METABOLIC DISORDERS: GENES AND MECHANISMS

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METABOLIC DISORDERS: GENES AND MECHANISMS

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MITOCHONDRIAL DISORDERS

Mitochondrial disorders constitute a subgroup of the large family of rare monogenic diseases called Inborn Errors of Metabolism (IEM), but defects in mitochondrial activity have also been reported in more common and complex pathologies. In the present thesis, I validate new disease-causing variants and study the underlying molecular defects in three different cases of IEM.

In paper I, I report the first individuals suffering from slowly-progressive, early-onset neurodegeneration caused by loss-of-function mutations in the autophagy adaptor Sequestosome 1 (SQSTM1, or p62). Analysis of fibroblasts from these patients suggests a defect in the early response to mitochondrial stress and in autophagosome formation.

In paper II, I further investigate the phenotype of two of the previously reported patients, using reprogrammed neuroepithelial stem (NES) cells and differentiated neurones. Loss of p62 in NES cells leads to an impairment in neuronal differentiation, possibly due to an inability to switch between glycolytic and oxidative metabolism.

In paper III, I study the consequences of the lack of Thioredoxin interacting protein (TXNIP) in myoblasts and fibroblasts of affected individuals. Oxygen consumption measurements revealed decreased mitochondrial activity, depending on the supplied carbon source. In particular, patient cells are unable to utilise the end product of glycolysis - pyruvate - but can efficiently use the tricarboxylic acid (TCA) cycle intermediate, malate, to fuel mitochondrial energy production. This expands the current view of the role of TXNIP in physiology and pathology.

In paper IV, I model a neurodegenerative disorder related to mutations in the mitochondrial tryptophanyl-tRNA synthetase (WARS2) gene, after assessing pathogenicity of the variants in yeast. Knock down of WARS2 in Drosophila melanogaster resulted in larval lethality and reduced aminoacylation of tRNA^{Trp}, much resembling what can be observed in the affected individuals. Although muscle biopsies and fibroblasts from patients did not reveal a mitochondrial dysfunction, reprogrammed NES cells displayed a combined Complex I and IV defect, strengthening the relevant tissue-specificity of mitochondrial disorders. Our results demonstrate the importance of using appropriate models for the characterisation of mutations and for the efficient validation of pathogenic variants.
LIST OF SCIENTIFIC PAPERS


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<tr>
<td>aaRS</td>
<td>Aminoacyl-tRNA synthetase</td>
</tr>
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<td>AARS2</td>
<td>Mitochondrial alanyl-tRNA synthetase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<tr>
<td>ARE</td>
<td>Antioxidant Response Elements</td>
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<tr>
<td>ATG</td>
<td>Autophagy Related Genes</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Avian myelocytomatosis virus oncogene cellular homolog</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR Associated Protein 9</td>
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<tr>
<td>CCCP</td>
<td>Carbonyl Cyanide m-Chlorophenyl Hydrazone</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
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<tr>
<td>CytC</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DARS2</td>
<td>Mitochondrial aspartyl-tRNA synthetase</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNP</td>
<td>2, 4-Dinitrophenol</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-Strand Break</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal Dementia</td>
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<tr>
<td>FTLD</td>
<td>Frontotemporal Lobar Dementia</td>
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<tr>
<td>HARS2</td>
<td>Mitochondrial Histidyl-tRNA synthetase</td>
</tr>
<tr>
<td>Hif1-α</td>
<td>Hypoxia Inducible Factor 1-alpha</td>
</tr>
<tr>
<td>IEM</td>
<td>Inborn Errors of Metabolism</td>
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<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<tr>
<td>KEAP1</td>
<td>Kelch-like ECH associated protein 1</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like Factor 4</td>
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<td>LARS2</td>
<td>Mitochondrial leucyl-tRNA synthetase</td>
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<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1 Light Chain 3 alpha</td>
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LDHA  Lactate Dehydrogenase A
MAPT  Microtubule-associated protein Tau
MnSOD/SOD2  Manganese Superoxide Dismutase
mtDNA  Mitochondrial DNA
mTORC1  Mammalian Target of Rapamycin Complex 1
NAC  N-Acetyl Cysteine
NAD⁺  Nicotinamide Adenine Dinucleotide
NES  Neuroepithelial Stem
NGS  Next-Generation Sequencing
NHEJ  Non-Homologous End Joining
NLRP3  NOD-like Receptor Protein 3
NQO1  NAD(P)H Quinone Oxidoreductase 1
Nrf2  Nuclear Factor (erythroid-derived 2)-like 2
Oct 3/4  Octamer-Binding Transcription Factor 4
OXPHOS  Oxidative Phosphorylation
PINK1  PTEN-Induced Putative Kinase Protein 1
ROS  Reactive Oxygen Species
SH2  Src Homology 2
Sox2  (Sex Determining Region Y)-Box 2
SQSTM1/p62  Sequestosome 1
TALEN  Transcription Activator-Like Effector Nucleases
TCA  Tricarboxylic Acid
TXNIP  Thioredoxin Interacting Protein
UQ/CoQ10  Ubiquinone/Coenzyme Q
WARS2  Mitochondrial tryptophanyl-tRNA synthetase
WES  Whole-Exome Sequencing
WGS  Whole-Genome Sequencing
ZFN  Zinc Finger Nuclease
α-KGDH  2-oxoglutarate dehydrogenase (α-Ketoglutarate Dehydrogenase)
1. INTRODUCTION

1.1 Introduction to metabolism

“For things to remain the same, everything must change”: what is written in the 1958 novel by Giuseppe Tomasi di Lampedusa “The Leopard” can easily be seen as a general rule of Nature. Balancing continuous changes is what maintains life as we know it, and for this reason every organism struggles towards homeostasis. The repertoire of choices at the disposal of living beings is to efficiently keep an equilibrium, while constantly modifying themselves, is metabolism. The word “metabolism” stems from Greek μεταβολή, “change” and was first used in 1839 by Schwann (Schwann 1839), before being adopted into English (Bing 1971). Yet, the concept of metabolism originates earlier, in 1836, when Tiedemann introduced the notion of Stoffwechsel, i.e. “the conversion of body substance into other forms and the rapid production of new and precisely similar body structures by means of the constant supplying of nourishing material” (Tiedemann 1836). With the years, the word “metabolism” has acquired the more specific meaning of a complicated network of biochemical and enzymatical reactions that sustains living organisms (Fig. 1).

Fig.1: schematic outline of the main pathways constituting the metabolic network.
Metabolic reactions can be divided into two major groups, depending on whether they are constructive or destructive: anabolic reactions promote the conversion of simple molecules into more complex ones, normally consuming energy, while catabolic reactions promote the breakdown of complex molecules into simple ones, and release energy. To coordinate this tangled system, each cell needs to finely tune its needs and regulate different pathways in an independent way; naturally, various compartments will have varying demands at different timepoints and thus communication within cellular environments cannot be underestimated.

1.2 Introduction to metabolic disorders

Under the broad class of “Inborn Errors of Metabolism” (IEM), a term coined by Sir Archibald Garrod in 1923, a group of pathological situations affecting around 1:1000 newborns is represented. These diseases are defined by a loss or toxic accumulation of metabolic intermediates, and normally emerge at a neonatal stage. Disease progression is usually rapid, although age of onset can vary from birth to adulthood (El-Hattab 2015). Mitochondrial dysfunctions form the largest subgroup within IEM, and are classically defined as deficiencies in oxidative phosphorylation (OXPHOS), leading to impaired ATP synthesis and, ultimately, to insufficient energy production. The clinical spectrum of the affected patients can vary greatly, but often includes myopathy, ataxia, vertical gaze palsy and, if any involvement of the central nervous system is present, a cerebellar syndrome.

Recent years have witnessed an explosion of investigations suggesting a critical role of mitochondria in more common disorders (Murphy and Hartley 2018), e.g. heart disease (Schwarz et al. 2014), diabetes (Karaa and Goldstein 2015), cancer (Porporato et al. 2018) and neurodegeneration (Martin 2012; Burté et al. 2015). Yet, the mitochondrial role in widespread pathophysiological conditions represents a main issue to be elucidated and the next challenge for future research (Nunnari and Suomalainen 2012; Vafai and Mootha 2012). The field of neurodegeneration has shown a particular interest in mitochondria. More and more studies are hinting at the essential function these organelles have for neuronal well-being (Kann and Kovács 2007; Mattson et al. 2008); not only because they are at the core of cellular metabolism, but also for their fundamental role in keeping production of Reactive Oxygen Species (ROS) in efficient balance. For example, reduced activity of Complex I is one of the landmarks of Parkinson’s (Bose and Beal 2016; Park et al. 2018) and deletions in mitochondrial DNA (mtDNA) might contribute to Alzheimer’s disease (Phillips et al. 2014). Furthermore, dysfunctional organelles and their accumulation have also been described in amyotrophic lateral sclerosis (ALS) (Martin 2011; Muyderman and Chen 2014; Khalil and Liévens 2017). The possibility that mitochondrial malfunctioning can affect neuronal health should not be surprising, taken into consideration the importance of energy production for our central nervous system. In fact, it is there that we require and consume most of the glucose that is taken in every day. Oxygen pressure and the access to it by the brain are of extreme importance; in fact, too low or too high concentrations can both cause cell damage...
and ultimately death. Furthermore, the post-mitotic status of neurones impedes any replacement of faulted cells with new ones, making it impossible to fully reconstitute their original pool.

1.3 Introduction to mitochondria

When Altmann first described mitochondria in 1890, he baptised them “bioblasts”. Only eight years later, in 1898, microbiologist Carl Benda named them from the Greek μήτρος, “thread” and χονδρίον, “granule” (Ernster and Schatz 1981). These organelles evolved from an alphaproteobacterium that started an endosymbiotic relationship with a prokaryotic cell. Even though the exact timing and mechanism of the onset of this relationship are matter of vivid debate (Roger et al. 2017), what is considered established is the inheritance mitochondria still retain of their past: in fact, they maintain a prokaryotic genome, a circular 16.5 kb DNA molecule containing 37 genes, among which 13 polypeptides, 22 tRNAs and 2 rRNAs, all essential for correct function of the organelles (Falkenberg et al. 2007; Gustafsson et al. 2016). These gene products are necessary but not sufficient for proper mitochondrial function. Besides the mtDNA-derived peptides over 1000 mitochondrial proteins are encoded in the nuclear genome and need to be imported into mitochondria (Schmidt et al. 2010; Wiedemann and Pfanner 2017). Mitochondria are thus under dual genetic control, requiring careful coordination.

Mitochondria are enclosed by an outer membrane, and their internal space is portioned into a matrix and an invaginated inner membrane, thus increasing the surface to volume ratio and optimising the space at disposal. As mentioned before, they constitute the central hub of cellular metabolism, as they are involved in numerous vital functions, such as steroid synthesis, initiation of apoptosis, Ca$^{2+}$ and ROS signaling, as well as the final steps of aerobic energy metabolism. In their matrix, oxidation of acetyl-CoA during the TCA cycle leads to the reduction of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD$^+$) to FADH$_2$ and NADH, respectively. A number of metabolic pathways converge to the TCA cycle, including glycolysis and beta-oxidation of fatty acids, that supply acetyl-CoA, or protein catabolism, that can provide the cycle with intermediates.

Reduced FADH$_2$ and NADH are then used in OXPHOS, a broad term that designates the coupling of an electron transport chain (ETC) with ATP production across the inner mitochondrial membrane. The hypothesis that the reducing power released by the ETC is not freed in the mitochondrial or cellular environment, but is, instead, efficiently used, was first conceived by Herman Kalckar in 1939. He demonstrated a correlation between the transport of electrons in mitochondria and ATP production (Kalckar 1939), but the exact mechanism by which this coupling can be accomplished remained for some years mysterious, until in 1961 Peter Mitchell formulated his now renowned chemiosmotic theory (Mitchell 2011). In this model, the ETC transfers electrons from reduced NADH and FADH$_2$ to molecular oxygen via a series of redox reactions (Krebs and Johnson 1937), while at the
same time generating an electrochemical proton gradient across the inner mitochondrial membrane. This gradient is then used as a driving force by an ATP synthase (Complex V) to synthesise ATP from ADP and phosphate.

Four complexes, sitting on the cristae in the inner membrane, compose the ETC, aided in their work by the two electrons-carriers ubiquinone (UQ, also known as Coenzyme Q, or CoQ10) and Cytochrome c (CytC) in the intermembrane space (Fig. 2):

**Complex I** (NADH dehydrogenase or NADH:ubiquinone oxidoreductase), consecutively transfers two electrons from NADH to a molecule of Coenzyme Q, thereby reducing ubiquinone to ubiquinol. It is the largest complex, consisting of 45 subunits, with seven encoded by mtDNA. The catalytic core of the complex constitutes 14 subunits, with an additional 31 subunits in mammals. Recently, the full structure was resolved by Zhu, Vinothkumar and Hirst, revealing the exact electron flow through the complex (Zhu et al. 2016). However, how the redox reaction of NADH to ubiquinol is coupled to proton pump remains to be solved.

**Complex II** (Succinate dehydrogenase or Succinate:ubiquinone oxidoreductase) is also a member of the TCA cycle and therefore sits on the matrix side. It oxidises succinate to fumarate, at the same time reducing the covalently bound FAD to FADH₂, which is in turn used to fuel sequential reduction of ubiquinone into ubiquinol. Unlike Complex I, the reduction of ubiquinone is not coupled to proton translocation across the inner mitochondrial membrane. Additionally, it is the only complex of OXPHOS with all of its four subunits being encoded in the nuclear genome.

**Complex III** (Cytochrome c reductase or Ubiquinol:cytochrome c reductase) oxidises ubiquinol into ubiquinone, while reducing CytC, freeing the coenzyme to accommodate new electrons from Complexes I and II. It consists of 11 subunits, one encoded in mtDNA and the rest in the nuclear genome.

**Complex IV** (Cytochrome c oxidase or ferrocytochrome c:oxygen oxidoreductase) oxidises reduced CytC and passes electrons on to molecular oxygen, finally producing water. It consists of 20 subunits, of which 3 are encoded by mtDNA.

These four complexes constitute the mitochondrial ETC. By pumping protons across the inner mitochondrial membrane, Complexes I, III and IV promote formation of the proton motive force that is used by the ATP synthase.

**Complex V** is also known as ATP synthase and works as a dynamo, synthesizing ATP from ADP and phosphate. In brief, the re-entry of protons to the mitochondrial matrix is coupled with rotation of its F0 subunit, which results in the physical condensation of ADP and phosphate in its F1 subunit. It is composed of 20 subunits of which two are encoded by mtDNA and the rest in the nuclear genome. The way these subunits are assembled and the final structure of the protein has been resolved over the years by the group of John Walker (see, e.g. (Watt et al. 2010)).
Fig.2: schematic overview of oxidative phosphorylation. CI: NADH dehydrogenase, CII: Succinate dehydrogenase, CIII: Cytochrome c reductase, CIV: Cytochrome c oxidase, CV: ATP synthase. Four protons are extruded in the intermembrane space at CI and CIII, two protons at CIV. Below each complex, the respective electron donor and receiver are listed.

As most things in biology, mitochondria are not static entities; rather, they endure in continuous phenomena of fusion and fission, that respectively decrease or increase the number of organelles at a cell’s disposal. These events are tightly controlled and require a strict interplay between different cellular microenvironments and signalling pathways (Youle and van der Bliek 2012; Archer 2013). Furthermore, mitochondria are highly mobile and continuously travel across the cell to reach the areas where they are more needed. In this way, a cell has always the chance to spatiotemporally coordinate its energy demands and supply.
1.4 Introduction to ROS and oxidative stress response

ROS constitute the byproduct of aerobic metabolism and include anion superoxide (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^•$). All these molecules exert a dual role: on one hand they are toxic and can interact with DNA, proteins and lipids promoting their damage, ultimately contributing to conditions as neurodegeneration and aging. On the other hand, they can be messengers, promoting communication between cellular compartments (Yoboue et al. 2018) and are important for the immune response (Yang et al. 2013).

The main origin of ROS is the ETC, where they are produced depending on the electrochemical proton potential across the inner mitochondrial membrane, the ratio between NADH/NAD$^+$ and between reduced and oxidized ubiquinone. Understandably, also local O$_2$ concentration plays a role (Murphy 2009). Numerous studies have been conducted during the years to pinpoint the major source of ROS production. Complex I and Complex III have been identified as the components of the ETC where most of O$_2^•$ is generated, even if contribution of the latter can have been overestimated (Adam-Vizi and Chinopoulos 2006), as it seems to have a more important role in the production of hydrogen peroxide (Chen et al. 2003). Both the flavin and the ubiquinone binding site of Complex I are involved in ROS formation (Pryde and Hirst 2011; Hirst and Roessler 2016), while in Complex III ROS seem to be produced at the ubiquinol oxidation centre (centre P, Q$_6$ site) (Bleier and Dröse 2013).

In particular, impaired or reduced mitochondrial respiration leads to O$_2^•$ formation via an increase in the NADH/NAD$^+$ ratio. Complex I can also produce large amounts of ROS when it operates in reverse, that is when a reduction in the UQ pool increases the proton motive force, favouring electron flux back through Complex I (Hinkle et al. 1967; Pryde and Hirst 2011). Small contribution from other mitochondrial enzymes, such as 2-oxoglutarate dehydrogenase (α-ketoglutarate dehydrogenase, αKGDH) can also, under some conditions, influence mitochondrial ROS content (Murphy 2009).

To counteract possible damages caused by ROS, cells and mitochondria have evolved efficient removal methods. In the mitochondrial matrix, manganese superoxide dismutase (MnSOD or SOD2) actively transforms potentially toxic O$_2^•$ into H$_2$O$_2$.

H$_2$O$_2$ is then in turn degraded by the thioredoxin and the glutathione systems, two disulphide reductase mechanisms, both existing also in a cytosolic form and utilising the reducing power of the NADPH/NADP$^+$ couple to efficiently eliminate the damaging molecules (Ren et al. 2017). The efficiency of this approach is what ultimately regulates the messenger capacity of ROS, so that proper signalling can take place (Collins et al. 2012). Many cellular responses, such as oxygen sensing (Guzy et al. 2005), differentiation (Tormos et al. 2011; Zhou et al. 2016), and mitochondrial homeostasis (St-Pierre et al. 2006), are influenced by the presence of ROS and thus controlled.
1.5 Introduction to autophagy

The process of autophagy consists of the removal of damaged or non-functional proteins and organelles. Autophagy can be classified into non-selective and cargo-specific autophagy. In the first case, nutrient deprivation promotes catabolism to supply the cell with building blocks. In the second case, autophagy is promoted even if there is no shortage of nutrients or energy to remove damaged organelles or potentially toxic protein aggregates. Alternatively, autophagy can be classified as either chaperone-mediated, micro-, or macro-autophagy. In particular, macroautophagy involves the sequestration of cargo into double-membraned vesicles, termed autophagosomes, whose formation is promoted by members of the autophagy-related genes (ATG) family (Mizushima et al. 2011), and which can fuse with lysosomes, where the low pH and different enzymes finally favour degradation of their faulty content.

Because autophagy has a very important role for cellular well-being, different studies have tried to identify ways to efficiently promote or inhibit it. On the one hand, the antibiotic rapamycin can promote autophagy by forming a complex with the intracellular receptor FKBP12 and physically interact with the main autophagy inhibitory signal in the cell; the mammalian target of rapamycin complex 1 (mTORC1) (Dumont and Su 1996), a well-studied pathway involved in various cellular processes. On the other hand, the autophagic flux can be blocked by inhibitors of the lysosomal proton pump, such as bafilomycin and chloroquine (Levine and Kroemer 2008).

Mitophagy is one example of cargo-specific autophagy that sustains elimination of mitochondria. It has been shown that this process has a fundamental role in maintaining the appropriate number of organelles and ensuring constant health of their network (Kim et al. 2007). The current mitophagic model (Fig.3) begins with the loss of the mitochondrial membrane potential, which impedes mitochondrial protein import, as well as degradation, of PTEN-induced putative kinase protein 1 (PINK1), which eventually accumulates on the outer surface of the organelle. PINK1, in turn, recruits the E3-ubiquitin ligase Parkin that links polyubiquitin chains to various targets on the outer mitochondrial membrane, ultimately sending the depolarised organelles to lysosomal degradation. This process is mediated via the microtubule associated protein 1 light chain 3 alpha (LC3), a protein responsible for the recruitment of the autophagosome (Youle and Narendra 2011). During autophagy, cytosolic LC3 (LC3-I) is conjugated to a molecule of phosphatidylethanolamine to obtain LC3-phosphatidylethanolamine conjugate (LC3-II) that can be recruited to autophagosomal membranes. Ubiquitinated mitochondria are recognised and bound by Sequestosome 1 (SQSTM1/p62), that interacts with activated LC3 to finally lead them to degradation (Park et al. 2014).
This paradigm has been supported by the scientific literature (Narendra et al. 2008); still, in recent years, the role of p62 in this process is a matter of vivid debate (Fon 2013; Strappazzon et al. 2015), and p62 is suggested to be only necessary for mitochondrial clustering and not for mitophagy per se (Narendra et al. 2010; Okatsu et al. 2010). Work presented in this thesis also investigated the role of p62 in mitophagy, proposing a different function during this process (Haack et al. 2016; Calvo-Garrido et al. 2019).

**Fig. 3:** schematic overview of the proposed mitophagic model.
1.6 Introduction to the projects

In the present thesis, I focus my effort on the proteins briefly discussed below:

1.6.1 SQSTM1/p62

SQSTM1 is also known as p62 or as A170. It was first discovered in 1996 as ligand of p56\textsuperscript{ck} and specifically binds its Src homology 2 (SH2) domain (Joung et al. 1996). Just a few months later, it was described as a member of a new class of ubiquitin-binding proteins. As the name suggests, it is a 62 kDa protein, composed of 440 amino acids, and it has various domains interacting with a number of different pathways. In particular, the N-terminal region seems to be important for dimerization, while the C-terminus is essential for binding of ubiquitin (Vadlamudi et al. 1996) (Fig.4).

\textbf{Fig.4: schematic overview of the structure of p62. The different domains are highlighted with different colours that also identify the corresponding interactors. PB1: Phox and Bem1. ZZ: Zinc finger. TB: TGF\beta-binding-protein-like. LIR: LC-3 interacting region. KIR: Keap1 interacting region. UBA: Ubiquitin associated. aPKC: Protein kinase C. ERK: Extra-cellular signal regulated kinase. NBR1: Neighbour of BRCA1 gene 1. RIP: Receptor-interacting protein kinase. TRAF6: TNF-receptor associated factor 6.}

Over the years, numerous studies have highlighted the importance of p62 in different processes, as diverse as cell proliferation (Qiang et al. 2014) or the removal of the microtubule associated protein Tau (MAPT) (Xu et al. 2019). This is of particular importance in neurodegenerative disorders, as abnormal phosphorylation of MAPT is a hallmark of Alzheimer’s, and mutations in its gene have been correlated with Frontotemporal Dementia (FTD) (Iqbal et al. 2016).
p62 also plays a role in the oxidative stress response, via the antioxidant response elements (ARE) present in its promoter (Jain et al. 2010). Furthermore, the oxidation of two cysteine residues (Cys105/103) in p62 has been proposed to act as oxygen sensor (Carroll et al. 2018). Additionally, p62 is known to regulate Kelch-like ECH associated protein 1 (KEAP1) expression, promoting its degradation and making it unable to inhibit nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Stabilisation of Nrf2 leads to its translocation to the nucleus and promotes transcription of genes related to the oxidative stress response, such as NAD(P)H quinone oxidoreductase 1 (NQO1) or other components of the glutathione system (Komatsu et al. 2007; Copple et al. 2010; Fan et al. 2010; Lau et al. 2010; Kwon et al. 2012). Last but not least, via its ubiquitin-binding domain, p62 is thought to play a major role in autophagy (Dikic and Elazar 2018; Zaffagnini et al. 2018).

Mutations in the last exons of p62 were causatively correlated to Paget’s disease of bones (Laurin et al. 2002), while others were related to frontotemporal lobar dementia (FTLD) (Arai et al. 2003) and ALS (Fecto et al. 2011). Moreover, p62 inclusions have been observed in post mortem tissues from individuals suffering from protein aggregation disorders (Zatloukal et al. 2002).

In mice, knock out of p62 leads to adult onset obesity, leptin resistance and glucose intolerance (Rodriguez et al. 2006). Ramesh Babu and colleagues have also reported hyperphosphorylated MAPT and neurofibrillary tangles, clear signs of neurodegeneration, in mice deprived of p62, closely resembling what is observed in humans (Haack et al. 2016). Interestingly, the mice also developed behavioural symptoms and showed signs of increased anxiety, depression and working memory loss (Ramesh Babu et al. 2008) that were not reported in patients.

As this demonstrates, it is very hard to pinpoint the exact mechanism by which decreased expression of a scaffold protein like p62 can impair cellular well-being and, ultimately, individual health, because of its considerable tissue specificity and multitude of interactions.
The thioredoxin system is essential for ROS scavenging and cellular well-being. Thioredoxin-interacting protein (TXNIP), a member of the α-arrestin family, binds to, and inhibits, thioredoxin activity, thus regulating the response to oxidative stress and redox homeostasis (Nishiyama et al. 1999). Nevertheless, the role of TXNIP does not seem to be only directly related to ROS management and oxidative stress response. Numerous reports relate TXNIP to the suppression of Nrf2 activity (Cebula et al. 2015) and to the activation of the NOD-like receptor Protein-3 (NLRP3) inflammasome (Abderrazak et al. 2015). Interestingly, Nrf2 and the inflammasome are also tightly connected via Nrf2 itself, which can inhibit TXNIP action, suggesting a complex relationship between their activities (Hou et al. 2018). Apart from contributing to the expression of ARE, and thereby reducing the burden of oxidative stress, Nrf2 also directs glucose towards anabolic pathways. It has therefore been implicated as an important factor in the protection against diabetes (Uruno et al. 2015).

Studies in HeLa cells suggest that TXNIP expression is regulated by the glycolytic flux, and, in turn, by mitochondrial aerobic metabolism. In particular, inhibition of the ETC and ATP synthase leads to decreased TXNIP levels at both mRNA and protein level (Yu et al. 2010).

The downstream metabolic consequences, resulting from a loss of TXNIP, are rather well demonstrated by full-body knock out mice. Although TXNIP knock out mice are viable, they are unable to efficiently retain normoglycemia during fasting, and present with a failure to regulate the switch between an unfed and fed state (Chutkow et al. 2008). This is in line with observations made in humans, were TXNIP expression has been proposed to be antithetically regulated by insulin and glucose (Parikh et al. 2007). Furthermore, and more importantly for the scope of this thesis, TXNIP deletion in mice has shown functional and structural mitochondrial deficiency (Yoshioka et al. 2012).
1.6.3 WARS2

Aminoacyl-tRNA synthetases (aaRSs) are proteins involved in the formation of an ester bond between an amino acid and its cognate tRNA. Two spatially separate pools exist, a cytoplasmic and a mitochondrial that can also be divided into two classes depending on their chemical properties. In mitochondria there are 19 aaRSs for the 23 mt-tRNAs, highlighting some redundancy.

Aminoacylation is a two-step process, requiring ATP (Ibba and Soll 2000), thus making it strongly dependent on the cellular metabolic state. All members of the aaRS family share a conserved structure, with one catalytic domain and one anticodon binding domain (Suzuki et al. 2011). Some aaRSs have also been shown to possess proofreading ability via a hydrolytic domain in their catalytic core that can remove mismatched aminoacids (Pang et al. 2014).

Mitochondrial aaRSs have been intensely studied and recently a number of laboratories have associated mutations in aaRSs to mitochondrial diseases. The clinical presentation of the affected patients is broad, with mutations in different aaRSs affecting different systems or organs (Sissler et al. 2017). For example, mutations in histidyl-tRNA synthetase (HARS2), or in leucyl-tRNA synthetase (LARS2) can lead to Perrault’s syndrome, with sensorineural hearing loss and ovarian dysgenesis (Demain et al. 2017), while mutations in aspartyl-tRNA synthetase (DARS2) lead to a leukoencephalopathy with cerebellar ataxia and cognitive impairment (Uluc et al. 2008; van Berge et al. 2014), and mutations in alanyl-tRNA synthetase (AARS2) give rise to cerebellar ataxia, hypertrophic cardiomyopathy and delayed motor development (Götz et al. 2011; Mazurova et al. 2017).

Mutations in the mitochondrial tryptophanyl-tRNA synthetase (WARS2) have been associated with an array of clinical symptoms, ranging from intellectual disability, speech impairment and ataxia (Musante et al. 2017), severe encephalopathy with infantile onset, epilepsy and spastic quadriplegia (Theisen et al. 2017), lactic acidosis, hypotonia, dystonia and cardiomyopathy (Wortmann et al. 2017), symptoms related to Parkinson’s disease (Burke et al. 2018), hypoglycaemia, and hepatotoxicity (Vantroys et al. 2018). In total, 13 different variants have been reported in 14 subjects from 10 families. In particular, p.Lys313Met has been reported in six different families (Wortmann et al. 2017; Theisen et al. 2017; Vantroys et al. 2018; Maffezzini et al. 2019), although a common ancestry of this variant has not been investigated. All patients carrying the p.Lys313Met variant presented with white matter involvement in the central nervous system and a muscle phenotype, leading to muscle weakness and movement impairment, but it is still unclear what molecular mechanisms lie behind the pathology.

Further studies are required to fully understand whether the tissue specificity in aaRSs-related disorders is just due to different energy demands and use or whether toxicity of non-processed intermediates could also play a role.
2 AIMS

Next-Generation Sequencing (NGS) has revolutionized diagnostics, resulting in a large number of novel disease-causing genes being identified. Especially IEM, which predominantly are caused by monogenic alterations, have gained from NGS, with hundreds of genes now associated with metabolic derangements. The aim of the presented studies was to validate pathogenicity of newly identified gene variants of patients with IEM, and to identify molecular mechanisms of disease progression.

The specific goals of each study are:

**Paper I** to validate mutations in SQSTM1/p62 causing a slowly progressive, early onset neurodegeneration, and study the process of autophagy in patient cell lines.

**Paper II** to further dissect the mechanism by which absence of SQSTM1/p62 can lead to a central nervous system pathology. For this, patient fibroblasts were reprogrammed to induced Pluripotent Stem cells (iPSCs) and further differentiated to NES cells and neurones.

**Paper III** to validate TXNIP as a novel metabolic disease-causing gene. The bioenergetic and metabolic consequences were studied in patient-derived fibroblasts and myoblasts.

**Paper IV** to validate two mutations in WARS2 as pathological and characterise their outcome. For this, genetically modified yeast models, as well as patient-derived NES cells were used. Furthermore, a *Drosophila melanogaster* model shed a light on the importance of WARS2 for life.
3 MATERIALS AND METHODS

All of the work here presented would not have been possible without the kindness of the families involved. Informed consent was obtained for all involved subjects and studies were conducted according to the guidelines of the institute.

3.1 Introduction to whole-genome and whole-exome sequencing

In recent years, the possibility to exploit the enormous amount of information contained in our DNA has become close to reality thanks to NGS technology, of which Whole-Exome Sequencing (WES) and Whole-Genome Sequencing (WGS) are common applications. Apart from the obvious curiosity some of us might have in knowing the details of their own molecular heritage, these approaches have the captivating potential of enabling the fast identification of possible pathologic mutations (Stranneheim et al. 2014).

The main difference between the two methods lies in the isolation procedure and in the amount of data they generate. WES provides researchers and clinicians with information only concerning the coding sequences of our genome, while WGS provides data on close to all our 3 billion base pairs. Technical and computational limitations had favoured WES as the preferred method in detecting clinically relevant variants, but recent advances in both sequencing and data handling has now allowed clinics to shift to WGS. Although the interpretation of variants in non-coding regions is in the majority of cases still difficult, sample preparation, downstream computational protocols, and a better coverage of potential splice sights, make WGS now the preferred method for clinical variant calling.

Ever since their first applications, both WES (Choi et al. 2009; Ng et al. 2010) and WGS (Nordström et al. 2013) have proved reliable in identifying possible causative mutations. In some way, the identification of the disease-causing variant in a sea of 3 billion base pairs can be compared to finding the preverbal needle in the haystack, and has only been made possible due to advances in molecular biology, genetics, and computational sciences. For the scope of this thesis, it should be noted that both WES and WGS have been shown to be of particular importance, as they provide a fast and efficient way to identify the mostly monogenic metabolic disorders (Stranneheim et al. 2014).
3.2 Introduction to cell reprogramming

The need for adequate models is a long-known issue in every field of research. Choosing the right one is, in fact, of invaluable significance to give the correct interpretation to the observed phenomena. This concern is especially amplified in the case of neuroscience and the study of neurodegeneration. In this case it is virtually impossible to acquire any material from patients, as the access to the affected tissues is impracticable in living individuals and neurones, being post-mitotic cells, cannot be efficiently cultured for many passages. The use of animal models, especially mice and rats, is today widespread, but the fact that not all syndromes are recapitulated in the observed species renders it complicated to design a satisfactory approach to the problem.

Tumour cell lines have also been exploited, but they are quite distantly related to the central nervous system and its cellular components, making it difficult at times to conclude anything from the observed results.

In 2012 the Nobel prize in Medicine was awarded to Sir John Gurdon and Shinya Yamanaka for the reprogramming of mature cells. According to Gurdon’s studies on frogs, transplanting the nucleus of a fertilised egg into an enucleated egg of the same species leads to full development of embryos, suggesting that there are some factors that, on their own, can influence and address cellular fate (Gurdon et al. 2017).

In 2006 Takahashi and Yamanaka published their seminal paper on the generation of induced iPSCs from mouse embryonic and adult fibroblasts. The group identified a minimum number of factors required to sufficiently induce regression towards stemness, i.e. toward a state of pluripotency, in mature, differentiated cells. These factors were the four transcription factors Oct3/4, Klf4, Sox2, and c-Myc. The such gained iPSCs resembled physiological embryonic stem cells in morphology and expression of embryonic markers. Furthermore, they could be differentiated into a number of different cell types from all three germ layers (Takahashi and Yamanaka 2006). Nowadays, the number of factors and technology used to obtain iPSCs has been reduced and simplified (Löhle et al. 2012), allowing for the adaptation of reprogramming in a variety of scientific fields.

The potential to differentiate iPSCs into neurones has proven to be of specific importance, and numerous disease models have since been reported, including models for Alzheimer’s (Kondo et al. 2013), Parkinson’s (Kikuchi et al. 2017), ALS (Park et al. 2016), or simply ageing (Mertens et al. 2018).

In 2009, Koch and colleagues proved it possible to obtain stable neural precursor cultures from human embryonic stem cells, allowing the constant proliferation of neuroprogenitor cells. Despite their long-term maintenance of plasticity, they could, if correctly stimulated, give rise to specific neuronal subpopulations (Koch et al. 2009).

In 2012, Anna Falk and her colleagues demonstrated the possibility of inducing previously reprogrammed iPSCs into lines of neuronal lineage (NES cells) that will show all neuronal precursors markers and can then be further differentiated into a mixed neuronal and glial population, much like what was seen with embryonic stem cells (Falk et al. 2012). With this
protocol, there is no need for culturing of human embryonic stem cells, which could rise ethical, as well as practical, issues, but neuronal precursors can be directly obtained from adult patients and their differentiated cell lines.

In our papers, NES cells were cultured in DMEM/F12 medium containing N-2 supplement (1:100), B-27 supplement (1:1000), 10 ng/µL basic Fibroblast Growth Factor (bFGF) and 10 ng/µL Epidermal Growth Factor (EGF). Removal of these factors and increase of B-27 concentration to 1:100 resulted in induction of neuronal differentiation. The cells thus obtained constituted a mixed pool of neuronal subtypes and only prolonged culturing originated astrocytic presence.

3.3 Introduction to CRISPR/Cas9

Before the advent of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated Protein (Cas) 9 method, Zinc Fingers Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) were the only ways to artificially edit a specific locus in the genome. Yet, designing the correct experiment was quite tricky and the need for an efficient approach became more and more urgent. The first discovery of CRISPR was in bacteria, where short, palindromic repeats seemed to be of particular importance for the immune system response (Hille and Charpentier 2016). In 2013 it was demonstrated that directing Cas9 with guide RNAs could efficiently be engineered to precisely cleave DNA in human cells (Cong et al. 2013). Guiding the nuclease Cas9 to the DNA region of interest promotes double-strand breaks (DSBs) that can then either be repaired with homologous recombination, or by non-homologous end joining (NHEJ). In the first case, an exogenous DNA template with homologous ends can be provided, allowing the integration of specific sequences or point mutations into the locus of interest. In the second instance, NHEJ often results in the deletion of short DNA stretches, providing the possibility to generate knock out models for the gene of interest (Adli 2018). The simplicity and high efficiency of CRISPR/Cas9 technology has resulted in its use in a wide range of applications, from drug screening to the generation of animal models (Ma et al. 2014; Motta et al. 2017; Qi et al. 2017).
3.4 Introduction to oxygen consumption measurements

Ever since the mid of the last century, the Clark electrode has been used to measure oxygen concentration. In this system, a silver (Ag/AgCl) anode and a platinum cathode, are covered by a membrane permeable to molecular oxygen. Reduction of oxygen at the cathode is dependent on the partial pressure of oxygen itself diffusing through the membrane, and can be considered a measure of oxygen concentration in the environment.

A number of commercially available systems have been adapted on the principle of the Clark electrode, to allow to measure changes in oxygen concentration over time and therefore respiratory rate in mitochondria. One of the most widely used systems is the Oroboros oxygraph (Oroboros Instruments GmbH). In this system, samples are injected in a closed, isolated chamber, with the possibility to supply various substrates to differentially activate or inhibit complexes of the ETC. The prime derivative of oxygen decrease over time represents a measure of oxygen consumption and, indirectly, of mitochondrial activity. Analysis of the decrease in oxygen in the chamber after each step of the protocol can thus help to identify mitochondrial deficiencies and dysfunctions. The main advantage of this method lies in its flexibility, with a wide range of applications and protocols possible to cater for a number of research questions.

Another commonly used system to evaluate mitochondrial function is the Seahorse (Seahorse XF Analyzers, Agilent). Although both systems are designed to evaluate mitochondrial oxygen consumption, the Oroboros measures respiration in cell suspension, while the Seahorse measures oxygen consumption in seeded cells, using fluorescent sensors that can measure both oxygen concentration and pH in the cell media. The main advantage of the Seahorse system is the automation and scalability of the system, with up to 96 samples measured in parallel, while the Oroboros requires manual manipulation with only two possible samples. Both systems described have been used in this thesis.
4 RESULTS AND DISCUSSION

4.1 Paper I

Paper I comprises the first description of patients with loss-of-function mutations in the autophagy adaptor protein SQSTM1/p62. Together, nine patients from four unrelated families were reported, all presenting with clear signs of central nervous system dysfunctions from the pre-adolescent age, suffering from gait abnormalities, ataxia, dysarthria, vertical gaze palsy, dystonia and cognitive decline. Clinical symptoms were slowly progressive, with the oldest affected patient now well above 40 years of age. Brain MRI revealed iron accumulation, typically associated to mitochondrial dysfunction with central nervous system involvement, in only one of the affected families, while cerebellar atrophy was evident in four of the subjects, albeit not in all. Mitochondrial function, when investigated, showed a mild decrease of Complex IV activity, but normal ATP production rates. WES identified three bi-allelic loss of function variants that led to complete absence of p62 at transcript and protein levels in the pool of nine patients.

The suggested role of p62 in mitophagy, together with the mild ETC dysfunction, led us to investigate the mitochondrial clearance upon mitochondrial stress exposure. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) has previously been suggested to induce p62-dependent mitophagy, as it depolarises mitochondrial membrane potential and induces PINK1 extrusion, resulting in fragmentation of the mitochondrial network in both patient and control fibroblast cell lines. Although prolonged exposure to CCCP resulted in the expected mitochondrial clearance, patient cell lines failed to sufficiently form mitochondrial perinuclear aggregates upon short CCCP exposure, typically associated with mitophagy (Narendra et al. 2010).

Overexpression of Parkin nicely co-localised with the mitochondrial network, but did not revert the observed phenotype, in agreement with the hypothesis of p62 being dispensable for disposal of damaged mitochondria by Parkin. Treating the cells with a combination of oligomycin (inhibitor of ATP synthase) and antimycin A (inhibitor of Complex III of the ETC) did not reveal any obvious defect in patient cells regarding autophagosome formation or mitochondrial clearance.

This manuscript described the first patients deficient of the p62 protein, presenting with a slowly-progressive neurodegeneration. Studies of autophagy in the fibroblasts of these patients highlight a defect in perinuclear translocation of mitochondria upon depolarisation, but no further impairment in clearance of defective organelles, suggesting not only a dispensable activity of p62 in the processes mediated by Parkin, but also hinting at a composite redundancy in the activity of different participants in the pathway.
4.2 Paper II

In Paper II I further investigate the molecular mechanisms underlying the clinical progression in two patients with homozygous null mutations in p62 (the two siblings presenting with the c.286C>T, p.Arg96* mutation) (Haack et al. 2016). The strictly neurological presentation led us to generate NES cells from both patients, as well as SQSTM1 knock out NES cells by CRISPR/Cas9 gene editing technology. Differentiation potential of NES cells was severely reduced in patient cell lines, which was accompanied by the reduced expression of several neuronal lineage markers. This phenotype was confirmed both in CRISPR/Cas9 p62 knock out cells, silencing of p62 by RNAi in control NES cells, and could be rescued by reintroducing full-length p62 into patient NES cells, which resulted in improved differentiation and long-range projection formation.

In agreement with observations made in patient fibroblasts, NES cells lacking p62 presented with reduced Complex I and IV activities, but normal ATP production, and had normal mitochondrial clearance in response to mitochondrial damage. However, NES cells lacking p62 presented with increased sensitivity to both CCCP and 2, 4-dinitrophenol (DNP), an additional protonophore, suggesting increased sensitivity to oxidative stress. The fluorescent dye CM-H2DCFDA, which is an indicator for ROS and thus for oxidative stress, did not show any difference in basal ROS levels between control and patient NES cells. However, increased ROS levels could be observed upon induction of differentiation, accompanied by decreased expression of genes involved in the glutathione system. This phenotype could be partially reverted by treatment with the antioxidant N-Acetyl Cysteine (NAC). The expression of KEAP1 and several other factors involved in ROS management were not changed in patient cells, suggesting a possible role of SQSTM1 in the oxidative stress response, independent from the KEAP1-Nrf2-ARE pathway.

Transcriptomic and proteomic analysis on CRISPR/Cas9 knock out, patient and control cells revealed impaired up-regulation of aerobic metabolism pathways upon neuronal differentiation induction. Neuronal differentiation requires the cells to undergo a metabolic shift from a glycolytic metabolism to a more aerobic profile. Zheng and colleagues previously demonstrated that reduction of lactate dehydrogenase A (LDHA) was essential for this process during neuronal differentiation (Zheng et al. 2016). Indeed, NES cells lacking p62 presented with an altered pyruvate metabolism, including a severe increase in LDHA expression, both on transcript and protein level. Surprisingly, though, fibroblasts from patients lacking p62 did not show increased LDHA expression.

Cell culture is usually performed at atmospheric oxygen concentrations, while oxygen pressure in the brain is much lower. The increased sensitivity to oxidative stress suggested a defect in oxygen sensing. We therefore chose to expose NES cells to 3% oxygen, a more physiologically relevant oxygen concentration, and could observe a defect in the dynamic response of the hypoxia inducible factor 1-alpha (Hif-1α).
In conclusion, we show that neuronal precursors lacking p62 exhibit a defect in maturation. This limitation is not caused by impairment in mitochondrial clearance, but due to the inability to activate their metabolic profile to a more aerobic energy metabolism, possibly because of increased sensitivity to oxidative stress and increased LDHA levels.

**4.3 Paper III**

In **Paper III** I present three siblings with mutations in the TXNIP gene. WES analysis identified two homozygous nucleotide variants close to each other (c.174G>T, p.Gln58His and c.175G>T, p.Gly59*), leading to no detectable TXNIP expression. The subjects presented with lactic acidosis and low serum methionine levels. Evidence of hypoglycemia, liver involvement, and muscle weakness were also noted but not in all three siblings. Diagnostic investigation of mitochondrial function on muscle biopsies failed to identify a defect in mitochondrial respiration or isolated respiratory chain enzyme activities.

As previously mentioned, TXNIP is an oxidative stress mediator by inhibiting thioredoxin activity. However, patient cell lines presented with normal thioredoxin activity in myoblasts from all three patients. In contrast, in agreement with previous reports, loss of TXNIP resulted in a robust up-regulation of the entire Nrf2 system.

Investigations in patient cell lines revealed increased lactate release into the extracellular medium, indicating a pronounced use of anaerobic energy pathways. This was confirmed when cells were treated with the Complex V inhibitor oligomycin. Measuring cellular respiration on intact patient cells, using a Seahorse analyzer, revealed a severe defect in mitochondrial respiration, when providing pyruvate as the sole carbon source. In contrast, supplementation with malate, an additional carbon source entering the respiratory chain at Complex I, showed normal mitochondrial respiration, suggesting a severe defect in pyruvate metabolism in the absence of TXNIP. Interestingly though, pyruvate dehydrogenase activity, the complex decarboxylating pyruvate to acetyl-CoA, which enters the TCA cycle, was normal in patient samples, suggesting either impaired pyruvate import into the cell, or into mitochondria.

In conclusion, I report the first patients with non-sense mutations in TXNIP, resulting in lactic acidosis and reduced serum methionine levels. Further, the results suggest an important role of TXNIP in regulating the use of pyruvate for aerobic energy metabolism. Failure to further oxidise pyruvate during aerobic metabolism leads to the observed lactate accumulation.
4.4 Paper IV

In Paper IV I report two pathogenic variants in the mitochondrial tryptophanyl-tRNA synthetase (WARS2) gene. Two siblings carrying the c.938A>T, p.Lys313Met and c.833T>G, p.Val278Gly compound heterozygous variants presented with a severe neurological syndrome, muscle weakness, hypotonia, ataxia, and developmental delay. The Lys313Met variant has previously been reported by Theisen et al. 2017, Wortmann et al. 2017 and Vantroys et al. 2018, while the Val278Gly variant has not been described previously.

To evaluate the pathogenicity of our mutations, the variants were modelled in yeast. Neither variant is conserved in yeast and therefore the equivalent yeast amino acids were mutated into both the human control, as well as patient amino acids. Humanisation of the p.Val278 (p.I297V in yeast) showed normal growth under non-fermentable growth conditions, with normal respiration at both 28°C and 37°C growth conditions. In contrast, the patient-specific variant (p.I297G in yeast) resulted in a failure to grow in non-fermentable conditions, and a severe respiratory rate defect. In contrast, humanisation, as well as introduction of the patient p.Lys313Met (p.E333M in yeast) variant, had no effect on growth on glycerol, nor affected the respiratory rate at 28°C. However, shifting growth to 37°C revealed a reduced respiratory rate, for both humanised and patient mutation, suggesting that any alteration at this position can result in a biochemical defect.

Neither the diagnostic investigation of fresh muscle biopsies, nor measurements of respiratory rate and isolated respiratory chain enzyme activities in patient fibroblasts, revealed a mitochondrial dysfunction in either patient, although an aminoacylation defect of mitochondrial tRNA_{\text{Trp}} could be observed by Northern blot analysis. In contrast, NES cells, derived from reprogrammed patient fibroblasts did present with a mild Complex I and IV defect. Finally, in an attempt to mimic the reduced aminoacylation of mitochondrial tRNA_{\text{Trp}}, WARS2 was silenced by RNAi in a Drosophila melanogaster animal model. Full-body knock down of Dm-wars2 resulted in reduced aminoacylation of tRNA_{\text{Trp}}, triggering larval lethality and reduced mitochondrial respiration. Together, this manuscript provides molecular and biochemical evidence from three different model systems that the observed variants in WARS2 indeed could be the case for the clinical symptoms observed in the patients.
5 CONCLUSIONS

Metabolism is a complex, intricate network of processes that sustains all cellular activities and ultimately life. This system is not infallible: problems and imbalances can always occur and lead to clinically relevant situations. The chances of solving peculiar cases and gaining insight into the genetic causes of multiple clinically relevant situations have in the last years escalated, thanks to the design of WES and WGS protocols.

In particular, mitochondria are protagonists of aerobic energy metabolism and in the past years have been connected with complex clinical presentations including neurodegeneration and ageing.

In Paper I and paper II I explore the aetiology of an early-onset, slowly-progressive neurodegenerative syndrome related to loss of the autophagy adaptor protein SQSTM1/p62. The results of this study demonstrate how important the ability to efficiently balance different metabolic pathways is to proper neuronal development.

In Paper III, I analyse the case of three siblings with loss of TXNIP. This study supports the idea that the ROS balance is also essential for metabolism and cellular well-being.

Lastly, in Paper IV I explore another neurodegenerative syndrome that affects two siblings presenting with mutations in the WARS2 gene, highlighting the importance of using the correct model for validation of novel disease variants.

Summing up, the studies collected here unveil a number of apparently unrelated pathological mechanisms, all bound to metabolism and energy management.

The impact rare disorders have is sometimes underestimated: the work here presented tries to highlight their possible implications into more common and widespread disorders.
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“Piglet noticed that even though he had a Very Small Heart, it could hold a rather large amount of Gratitude.”

A.A. Milne

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