UNIVERSITÀ DI PARMA

UNIVERSITY OF PARMA

Ph.D IN BIOTECHNOLOGY AND LIFE SCIENCES

XXXIII CYCLE

Saccharomyces cerevisiae as a system for studying molecular basis of mitochondrial diseases and evaluating the effects of potentially beneficial molecules

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Years 2017-2020

Table of contents

1. Introduction

1.1 Mitochondria architecture and function

Mitochondria are tiny organelles sitting inside eukaryotic cells and classically known as "powerhouse of the cell" thanks to their capacity of producing ATP (adenosine triphosphate), the main energy substrate of life. Mitochondria are thought to have evolved around two billion years ago from the engulfment of aerobic bacteria by an ancestor of eukaryotic cell, thus generating an endosymbiotic relationship highly advantageous to their partners: bacteria lived in a protected and nutrient-rich environment (the inside of the cell) conferring on its host the ability to carry out aerobic respiration, a much more efficient way of generating energy from organic substrates (Margulis, 1970; Herst *et al.,* 2017). Although during eukaryotic evolution many structural and compositional changes have occurred, including a broad transfer of genes from the bacteria to the host nuclear DNA, mitochondria remain double-bounded membrane organelles which contain their own genome.

Each mitochondrion is surrounded by two membranes which have very different features and functions. One of the most important differences between the two membranes is that the outer mitochondrial membrane (OMM), which separates the mitochondrion from the cytoplasmic environment, is permeable to ions and small molecules thus making the intermembrane space chemically equivalent to the cytosol. Instead the inner mitochondrial membrane (IMM) that delimits the mitochondrial matrix is an extremely selective barrier mainly constituted by cardiolipin, a unique phospholipid that may contribute to make the membrane impermeable to ions and smallest charged molecules, whose passage is allowed by specific transport protein. Thanks to its high selectivity, the IMM is an excellent electrical insulator that contributes to the maintenance of the electrochemical membrane potential. Moreover, the IMM forms a series of invaginations, known as cristae that expand the surface of the membrane and contain the protein complexes of the respiratory chain and the ATP synthase that catalyzes the synthesis of the ATP starting from adenosine diphosphate (ADP) and inorganic phosphate (Pi) exploiting the transmembrane proton gradient generated by the electron transport chain.

Mitochondria, although often illustrated as cylinders, are dynamic organelles continually changing their shape and constantly in communication with each other via fusion and then separating again by fission (division). For this reason, mitochondria are not to be considered as discrete and isolated entities but rather as a network in which the balance between two opposite events, fusion and fission, is crucial for maintaining of morphology and distribution of mitochondria and also for the distribution and maintenance of mitochondrial DNA (mtDNA) (Friedman and Nunnari, 2014).

The number of mitochondria vary not only among species but also among different cell types and in the same cell depending on their metabolic state, cell cycle phase and energetic demand. For example in skeletal muscle cells the number of mitochondria increases by 5 to 10 times when they are stimulated to contract. In yeast it has been shown that the number of mitochondria changes according to the metabolic activity: in respiratory conditions, so in the presence of ethanol, yeast cells contain a large number of tubular-shaped mitochondria. Conversely in the presence of glucose, so under the fermentative metabolism, the mitochondria are less and branched (Visser *et al.,* 1995).

Although the role of mitochondria is mainly associated with the production and stock of energy derived from oxidative phosphorylation (OXPHOS), their importance in other cellular processes cannot be overlooked. Mitochondria are signaling organelles whose intimate interaction with other cellular compartments is crucial for the regulation of many cellular functions such us the maintenance of cellular ion homeostasis, apoptosis, autophagy. Moreover mitochondria provide biosynthetic intermediates required for other metabolic pathways, such us amino acids and nucleotides (**Figure 1**.**1**).

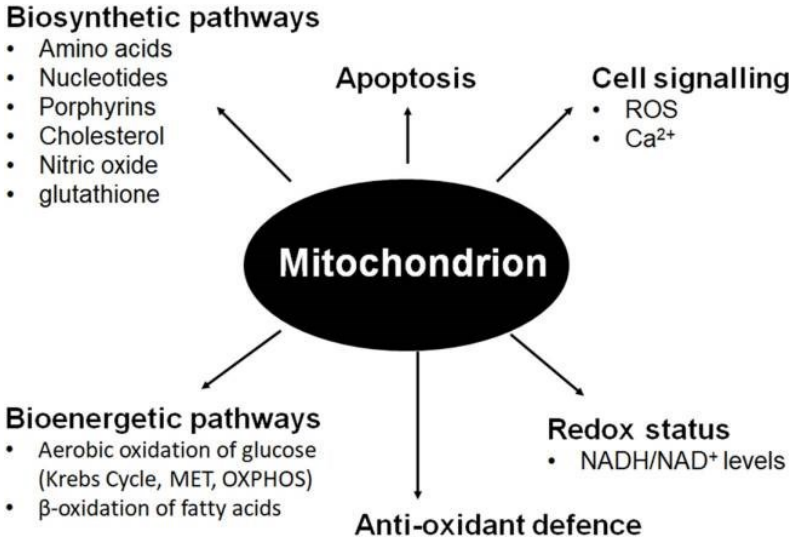


Figure 1.1. The most important cellular pathways and processes in which the mitochondrion is involved (from Herst *et al.,* 2017).

Beyond the role of the mitochondrion in providing the precursors of dNTP synthesis, its involvement in this biosynthetic pathway also occurs at other stages. For example mitochondria contribute to the formation of the ribonucleotide reductase complex (RNR), the key enzyme in dNTP synthesis, providing some components (iron prosthetic group, glutaredoxin heme protein complex) necessary for the assembly of the complex (Zhang *et al.,* 2014). Numerous studies (reviewed in Desler *et al.,*

2010) have shown that failure of different mitochondrial functions, not only associated to a reduction in ATP production caused by an OXPHOS impairment, are related to an aberrant synthesis of nucleotides, thus demonstrating that mitochondria are pivotal also for the nuclear genome stability. Since a mitochondrial dysfunction resonates in other biosynthetic pathways and can also perturb other organelles within the cell, interfering with their function or biosynthesis, it is not surprising that impairment of mitochondrial functionality has emerged as a critical factor in a large number of pathologies, including metabolic, degenerative, and age-related inflammatory diseases.

1.1.1 A global view of the Citric Acid Cycle and the Oxidative Phosphorylation

The tricarboxylic acid cycle, (TCA cycle), also called Krebs cycle and citric acid cycle is the central metabolic process through which all fuel molecules, pyruvate and fatty acids, derived from glucose and fats respectively, are catabolized in aerobic organisms. Both molecules are degraded to the acetyl coenzyme A (acetyl CoA), the key molecule that is metabolized by the TCA cycle. It takes place in mitochondrial matrix and consists of eight steps (**Figure 1.2**) that lead to the oxidation of acetyl CoA into CO_2 and to the production of energy stored in the NADH and $FADH_2$ molecules.

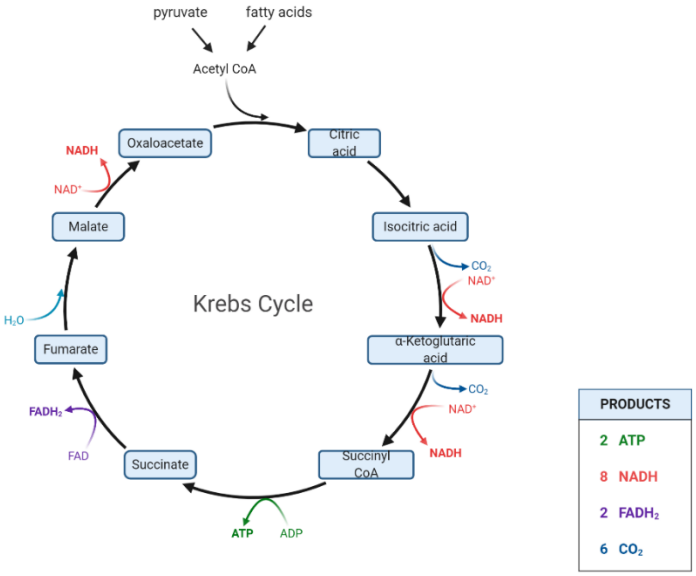


Figure 1.2. Schematic representation of the Krebs cycle created with Biorender.com.

The high energy electrons carried by these two molecules are then passed, through the electron transport chain (ETC) localized in the IMM, to oxygen as the terminal electron acceptor (**Figure 1.3**). NAD^+ and FAD are thus regenerated and oxidative metabolism can thus continue. The energy released during the transfer of electrons along the ETC is used to pump protons (H^+) from the matrix

to the intermembrane space, thus generating an electrochemical proton gradient or proton motive force accross the IMM. The “electrochemical” term refers to the two components that make it up, the membrane potential (ΔΨ) and the pH gradient (ΔpH). The proton gradient drives the ATP synthesis through the oxidative phosphorylation via the transmembrane protein complex F_0F_1 ATP synthase (respiratory complex V). This enzyme allows H^+ to flow back into the matrix and exploits the energy of H^+ flow to synthesize the ATP from ADP and P_i in the matrix.

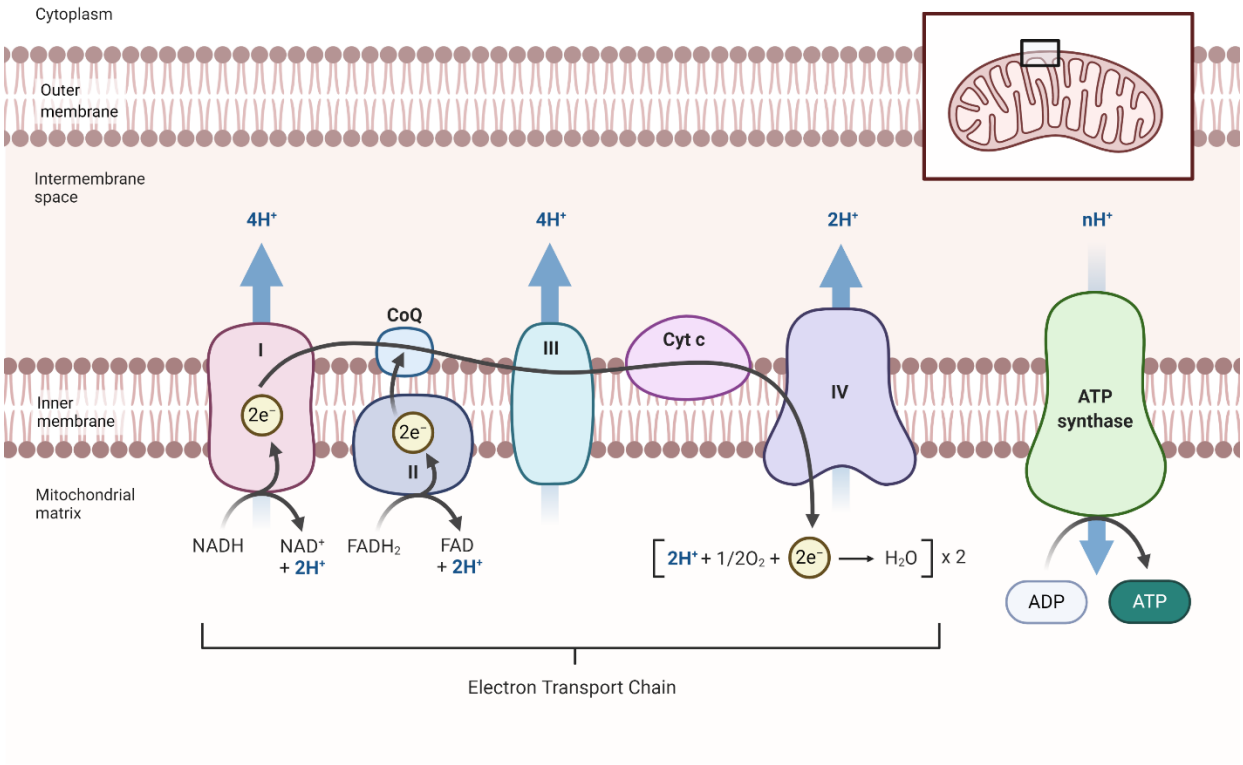


Figure 1.3. Electron Transport Chain created with Biorender.com

It is important to underline that the electrochemical proton gradient is not only crucial for the synthesis of ATP, but it represents also a driving force for non-energy-producing functions such as influx of Ca^{2+}, known as a regulator of mitochondrial respiration (Hansford, 1994) and import of nuclear-encoded protein essential for mitochondrial viability. Moreover, in yeast it has been shown that a loss of membrane potential, consequent to a mitochondrial dysfunction, represents the main signal that triggers the retrograde response, that is a communication pathway between the nucleus and the mitochondrion through which the cell tries to compensate for the mitochondrial dysfunction (Miceli *et al.,* 2012).

Beyond energy, the OXPHOS process also generates reactive oxygen species (ROS). In normal conditions, 0.2-2% of electrons passing through respiratory complexes do not completely reduce

oxygen in water, but lead to the formation of superoxide, which can be further reduced generating other reactive oxygen species. If kept at low levels, thanks to the action of antioxidant systems, ROS play a critical role in homeostasis and in cell signaling. However impairment of electron transport through the respiratory complexes lead to an accumulation of electrons thus enhancing ROS production and causing oxidative damage which can contribute to the onset of degenerative disorders, cancer and aging (Schieber and Chandel, 2014).

Other fundamentals components of the mitochondrial OXPHOS system are the ADP/ATP carrier proteins (encoded by the ANT genes in humans and *AAC* in yeast) that exchange ATP, generated in the matrix, and ADP across the inner mitochondrial membrane. Since one molecule of free ATP contains four negative charges and one molecule of free ADP contains three negative charges, the equimolar exchange is driven by the membrane potential. The ADP/ATP carrier can play a critical role in maintaining ΔΨm in cells lacking mitochondrial DNA (Appleby *et al.,* 1999). In this case, ANT works in the reverse mode: the cytosolic ATP produced from glycolysis is imported into the mitochondrial matrix and so the import of four negative charges in exchange for three, generates a membrane potential. The role of this carrier and the pathological effects caused by its deficiency will be later deepened.

1.2 The mitochondrial DNA (mtDNA)

As previously mentioned, mitochondria contain their own genome, whose copy number varies among species and, in the case of multicellular organisms, among tissues depending on their bioenergetics demand. For istance, muscle and cardiac cells, which require a lot of energy for the excitation-contraction cycle, contain between 2000 and 5000 copies of mitochondrial DNA, while tissues such as liver, kidneys have a copy number ranging from 500 to 2000 (D'Erchia *et al.,* 2015). Moreover, even within the same cell, the number of copies of mtDNA can change depending on environmental stimuli or as a consequence or cause of the onset of physiological or pathological conditions, such us aging (He *et al.,* 2014), cancer (Reznik *et al.,* 2016), diabetes (Chien *et al.,* 2012). The structure of mtDNA is different from the nuclear DNA (nDNA) one. First of all the mtDNA is generally a highly compact circular doubled-stranded molecule, lacking introns and whose length varies greatly depending on the species. Unlike nDNA, it is not associated with histones but it is organized in highly compacted structures, termed *nucleoids*, anchored to the IMM. Packaging of mtDNA is made possible by its association with mitochondrial transcription factor A (TFAM) (Rubio-Cosials and Solà, 2013), mtDNA helicase Twinkle and mitochondrial single-stranded DNA-binding

protein (mtSSB) (Garrido *et al.,* 2003). Given their pivotal role in the maintenance and transcription of mtDNA (Ekstrand *et al.,* 2004), mutations in these genes lead to the onset of several mitochondrial diseases (Goffart *et al.* 2009; Stiles *et al.,* 2016; Gustafson *et al.,* 2019).

Lack of histones and of efficient repair systems togheter with the proximity of mtDNA to the ROS-generating electron transport chain make the mtDNA more susceptible to mutations than nDNA. However, as mentioned above, each mitochondrion contains multiple copies of mitochondrial genome and so it can contain a mix of both mutant and wild-type mtDNA, a condition called heteroplasmy, typical of mammalian cells. This means that a mutation in mitochondrial DNA has consequences on metabolic functionality only when the ratio between mutated/wt copies exceeds a certain threshold which varies according to the mutation itself and the affected tissue (Stewart and Chinnery 2015).

Unlike nuclear DNA, inherited from both parents, mtDNA is maternally transmitted although there are several exceptions to this rule (Ladoukakis and Zouros, 2017). Therefore, mitochondria-associated disease mutations don't follow Mendelian rules of inheritance.

In human cells, mtDNA measures 16569 base pairs (bp) and contains 37 genes that encode 13 subunits of the respiratory complexes, 22 tRNAs, and 2 rRNAs required for mitochondrial protein synthesis. All other mitochondrial proteins (∼1300) needed for mitochondrial functionality are encoded by nuclear genes, synthesized by cytosolic ribosomes, folded upon entry and subsequently transported in the mitochondrion by the TOM/TIM super-complex, the mitochondrial protein import machinery highly regulated at multiple levels (Harbauer *et al.,* 2014). Because of their dependence on the nucleus, mitochondria are defined as semi-autonomous organelles and this explains why the majority of mitochondrial disorders are caused by mutations in nuclear DNA.

In *Saccharomyces cerevisiae*, mtDNA consists typically of linear molecules of variable length ranging from ∼68 (*short strain*) to 86 kb (*long strain*), even if small circular DNA are also present (Williamson, 2002; Nosek and Tomáska, 2003). Unlike the human mitochondrial genome, yeast has a low degree of compaction and three genes contain introns (*COX1, COB, RNL*) (Foury *et al.,* 1998). *In S. cerevisiae*, mtDNA encodes 8 proteins, of which 7 are subunits of the respiratory complexes and oxidative phosphorylation, and one is a ribosomal protein. Furthermore, it also contains genes encoding 24 tRNAs and large and small rRNAs (15S and 21S) needed for mitochondrial translation (Foury *et al.,* 1998). In yeast mtDNAs is biparentally inherited and unlike human tissues that can be

heteroplasmic, in yeast the homoplasmy is the natural condition and heteroplasmic state is only a transient phase (Solieri, 2010).

1.3 Mitochondrial diseases

The term “Mitochondrial diseases” (MDs) refers to a group of pathologies resulting from either inherited or spontaneous mutations in mitochondrial DNA or nuclear DNA which lead to an impairment in the process of oxidative phosphorylation responsible for the synthesis of ATP, the principal energy-carrying molecule in living cells (Chinnery, 2000). Currently MDs affect 1 in 5000 individuals making these pathologies the most common among the genetic ones (Ng and Turnbull, 2016). A peculiar feature of MDs is the variability of clinical manifestations, an aspect that makes their diagnosis quite challenging. Indeed the symptoms can arise in childhood or later in adult life, can affect a single tissue or multiple organs causing a multisystemic disease. Moreover the same mutation can cause different symptoms and on the contrary different mutations can cause the same symptomatology (Schon *et al.,* 1997). Generally the most affected systems are those with high energy demand such as the muscular and central nervous system, but other systems (visual, auditory, cardiocitcolatory, gastrointestinal ecc) can also be involved, with varying severity of involvement and in different combinations. To date not all patients have a genetic diagnosis but it is important to underline that in recent years the application of next-generation sequencing strategies (NGS), such as whole exome sequencing (WES) and whole genome sequencing (WGS) has vastly improved the genetic diagnosis and speeded up novel disease gene discovery (Stenton and Prokisch, 2020). Although until a few years ago the term “mitochondrial disease” referred only to a group of disorders resulting from an impairment of the respiratory chain and therefore to an insufficient energy production, to date the term is also extended to another series of pathologies, known as secondary mitochondrial dysfunction (SMD), caused by mutations in genes that are not involved in the production or functionality of respiratory complexes (Niyazov *et al.,* 2016). For example defects in the mitochondrial fission/fusion processes are implicated in the onset of multifactorial disorders such as diabetes (Yoon *et al.,* 2011), or cardiovascular disease (Ahuja *et al.,* 2013), thus underlining the pivotal role of the mitochondrion in many cellular functions, beyond energy production.

In general, mitochondrial diseases are classified into two groups depending on the mutation localization in mitochondrial or nuclear DNA.

Mitochondrial diseases caused by mutations in the mitochondrial DNA

As mentioned before mtDNA is maternally transmitted so these disorders follow maternal inheritance laws. The mtDNA-related diseases can be caused by point mutations and by mtDNA rearrangements (single, large-scale mtDNA deletions). Because of its high mutation rate, pathogenic mtDNA point mutations are reported in approximately 1 in 200 individuals but in most cases they are heteroplasmic. This means that these mutations may or may not lead to mitochondrial dysfunction and so to the onset of clinical symptoms depending on the ratio of normal/mutated mtDNA and on the tissue affected. Some of the representative heteroplasmic mutations are associated with MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibres), NARP (neurogenic weakness, ataxia and retinitis pigmentosa). Point mutations have been identified both in mitochondrial genes encoding respiratory chain subunits thus damaging the functionality of the complex in which the protein takes part, and in the genes encoding tRNA and rRNA thus compromising mitochondrial protein synthesis. Otherwise, single and large-scale mtDNA deletions, arising sporadically during embryonic development, cause the removal of one or more mitochondrial genes leading to the onset of different, in terms of severity and population frequency, manifestations: chronic progressive external ophthalmoplegia (PEO), Kearns–Sayre syndrome (KSS) and Pearson syndrome.

Mitochondrial diseases caused by mutations in the nuclear DNA

Most of the proteins needed for mitochondrial functionality are nuclear encoded. Moreover nuclear genes encode not only OXPHOS system subunits but also proteins involved in mitochondrial dynamics, in mitochondrial protein import, in mtDNA maintenance, duplication and transcription. This explains why the majority of mitochondrial disorders, with varied inheritance pattern (de novo, X-linked, autosomal dominant or autosomal recessive) are caused by mutations in nuclear DNA. To date, over 300 nuclear genes associated to mitochondrial diseases have been reported and they have been classified in different categories according to their function (Thompson *et al.,* 2020).

1.4 Mitochondrial DNA maintenance defects

The stability of mtDNA depends both on gene encoding proteins involved in mtDNA synthesis (*POLG, POLG2, TWNK, TFAM, RNASEH1, MGME1, and DNA2*) and genes encoding protein involved in a balanced supply of mitochondrial nucleotide pool (*TK2, DGUOK, SUCLG1, SUCLA2, ABAT, RRM2B, TYMP, SLC25A4, AGK*) (reviewed in El-Hattab *et al.,* 2017a). Mutations in these nuclear genes result both in quantitative abnormalities (mtDNA depletion) leading to Mitochondrial Depletion

Syndromes (MDS) and qualitative defects (multiple mtDNA deletions) leading to syndromes generally less severe than the first ones. However, defects in the mtDNA maintenance are also caused by mutations in genes encoding proteins not directly involved with mtDNA metabolism, such proteins engaged in mitochondrial dynamics (*OPA1, MFN2,* and *FBXL4*).

In this background, *ANT1* (*SLC25A4*) and *MPV17*, objects of my Phd research, are included in the class of genes whose mutations lead respectively to Multiple Mitochondrial DNA Deletions Syndromes, and Mitochondrial DNA Depletion Syndromes (MDS) even if the role of these two protein in mtDNA stability remains unclear.

1.4.1 An overview of mtDNA synthesis machinary

Unlike nuclear DNA whose synthesis and repair processes are catalyzed by different polymerases, the holoenzyme of DNA polymerase γ (Pol γ) is the only polymerase involved in mitochondrial genome replication and repair. It consists of a catalytic subunit encoded by *POLG* and a processing subunit, encoded by *POLG2* that enhances DNA binding and increases the processivity of Polγ. Mutations in *POLG*/*POLG2* can compromise the formation of the holoenzyme or decrease its processivity and proofreading activity and they have been associated with a broad spectrum of pathologies (reviewed in Rahman and Copeland, 2019). However, Pol γ is not the only component of the mitochndrial DNA synthesis machinery but interacts with other additional proteins including:

- Twinkle mtDNA helicase (encoded by *TWNK*) unwind the double-stranded DNA thus allowing the Pol γ to proceed with DNA replication;
- Mitochondrial single-stranded DNA-binding protein (encoded by *SSBP1*) needed for stabilizing the single stands and interacts with Polγ and Twinkle to stimulate their activity;
- Mitochondrial RNA polymerase (encode by *POLRMT*) catalyse the synthesis of short RNA primers that serve as starting points for DNA synthesis;
- DNA polymerase-primase (encoded by *PRIMPOL*), characterized by primase and DNA polymerase activity that allow to bypass and to rescue a stalled replication fork. Therefore it is necessary for mtDNA repair.
- Ribonuclease H1 (encoded by *RNASEH1*) degrades the RNA of RNA-DNA hybrids and plays a key role in transcription termination;
- DNA replication helicase/nuclease 2 (encoded by *DNA2*), Mitochondrial Genome Maintenance Exonuclease 1 (encoded by *MGME1*) and Flap Structure-specific Endonuclease 1 (encoed by *FEN1*), nucleases which help RNASEH1 to eliminate the remaining RNA from the RNA-DNA hybrids.

Mutations in all these genes are responsible for several mtDNA maintenance disorders characterized by mitochondrial DNA depletions or deletions with a consequent impairment of OXPHOS process (reviewed in Young and Copeland, 2016).

1.4.2. An overview of mitochondrial dNTP metabolism

An adequate and balanced amount of each dNTP is critically important for DNA integrity. Altough until 20 years ago many studies were focused on investigating the effect of dNTP pool alterations on nuclear DNA, several mutations in nucleotide metabolism genes are found to be associated with mitochondrial DNA instability, thus extending the importance of a balanced dNTP pool also for mitochondrial DNA manteinance.

What is currently known about the origin of the mitochondrial nucleotide pool?

For many years the nature and source of mitochondrial nucleotides have been the topic of intense debate. The earliest studies suggested that mitochondrial dNTP pool are strictly separated and independent from the cytosolic dNTP pool since (i) in mouse cells lacking cytosolic thymidine kinases (TK1) treated with antimetabolites (5-fluorodeoxyuridine and methotrexate) and with radioactive thymidine, mtDNA replication continued while nuclear DNA replication was strongly inhibited (Bogenhagen and Clayton 1976), (ii) in HeLa cells, after treatment with antimetabolites, only cytosolic dNTP pool were severely depleted whereas mitochondrial dNTP pool expanded, suggesting that independent regulation of mt nucleotide may be due to the fact that the mtDNA, unlike the nuclear one, replicates throughout the cell cycle (Bestwick *et al.,* 1982). However other works don't confirm these data but instead demonstrate a rapid movement of dNTPs both from the cytosol to the mitochondria and in the opposite direction, suggesting a dynamic relationship between the two compartments (Pontarin *et al.,* 2003; Leanza *et al.,* 2008).

Mitochondrial dNTP pool can be fed by two different routes: (i) deoxyribonucleotide transport as mono-, di-, tri-phosphates, synthesized *de novo* in the cytosol by the key enzyme ribonucleotide reductase (RNR), (ii) deoxyribonucleoside uptake followed by intra-mitochondrial kinase activity of *salvage* pathway enzymes. The use of one pathway over the other depends on the cell-cycle phase: in cycling cells with *de novo* synthesis of dNTPs, mt dNTPs are mainly imported from the cytosol; in non cycling cells, since *de novo* synthesis is downregulated, mitochondrial dNTPs synthesis depends mainly on *salvage* pathway enzymes.

Deoxyribonucleotide exchange between the cytosol and mitochondria

The first evidence that the dNTPs synthesized in the cytosol could be imported into mitochondria derives from reports in which it was shown that isolated mitochondria were able to use exogenous dNTPs to fully synthesize the mt DNA in an ATP dependent manner (Karol and Simpson, 1968; Enríquez *et al.,* 1994). These data suggested the existence of dNTPs transporters but the real evidence of their existence came from the isolation and functional characterization of mitochondrial dNTPs carrier. To date several deoxynucleotide mitochondrial transport activity have been described even if the knowledge about the identity of the carriers and their molecular mechanisms are not yet fully defined.

The dNTP mitochondrial carriers known so far are listed below:

- dCTP transport system of human acute lymphocytic leukemia cells was characterized in human mitochondria (Bridges *et al.,* 1999): it seems to prefer nucleoside at the triphosphate level (dCTP) compared to diphosphate (dCDP), monophosphate (dCMP) or nucleoside deoxycytidine (dCyd); however, its Ca^{2+} - dependent activity may not be specific for dCTP but may represent a general carrier since other triphosphate nucleosides reduce the uptake of dCTP. It is also not clear whether the transport is unidirectional or bidirectional.
- dTMP highly selective import was described in isolated mouse liver mitochondria (Ferraro *et al.,* 2006): dTMP but not thymidine, dTDP, or dTTP is quickly imported into the mitochondria, while the export, which seems to take place at diphosphate level, occurs more slowly.
- PNC1: a SLC25 family member, was found to be involved the import of thymidine nucleotides even if the phosphorylation level of the imported nucleotide remains to be clarified (Floyd *et al.,* 2007; Franzolin *et al.,* 2012).

The dNTPs entering the mitochondrion via these transporters are produced *de novo* in the cytosol by the RNR activity which reduce rNDPs in dNDPs. Eukaryotic RNR consists of a large subunit, R1, whose levels are constant throughout the cell cycle (Engström *et al.,* 1985), and of a small subunit, R2 whose expression starts at the beginning of S phase thus ensuring the right supply of dNTPs necessary for DNA replication. At the S/G2 border R2 levels decrease by selective proteolysis and this degradation shut off dNTPs synthesis (Chabes *et al.,* 2003; D'Angiolella *et al.,* 2012; Guarino *et al.,* 2014). Therefore the control of RNR activity during cell-cycle depends on R2 levels. However nucleotides must also be available for DNA repair or mitochondrial DNA (mtDNA) synthesis, processes that are not limited to the S phase but occur throughout the cell cycle. Mammalian cells

respond to this request by expressing a p53-inducible gene, *RRM2B*, that encodes for another variant of R2 subunit able to associate with R1 subunit to form an active RNR complex. The *RRM2B* gene was originally thought to be induced only by DNA damage, via p53 transcriptional activation, to supply dNTPs for DNA repair (Tanaka *et al.,* 2000). However, Håkansson *et al.* (2006) showed that p53R2 has low level constitutive expression suggesting its critical role not only for DNA repair but also for mtDNA synthesis. This hypothesis is strongly supported by the observation that the loss of p53R2 lead to mtDNA depletion in quiescient cells where p53R2 function can not be compensated with R2 activity, whereas in cycling cells, where R2 is highly expressed, mtDNA synthesis is not disturbed (Pontarin *et al.,* 2012).

As in mammalian cells*,* also in the yeast *Saccharomyces cerevisiae* RNR activity is cell cycle-regulated. It consists of four RNR proteins: Rnr1 and Rnr3 that are components of the catalytic R1 subunits and Rnr2 and Rnr4 that are component of the regulatory R2 subunits. Rnr1 expression is strictly dependent on the cell cycle and specifically induced in phase S; Rnr3 transcript levels, on the other hand, increase by about 100 times following DNA damage (Elledge and Davis, 1990). Recently it has been demonstrated that the expression of these two genes is regulated by the carbon source available: the abundance of glucose which drives a rapid fermentative proliferative process, induces Rnr1 expression probably to ensure an adequate supply of dNTPs necessary for DNA replication. Oppositely, a limited amount of glucose which trigger slower respiratory proliferation, down regulate Rnr1 and induce Rnr3 expression. In this way, the reduction of the Rnr1 levels prevents an accumulation of dNTPs, which have harmful effects as much as the dNTPs deficiency (Corcoles-Saez *et al.,* 2019).

Beyond transcriptional mechanisms, RNR activity is also subjected to allosteric regulation, degradation and control of the cellular localization of RNR subunits. For example *S. cerevisiae* possesses three proteins Sml1, Dif1 and Hug1 that bind to RNR and inhibit it or alter the localization of its subunits, thus regulating RNR activity (Guarino *et al.,* 2014).

The allosteric regulation of RNR is crucial to maintain the physiological intracellular pool of dNTPs. Binding of ATP or dATP to active site in the R1 subunit controls the general enzymatic activity of RNR: ATP turn the enzyme on, dATP turn the enzyme off. Binding of ATP, dATP, dTTP, and dGTP to the specificy site in the R1 subunit, determines which rNDPs must be reduced at the catalytic site (Brown and Reichard, 1969).

Nucleoside *salvage* pathway within the mitochondria

As mentioned above mt dNTP pool in non-dividig cell depends maily on nucleoside *salvage* pathway. This pathway begins with import of deoxynucleosides within mitochondria through human equilibrative deoxynucleoside transporters, hENT1-4 (Baldwin *et al.,* 2004) expressed on the mitochondrial membrane (Lai *et al.,* 2004; Govindarajan *et al.,* 2009) followed by phosphorylation by mt kinase deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2) which lead to the synthesis of all four deoxymonophosphate nucleosides (dNMP) then converted to the corresponding deoxyribonucleoside triphosphates (dNTP) by other kinases. Mitochondrial nucleoside monophosphate kinases (NMPKs) idenfied and characterized so far are as follows: adenylate kinase (AK) acting on dAMP (Panayiotou *et al.,* 2014); uridylate-cytidylate kinases 2 (UMP-CMPK2) acting on dUMP which represents its preferred substrate followed by dCMP (Xu *et al.,* 2008); thymidine monophosphate kinase 2 (TMPK2) acting on dTMP even if attempts to define the enzyme activity (substrate specificity and kinetic properties) were unsuccessful (Chen *et al.,* 2008).

Nm23-H4 protein, essentially bound to the IMM (Tokarska-Schlattner *et al.,* 2008) has been idenfied as mitochondrial nucleoside diphosphate kinase (Milon L. *et al.,* 2000).

Also in the mitochondria, as occurs in the cytosol, the availability of dNTPs is regulated by the phosphorylic activity of kinases opposing the hydrolytic activity of 5’-NT nucleotidases. To date, only one mitochondrial nucleotidase has been identified: mitochondrial 5'(3')-deoxyribonucleotidase (mdN) dephosphorylating dTMP and dUMP, creates a substrate cycle with TK2 thus avoiding dTTP overproduction (Rampazzo *et al.,* 2000; Rampazzo *et al.,* 2004).

Is the contribution provided by *salvage* pathway sufficient to support the mtDNA replication in quiescient cells? As already mentioned above, in cycling cells the greatest contribution of mt dNTP is provided by the cytosol through *de novo* synthesis, but even in non-proliferating cells a substantial contribution of cytosolic dNTPs, produced by R1-p53R2 isoform of ribonucleotide reductase, have been demonstrated (Rampazzo *et al.,* 2007; Leanza 2008). Indeed kinetic properties of the enzymes of mitochondrial *salvage pathway* would seem to be inadequate to support replication of mitochondrial genome (Gandhi and Samuels, 2011). Furthermore, if the *salvage* pathway alone could provide the right amount of dNTPs needed for the mtDNA replication, deficiency in cytoplasmic enzymes of nucleotide metabolism (such as p53R2), should not led to mtDNA depletion. On the other hand, defects in the *salvage* pathway enzymes (such as TK2, dGK) also cause mtDNA depletion with more serious effects in post-mitotic tissues, thus underlining that even the activity of p53R2 alone is not able to provide sufficient dNTPs for mtDNA replication (Leanza *et al.,* 2008).

Probably the mt dNTP pool is the result of a complex enzyme network involving *de novo*, *salvage* pathway, catabolic enzymes and transporters and it is also probable that our knowledge about components of the mitochondrial metabolism of nucleotides are not yet complete. In the literature, one report describes the existence of RNR activity in mammalian tissue mitochondria (Chimploy *et al.,* 2013) thus suggesting a mitochondrial *de novo* synthesis pathway as a possibly significant contributor to mitochondrial dNTP pool but this hypothesis requires further investigations.

The situation is less complex in *S. cerevisiae* because it lacks the enzymes of the *salvage* pathway thus making the mitochondrial dNTP pool completely dependent on the direct transport of dNTPs from the cytosol. This dependence on the cytosolic dNTP pool is proven by the fact that *RNR1* overexpression or *RNR1* inhibiting protein (*SML1*) deletion lead to an increase of mtDNA stability (Baruffini *et al.,* 2006). Furthmore mitochondrial transporter for pyrimidine nucleoside triphosphates (Rim2) and mitochondrial GTP/GDP transporter (Ggc1) have been identified in *S. cerevisiae* (Vozza *et al.,* 2004; Marobbio *et al.,* 2006).

1.5 Mitochondrial DNA maintenance defects related to ANT1 mutations

Adenine nucleotide translocator (ANT), belonging to the large family of mitochondrial carriers, is one of the most abundant proteins located in the inner mitochondrial membrane (Palmieri 2004, 2014). Its primary function is to import cytosolic ADP into the mitochondrial matrix to fuel the ATP production by ATP synthase (Complex V), and to exports ATP, produced by OXPHOS process and necessary to support all cellular activities, from the matrix to the cytosol. ANT is encoded by nuclear genome, synthesized by cytosolic ribosomes, transported to the mitochondrion by the TIM/TOM complex and then inserted into the inner mitochondrial membrane, where it creates a transmembrane channel consisting of six alpha helices, whose amino acid sequence includes three repeats of 100 amino acids each of which forms two transmembrane α-helices domains (Saraste and Walker, 1982; Walker and Runswick, 1993; Pebay-Peyroula *et al.,* 2003;). This channel, large enough to allow ATP/ADP passage through the IMM, cyclically assumes two different conformational states due to the destruction and formation of a triple salt bridge (**Figure 1.4**) present both on the matrix side and on the cytosol side (Kunji *et al.,* 2016; Ruprecht *et al.,* 2020):

1. in the cytoplasmic state, the triple salt bridge is destroyed on the cytoplasmic side and is formed on the matrix side so that the binding site for ADP is accessible to the intermembrane space confluent with the cytosol; therefore the site binds ADP^{3-} and catalyzes its extrusion from the cytosol to the mitochondrion;

2. in the matrix state, the triple salt bridge is destroyed on the matrix side and is formed on the cytoplasmic side so that the binding site for ATP is accessible to matrix; therefore the site binds ATP^{4-} generated by oxidative phosphorylation and catalyzed its extrusion from the mitochondrial matrix to the cytosol.

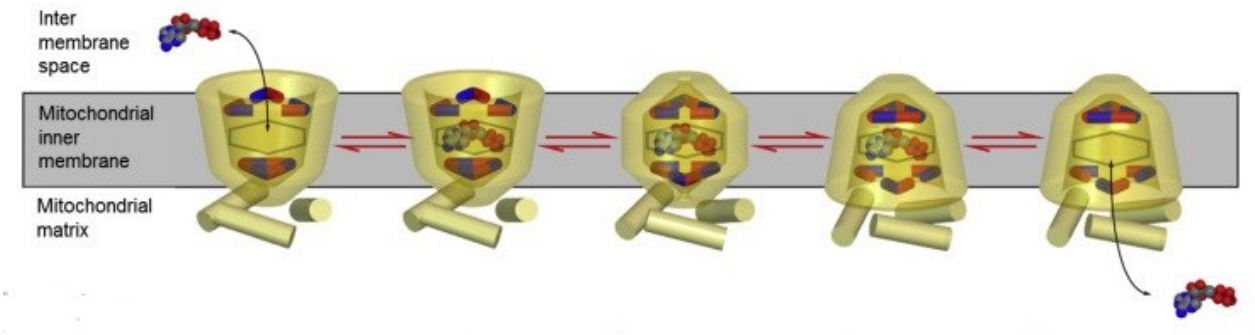


Figure 1.4. Representation of the transport cycle of ADP/ATP through the formation and destruction of a triple salt bridge present both on the matrix and on the cytosol side (from Thompson *et al.,* 2016)

However, the role of ANT is not limited to ADP/ATP transport across the inner mitochondrial membrane. As previously anticipated, in cells lacking mitochondrial DNA, ANT plays a crucial role in maintaining membrane potential by reversing the transport direction (Appleby *et al.,* 1999).
Moreover it has been shown that ANT amount also plays a role in the basal proton conductance of mitochondria. In fact, it has been demonstrated that ANT deletion in the mitochondria of mouse muscle and ANT overexpression in Drososophila cause a change in the proton conductance (a decrease and an increase respectively) compared to wild type (Brand *et al.,* 2005). This suggests that the ANT content contribute to the proton loss across the inner mitochondrial membrane, thus mildly uncoupling the membrane and avoiding hyperpolarization and overproduction of ROS.
Recently, a role of ANT in mitophagy, selective elimination of damaged mitochondria, has also been proposed, because it has been observed that mice lacking ANT accumulate harmed mitochondria (Hoshino *et al.,* 2019).
In humans there are 4 isoforms of the ADP/ATP carrier, encoded by 4 nuclear genes, among which there is no functional difference but only a distinct pattern of expression depending on the tissue and the metabolic state of the cell (Stepien *et al.,* 1992). Ant1, encoded by SLC25A4, is expressed highly in the mitochondria of the heart and skeletal tissue; Ant2, encoded by SLC25A5, is expressed at low level in differentiated tissues but it is particularly abundant in proliferative, undifferentiated cells so much to be considered a key protein in carcinogenesis (Chevrollier *et al.,* 2011). Ant3,

encoded by SLC25A6 is ubiquitously expressed at variable levels depending on the oxidative metabolism. Ant4, encoded by, is exclusively present in liver, testis and brain (Dolce *et al.,* 2005).
Given Ant1 is predominantly expressed in tissues with greater energy demand, it is not surprising that several mutations in ANT1 lead to mitochondrial diseases.
To date, a total of 8 missense mutations have been identified in ANT1, divided into different classes according to the clinical phenotype:

- Dominant missense mutations (A90D, L98P, D104G, A114P and V289M) have been found in patients affected by adult-onset autosomal dominant Progressive External Ophtalmoplegia (adPEO), clinically characterized by ptosis and impairment of eye movements (Kaukonen *et al.,* 2000; Napoli *et al.,* 2001; Komaki *et al.,* 2002; Siciliano *et al.,* 2003; Deschauer *et al.,* 2005)
- recessive loss of function mutations (A123D and R236P) have been found in subjects affected by mitochondrial myopathy and cardiomyopathy (Palmieri *et al.,* 2005; Korver-Keularts *et al.,* 2015);
- *de novo* dominant missense mutations (R80H and R235G) leading to a more severe early-onset mitochondrial disease have been described in seven affected individuals (Thompson K. *et al.,* 2016), whereas a new case of *de novo* variant (K33Q) was found in a patient with mild symptoms (King *et al.,* 2018).

A common feature to all these patients is the accumulation of multiple mitochondrial DNA deletions in their tissues and, in the case of patients with the two *de novo* mutations (R80H and R235G), a dramatic reduction of mitochondrial DNA copy number in skeletal muscle has been observed. How ANT1 mutation induces mtDNA instability is not yet completely understood and so the role of Ant1 in the maintenance of mtDNA remains unclear and highly debated. As said before, defects in mitochondrial DNA maintenance are generally caused by mutations in genes involved in mitochondrial DNA synthesis or those involved in mitochondrial dNTPs metabolism. Since in mammalian cells mitochondrial dNTP pool is partially fed by direct import of deoxyribonucleotide synthesized *de novo* in the cytosol, mitochondrial nucleotide carriers are pivotal to maintain an adequate balance of mt dNTP. About this, it has been proposed that Ant1 could regulate intramitochondrial dATP levels; in fact, although dATP/dADP are not the physiological substrates of Ant1, the ADP imported into the mitochondrial matrix could be converted to its corresponding deoxy-form by mitochondrial RNR, and then phosphorylated into dATP (Kaukonen *et al.,* 2000). Fontanesi *et al.* (2004) have demonstrated that proteoliposomes reconstituted with adPEO-type

Ant1 mutant form are involved in a futile ATP/ATP homoexchange, binding preferentially ATP over ADP, thus leading to a decrease in ADP import and probably lowering mt dATP levels with a consequent loss in the replication fidelity of the mt genome. Furthmore the impairment of ADP/ATP transport activity through the IMM could lead to a ROS overproduction within mitochondria with a consequent damage on mtDNA as shown in ANT1 KO mice (Esposito *et al.,* 1999). It has been proposed that a reduced level of matrix ADP, due to loss of ADP/ATP translocation activity, could inhibit the ATP synthase thereby blocking the proton influx into the matrix mediated by ATP synthase activity. As a consequence, the electrochemical gradient is not dissipated and reaches its maximum level, thus blocking the proton pumping system by the respiratory complexes with stalling of electron flow and consequent accumulation of electrons that enhance ROS production.

Other hypotheses, not related to a dysfunction of ADP/ATP transport activity, have been suggested to explain the onset of mitochondrial DNA instability resulting from mutations in ANT1. Formation of unregulated channel on the IMM, induced by mutated form of Ant1, could cause mitochondrial membrane depolarization with a consequent mitochondrial dysfunction that in turn could lead to instability of mtDNA. In fact, as already said before, the maintenance of mitochondrial membrane potential, is not only crucial for the synthesis of ATP, but it represents also an important factor for mitochondrial biogenesis and fuctionality. Therefore, according to this hypothesis, the defects in the maintenance of mitochondrial DNA, would be consequent to the formation of an uncontrolled channel and not to the altered ADP/ATP transport activity (Chen, 2002).

The hypothesis that mtDNA instability could be a consequence of general mitochondrial damage, rather than nucleotide imbalance following impairment of ADP/ATP transport activity, is also supported by the demonstration that Ant1 mutant protein tends to form large aggregates thus inducing proteostatic stress on the IMM that in turn could affect mitochondrial biogenesis and could damage the electron transport chain assembly and mtDNA integrity (Liu and Chen, 2013; Liu *et al.,* 2015; Coyne and Chen, 2019).

Despite all these observations, the pathogenesis of the ANT1-induced pathologies and in particular the mechanism by which mutations in this gene lead to instability of the mitochondrial DNA remains highly unsolved issues.

1.6 Mitochondrial Depletion Syndromes (MDS) related to MPV17 mutations

Mitochondrial DNA depletion syndromes (MDS) are a remarkable group of diseases characterized by a notable drop in the mitochondrial DNA content, resulting in deficiency of the respiratory chain components and so in impairment of energy production in one or several tissues (El-Hattab and Scaglia, 2013). Like other mitochondrial diseases, these syndromes are genetically and clinically heterogeneous, but MDS are typically characterized by early-onset in the first days or months of life and, in many cases, by the rapid worsening of symptoms. However, there are cases in which an adult-onset progressive manifestation of symptoms is observed (Béhin *et al.,* 2012; Ronchi *et al.,* 2012; Blakely *et al.,* 2012). Depending on the clinical manifestations and the affected tissues, these disorders are generally classified into 4 categories: a myopathic form (affects the skeletal muscles), encephalomyopathic form (affects both the musculature and the nervous system), hepatocerebral form (affects the liver and brain) and neurogastrointestinal form (affects stomach and small intestines muscles).

Hepatocerebral type of MDS is associated with mutations in the nuclear genes TWNK, POLG, DGUOK, TFAM and MPV17. While for the first 4 genes, involved in the mitochondrial DNA synthesis machinery or in the metabolism of mt nucleotides, the association with mitochondrial DNA stability is clear, the role of MPV17 in the maintenance of mitochondrial genome remains unclear.

The human MPV17 gene maps on chromosome 2p23-21 and encodes a small hydrophobic protein of 176 amino acids embedded in the IMM and characterized by four predicted hydrophobic transmembrane domains and short hydrophilic stretchs in the intermembrane space and matrix regions (Spinazzola *et al.,* 2006; Wong *et al.,* 2007).

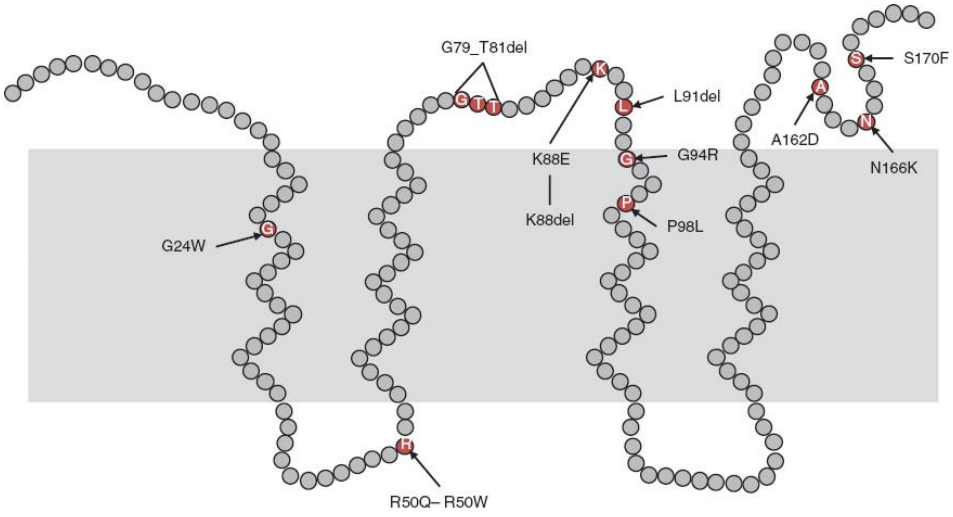


Figura 1.5. Molecular modeling of the MPV17 protein (from Löllgen and Weiher, 2015).

Mutations in MPV17 were initially identified in three families with hepatocerebral MDDS (Spinazzola *et al.,* 2006) and in individuals with Navajo neurohepatopathy, an autosomal recessive multisystem disorder (Karadimas *et al.,* 2006). So far, 39 pathogenic variants have been reported in MPV17 gene on The Human Gene Mutation Database (HGMD): 20 missense/nonsense mutations, 6 splicing variants, 6 small deletions, 2 small insertions, 1 small indel and 4 gross deletions. Although the clinical presentations associated with MPV17 mutations are highly varied, hepatopathy and neurologic abnormalities are the most recurrent clinical features (AlSaman *et al.,* 2012).

Symptoms generally occur in the first months of life or in infancy although cases of adult onset neuropathy and leukoencephalopathy, characterized by multiple mitochondrial DNA deletions rather than depletion, have been reported (Blakely *et al.,* 2012; Garone *et al.,* 2012).

The high degree of conservation between human MPV17 and its mouse (MPV17), zebrafish (MPV17) and yeast (*SYM1*) orthologs made possible to study the phenotypic effects of Mpv17/Sym1 mutations in different model organisms. Studies carried out in yeast and in an artificial membrane reconstituted with purified recombinant human Mpv17 protein, demonstrated that the protein forms a non selective channel that resides in the IMM mostly in a closed state and whose opening occurs only in conditions of harmed mitochondria, thus lowering the membrane potential and preventing ROS overproduction (Reinhold *et al.,* 2012; Antonenkov *et al.,* 2015).

Although biochemical function(s) of Mpv17 remain elusive, it appears to be essential for mtDNA copy number maintenance since that loss of function of this protein cause mtDNA depletion in *MPV17* knock out mice (Viscomi *et al.,* 2009) and mtDNA instability in *S. cerevisiae* (Spinazzola *et al.,* 2006; Gilberti *et al.,* 2018). However, the role of Mpv17 in mtDNA maintenance is not yet completely understood. About this, several hypothesis have been proposed. The enhanced ROS production observed in glomeruli of *MPV17* knock out mice suggest an involvement of Mpv17 in the regulation of ROS levels (Binder *et al.,* 1999), even if it is not clear whether the ROS increase is a consequence of impaired OXPHOS process, resulting from reduction of mitochondrial DNA content, or if the ROS increase is the cause of the mtDNA damage (Löllgen and Weiher, 2015).

The decrease of mitochondrial dNTP pool, observed in liver mitochondria of rats tissues and fibroblasts derived from patients with mutations in MPV17 gene and the demonstration that supplementation of dNTPs prevents and rescue mtDNA depletion in patients fibroblasts, indicate that insufficient availability of mitochondrial dNTP is the principal cause of mtDNA depletion (Dalla Rosa *et al.,* 2016). At a molecular level, it seems that Mpv17 supports the mitochondrial purine *salvage* pathway, since a decreased expression of enzymes involved in this pathway was observed

in the *MPV17* KO mouse and in patients derived fibroblasts (Dalla Rosa *et al.,* 2016). The hypothesis that Mpv17 may be involved in mitochondrial nucleotide metabolism is also supported by the observation that deficiency of *MPV17* orthologous gene in zebrafish results in a strong reduction of pigment cell iridophores, mainly constituted by guanine (Krauss *et al.,* 2013). It has been proposed that lack of protein leads to a reduction in the uptake of guanosine or its phosphate derivatives, resulting in mitochondrial dysfunction and in iridophores death. Moreover iridophore and melanophore loss in zebrafish embryos can be caused by the chemical inhibition of pyrimidine *de novo* synthesis (White *et al.,* 2011). Interestingly it has been demonstrated that supplementation with dNTPs and pyrimidine precursors, as orotic acid (OA), lead to a significant increase of both iridophore number and mtDNA content in *mpv17−/−* zebrafish mutants, thus linking the loss of Mpv17 to pyrimidine *de novo* synthesis (Martorano *et al.,* 2019).

More recently, the Mpv17 deficiency in Hela cells has been shown to be associated with a reduction in folate levels and with an increase in the uracil level, a marker of impaired dTMP synthesis, without compromising either *de novo* or *salvage* pathway. This suggests that Mpv17 can provide a third dTMP source and prevents uracil misincorporation in mtDNA (Alonzo *et al.,* 2018), which could lead to DNA strand breaks and genome instability (Blount *et al.,* 1997). On the other side in *S. cerevisiae* the ortholog Sym1 has been related to a homeostatic control of tricarboxylic acid cycle (TCA) intermediates, such as oxalacetate and alpha-ketoglutarate (Dallabona *et al,* 2010).

Despite all these findings, the function of Mpv17 protein and in particular its role in mitochondrial genome maintenance, remains to be clarified.

1.7 Yeast *S. cerevisiae* as a model organism to study MD and to discovery new potential therapies

S. cerevisiae has played a pivotal role in over 60 years in understanding the molecular mechanisms underlying the onset of mitochondrial mutations that impair the respiratory metabolism, thanks to its ability to survive in absence of mtDNA, when provided with fermentable carbon source (Ephrussi and Slonimski, 1955). Therefore, yeast represents an excellent model for the study of MD, because in this organism it is possible to study the phenotypic effects of mutations in mitochondrial or nuclear genes that damage mitochondrial functionality and that are lethal in other eukaryotes. In *S. cerevisiae*, phenotypes related to the mitochondrial metabolism dysfunction, such as reduction or inhibition of growth on oxidizable/respirable sources, alteration of the absorption spectra of respiratory cytochromes or alteration of respiratory activity, are easily detectable. Moreover, yeast can tolerate defects in mitochondrial DNA maintenance and thus offers the possibility of

determining whether a mutation is associated with mtDNA instability. In particular yeast cells with abnormalities in the mtDNA and so unable to use oxidative carbon sources, generate colonies of smaller size, known as *petite,* in the presence of a low concentration of a fermentable source (such as glucose) and a high concentration of a respirable source (such as ethanol, glycerol, lactate). Therefore the frequency of the *petite* colonies onset is an index of the mitochondrial DNA instability. Certainly another important aspect which makes possible to decipher in yeast the molecular basis underlying MD is the high level of mitochondrial function conservation and the fact that several human genes involved in mitochondrial diseases have an orthologue in yeast. So a yeast model-disease can be easily obtained. In case the human gene complements the OXPHOS phenotype of the yeast deleted of the gene of interest, the pathogenic mutation can be introduced in the human cDNA then expressed in yeast. Alternatively the pathogenic mutation can be inserted directly in the yeast orthologous gene. Furthermore the ability of yeast to grow as both haploid and diploid cells, offers the advantage to obtain information also on the dominace/recessivity of mutations, which is not always straightforward in patients.

Some of the mitochondrial disorders for which yeast has proved an excellent model in understanding the pathogenetic mechanisms are reviewed in Rinaldi *et al.,* 2010, Lasserre *et al.,* 2015, Francisci and Montanari, 2017.

Importantly, yeast has been proposed as a skilled model for the discovery of new potential therapies for mitochondrial diseases. This aspect should not to be overlooked since to date there are no pharmacological therapies for MDs. Despite the advances of gene-therapy for treatment of some mitochondrial disorders, such as LHON (Feuer *et al.,* 2016), for almost all mitochondrial diseases therapeutic approaches remains symptomatic and does not alter the course of the diseases (El-Hattab *et al.,* 2017b). Therefore the development of a curative treatment for mitochondrial diseases represents an enormous challenge.

To this purpose, yeast has been proposed as a valuable tool to identify new drugs active against mitochondrial diseases thanks to the establishment of a two-step yeast-based screening assay called “drug drop test”, a high throughput screening which allows to analyze in short time an extremely high number of molecules (Couplan *et al.,* 2011; Lasserre *et al.,* 2015). Developed for the first time to search active compounds against NARP syndrome, a mitochondrial disease caused by deficiency in fully assembled ATP synthase (Couplan *et al.,* 2011), until now several groups used this method to identify candidate drugs for treatment of different MDs, such us Friedreich’s ataxia (Cotticelli *et*

al., 2012), POLG-related diseases (Pitayu *et al.,* 2016), optic atrophies (Delerue *et al.,* 2019; Aleo *et al.,* 2020).

How does drug drop test work? The mutant strain is spread on plates containing a non fermentable carbon source, where it is not able to grow, and small filter disks, which are then spotted with the different compounds to be tested, are placed on the agar surface. The choice of the mutant strain to be used for screening is very important: ideal is a strain with an evident but not irreversible phenotype. Depending on the presence/absence of a halo of growth and its position around the filters, the compounds are classified into three groups:

- compounds that lead to formation of a halo of growth near the filter. These molecules have a rescuing effect starting from the maximum concentration tested.
- compounds that lead to formation of an external crown of growth, whereas no growth is observed near the filter. These molecules are toxic at high concentrations (near the filter) and active at lower concentration (far from the filter).
- compounds without any effect.

A schematic representation of yeast-based assay is reported in **Figure 1.6.**

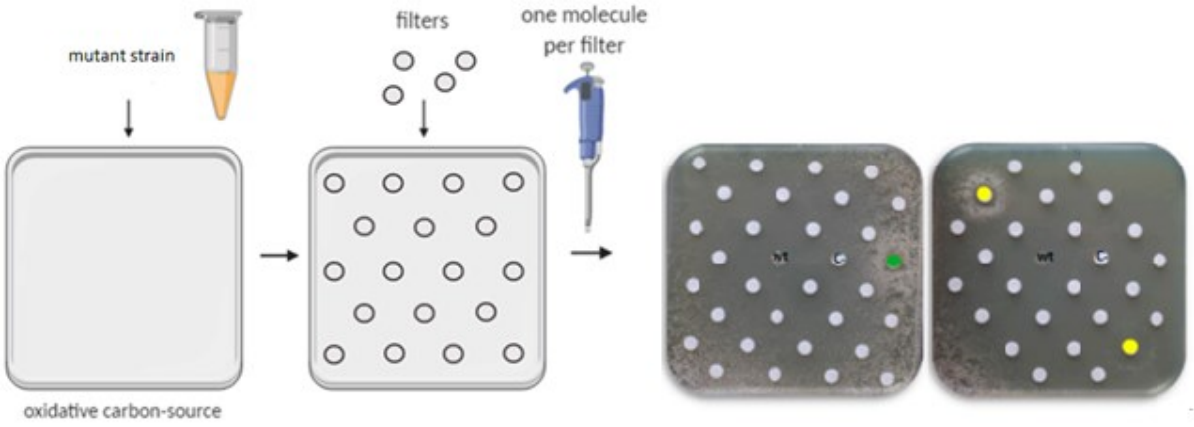


Figure 1.6. A schematic representation of yeast-based assay. In green is indicated a beneficial drug, with its halo of growth around, while in yellow it can be observed drug actives only at low concentrations (external halo of growth). WT/C+: wild-type/positive control; C-: negative control (adapted from Gilberti, PhD thesis).

To confirm the rescuing effect of positive molecules identified with the primary screening and to minimize the number of false-positive molecules, the active compounds are then subjected to a secondary screening which differs from the primary one only for the number of filters placed on the plate.

The possibility to screen collections of thousand FDA-approved drugs and so available de-risked compounds, allows a drug repurposing approach, a promising strategy in drug discovery field

because it provides a new alternative use for existing drugs (Ashburn and Thor, 2004; Aubé, 2012; Pushpakom *et al.,* 2019). Repurposing the use of an "old" compund means reducing development cost and speeding up an eventual administration in humans, characteristics that make this strategy attractive especially in the field of rare diseases and so also in that of mitochondrial diseases.

1.8 *S. cerevisiae* as a model for the study of pathological mutations in the ANT1 gene

The study of the molecular basis of the pathologies caused by alterations in the ANT1 gene cannot be carried out in human cell lines, since the ANT1 gene is not expressed in fibroblasts or in cultured myoblasts, and furthermore its overexpression induces apoptosis (Kaukonen *et al.,* 2000; Bauer *et al.,* 1999).

ANT1 genes is highly conserved in all eukaryotic organisms, including the yeast *S. cerevisiae* which has proved to be an excellent model to get insight into the pathogenetic mechanisms underlying mitochondrial dysfunctions caused by mutations in this gene. Three genes coding for the mitochondrial carrier ATP/ADP (*AAC1, AAC2, AAC3*) have been identified in *S. cerevisiae* (Adrian *et al.,* 1986; Lawson and Douglas 1988; Kolarov *et al.,* 1990; Drgon *et al.,* 1992). Among these, *AAC2* is considered the major transporter of ADP/ATP related to oxidative phosphorylation, and so essential for the growth on respirable carbon sources, because the destruction of this gene, but not of *AAC1* and/or *AAC3*, leads to a reduction of oxidative growth (Lawson and Douglas, 1988; Drgon *et al.,* 1992). The protein sequence of yeast Aac2 shares 54% identity with human Ant1 protein and all the pathological substitutions identified in Ant1 are conserved or are localized in conserved regions between the human and yeast sequence (with the exception of D104G). Therefore it was possible to obtain mutant *aac2* alleles containing substitutions corresponding to the ANT1 mutations. The mutant alleles were introduced in a *S. cerevisiae* strain devoid of both *AAC2* and *AAC1* (WB-12), thus making it possible, by complementation studies, to analyze the effects of each pathogenic mutation on the OXPHOS metabolism, through analysis of growth on non-fermentable carbon sources, oxygen consumption, content of respiratory cytochromes, ADP/ATP transport (Kaukonen *et al.,* 2000; Fontanesi *et al.,* 2004; Palmieri *et al.,* 2005; Lodi *et al.,* 2006; Thompson *et al.,* 2016).

In our laboratory, WB-12 strains harbouring the pathological mutations M114P, A128P, S303M, A106D, R96H, R252G have been fully characterized (**Table 1**). All these mutant strains showed a pronounced OXPHOS defect, with marked reduction of the cytochrome content, in agreement with a significative decrease in oxygen consumption. Furthermore, the insertion of the mutated alleles in presence of the wild-type *AAC2* allele demonstrated that M114P, A128P, S303M, R96H, R252G

mutations are dominant, while A137D is recessive in yeast as in human (Fontanesi *et al.,* 2004; Palmieri *et al.,* 2005; Thompson *et al.,* 2016).

Clinical phenotypes	**Pathological mutations in *ANT1***	**Corresponding residues in *AAC2***
adPEO	L98P	M114P
adPEO	A114P	A128P
adPEO	V289M	S303M
severe early-onset disease	R80H	R96H
severe early-onset disease	R235G	R252G
Myopathy, cardiomyopathy	A123D	A137D

Table 1. Pathological variants in h*ANT1*, the related pathological phenotypes and their equivalent substitutions in y*AAC2*.

As mentioned before, a clinical phenotype associated with ANT1 mutations is the presence of multiple deletions of the mitochondrial DNA in the affected tissues, indicating an alteration of mtDNA maintenance. Analysis of the mtDNA instability cannot be carried out in the yeast strain WB-12 because it is *petite-negative*, i.e. it is not viable in the presence of multiple deletions or complete loss of mtDNA. In contrast, the heteroallelic *AAC2/aac2* strain, containing both the wild type *AAC2* and the mutant *aac2* copy, is *petite-positive*, enabling this analysis. It was thus demonstrated that even in yeast the pathological mutations lead to mtDNA deletions as in patients (Fontanesi *et al.,* 2004; Dallabona *et al.,* 2017).

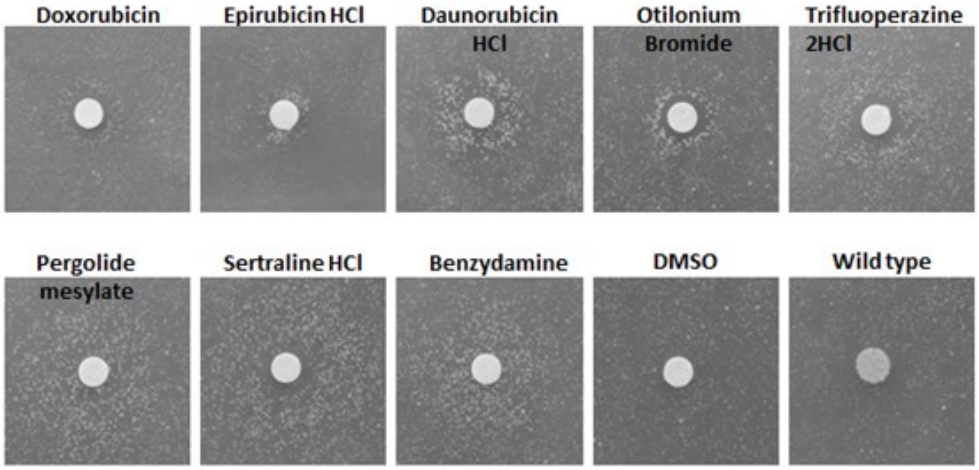
1.8.1 *S. cerevisiae* as a model for the identification of potentially therapeutic molecules for adPEO caused by mutation in the ANT1 gene

In order to identify potentially therapeutic drugs for the mitochondrial disorder autosomal dominant external progressive ophthalmoplegia (adPEO) due to ANT1 mutations, in our laboratory a yeast-based pharmacological screening was performed by “drug drop test” described above.

The screening of 1018 chemical molecules, belonging to the Selleck "FDA approved Drug Library", consisting of molecules already used in the treatment of several human diseases, was carried out using the *S. cerevisiae* WB-12 strain, deleted of the *AAC1* and *AAC2* genes and transformed with pFL38 centromeric vector carrying the mutant allele $aac2^{M114P}$ (WB-12/$aac2^{M114P}$), equivalent to the human pathological mutation leading to the aminoacidic substitution L98P in the Ant1 protein. Oxidative growth of this mutant strain is severely affected, but not completely abolished and therefore this mutant is ideal for the identification of rescuing molecules.

Secondary screening led to the identification of 8 active compounds: Doxorubicin, Epirubicin, Daunorubicin, Otilonium Bromide, Trifluoperazione, Pergolide mesylate, Sertraline and Benzydamina (**Figure 1.7**). Three of them (Doxorubicin, Epirubicin and Daunorubicin), are chemotherapy medications used to treat cancer and were excluded for further analyses. In fact, due to their high toxicity and side effects, their use is unachievable to treat mitochondrial diseases that require long-term administration.

A



B

Drug	OXPHOS rescue	Growth intensity
Doxorubicin	Inibition+growth	+
Epirubicin HCl	Growth	+
Daunorubicin HCl	Growth	++
Otilonium Bromide	Inibition+growth	+
Trifluoperazine 2HCl	Inibition+growth	++
Pergolide mesylate	Growth	+++
Sertraline HCl	Inibition+growth	+++
Benzydamine HCl	Growth	+++

Figure 1.7 A) Rescue of the oxidative growth defect of the WB-12/*aac2*^{M114P} mutant strain induced by active compounds. Wild-type strain WB-12/*AAC2* was used as a positive growth control. DMSO, the solvent in which molecules are dissolved, was used as a negative control. **B)** Rescue effect of the active compounds identified on the oxidative growth defect of the WB-12/*aac2*^{M114P} mutant strain. +++ Strong effect; ++ Medium effect; + Mild effect.

To better characterize the potentially therapeutic application of the molecules identified using the WB-12/*aac2*^{M114P} strain, the beneficial effect of the 5 molecules was subsequently tested also on other *aac2* mutants. Treatment with molecules allowed rescue of oxidative growth in the case of other yeast models for adPEO disease *aac2*^{A128P} and *aac2*^{S303M}, while no improvement was observed in strains carrying the empty pFL38 (null mutant strain) or mutant alleles *aac2*^{A106D}, *aac2*^{R96H} and

aac2^{R252G}, equivalent to the human A123D, R80H and R235G mutations respectively, associated with a more severe clinical phenotype (**Figure 1.8**).

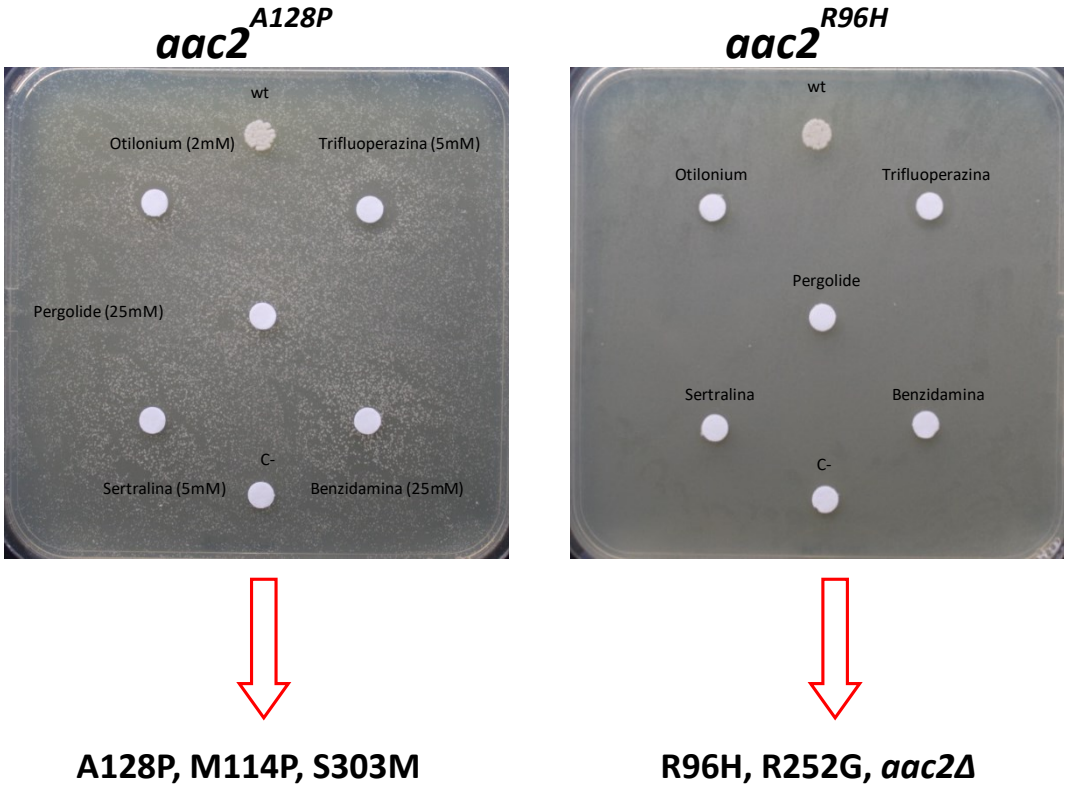


Figura 1.8 Rescue of the oxidative growth defect induced by active compounds on the WB-12/*aac2*^{A128P} mutant strain (on the left). Similar result was obtained with the WB-12/*aac2*^{M114P} and WB-12/*aac2*^{S303M} strains. The same drugs were not effective on the WB-12/*aac2*^{R96H} strain (on the right), nor on the WB-12/*aac2*^{R252G} and on the null *aac2Δ* mutants. Wild-type strain (wt) was used as a positive growth control. DMSO, the solvent in which molecules are dissolved, was used as a negative control (C-).

The fact that the molecules are able to exert their beneficial effect only on leaky mutants and neither on strong/severe mutants nor on the null mutant, on the whole, indicates that the molecules do not act through a bypass, but on the contrary they act only if the Aac2 protein is present and is at least partially functional. The drugs could act directly on the mutant protein (e.g. changing the protein stability or the affected catalytic activity) or indirectly through a compensatory mechanism.

1.9 *S. cerevisiae* as a model for the study of pathological mutations in the MPV17 gene.

The study of the molecular mechanisms underlying the Mitochondrial DNA Depletion Syndrome (MDS) caused by mutations in MPV17, taked advantage of the use of different model organisms, including the yeast *S. cerevisiae*. The functional ortholog of MPV17 in yeast was identified as gene induced by heat stress and so called *SYM1* (*Stress-inducible Yeast MPV17*) by Trott and Morano more than 15 years ago (Trott and Morano, 2004). The protein sequence of yeast Sym1 shares 48%

similarity and 32% identity with human Mpv17 protein and the expression of wild-type human MPV17 gene, but not the mutated one, rescues the growth defect of *S. cerevisiae* lacking *SYM1* (*sym1Δ* strain) thus demonstrating that *SYM1* is a functional orthologous of MPV17 (Trott and Morano, 2004; Spinazzola *et al.,* 2006). Beyond the sequence identity, the Sym1 protein shares the localization in the inner mitochodondrial membrane and similar design of transmembrane domains with Mpv17 protein (Trott and Morano, 2004; Reinhold *et al.,* 2012).

Sym1 was identified as a heat-induced protein required for OXPHOS metabolism under stress conditions such as high temperature (37 °C) and 2% ethanol (Dallabona *et al.,* 2010). Interestingly, it was demonstrated that supplementation of *sym1Δ* mutant with glutamine, glutamate, asparagine, aspartate and overexpression of two genes, *YMC1* and *ODC1*, encoding mitochondrial transporters of TCA intermediates, rescued the metabolic defect of *sym1Δ*, thus suggesting a role of Sym1 in controlling the flux of Krebs' cycle intermediates, e.g. alpha-ketoglutarate and/or oxalacetate, across the IMM (Dallabona *et al.,* 2010). Moreover deficiency of Sym1 causes depletion of glycogen storage that is dependent on gluconeogenesis, which in turn depend on the anaplerotic flux of tricarboxylic acid (TCA) intermediates from mitochondria to the cytosol (Dallabona *et al.,* 2010). Curiously, patients with MPV17 mutations are mostly diagnosed with a severe hypoglycemia (Spinazzola *et al.,* 2006; Mahjoub G. *et al.,* 2019), similar to those of glycogen storage disease type I where both glycogenolysis and gluconeogenesis are impaired (Parini *et al.,* 2009).

Moreover, studies performed in our laboratory showed that, *in vivo*, Sym1 is part of a high molecular–weight complex the composition of which is, however, unknown (Dallabona *et al.,* 2010). As for human Mpv17 (Antonenkov *et al.,* 2015), also for Sym1 it was demonstrated that it forms a pore in the IMM whose diameter is large enough to enable the passage of metabolites whose nature are not yet known (Reinhold *et al.,* 2012).

The high degree of homology between MPV17 and *SYM1* and above all the fact that some pathological mutations in MPV17 concern amino acid residues conserved in the two proteins, made it possible to validate these mutations in yeast and to elucidate their molecular consequences. All the mutations analysed (**Table 2**) resulted in an oxidative growth defect and in a significant increase of *petite* colonies indicative of mtDNA instability (Spinazzola *et al.,* 2006; Gilberti *et al.,* 2018). Therefore, similar to its mammalian ortholog MPV17, Sym1 seems to be essential for mtDNA stability, even if the role of the protein in mitochondrial genome maintenance remains unclear.

Pathological mutations in *MPV17*	**Corresponding residues in *SYM1***
G24W	G24W
R50Q	R51Q
R50W	R51W
P98L	P104L
N166K	N172K
S170F	S176F

Table 2. Pathological mutations in hMPV17 and the corresponding substitutions in y*SYM1*.

Another important result obtained in our laboratory is that all mutations, with exception of G24W (hG24W) that affects protein stability, compromised the formation of the high molecular weight complex of which Sym1/Mpv17 is part, thus suggesting that the origin of MPV17-related disorders is to be searched in capability of Sym1/Mpv17 in participating in a functional high weight molecular complex (Gilberti *et al.,* 2018). Furthermore, given the mitochondria of *sym1* mutants, as well as those of *mpv17−/−* mouse and zebrafish, show large structural alterations, such as flattening or disappearance of mitochondrial cristae, accumulation of electrodense particles, impairment and reduction of mitochondrial respiratory chain, (Viscomi *et al.,* 2009; Dallabona *et al.,* 2010; Martorano *et al.,* 2019) it has been proposed that Mpv17/Sym1 could play a role in preserving the integrity of the IMM. In our laboratory it has been shown that these structural changes are not consequent to the instability of the mitochondrial genome as they arise in growth conditions in which no instability of the mtDNA is observed (Dallabona *et al.,* 2010). Also in *mpv17−/−* zebrafish, impairment of mitochondrial ultrastructure and RC complexes arise before the reduction of the number of copies of mitochondrial DNA (Martorano *et al.,* 2019). Therefore all together these results suggest that the presence of Sym1/Mpv17 is essential for the correct formation of the high molecular weight complex of which it is part and for preserving mitochondrial structure and functionality and that mitochondrial DNA instability could be subordinated to the loss of the integrity of IMM.

As mentioned before, studies performed in mouse cells, in fibroblasts of patients with mutations in MPV17 and also in *mpv17−/−* zebrafish mutants, suggest that the depletion of mitochondrial DNA may be caused by a reduction in the pool of mitochondrial nucleotides, thus linking the Mpv17 protein to the metabolism of mt dNTPs (Krauss *et al.,* 2013; Dalla Rosa *et al.,* 2016; Martorano L. *et al.,* 2019). Preliminary studies performed in our laboratory on yeast showed deletion of *RNR*

inhibitor, *SML1,* and supplementation of intermediates of dNTPs synthesis (i.e guanosine, adenosine, uridine, orotic acid and cytidine at different concentrations), was able to reduce the frequency of *petite* mutants in the null mutant *sym1Δ* and in *sym1*^{R51W} mutant. This suggests that dNTP pool is defective also in *sym1* yeast mutants (Gilberti, PhD thesis).

Taken together these considerations evidence that the *sym1* yeast mutant perfectly emulates the mitochondrial phenotypic characteristics found in humans and other model organisms, confirming yeast as an excellent, albeit simple, model for MPV17-related MDS.

1.9.1 *S. cerevisiae* as a model for the identification of potentially therapeutic molecules for MDS caused by mutation in the MPV17 gene

In order to identify potentially therapeutic drugs for MDS due to MPV17 mutations, in our laboratory a yeast-based pharmacological screening was performed. The screening of 1018 chemical molecules belonging to the Selleck "FDA approved Drug Library" and of 6 molecules belonging to Prestwick Chemical Library, already identified in our laboratory as beneficial in another yeast model, was carried out using the *S. cerevisiae* BY4741 strain, deleted of the *SYM1* gene and transformed with pFL38 carrying the mutant allele *sym1*^{R51W} (BY4741*Δsym1*/pFL38*sym1*^{R51W}). The *R51W* substitution in yeast is equivalent to the aminoacidic substitution R50W in the Mpv17 human protein. Oxidative growth of this mutant strain is severely affected, but not completely abolished and therefore it is ideal for identification of rescuing molecules (Gilberti, PhD thesis). Secondary screening led to the identification of 12 active compounds: Posaconazole, Haloperidol, Fenticonazole nitrate, Itraconazole, Otilonium bromide, Sertaconazole nitrate, Sertraline HCl, Benzethonium chloride, Domiphen bromide, Alexidine HCl, Thonzonium bromide, Imazalil (**Table 3**).

Drug	Oxphos phenotype rescue	Growth rescue
Posaconazole	Inhibition+growth	+++
Haloperidole	Growth	+++
Fenticonazole nitrate	Growth	+
Itraconazole	Inhibition+growth	+++
Otilonium bromide	Inhibition+growth	+++
Sertaconazole nitrate	Inhibition+growth	+
Sertraline HCl	Inhibition+growth	+
Benzethonium chloride	Inhibition+growth	+++
Domiphen bromide	Inhibition+growth	++
Alexidine HCl	Inhibition+growth	+
Thonzonium bromide	Growth	++
Imazalil	Inhibition+growth	++

Table 3. Rescue effect of the active compounds identified on the oxidative growth defect of the BY4741*Δsym1*/pFL38*sym1*^{R51W}. +++ Strong effect; ++ Medium effect; + Mild effect (from Gilberti, PhD thesis).

Since five molecules (Posaconazole, Fenticonazole nitrate, Itraconazole, Sertaconazole nitrate and Imazalil) belong to the same class of azoles, and they act through the same mechanism, some of them (Itraconazole, Sertaconazole) were excluded from further analyses. To deepen the effects of the active compounds, the beneficial molecules were then tested on mtDNA instability of the *sym1*^{R51W} mutant strain. All the drugs were able to significatively reduce *petite* percentage in the *sym1*^{R51W} mutant strain (Gilberti, PhD thesis).

Furthermore to better understand if the beneficial effect was due to a direct action on the Sym1 protein or bypassing the function of Sym1, it was evaluated whether the molecules were able to rescue even the oxidative growth defect of the null mutant strain (BY4741*sym1Δ*/pFL38).

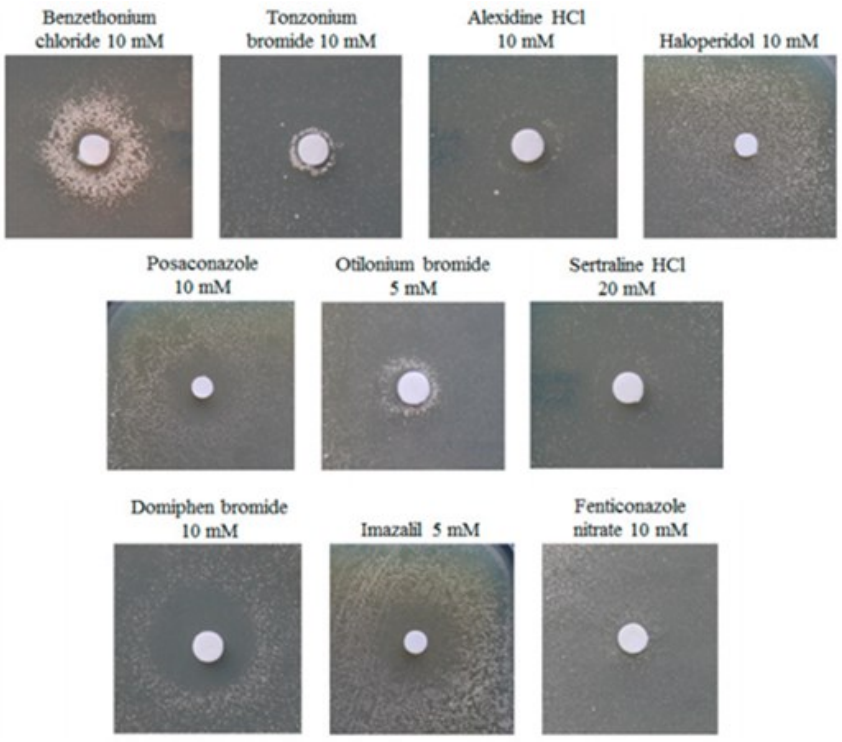


Figura 1.9. Rescue of the oxidative growth defect induced by active compounds on the null mutant *sym1Δ.* (from Gilberti, PhD thesis).

All tested molecules were able to exert their beneficial effect even in the absence of the protein Sym1, indicating that the drugs act through a bypass (**Figure 1.9**).

The function of these molecules and their mechanism of action will be later deepened.

AIM OF THE RESEARCH

The general aim of my thesis was to deepen the effects of beneficial molecules previously identified through the drug drop test in yeast models of mitochondrial diseases associated with mutations in human genes ANT1 and MPV17.

The first aim was to evaluate the effect of the identified beneficial drugs on several phenotypes of the yeast model of ANT1 human mutations that cause Autosomal Dominant Progressive External Ophthalmoplegia (adPEO). The purpose of these experiments was to try to identify the pathway targeted by these molecules, aspect which may provide useful information to understand the molecular basis underlying the disease.

The second aim of my thesis was to get insight into the molecular mechanisms that could cause mitochondrial DNA instability in the yeast model of MPV17 human mutations that cause an hepatocerebral form of Mitochondrial DNA Depletion Syndrome (MDS). Experiments carried out in mice, zebrafish, and fibroblasts suggest that depletion of mtDNA is due to a deficiency of the mitochondrial nucleotide pool, thus linking the Mpv17 protein to the metabolism of mt dNTPs. To evaluate this hypothesis in yeast, I had set up, in yeast, an enzymatic assay that enables the quantification of low concentrations of dNTPs such those found in mitochondria and I applied this method on yeast model for hepatocerebral MDS. Moreover, having demonstrated a remarkable decrease of mtDNA dNTP pool, I investigated if the beneficial molecules, previously identified through the drug drop test, could improve mtDNA stability increasing the dNTP pool. The fact that all identified beneficial molecules increased both the mtDNA stability and the dNTP pool suggests that the decrease of dNTPs is the cause of mitochondrial DNA instability.

The third aim of my thesis was, then, to test the beneficial molecules identified for this MDS on other MDS yeast models available in our laboratory in order to extend their potential use to other patients affected by MDS.

2. Results and Discussion-Section I

2.1 Analyses of the identified drugs on WB-12/*aac2*^{M114P} phenotypes

In our laboratory screening of 1018 FDA-approved molecules led to the identification of 8 drugs (Doxorubicin, Epirubicin, Daunorubicin, Otilonium Bromide, Trifluoperazine 2HCl, Pergolide mesylate, Sertraline HCl and Benzydamine HCl) able to rescue the oxidative growth defect of the haploid strain WB12 harboring the allele *aac2* carrying the mutation M114P (WB-12/*aac2*^{M114P}) equivalent to the human mutation L98P responsible for adPEO. The drug drop test leads to the identification of beneficial molecules but does not provide information about the molecular pathway through which these molecules act. Therefore, to deepen the effect of the identified drugs, I evaluated their impact on other phenotypes of the WB-12/*aac2*^{M114P} mutant to assess whether they were able to restore all the defects or only some of them and to what extent.

Three of the identified molecules, Doxorubicin, Epirubicin and Daunorubicin, were excluded from the analysis because they belong to the class of anticancer drugs and their long-administration, such as that required for patients with mitochondrial diseases, is unachievable due to their toxic and side effects.

2.1.1 Effect of the identified drugs on mitochondrial respiration

The respiratory analysis of the WB-12/*aac2*^{M114P} mutant strain revealed that M114P mutation caused a significant decrease of the respiratory activity (Fontanesi *et al.,* 2004). Therefore, I wondered if the identified drugs were able to increase the respiratory rate of the *aac2*^{M114P} mutant strain.

Firstly, it was necessary to identify, for each molecule, the minimum growth inhibiting concentration (MIC) i.e. the lowest concentration of the compound that completely inhibits the growth. This analysis was carried out as described in Material and Methods.

The analysis of respiratory activity was performed in the strains WB-12/pFL38*AAC2* and WB-12/pFL38*aac2*^{M114P} grown at 28°C in YP supplemented with 0,6% glucose. The molecules were added in the medium at different concentrations starting from the previously determined sub-MIC. All molecules were dissolved in dimethyl sulfoxide (DMSO). To ensure that an alteration in respiratory rate in the cells treated with the molecules was not due to the compound vehicle, WB-12/*aac2*^{M114P} cells were also treated with DMSO. The oxygen uptake rate was measured after total exhaustion of glucose present in the medium to avoid glucose-mediated catabolic repression and thus promoting respiratory metabolism. **The Figure 2.1** shows the results obtained.

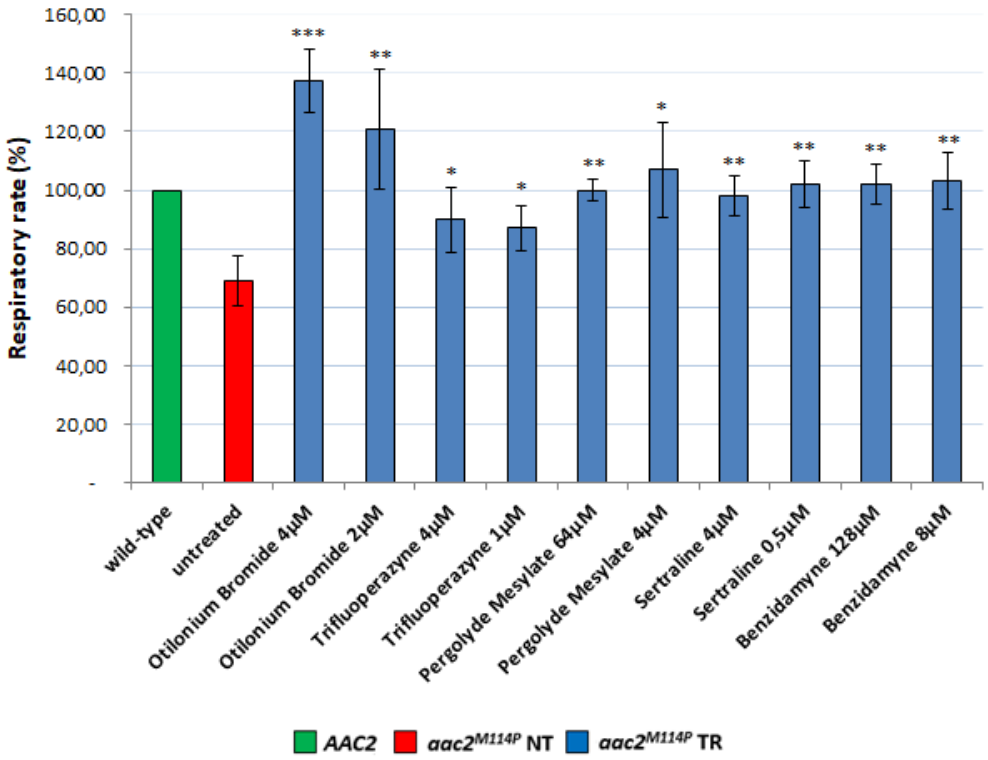


Figure 2.1. Oxygen consumption rate of wild type WB-12/*AAC2* (green bar) and WB-12/*aac2*^{M114P} mutant strains with (blue bars) or without (red bar) the supplementation of active compounds. Cells were grown in YP medium supplemented with 0.6% glucose and respiratory rate was measured after the total exhaustion of glucose. Values were normalized to the wild type strain and represented as the mean of at least four values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

Supplementation with all molecules led to a marked increase of the respiratory rate, bringing the percentage of oxygen consumption from about 70% to the level of the wild-type strain. Otilonium Bromide resulted the most active compound leading to an increase that exceeded the wild type respiratory activity. The highest and lowest effective concentrations are reported for each molecule. Pergolide mesylate, Sertraline HCl and Benzydamine HCl were effective in a wide range of concentrations. Instead the efficacy of Trifluoperazine and especially of Otilonium bromide were limited to a smaller spectrum of concentrations. These findings perfectly fitted with results obtained with the drug drop test (**Figure 2.2**). In the case of Pergolide mesylate, Sertraline HCl and Benzydamine HCl no inhibition halo was observed near the disk and the growth halo remained observable in a wide concentration range, thus suggesting that these molecules have a rescuing effect from the highest tested concentration to much lower concentrations. Conversely, around the disk spotted with Otilonium Bromide and, to a lesser extent, with Trifluoperazine 2HCl it was possible to observe a halo of inhibition followed by a thin crown of growth, thus indicating that the phenotypic rescue effect exerted by these molecules is limited to very specific concentrations.

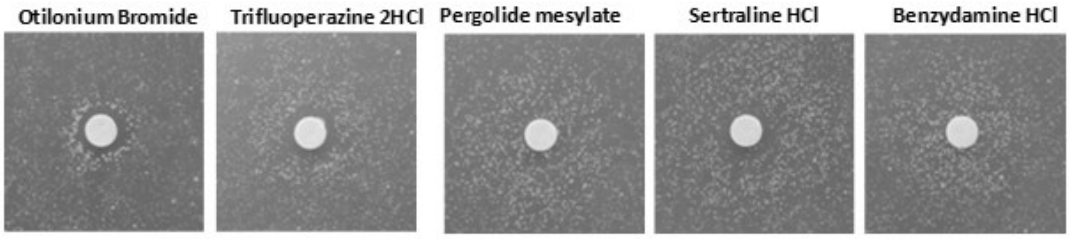


Figure 2.2. Rescue of the oxidative growth defect of the *aac2*^{M114P} mutant strain induced by active compounds.

To investigate whether the increase of oxygen consumption rate was due to a beneficial effect on respiration or to the decoupling action of the molecule itself, I repeated the experiment by adding the mitochondrial potential uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in the oxygraph chamber, thus obtaining the maximal respiration rate (non-coupled state). Addition with CCCP resulted in 2-fold increase of respiratory activity of both wild type *AAC2* and *aac2*^{M114P} mutant (**Figure 2.3**). In contrast, no increase was observed in the WB-12 strain carrying the empty plasmid (null mutant) indicating that the respiration activity observed in this strain is not coupled to ATP production, thus explaining why the strain is unable to grow on respirable carbon sources. The addition of the CCCP led to a significant increase in oxygen consumption for all the tested drugs with the exception of Otilonium Bromide (**Figure 2.3**), thus suggesting that this drug partially dissipates the mitochondrial membrane potential.

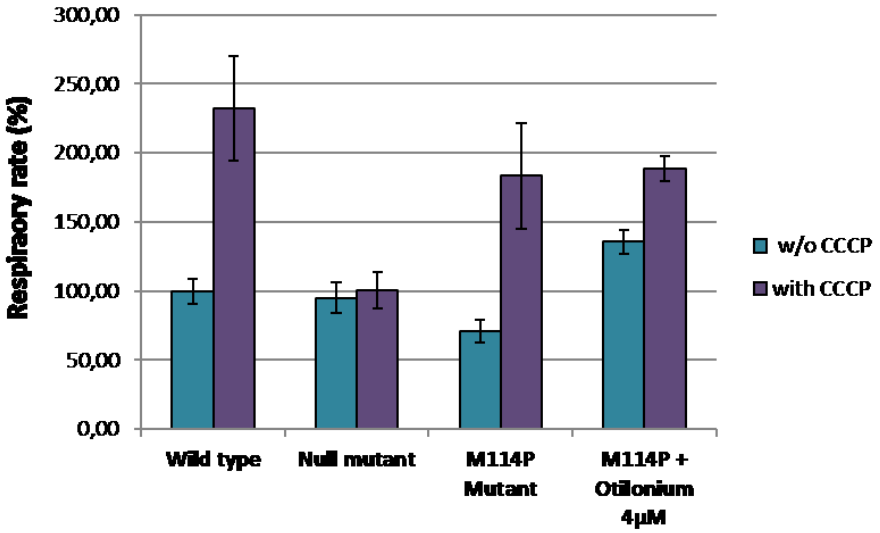


Figure 2.3. Oxygen consumption rate of wild type (WB-12/*AAC2*), null mutant (WB-12/pFL38), M114P mutant (WB-12/*aac2*^{M114P}) untreated and treated with Otilonium Bromide, under normal (blue bars) and uncoupled state conditions using the mitochondrial uncoupler CCCP (violet bars).

2.1.2 Effect of the identified drugs on ROS overproduction

A possible consequence of damage or stalling of the respiratory chain could be the increasing production of reactive oxygen species (ROS). In particular, mutations in the Aac2 protein can compromise ADP/ATP translocation activity leading to a diminished intramitochondrial ADP level, that can in turn inhibit the ATP synthase, thereby blocking the proton flux through the Inner Mitochondrial Membrane (IMM) via the ATP synthase. Consequently, the electrochemical gradient may not be dissipated, thus stalling electron flow with a consequent accumulation of electrons that can be transferred directly to oxygen leading to increased ROS production (Palmieri *et al.,* 2005). Interestingly, it has been demonstrated a marked increase of mitochondrial H_2O_2 production in mitochondria of Ant1-deficient mice (Esposito *et al.,* 1999).

To test this hypothesis in yeast, I measured ROS production in the WB-12/*aac2*^{M114P} mutant strain, compared to the WB-12/*AAC2* wild type strain, by a cytofluorimetric analyses using the fluorescent ROS indicator dihydrorhodamine 123 (DHR123), an uncharged non-fluorescent probe derivative of rhodamine 123 (R123). DHR123 passively diffuses through cell membrane, and is oxidized by ROS (mainly by H_2O_2) (Gomez *et al.,* 2014) to form R123. R123 is a cationic probe that can store and localize into the mitochondria exhibiting green fluorescence (Kiani-Esfahani *et al.,* 2012). To set up the optimal conditions for quantification of ROS by DHR123 (concentration and the treatment time of the cells with DHR123), I used a panthotenate kinase mutant (*cab1*^{N290I}) whose overproduction of ROS had been previously demonstrated in our laboratory using Dichlorofluorescein (DCFH-DA) and whose fluorescence was quantified by Fluorescence spectroscopy (Ceccatelli Berti, PhD thesis). **Figure 2.4** shows the fluorescence intensity in a log unit of *CAB1* wild type strain and of *cab1*^{N290I} mutant strain incubated for 2 hours (optimal treatment time) in the dark with different concentration of DHR123.

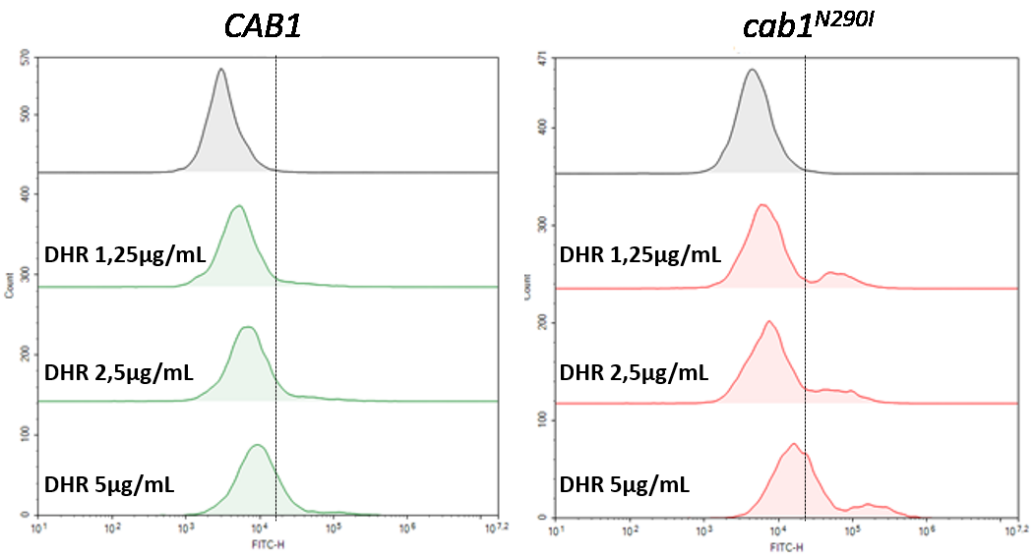


Figure 1.4. Flow cytometric curves of wild-type *CAB1* (on the left) and *cab1*^{N290I} mutant strain (on the right) stained with different DHR123 concentrations for 2 hours in the dark. Each curve represents distribution of the measured events (counts) according to their fluorescence intensity expressed in a log unit. Grey curve represents auto-basal fluorescence.

A sample without DHR (grey curve) was prepared for both strains to be used as a reference to set the threshold index and thus delimiting an auto-basal fluorescence area (to the left of the dotted line) and a R123 positive fluorescence area (to the right of the dotted line). All tested concentrations allowed to discriminate the higher production of ROS in the mutant strain compared to the wild-type. However, increasing DHR123 concentration increased the percentage of R123 positive cells in both wild-type and mutant strains. For this reason the lowest concentration of DHR123 (1,25µg/ml) was chosen for subsequent analyses.

Once set up, I applied this method on the *aac2*^{M114P} mutant strain. The analysis was carried out in WB-12/pFL38*AAC2* and WB-12/pFL38*aac2*^{M114P} grown at 28°C in YP supplemented with 0,6% glucose. Cells were harvested after the total exhaustion of glucose present in the medium (after about 18 hours of growth) and incubated for 2 hours in the dark with 1,25 µg/ml of DHR123. Then cells were harvested and the fluorescence was quantified by flow cytometry. The *aac2*^{M114P} mutant strain displayed a significant increment (about 2-fold) of R123 positive cells compared to wild-type strain thus demonstrating an increase of ROS production due to M114P mutation (**Figure 2.5**)

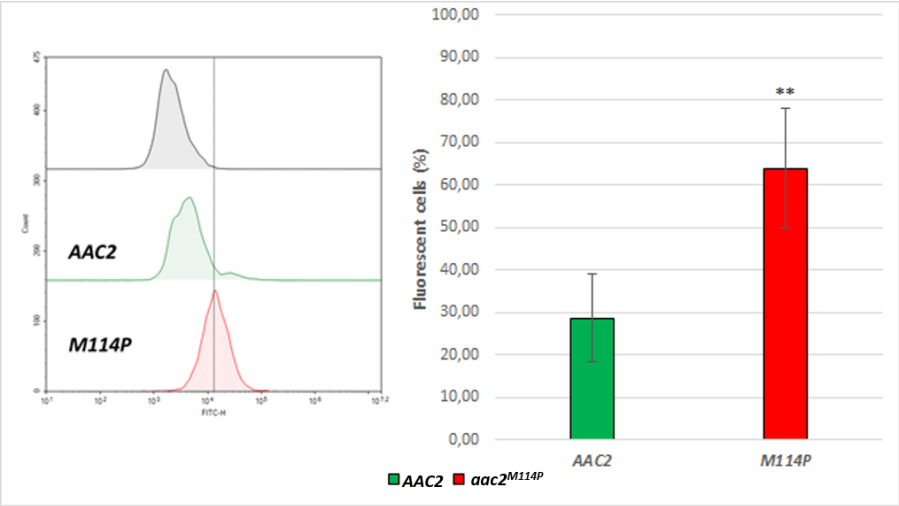


Figure 2.5. Determination of ROS production in wild-type WB-12/*AAC2* (green curve/bar) and WB-12/*aac2*^{M114P} mutant (red curve/bar) strains stained with 1,25 µg/ml of DHR123 for 2 hours in the dark. ROS production was determined as the percentage of fluorescent cells (PFC) corresponding to the cells producing an increase in fluorescence intensity of at least one logarithmic unit. All values are means of five independent experiments ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

To investigate whether the treatment with the molecules could reduce the ROS increase, I repeated the cytofluorimetric analysis after incubation of the mutant strain with two different concentrations of each drug and, as a positive control, with the well-known antioxidant agent N-acetil-cysteine. As expected, addition with N-acetil-cysteine led to a strong dosage-dependent reduction of ROS. Conversely none of the molecules decreased the percentage of fluorescent cells, indicating that they do not act as antioxidant (**Figure 2.6**).

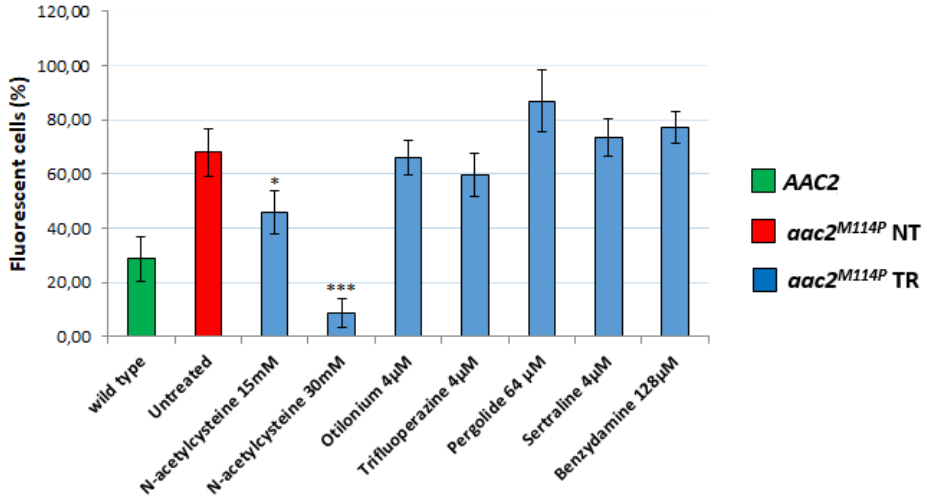


Figure 2.6. ROS generation in wild type WB-12/*AAC2* (green bar) and WB-12/*aac2*^{M114P} mutant strains with (blue bars) or without (red bar) the supplementation of active compounds. In addition to the showed concentrations, halved concentrations were also tested and similar results were obtained. The well-known antioxidant agent N-acetil-cysteine, was used at two different concentrations (15mM and 30mM) as a positive control. All values are means of three independent experiments ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

2.1.3 Effect of the identified drugs on Mitochondrial Membrane Potential

In yeast cells the mitochondrial membrane potential (MMP) can be generated in two different ways. In respiring cells it is generated by the activity of respiratory complexes that pump protons from the mitochondrial matrix to the intermembrane space thus generating an electrochemical proton gradient across the Inner Mitochondrial Membrane. When MMP cannot be built up by the respiratory complexes (growth in anerobosis, presence of glucose that represses the expression of respiratory complexes or in cells devoid of mtDNA), the Aac2 protein play a critical role in maintaining the MMP. Indeed, in these conditions Aac2 works in the reverse mode: the cytosolic ATP produced from glycolysis is imported into the mitochondrial matrix in exchange for mitochondrial ADP and so the import of four negative charges in exchange for three, generates MMP (Traba *et al.,* 2009). I measured MMP in the WB-12/*aac2*^{M114P} mutant, where both mitochondrial respiration and ADP/ATP transport are affected (Fontanesi *et al.,*2004), using the lipophilic green-fluorescent dye 3,3P-dihexyloxacarbocyanine iodide ($DiOC_6$) whose fluorescence was quantified by flow cytometry. Used at low concentrations (≤ 0.1µM) $DiOC_6$ is selective for mitochondria of living yeast cells (Koning *et al.,* 1993) and its uptake by mitochondria, and consequently its fluorescence emission, is modulated by the MMP (Miceli *et al.,* 2012). This analysis was performed on WB-12/pFL38*AAC2* and WB-12/pFL38*aac2*^{M114P} grown in the same conditions previously described for ROS quantification. Cells were harvested after the total exhaustion of glucose present in the medium (after about 18 hours) and incubated for 30 minutes with 0,05 µM of $DiOC_6$ and then fluorescence was quantified by flow cytometry. As a control, I measured the MMP also in cells devoid of mtDNA (*rho*^{0} cells) characterized by a severe reduction of MMP compared to *rho*$^{+}$ cells (**Figure 2.7**).

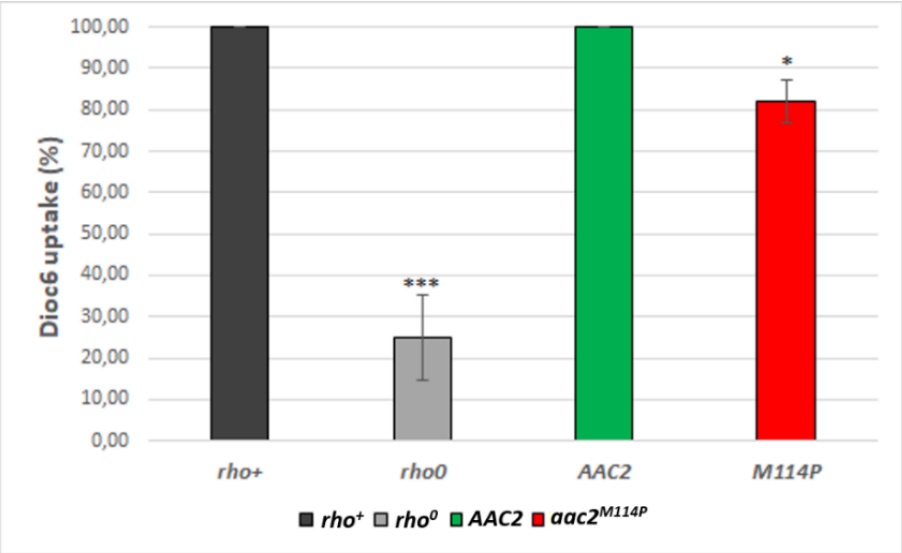


Figure 2.7. Mitochondrial membrane potential (MMP) of wild type WB-12/*AAC2* (green bar) and WB-12/*aac2*^{M114P} mutant (red bar). MMP of *rho*$^{+}$ (black bar) and *rho*^{0} (grey bar) cells was measured as a control. MMP was measured by the uptake of the fluorescent dye $DiOC_6$. Values were normalized to the wild type strain values and represented as the mean of three independent experiments ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

In the *aac2*^{M114P} mutant strain, the uptake of the fluorescent probe $DiOC_6$ was reduced by about 20% compared to the wild-type isogenic strain indicating that the mutation has a weak negative impact on the MMP. A more marked depolarization was observed by Chen (2002) in a strain carrying another adPEO-associated mutation (*aac2*^{A128P}) but in that case the analysis was performed in a strain over-expressing the mutated allele, and therefore under not physiological conditions.

To investigate whether the treatment with the molecules could restore the MMP of the mutant strain, I repeated the cytofluorimetric analysis after addition of the molecules to the culture medium. None of the drugs was able to restore MMP of the mutant strain. Supplementation with Otilonium Bromide even led to a drastic reduction of $DIOC_6$ uptake, indicating a depolarizing activity of this drug (**Figure 2.8**). This data is in agreement with the hypothesized uncoupling effect induced by Otilonium bromide.

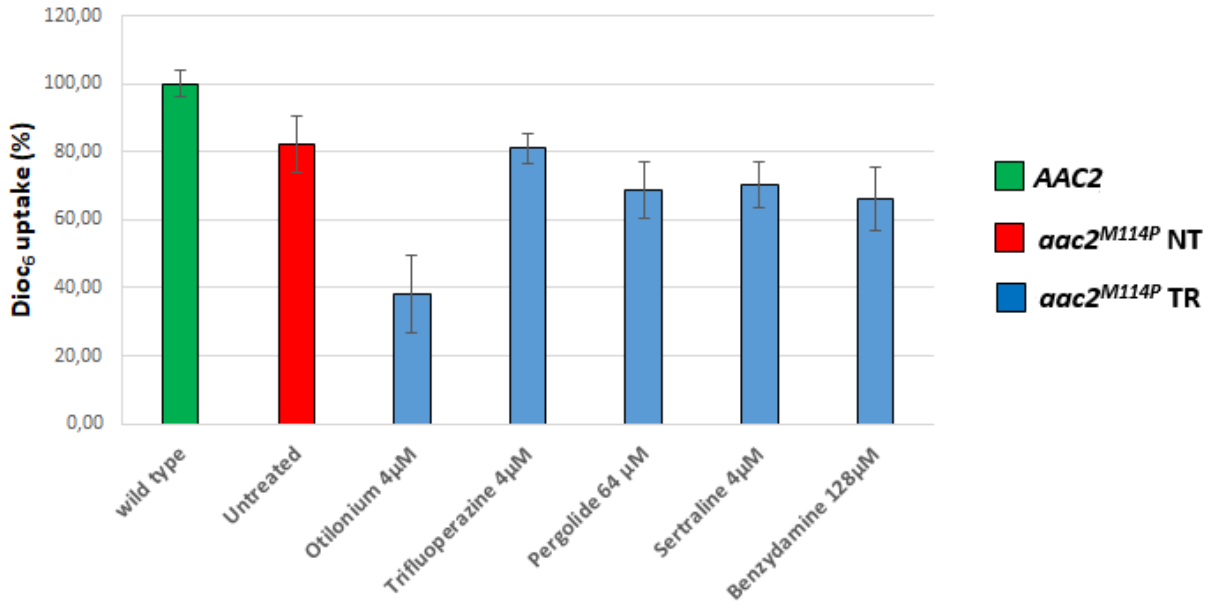


Figure 2.8. Mitochondrial membrane potential (MMP) of wild type WB-12/*AAC2* (green bar) and WB-12/*aac2*^{M114P} mutant strains with (blue bars) or without (red bar) the supplementation of active compounds. MMP was measured by the uptake of the fluorescent dye $DiOC_6$. Values were normalized to the wild type strain values and represented as the mean of three independent experiments ± SD.

Why is a mitochondrial oxidative phosphorylation uncoupler able to rescue the OXPHOS growth defect of *aac2*^{M114P} mutant strain? As said before, mutations in the Aac2 protein can compromise ADP/ATP translocation activity leading to a decreased intramitochondrial ADP level, that can in turn inhibit the ATP synthase thereby blocking the proton flux through the IMM via the ATP synthase. As a consequence the electrochemical gradient is not dissipated and is driven to a maximum level. The excess of electrons that accumulates on respiratory complexes can be passed directly to O_2 enhancing the ROS production. In this background, a molecule that dissipates partially the electrochemical gradient could protect the cell from oxidative stress. According to this, it has been demonstrated that mice with longer lifespan have a greater mitochondrial uncoupling (Speakman *et al.,* 2004), supporting the 'uncoupling to survive' hypothesis (Brand, 2000). Therefore, the beneficial effect of Otilonium Bromide could be due to its ability to induce proton loss through the IMM thus decreasing the electrochemical gradient and protecting the cell from oxidative damage.

2.2 Analyses of the identified drugs on heteroallelic *AAC2*/*aac2*^{M114P}

Heteroallelic strain, containing both the wild type copy of the *AAC2* gene and the mutant *aac2*^{M114P} copy, represents the correct model of the adPEO human condition, since the M114P mutation (as well as other mutations associated with adPEO) is dominant in humans. The heteroallelic *AAC2/aac2*^{M114P} strain, previously constructed in our laboratory, is characterized by a significant

reduction of the cytochrome content and a consequent decrease of the respiratory activity indicating that even in yeast the M114P mutation behaves as dominant for these phenotypes (Fontanesi *et al.,* 2004). Moreover, it was observed that the introduction of the M114P mutation in one of the two *AAC2* alleles, resulted in a 4-fold increase of *petite* mutants compared to homoallelic strain (*AAC2*/*AAC2*), thus demonstrating that this mutation led to an alteration of mtDNA maintenance, a typical feature of patients affected by adPEO.

Here I evaluated the effect of the identified drugs both on the respiratory phenotype and on the mtDNA stability of the *AAC2*/*aac2*^{M114P} heteroallelic strain.

2.2.1 Effect of the identified drugs on AAC2/*aac2*^{M114P} respiration activity

Before describing the experiments carried out in the heteroallelic strain it is necessary to make some considerations. Analyses previously performed in the haploid strain WB-12 carrying the mutant allele *aac2*^{M114P} revealed that growth in rich medium (YP) was the optimal culture condition to test the effect of the molecules. In fact, in synthetic medium (SC) the *rescue* effect was barely visible and only Otilonium Bromide and Sertraline HCl were able to determine an evident *rescue* of the respiratory activity of the mutant *aac2*^{M114P}. This different efficiency of the molecules could be explained by a minor biodisponibility of the drugs in SC saline medium or by a minor uptake of drugs in SC medium due to a different composition of the cellular wall. On the other hand, the heteroallelic strain *AAC2*/*aac2*^{M114P} displayed a respiratory defect detectable only in SC medium at 37°C. In view of these considerations, the analysis of respiratory activity was performed in homoallelic *AAC2*/*AAC2* and heteroallelic *AAC2*/*aac2*^{M114P} strains grown at 37°C in SC medium supplemented with Otilonium Bromide and Sertraline HCl, i.e the only two effective drugs in SC medium, at different sub-MIC concentrations. Treatment with both molecules brought the oxygen consumption rate to the level of wild-type strain (**Figure 2.9**).

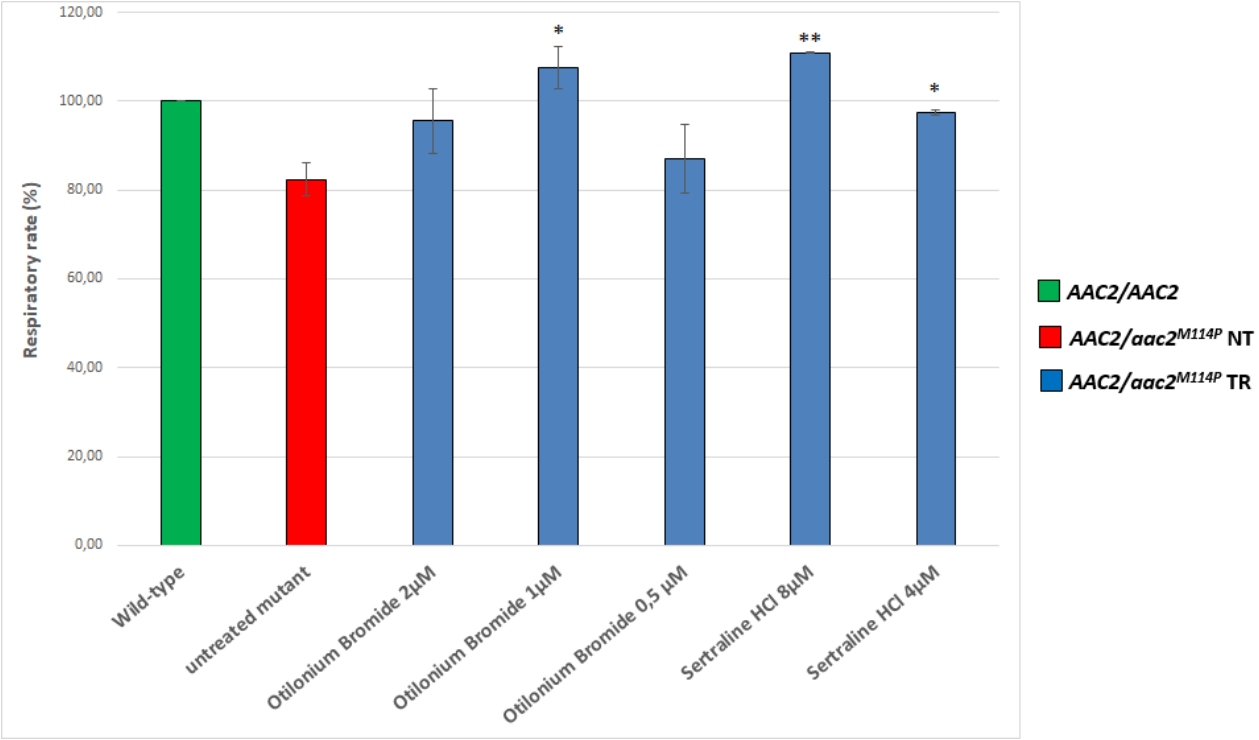


Figure 2.9. Oxygen consumption rate of homoallelic *AAC2/AAC2* (green bar) and heteroallelic *AAC2/aac2*^{M114P} mutant strains with (blue bars) or without (red bar) the supplementation of active compounds. Cells were grown in SC medium supplemented with 0.6% glucose and respiratory rate was measured after the total exhaustion of glucose. Values were normalized to the wild type strain and represented as the mean of at least four values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

2.2.2 Effect of the identified drugs on AAC2/*aac2*^{M114P} mtDNA instability

Patients affected by adPEO due to ANT1 mutations present multiple deletions of mtDNA in affected tissues, indicating an alteration of mtDNA maintenance. In yeast it is possible to analyse whether a mutation can affect the structural integrity of the mtDNA by determining the frequency of *petite* colonies. The *petite* mutants, arising spontaneously after large deletions or loss of mtDNA, are respiratory-deficient (RD), so they are not able to grow on respiratory carbon sources, but they are viable and can grow on fermentable source (such us glucose). In the haploid strain it was not possible to test the effect of the M114P mutation on the stability of mtDNA because *aac2*^{M114P} mutant is *petite negative*, i.e. it is not able to survive with abnormalities of mitochondrial genome (deletions or total loss). Conversely, the heteroallelic *AAC2/aac2*^{M114P} strain is *petite-positive*, allowing this analysis. As said before, in *AAC2/aac2*^{M114P} strain the frequency of *petite* was increased by about 4-fold compared to homoallelic wild-type strain.

Here I investigated whether the addition of the molecules could lead to reduction of mtDNA instability. This analysis was performed in *AAC2/AAC2* strain and *AAC2/aac2*^{M114P} grown at 28°C for 48 hours in YP medium supplemented with 2% glucose, thus allowing even OXPHOS-defective cells

to proliferate. The molecules were added in the medium at different sub-MIC concentrations. **Figure 2.10** shows the results obtained.

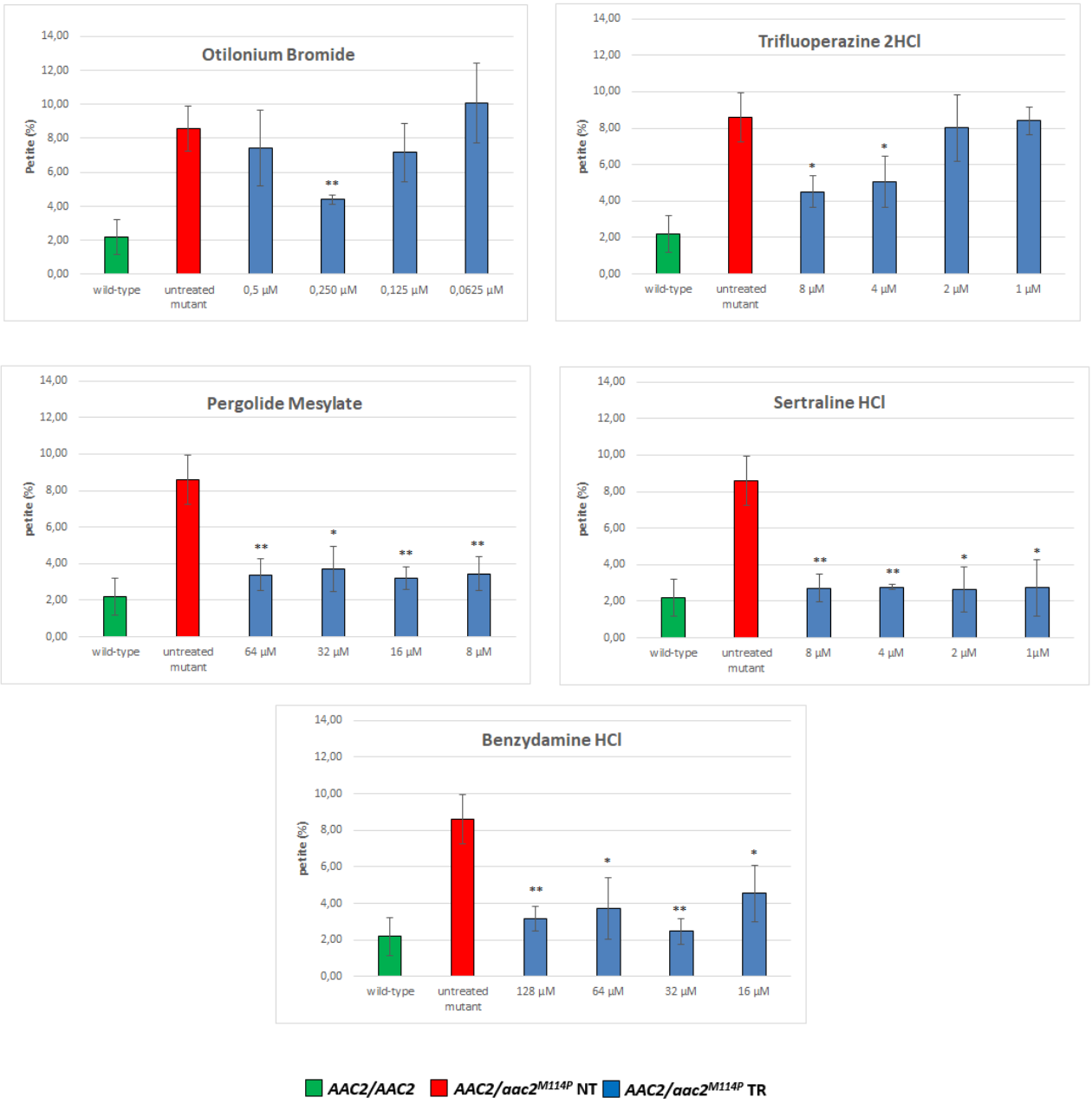


Figure 2.10. Determination of *petite* frequency of homoallelic (*AAC2/AAC2*) strain (green bar), and heteroallelic (*AAC2/aac2*^{M114P}) mutant strains with (blue bars) or without (red bar) the supplementation of active compounds. For each molecule different concentrations were tested. More than 4000 colonies for each strain were scored. Data are represented as the mean of at least three values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

All the drugs were able to significatively reduce the *petite* percentage of the heteroallelic *AAC2/aac2*^{M114P} strain. In particular, the *petite* frequency was reduced by about 50% after

supplementation of Otilonium Bromide and Trifluoperazine 2HCl and by about 70% after addition of Sertraline HCl, Pergolide Mesylate and Benzydamine HCl, thus resulting the most active compounds.

A possible decrease in *petite* mutants after treatment with molecules could derive from an effective reduction in mitochondrial mutability but also from a selective toxicity of molecules for RD cells. In this latter case, the reduction in the frequency of *petite* would not be ascribable to a rescue operated by the treatment with the molecules. Therefore, to exclude that the beneficial effect observed was due to a selective induction of the *petite* mortality, a fitness test was performed by growing together *rho*$^{+}$ and *rho*^{0} cells, in an equal amount, in presence or in absence of the molecules to test. After 24 hours of growth cells were plated to determine the ratio of respiratory sufficient and respiratory deficient colonies in presence and in absence of molecules. The fitness test was performed starting from the highest beneficial concentration. In the case of Otilonium Bromide it was performed by testing only 0,250 µM since it was the only one able to decrease the percentage of *petite*. As shown in **Figure 2.11,** for all drugs tested, the frequency of *petite* was similar between treated and untreated strain, thus indicating that the beneficial effect observed should be ascribed to the diminished onset of *petite* cells and so to a real improvement of mtDNA stability. In the case of Trifluoperazine and Benzydamine, the highest beneficial concentrations, 8 µM and 128-64 µM respectively are toxic for *petite* mutants (data not shown). However, at lower concentrations (Trifluoperazine 4 µM and Benzydamine 32 µM) the toxic effect was no observable thus indicating that for these concentrations the reduction of *petite* frequency should be attributed to a positive effect on mtDNA stability.

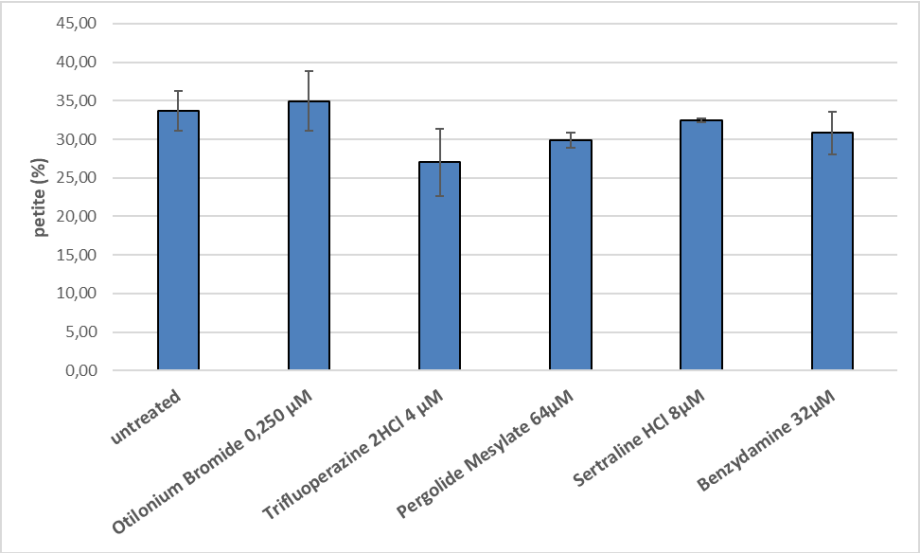


Figure 2.11. *Petite* frequency obtained by competition fitness test between *rho*$^{+}$ e *rho*^{0} yeast strains in presence or in absence of the active compounds.

Altogether these results showed that all the molecules were able to increase mtDNA stability in *AAC2*/*aac2*^{M114P}. Also in this case the beneficial effect of Otilonium Bromide and Trifluoperazine 2HCl was limited to a specific concentration while Sertraline HCl, Pergolide Mesylate and Benzydamine were effective in a wide range of concentrations.

2.3 Discussion

The high degree of conservation between yeast mitochondrial ADP/ATP carrier Aac2 and human Ant1, made possible the use of *S. cerevisiae* as a model to mimic human ANT1 mutations. Moreover in our laboratory, yeast has been exploited as a suitable tool to identify potentially therapeutic drugs for adPEO due to ANT1 mutations. Through the use of a mutant carrying an adPEO-associated mutation (*aac2*^{M114P}) 5 potentially therapeutic molecules were identified that were able to rescue the OXPHOS growth defect of *aac2*^{M114P} mutant strain. Interestingly these drugs allowed even the rescue of oxidative growth of other yeast models for adPEO disease (*aac2*^{A128P} and *aac2*^{S303M}). Conversely no improvement was observed in strains carrying the empty pFL38 (null mutant strain) or mutant alleles correspondent to human mutations associated with a sever clinical phenotype (*aac2*^{A106D}, *aac2*^{R96H} and *aac2*^{R252G}), suggesting that these compounds act only if the Aac2 protein is present and is at least partially functional. Although the drug drop test is an excellent tool to identify rapidly putative beneficial molecules, it does not provide information about the molecular pathway through which these molecules act. The identification of the molecular pathway is an important aspect as it could provide useful information for understanding the molecular mechanisms

underlying the disease and could also be the starting point for research and development of other therapies.

Therefore, here I investigated the effect of the identified drug on several phenotype of the mutant *aac2*^{M114P} (respiratory activity, ROS production, mitochondrial membrane potential, mtDNA instability) to assess whether these molecules were able to restore all the defects or only some of them and to what extent. The first result emerging from these analysis is that all molecules, although with different intensity, were able to increase the respiratory activity and to strongly reduce the mtDNA instability of *aac2*^{M114P}. However, none of them was able to decrease ROS production or change the MMP (except Otilonium Bromide) in the haploid strain. The fact that treatment with all molecules increased mtDNA stability but it did not decrease ROS levels or restore MMP suggests that ROS overproduction and IMM depolarization cannot be the only mechanisms that lead to the onset of mtDNA instability and that the identified molecules probably exert their beneficial effect acting by another mechanism. Otilonium Bromide was the only compound whose supplementation decreased the MMP, thus indicating a depolarizing activity of this drug. As said before, its beneficial effect could be due to its ability to reduce the electrochemical gradient across the IMM thus protecting the cell from oxidative stress. The reason why no reduction of ROS levels was observed after treatment with Otilonium Bromide remains to be investigated.

In human cells, the molecular target of all five compounds is known and to date they are used for treatment of several kind of pathologies: irritable bowel syndrome (Otilonium Bromide), infections (Benzydamine HCl), Parkinson's disease (Pergolide Mesylate), schizophrenia (Trifluoperazine) and depression (Sertraline HCl). The observation that these molecules are able to rescue several phenotypic defects of a yeast mutant suggests that these compounds are also biologically active in yeast. However, yeast does not express the molecular target of these drugs, thus suggesting the presence of one or more unknown secondary drug targets.

DRUGS	Target in mammalian	Target in yeast
Otilonium Bromide	Ca^{2+} channels muscarinic and tachykinin receptors (NK1r and NK2r) blocker	unknown
Trifluoperazine 2HCl	Dopamine D2 receptor antagonist	Calmodulin, membrane lipids
Pergolide Mesylate	Dopamine D1 and D2 receptor agonist	unknown
Sertraline HCl	Serotonin/5-HT transporter	Phospholipid membranes
Benzydamine HCl	Membrane-stabilizing properties	unknown

Table 4. Beneficial molecules identified through drug drop test and their targets in yeast and mammals.

Interestingly, in yeast it has been demonstrated that Sertraline HCl, in the absence of the serotonin/5-HT transporter, targets phospholipid membranes of the organelles of the intracellular vesicle transport system (Rainey *et al.,* 2010). Accordingly, Sertraline HCl is placed in the class of drugs called CADs, cationic amphiphilic drugs that interact with phospholipid membranes (Halliwell, 1997). The most abundant phospholipid in the IMM is cardiolipin, a critical molecule for formation and stability of respiratory supercomplexes (Zhang *et al.,* 2002; Pfeiffer *et al.,* 2003), including those containing Aac2 protein (Claypool *et al.,* 2008). In-fact in yeast devoid of cardiolipin, Aac2 function is compromised (Jiang *et al.,* 2000). Notably, *aac2* mutant alleles cause a marked reduction of cytochrome activity suggesting that, beyond its function on ATP/ADP transport activity, the Aac2 protein could play a 'structural' role contributing to the maintenance of the integrity of respiratory complexes in the IMM (Fontanesi *et al.,* 2004). Based on these considerations, one intriguing hypothesis to explain the beneficial effect of Sertraline is that it may induce a change in the cardiolipin content in the IMM, that at least could favour Aac2 assembly and interaction with other respiratory complexes, resulting in improving the respiratory activity and rescuing the OXPHOS growth defect of the *aac2* mutant. Furthermore, since that cardiolipin plays a critical role in stabilizing the carrier's fold state and its transport-related activity (Senoo *et al.,* 2020), a change in its content could partially compensate the defective transport activity of Aac2 carrier. Analyzing the cardiolipin content and the transport activity in the *AAC2* wild-type and in $aac2^{M114P}$ mutant with and without Sertraline could be a good strategy to test this hypothesis. To this aim, analysis of the transport activity in proteoliposomes reconstituted with extracts from mitochondria of $aac2^{M114P}$ mutant in the presence or in the absence of Sertraline HCl are currently in progress at the Department of Biosciences, Biotechnology and Biopharmaceutics in University of Bari.

Since even Benzydamine display membrane-stabilizing properties (Quane *et al.,* 1998), it is reasonable to speculate that it may act by the same mechanism. Intriguingly the Benzydamine molecular target is not known but the structure similarity between this drug and the neurotransmitter serotonin and also the psychedelic effects caused by high doses of benzydamine, suggest that this compound may be an agonist of the 5H-T transporter (Meringolo, PhD thesis), the same target of Sertraline.

The other two molecules, Trifluoperazine 2HCl and Pergolide Mesylate, are dopamine receptors antagonist and agonist respectively. Although Trifluoperazine is primarily used as an antipsychotic, its antifungal activity makes it useful for treating infection resistant to other antifungals (Eilam *et al.,* 1987; Siavoshi *et al.,* 2012). To date, it remains unclear whether its cytotoxicity is mediated by

inhibition of calmodulin, a Ca^{2+}binding protein known to be engaged in many regulatory processes, or by an effect on cellular/membrane lipids (Sharma *et al.,* 2001). Its antifungal action could explain the toxic effects observed on the *aac2* mutant treated with high concentrations of the molecule. On the other hand at lower and specific concentration, addition with Trifluoperazine rescues the OXPHOS defects and improve mtDNA stability of the *aac2*^{M114P} mutant strain. Interestingly, treatment of the yeast *S. cerevisiae* cells with trifluoperazine has been shown to induce a marked increase in intracellular levels of Ca^{2+} (Eilam *et al.,* 1983), the most important signalling molecule that allows cells to adapt to environmental changes. Notably Ca^{2+} is fundamental for the adenine nucleotide transport exerted by mitochondrial ATP-Mg/Pi carrier *SAL1*, able to rescue the growth defect of cells with an impaired ADP/ATP transport activity (Chen, 2004; Traba *et al.,* 2008; Laco *et al.,* 2010). In light of these considerations, one intriguing hypothesis about the beneficial effect of Trifluoperazine is that it may increase Ca^{2+} levels that in turn could stimulate *SAL1* activity thus recovering the defects of *aac2* ^{M114P} mutant. To assess this hypothesis it might be interesting to measure Ca^{2+} levels and Sal1 transport activity in *aac2* ^{M114P} mutant in the absence and presence of Trifluoperazine.

Certainly, understanding of the molecular mechanism or generally the pathway through which these molecules act requires further studies. Nevertheless, the identification of molecules able to recover the OXPHOS growth defect of yeast models of adPEO, and especially capable of significantly reducing the instability of mtDNA, phenotypic defect of adPEO patients, could be a starting point for the development of pharmacological therapies for the treatment of adPEO disorder due to ANT1 mutations.

3. Results and Discussion-Section II

3.1 Setting up a polymerase assay for the measurement of low quantity of dNTPs

As described in the Introduction, studies performed in mice, zebrafish and fibroblasts characterized by MPV17 deficiency, suggested that depletion of mtDNA is due to a limited mitochondrial dNTP availability thus linking the Mpv17 protein to the metabolism of mt dNTPs (Krauss *et al.,* 2013; Dalla Rosa *et al.,* 2016; Martorano *et al.,* 2019). Preliminary studies performed in our laboratory on yeast have shown that the increase of cytosolic dNTP pool, obtained through different strategies, was able to ameliorate the frequency of *petite* mutants in the null mutant *sym1Δ* (Gilberti, PhD thesis). These data suggested that dNTP pool is defective also in yeast but the real evidence of a decrease of the mitochondrial dNTP pool and, above all, the extent of the reduction can only derive from the direct measurement of dNTPs comparing wild-type and mutant yeast strains. Furthermore, since yeast lacks deoxyribonucleoside kinase activities of *salvage* pathway making the mitochondrial dNTP pool entirely dependent on the direct transport of dNTPs synthesized *de novo* in the cytoplasm by the RNR activity, I decided to extend the analysis also to the whole-cell dNTP pool in order to understand if it could be affected by the *SYM1* deletion. For this purpose I have set up in yeast an enzymatic assay that enables the quantification of low concentrations of dNTPs such those found in mitochondria and allows the simultaneous determination of two pyrimidine or two purine deoxyribonucleoside triphosphates. I have set up this technique starting from the indications reported in Roy *et al* (1999). This method is based on elongation, catalyzed by the Klenow fragment of *Escherichia coli* DNA polymerase I, of 5'-end-labeled synthetic primers annealed to complementary templates (**Table 5**). Thanks to the different combinations of two primers (p13 and p27) and four different templates (tA, tG, tT and tC) of different lengths, it is possible to quantify simultaneously the two pyrimidines and the two purines after a separation on 12,5% polyacrylamide-urea gel, after the elongation reaction in excess of dATP and dTTP respectively. Visualization detected by ChemiDoc MP Imaging System (BioRad s.r.l.) is possible thanks to the use of primers labelled with DY-682 fluorophore.

dNTP quantified	Primer/ template	Oligonucleotide sequence
dTTP	p13 tT	● — 5' TCG CAG CCG TCC A 3' 3' AGC GTC GGC AGG T**AT TAT TAT T** 5'
dCTP	p27 tC	● — 5' GGT AGG GCT ATA CAT CGC AGC CGT CCA 3' 3' CCA TCC CGA TAT GTA GCG TCG GCA GGT **GTT GTT GTT** 5'
dATP	p13 tA	● — 5' TCG CAG CCG TCC A 3' 3' AGC GTC GGC AGG T**AA TAA TAA TAA** 5'
dGTP	p27 tG	● — 5' GGT AGG GCT ATA CAT CGC AGC CGT CCA 3' 3' CCA TCC CGA TAT GTA GCG TCG GCA GGT **CAA CAA CAA** 5'

Table 5. Combinations of primers/templates (p/t) utilized for dNTPs quantification. The red circle represents the DY682 fluorophore bonded to the 5'end of the primers.

All the details related to the primer/template annealing, reaction mix for DNA polymerase assay, samples preparation and gel run, visualization and analysis are reported in the Materials and Methods section.

To determine the amount of nucleotides incorporated into the primer/template dimers (p/t), first of all I assembled reactions with different amounts of dNTPs. **Figure 3.1** shows a typical separation on 12,5% polyacrylamide-urea gel obtained with dNTPs quantity ranging from 0.2 to 15 pmol.

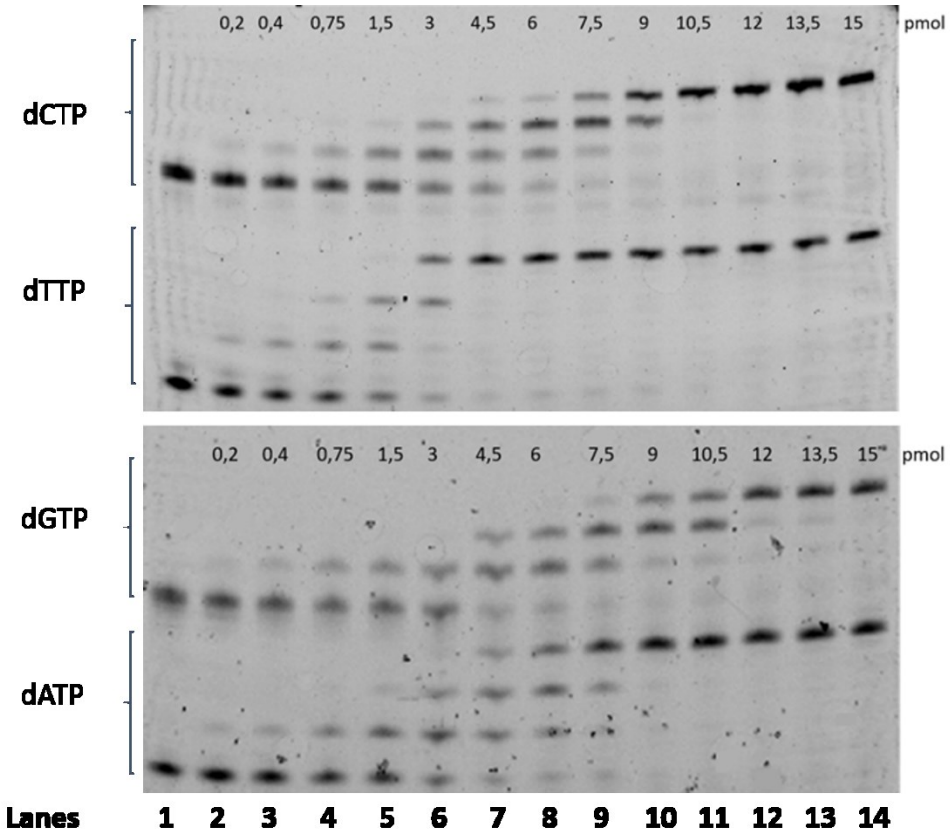


Figure 3.1 Visualization of a urea-PAGE gel of elongated products obtained with the indicated amount of dNTPs.

The elongation products obtained with standard quantities of dTTP and dCTP are shown in the upper gel, while the lower gel displays those obtained with standard quantities of dATP and dGTP. The non-elongated products displayed in lane 1 of both gels, were detected when the dimers for the quantification of the two pyrimidines (p13/tT and p27/tC) and those for the quantification of the two purines (p13/tA and p27/tG) were incubated with an excess of dATP and dTTP respectively, but without the nucleotide to be quantified, thus the p/t dimers remained of its original length. These non-elongated products were used as markers in the gels. Depending on the quantities of dNTPs, primers can be elongated to one, two or three positions leading to products of different lengths

corresponding respectively to the second, third or fourth band starting from the bottom of the gel. The reaction with standard amounts of dTTP reached its saturation level with 4,5 pmol of dTTP, while other reactions reached their saturation level with a higher quantity of dNTPs, 9 pmol for dATP, 10,5 pmol for dCTP and 12 pmol for dGTP. These data suggested that not for all 4 nucleotides there was a perfect proportionality between the amount of dNTP added and that incorporated in the primer by the Klenow fragment, thus indicating that the inherent specificity of the Klenow enzyme is different for the 4 dNTPs. To determine the amount of dNTPs incorporated into the primers, I used the following formula, reported by Roy *et al* (2009):

$$[\sum_{i=1}^{3} x_1 + 2\sum_{i=4}^{6} x_1 + 3\sum_{i=7}^{9} x_1]/[x_0 + \sum_{i=1}^{9} x_1]$$

in which, X_0 represents the fluorescence intensity of the non-elongated product and X_i represents the fluorescence intensity of elongated products. From the values obtained from this formula it was possible to construct the calibration curves.

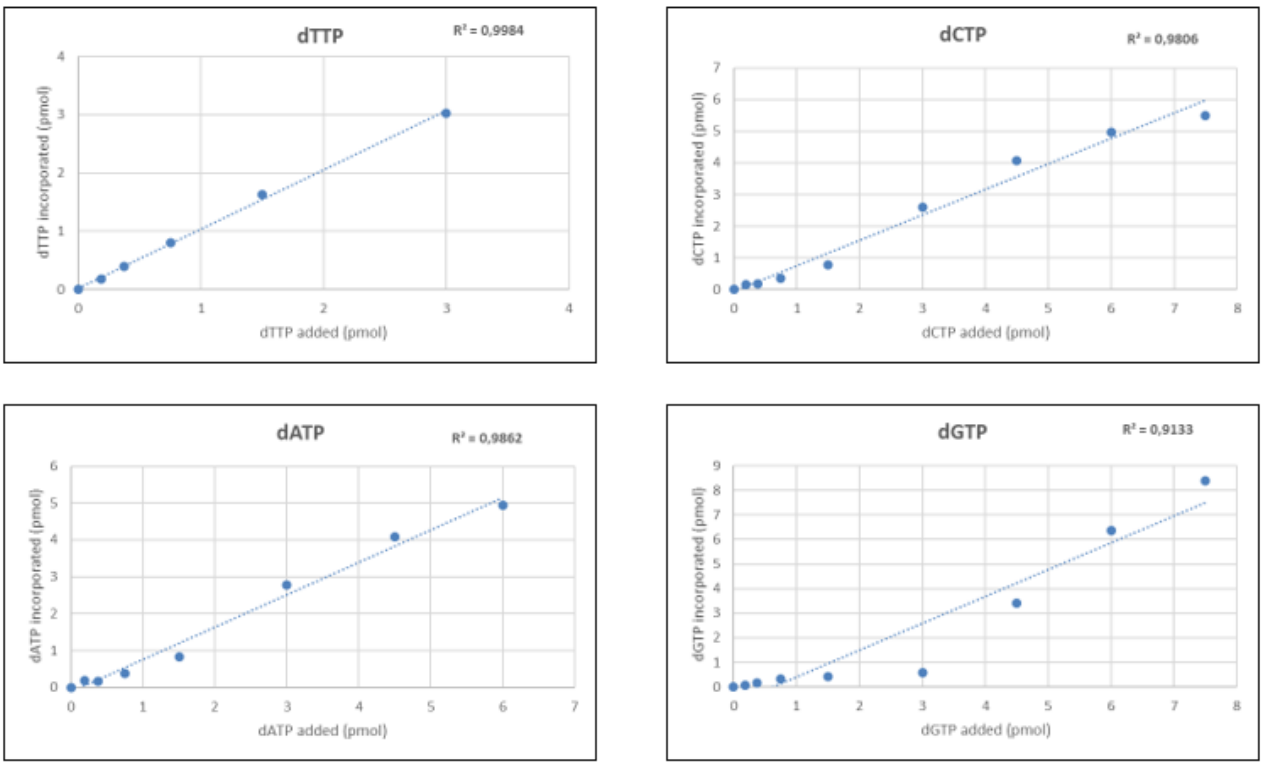


Figure 3.2 Calibration curves for pyrimidines (in the upper part) and for purines (in the lower part). Data are represented as mean values of two independent experiments. For each curve the related r^2 is reported.

As shown in **Figure 3.2** calibration curve for dTTP is linear from 0.1 to 3 pmol dTTP with a r^2 value close to 1 thus indicating that very low amounts of dTTP can be measured with great accuracy.

For dATP and dCTP, the proportionality between added and incorporated dNTPs is lower than that observed for dTTP, especially for very low quantities, but in any case the r^2 value is higher than 0.98. Instead, due to the low affinity of the Klenow fragment for dGTP, there is no linear correlation between dGTP added and dGTP incorporated thus compromising their correct measurement. Although the use of the Klenow fragment may lead to an overestimation of the amount of dGTP and, to a lesser extent also of the dCTP, on the other hand the advantage offered by this method is that of being able to use the same extract to simultaneously quantify the two pyrimidines and the two purines. Furthermore, the main purpose of this thesis was to understand how depletion of *sym1* could interfere with dNTP pools rather than giving an accurate absolute quantification of each dNTP.

3.2 Setting up the nucleotides extraction procedures

Nucleotides can be extracted by various methods which generally involve the use of very strong acids (typically perchloric or trichloroacetic) or use of an alcohol–water mixtures (60% methanol). However, the use of acids is more complex because it requires a phase of neutralization and removal of the acid and furthermore it can lead to degradation of dNTPs (Mathews and Wheeler, 2009). For this reason I decided to use the methanol extraction and boiling method which basically involves a re-suspension of sample in 1 ml of 60% cold-methanol following by a heating of the extract to destroy residual enzymatic activity of nucleases and nucleotide kinases, which could interfere with the enzymatic dNTP assay (Mathews and Wheeler, 2009). To set up the right amount of cells to extract, the optimal extraction time, the volume of water to re-suspend either the whole-cell or the mitochondrial dNTP pellet obtained after methanol evaporation and also the quantity of extract to be used in the reaction mix for DNA polymerase assay, I performed tests using two control mutants.

3.2.1 Setting up the whole-cell dNTPs extraction procedure

First of all I used the mutant lacking *SML1,* the ribonucleotide reductase inhibiting protein, to choose the most suitable conditions for the whole-cell dNTPs extraction. In fact, it had been shown that deletion of *SML1* resulted in dNTP pool increase (Zhao *et al.,* 1998) thus making this strain an excellent test to validate the whole-cell dNTPs extraction conditions. Total dNTP pool was extracted from BY4741 and BY4741/*sml1Δ* cells exponentially grown at 28°C in YP medium supplemented with 2% glucose. $1*10^9$ cells were harvested and dNTPs were extracted as described in "Materials and Methods". The resulting dNTP pellet was resuspended in 200 µl of cold H_2O and several volumes of whole-cell dNTP extracts were used for quantification of dNTPs by the DNA polymerase assay. The volume of water chosen to re-suspend the whole-cell dNTP pellet obtained after methanol

evaporation, and also the quantity of extract to be used in the reaction mix for DNA polymerase assay, were the result of several tests, in order to identify which was the right combination to obtain the right quantity of dNTPs measurable by the assay. **Figure 3.3** shows the results obtained on whole-cell dTTP pool of wild type and *sml1Δ* strains.

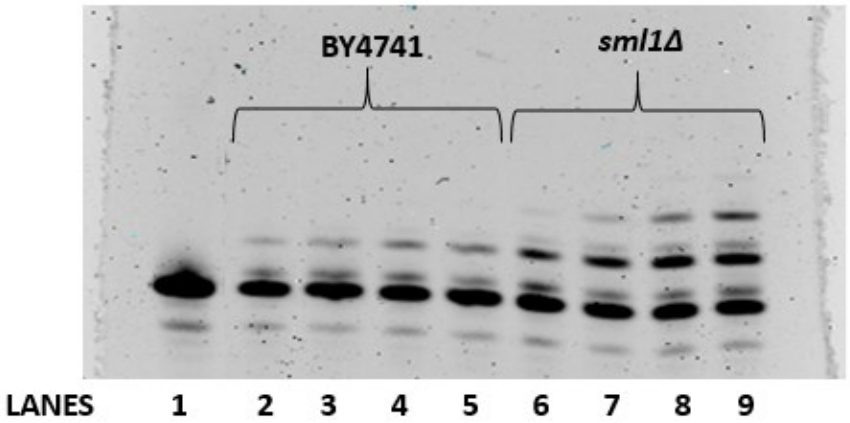


Figure 3.3. Visualization of a urea-PAGE gel of elongated products obtained with whole-cell dTTP pool of BY4741 wild-type and BY4741/*sml1Δ* strains. Lanes 2-5, elongation performed with 1,5 µl (lane 2), 3 µl (lane 3), 4,5 µl (lane 4) and 6 µl (lane 5) of whole-cell dNTP extracts of wild-type strain. Lanes 6-9, elongation performed with 1,5 µl (lane 6), 3 µl (lane 7), 4,5 µl (lane 8) and 6 µl (lane 9) of whole-cell dNTP extracts of *sml1Δ* strain. The non-elongated product was used as marker in lane 1.

In the lanes 2-5, where I loaded reaction mix with whole-cell dNTP extract of wild-type strain, two bands, corresponding respectively to the non-elongated p/t dimer and to product elongated of one position, were observed. In the lanes 6-9, where I loaded reaction mix with whole-cell dNTP extract of null mutant strain (*sml1Δ*), also the third band corresponding to dimer elongated of two position was observed, thus indicating that deletion of *SML1* resulted in dTTP pool increase. Moreover, it is possible to note that the third band (for *sml1Δ*) and the second band (for wild-type) intensity increased using a higher volume of extract, thus demonstrating a proportionality between added extract volume and quantitative incorporation into the p/t dimer. Therefore, these observations validate this enzymatic assay and the nucleotides extraction procedure.

3.2.2 Setting up the mitochondrial dNTPs extraction procedure

Since the mitochondrial dNTP pool amounts to a small percentage of the whole-cell dNTP, the mt dNTPs measurement requires a very sensitive method which also avoids cytosolic dNTPs contamination. For this purpose I used a rapid method that allows a complete separation of mitochondrial and cytosolic nucleotides (Pontarin *et al.,* 2003). It had been set up in human and mouse cells and I optimized the procedure for yeast cells. To validate this protocol I used a mutant lacking *POS5*, a mitochondrial NADH kinase, which showed a higher mitochondrial dNTP pool

compared to wild-type strain while cytosolic dNTP pool remained unchanged (Wheeler and Mathews, 2012). Thus, the difference observed between these two nucleotides pools makes this strain an excellent test to validate the method.

BY4741 wild type and BY4741/*pos5Δ* were grown in lactate medium at 28°C for 15 hours; $1*10^9$ cells were harvested to extract the whole-cell dNTPs and $1*10^9$ cells were harvested to extract the mitochondria and the relative dNTPs. For the mitochondria extraction I performed an enzymatic digestion with zymoliase at a concentration of 6mg/ml, in order to degrade yeast cell wall (see for the details Materials and Methods). The spheroplasts obtained from enzymatic digestion were subsequently subjected to differential centrifugation as described by Pontarin *et al* (2003). The first centrifugation was necessary to separate nuclei and mitochondria from the cytosolic fraction. The second centrifugation aimed to remove any remaining cytosolic dNTPs so that the pellet contained only mt dNTPs. Because the most common unit used in literature to report mitochondrial dNTP pool are picomoles per milligram mitochondrial proteins, I prepared from each mitochondrial pellet an aliquot of protein (1mg) subsequently precipitated and re-suspended in 1ml of ice-cold 60% methanol to extract dNTPs. The resulting mitochondrial dNTP pellet was resuspended in 100 µl of cold H_2O and different volumes of mt dNTP extracts were used for the quantification of dNTPs by the DNA polymerase assay. Instead, the residue obtained from the whole-cell extract was re-suspended in 200 µl of water since it should contain more nucleotides. **Figure 3.4** shows the results obtained on whole-cell and mitochondrial dTTP pool of wild type and *pos5Δ* strains.

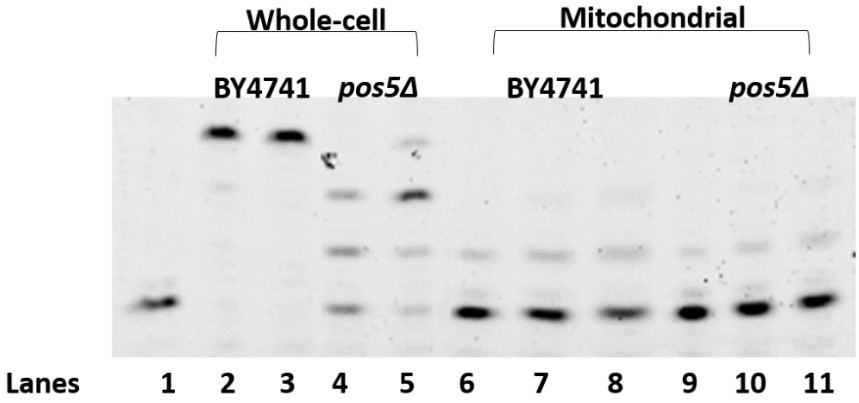


Figure 3.4 Visualization of a urea-PAGE gel of elongated products obtained with whole-cell (lanes 2-5) and mitochondrial (lanes 6-11) dTTP pool of BY4741 wild-type and BY4741/*pos5Δ* strains. Lanes 2-3, elongation performed with 2,5 µl (lane 2), 5 µl (lane 3) of whole-cell dNTP extracts of wild-type strain. Lanes 4-5, elongation performed with 2,5 µl (lane 4), 5 µl (lane 5) of whole-cell dNTP extracts of *pos5Δ* strain. Lanes 6-8, elongation performed with 5 µl (lane 6), 10 µl (lane 7), 15 µl (lane 8) of mitochondrial dNTP extracts of wild-type strain. Lanes 9-11, elongation performed with 5 µl (lane 9), 10 µl (lane 10), 15 µl (lane 11) of mitochondrial dNTP extracts of *pos5Δ* strain. The non-elongated product was used as marker in lane 1.

Starting from the part of the gel related to the whole-cell extracts, only a fourth band corresponding to the maximum elongation of p/t dimers was observed in lanes 2-3, while different elongation products were visible in lanes 4-5 suggesting that in *pos5Δ* yeast cells there was a lower amount of dTTP than wild-type strain. No such differences were instead evident in the part of the gel related to the elongation products obtained with the mitochondrial extracts. Indeed, the same bands pattern was visible in both the wild-type strain (lanes 6-8) and in the deleted strain (lanes 9-11). Although my results did not match those reported by Wheeler and Mathews, the dTTP reduction observed in the whole-cell extract of *pos5Δ* strain, but not in the mitochondrial one, suggest that in the latter there was no contamination with total dNTPs. Therefore, this experiment validated a simple and a rapid method to completely separate the mitochondrial dNTP pool from the cytosolic one.

3.3 Consequence of *SYM1* deletion on mitochondrial and whole-cell dNTP pools

Once set up the enzymatic assay and validated the dNTPs extraction method, I investigated the effects of *SYM1* deletion on whole-cell and mitochondrial dNTP pool, in order to understand how they could be affected.

Total and mitochondrial dNTPs were extracted from BY4741*sym1Δ*/pFL38*SYM1* and BY4741*sym1Δ*/pFL38 pre-grown at 28°C in SC medium supplemented with 2% ethanol, then exponentially grown in SC medium supplemented with 2% glucose and transferred to SC medium supplemented with 0.6% glucose and 2% ethanol for 24 hours at 37°C, stress conditions necessary for the manifestation of *sym1Δ* phenotype. In these stringent growth conditions the *petite* frequency (indicative of mtDNA instability) was similar between the *sym1Δ* mutant and the wild-type *SYM1* strain, a critical requirement to compare the two strains in a condition where the phenotype of the mutant is not a consequence of the mtDNA loss. Indeed, a larger percentage of cells with mtDNA instability in the null mutant compared to the wild type could alter the results, since in the absence of Sym1, about 50% of *petite* mutants is devoid of mtDNA (*rho*^{0}) (Dallabona, PhD thesis) and I have observed that in *rho*^{0} cells the amount of dNTPs is drastically reduced compared to *rho* $^{+}$ cells (**Figure 3.5**).

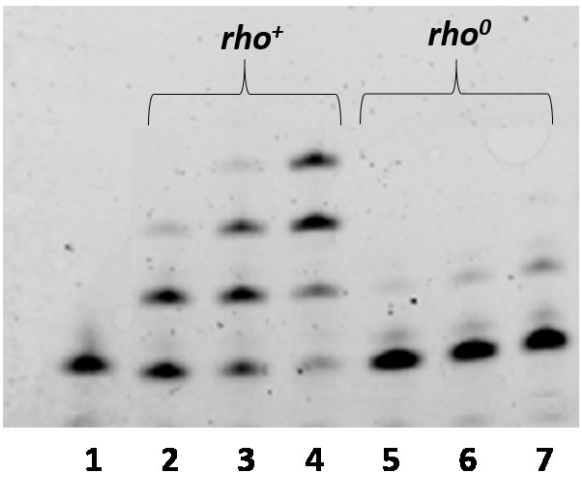


Figure 3.5. Visualization of a urea-PAGE gel of elongated products obtained with whole-cell dTTP pool of *rho*$^{+}$ and *rho*^{0} strains. Lanes 2-4, elongation performed with 2,5 µl (lane 2), 5 µl (lane 3), 10 µl (lane 4) of whole-cell dNTP extracts of *rho*$^{+}$ strain. Lanes 5-7, elongation performed with 2,5 µl (lane 5), 5 µl (lane 6), 10 µl (lane 7) of whole-cell dNTP extracts of *rho*^{0}strain. The non-elongated product was used as marker in lane 1.

Mitochondria were extracted with the zymoliase method as described before, but I had to make some changes because the growth conditions used for *sym1* made difficult the digestion of cell wall and risked compromising the dNTPs extraction. The inability to quickly lyse the cell wall could be due to both the high temperature and the presence of ethanol. Indeed in these severe conditions yeast cells accumulate trehalose, a disaccaride that serves to preserve the cellular integrity (Attfield, 1987) and which therefore could make digestion harder. Thus, since I could not change the growth conditions, I had to reduce the quantity of cells harvested, that was $2x10^{8}$ (instead of $1x10^{9}$ cells pelleted for *pos5Δ*), and doubling the concentration of zymoliase used (from 6 to 12mg/ml). These devices made the digestion easier and faster. Certainly, having drastically reduced the number of cells collected compared to *pos5Δ*, I had to consequently adapt other conditions: after subjecting spheroplasts to differential centrifugation, I prepared, from each mitochondrial pellet, two aliquot of proteins (50 µg each instead of 1 mg) subsequently precipitated and re-suspended in 1 ml of ice-cold 60% methanol to extract dNTPs. The resulting mt dNTPs were resuspended in 10 µl of cold H_2O and the entire volume was used for quantification of dNTPs by the DNA polymerase assay. One of the two aliquots was used for the quantification of pyrimidine nucleotides and the other for purine nucleotides (**Figure 3.6 A**). From quantification of the amount of fluorescence in each band and the subsequent interpolation from the calibration curves previously set up with standard quantity of dNTPs, it was possible to measure the extent of the decrease and understand if one nucleotide was more compromised than the others **(Figure 3.6 B).**

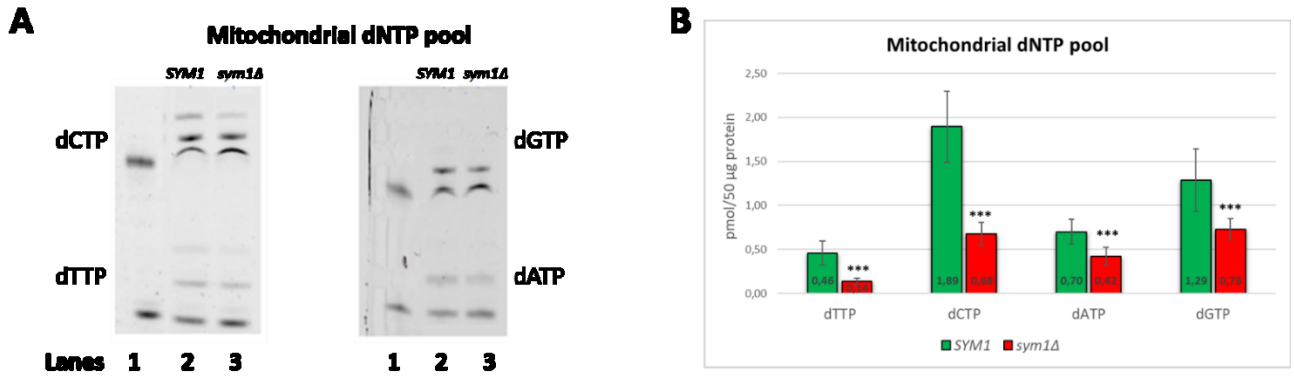


Figure 3.6. A) Visualization of a urea-PAGE gel of elongated products obtained with mitochondrial dNTP pool of BY4741*sym1Δ*/pFL38*SYM1* (lane 2) and BY4741*sym1Δ*/pFL38 (lane 3). The non-elongated product was used as marker in lane 1. **B)** mitochondrial dNTP amount of wild-type strain (green bar) and of null mutant strain (red bar). Data are represented as the mean of at least eight values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. Mitochondrial dNTP pool was reported as the amount of dNTP (in picomoles) per 50 µg mitochondrial protein.

Especially in the gel related to the elongation products obtained with pyrimidine nucleotides it is clearly visible that the fluorescence intensity of the bands corresponding to the major elongation of p/t dimers (the third band starting from the bottom of the gel) was more pronounced in wild-type compared to null mutant strain, thus indicating that *sym1Δ* strain contained less dTTP and dCTP than the *SYM1* strain. The same interpretative pattern is valid for the gel related to products obtained with purine nucleotides even if the difference in intensity was less evident. According to this, quantification of the fluorescence intensity in each band showed that ablation of *SYM1* resulted in a significant decrease in all four mitochondrial dNTP pool with pyrimidine nucleotides appearing to be the most compromised. Indeed, in the null mutant strain dTTP and dCTP decreased respectively to about 30% and 35% relative to the *SYM1* wild-type, while dGTP and dATP decreased to about 50% of corresponding values in wild-type strain **(Figure 3.6 B).** However, the extent of the reduction may not be completely correct for dGTP since there is not a perfect proportionality between the added and incorporated nucleotide. Anyway, although the technique did not allow a precise quantification for all dNTPs, it certainly enabled to demonstrate that the deletion of *SYM1* leads to a reduction of all 4 nucleotides, as shown by the results on the gels. Therefore, the Sym1 deficiency cause a mitochondrial dNTP pool decrease. Once again *sym1* yeast mutant perfectly recapitulates the mitochondrial phenotypic traits of humans and other model organisms, confirming yeast as an excellent, albeit simple, model for MPV17-related MDS.

As said before, since in yeast the mt dNTP pool is completely dependent on the cytoplasmic *de novo* pathway, I decided to extend the analysis also to the whole-cell dNTP pool in order to understand if

it could be affected by the *SYM1* deletion. Therefore, starting from the same cell cultures used for the extraction of mitochondria and related dNTPs, $2*10^8$ cells were harvested and re-suspended in 1ml of ice-cold 60% methanol to extract dNTPs. The resulting dNTP pellet was resuspended in 50 µl of cold H_2O and 5 µl of whole-cell dNTP extracts were used for quantification of dNTPs by the DNA polymerase assay (**Figure 3.7**).

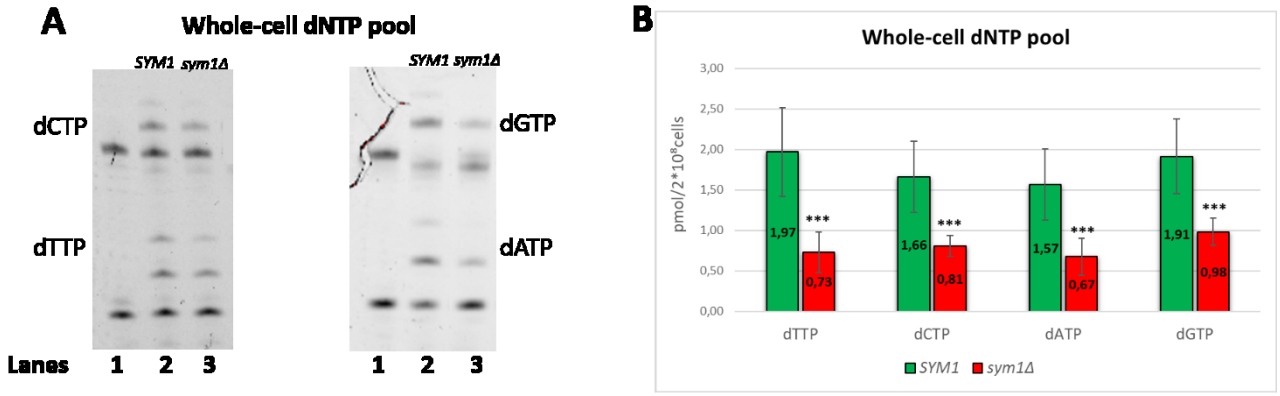


Figure 3.7 A) Visualization of a urea-PAGE gel of elongated products obtained with whole-cell dNTP pool of BY4741*sym1Δ*/pFL38*SYM1* (lane 2) and BY4741*sym1Δ*/pFL38 (lane 3). The non-elongated product was used as marker in lane 1. **B)** whole-cell dNTP amount of wild-type strain (green bar) and of null mutant strain (red bar). Data are represented as the mean of at least eight values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. Whole cell dNTP pools was reported as the amount of dNTP (in picomoles) per $2*10^8$cells.

As in mitochondrial nucleotide pool, deletion of *SYM1* led to a general and marked reduction in all four total nucleotides: dTTP decreased by about 3 fold, while the amount of dCTP, dGTP and dATP halved compared to the corresponding wild-type values. Therefore, these analyses demonstrated that the decrease of dNTPs, due to Sym1 deficiency, is not limited to the mitochondrial compartment but it is also extended to the whole cell dNTP pool. These data are not surprising since mitochondria play a pivotal role in maintaining a balanced supply of nucleotides (Desler *et al.,* 2007) and several studies (reviewed in Desler *et al.,* 2010) have demonstrated a correlation between different types of mitochondrial dysfunctions and abnormal synthesis of cytosolic ribonucleotides and deoxyribonucleotides.

Beyond the fact that a decrease in the dNTPs levels could be due to an aberrant production of ATP caused by an OXPHOS impairment that in *sym1Δ* yeast cells occurs independently from mtDNA instability (Dallabona *et al.,* 2010), it is possible that other factors lead to a whole-cell dNTPs decrease in *sym1Δ* strain. Studies performed in our laboratory showed that the *sym1Δ* mutant was characterized by a reduced succinate dehydrogenase activity, which is part of the TCA cycle, and that the metabolic defect of *sym1Δ* strain was rescued by overexpression of mitochondrial

transporters of TCA intermediates (*YMC1* and *ODC1*). Taken together these results suggested a role for *SYM1* in a homeostatic control of TCA cycle intermediates (Dallabona *et al.,* 2010). TCA cycle is defined as an amphibolic cycle because it is essential not only for catabolism of carbohydrates, fatty acids and aminoacids, but also it provides precursors for many biosynthetic (anabolic) pathways. For example, α-ketoglutarate (αKG) and oxaloacetate (OAA) are subtracted from the TCA cycle to be used as precursors of glutamate and aspartate by simple transamination. Through glutamate and aspartate, the carbon atoms of αKG and OAA are used to build other aminoacids, as well as purine and pyrimidine nucleotides. According to this, supplementation with either glutamate, aspartate, glutamine or asparagine rescued oxidative growth defect of *sym1Δ* (Dallabona *et al.,* 2010). Therefore, Sym1 deficiency could led to a reduction or an imbalance in TCA cycle intermediates that in turn could impair cytosolic nucleotide biosynthesis. Diminishing of all 4 dNTPs synthetized in the cytosol could be reflected in a decrease of mitochondrial dNTP pool which in yeast depends entirely on direct transport of cytosolic dNTPs. Notably with these measurements of dNTP pools size, I obtained only snapshots of cells which does not provide information about the dynamic aspects of nucleotides metabolism. Indeed, the size of the dNTP pool depends not only by its synthesis but also by its consumption (incorporation of a nucleotide in DNA) and its catabolism.

3.4 Drugs effect on *petite* mutants frequency of *sym1Δ* strain

In our laboratory, screening of two Chemical Library led to the identification of ten molecules able to rescue the oxidative growth defect and ameliorate the mtDNA instability of the $sym1^{R51W}$ mutant strain. Moreover, all these molecules were able to rescue the oxidative growth defect also in the absence of the Sym1 protein, indicating that the drugs act through a bypass (Gilberti, PhD thesis). Having demonstrated a remarkable decrease of mitochondrial and whole-cell dNTP pool, I investigated if the beneficial molecules, previously identified through the drug drop test, could act by increasing the dNTPs levels in *sym1Δ* strain. Firstly, I evaluated the molecules effect on mtDNA instability of *sym1Δ* strain by measuring the *petite* frequency with and without molecules. This analysis was carried out in BY4741*sym1Δ*/pFL38*SYM1* and BY4741*sym1Δ*/pFL38 grown at 37°C in SC supplemented with both 2% ethanol and 2% glucose to enable to OXPHOS-defective cells to proliferate. For each molecule different concentrations were tested and in the **Figure 3.8** are shown the results obtained with the most effective dosage. All molecules were dissolved in DMSO, so to ensure that an alteration in the *petite* frequency in the cells treated with the molecules was not due to the compound vehicle, *sym1Δ* cells cell were also treated with DMSO.

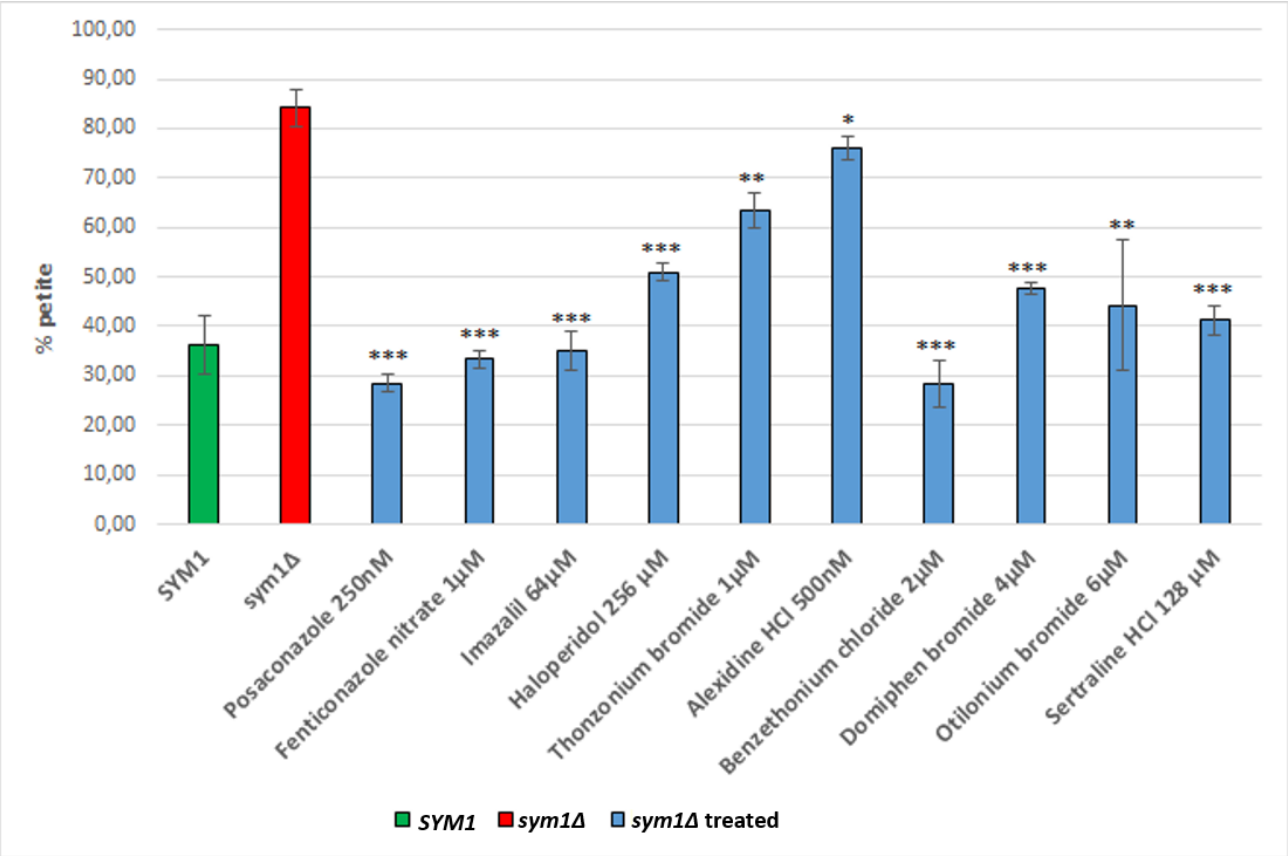


Figure 3.8 Determination of *petite* frequency of wild-type SYM1 (green bar) and null mutant *sym1Δ* with (blue bars) or without (red bar) the supplementation of beneficial compounds. More than 4000 colonies for each strain were scored. Data are represented as the mean of three values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001.

Addition of all molecules resulted in a significant reduction of *petite* percentage in *sym1Δ* strain. Except for Alexidine HCl and Thonzonium bromide that led to a mild reduction of *petite* frequency (about 15% and 25% respectively) all other molecules showed a marked effect. Indeed, *petite* percentage was reduced by about 50% after supplementation of Haloperidol, Domiphen Bromide and Otilonium Bromide, and addition of Posaconazole, Fenticonazole nitrate, Imazalil, Benzethonium Chloride and Sertraline HCl brought the *petite* frequency to a level equal or even lower than the wild type, thus resulting the most active compounds. The **table 6** displays a summary of the effects of the beneficial molecules on mtDNA instability of *sym1Δ* strain.

Drug	mtDNA instability rescue
Posaconazole	+++
Fenticonazole nitrate	+++
Imazalil	+++
Haloperidol	++
Thonzonium bromide	+
Alexidine HCl	+
Otilonium bromide	++
Benzethonium chloride	+++
Domiphen bromide	++
Sertraline HCl	+++

Table 6. Effects of the beneficial molecules on mtDNA instability of *sym1Δ* strain measured as reduction of *petite* frequency. +++ Strong effect (greater than 50% reduction); ++ Medium effect (reduction between 50% and 30%); + Mild effect (less than 30% reduction)**.**

To exclude that the beneficial effect observed was due to a selective induction of the *petite* mutants mortality, a fitness test was previously performed growing together *rho*$^{+}$ and *rho*^{0} cells in an equal amount in presence or in absence of the molecule to test (Gilberti, PhD thesis). Since that the ratio of *rho*^{0} to *rho*$^{+}$ was similar with and without molecules, the reduction of *petite* percentage was ascribed to a positive effect of drugs on mitochondrial genome stability.

3.5 Drugs effect on whole-cell and mitochondrial dNTP pool of *sym1Δ* strain

In our laboratory it had already been shown that deletion of *SML1*, the *RNR* inhibiting protein, led to a very strong reduction in the *petite* frequency of *sym1Δ* (Gilberti, PhD thesis). I further analysed the effect of *SML1* deletion on dNTP pool content in the absence of Sym1 and I demonstrated that the ablation of Sml1 improved mtDNA stability, increasing the cellular availability of the dNTP pool (**Figure 3.9**).

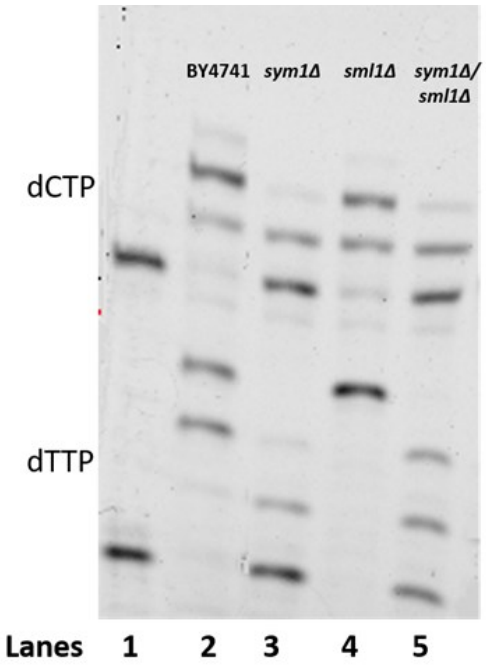
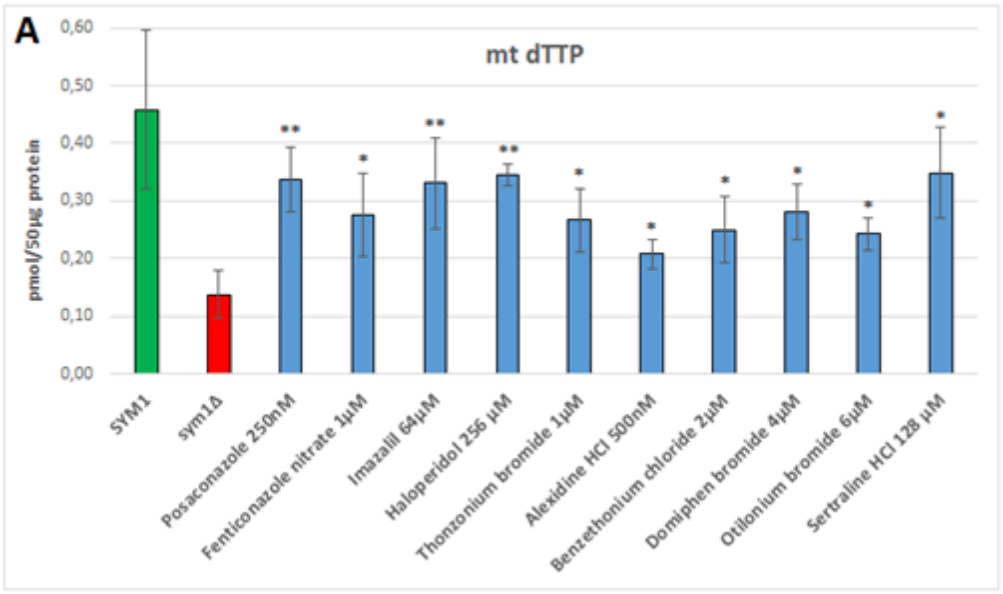


Figure 3.9. Visualization of a urea-PAGE gel of elongated products obtained with whole-cell dTTP pool (lower part of the gel) and dCTP pool (upper part of the gel) of BY4741 (lane 2), BY4741/*sym1Δ* (lane 3) and BY4741/*sml1Δ* (lane 4), BY4741/*sym1Δ*/*sml1Δ* (lane 5). The non-elongated product was used as marker in lane 1.

It is evident that the deletion of *SML1* led to increase of dTTP and dCTP pools in *sym1Δ* strain. Indeed, the fluorescence intensity of the bands corresponding to the dimer elongated of one or two position (the second and the third band starting from the bottom of the gel) was more pronounced in *sym1Δ*/*sml1Δ* (lane 5) compared to *sym1Δ* (lane 3). Conversely, the intensity of the band corresponding to the non-elongated product was more marked in *sym1Δ* compared to *sym1Δ*/*sml1Δ*, thus demonstrating that amount of dTTP and dCTP in sample derived from *sym1Δ* did not permit a dimer elongation similar to that observed in *sym1Δ*/*sml1Δ* strain.

I then investigated if the reduction of the mitochondrial instability achieved adding the beneficial molecules in *sym1Δ*, could be the result of an increase in mitochondrial dNTP pool. This analysis was performed in the same conditions previously described for the determination of the effect of deletion of *SYM1* on mitochondrial dNTP pool and adding the compounds to test in the medium at the concentrations used for mt DNA instability analysis. In the **Figure 3.10** are reported the results obtained.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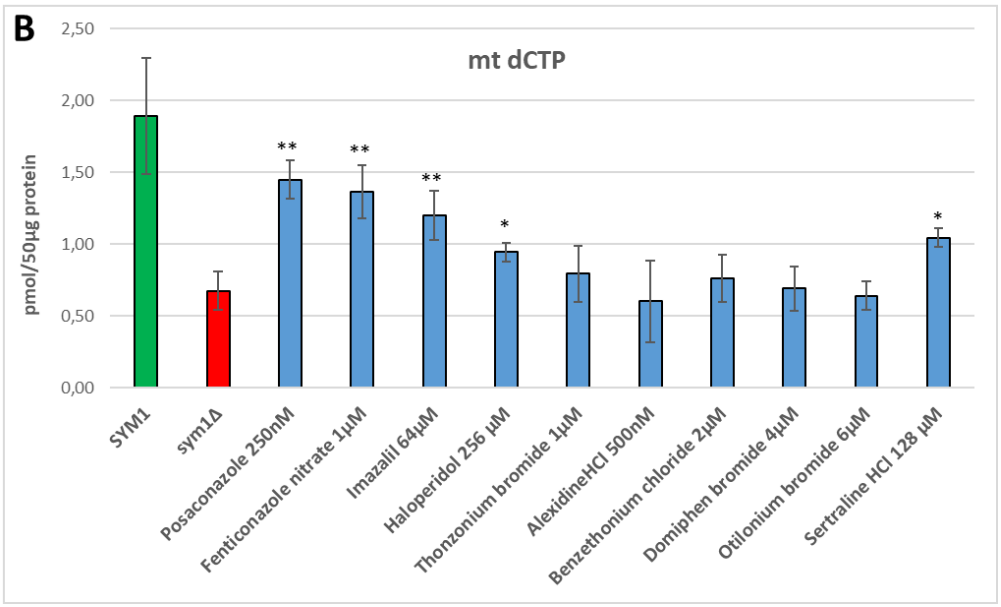
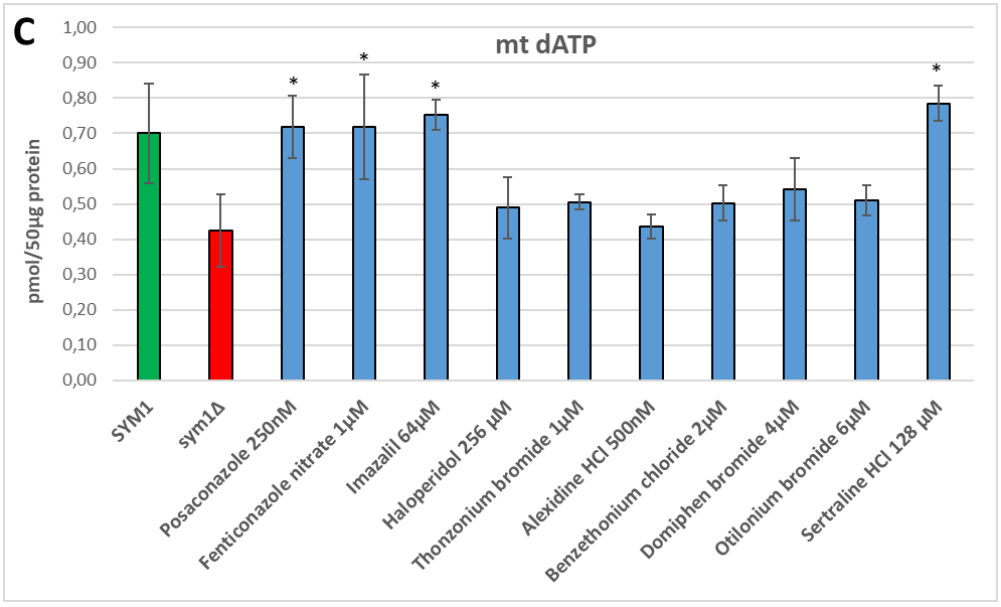
mt dTTP
pmol/50µg protein
0,60
0,50
0,40
0,30
0,20
0,10
0,00
SYM1
sym1Δ
Posaconazole 250nM
Fenticonazole nitrate 1µM
Imazalil 64µM
Haloperidol 256 µM
Thonzonium bromide 1µM
Alexidine HCl 500nM
Benzethonium chloride 2µM
Domiphen bromide 4µM
Otilonium bromide 6µM
Sertraline HCl 128 µM


B
mt dCTP
pmol/50µg protein
2,50
2,00
1,50
1,00
0,50
0,00
SYM1
sym1Δ
Posaconazole 250nM
Fenticonazole nitrate 1µM
Imazalil 64µM
Haloperidol 256 µM
Thonzonium bromide 1µM
AlexidineHCl 500nM
Benzethonium chloride 2µM
Domiphen bromide 4µM
Otilonium bromide 6µM
Sertraline HCl 128 µM


C
mt dATP
pmol/50µg protein
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
SYM1
sym1Δ
Posaconazole 250nM
Fenticonazole nitrate 1µM
Imazalil 64µM
Haloperidol 256 µM
Thonzonium bromide 1µM
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Otilonium bromide 6µM
Sertraline HCl 128 µM

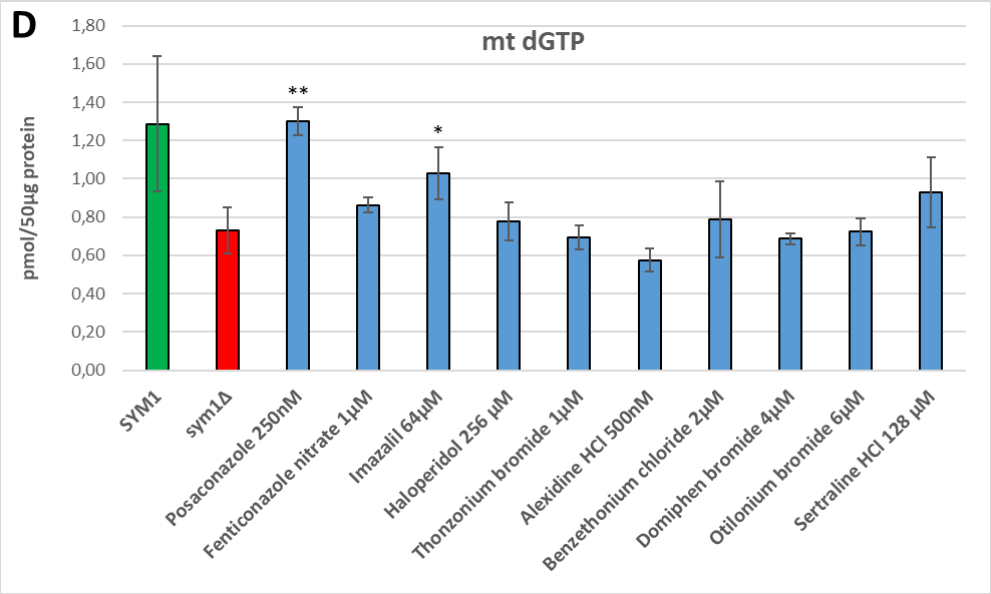


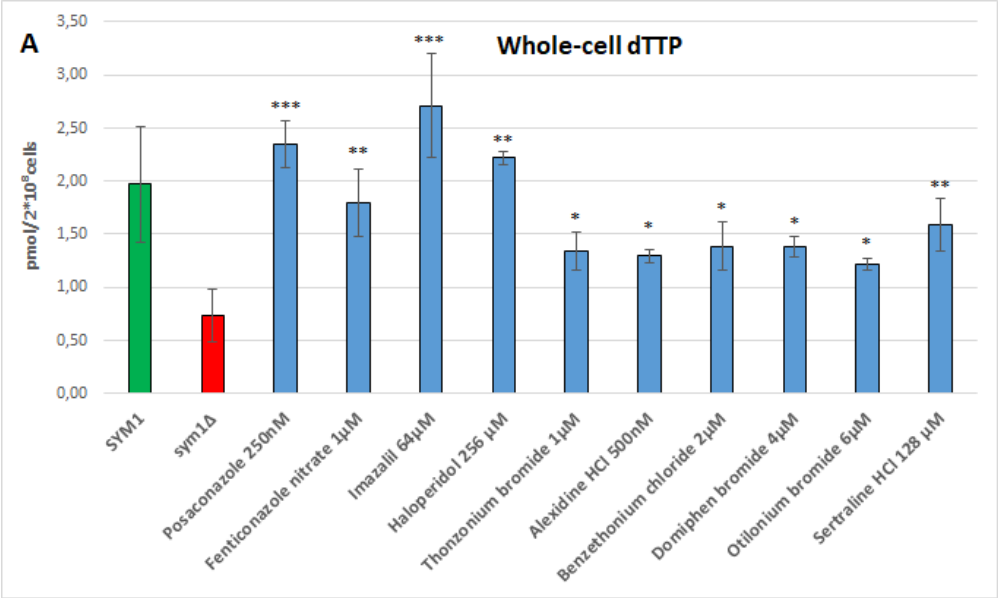
Figure 3.10 Mitochondrial dTTP **(A),** dCTP **(B)**, dATP **(C)** and dGTP **(D)** amount of wild-type strain (green bar) and null mutant *sym1Δ* with (blue bars) or without (red bar) the supplementation of beneficial compounds. Data are represented as the mean of at least three values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. Mitochondrial dNTP pools was reported as the amount of dNTP (in picomoles) per 50 µg mitochondrial protein.

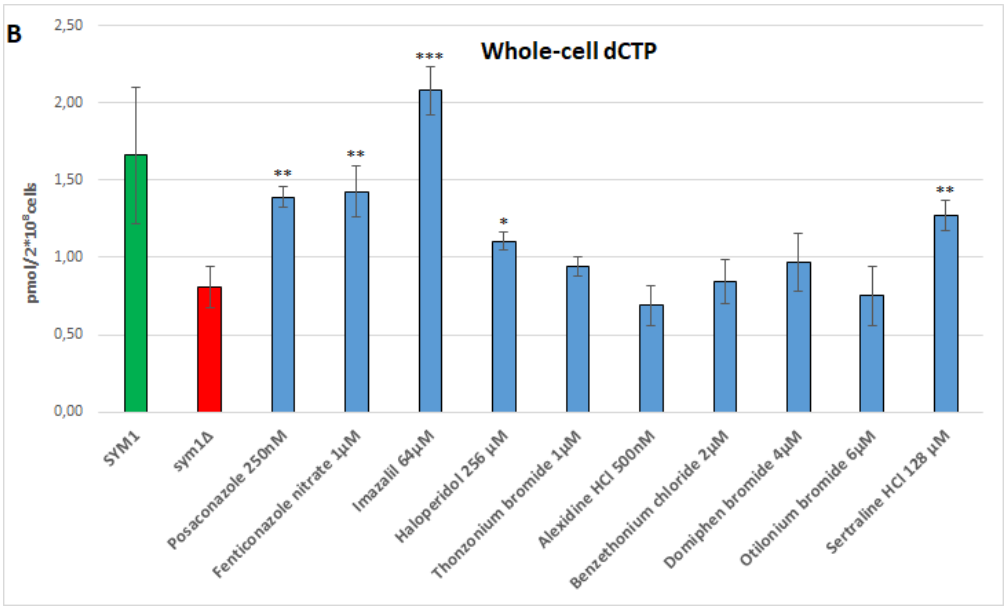
Addition of all molecules resulted in a significant increase of mitochondrial dTTP pool in *sym1Δ* strain. As said before, the bigger alteration of the dTTP compared to the other 3 nucleotides could be due to the greater sensitivity of the polymerase assay to detect very small variations of the dTTP. In particular, Alexidine HCl was the less effective molecule, leading to a moderate increase of dTTP (about 1.5 fold). Addition with Fenticonazole Nitrate, Thonzonium Bromide, Benzethonium Chloride, Domiphen Bromide and Otilonium Bromide, doubled the level of dTTP wheras addition with Posaconazole, Imazalil, Haloperidol and Sertraline HCl tripled the levels of this nucleotide respect to untreated mutant strain. The beneficial effects of Posaconazole, Fenticonazole nitrate, Haloperidol, Imazalil and Sertraline was also evident for the other nucleotides.

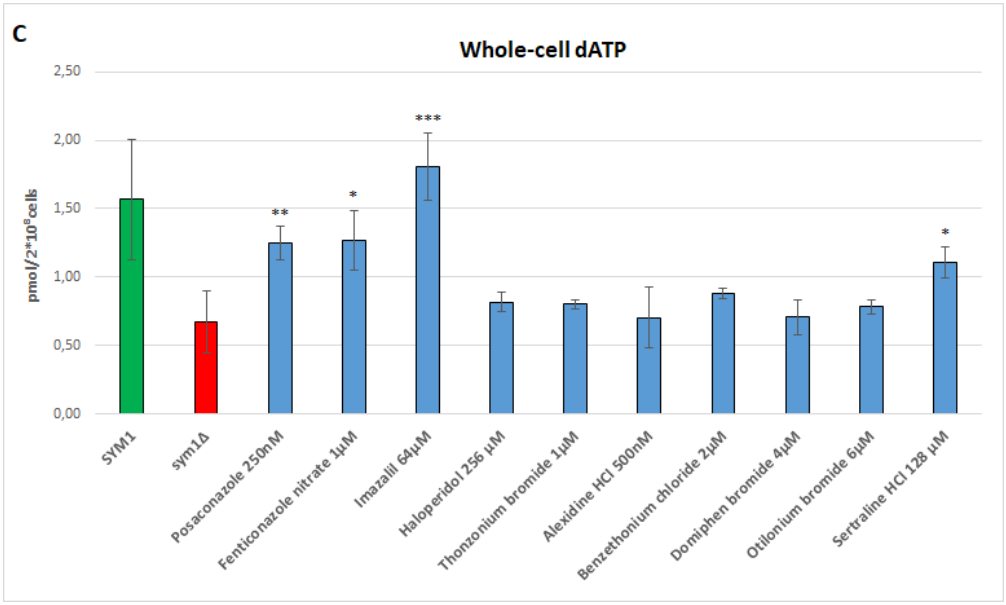
Overall, from these experiments it emerged that all the molecules, although with a different intensity, were able to determine an increase of mitochondrial dNTP pool in *sym1Δ* strain. The concomitant increase of both mt dNTP pool and mtDNA stability suggested the reduced availability of DNA synthesis precursors as the cause of the mtDNA deletion/depletion in Sym1 deficiency. Notably, also in quiescent fibroblast of patients with *MPV17* deficiency, the mtDNA loss was prevented and rescued by deoxynucleoside supplementation, thus identifying the administration of deoxyribonucleosides as a possible pharmacological approach for *MPV17*-related MDS (Dalla Rosa *et al.,* 2016). Since all molecules tested were able to increase mt dNTP pool, they could represent a starting point of new potential therapies for *MPV17*-related disease. Furthermore, all these

molecules are FDA-approved so their use could minimize development costs and facilitate the timing for an eventual administration in humans.

Starting from the same cell cultures used for the extraction of mitochondria and related dNTPs, I investigated the effect of molecules even in whole-cell dNTP (**Figure 3.11**).







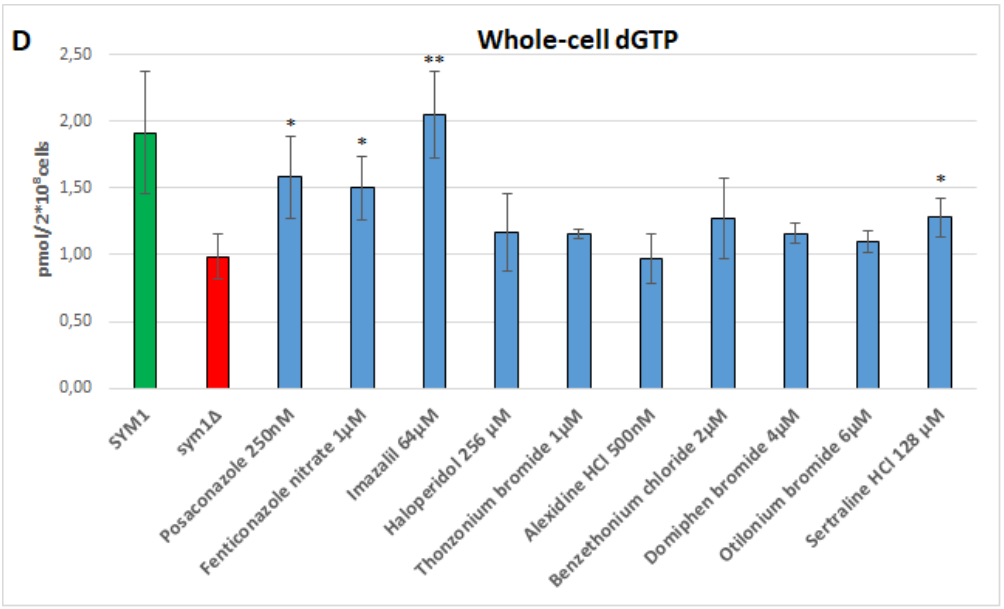


Figure 3.21 Whole-cell dTTP **(A),** dCTP **(B)**, dATP **(C)** and dGTP **(D)** amount of wild-type strain (green bar) and null mutant *sym1Δ* with (blue bars) or without (red bar) the supplementation of beneficial compounds. Data are represented as the mean of at least three values ± SD. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. Mitochondrial dNTP pools was reported as the amount of dNTP (in picomoles) per $2*10^8$cells.

Supplementation with all compounds resulted in a remarkable increase of whole-cell dTTP that reached wild-type levels following the administration of Posaconazole, Fenticonazole Nitrate, Imazalil and Haloperidol thus resulting the most active drugs. Their beneficial effect and also that of Sertraline HCl was evident even for the other nucleotides. As in the case of mt dNTP pool, the other molecules determined a less marked but however significant increase of dTTP whereas no effect was observable for the other nucleotides.

Since in yeast the mitochondrial dNTPs amount entirely depends on *de novo* cytosolic synthesis by the *RNR*, it is plausible to speculate that addition of these molecules could enhance DNA synthesis precursors production and in turn the increase in total dNTPs could be reflected in an increase in mt dNTP pool. Thus, the administration of these drugs could be sufficient to counteract dNTP deficiency and mtDNA instability in *sym1Δ* cells.

3.6 Effect of the beneficial molecules on other MDS yeast models

The third aim of my thesis was to test the beneficial molecules identified for *MPV17*-related MDS on other MDS yeast models available in our laboratory in order to extend their potential use to other patients affected by MDS. In particular, I investigated the effect of all ten drugs on mtDNA instability of two yeast models characterized by mutations in *MIP1* and *RNR2*, orthologs of the human genes *POLG* and *RRM2B* respectively.

3.6.1 Effect of the beneficial molecules in yeast model of *POLG*-related MDS

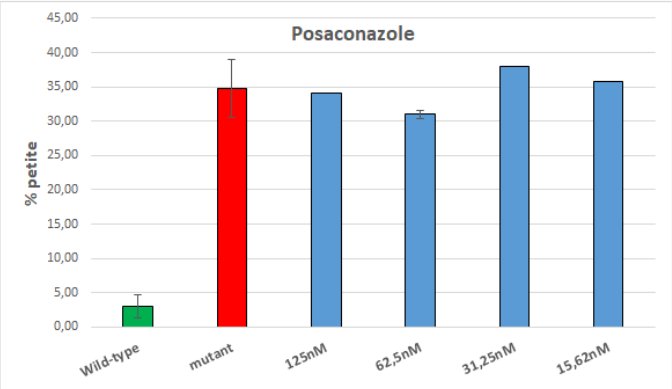
POLG, encoding the catalytic subunit of human mtDNA polymerase γ, is included in the class of nuclear genes whose mutations result in mtDNA depletion or deletion disorders. The clinical phenotypes associated with *POLG* pathogenic mutations are very heterogeneous ranging from aggressive and childhood-onset Alpers–Huttenlocher syndrome (AHS) to mild and adult-onset progressive external ophthalmoplegia (PEO). Until now, more than 150 pathological variants in *POLG* have been identified in different *POLG* domains (https://tools.niehs.nih.gov/polg/), thus making *POLG* mutations the most frequent cause of inherited mitochondrial diseases.

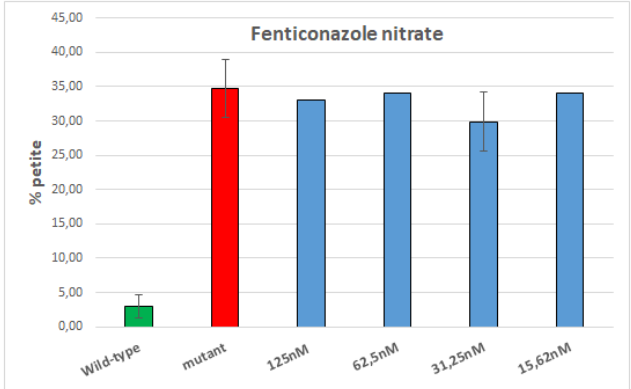
The high degree of conservation between human *POLG* and its yeast ortholog *MIP1* made possible to study in *S. cerevisiae* the phenotypic effects of *MIP1* mutations corresponding to human pathological mutations (Foury, 1989; Baruffini *et al.,* 2006; Stuart *et al.,* 2006; Baruffini *et al.,* 2007; Stumpf *et al.,* 2010; Baruffini *et al.,* 2012; Kaliszewska *et al.,* 2015).

A successful therapy for *POLG*-related disorders is not yet available and treatment remain largely symptomatic. Since *POLG* mutations are associated with 10–25% of PEO and >10% of ataxia cases and are the most common cause of mitochondrial epilepsy (Rahman and Copeland, 2019) finding a strategy that provides a relevant improvement in life of the subjects affected is certainly a stimulating challenge. In our laboratory, a yeast-based screening was performed in order to identify beneficial molecules against these mitochondrial disorders. Interestingly one of these molecules, clofilium tosylate (CLO) was able to increase mtDNA content of *Caenorhabditis elegans* and quiescent fibroblasts characterized by *POLG* deficiency. Therefore, this molecule could represent a

possible pharmacological approach for the treatment of *POLG*-associated diseases (Pitayu *et al.,* 2016). Moreover, in our laboratory it has been demonstrated that *RNR1* overexpression or *SML1* deletion, caused a marked decrease of the *petite* frequency in different Mip1-deficient mutants (Baruffini *et al.,* 2006; Pitayu *et al.,* 2016). Interestingly deoxyribonucleoside supplementation restores mtDNA copy number in cells with *POLG* deficiency suggesting that the administration of deoxyribonucleosides could be a possible pharmacological therapy for *POLG*-related MDS (Bulst et al., 2012; Blázquez-Bermejo *et al.,* 2019).

Here I investigated whether the beneficial molecules identified for *MPV17*-related MDS, capable to increase dNTPs levels in *sym1Δ* strain, were able to reduce the mtDNA instability of the *mip1*^{G651S} mutant used for the previous analysis. The G651S mutation corresponds to the human G848S mutation, which affect a very conserved residue in the polymerase domain and it is associated with PEO and Alpers syndrome (Kollberg *et al.,* 2005; Ferrari *et al.,* 2005; Davidzon *et al.,* 2005; Nguyen *et al.,* 2005; Horvath *et al.,* 2006; Kollberg *et al.,* 2006;). In yeast, the introduction of the G651S mutation in *MIP1* allele in a specific genetic context allowed the maintenance of mtDNA in about 50% of cells (Baruffini *et al.,* 2007). The mtDNA instability analysis was carried out in DWM-5A*mip1Δ*/pFL39*MIP1* and DWM-5A*mip1Δ*/pFL39*mip1*^{G651S} grown at 28°C in SC supplemented with 2% glucose for 48 hours as described in Materials and Method. For every drug I tested different concentrations below the minimum inhibitory concentration (MIC), i.e. the lowest concentration of the compound that completely inhibits the growth. The **Figure 3.12** shows the results obtained. The analysis was repeated at least three times for concentrations where standard deviation is indicated.





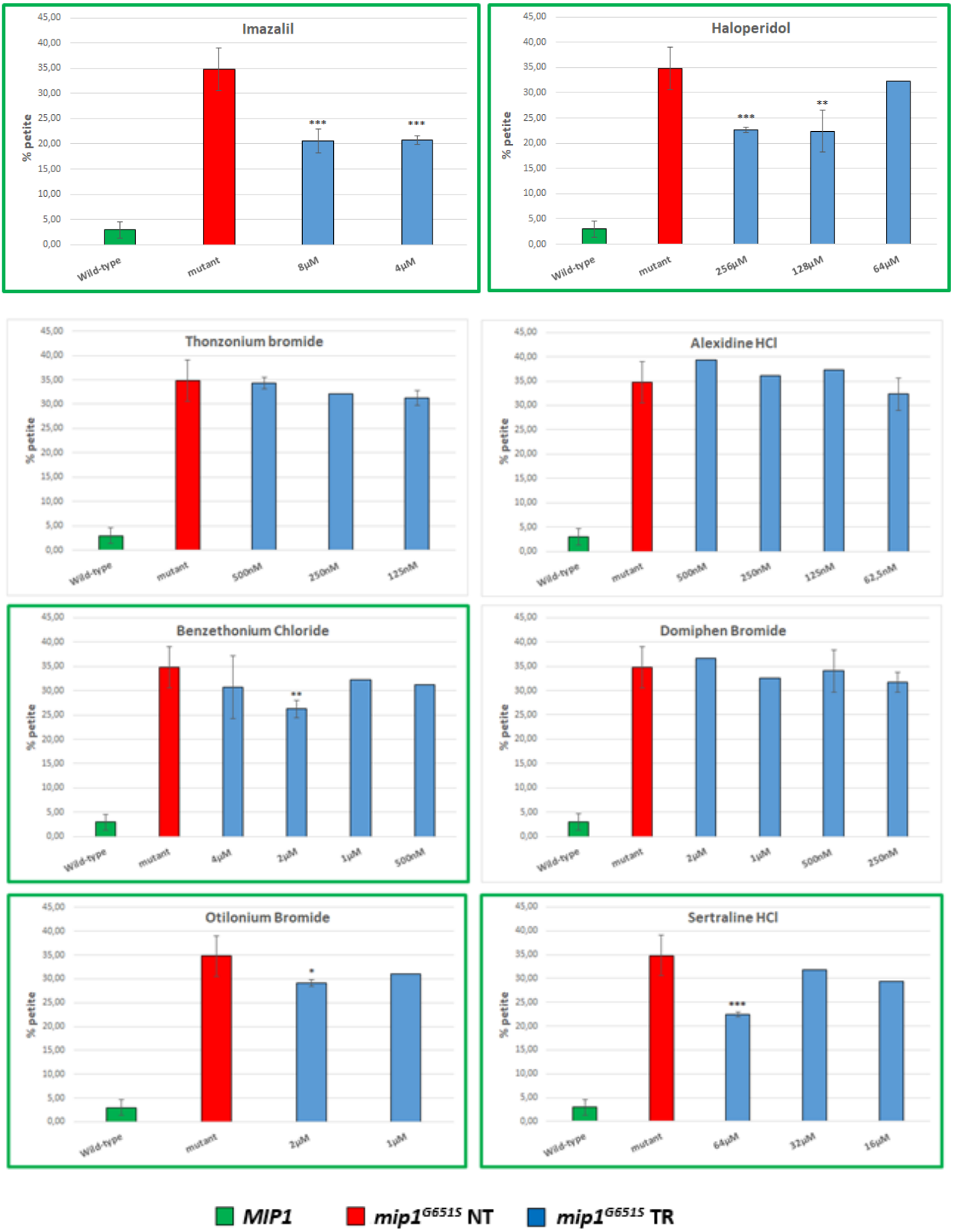


Figure 3.12 Determination of *petite* frequency of wild-type *MIP1* (green bar) and *mip1*^{G651S} with (blue bars) or without (red bar) the supplementation of beneficial compounds. More than 4000 colonies for strain were scored. All values with error bar are means of at least three independent experiments. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. The green boxes represent the active compounds.

Posaconazole, Fenticonazole Nitrate, Thonzonium Bromide, Alexidine HCl and Domiphen Bromide did not result in a decrease in *petite* frequency of *mip1*^{G651S} mutant strain. On the contrary, addition of Haloperidol, Imazalil, Benzethonium Chloride, Otilonium Bromide and Sertraline HCl resulted in a significant improvement of mtDNA stability. In particular, Imazalil, Haloperidole and Sertraline HCl were the most active compounds, leading to almost 2-fold reduction in the *petite* frequency compared to the untreated mutant. Benzethonium Chloride and Otilonium Bromide led to a mild reduction of mtDNA instability. For all molecules, except for Otilonium Bromide, these data perfectly reflected the results obtained by testing the ability of these molecules to rescue the oxidative growth defect of the same mutant. **Figure 3.13** shows the results obtained with compounds able to rescue the growth defect of the *mip1*^{G651S} mutant strain.

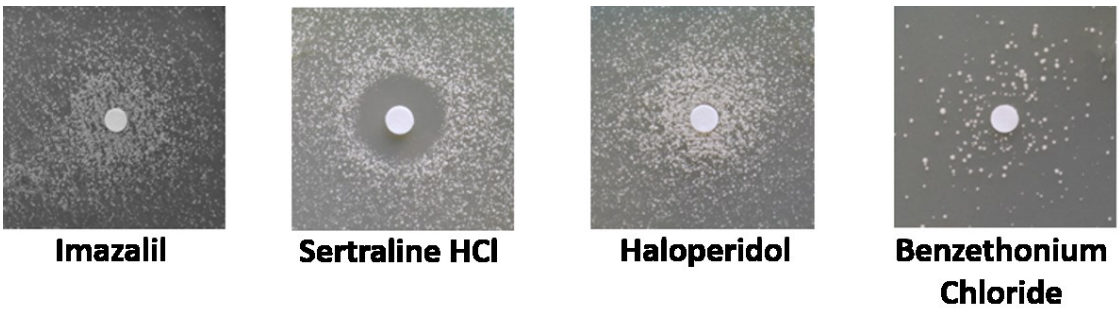


Figure 3.13 Rescue of the oxidative growth defect of the *mip1*^{G651S} mutant strain induced by active compounds.

Imazalil and Haloperidol led to formation of a marked and widespread halo of growth suggesting that these molecules are active on a broad range of concentrations. Sertraline HCl led to a formation of an external crown of growth, whereas no growth was observed near the filter, thus indicating that Sertraline HCl was toxic at high concentrations (near the filter) and active at lower concentration (far from the filter). The addition of Benzethonium Chloride had a milder effect also on the growth. Otilonium Bromide, as well as Posaconazole, Fenticonazole Nitrate, Thonzonium Bromide, Alexidine HCl and Domiphen Bromide, did not rescue significatively the oxidative growth defect of the mutant strain (data not shown).

Overall, the results obtained showed that not all molecules were able to determine an improvement in the mtDNA stability of this mutant. Whether the increase of mtDNA stability achieved with addition of Imazalil, Sertraline HCl, Haloperidol and, to a lesser extent of Benzethonium Chloride, is due to an increase in dNTP pool, to an improvement of the polymerase fidelity or to a Mip1 stabilization requires further analysis.

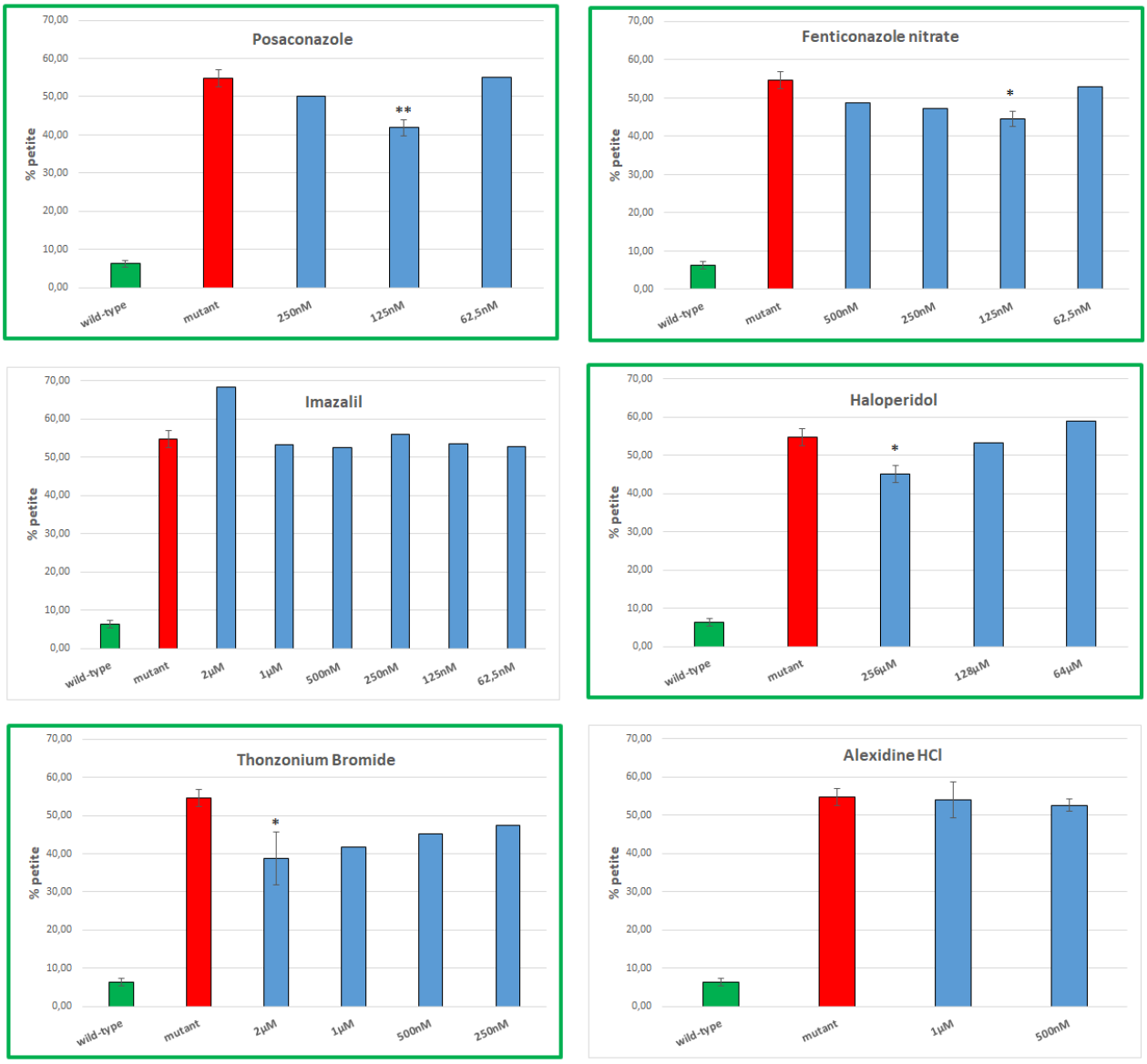
3.6.2 Effect of the beneficial molecules in yeast model of *RRM2B*-related MDS

RRM2B encodes the small p53-inducible R2 subunit (p53R2) able to associate with R1 subunit to form an active ribonucleotide reductase (RNR), the key enzyme for the biosynthesis of dNTPs. p53R2 expression is essential for DNA repair and mtDNA (mtDNA) synthesis in postmitotic cells, since in cycling cells, another small subunit called R2 interact with R1 to form another type of RNR complex (Pontarin *et al.,* 2012). Therefore, mutations in the *RRM2B* gene are an obvious cause of insufficient dNTP supply in the mitochondrion resulting in mtDNA deletions or depletions. Until now 43 different mutations in 81 patients have been described (Finsterer and Zarrouk-Mahjoub, 2018). The clinical manifestations associated with *RRM2B* mutations are very heterogeneous both in terms of severity of symptoms and age of onset. Indeed as all mitochondrial diseases caused by mutations in genes involved in mtDNA maintenance, mutations in *RRM2B* generally can result in quantitative abnormalities (mtDNA depletion) associated with a severe and childhood-onset multisystemic disease (Bourdon *et al.,* 2007; Bornstein *et al.,* 2008; Kollberg *et al.,* 2009; Acham-Roschitz *et al.,* 2009; Stojanovic *et al.,* 2013; Kropach *et al.,* 2017), or in qualitative defects (accumulation of multiple mtDNA deletions) associated with a milder and adult-onset tissue-specific, such as PEO (Pitceathly *et al.,* 2012). The most frequent signs include weakness of extraocular muscle, ptosis, hypotonia, lactic acidosis and pediatric patients manifest in most cases failure to thrive and a severe respiratory distress requiring intubation and assisted ventilation. Unfortunately, in the latter case the disease progresses very quickly and in most cases causes death in a few months from the onset of symptoms.

The disease pathogenesis can be caused by mutations that affect residues involved in iron binding, crucial for the catalytic activity of the enzyme, residues essential for the conformation and stability of the active site, or residues that allow the interaction of R2 with R1 subunit, thus interfering with RNR assembly (Bourdon *et al.,* 2007; Bornstein *et al.,* 2008; Pitceathly *et al.,* 2011).

In our laboratory, the high degree of identity between human *RRM2B* and its yeast orthologs *RNR2* has been exploited to deepen in *S. cerevisiae* the phenotypic effects of several *RNR2* mutations corresponding to human pathological variants. The L362V mutation equivalent to the human mutation L317V was the alteration associated with a greater accumulation of mtDNA instability, increasing the percentage of *petite* by about 7-fold compared to wild-type strain (personal communication). This mutation changes Leu362 (hLeu317), that is not only a highly conserved residue between different species, but it is also located close to two other residues, Ser361 (hSer316) and Tyr376 (hTyr331), which are considered crucial for the interaction between p53R2

and R1 and therefore for the proper formation of the RNR complex (Bornstein *et al.,* 2008). Interestingly in our laboratory it has been demonstrated that *RNR1* overexpression, encoding R1 subunit, led to a significant reduction of the mtDNA instability in *rnr2*^{L362V} mutant strain. For this reason I decided to investigate whether the beneficial molecules identified for *MPV17*-related MDS were able to reduce the mtDNA instability even in *rnr2*^{L362V} mutant strain. The mtDNA instability analysis was performed in W303-1A*rnr2Δ*/pFL39*RNR2* and W303-1A*rnr2Δ*/pFL39*rnr2*^{L362V} grown at 37°C in SC supplemented with 2% glucose for 48 hours as described in Materials and Method. For every drug I tested different concentrations starting from the sub-MIC concentration. The **Figure 3.14** shows the results obtained. The analysis was repeated at least three times for concentrations where standard deviation is indicated.



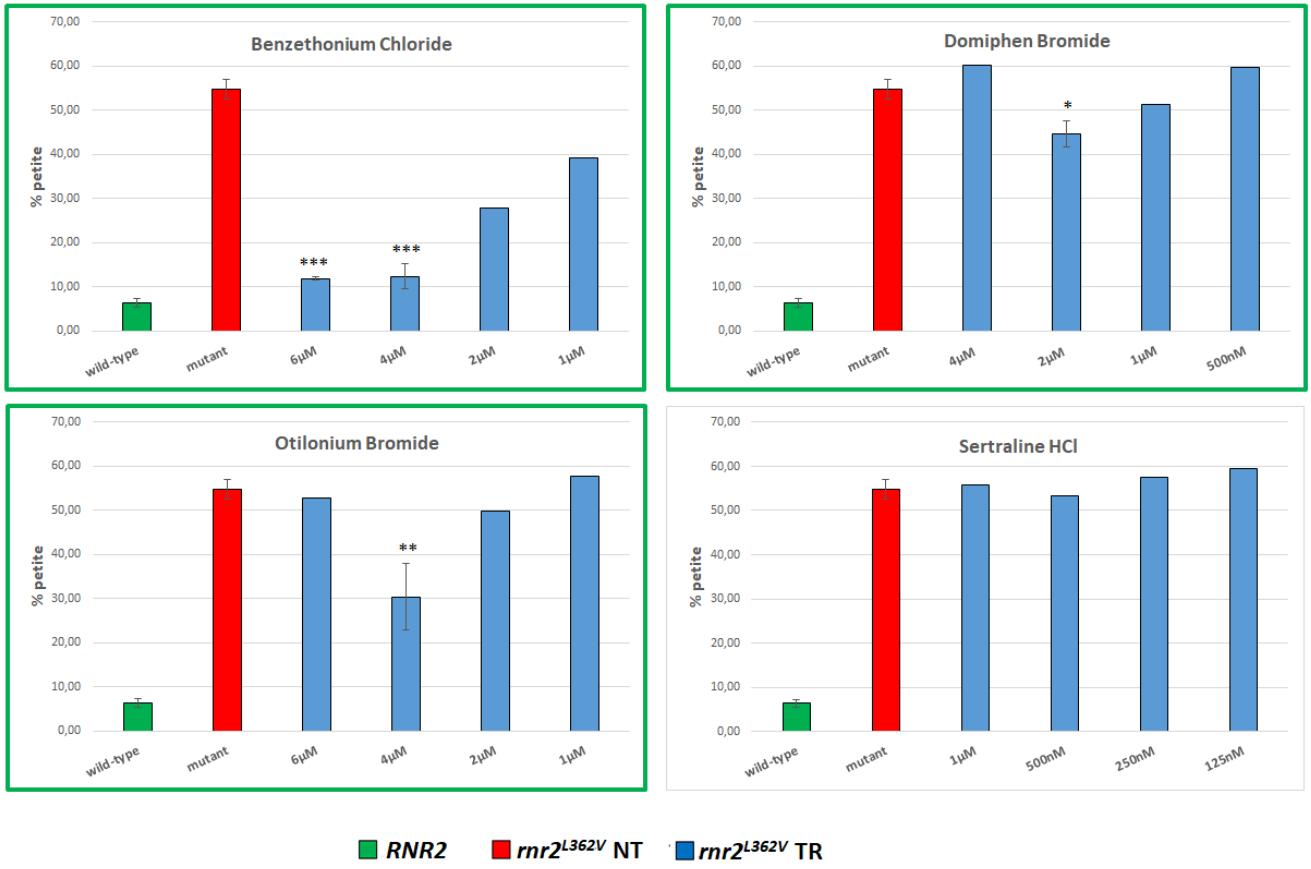


Figure 3.14. Determination of *petite* frequency of wild-type *RNR2* (green bar) and *rnr2*^{L362V} with (blue bars) or without (red bar) the supplementation of beneficial compounds. More than 4000 colonies per strain were scored. All values with error bar are means of at least three independent experiments. P values were obtained using Student's t test: *P<0.05; **P<0.01; ***P<0.001. The green boxes represent the active compounds.

The addition with almost all molecules, except Imazalil, Alexidine HCl and Sertraline HCl, led to a significant decrease of mtDNA instability in the *rnr2*^{L362V} mutant strain. In particular supplementation with Posaconazole, Fenticonazole Nitrate, Haloperidol, Thonzonium Bromide and Domiphen Bromide caused a reduction in the frequency of *petite* by about 30% compared to untreated strain. Otilonium bromide and especially Benzethonium chloride showed a more marked effect: indeed, the percentage of *petite* in the mutant treated with Otilonium Bromide was halved compared to the untreated mutant, and was brought to the levels of wild-type in the mutant treated with Benzethonium Chloride, thus resulting the most active compound. Moreover, all effective molecules were active at only one specific dosage, with the exception of Benzethonium Chloride, whose beneficial effect was evident in wider spectrum of concentrations. To deepen the effect of these molecules it would be interesting to understand if they act directly on the mutated protein (for example by stabilizing the protein or allowing its interaction with the R1 subunit, thus improving the affected catalytic activity) or indirectly through a compensatory mechanism.

3.7 DISCUSSION

Mitochondrial DNA depletion syndromes (MDS) encompass genetically and clinically heterogeneous group of autosomal-recessive mitochondrial disorders, characterized by a profound reduction in mtDNA copy number and consequent impaired energy production in affected tissues and organs (Suomalainen *et al.,* 2010; Nogueira *et al.,* 2014). Most of the MDS causative proteins are involved in the mtDNA replication machinery, such as Polg, or in mitochondrial nucleotide synthesis, such as p53R2. Besides mutations in these genes for which the mechanism linking mutations and mtDNA depletion is clear, mutations in the MPV17 gene were described as cause of an hepatocerebral form of MDS. Discovered about 30 years ago, MPV17 encodes a protein embedded in the IMM and whose role in mtDNA maintenance remains an open question. So far the leading hypothesis is that MPV17 deficiency might affect mitochondrial dNTP metabolism, leading to inadequate dNTP availability and consequent mtDNA depletion, thus placing MPV17-related disease in the group of mtDNA disorders caused by mt dNTP perturbation (Dalla Rosa *et al.,* 2016; Martorano *et al.,* 2019). Here I showed that even *in S. cerevisiae* deletion of the MPV17- ortholog, *SYM1*, resulted in a general decrease in all four mitochondrial nucleotides confirming yeast as a valuable model for the study of the molecular mechanisms underlying MPV17-related diseases. I also demonstrated that the nucleotide reduction is not limited to mitochondria but is extended to the entire cell compartment. As widely discussed above, a decrease of whole-cell dNTPs could be due to an aberrant energy production caused by an OXPHOS damage, but could also be subordinated to an impairment of TCA cycle. Coherently, supplementation with several amino acids, precursors of dNTPs synthesis and produced from TCA intermediates, rescued oxidative growth defect of *sym1Δ* (Dallabona *et al.,* 2010). Since yeast lacks deoxynucleoside *salvage* pathway making the mitochondrial dNTP pool entirely dependent on cytosolic dNTP transport, a reduction of the cytosolic dNTP pool can be reflected in a decrease of mt nucleotides.

To date, the treatment of MDS is mainly palliative based on the prescription of specialized diets and the administration of metabolic substrates, which provide symptomatic relief but do not stop the progression of the disease (El-Hattab *et al.,* 2017b). In recent years, supplementation of deoxyribonucleosides (dNs) has been proposed as a powerful strategy for the treatment of MDS caused either to defective nucleotide metabolism (such as RRM2B and MPV17-relatated diseases) or an impairment of mtDNA synthesis machinery (such as POLG-related disease) (Bulst *et al.,* 2012; Cámara *et al.,* 2014; Dalla Rosa *et al.,* 2016; Blázquez-Bermejo *et al.,* 2019). If the mtDNA maintenance defect is caused by a perturbation of nucleotide homeostasis, administration of

appropriate dNs depending on the genetic defect, can restore dNTP pool thus preventing depletion and restoring mtDNA copy number. If instead it is caused by defects in enzymes involved in mtDNA synthesis, dNs supplementation can at least partially compensate the defective catalytic activity of the enzyme. The response to dNs treatment depends however on the localization and type of mutation and consequently on the residual activity of the enzyme (Bulst *et al.,* 2012).

Investigation of putative therapeutic strategies for mitochondrial diseases can greatly benefit from the use of a simple, fast growing and low-cost organism like the yeast *S. cerevisiae*. Indeed, thanks to the development of a two-step yeast-based screening assay called "drug drop test", a large number of molecules can be analysed in short time. In our laboratory screening of two Chemical Library, performed on *sym1*^{R51W} mutant strain, led to the identification of ten potentially beneficial molecules for MPV17-related disease. Interestingly these drugs even rescued the oxidative growth defect of the *sym1Δ* mutant strain thus indicating that these drugs bypass Sym1 function (Gilberti, PhD thesis). Here I proved that all molecules were able to both determine a significant increase in the dNTP pool and to strongly improve the mtDNA stability, thus suggesting a decreased availability of DNA building block as the cause of the mtDNA maintenance defect in Sym1/Mpv17 deficiency.

In order to extend the potential use of these drugs to other MDS patients, I also evaluated the molecules effect on mtDNA stability of *mip1* (ortholog of human POLG) and *rnr2* (ortholog of human RRM2B) mutant strains for which it had already been demonstrated that *RNR1* overexpression or deletion of its inhibitor *SML1* led to a significant improvement in mtDNA stability.

The **Table 7** shows an overview of the effect of the compounds on mtDNA instability of *sym1Δ*, *mip1*^{G651S} and *rnr2*^{L362V} mutant strains.

	Rescue on mtDNA instability		
DRUG	***sym1Δ***	***mip1^{G651S}***	***rnr2^{L362V}***
Posaconazole	+++	n.e	+
Fenticonazole nitrate	+++	n.e	+
Imazalil	+++	++	n.e
Haloperidol	++	++	+
Thonzonium bromide	+	n.e	+
Alexidine HCl	+	n.e	n.e
Benzethonium chloride	+++	+	+++
Domiphen bromide	++	n.e	+
Otilonium bromide	++	+	++
Sertraline HCl	+++	++	n.e

Table 7. Effects of the molecules on mtDNA instability of sym1Δ, *mip1^{G651S}* and *rnr2^{L362V}* mutant strains measured as reduction of petite frequency. +++ Strong effect (greater than 50% reduction); ++ Medium effect (reduction between 50% and 30%); + Mild effect (less than 30% reduction); n.e no effect.

By comparing the results obtained in these different MDS models, it is possible to note that almost all molecules that had a beneficial effect in the *sym1Δ* cells were also able to determine a rescue of mtDNA instability in the *rnr2^{L362V}* mutant strain, whereas fewer molecules were effective for the *mip1* mutant strain. This different response of *mip1* and *rnr2* mutants to treatment with *sym1* beneficial molecules may be due to different pathomechanisms of *MIP1*/*RNR2*-related mtDNA instability. In-fact, whereas in *rnr2* mutant strain the reduction of mitochondrial genome stability is due to a limited availability of dNTPs, as in *sym1Δ* cells, in Mip1-deficient cells mtDNA instability is the result of a defective DNA polymerase. Therefore, in the *rnr2* mutant strain, an increase in the missing or insufficient nucleotides, determined by the addition of these molecules, may be sufficient to counteract the instability of mtDNA. Conversely, in the *mip1* mutant strain, increasing dNTPs amount may not be sufficient to bypass the catalytic defect of the enzyme and therefore not able to improve the stability of the mitochondrial genome. Whether in this mutant the beneficial effect on mtDNA exerted by Imazalil, Haloperidol, Sertraline HCl, and to a lesser extent by Benzethonium Chloride and Otilonium Bromide, was due to a dNTPs levels increase or an increase in polymerase stability/catalytic activity/fidelity remains to be investigated.

As already mentioned in the Introduction, all these molecules are FDA-approved and so their declared *safety* makes their use potentially translated into a treatment, even if some of them are more suitable then others. So far they are used for treatment of several kind of diseases including mycoses, bacterial infection, psychotic disorders, depression, irritable bowel syndrome. Of all these molecules the target in mammals is known although not in all cases their mechanism of action is clear. In yeast some of these targets do not exist, thus suggesting that the biological activity of these drugs in yeast is probably due to action on one or more secondary targets.

Drug	Target in yeast	Target in mammals
Posaconazole	Lanosterol 14 alpha-demethylase (ergosterol pathway)	Lanosterol 14 alpha-demethylase (cholesterol pathway)
Fenticonazole nitrate	Lanosterol 14 alpha-demethylase (ergosterol pathway)	Lanosterol 14 alpha-demethylase (cholesterol pathway)
Imazalil	Lanosterol 14 alpha-demethylase (ergosterol pathway)	Lanosterol 14 alpha-demethylase (cholesterol pathway)
Haloperidol	Sterol C8-C7 isomerase (ergosterol pathway)	Dopamine receptors; sigma-1 receptor; 3-beta-hydroxysteroid Δ8,Δ7 isomerase (cholesterol pathway)
Thonzonium bromide	Vacuolar ATPase proton transporter	Vacuolar ATPase proton transporter
Alexidine HCl	Vacuolar ATPase proton transporter	Vacuolar ATPase proton transporter
Benzethonium chloride	unknown	HERG K-channel
Domiphen bromide	unknown	HERG K-channel
Otilonium bromide	unknown	Muscarinic receptor, VD Ca-Channel, NK receptor
Sertraline HCl	Phospholipids membranes	Serotonin 5-HT transporter

Table 8. Beneficial molecules found through drug drop test on *sym1*^{R51W} mutant strain and their targets in yeast and mammals (adapted from Gilberti, PhD thesis).

Interestingly Posaconazole, Fenticonazole nitrate and Imazalil, belonging to the class of azoles with antifungal activity, inhibit the CYP51 enzyme, encoded by *ERG11* gene (lanosterol 14 alpha-demethylase) a key enzyme involved in the biosynthesis of ergosterol. Sterols are the most important hydrophobic lipids of eukaryotic cell membranes, playing a central role in many biological

processes such as endocytosis, (Souza and Pichler, 2007), stabilization of membrane proteins (Gimpl and Fahrenholz, 2002) and they are undoubtedly key molecules for maintaining proper membrane permeability and fluidity so much to be defined as "membrane dynamic regulators" (Dufourc, 2008). The major fungal sterol is ergosterol which is particularly abundant in the plasma membrane and secretory vesicles (Zinser *et al.,* 1993). Much of the mitochondrial ergosterol is contained in the IMM membrane, whereas other sterols are present in the OMM (Zinser *et al.,* 1991). Interestingly, also Haloperidol, used in human therapy as antipsychotic medication thanks to its strong antagonism of the D2 dopamine receptor (Dold *et al.,* 2015), in yeast acts on the ergosterol pathway inhibiting both C8-C7 sterol isomerase, encoded by *ERG2* gene, and C-14 reductase, encoded by *ERG24* gene, thus reducing ergosterol content (Moebius *et al.,* 1996; Lum *et al.,* 2004). Given its important role as a modulator of membrane properties, a severe reduction in ergosterol levels, due to *ERG* genes deletion or to the presence of inhibitors of sterol biosynthesis (e.g azoles), is deleterious for the yeast cell (Rodriguez *et al.,* 1985; Parks and Casey, 1995). However, the fact that addition of Posaconazole, Fenticonazole Nitrate, Imazalil and Haloperidol were able to rescue mtDNA instability of *sym1Δ* mutant strain, and that some of them also had positive effects on *mip1* and *rnr2* mutant strains, suggests that modulation of ergosterol levels can lead to a beneficial effect in mutant cells. In *sym1* null mutant strain it is reasonable to speculate that a moderate reduction of ergosterol content could increase the membrane permeability thus allowing transmembrane flows that are impaired in these mutant cells and so rescuing the mitochondrial defects (Gilberti, PhD thesis). Sym1/Mpv17 encodes, in fact, a channel protein with gating properties under the regulatory control of a combination of several factors reflecting mitochondria conditions and it has been hypothesized that a transient opening of the channel may be beneficial for mitochondrial homeostasis (Antonenkov *et al.,* 2015). In mammalian cells cholesterol performs many of the functions that ergosterol performs in fungal cells membranes (Weete *et al.,* 2010), being a modulator of the bilayer structure of mammalian membranes and affecting several membrane proteins such as ion channels (e.g Ca^{2+}, channels), transporters of several molecules as adenine nucleotides (Haslam *et al.,* 1977), 2-oxoglutarate, glutathione (Coll *et al.,* 2003) citrate (Parlo and Coleman, 1984), phosphate (Paradies *et al.,* 1992), pyruvate (Paradies *et al.,* 1999). Therefore, also in human cells characterized by MPV17 deficiency a moderate reduction of cholesterol could have beneficial effects. Currently statins are used as cholesterol-lowering drugs for cholesterol-related diseases. However due to their side effects, their use is unachievable to treat MPV17-related disease that require long-term administration. Indeed, these drugs, inhibiting HMG-CoA reductase, the rate-limiting enzyme of the

mevalonate pathway, prevent the biosynthesis of other biologically important substances derived from mevalonate (**Figure 3.15**).

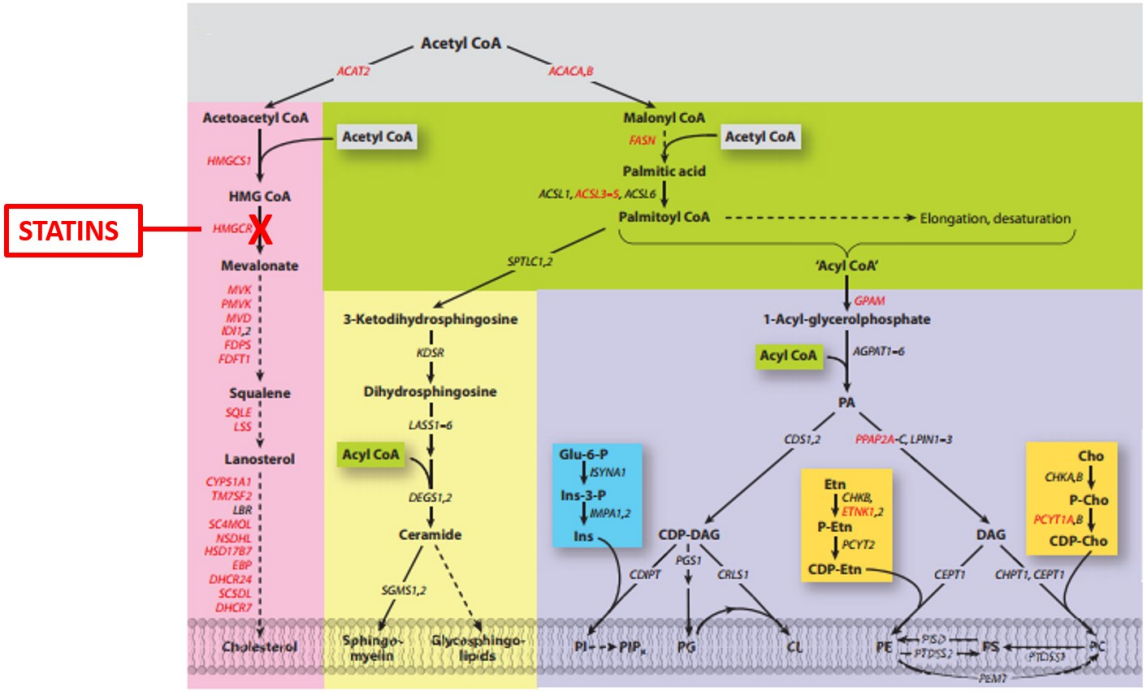


Figure 3.15. Main pathways for membrane lipid synthesis. (modified from Nohturfft and Zhang, 2009).

The human *CYP51A1*, orthologus gene of yeast *CYP51*, could be a more specific molecular drug target because its moderate inhibition could specifically reduce cholesterol synthesis. In spite of a high degree conservation between fungal and human enzyme, to date many of the Cyp51 inhibitors have exhibited low affinity for the human enzyme. Indeed, they are used as antifungal agent in human therapy. For an effective treatment of the disease associated with mutations in MPV17 specific inhibitors of the activity of the human enzyme should therefore been identified. Recently through comparative structure/functional studies of CYP51 orthologs from different biological kingdoms it was possible to analyse the structural features that make the human enzyme resistant to inhibition and on the basis of these observations it was possible to synthesize potent stoichiometric and functionally human Cyp51 inhibitors: (R)-N-(1-(3,4'-difluorobiphenyl-4-yl)-2-(1H-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide (VFV), (R)-N-(1-(3-Chloro-4'-fluoro-[1,1'-biphenyl]- 4-yl)-2-(1H-imidazol-1-yl)ethyl)-4-(5-(3-fluoro-5-(pyridin-2-yl)- phenyl)-1,3,4-oxadiazol-2-yl) benzamide and (R)-N-(1-(3-Chloro-4'-fluoro-[1,1'-biphenyl]-4-yl)-2-(1H-imidazol-1-yl)ethyl)-4-(5-(3-fluoro-5-(5-fluoropyri-midin-4-yl)phenyl)-1,3,4-oxadiazol-2-yl)benzamide (Hargrove *et al.,* 2016; Friggeri *et al.,* 2019). It would be interesting to test these compounds in animal models or in human cells to define whether their supplementation may become an

achievable approach for therapy of MPV17. Since some of the molecules acting on the ergosterol patwhay had a positive effect also on the *mip1* and *rnr2* mutant strains it would be interesting to test these new compounds also in animal models or human cells characterized by POLG and RRM2B deficiency. The fact that not all molecules targeting the ergosterol pathway are able to rescue the mtDNA stability in *mip1* and *rnr2* deficient cells may be due to the use of unsuitable concentrations. In fact, as discussed before, a drastic reduction or especially depletion of ergosterol has deleterious effect on yeast. Therefore, identifying the optimal working concentration is not so obvious and it is reasonable to speculate that the rescuing concentrations of Posaconazole and Fenticonazole nitrate in *mip1* and of Imazalil in *rnr2* mutant strain have not been yet identified. Further analysis will be required to solve this issue.

Regarding the other molecules, Alexidine HCl and Thonzonium Bromide are generally used as antiseptic in mouthwashes and detergent, respectively. For both compounds antifungal activities have been reported (Siles *et al,* 2013; Mamouei *et al.,* 2018). In particular, they act inhibiting the vacuolar V-ATPase proton pump that contributes to regulating the cytosolic pH, by pumping cytosolic protons into intracellular organelles (Chan *et al.,* 2012). Interestingly impairment of V-ATPase lead to phenotypic defects similar to those observed in *ERG2* mutants (Serrano *et al.,* 2004). Based on these observations, one intriguing hypothesis about the beneficial effect of these two compounds in *sym1Δ* cells is that inhibiting the V-ATPase they could induce, beyond a cytosolic acidification, an alteration of the content of ergosterol in the mitochondrial membrane, thus enabling flows that are impaired in *sym1* null mutant cells. To evaluate this hypothesis it might be interesting to measure ergosterol content in *sym1*-deficient cells in the absence and presence of Alexidine HCl and Thonzonium Bromide. Notably Alexidine HCl was the less effective molecule both on mtDNA stability and mt dNTP pool in *sym1Δ* mutant and this could also explain the absence of beneficial effect in the *rnr2* mutant strain.

Benzethonium Chloride and Domiphen Bromide are synthetic quaternary ammonium with a wide spectrum of antimicrobial activities. Both are also powerful inhibitor of human HERG potassium channels (Long *et al* 2013; Long *et al.,* 2014). There is little information about their action in yeast. Interestingly, cationic surfactant as Benzethonium Chloride induce membrane perturbation in yeast, probably by intercalating into the lipid bilayer (Schreier *et al.,* 2000; Uesono *et al.,* 2008). Benzethonium Chloride was one of the most active molecules in the *sym1Δ* mutant strain and strongly improved mtDNA instability even in *rnr2* mutant cells. Whereas in *sym1*-deficient cells, the beneficial effect exerted by this molecule could always be attributed to a change in membrane

permeability, in the *rnr2* mutant strain it would be interesting to investigate the effect of this molecule to understand if it can act directly on the protein, favoring its stability or allowing the interaction with the Rnr1 subunit, or through a compensatory mechanism. Benzethonium Chloride showed a beneficial effect, although less strong, even in the *mip1* mutant.

The remaining two molecules, Sertraline HCl and Otilonium Bromide were identified as beneficial also for the *aac2* mutant strain. As widely discussed before, Sertraline HCl belongs to the class of drugs called CADs, cationic amphiphilic drugs that interact with phospholipid membranes (Halliwell, 1997). Given its ability to induce membrane ultrastructural changes in yeast, it is reasonable to speculate that even this drug may favour flows in *sym1Δ* mutant cells. Interestingly enough Sertraline HCl was one of the most effective molecules in both the *sym1Δ* and *mip1*^{G651S} mutants but did not improve mtDNA stability in *rnr2*^{L362V} mutant strain. One explanation can be that this drug could act only if the Rnr2 protein is completely functional. Analysing the effect of Sertraline HCl on dNTP pool of *RNR2* wild-type and *rnr2* mutant strains could be a good strategy to solve this issue.

Otilonium Bromide is commonly used in human therapy as antispasmodic drug for the treatment of Irritable Bowel Syndrome. Its beneficial effect is mediated by a complex mechanism of action that involves interaction of this drug with several cellular targets (Muscarinic receptor, VD Ca-Channel, NK receptor) (Evangelista *et al.,* 2018). However, nothing is known about the target in yeast.

Certainly more studies will be needed to further investigate the mechanism of action of some of these molecules in yeast. Moreover, being yeast a unicellular organism, it will be very useful to demonstrate the beneficial effect of these drugs at the multi-organ level modelling the same mitochondrial dysfunction in invertebrate (*Caenorhabditis elegans*) or vertebrate animal models (*Danio rerio*) and in cells derived from patients. However, the capability of these molecules to reduce the mtDNA instability in different MDS-yeast models, and their declared safety for the use in humans make them good candidates for the treatment of MPV17-POLG-RRMB2-related MDS moving from yeast into translational research.

4. Materials and Methods

4.1 Strains used:

S. cerevisiae strains used in this work are reported below:

Strain	Genotype	Reference
W303-1B	*Matα ade2-1 leu2-3 ura3-1 trp1-1 his3-11 can1- 100*	Thomas and Rothstein, 1989
W303-1B ρ^0	*Matα ade2-1 leu2-3 ura3-1 trp1-1 his3-11 can1- 100* ρ^0	Our laboratory
WB12/pFL38 *AAC2*	*Matα ade2-1 trp1-1 ura3-1 can1-100 aac1 :: LEU2 aac2 :: HIS3*	Fontanesi *et al.,* 2004
WB12/pFL38 *AAC2*^{M114P}	*Matα ade2-1 trp1-1 ura3-1 can1-100 aac1* :: LEU2 *aac2 :: HIS3*	Fontanesi *et al.,* 2004
W303-1B/pFL38 *AAC2*	*Matα ade2-1 leu2-3,112 ura3-1 his3-22,15 trp1-1 can1-100 AAC1 AAC2*	Fontenesi *et al.,* 2004
W303-1B/pFL38 *AAC2*^{M114P}	*Matα ade2-1 leu2-3,112 ura3-1 his3-22,15 trp1-1 can1-100 AAC1 AAC2*	Fontenesi *et al.,* 2004
W303-1B *cab1Δ*/pFL39 *CAB1*	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 cab1::kanMX4*	Ceccatelli Berti, PhD thesis
W303-1B *cab1Δ*/pFL39 *CAB1*^{N290I}	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 cab1::kanMX4*	Ceccatelli Berti, PhD thesis;
BY4741	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al.,* 1998
BY4741 ρ^0	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ρ^0	Gilberti, PhD thesis
BY4741 *pos5Δ*	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pos5::kanMX4*	Euroscarf collection; Brachmann et al., 1998
BY4741 *sym1Δ*	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sym1::kanMX4*	Euroscarf collection; Brachmann *et al.,* 1998
BY4741 *sml1Δ*	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sml1::kanMX4*	Euroscarf collection; Brachmann et al., 1998
BY4741 *sym1Δ sml1Δ*	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sml1::kanMX4 sym1::HigR*	Gilberti, PhD thesis
BY4741 *sym1Δ*/pFL38 *SYM1*	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sym1::kanMX4*	Gilberti, PhD thesis
BY4741 *sym1Δ*/pFL38	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sym1::kanMX4*	Gilberti, PhD thesis
DWM-5A *mip1Δ*/pFL39 *MIP1*	*Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11 can1- 100 mip1::KanR*	Baruffini *et al.,* 2007
DWM-5A *mip1Δ*/pFL39 *mip1*^{G651S}	*Matα ade2-1 leu2-3,112 ura3-1 trp1-1 his3-11 can1- 100 mip1::KanR*	Baruffini *et al.,* 2007
W303-1A *rnr2Δ*/pFL39 RNR2	*Mata ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rnr2::kanMX4*	Our laboratory
W303-1A *rnr2Δ*/pFL39 *rnr2*^{L362V}	*Mata ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 rnr2::kanMX4*	Our laboratory

4.2 Plasmid used:

Plasmid	Marker in *S.cerevisiae*	Type	Reference	Figure
pFL38	URA3	Centromeric	Bonneaud *et al.*, 1991	4.1
pFL39	TRP1	Centromeric	Bonneaud *et al.*, 1991	4.2

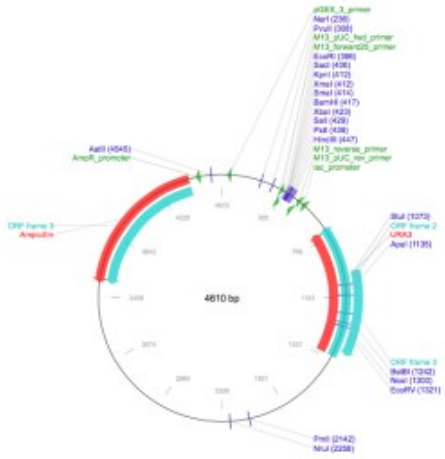


Figure 4.1. pFL38 plasmid

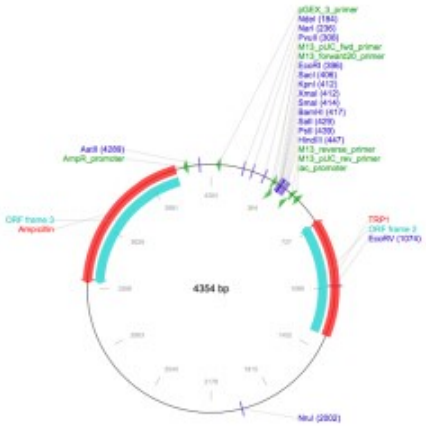


Figure 4.2. pFL39 plasmid

4.3 Media and growth conditions

For *S. cerevisiae* growth the following media were used:

- **Rich medium YP**: 0.5% yeast extract, 1% bactopeptone
- **Minimal medium YNB**: yeast nitrogen base ForMedium™ without aminoacids without 1,9 g/L NH_4SO_4, 5 g/l NH_4SO_4
- **Synthetic Complete medium SC:** YNB media enriched with drop-out powder (Kaiser *et al.* 1994). If necessary singles amino acids could be excluded from complete drop-out powder to maintain selective pressure.
- **Semisynthetic lactate medium:** 3 g/litre yeast extract, 0.5 g/litre glucose, 0.5 g/litre CaCl2·2H2O, 0.5 g/litre NaCl, 0.9 g/litre $MgCl_2 \cdot 6H_2O$, 1.0 g/liter KH_2PO_4, 4 g/litre NH_4Cl, 24 ml of 85% DL-lactic acid and 8 g/litre NaOH with pH adjusted to 5.5 with NaOH (Wheeler and Mathews, 2012);

One or more carbon source at different concentrations were added in each medium. Glucose (D) and ethanol (E) were used. If necessary medium was solidified by adding Agar ForMedium™ 2%.

Yeast cultures were grown at 28°C in constant shaking 120 rpm if liquid media was used. To induce heat stress, cultures were incubated at 37°C in thermostat or water bath.

4.4 Phenotypic analysis

4.4.1 Determination of the minimal inhibition concentration (MIC)

MIC had already been previously determined for *SYM1* cells (Gilberti, PhD thesis).

AAC2, MIP1, RNR2: strains were pregrown at 28°C in SC liquid medium supplemented with 2% glucose and then inoculated at a final concentration of 0.05 OD_{600}/ml in YP or SC supplemented with 2% glucose and molecules at different concentrations starting from 512 µM and sequentially halved. Cultures were incubated at 28°C or 37 °C in constant shaking and growth was determined after 24 hours by measuring absorbance at 600 nm. MIC was defined as the dosage at which no growth was detectable. The drugs of the Selleck chemical library and Prestwick chemical library were dissolved in dimethyl sulfoxide (DMSO), so as control cells were treated also with this compound vehicle.

4.4.2 Mitochondrial respiration

Oxygen consumption rate was measured at 28°C using a Clark-type oxygen electrode (Oxygraph System Hansatech Instruments England). Cells were grown for 18 hours at 28˚C (or 16 hours at 37°C) in YP or SC medium supplemented with 0.6% glucose in presence of molecules or DMSO (as a control). Respiratory rate was measured after the total exhaustion of glucose by adding in the oxigraph chamber 50 µl of cells, 850 µl of air-saturated respiration buffer (0,1M phthalate-KOH, pH 5,0) and 100 µl 5% glucose. Values were normalized to the wild type strain. Statistical analysis was performed on the values before the normalization through unpaired, two-tailed Student's t test. Only p values < 0.05 were considered statistically significant *p<0.05, **<0.01, ***p<0.001.

4.4.3 Mitochondrial DNA mutation frequency

SYM1: analysis was performed as previously described (Gilberti, PhD thesis).

AAC2-MIP1-RNR2: cells were pregrown at 28°C in SC medium supplemented with 2% ethanol for 48 hours to counterselect the *petite* cells. Then cells were inoculated at a final concentration of 0.02 OD_{600}/ml in YP or SC liquid medium supplemented with 2% glucose and in presence of molecules or DMSO (as a control). After 24 hours of growth at 28°C or 37°C, cells were inoculated as before and incubated again for 24 hours at 28°C or 37°C. Then cells were plated on SC agar plates supplemented with 2% ethanol and 0.4% glucose to a final concentration of 200–250 cells/plate. *Petite* frequencies were defined as the percentage of colonies showing the *petite* phenotype after 4-5 days incubation at 28°C (Baruffini *et al.,* 2010). Statistical analysis was performed through unpaired, two-tailed

Student's t test. Only p values < 0.05 were considered statistically significant *p<0.05, **<0.01, ***p<0.001.

4.4.4 Fitness test ρ^+/ρ^0

W303-1B ρ^+ and W303-1B ρ^0 were pregrown in SC liquid medium added with 2% glucose and then were inoculated at the final concentration of 0.05 OD_{600}/ml (50% ρ^+ and 50% ρ^0) in SC medium supplemented with 2% glucose in the presence of molecules or DMSO (as control). After 24 hours of growth at 28 °C cells were plated on SC agar plates supplemented with 2% ethanol and 0.4% glucose to a final concentration of 200–250 cells/plate to determine the ratio of respiratory sufficient (ρ^+) and *petite* (respiratory deficient) colonies.

4.4.5 ROS quantification:

Cells were pregrown at 28°C in SC liquid medium supplemented with 2% glucose and then inoculated at a final concentration of 0.1 OD_{600}/ml in YP supplemented with 0.6% glucose and if necessary added of molecules or DMSO (as a control). After 18 hours of growth at 28 °C samples were incubated with 1.25 µg/ml of dihydrorhodamine 123 (DHR123; Sigma-Aldrich) for 2 hours at 28 °C. Then cells were centrifuged (30 seconds at 14000 rpm) and resuspended in H_2O and the fluorescence was quantified by NovoCyte flow cytometer. An unstained sample (without DHR123) was prepared as a negative control to set the threshold index and thus delimiting an auto-basal fluorescence area and positive fluorescence. Oxidation of DHR123 by ROS (mainly H_2o_2) produces green fluorescent R123 (excitation/emission spectra of 488/530 nm) detected by the fluorescence channel (FL-1) with a 530/30 nm band pass filter. For each sample 10,000 cellular events were analysed. Data achieved from flow cytometer were analyzed using NovoExpress software. ROS generation was measured as the percentage of fluorescent cells (PFC), corresponding to cells that produced ROS level increments of at least one log unit (Gomez *et al.,* 2014). Statistical analysis was performed through unpaired, two-tailed Student's t test. Only p values < 0.05 were considered statistically significant *p<0.05, **<0.01, ***p<0.001.

4.4.6 Mitochondrial Membrane Potential (MMP) determination:

Cells were grown in the same conditions described for ROS quantification. After 18 hours of growth at 28 °C samples were loaded with 0.05 µM of the 3,3P-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma-Aldrich) for 30 min at 28 °C. Then cells were centrifuged (30 seconds at 14000 rpm) and resuspended in H_2O and the fluorescence was quantified by NovoCyte flow cytometer. An unstained sample (without $DiOC_6$) was prepared as a negative control. $DiOC_6$ fluorescence (excitation/emission

spectra of 484/501 nm) was monitored by the fluorescence channel (FL-1) with a 530/30 nm band pass filter. Data achieved from flow cytometer were analyzed using NovoExpress software. The geometric mean fluorescence intensities of 10,000 cells were used to calculate relative MMP (Miceli *et al.,* 2012). Values were normalized to the wild type strain. Statistical analysis was performed through unpaired, two-tailed Student's t test, before the normalization. Only p values < 0.05 were considered statistically significant *p<0.05, **<0.01, ***p<0.001.

4.5 Nucleotide pools extraction

4.5.1 Whole-cell Dexoxyribonucleoside Triphosphate Pool (dNTPs) extraction

Cells, both wild-type and null-mutant *sym1Δ*, were pregrown at 28°C in SC supplemented with 2% ethanol to counterselect the *petite* cells that could be present in the population. Subsequently, cells were exponentially grown in SC supplemented with 2% glucose and transferred to SC medium supplemented with 0.6% glucose and 2% ethanol at 37°C for 24 h. A total of $2x10^8$ cells were harvested by centrifugation (5 min at 6000 rpm) and nucleotide extracts were prepared by methanol extraction and boiling according to method reported (Mathews and Wheeler 2009). The harvested cells were re-suspended in 1 ml of 60% cold-methanol, then vortexed for 30 seconds and incubated for 2 hours at -20°C. They were subsequently heated at 99 °C for 3 minutes to destroy residual enzymatic activity of nucleases and nucleotide kinases, which could interfere with the enzymatic dNTP assay, and then centrifuged at 17.000 g for 15 minutes. Extracts were dried for almost 3 hours using a SpeedVac concentrator and the residues re-suspended in 50µl of cold deionized water and later used for quantification of the four dNTPs by the DNA polymerase assay (see below).

4.5.2 Mitochondria extraction and separation of mitochondrial and cytosolic dNTPs

Working conditions until the first centrifuge were the same as described in paragraph 4.5.1. After two water washes, each pellet was re-suspended in 400 µl of 0.6 M sorbitol and 5 mM dithiotrehitol (DTT), then incubated for 20 minutes at room temperature. Samples are centrifuged at 7.000 rpm for 30 seconds and pellet re-suspended in 400 µl of 1,2 M sorbitol, 10 mM Tris-HCl pH 7,5 and zymoliasi 20T (12mg/ml); enzymatic digestion can be carried on and digestion of cell wall was monitored at Cary spectrophotometer at 800 nm. Meanwhile extracts were kept in a Thermoblock at 29°C and digestion was stopped when an 80-90% decrease in optical density (OD) was observed. All subsequent steps should be carried out in ice. To separate the cytosolic nucleotides from the mitochondrial one differential centrifugation were performed as described by Pontarin *et al.,* 2003. In particular, spheroplasts formed after enzymatic digestion were centrifuged for 25 minutes at 17.000 g at 4 °C to separate nuclei and mitochondria from the cytosol. The obtained pellet (nuclei

and mitochondria) was washed with 0.5 ml of extraction buffer (0.6 M Sorbitol, 20 mM Tris-HCl pH 7,5, 1mM EDTA) and then centrifuged as above. This washing step is necessary to remove any remaining cytosolic dNTPs from the nuclei. The pellet, now containing mt dNTP pool, was re-suspended in 50 µl of extraction buffer, followed by quantification of protein concentration by Bradford's method (Bradford, 1976). Measurements of mt dNTP pool is reported as the amount of dNTPs (in picomoles,) per 50 µg of protein. Subsequently, a centrifugation at 4 °C at 17.000 g for 25 minutes was performed, and the resulting pellet was re-suspended in 1 ml of ice-cold 60% methanol and incubated at -20 °C for 2h. The remaining steps are the same as the one used for whole-cell dNTP pool (reported in paragraph 4.5.1), except for the volume in which extracts were re-suspended, that was 10 µl of cold de-ionized water.

4.6 Protein quantification

Bradford assay is a colorimetric procedure used to measure the concentration of protein in a solution (in this case, proteins in yeast samples). We used Bio-Rad protein assay following the manufacturer's instructions. In our experiments, dilution of the protein sample of 1:200 was required for the resulting absorbance to fall within the linear range of the assay. For each sample 200 µl of Bradford reagent (an acidified solution of Coomassie G-250 dye) was added in a total volume of 1 ml. Extracts were left at room temperature for 15 minutes and absorbance where measured with a Cary spectrophotometer at λ = 595nm.

4.7 DNA polymerase assay

To quantify dNTP pool, both in *SYM1* and *sym1Δ* extracts, a DNA polymerase assay was performed (Roy *et al.,* 1999). Thanks to this method, subsequently to dNTPs extraction of both pools (whole-cell and mitochondrial) it is possible to simultaneously quantify purines or pyrimidines in excess of, respectively, dTTP or dATP, through a reaction catalysed by Klenow fragment of *Escherichia coli* DNA polymerase I (BioLabs DNA Polymerase I, Large (Klenow) Fragment). Two primers labelled at 5'-end with DY682 fluorophore, annealed to their complementary oligonucleotide, were used:

- p13-mer for the determination of dTTP and dATP;
- p27-mer for the determination of dCTP and dGTP.

Different length of the two primers and of the four templates (see **table 9**) allows elongations products to do not overlap one another after a separation on 12,5% polyacrylamide-urea gel.

dNTP quantified	Primer/ template	Oligonucleotide sequence
dTTP	p13 tT	5' TCG CAG CCG TCC A 3' 3' AGC GTC GGC AGG T**AT TAT TAT T** 5'
dCTP	p27 tC	5' GGT AGG GCT ATA CAT CGC AGC CGT CCA 3' 3' CCA TCC CGA TAT GTA GCG TCG GCA GGT **GTT GTT GTT** 5'
dATP	p13 tA	5' TCG CAG CCG TCC A 3' 3' AGC GTC GGC AGG T**AA TAA TAA TAA** 5'
dGTP	p27 tG	5' GGT AGG GCT ATA CAT CGC AGC CGT CCA 3' 3' CCA TCC CGA TAT GTA GCG TCG GCA GGT **CAA CAA CAA** 5'

Table 9: combinations of primers/templates (p/t) utilized for dNTPs quantifications.

4.7.1 Template-primer annealing

Single strand templates were mixed with primers in a molar ratio of 2:1 in order to obtain double-stranded oligonucleotides (dimers). Reactions were performed in 80 mM Tris–HCl pH 7.8 and 40 mM NaCl, heated at 85°C for 5min and then allowed to slowly cool to 26°C (a decrease of 1°C for each cycle). Obtained dimer were stored at -20°C.

4.7.2 Reaction mix for DNA polymerase assay

The enzymatic assay was carried out using a 4:1 molar ratio of unlabelled dimer to labelled dimer.

Reaction conditions for pyrimidines triphosphates determination:

Reaction mix components	**Quantities required**
p13/tT	3,6 pmol
p13-DY682/tT	0,9 pmol
p27/tC	3,6 pmol
p27-DY682/tC	0,9 pmol
Klenow fragment	0,6 U
10X Klenow buffer	50 mM NaCl 10 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9@25°C
dATP (exceeding)	150 pmol
Whole-cell or mitochondrial extract	5µl or 10 µl

Table 10: reaction mix for pyrimidine triphosphate determination.

Reaction conditions for purine triphosphates determination:

Reactions mix components	**Quantities required**
p13/tA	3,6 pmol
p13-DY682/tA	0,9 pmol
p27/tG	3,6 pmol
p27-DY682/tG	0,9 pmol
Klenow fragment	0,6 U
10 X Klenow buffer	50 mM NaCl 10 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9@25°C
dTTP (exceeding)	150 pmol
Whole-cell or mitochondrial extract	5µl or 10 µl

Table 11: reaction mix for purine triphosphate determination.

In both reactions mix, final volume of 30 µl was obtained with addition of deionized water. Lastly, reactions were performed at 26°C for 20 min and stopped by addition of an equal volume of cold stop-dye solution (80% formamide, 5mM EDTA.)

4.8 Denaturing Urea Polyacrylamide Gel Electrophoresis (Urea Page)

To separate and view the elongated products derived from DNA-polymerase assay, a 12,5% polyacrylamide – 7 M urea gel was prepared. Separation is based on molecular weight of nucleic acids and denaturation is given by urea. Fragments ranging from 2-500 bases, which differ from each other even by a single nucleotide, can be separated with this method. These gels are therefore ideal to analyse our elongated products.

4.8.1 Gel preparation and pre-run

The apparatus used for gel assembly is composed by:

- glass plates: 14 x 20 cm (inner plate), 14 x 22 cm (outer plate);
- 1 mm gel comb
- 1 mm spacers.

For a denaturing acrylamide gel of these dimensions, 30 ml of gel solution is sufficient:

Reagents	Quantity
Urea	12,6 g
40% Acrylamide (29:1)	9,375 ml
10% APS	300 µl
TEMED	12 µl
5x TBE	6 ml
Water	to fill volume

Table 7: reagents and solution required for preparation of 30ml of 12,5% urea-PAGE gel.

To facilitate polymerization a heater at 28°C was used. When gel was solified, it was positioned in the electrophoretic apparatus with TBE 1X as running buffer and it was pre-heated for 30 minutes to heat the gel and remove possible fragments of urea.

4.8.2 Sample preparation and run

Before the samples can be loaded on the gel, they had to be boiled at 95°C for 4 minutes. 0,21 pmol of each sample was loaded and loading buffer (80% formamide, 5 mM EDTA, 0,1% xylene cyanol and 0.1% bromophenol blue) was added to empty pockets to maintain similar conditions during the run, which was performed at 16 W until the bromophenol blue reached the lower end of the gel (about 2 hours).

4.8.3 Gel visualization and analysis

Apparatus was disassembled and gel was subsequently visualized at ChemiDoc MP Imaging System (BioRad s.r.l.) in the far red spectrum/DyLight680 setting (excitation: 650-675 nm, emission: 700-730nm), optimal for the fluorophore DY682.

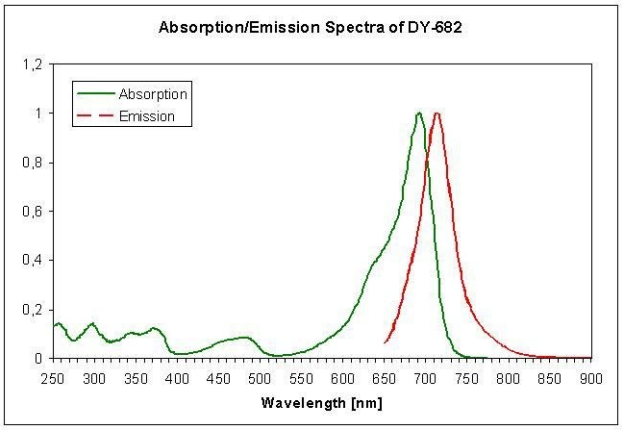


Fig. 4.1: absorption and emission curves of the fluorophore DY682.

The amount of fluorescence of each band was later quantified with Image Lab Software (BioRad s.r.l.) and since the number of moles of the elongated products is proportional to the observed fluorescence intensity, to determine the quantity of dNTP incorporated in the primer/template the following formula (Roy *et al.,* 1999) was used:

pmol dNTP incorporated = pmol primer/template

$$[\sum_{i=1}^{3} x_1 + 2\sum_{i=4}^{6} x_1 + 3\sum_{i=7}^{9} x_1]/[x_0 + \sum_{i=1}^{9} x_1]$$

in which

X_0*:* represents the fluorescence intensity of non-elongated product;

X_i*:* represents the fluorescence intensity of elongated product.

Quantity of dNTPs in each sample was obtained by interpolation from the calibration curves previously set up with standard quantity of dNTPs. Statistical analysis was performed through unpaired, two-tailed Student's t test. Only p values < 0.05 were considered statistically significant *p<0.05, **<0.01, ***p<0.001.

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