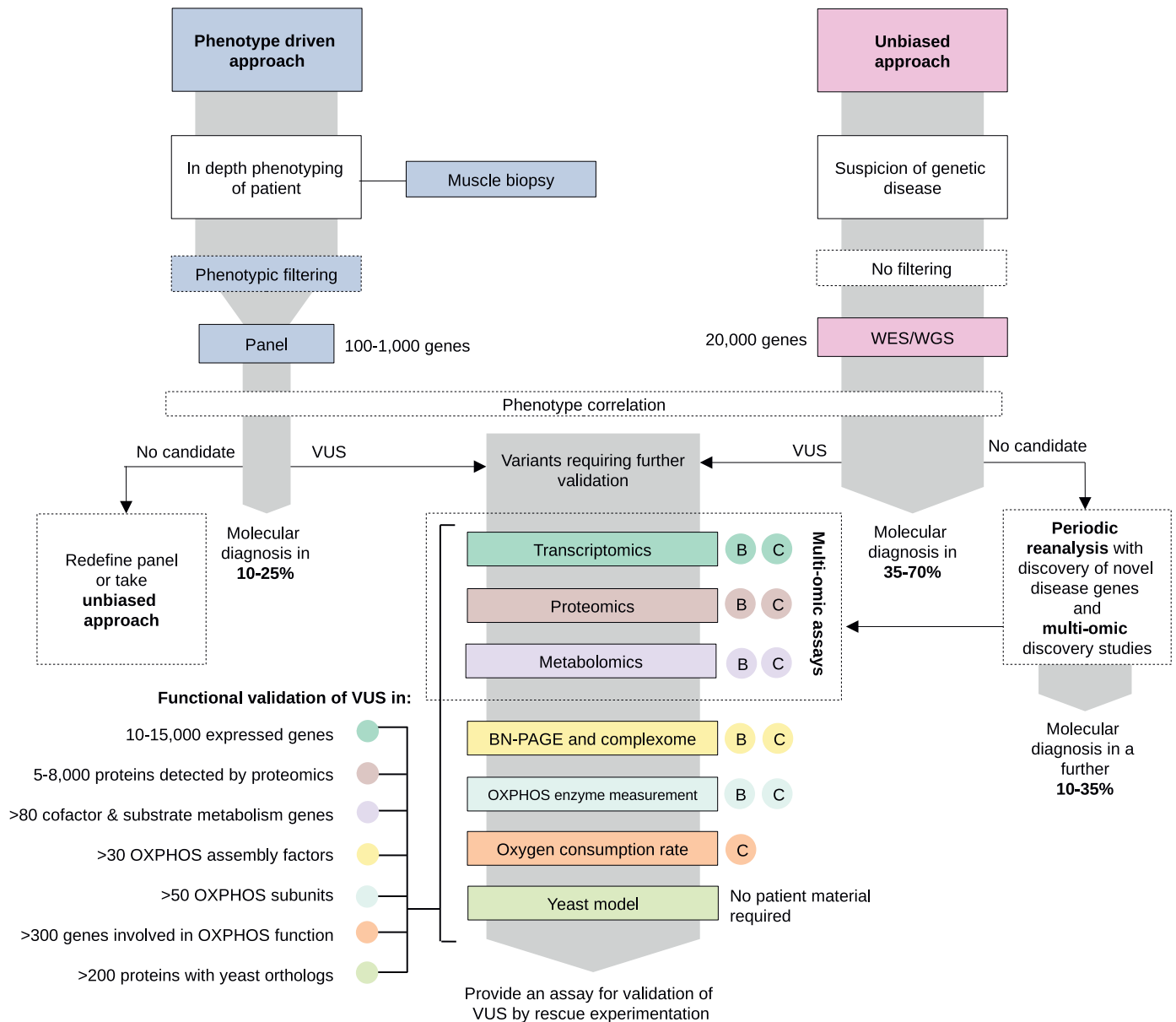
Fig. 4. Evolution of the diagnostic approach. Schematic comparing a conventional phenotype driven approach to molecular diagnostics, where deep phenotyping of the patient in combination with invasive muscle biopsy determines choice of genetic investigation, to the unbiased approaches of WES and WGS. Variants of uncertain significance (VUS) resulting from the analyses require functional validation and often necessitate patient materials such as a biopsy (B) or cell line (C), optimally undertaken in the affected tissue. Beyond the global "omic" methodologies, depicted here are a selection of assays capable of capturing the consequence of substantial subgroups of mitochondrial disease genes.

the benefits to parental reproductive outcomes were taken into consideration [52,53].

5. Variant of uncertain significance: "Innocent until proven guilty"

The overarching issue of all sequencing approaches is the discovery of variants of uncertain significance (VUS). With genomic advancement, came the shift in the diagnostic bottleneck from variant discovery to interpretation, which must be rigorous to avoid misinterpretation. Bringing clarity to large variant-rich genes, frequently harbouring synonymous and missense VUS, is notoriously challenging especially when different modes of inheritance are reported. *POLG* is a prime example, where an expertly curated database of reported variation is necessitated (Human DNA Polymerase Gamma Mutation Database, https://tools.niehs.nih.gov/polg/). As an initial step to structure diagnostics, the American College of Medical Genetics (ACMG) provides guidelines incorporating 28 criteria. These

criteria span population, computational and predictive, functional, segregation, and allelic data to position a variant in a 5-tier system from "Pathogenic" through to "Benign" interpretation. Functional validation has prominent weighting in such criteria.

Here, we review the most profitable choices of functional assay in mitochondrial disease, for both validation of VUS and for variant discovery in unsolved WES and WGS cases. We focus on recent advances in the application of unbiased transcriptomic, proteomic, and metabolic methodologies. Beyond these, methylomics, lipidomics, and glycomics could shed further light on the pathogenesis of mitochondrial disease and improve the outlook for the discovery of precision medicines in this largely "untreatable" collection of diseases.

5.1. Transcriptomics

Transcriptome analysis facilitates genome-wide DNA variant interpretation, both coding and non-coding. Non-coding variants are often located deep within the intronic sequence or in regulatory

Table 2
The diagnostic yield of RNA-sequencing studies in rare Mendelian disease.

Study	Patient group	Size of cohort	Control source*	Tissue	Analyses			Search space	Diagnostic yield	Candidate yield
					Aberrant splicing	Allelic imbalance	Aberrant expression			
Kremer et al. [42]	Mitochondrial diseases	48	1	Fibroblasts	✓	✓	✓	Genome-wide	10% (5)	75% (36)
Cummings et al. [43]	Rare muscle disorders	50	2	Muscle	✓	✓	✗	Muscle disease genes	34% (17)	6% (3)
Fresard et al. [44]	Rare Mendelian diseases	80	2,3,4	Blood	✓	✓	✓	Genome-wide	8% (6)	6% (5)
Gonorazky et al. [45]	Monogenic neuromuscular disorders	25	2	Muscle	✓	✓	✓	Genome-wide	36% (9)	–
Lee et al. [46]	Rare mendelian diseases	48	1	Blood, fibroblasts, muscle*	✓	✓	✗	Genome-wide	10% (5)	–

* Tissue selection based on tissue readily available without a priori knowledge of most appropriate tissue.
1 Internal collection of transcriptomic data.
2 Genotype-Tissue Expression consortium (GTEx).
3 Depression Genes and Network (DGN) cohort data.
4 Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) project data.

regions and are influential in mRNA expression and splicing. Discarding lowly expressed reads, around 10−15,000 transcribed genes are detectable [42−46]. For the diagnosis of Mendelian disease, three aberrant events may be analysed: (1) aberrant splicing, (2) aberrantly low expression, (3) imbalance in allele specific expression, and variants often present as a combination of these events. Allele imbalance may arise secondary to a variant that results in non-sense mediated decay (NMD) of one allele, arises in a regulatory region resulting in decreased expression of one allele, or can be the consequence of phenomenon such as X-chromosome inactivation and imprinting. By capturing these events, RNA-sequencing provides functional validation and is often the missing link in a diagnosis, as it provides evidence of a second variant to complement a rare heterozygous mutation already identified by NGS [42,46].

To date, five systematic transcriptomic discovery studies in suspected Mendelian diseases have been described. The field is still evolving and a standardised protocol with concrete diagnostic criteria is not yet established. Given the differences in experimental design and in disease cohort, diagnostic rates range from 8% to 35% following negative WES or WGS analysis, with additional steps made in identifying strong candidates in the majority (Table 2). In the study of mitochondrial diseases, a median of five aberrant splicing events, one aberrantly expressed gene, and six mono-allelically expressed rare variants were called in patient-derived fibroblasts, a manageable number for manual curation and downstream validation studies [42]. Across all studies, diagnoses were made primarily by detection of aberrant splice events, with allele imbalance and aberrantly low expression playing a complementary role (Fig. 5). Such high throughput validation of splice variants by transcriptomics is essential due to the low specificity of splicing algorithms for true splice disrupting events [54], as often the causative variant is detected by WES or WGS but lost amongst a vast list of variants predicted to affect splicing. Moreover, in combination with WGS, transcriptomics was instrumental in establishing a new mitochondrial disease gene, *TIMMDC1*, where a homozygous deep intronic variant led to pseudoexon creation, a downstream premature stop codon, and subsequent NMD [42].

The current challenges of RNA-sequencing arise in the tissue selection and data analysis. Tissues are known to demonstrate unique expression patterns. Therefore, the tissue selected needs to be representative of the expression patterns in the disease relevant tissue to circumvent an underpowered analysis. In mitochondrial disease, we are in a privileged position as the culprit genes are housekeeping, allowing high numbers to be captured across a range of different

tissues (Fig. 5). Fibroblasts are obtainable by minimally invasive skin biopsy or as a by-product of a diagnostic muscle biopsy and can be utilised in further downstream functional validation, where required. Moreover, selection of such a non-affected tissue may have advantages, as the regulatory consequences on other genes are minimal allowing the causal defect to be a more prominent outlier. As we move away from the phenotype driven approach to diagnosis, negating the need for muscle biopsy, the most readily available tissue is blood. However, in corroboration with other studies, across tissues obtainable by biopsy we find blood to be the least informative (Fig. 5) [45,46]. Regarding the analysis, selection of control cohorts is an important consideration. Publicly available databases are rich in RNA-sequencing data, however given platform-specific biases, Kremer and colleagues argue for the optimal use of an in-house database where matched controls are generated overtime [42]. As outlined by the five studies, which each elected different control cohorts, both choices see success (Table 2). Key to all approaches is normalisation of the data, optimally for both known and unknown cofounders. OUTRIDER, a statistical method for detecting aberrantly expressed genes, makes steps in overcoming these hurdles [55].

5.2. Proteomics

Proteomic methodology was pivotal in cataloguing the 1500 predicted mitochondrial proteins [56]. It allows quantification of all detected proteins in one assay, removing the need for individual Western blot analyses of predefined proteins of interest. In patient-derived fibroblast cell lines approximately 5,000−8,000 proteins are quantified, encompassing over 65% of mitochondrial disease proteins [42,57,58]. Missense variants are by far the most commonly occurring variant in mitochondrial disease, accounting for over 50% of all mutations reported in the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk, accessed March 2020). Missense variants may have no functional consequence or may exercise their pathogenicity by disrupting interactions or destabilising the protein structure. Destabilisation occurs in approximately 40% of missense variants [59] and can be detected by proteomics as aberrantly low expression [57].

The application of proteomics as a complementary diagnostic tool to WES and WGS has not been studied systemically in mitochondrial disease to date, or indeed any rare disease cohort. However, there are a handful of examples in the literature of the role of proteomics in validation of VUS. Kremer and colleagues evaluated the consequence of diminished RNA expression on the protein level, confirming
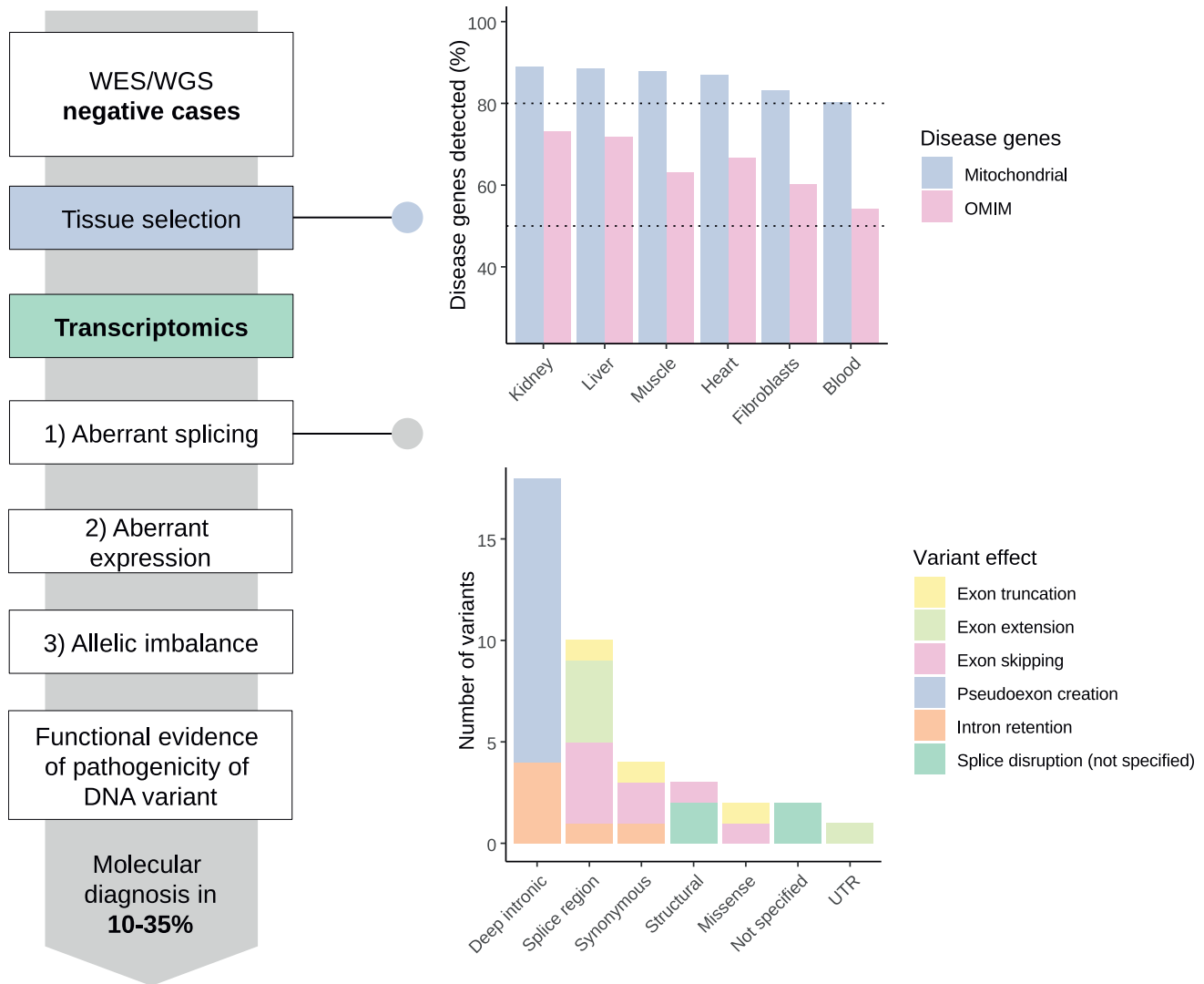
Fig. 5. Integration of transcriptomic data with genetic data. Tissue selection in transcriptomics influences disease gene detection. Over 80% of the mitochondrial disease genes and 50%−75% of OMIM disease genes are captured across a range of different tissues. In the captured transcripts, aberrant splicing, aberrant expression, and allelic imbalance may be analysed. Of 46 identified pathogenic variants in diagnostic studies to date (Table 2), 40 induce aberrant splice events. The majority of these events were pseudoexon creation or intron retention resulting from deep intronic variants, and exon creation or exon skipping as a result of splice region variants.

complete or near complete (98%) loss of protein in patients with *TIMMDC1* VUS [42]. Moreover, by the unbiased detection of cellular protein changes, the proteomic analysis revealed global reduction of complex I subunits consistent with the function of TIMMDC1 as a complex I assembly factor. Similarly, Lake and colleagues utilised proteomics to detect a mitochondrial translation defect caused by destabilisation of the small mitoribosomal subunit to validate biallelic VUS in the small ribosomal subunit encoding gene *MRPS34*, where the collective abundance of all detected small mitoribosomal subunit proteins was significantly decreased [58]. Interestingly, both studies remark on the ability of the reduction in subunits of specific OXPHOS enzymes on proteomics to closely resemble the residual OXPHOS enzyme activity in the respective cell line by biochemical measurement [42,58].

A limitation of proteomics arises in the lack of detection of the functional consequence of the majority of missense variants which do not lead to degradation of the defective protein. Indeed, a study systematically phenotyping a library of over 2850 rare disease-causing missense variants across almost 1150 genes (the human mutation open reading frame collection; hmORF), found that approximately 60% of missense variants resulted in preserved folding or stability [59].

5.3. Metabolomics

Measurement of lactate, amino acids, and organic acids in blood and urine reveal the initial biomarkers of mitochondrial disease. These metabolites provide diagnostic clues but have low sensitivity and specificity, and cannot pinpoint a diagnosis on the gene-level with the exception of select diagnoses, such as ethylmalonic aciduria indicative of *ETHE1* mutations (Ethylmalonic encephalopathy, OMIM 602,473). Newborn screening is another example of a metabolite driven analysis, where a clear-cut answer is provided. However, with the exception of MCADD (medium-chain Acyl-CoA dehydrogenase deficiency, OMIM 607,008) due to *ACADM* mutations, severe and often lethal neonatal-onset mitochondrial disease are unfortunately inadequately represented in these screening initiatives.

Metabolomics is a mass spectrometry-based approach for the systematic capture and quantification of up to thousands of small molecule metabolites within a given tissue [60]. The approach allows analysis of the up- and downstream effects of mitochondrial dysfunction on cellular processes and can illuminate disease-distinguishing subsets of metabolites, termed "metabolite fingerprints". The generation and analysis of the data is complex, not least due to the differing properties of the metabolites in concentration, charge,

hydrophobicity, and solubility, but in the need for complex enrichment and pathway analyses of the data. Moreover, the metabolome is under the combined influence of the primary genetic defect, the genetic background, and environmental exposures. The diagnostic value of metabolomics in certain subsets of genetic diseases such as inborn errors of metabolism (IEM) has proven to be high [61]. However, though its use has been reported in individual mitochondrial disease cases for the validation of VUS [42,62,63], a large systematic metabolomic study is needed to establish the true value of metabolomics as a complementary diagnostic tool to WES and WGS.

6. Focused functional validation assays

Beyond the global "omic" methodologies, advances in gene discoveries by WES and WGS have far outpaced the development of high-throughput functional studies. Often the choice of a specific functional validation assay is necessary and fluctuates from gene to gene. Details on specific assays are beyond the scope of this review and are explored in detail elsewhere [64]. Here, we highlight assays in the experimental toolkit capable of capturing the consequence of substantial subgroups of mitochondrial disease genes (Fig. 4).

Blue-native electrophoresis (BN-PAGE) is a traditional way to study the integrity of OXPHOS complexes in subunit and assembly factor defects, and more recently in combination with quantitative proteomics in complexome analysis [65]. Its development has provided valuable insight into the formation of the OXPHOS complexes, most notably the step-wise assembly of complex I, followed by the formation of suprastructures known as the "supercomplexes" [66]. OXPHOS enzyme assays measure the amount of residual enzyme activity of the respective complexes in a given tissue or cell line. Generally speaking, an isolated OXPHOS enzyme deficiency can be utilised as proof of pathogenicity for VUS within OXPHOS subunit encoding genes. These biochemical assays are complex procedures and must be performed in expert centres with good quality control, experience, and a certain turnover of samples. Measurement of oxygen consumption rate (OCR) by microscale oxygraphy provides an impression of the overall bioenergetic state of the mitochondrial in vivo and assesses the capability of the mitochondrial to respond to stress [67]. Notably, mutations can also be modelled in the yeast system without the need for a patient-derived tissue or cell line. Of the 338 currently described disease genes, 217 have orthologs in *Saccharomyces cerevisiae* according to the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) [68]. Moreover, where a functional validation assay reveals a phenotype, rescue complementation with wild-type cDNA can cement the pathogenic designation of a variant [69].

7. Outstanding questions

The days of rapid-paced gene discovery with gathering of the so called "low-hanging fruit" have paved the way to the future diagnostic challenges. The complexities of inheritance almost untouched in mitochondrial disease research include the contribution of digenic, oligogenic, and polygenic inheritance, the potential for genetic modifiers in variable penetrance and phenotype expressivity, tissue specificity, and gene-environment interactions, amongst others. We foresee these to be the next frontiers in mitochondrial disease genetics.

With the exception of a number of mt-DNA encoded diseases, where penetrance is reported to be as low as 10%−50%, nearly all molecularly confirmed mitochondrial diseases result from highly penetrant alleles [6,70]. Such incomplete penetrance clouds the water for determination of variant pathogenicity given the absence of segregation with affected individuals in the pedigree, and higher

population allele frequency than expected, two criteria pointing towards a likely benign variant. It is also proposed that milder phenotypes in mitochondrial disease may be attributable to a collective interaction of "weaker" alleles, akin to a complex disease, or from the influence of modifier loci. To delineate these subtle interactions, we require an immense volume of harmonised genotypic and phenotypic data, in addition to large pedigrees, underlining the importance of collaboration and data sharing. GENOMIT, a global registry initiative, works towards this objective in mitochondrial disease (www. GENOMIT.eu). Given the prevalence of the individual mitochondrial diseases, such analyses may struggle to reach the statistical power needed. Approaches are therefore emerging that utilise data from large genome-wide association studies (GWAS) of complex traits, and demonstrate the involvement of common polygenic factors in the variable expressivity of rare disease [71−73].

8. Conclusion

As we move further into the genomic and "multi-omic" era, the opportunity to understand the cause of each and every mitochondrial disease and to provide a diagnosis to the majority becomes ever more tangible. Though caution should be exercised in variant interpretation and actionability, the opportunity for every patient to be explored in the research environment may overcome the bottleneck in clinical translation of these methodologies. Moreover, to realise their true diagnostic capability we must increase willingness to pool resources, such as (un)solved WES and WGS data, to increase the signal of pathogenic variant recurrence. Overall, given the growing complexity of mitochondrial disease genetics, we endorse a wide-angle unbiased approach to diagnostics.

9. Search strategy and selection criteria

Data for this review were identified by a systematic search of PubMed using the term ("mitochondrial disease") AND (mutation OR diagnostic OR omic) or were manually sought after from relevant articles. Preference was given to articles published over the last three years and each was academic and peer-reviewed.

Author contributions

Sarah L. Stenton wrote the manuscript and created the figures and tables. Holger Prokisch supervised the writing and editing of the manuscript.

Declaration of Competing Interest

The authors declare no competing interest.

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