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Cholesterol and mitochondrial involvement in Rett Syndrome pathogenesis

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To you, who believed it was possible.

And made it possible.

Index

Abbreviations

ASD	Autism Spectrum Disorder
BBB	Brain blood barrier
CE	Cholesterol Ester
CNS	Central nervous system
2,4-DNP	2,4-Dinitrophenol
DRP1	Dynamin-related protein 1
FCCP	Carbonilcyanide p-trifluoromethoxyphenylhydrazone
FIS1	Mitochondrial fission 1 protein
HDL	High Density Lipoprotein
IMM	Inner mitochondrial membrane
LDL	Low Density Lipoprotein
Lp-PLA2	Lipoprotein associated phospholipase A2
MECP2	Methyl-CpG-binding protein 2
MFN1	Mitofusin-1
MFN2	Mitofusin-2
MTS	Mitochondrial targeting signal
OxS	Oxidative Stress
OMM	Outer mitochondrial membrane
PARKIN	Parkin E3 Ubiquitin Protein Ligase
PINK-1	PTEN-induced kinase 1

PON-1 Paraoxonase 1

RCT Reverse cholesterol transport

ROS Reactive Oxygen Species

RTT Rett Syndrome

SR-B1 Scavenger Receptor class B type 1

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ABSTRACT

Rett Syndrome (RTT) is an X-linked neurodevelopmental disorder that primarily affects females, associated in 95% of cases with loss-of-function mutations in MECP2 gene, encoding for methyl-CpG-binding protein 2 (Mecp2). Even if the pathology was ascribed to the gene mutation many years ago and enormous scientific progress have been done, the precise pathologic mechanism that lead from MECP2 mutations to RTT manifestation is still a lack. Among several clinical aspects of RTT, growing evidence suggest a possible key role exerted by metabolic dysfunctions linked to a detrimental vicious cycle of chronic OxInflammation state. In our previous works we have demonstrated that RTT fibroblasts showed an altered cholesterol metabolism, characterized by a strong reduction of the scavenger HDL receptor SR-B1 due to oxidative post-translation modifications. HDL's ability to perform its functions in cholesterol transport as well as in exerting antioxidant and anti-inflammatory activity, may be related to the presence of some accessory proteins like Paraoxonase-1 (PON-1) and Lipoprotein associated phospholipase A2 (Lp-PLA2).

We found also an impaired redox homeostasis represented by mitochondrial dysfunctions that parallels with a lower antioxidant defence activity.

Therefore, in this work we investigated a possible involvement of cholesterol and mitochondrial impairment in RTT pathogenesis addressing the following topics:

i) The role of SR-B1 receptor, PON-1 and Lp-PLA2 enzymes in cholesterol dysregulation in RTT and the determination whether the two enzymes might be used to discriminate RTT from Autism Spectrum Disorder (ASD) patients.

ii) The functional status of mitochondria in RTT, with specific focus on morphology and mitophagy, taking into account also their relationship with mitochondrial dynamics (e.g. fusion and fission) as well as apoptosis, a cell death pathway involving also mitochondria.

Our findings showed a potential involvement of cholesterol dysregulation via SR-B1 loss in RTT brain mice, presenting also Lp-PLA2 as a new possible biomarker for RTT. Moreover, we found alterations in the morphology of RTT mitochondria, which were characterized by a defect in PINK1/Parkin dependent-mitophagy possibly due to unbalanced mitochondrial fusion and Drp1/Fis1-mediated fission, which in turn could be related to an aberrant apoptosis. Therefore, our results highlighted a possible role for this fascinating organelle in RTT pathogenesis.

GENERAL INTRODUCTION

The present thesis is focused on Rett Syndrome (RTT), an orphan neurodevelopmental disease with still unclear pathogenesis. Despite being classically referred to a brain pathology, RTT is characterized by evident systemic and inter-connected physiopathological conditions such as metabolic dysregulation and oxidative stress (OxS), that appear to contribute to the clinical progression of the disease.

The first chapter describes our findings regarding cholesterol homeostasis perturbation in this rare disease. In the same section we also present data regarding a biomarker related to cholesterol metabolism that can be used to discriminate between RTT and Autism Spectrum Disorder (ASD).

RTT exhibits also the derangement of redox homeostasis, which seems to be mostly related to mitochondrial dysfunction. Nevertheless, little is still known about the functionality of mitochondria in the disease pathogenesis. With the intent to shed light on this topic, in the second chapter we discuss our result regarding mitochondrial structural alterations in RTT fibroblasts and how changes at this level might influence the physiological degradation of mitochondria through mitophagy, and its connection with mitochondrial fusion and fission as well as apoptosis.

INTRODUCTION

Rett Syndrome

1. History

RTT Syndrome (OMIM 312750) is a pervasive neurodevelopmental disorder that was first discovered by the Viennese pediatrician Andreas Rett. In 1965, he observed two girls sitting in his waiting room who were intellectually disabled and continuously wringing their hands in a such unusual manner. After examining several other patients, Dr. Rett understood that these symptoms represented something different from the common cerebral palsy, describing for the first time in 1966 the disorder now bearing his name [1]. Unfortunately, his paper remained unnoticed by the medical community until the 1980, when the Swedish child neurologist Bengt Hagberg published the same clinical findings and named the disorder RTT Syndrome [2]. Afterwards, the disease became recognized worldwide by paediatricians, neurologists, geneticists and neuroscientists. The gene involved in the large majority of RTT cases, the methyl-CpG-binding protein 2 (MECP2) was finally discovered in 1999 [3]. This finding allowed the molecular confirmation of clinical cases and contributed to amendments of the diagnostic criteria [4]. Therefore, it started a widespread investigation into the molecular mechanisms that underlie this such complex pathology.

2. Epidemiology

RTT is a rare disease that occurs almost exclusively in girls with an incidence around 1 in 10.000 girls by age 12 in the United States. Cases of RTT can be undiagnosed or misdiagnosed, making it difficult to determine the disorder's true frequency in the general population. It is the second most common genetic cause of severe intellectual disability and retardation after Down syndrome (https://rarediseases.org/rare-diseases/rett-syndrome/). Originally it was thought that RTT could affect only females. However, a systematic review conducted in Texas in 2015 reported a total of 57 males cases of RTT and a population study showed that the incidence of RTT in males was not more predominant in any particular race. Further investigations on the incidence and prevalence of RTT in males are needed [5].

3. Clinical aspects

3.1 Symptoms and stages

The classic form of RTT is associated with mutations in MECP2 gene, begins to manifest during early childhood and is characterized by neurodevelopmental regression that severely affects motor, cognitive and communication skills. Prenatal and perinatal period are usually normal, the affected girls seemingly having a physiological development from the first 6 to 18 months of life. Nevertheless, retrospective analyses of home videos often show that infants with RTT display some suboptimal development even during the first period of life. This underdevelopment may include subtle motor and behavioral abnormalities, as well as hypotonia and feeling problems. General mobility and eye-hand coordination may be insufficient and excess of repetitive hand washing can be observed. However, the overall developmental pattern is not so clearly disturbed. In fact, the child is usually quiet and placid [6]. The characteristic clinical features appear successively over several stages, outlining a distinctive disease progression pattern even if it can change in time and also in symptoms and severity (Figure 1).

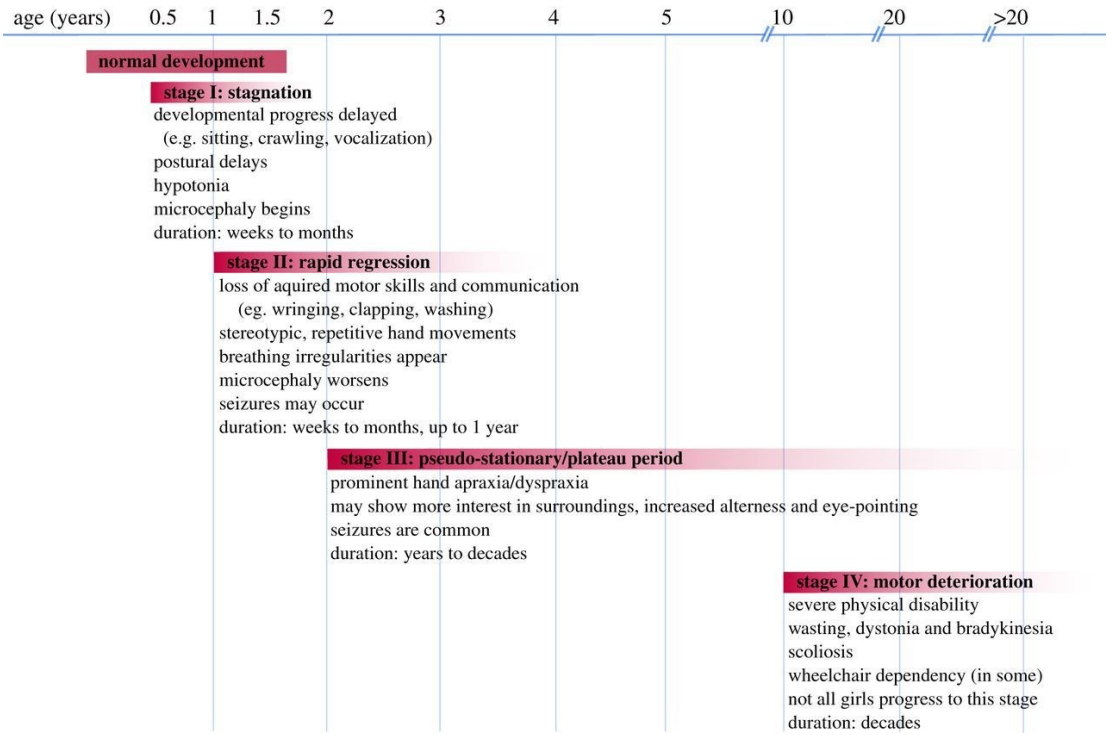


Figure 1: ***Stages of Rett Syndrome.***

Reported from *Kyle et al.* 2018 (137).

Stage I: Early onset stagnation (age of onset: 6-18 months).

Even if the general developmental pattern is not significantly altered, psychomotor development begins to slow. In most patients, head growth slows down, leading to microcephaly and growth retardation. The child has delay or interruption in the acquisition of skills. The language skills usually remain poor. A girl affected by RTT may become irritable and restless, and she may begin to display some autistic features, such as emotional withdrawal and indifference to the surrounding environment.

Stage II: Developmental regression (age of onset: 1-4 years, after stage I).

The second stage may last from days to weeks; it is characterized by a rapid reduction or loss of acquired skills, like purposeful use of hands, speech and interpersonal contact. In some patients, the decline of motor and communicative performances can be more gradual. A decrease of interest in people and objects can also occur, but eye contact may be preserved. Voluntary hand use, such as grasping and reaching out for toys, is replaced with repetitive stereotypic hand movements, the hallmark of RTT. During waking hours wringing, hand washing, mouthing, clapping, rubbing, squeezing and other hand automatism can occur. Febrile seizures are often present which severity can vary, ranging from relatively mild or easily controlled by medication to severe drug-resistant episodes. Epileptic paroxysms may also occur in most patients. In the last phase of the regression period, the patients usually develop irregular breathing patterns, such as episodes of hyperventilation, breath holding and aerophagia. Other frequent symptoms are panting, spitting and hypersalivation.

Stage III: Pseudostationary period (age of onset: 2-10 years, after stage II).

This stage can last for years or decades and is characterized by a relative stabilization of the disorder course. Girls may recover some skills which were lost during the previous stage. The girls can become more joyful and sociable, and they may use eye pointing as a typical way to communicate and to express their needs. Some patients may even learn new words and use simple phrases in a meaningful way. Nevertheless, they continue to be affected by gross cognitive impairments. Despite improved eye contact and non-verbal communication ability, during this stage occurs a progressive motor functions loss. Stereotypic hand movements and breathing irregularities become prominent. Many patients develop scoliosis, which is often rapidly progressive and eventually requires surgical treatment. Also cold feet and lower limbs, with or without color and atrophic changes, can be common; these conditions are due to poor perfusion, which is a consequence of altered autonomic

control. Sleeping patterns are often altered and characterized by frequent nighttime waking and daytime sleeping. Unexplained night laughing, agitation and crying spells may also be present.

Stage IV: Late motor deterioration (age of onset: 10 years, after stage III).
During this stage there is a continuous and gradual loss in non-verbal communication and social skills. Despite persistent serious cognitive impairment, older patients with RTT are usually sociable and pleasant with others. Seizures become less frequent and less severe, and stereotypic hand movements become less intense. However, motor deterioration continues and progresses with age. Most of the patients become nonambulatory and wheelchair-dependent; this decrease in mobility inevitably leads to pronounced muscle wasting and rigidity and, at older ages, the patients often develop Parkinsonian features.

Females affected by RTT often survive into adulthood and older age, but their life expectancy is less than that of the healthy population [5]; males survival with MECP2 mutations depends on the underlying mutation and/or other associated genetic problems. Approximately 25% of the annual death from RTT are sudden and they may occur due to autonomic nervous system disturbances or cardiac abnormalities.

Many other important features appear to be associated with RTT. The patients are generally small for their age, and this may be due to poor gastroesophageal reflux and bloating. Decreased intestinal motility often lead to severe constipation. Electroencephalogram results tend to be abnormal but without any clear diagnostic pattern. A prolonged QT_c interval is observed in many patients and show a risk for cardiac arrhythmia [6].

3.2 Variants

RTT is associated with a complex phenotype and MECP2 mutations cover the 95-97% of individuals with typical RTT [7]. Therefore it has been classified into typical and atypical or variant presentations [5]. These types may differ by symptoms, the age at which the symptoms manifest or the specific gene mutation (https://www.nichd.nih.gov/health/topics/rett/conditioninfo/types).

At least five variants have been delineated in addition to classic RTT to date:

- The *congenital variant (Rolando Variant)* is the most severe form of atypical RTT, with clinical presentations similar to the classic form, even if the distinction between this variant and classic RTT is not unanimously accepted. This variant is generally caused by mutations in the Forkhead Box G1 (FOXG1) gene (14q11-q13). It affects both females and males and is characterized by the absence of the usual period of apparently normal development; the onset of classic RTT features start during the first three months of life.

- The *early-onset seizure type (Hanefeld Variant)* is a severe and rare variant characterized by seizures in the first months of life with later development of RTT features (including developmental problems, loss of language skills, and repeated hand wringing or hand washing movements). It is frequently caused by mutations in the X-linked CDKL5 gene (Xp22) and is lacking of some distinctive clinical features of typical RTT such as the clear period of regression and the characteristic intense eye-gaze. [7].

- The *late childhood regression* form is a mild form characterized by a normal head circumference and by a more gradual and later onset (late childhood) regression of language and motor skills.

- The *forme fruste* is the most common atypical variant of RTT representing a milder variant with an early childhood onset and an incomplete and prolonged course with partially preserved communication skills and gross motor functions. Other neurological abnormalities that are typical of RTT are subtler and can be easily overlooked in this variant.

- The *preserved speech variant (Zappella Variant)* is another mild form marked by recovery of some verbal and manual skills and is caused in at least some cases by mutations in the *MECP2* (Xq28) gene, which is also responsible for the majority of cases of classic RTT (https://rarediseases.info.nih.gov/diseases/4694/disease).

3.3 Diagnostic criteria

Despite a known genetic cause, RTT remains a clinical diagnosis. This is due to the consideration that: i) some RTT patients do not have the MECP2 mutations [7], [8]; ii) MECP2 mutation can also be found in individuals who do not have the clinical features of RTT; iii) this mutation covers only the 50-70% of atypical forms.

Therefore, RTT diagnosis is based on many well-defined criteria (Table 1), revised several times over the past few decades. The disease was originally classified as ASD and this definition lasted until 2010, when RTT was formally removed from this disease group [9].

RTT Diagnostic Criteria 2010

Consider diagnosis when postnatal deceleration of head growth observed.

Required for typical or classic RTT

1. A period of regression followed by recovery or stabilization
2. All main criteria and all exclusion criteria
3. Supportive criteria are not required, although often present in typical RTT

Required for atypical or variant RTT

1. A period of regression followed by recovery or stabilization
2. At least 2 out of the 4 main criteria
3. 5 out of 11 supportive criteria

Main Criteria

1. Partial or complete loss of acquired purposeful hand skills.
2. Partial or complete loss of acquired spoken language
3. Gait abnormalities: Impaired (dyspraxic) or absence of ability.
4. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms

Exclusion Criteria for typical RTT

1. Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems
2. Grossly abnormal psychomotor development in first 6 months of life

Supportive Criteria for atypical RTT

1. Breathing disturbances when awake
2. Bruxism when awake
3. Impaired sleep pattern
4. Abnormal muscle tone
5. Peripheral vasomotor disturbances
6. Scoliosis/kyphosis
7. Growth retardation
8. Small cold hands and feet
9. Inappropriate laughing/screaming spells
10. Diminished response to pain
11. Intense eye communication - “eye pointing”

Table 1: ***Diagnostic criteria for RTT Syndrome diagnosis*****.**

Reported from *Neul et al.* 2010 (7).

4. Genetic aspects

The search for a gene for RTT was hampered by a lack of familial cases as > 95% of those are sporadic. By performing linkage analysis on the few available familial cases and using a systematic genetic screening approach, the region of interest was localised to Xq28. In 1999 Amir *et al*. published the first report linking the syndrome to mutations in the *MECP2* gene encoding X-linked methyl-CpG-binding protein 2 (Mecp2) (https://ghr.nlm.nih.gov/condition/rett-syndrome) [10].

4.1 MECP2 gene

MECP2 gene is 112 756 bp long and contains four exons that encode for two isoforms generated by alternative splicing of exon 2, Mecp2_e1 and Mecp2_e2 (Figure 2).

MECP2 has a large, highly conserved 3' UTR (untranslated region) that contains multiple polyadenylation sites. Alternative 3' UTR usage leads to three distinct transcripts, one short transcript of 1.8 kb and one long of 10 kb, with the latter including a highly conserved (8.5 kb) 3' UTR, and a third additional low abundance transcript of approximately 5–7 kb [11].

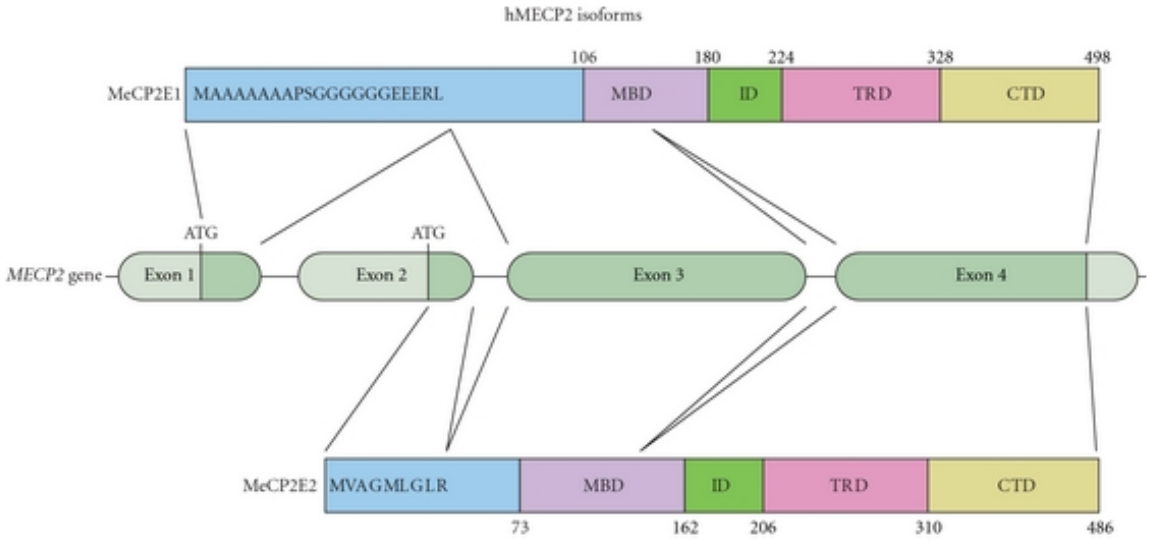


Figure 2: ***Representation of MECP2 gene and its two isoforms.***

Reported from *Zachariah and Rastegar* 2012 (11).

4.2 Mecp2 protein

Mecp2 is an ubiquitously expressed nuclear protein that is mainly co-localized with densely methylated heterochromatin in mouse cells.

The highest levels of this protein are found in the central nervous system (CNS). Mecp2 concentration increases during the post-natal development, suggesting that it could be related with the neuronal maturation grade [12]. In addition, the amount of Mecp2 protein varies among the different cell populations in the brain; indeed, as compared with normal brain, RTT glial cells, astrocytes and microglia have lower detectable levels while mature neurons have higher levels of Mecp2 protein [13]. Some recent studies have highlighted that Mecp2 may be the most abundant protein in the nucleus of neurons, with a level that is comparable to that of nucleosome, the structural repetitive and fundamental units of chromatin.

Mecp2 protein contains several functional domains:

- a methyl-CpG-binding domain (MBD) of 85 amino acids splitted between exon 2 and 3 which give the high affinity binding of Mecp2 to methylated DNA [14];
- a transcriptional repression domain (TRD) of 104 amino acids lying entirely in exon 4 that interacts with the histone deacetylates complex (HDACs) and the transcriptional co-repressor switch independent 3A (SIN3A) [15], [16];
- two nuclear localization signals (NLS) one lying between nucleotides 173 and 193 and the other in the TRD that import part of the total protein into the nucleus;
- a C-terminal sequence involved in the interactions between DNA and its protein partners [16].

In addition, three AT-hook-like domains in Mecp2 which are shared with chromatin-associated proteins of the high-mobility group AT-hook (HMGA) family were recently identified, and are involved in DNA binding [17].

The first identified form, Mecp2-e2 has 486 amino acids, is ubiquitously expressed and derives from the splicing of exons 2,3 and 4; the second, Mecp2-e1 has 498 amino acids and it was discovered in 2004, it lacks of exon 2 and derives from the alternative splicing of exons 1,3,4. Therefore, the two forms are substantially equal, differing only for the N-terminal sequence [18], [19].

The whole physiological meaning of the two isoforms has not been yet completely elucidated. However, it is known that Mecp2 transcripts undergo on a differential expression and that can be subjected to tissue- and developmental stage specific regulation and to a particular expression pattern in the brain [20]. Moreover, a recent study suggests that there could be functional differences between these two isoforms. In particular, the overexpression of Mecp2_e2 leads to neuronal death, whereas Mecp2_e1 has no effect [21].

4.3 Mecp2 functions

It is possible that the functions of Mecp2 are very complex and not completely understood. Thus led to the consideration of Mecp2 as a multifunctional protein involved in the transcriptional activation/repression, in the chromatin architecture remodelling and also in the control of m-RNA splicing [22].

The original model suggested that Mecp2 was a transcriptional repressor [16]. However, recent findings highlighted to a more complex picture in which Mecp2 acts both as activator and a repressor of target genes (Figure 3) [23].

Mecp2 can act as a transcriptional repressor through two different mechanisms. The first one is chromatin-dependent: the TRD interacts with the co-repressor SIN3A; then, Mecp2 and SIN3A recruit histone deacetylases (HDACs) and histone methyl transferases (HMTs), making the chromatin more packed and unreachable to the transcriptional machinery. The second mechanism is chromatin-independent: the transcription is inhibited directly through the interaction of TRD domain with the general transcription factor IIB (TFIIB) [24].

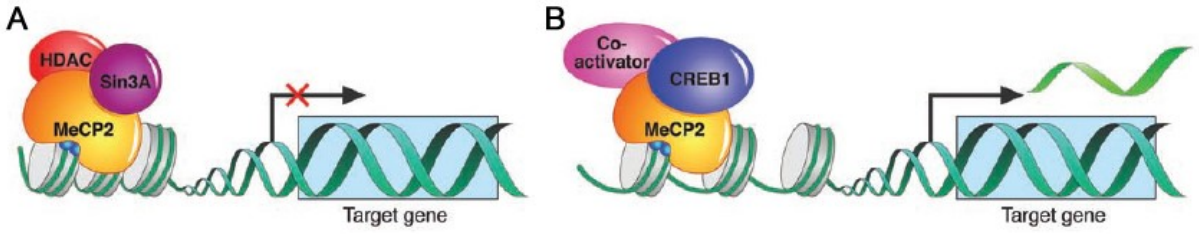


Figure 3: ***Representation of some of the possible MECP2 protein functions.***

Reported from *Chahrour et al. 2008* (25).

Recently, Chahrour *et al.* demonstrated that Mecp2 is able to promote the gene expression of cAMP response element-binding protein (CREB1), one of the main transcriptional activator, by binding and acting synergically into a complex in order to activate the transcription of specific genes [25].

In addition, it has been proposed that Mecp2 may be subjected to several post-translational modifications like acetylation, phosphorylation, ubiquitination and sumoylation. In particular, it is widely known that the phosphorylation of Mecp2 at Ser 229 or Ser 80 selectively influences its interaction with factors like heterochromatin protein-1 (HP1) and structural maintenance of chromosome 3 (SMC3). These proteins are involved in the structural organization of chromatin and chromosome, with the co-factor SIN3A and YB-1, having a role in the modulation of gene expression and in the splicing of RNA. Moreover, the phosphorylation of Mecp2 in S229 has high affinity for the promoter of proto-oncogene ret (RET), and its phosphorylation is required for the activation or the repression of target genes like RET and early growth response 2 (EGR2), which take part in the signalling pathway of cell growth, differentiation and proliferation [26].

The current knowledge on Mecp2 functions underlines the complex role of Mecp2 in the regulation of the gene expression, by molecular mechanism that has been not definitely clarified yet. This uncertain scenario makes difficult to understand how mutations in MECP2 might be responsible of the RTT phenotype. To fill this gap in knowledge, several researchers have been focusing on the role of Mecp2 in the post-natal brain development.

4.4 MECP2 target genes

The identification of MECP2 as the disease-causing gene led rapidly to the development of mouse model of RTT that recapitulate the pathology. Established models include mice carrying either global alleles (null, hypomorphic, large deletions and point mutations) or conditional null alleles. Among those, Mecp2-deficient mice carrying global mutant alleles exhibit phenotypes that resemble some of the symptoms that characterized RTT patients, including shortened lifespan, motor and sensory impairments, breathing abnormalities, cognitive and behavioural dysfunction as wells as cellular and synaptic defects. Since their phenotype partially recapitulate the RTT features, the use of animal model has been of great help for the comprehension of the molecular mechanism of the disease, permitting the identification of some target genes of MECP2 (Table 2) [27], [28]. Their expression is positively or negatively regulated in the several areas of the brain as a consequence of the protein loss, determining the clinical phenotype in a direct or indirect way [29], [30].

The first target gene identified is the brain-derived neurotrophic factor (BDNF), which is an essential growth factor for neurogenesis, neuronal maturation, survival. plasticity, learning, memory and Ca^{2+} homeostasis [31].

MECP2 target genes	Function	Target genes expression in RTT patients or in MeCP2-null mice	
BDNF	Neuronal plasticity, learning process and memory	↑	Chang et al., 2006
Ddc	Biosynthesis of monoaminergic neurotransmitters (serotonin and catecholamine)	↑	Urdinguio et al., 2008
DLX5	Promotions of GABAergic interneuron differentiations; mutations in these genes are responsible of rare genetic diseases characterized by skeletal morphological defects from the fetal development	↑	Bienvenu & Chelly, 2006
DLX6		↑	Bienvenu & Chelly, 2006
Fkbp5	Hormone signalling	↑	Nuber et al., 2005; Urdinguio et al., 2008
Sgk1	Ion channel activation	↑	Nuber et al., 2005
FXYD1	Na^+/K^+-ATPase activity	↑	Deng et al., 2007
Irak1	Immune system and local response against pathogens.	↑	Urdinguio et al., 2008
Prodh	Glutamate synthesis and degradation of proline, protecting against oxidative stress.	↑	Urdinguio et al., 2008
S100a9	Possible involvement in chronic inflammatory disorders.	↑	Urdinguio et al., 2008
CREB	Transcriptional co-activator.	↓	Urdinguio et al., 2008; Chahrour et al., 2008
Gap43	Synaptic plasticity, learning and memory, regeneration of CNS.	↓	Pelka et al., 2006
Kif1b	Axonal-transport associated protein.	↓	Pelka et al., 2006
Hairy2A	Neuronal repressor	↓	Stancheva et al., 2003
UBE3A	Proteolysis	↓	Makedonski et al., 2005; Samaco et al., 2005

Table 2: ***Functions of some MECP2 target genes and relative expression.***

Modified from *Singh et al.* 2008 [32].

The functional and genetic relationship between BDNF and MECP2 led to define the specific mechanism of BDNF expression MECP2-dependent. Indeed, two models were initially proposed: a repression and an activation model followed by a more recent integration into a "dual operation" model.

The repression model says that in the absence of membrane polarization, MECP2 binds the promotor of BDNF repressing the transcription [29], [33]. After a stimulus, through membrane depolarization, MECP2 is phosphorylated leading to its release from the promoter of the target gene, determining the transcription's activation [29]. Therefore, mutations in MECP2 gene lead to an abnormal transcription of BDNF gene. However, in some regions of the brain reduced levels of BDNF protein have been found, failing to exclusively validate the repression model [34]. Chahrour *et al.* showed that the transcriptional activator CREB1 is co-localized together with MECP2 on multiple activated target genes, suggesting that MECP2 could also act as an activator of BDNF expression. In addition to the direct mechanism, several microRNAs have been discovered to directly target BDNF transcripts, regulating in an indirect and negative manner the BDNF mRNA translation [35]. Furthermore, the BDNF protein expression levels can be lower due to a reduction of the neuronal activity, which rate has been found lower in some region of the brain like the cerebral cortex of Mecp2 knockout mice [35].

It has been hypothesized that the reduction of BDNF may contribute to the symptoms or to the pathogenesis of RTT. In fact, when BDNF levels are restored, there is a parallel extension of the survival and a recovery of locomotor and electrophysiological deficit in $Mecp2^{-/y}$ mice [34]. In addition, it has been demonstrated that low BDNF expression in the encephalic trunk is correlated with respiratory dysfunction, and the increase of its level improves the respiratory symptoms [36]. Altogether, these findings suggest that the control of BDNF expression by MECP2 can dynamically switch between repression and activation.

After the identification of BDNF, other genes which expression is normally repressed by MECP2, such as Dlx5, Dlx6 [37], Fxyd1, Reln and Gtl2 [38] were found. In particular, Dlx5 gene encodes for proteins involved in the osteogenesis and GABAergic transmission, their altered functions can be linked with epileptic crisis, osteoporosis and the reduced somatic evolution observed in RTT patients. Interestingly, significant high levels of Dlx5 and Dlx6 have been found in the frontal cortex of MECP2 null mice [37]. Moreover, the upregulation of Dlx5 induced an increase of glutamic decarboxylase responsible of an altered neuronal differentiation [39].

Also the expression of GABRB3 and ubiquitin protein ligase 3A (UBE3A) is significantly reduced in MECP2 null mice, as well as in the brain of RTT patients [40]. These genes are important for the normal development of the dendritic morphology and for the regulation of the GABAergic function. In 2007, Deng *et al.* identified another target gene of MECP2, FXYD1, which encodes for the

transmembrane modulator of Na+/K+ ATPase and is highly expressed in frontal cortex neurons of RTT patients and in MECP2 null mouse models [41]. Cortical neurons of MECP2 null mice present reduced activity of Na+/K+ ATPase, suggesting a correlation between the altered expression of FXYD1 and the very high neuronal activity observed in RTT [41]. These changes in genes associated with the GABAergic function correlates with the signalling dysfunction observed in RTT patients, whereas the alteration in the expression of genes involved in the dendritic morphology can explain the dendritic modifications found in these patients (as well as in MECP2 null mice) [42].

MECP2 is also able to modulate the expression of glucocorticoid-responsive genes, like serum glucocorticoid-inducible kinase 1 (Sgk1) and FK506-binding protein 5 (Fkbp5). Increased mRNA levels of these two genes were observed before and after the neurological symptom's appearance in MECP2 null mice [43]. The over-expression of SGK1 induces a reduction in the cell survival and activation of specific ions channel responsible of the neuronal excitability. Other target genes like Kif1b and Gap43 were identified in the hippocampus and amygdala. The reduction of their protein levels, due to MECP2 gene deficit, lead to the loss of energetic and chemical requirement of the hippocampal neuron, compromising the cognitive functions under the control of this region [44].

4.5 MECP2 mutations

More than 620 mutations of MECP2 gene have been identified in females with RTT, with more than 99% occurring de novo mostly on the paternal X chromosome, which explains the high occurrence of RTT Syndrome in the female gender (https://ghr.nlm.nih.gov/gene/MECP2).

The spectrum of MECP2 mutations is heterogeneous including missense and nonsense mutations, frameshift, deletions, insertions, duplications, splice-site mutations and large deletions of several exons or the entire MECP2 gene [6].

Mutations located within the MBD reduce the affinity of the protein for methylated DNA [45], [46]. However, several proteins with mutations in MBD have been shown to bind to heterochromatin [47]. Proteins with an intact MBD but with a mutated TRD retain their ability to bind to methylated DNA, but they have impaired repressing activity [45]. Other mutations may affect the stability or the structure (secondary or tertiary) of the protein, and they may interfere with other functions of the protein itself.

Generally, MECP2 mutations can be divided into three groups, on the basis of the consequence on the protein:

- Early truncated: lead to the loss of the MBD and the TRD domain with a total loss of function of the protein
- Late truncated: lead to the loss of C-terminal region (MBD and TRB remain intact)
- Missense: localized in the MBD and in the last part of the TRD

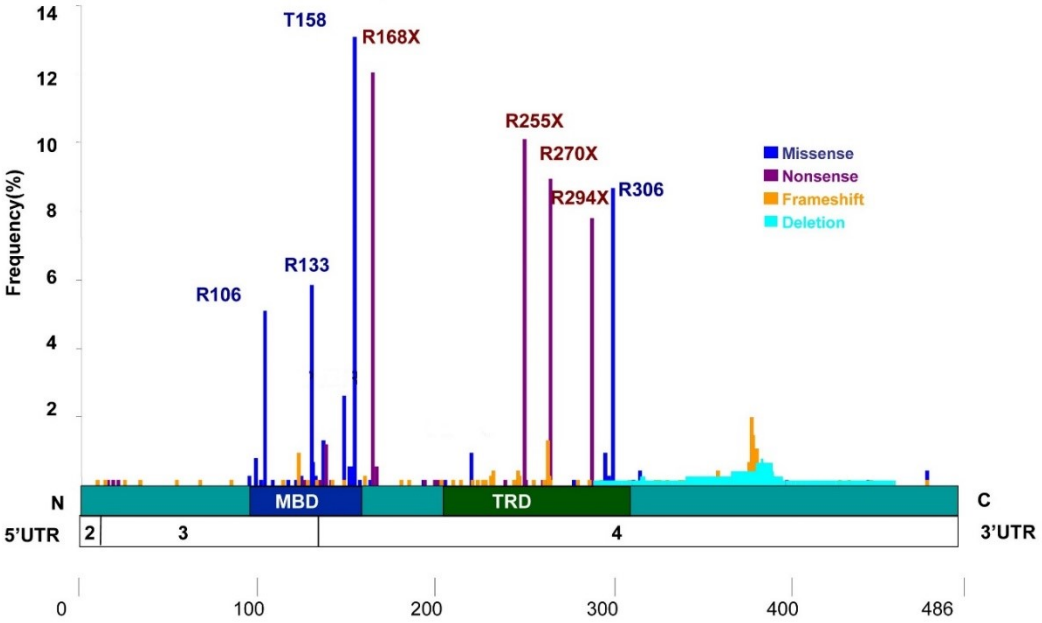


Figure 4: ***Representation of some of the most frequent mutations causing RTT Syndrome.***
Reported from RettBASE: http://mecp2.chw.edu.au/

Among the high number of MECP2 mutations, 8 are the most common and account for approximately 70% of RTT cases (Figure 4). Approximately 10% of cases are due to deletions, which are mostly clustered in the terminal segment of the coding region [23].

These recurrent mutations are R106W, R133C, T158M, R306C, R168X, R255X, R270X, R294X, formed mostly by C>T transitions presumably resulting from the spontaneous deamination of methylated cytosines [48]. The mutations are scattered throughout the coding sequence and splice sites, with the exception of exon 2.

All the missense mutations involve the conserved amino acids in the functional domains of the protein, compromising its ability to bind the DNA or its structure and/or the interaction with other proteins. The nonsense and frameshift mutations cause the formation of truncated proteins, which are

able to bind the DNA in the methylated regions but are not able to interact with the nuclear co-repressor [49].

4.6 Genotype-phenotype correlation

The general genotype-phenotype correlation was confirmed by numerous studies. As expected, patients with early truncating mutations (specifically R168X, R270X, R255X), large deletions of several exons or of the entire MECP2 gene, usually show the most severe clinical presentations.

In particular, R270X is correlated with high mortality compared to the other seven most common mutations [50].

A milder phenotype is often associated with late truncating mutations, such as R294X, that do not affect the MBD or the TRD [9], [51]–[53].

R133C is prevalently associated with autistic features whereas deletions in the C-terminal domain are mostly characterized by a rapid progression of scoliosis and a slow clinical progression. The persistence of some cognitive function during the teenager and the adulthood is accompanied by a marked dystonia and decline of the motor skills during the childhood [54]. There are more divergences regarding T158M, R306C and R255X mutations. The majority of R133C, T158M, R306C mutations are clinically associated with absence of microcephaly, normal weight, light or absence of scoliosis and the persistence of some words also during the regression period with a more favourable course of disease. In addition, it is known that missense mutations lead to minor alterations in the language skills (Table 3) [55], [56].

Most frequent MECP2 missense mutations	**Functional/clinical alteration**
R133C	Autistic features
R133C T158M R306C	Absence of microcephaly Normal weight Light or absence of scoliosis Persistence of some words during the regression period More favourable course of the disease

Table 3: ***Genotype-phenotype correlation of the most frequent missense mutations in MECP2 gene.***

4.7 X-chromosome inactivation (XCI) in RTT Syndrome

The studies published so far have not been able to clearly demonstrate a direct relationship between MECP2 mutations and the high variability observed in RTT phenotype. Other factors, such as X-chromosome inactivation may influence the severity of the clinical phenotype.

X-chromosome inactivation is the complex and highly regulated transcriptional silencing of one X chromosome occurring in female mammalian cells in order to equalize dosage of gene products from the X chromosome between XX females and XY males, preventing a potentially toxic double dose of X-linked genes. X-chromosome inactivation in the embryo proper occurs early during the development. The two X chromosomes have an equal probability of being silenced. Once established, silencing is stable: the same X chromosome remains inactivated in all subsequent cell generations. As a result, each female is a mosaic of cells in which either the maternally- or the paternally inherited X is silenced [57], [58].

Interestingly, girls with RTT are functionally considered mosaic for expression of the heterozygous MECP2 mutation [59], [60].

Normally, XCI occurs randomly, with the consequence that almost half of the cells express the functional MECP2 gene and the other half the defective MECP2. When this process is non random, is called *skewed*: the pattern is characterized by 80% or more of the cells that show a preferential inactivation of one X chromosome. This leads to the presence of an X-chromosome with either the normal or defective MECP2 gene that may be preferentially active in the majority of the cells in the body [60].

In some cases, skewed X-inactivation patterns were correlated with less severe clinical phenotypes [61], mild learning disability, or incomplete diagnostic features of RTT [10]. In at least two of these cases, the mutant X-chromosome was shown to be preferentially inactivated [61]. Further studies may clarify if tissue-specific differences exist in X-inactivation patterns [60]. Non random patterns of X-inactivation have been reported in asymptomatic carriers of MECP2 mutations [61]–[66]. In several families, asymptomatic mothers of girls with RTT are “silent” carriers of MECP2 mutations due to preferential inactivation of the mutated X-chromosome. Girls with RTT usually show random patterns of X-inactivation, although several studies have reported skewed patterns of X-inactivation in females with RTT [67].

However, XCI may not be used as the single predictor because, according to some studies, it may be responsible of just 20% of the high phenotypic variability observed among RTT patients [67]. This marked heterogeneity might be the result of a number of other factors including the type and the site

of the mutation, functional alterations of genes involved in the same metabolic pathway of MECP2 or still unknown environmental modulators.

5. Current therapies

Currently, there is no cure for RTT. The strategies followed by the clinicians are essentially two: i) pharmaceutical treatments to offset the inactivity of MECP2 gene; ii) gene therapy [68].
Several potential therapeutic approaches are under development, as 45 clinical trials are ongoing and have been registered in *ClinicalTrials.gov*. The majority of these are direct toward the modulation of neurotransmitters, with the aim to alleviate the symptoms associated to the disease. Unfortunately, these treatments can help to manage, but not to cure the disease [68].
Gene therapy may represent a candidate curative approach. One of the most investigated options in this frame, is the hot technology called CRISPR/Cas9 based on gene editing which uses molecular scissors to find and remove a specific DNA sequence. Even if some successful results have been reported in human embryos [69], there is still no conclusive evidence supporting the use of this approach in living patients [68]. In this regard, previous studies in mice shown that sometimes the technology is not sufficiently precise causing off-target mutations. Some researchers even suggest that a complete genome sequencing of each patient would be required to ensure that the CRISPR/Cas9 would not target unintended sites of the patient's genome [68].

Most people with RTT benefit from well-designed interventions based on a multidisciplinary approach to provide symptomatic relief for those patients. In fact, with therapy and assistance, people with RTT can participate in school and community activities. These treatments, forms of assistance, and options for medication generally aim to slow the loss of abilities, improve or preserve movement, encourage communication and social contact. The need for these treatments depends on the severity of different symptoms and include
(https://www.nichd.nih.gov/health/topics/rett/conditioninfo/treatments):

- Physical therapy/hydrotherapy: to improve the mobility, reduce misshapen back and limbs and provide weight-bearing training when scoliosis is present.
- Occupational therapy: to improve or maintain the use of hands reducing the stereotypic hand movements.

- Speech-language therapy: to teach nonverbal communication and to increase social interaction.
- Feeding assistance: to add supplements to the diet like calcium and minerals to strengthen bones and slow scoliosis as the adoption of high-calories or high-fat diet to increase the height and the weight.
- Medication: to reduce breathing irregularities, cardiac and gastrointestinal dysfunctions, behavioural alterations and sleep disorders.

It has been proved that management of these large amount of symptoms can substantially improve the quality of life of RTT patients and should not be overlooked [5].

CHAPTER I

Cholesterol metabolism in RTT Syndrome

1. Introduction

In the last decade, several novel aspects of RTT pathogenesis have been elucidated. The recent experimental and epidemiological/clinical evidence strengthen the idea of a pleotropic role of Mecp2 protein, which is now believed to regulate multiple functions of central nervous system (CNS) and non-CNS cells.

Metabolic, inflammatory and redox dysregulation chiefly characterize RTT, and this cluster of abnormalities reverberate both in neurons, systemic circulation and peripheral cells. One of the most striking example in this frame, is the cholesterol and lipid metabolism, which have been found to be perturbed in RTT patients [70].

2. Cholesterol metabolism

The word cholesterol derives from the ancient Greek, *chole* – (bile) and *stereos* (solid). It was first recognized as the solid component of gallstones when the French chemist, Michel Eugéne Chevreul, identified and purified this crystalline substance from biliary calculi naming it as cholesterine in August 1816 [71].

Cholesterol is an essential biological molecule being the vital constituent of cell membranes and representing the precursor of steroid hormones, bile acids and Vitamin D [72].

Cholesterol balance is maintained by the fine regulation of synthesis, transport, storage and catabolism.

It can derive from the diet, approximately 400 mg/die, as well as from endogenous biosynthesis in liver, brain or intestine and peripheral tissues. The cholesterol present in human organism is mainly (approximately 80%) derived by endogenous synthesis [72], [73], which primarily takes place in the liver [73], [74].

Dietary and endogen cholesterol enter in the cells where its synthesis is modulated by 3-hydroxy-3-methylglutaryl CoA reductase, taken up by LDL receptor and stored through esterification by acetyl coenzyme A cholesterol acyltransferase (ACAT). Only in the liver, the excess of cholesterol can be excreted as bile acids. Alternatively, free cholesterol (FC) can be eliminated from cells by oxidation forming 27-carbon oxidized derivatives of cholesterol or by-products of the cholesterol biosynthetic

process called oxysterols [75]. Both FC and cholesterol esters (CEs) are carried by lipoproteins to target tissues.

2.1 Cholesterol metabolism in the brain

The brain contains a large amount of cholesterol, about 20% of the whole body's cholesterol, including unesterified cholesterol, desmosterol and cholesteryl ester. The cholesterol metabolism in the brain and its complex regulation are currently poor understood. However, it is separated from the rest of the body due to the presence of an intact blood brain barrier (BBB), which allows a minimal exchange of lipoprotein particles between the systemic circulation and the CNS, although some smaller high-density lipoprotein HDL-like particles are able to traverse (Figure 5) [76]. For this reason, brain cholesterol homeostasis is maintained by independent processes that include in situ synthesis, storage, transport and removal. De novo synthesis of cholesterol is primarily made by neurons, astrocytes and microglia cells. The majority of brain cholesterol accumulates between the perinatal period and adolescence and the synthesis rate closely correlates with the ultimate cholesterol level in different brain region [76].

Brain cholesterol metabolism must be accurately maintained to keep brain function well. Indeed, defects of cholesterol metabolism at this level have been shown to be implicated in structural and functional nervous system diseases such as Parkinson, Alzheimer, Huntington and Niemann-Pick C disease [74], [77].

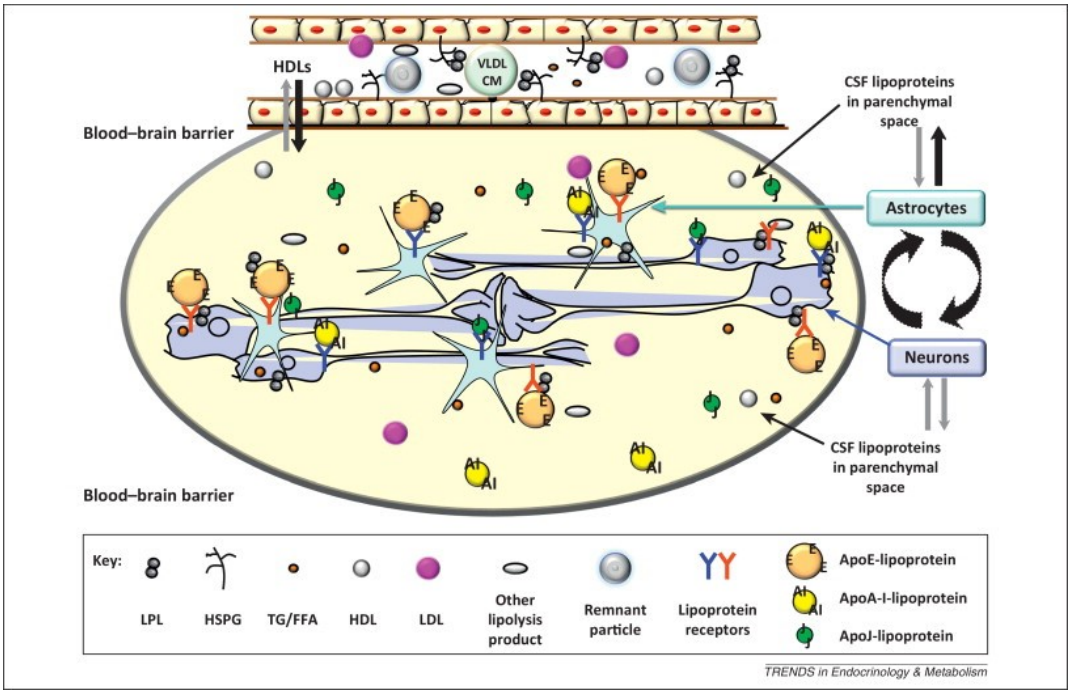


Figure 5: ***Lipoprotein metabolism in the CNS.***

Reported from *Wang and Eckel* 2014 (75).

2.2 Cholesterol transport

Cholesterol, like the other lipids, is water-insoluble. Thus, to be transported through the blood stream it needs to be associated with proteins (apolipoproteins) forming the so-called lipoproteins [78].

2.2.1 Lipoproteins

Lipoproteins exercise a fundamental role in the metabolism of cholesterol, being the carrier for this essential molecule. They are complex particles with a central hydrophobic core of non-polar lipids, primarily cholesterol esters and triglycerides. The hydrophobic core is surrounded by an hydrophilic membrane consisting of phospholipids, free cholesterol, and apolipoproteins (Figure 6).

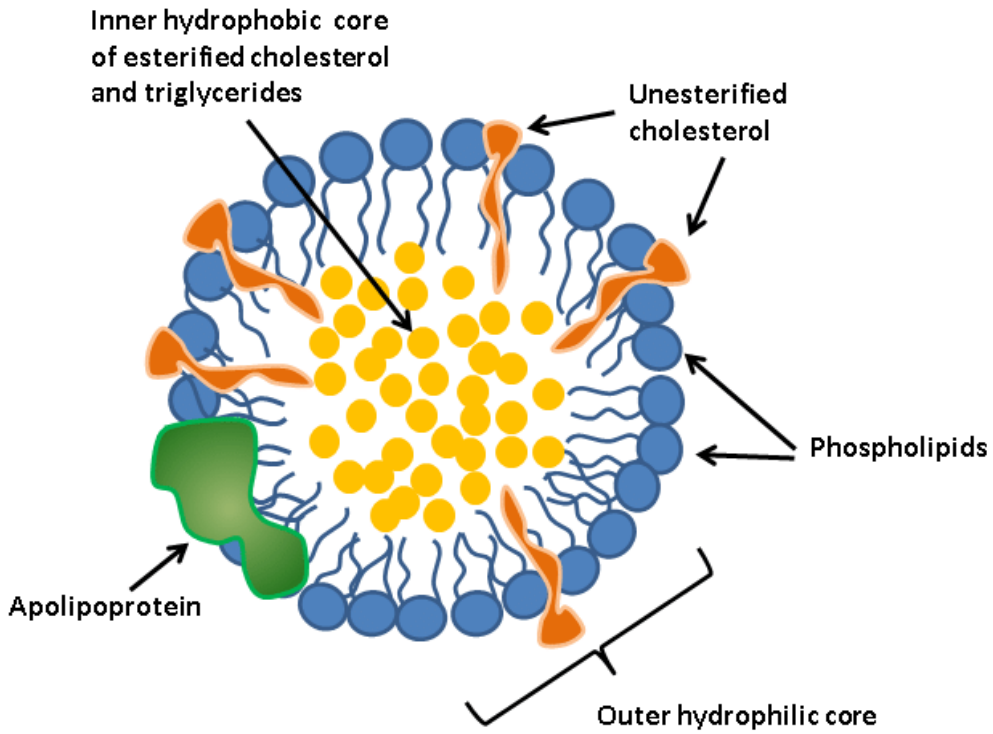


Figure 6: ***General structure of lipoprotein*****.**

Reported from *Karenein* 2015 [79].

Apolipoproteins are fundamental components of lipoproteins playing a crucial role in their metabolism. The apolipoproteins are essential for plasma lipid homeostasis, serving as ligands for lipoprotein receptors and modulators of enzymes involved in lipoproteins metabolism.

There are seven main classes of lipoproteins, which differ among each other for size, density, lipid composition and apolipoproteins as well as biological function: chylomicrons, chylomicrons remnants, VLDL, IDL, LDL, HDL, Lp (a) (Table 4) [78].

Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	Major Apoproteins
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
LDL	1.019- 1.063	18- 25	Cholesterol	Apo B-100
HDL	1.063- 1.210	5- 12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
Lp (a)	1.055- 1.085	~30	Cholesterol	Apo B-100, Apo (a)

Table 4: *Lipoprotein classes*.

Reported from *Feingold and Grunfeld* 2018 (77).

2.2.2 Transport of cholesterol between the liver and the peripheral tissues

As shown in Figure 7, dietary cholesterol is carried through blood by chylomicrons, which are lipoproteins formed in the intestinal cells; triacylglycerol is extracted from chylomicrons by lipoprotein lipase (LPL), forming chylomicron remnants. Then, liver cells package esterified cholesterol, together with triacylglycerol, into particles of very-low density lipoproteins (VLDLs). Like chylomicrons, also VLDL interacts with LPL and, after the release of triglycerides, thereby turn into intermediate density lipoproteins (IDLs). One of the fates of this lipoprotein is the definitive transformation into low-density lipoproteins (LDLs). LDL is taken up by cells in the periphery through endocytosis, which is mediated by LDL receptor. Excess cholesterol is exported from the cell by the active ATP-binding cassette transporter (ABCA1) and delivered to high density lipoproteins, which then carries it back to the liver where it can be recycled to synthesize new plasma lipoproteins, excreted in the bile as free cholesterol or removed in the form of bile acid by bile ducts (http://watcut.uwaterloo.ca/webnotes/Metabolism/Cholesterol.html).

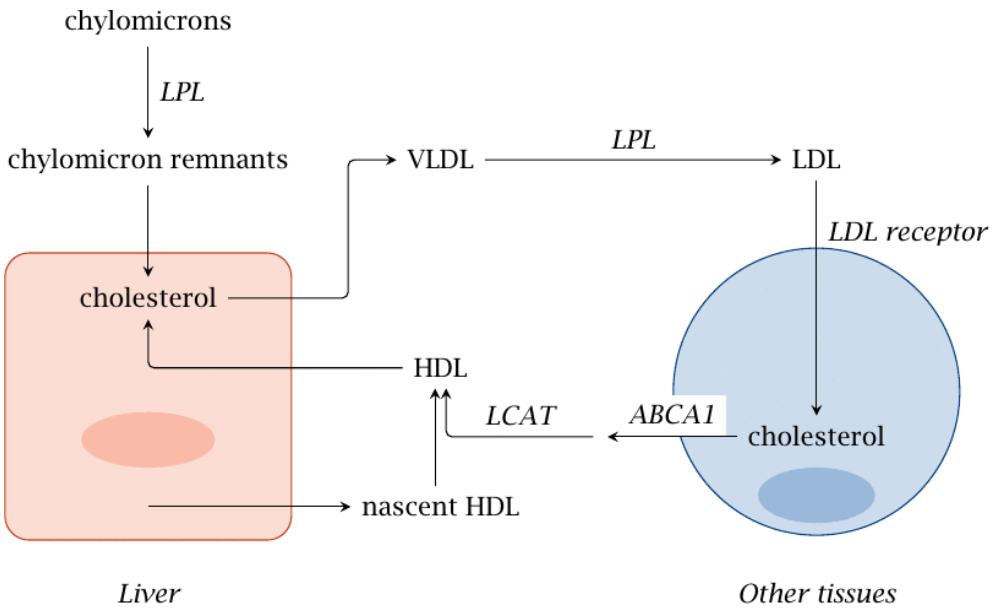


Figure 7: ***Transport of cholesterol between the liver and the peripheral tissues.***
Reported from http://watcut.uwaterloo.ca/webnotes/Metabolism/Cholesterol.html

2.2.3 Reverse cholesterol transport: role of HDL

Reverse cholesterol transport (RCT) is a multi-step process resulting in the net movement of cholesterol from peripheral tissues (including macrophages) back to the liver via the plasma compartment. Cellular cholesterol efflux is the main step of this pathway. This process is primarily promoted by HDL thanks to its protein constituents [80].

HDL represents an heterogeneous collection of lipoprotein particles with a density between 1.063 and 1.21 g/ml; compared with other lipoproteins, it has the highest relative density while being smallest in size [78], [81]. HDL is mainly secreted by the liver and small intestines; the liver, which secretes ~70–80% of the total HDL in plasma, is the main source of HDL in the circulation [81]. The HDL proteomics is very complex, but the majority of HDL particles contain the most abundant apolipoprotein in normal human plasma, Apo A1 (A-I) [82], covering approximately the 70% of the HDL protein itself [78].

HDL stimulates the efflux of free cholesterol from macrophages and endothelial cells, thus preventing from the accumulation of this steroid within intima layer of artery. This process occurs via interaction of HDL apolipoproteins (in primis, Apo A1) with carriers present on the surface of the tissue/cells, such as ATP-binding cassette transporter 1 (ABCA1) or via ATP-binding cassette sub-family G member 1 (ABCG1). ABCA1 is located on the cell membrane mediating cholesterol and

phospholipid efflux to Apo A1, which works as acceptor to form the nascent disc-shaped HDL. The nascent HDL is then transformed into spherical mature HDL by the regulation of Lecithin-cholesterol acyltransferase (LCAT), cholesterylester transfer protein (CETP) and other factors. ABCG1 is not involved in the assembly of nascent HDL but it only promotes free cholesterol efflux to mature HDL, increasing HDL cholesterol contents [81] and in the same time reducing the amount of this lipid in the cells. Cholesterol efflux is also mediated by the so-called aqueous diffusion scavenger receptor class B type 1 (SR-B1), a cell surface glycoprotein that binds HDL, LDL, VLDL, modified LDL, and anionic phospholipids [83].

More specifically, there are two ways by which cholesterol is delivered to the liver: direct and indirect. In the first, mature molecules of HDL interact with SR-B1 in the liver, which allows the transfer of its cholesterol content. The resulting HDL cholesterol molecule (HDL-c) can resume circulation and repeat the reverse cholesterol transport (RCT) process. Indirectly, mature molecules of HDL-c transfer its cholesterol content to apolipoproteins B-100 (Apo B-100), especially to LDL, in exchange for triacylglycerol molecules. This process is catalysed by the enzyme CETP. Thus, these lipoproteins can be associated with their liver receptors and deliver the respective cholesterol content [84].

2.2.3.1 Functions of HDL

Several epidemiological evidence have demonstrated the protective role of HDL in the development of atherosclerosis and coronary artery disease. This beneficial effects are the result of the multiple roles exerted by this lipoprotein in various physiological contexts, including cholesterol metabolism, redox processes and inflammation.

As described above, the HDL primary function is represented by transporting cholesterol from peripheral tissues back to the liver for biliary secretion, in a process referred to RCT. The anti-atherosclerotic actions of HDL stem from its ability to contrast the oxidation of LDL, that is one of the main downstream step of atheroma formation, and of membranes of cells such as macrophages and endothelial cells. Furthermore, HDL possesses anti-inflammatory function, being able to inhibit the expression of adhesion molecules, with the final blocking of NF-kB functions in the nucleus [81]. They possess also vascular protective effects by upregulating the expression of endothelial nitric oxide synthase (eNOs), maintaining therefore the caveolae lipid environment, in which eNOs are located. Furthermore, HDL can inhibit the platelet-activating factor/cyclooxygenase A2, as well as lower APC protein and the thrombomodulin to reduce the formation of thrombin in endothelial cells

[81]. It has been reported that after an oxidant stimulus, HDL exerts an antiapoptotic role when associated with ApoM in endothelial cells, with also antioxidant properties via PON-1 binding [85]. Finally, recent evidence showed that HDL might be involved in the transport process of miRNA in the cell. In fact, some studies have demonstrated that HDL can be combined with miRNAs by a divalent cation binding and that HDL purified by fast protein liquid chromatography showed the presence of small RNAs. It was also found that the lipoprotein is able to transport endogenous miRNA to recipient cells, even if the specific loading mechanism remains to be elucidated [81].

3. SR-B1: not just an HDL receptor

3.1 SR-B1 receptor: localization and structure

SR-B1 is ubiquitously expressed, reaching the maximum levels in the liver and representing the physiologically relevant HDL receptor.

SR-B1 was identified for the first time in hepatocytes [86]; it is well conserved between species and expressed in many mammalian tissues and cell types, like steroidogenic tissues, breast, myeloid cells, lymphoid cells, lung and interestingly, it has been found also in the skin and in the brain [87], [88]. Although a detailed study on the expression and regulation of brain SR-B1 has not been carried out yet, it has been reported that SR-B1 receptor is expressed in murine brain, where it is regulated by hormonal and nutritional stimuli, leading to a possible influence of the pathophysiology of neurological disorders like Alzheimer's disease [89]. Interestingly, it has been shown the presence of SR-B1 in astrocytes and vascular smooth muscle cells of Alzheimer's disease brain, where its decrease is correlated to the Aβ-related phenotype and cerebral amyloid angiopathy in J20 mice [90].

Of note, many studies have shown a conserved function of SR-B1 across species, proving its role as an important regulator for cholesterol efflux and steroid hormone production [91].

Mammalian SR-B1 is an 82 kDa (509 amino acid) multiligand membrane glycoprotein receptor located in *locus* 12q24.31 on chromosome 12 [92]. As the other class B scavenger receptor family members, it has a typical hairpin-like membrane topologies that contains two shorts N- (aa 1-11) and C-terminal (aa 462-509) cytoplasmic domains, two-transmembrane domains (aa 12-32 and 441-461), a large extracellular domain including 5-6 cysteine residues in addition to a multiple site for N-linked glycosylation (Figure 8) [88], [91]. It has been demonstrated that some of the structural components of SR-B1 are important for its function. The N-terminal transmembrane glycine (Gly) dimerization motif (Gly 15_Gly_18_Gly25) is required for the normal oligomerization of the receptor itself and for lipid transport [88]. The four terminal residues of the C-terminal cytoplasmic tail interact with a cytosolic protein of 70 kDa known as PDZK1, which is essential for the stability of SR-B1 [88], [93], [94]. Nearly all Cysteine are conserved among species, and four of these which are located in the extracellular domains are required for SR-B1 (HDL) binding activity, selective CE uptake and trafficking to the cell surface [88].

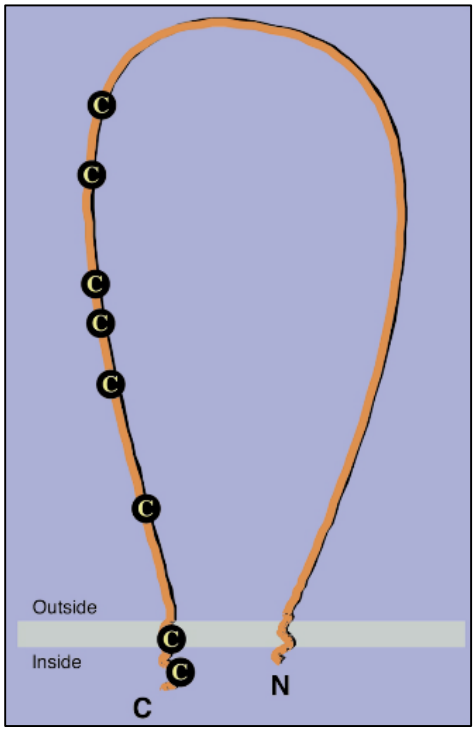


Figure 8: *SR-B1 structure*.

Reported from *Krieger et al. 2001 (89).*

3.1.1 SR-B1 ligands

SR-B1 possesses multiple binding sites and different ligands that can be bind and transported, including lipid-soluble vitamins, silica, maleylated BSA, advanced glycation end product (AGE) modified proteins and anionic phospholipids [88], [95].

Among these variety of ligands, SR-B1 has an high affinity for high density lipoprotein (HDL) [86], [95]–[97]; even if it is involved in the bidirectional movement of lipid between cells and lipoproteins [98], its primary function is to mediate the uptake of CE from HDL, without the internalization of the particle itself.

3.1.2 Functions of SR-B1

The functions of SR-B1 that have been demonstrated to date are multi-faceted, like reverse cholesterol transport, virus and bacteria recognition [99], liposoluble vitamins uptake [100], cell entry of hepatitis C virus (HCV) [101], modulation of platelet reactivity [102], phagocytosis of apoptotic cells [103], protection against female infertility [95], diet-induced atherosclerosis and myocardial infarction [104].

One of the presumptive protective effect of HDL is attributed to its role in RCT (Figure 9), process that involves the movement of cholesterol via HDL from peripheral tissues back to the liver for cholesterol excretion or steroid hormone synthesis in steroidogenic organs. The first step is the binding of CE-rich lipoproteins to SR-B1 extracellular domain through the amphipathic α-helix domain. After the reconstruction of the extracellular domain by an high-resolution crystal structure, it has been proposed that the extracellular domain of the receptor forms an hydrophobic “channel” along which CE molecules can diffuse [88]. In addition, there is also an hydrophobic tunnel and a cavity located at the centre of the β-barrel core that traverses the entire length of the molecule, allowing the passage of CE and FC molecules. While it promotes cholesteryl esters uptake and the incorporation of FC in lipoproteins, the efflux of free cholesterol from cells and its incorporation in lipoproteins might depend on the lipid composition of the lipoprotein that binds to SR-B1 [105]–[107].

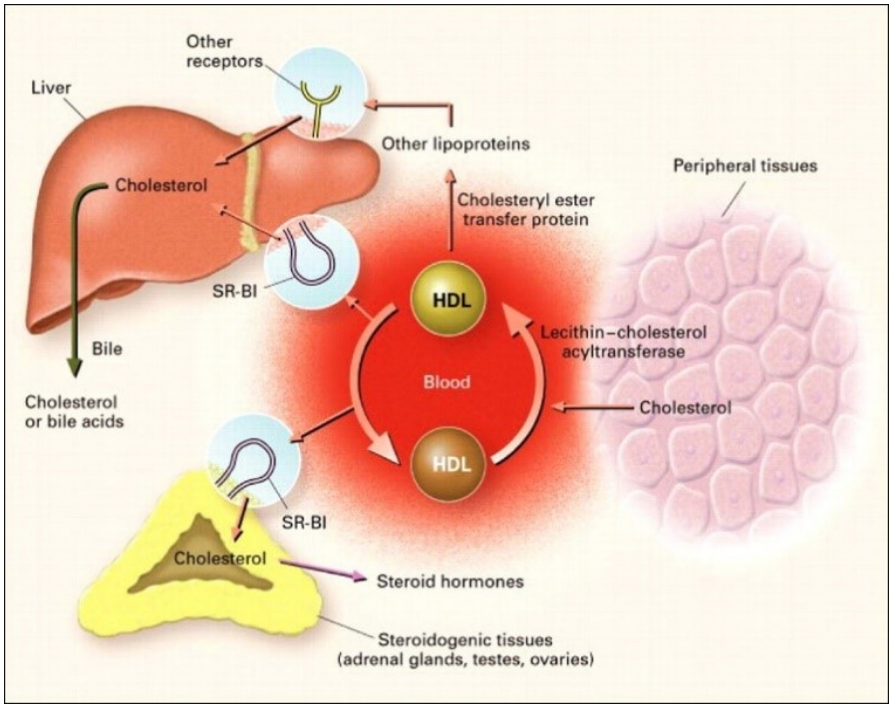


Figure 9: ***Involvement of SR-B1 in reverse cholesterol transport.***

Reported from *Rigotti et al. 2003* (93).

3.1.3 HDL-mediated link between SR-B1 and Paraoxonase-1 enzyme

HDL composition and atheroprotective functions of mediating cholesterol efflux reducing inflammation and oxidation, are modulated by hepatic SR-B1 [108] .

Interestingly, it has been recently shown that SR-B1 acts as the principal mediator of HDL ability to acquire PON-1 enzyme, thus maintaining its antioxidant property in two in vitro models of PON-1 secretion. Moreover, SR-B1 deficient mice have decreased plasma PON-1 activity, being essential to HDL preventing LDL oxidation [109].

An important determinant of PON-1 serum activity is HDL; in fact, HDL is the serum transport vector for PON-1, stabilizing and sustaining in the same time the activity of the enzyme. On the other side, PON-1 makes an important contribution to the antioxidant capacity of the lipoprotein, as suggested by animal studies. In humans, the reduced serum PON-1 activity is linked to increased serum levels of oxidized lipids and may be a determinant of the recently described phenomenon of dysfunctional HDL [109].

It has already been demonstrated by several in vitro and in vivo studies that the anti-inflammatory capacity of HDL is reduced when the PON-1 content of HDL is lower, an effect probably linked to the antioxidant potential of PON-1 [109].

3.2 Paraoxonase

Recent findings suggest that the sole cholesterol content of HDL particles does not fully cover the HDL-related atheroprotective functions. Indeed, the functionality of HDL also stem from their capacity to exert anti-inflammatory and antioxidant activity. This well-recognized pleiotropic nature of HDL mostly relates to its major non-lipid constituents, i.e. Apo A1, Apo A-2, Apo E and other accessory proteins, in primis Paraoxonase 1 (PON-1).

In this regard, the complex Paraoxonases-HDL has a key role in the LDL-oxidized removal acting as antioxidant [110] and anti-atherosclerotic mediator [111].

Paraoxonases family includes three enzymes called PON-1, PON-2 and PON-3, which are expressed by three different genes located on chromosome 7. They are able to perform a multitude of autonomous and often unrelated functions, explaining their consideration as “moonlighting proteins” [112].

PON-1 is the most studied enzyme of the family and together with PON-3 is expressed in the liver and bound to the circulatory HDL. PON-2 is ubiquitously expressed and intracellular located [113].

PON-1 exerts its properties based on three different enzymatic activities: arylesterase, lactonase and paraoxonase. Differently, PON-2 and PON-3 possess exclusively the first two activities.

3.2.1 PON-1 enzyme

The interest towards human PON-1 started since the discovery of its ability to protect from poisoning by organophosphate derivatives. More recently, research has been mostly shifted to its protective role in vascular and neurological diseases, and more in general to the pathological conditions involving OxS, inflammation and liver diseases [114]–[116].

3.2.1.1 Localization and structure

PON-1 is a serum calcium dependent esterase of 43–45 kDa associated with HDL and synthesized primarily in the liver.

PON-1 is a six-bladed β-propeller and each blade contains four strands (Figure 10). The 'velcro' closure characteristic of this fold is complemented by a disulphide bridge between Cys42 (strand 6D) and Cys353 (strand 6C). This covalent closure of the N and C termini, conserved throughout the PON family, is rarely seen in β-propellers with more than four blades.

Two calcium ions are seen in the central tunnel of the propeller, one at the top (Ca1) and one in the central section (Ca2). Ca2 is most probably a 'structural calcium' whose dissociation leads to irreversible denaturation. PON-1 retains its hydrophobic N terminus, which resembles a signal peptide and is thought to be involved in anchoring of the enzyme to HDL. The interface with HDL was further defined by a characteristic 'aromatic belt' rich in tryptophan and tyrosine side chains, and by a lysine side chain on H1 [117].

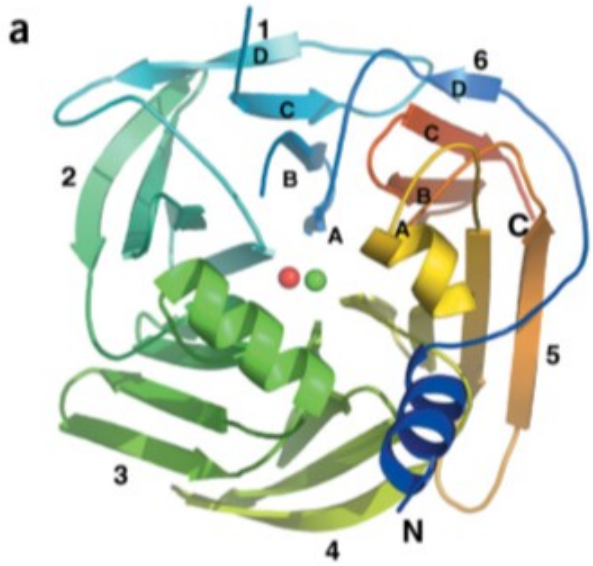


Figure 10: ***Overall structure of PON-1.***
Reported from *Harel et al. 2004* (115).

Despite the interest in PON-1 and HDL as potential therapeutic agents, the structural basis of the PON-1-HDL interface has not been clearly described yet. Using a combination of novel biophysical approaches, a study displayed the ternary complex of an HDL-MPO-PON-1 showing that APO-A1 possesses critical residues in supporting HDL-PON-1 interaction (Figure 11) [118].

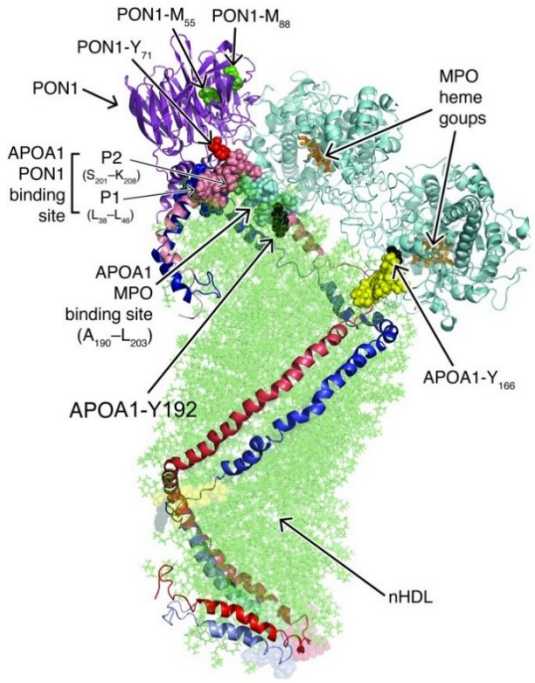


Figure 11: ***Illustration of an HDL-MPO-PON1 ternary complex.***

Reported from *Huang et al.* 2013 (116).

3.2.1.2 Functions of PON-1

The functions exerted by PON-1 are multiple and include the systemic protection against toxic agents deriving from OxS, exacerbating inflammatory response, dyslipidemia and pollution exposure [119], [120]. The ability to counter the effects of so many noxious agents appears to be linked to the broad substrate specificity that distinguish this enzyme [121].

In fact, PON-1 possesses three different hydrolytic activities: i) paraoxonase, towards toxic organophosphates such as paraoxon, the toxic oxon metabolite of parathion and insecticide [122]; ii) arylesterase towards nonphosphorous aryl esters, such as phenyl acetate; iii) lactonase, towards lactones [121].

While paraoxonase and arylesterase are considered as promiscuous activities, lactonase is widely suggested to be the primary and physiological activity of PON-1 [117].

Natural substrates of lactonase are lactones derived from food hepatic catabolism, drugs metabolism and fatty acid oxidation [123]. The ability to hydrolize specific lipid peroxidation products [124], [125], may at least in part accounts for the athero-protective action of HDL: this antioxidant-like activity also explains the abundant evidence showing that PON-1 levels are altered in diseases

apparently characterized by the detrimental crosstalk between impaired redox homeostasis and chronic inflammation [126].

The bond of PON-1 to both lipid and protein moiety of HDL (in particular Apo A1) is essential for its stability and catalytic activity.

3.3 Lp-PLA2 enzyme

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), originally named as platelet activating factor acetylhydrolase (PAF), is a member of phospholipase A2 superfamily synthesized by a variety of cells involved in host defence such as endothelial cells, platelets, neutrophils, monocytes and macrophages [127]–[129]. It is another enzyme circulating in complex with HDL (30%) and mostly with low density lipoprotein (LDL) (70%).

3.3.1 Localization and structure

Lp-PLA2, encoded by PLA2G7 gene, is secreted predominantly by macrophages [130] and is abundant in coronary atherosclerotic plaque generating pro-inflammatory mediators from oxidized LDL particles [131]. Lp-PLA2 is found predominantly bound to lipoproteins in human plasma and a small amount may also be linked to other microparticles [132]. The location of the enzyme in LDL or HDL may alter the protein's catalytic behaviour. In fact, the enzyme associated with LDL appears to be more active than the same enzyme associated with HDL [133]. From x-ray diffraction data, it has been discovered that the enzyme has a classic lipase α/β-hydrolase fold (Figure 12), containing a catalytic triad of Ser273, His351 and Asp296. Two clusters of hydrophobic residues define the potential interface-binding region, and a prediction is given of how the enzyme is bound to lipoproteins. Additionally, an acidic patch of 10 carboxylate residues and a neighboring basic patch of three residues are suggested to play a role in HDL/LDL partitioning. A crystal structure is also presented of PAF acetylhydrolase reacted with the organophosphate compound paraoxon via its active site Ser273 [127].

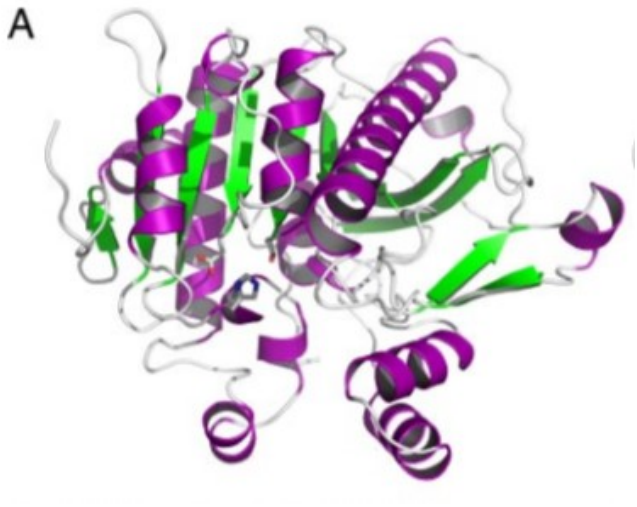


Figure 12: ***Stereo ribbon model of the Lp-PLA2 structure.***
Reported from *Samanta and Bahnson* 2008 (125).

3.3.2 Functions of Lp-PLA2

Lp-PLA2 plays multiple roles in redox and inflammatory processes [134] even if its mechanism of action and the biological role are still far to be clear.

The physiological role of Lp-PLA2 identified so far is to hydrolyze platelet activating factor (PAF) and phosphatidylcholine with short sn-2 chain including oxidized phosphatidylcholine, giving rise to lysophosphatidylcholine (lysoPC) and oxidized fatty acid (OxFAs). This catalytic activity may positively or negatively contribute to certain diseases, such as cardiovascular disease, asthma, and sepsis, likely due to the variety of pathological properties of both substrate and the catalytic products of Lp-PLA2 in different disease settings.

The diverse roles that PAF plays include the stimulation of the contraction of smooth muscle and myocytes, the promotion of oxygen species generation, platelet aggregation, and the increase leukocyte adherence to endothelial cells. PAF can effectively be hydrolyzed by PAF-acetylhydrolyse into lysoPAF and unesterized fatty acid, which can reduce some of the inflammatory effects of PAF. On the other side, oxidized phospholipids upregulate cytokine and chemokine expression by a variety of cell types. It has been shown that they are also able to switch endothelial cells to a procoagulant status and induce reactive oxygen species (ROS) generation. Signalling pathways that have been

associated with oxidized phospholipid functions include cAMP, early growth response factor (EGR), signal transducer, activator of transcription 3 (STAT3) and sterol regulatory element binding protein (SREBP).

LysoPC plays a variety of proatherogenic roles by increasing chemotaxis, generating ROS, stimulating inflammatory cytokine and chemokine secretion, inducing cell proliferation in vascular smooth muscle cells and apoptosis in macrophages, T-cells, endothelial cells and even smooth muscle cells in vasculature.

OxFAs have potent monocyte chemotactic activity even if little is known about how they enter cells, are metabolized and which metabolic pathways may be affected. However, despite the abundant studies on the topic, it is still not clear whether high levels of Lp-PLA2 are beneficial or detrimental for human health, leading to a related positive or negative effect in cardiovascular disease, asthma and sepsis. Indeed, this protein is highly expressed in nascent atherosclerotic lesions and is positively associated with an increased risk of coronary heart disease [135], [136]; other studies have clearly shown that overexpression of this enzyme reduces atherosclerosis in mice [137] and in rabbits [138].

4. Dysregulation of cholesterol and lipid metabolism in RTT Syndrome

The evidence supporting the idea that RTT is characterized by systemic metabolic dysfunctions, including dysregulation of metabolism of lipids and in particular of cholesterol, are continuously rising [139], [140]. A paper published from our group showed an altered plasma lipid profile in RTT patients, who especially presented a significant increase in total cholesterol, both LDL and HDL-cholesterol. In addition, we found a decrease in SR-B1 levels and a concomitant increase of 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation, in RTT fibroblasts. The oxidative modification of the receptor is one of the cause of the decreased SR-B1 levels, since it leads to its ubiquitination and degradation by proteasome [141].

Furthermore, in the same year Buckovecky *et al.* highlighted an abnormal lipid metabolism in the brain and in the liver of MECP2 null male mouse model, and also the ability of statin drug to improve systemic perturbations at this level. The treatment was also able to alleviate motor symptoms and to confer increased longevity in MECP2 mutant mice [70]. The authors reported a premature nonsense mutation in the gene encoding squalene epoxidase (SQLE), one of the rate-limiting enzyme in the cholesterol synthesis [70]. The increased levels of total cholesterol and LDL in RTT plasma compared to healthy controls were confirmed from another paper of our group. Corroborating the results obtained from Buchovecky *et al.,* it was reported a profound alteration in the protein network of

cholesterol homeostasis, like HMGR, SREBPs, LDLr and PCSK9 [142]. A very interesting paper associated MECP2 gene with lipid homeostasis. In fact, the authors showed that MECP2 deletion in mice resulted in a host of severe metabolic defects caused by lipid accumulation and that these effects were due to the anchoring between MECP2 and the repressor complex NCoR1 and HDAC3 to its lipogenesis targets in hepatocytes [143].

Some recent papers showed that the brains of $Mecp2^{-/y}$ mice had significantly lower concentrations of all three cholesterol precursors, campesterol and both oxysterols, with levels of 24S-OHC ~20% less than in their $Mecp2^{+/y}$ controls [144], a disrupted cholesterol synthesis in proteome of $Mecp2^{Jae/y}$ cortex [145] as well as a suppression of brain cholesterol synthesis in male $Mecp2^{-/y}$ mice in an age-dependent manner [146].

5. Rationale and aims

Despite decades of intense research have led to important progress in the knowledge of RTT pathogenesis, the precise molecular/biochemical mechanism linking MECP2 mutations to the disease manifestation is still lacking. In fact, MECP2 is able to operate as a gene modulator acting both as activator and repressor of many genes, leading even to the hypothesis that it has a role in maintaining cellular homeostasis [25]. Growing evidence suggest a possible key role exerted by metabolic alterations in this such complex disease, presenting metabolic dysfunctions as a component of RTT [139]. Dyslipidemia [140], [142], elevated ammonia [1] as well as plasma leptin and adiponectin [147], [148] and inflammation of the gallbladder [149] have been observed in many patients. In addition, cells of RTT patients had abnormal structure of mitochondria which are involved in the production of energy [150], altered function of the electron transport chain complex [151], increased OxS [152], elevated levels of lactate and pyruvate in blood and cerebrospinal fluid [151]. Also changes in brain carbohydrate metabolism [153] and neurometabolites associated with cell integrity and membrane turnover have been reported [154]. In vivo studies suggest that MECP2 mutations affect enzymes involved in cholesterol synthesis, leading to alterations in brain and liver lipid metabolism [70], [139].

Cholesterol is a fundamental molecule for a proper neurodevelopment, being the major component of the brain. It is essential during development but also in the adult stage to build up membrane surface for axons, dendrites and synapses as well as in signal transduction, membrane trafficking, myelin formation, synaptogenesis, dendrite remodelling and neuropeptide formation [74], [139]. A body of evidence suggest that homeostasis of cholesterol is deranged in RTT brain of mice models [144], [145],[146].

Dysregulation of the metabolism of this lipid has been also found to occur in the periphery, and seemed to be mostly related to a defective crosstalk between HDL and proteins involved in the efflux/influx of cholesterol from/to tissues. Cellular responses to HDL entail both its capacity to invoke cholesterol efflux that causes signal initiation via SR-B1 and other plasma membrane receptor activation by HDL cargo molecules.

In particular, SR-B1 is a membrane glycoprotein identified as the main physiological receptor for HDL [92]. It has been recently shown that SR-B1 acts as the principal mediator of the ability of HDL to acquire PON-1 enzyme, thus maintaining its antioxidative capacity in two in vitro models of PON-1 secretion [109]. Despite the abundant studies on the topic and controversial opinion, it is possible

that, as PON-1, also Lp-PLA2 exerts an antioxidant role [85], even if it is still not clear whether high levels of Lp-PLA2 are beneficial or detrimental for human health. Indeed, this protein is highly expressed in nascent atherosclerotic lesions and is positively associated with an increased risk of coronary heart disease [135], [136]; other studies have clearly shown that overexpression of Lp-PLA2 reduces atherosclerosis in mice [137] and in rabbits [138].

Altered levels of SR-B1, PON-1 and Lp-PLA2 have been found in many pathologies such as cardiovascular disease (CVD), diabetes mellitus, cancer, obesity, metabolic syndrome, atherosclerosis and neurological disorders [155], [156]. However, a special connection has been found with diseases characterized by a detrimental crosstalk between altered redox homeostasis and chronic inflammation [119], [136], [156]–[158], condition that, as demonstrated from our group, can also characterize RTT.

Therefore, the aim of our study was to evaluate whether RTT could be affected by an impairment of SR-B1, PON-1 and Lp-PLA2.

The first step was inspired by our previous results that demonstrated an alteration in SR-B1 expression in RTT fibroblasts. Following this track, we performed a cellular study to identify the expression of SR-B1 in cortex sample of RTT mouse model. Brain is the most cholesterol-enriched organ and as already underlined, dysregulation of this steroid has been detected in neuronal tissue of RTT transgenic animals.

The second step was to measure the serum levels of PON-1 and LpPLA2 in RTT, to check whether these two activities were altered in patients with this disease. Moreover, we sought to determine if these two enzymes activities might be used to discriminate RTT from ASD, another pathology chiefly characterized by brain, systemic redox and cholesterol unbalance. In fact, ASD typically share some clinical features with RTT. Moreover, MECP2 mutation is not sufficient to make a diagnosis of RTT, because this mutation can also be found in other neurodevelopmental disorders and additionally, some RTT children do not have MECP2 mutations.

Therefore, recognizing new aspects of RTT pathogenesis could possibly help the development of new therapeutic strategy for this orphan disease.

6. Methods

6.1 Animal model

Mecp2$^{-/y}$ mice were maintained under standard conditions and in accordance with Home Office regulations and licenses. The animals were sacrificed and the tissues were recovered and stored at −80 °C. The experimental procedures were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, the veterinary office regulations of the National Institute of Health (Italy) and Edinburgh (United Kingdom) and approved by the local Animal Care and Use Committees of the Italian Ministry of Health (EPIGENOME, CNR number 234/2011) and the United Kingdom Home Office.

6.2 Brain tissue collection

After transcardial perfusion with saline, brains were removed and bisected on the sagittal plane. Brain hemispheres were immediately frozen in dry ice and stored at −80 °C until analyses.

6.3 Subjects and ethic statement

The subjects enrolled in the study included n=95 female patients with clinical diagnosis of RTT (all with MECP2 mutations), n=76 patients with ASD, and n=78 healthy controls (age and gender prevalence across the groups are presented in Table 3). This research protocol was carried out accordingly to the Declaration of Helsinki (World Medical Association, http:// www.wma.net) and the European Guidelines for Good Clinical Practice (European Medicines Agency, http://www.ema.europa.eu). The study did not modify the routine implemented for the diagnosis of RTT or ASD nor conditioned any decision about the treatments of the enrolled individuals; it was approved by the local institutional review board. Written informed consent was obtained from each patient during the first office visit at baseline before the possible inclusion in the study. Blood sampling from controls was performed using routine health checks, sports checkups, or through blood donations. All the patients (ASD and RTT) were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Azienda Ospedaliera Universitaria Senese) and blood samplings were performed during periodic clinical checkups. RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [9]. Of note, 87 of 95 (91%) girls with RTT were unable to speak. The autistic patients were diagnosed by DSM-5 and evaluated using Autism Diagnostic Observation Schedule (ADOS) and Autism Behavior Checklist (ABC). ASD patients with diagnosed X-fragile or tuberous sclerosis, with perinatal adverse events and/or brain abnormalities

on magnetic resonance imaging (MRI) were excluded from the present study. ASD patients under medication or pharmacological treatment at the time of blood withdrawal were not included in the study.

6.4 Immunohistochemical analysis

Brains were dissected out, fixed in ethanol (60%), acetic acid (10%) and chloroform (30%) and included in paraffin. Paraffin embedded tissue sections of a thickness of 4 μm were deparaffinized in xylene and rehydrated in graded ethanol solutions (100%, 95%, 80% and 70%) for 5 min each. Sections were rinsed twice in dH2O for 5 min each. Briefly, antigen retrieval was obtained by incubation with buffer 10 mM citrate pH 6.0, at a temperature sub-boiling for 20 min. Slides were left to cool for 10 min. After blocking with PBS containing 5% BSA for 60 min, the sections were incubated with primary antibody (rabbit anti-SRB1 Novus Biologicals, mouse anti-βIII tubulin isoform clone TU-20 Millipore 1:50) overnight at 4°C. Incubation with secondary antibody fluorochrome conjugate (goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 568) diluted 1:100 in antibody dilution buffer was performed for 1 h at room temperature in the dark. The nuclei were counterstained by incubating the sections for 10 min with 4′,6-diamidino-2-phenylindole (DAPI). Slides were washed with PBS and mounted with Antifade. Negative controls were generated by omitting the primary antibody. The fluorescence was observed under a microscope Leica AF CTR6500HS (Microsystems).

6.5 Immunoblot analysis

Protein extracts for immunoblot analysis were obtained from cerebral cortex. Tissues were collected in ice cold PBS, then homogenized in RIPA buffer (20 mM Tris–Cl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) with Protease Inhibitor Cocktail by Turrax homogenizer. After 20 min of incubation in ice, the homogenate was centrifuged at maximum speed for 20 min at 4 °C, and the supernatant was stored at −80 °C. Protein extracts were run on 10% SDS-PAGE gel with 50 μg protein per lane. Western blot assays were performed with 1:1000 dilution of SR-B1 rabbit polyclonal antibody (Novus Biologicals, Inc.; Littleton, CO). β-Actin rabbit polyclonal antibody (Sigma-Aldrich, 1:2500 dilution) was used as loading control. Following washes in PBS–Tween 0,01% and incubation with specific secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at RT, the membranes were incubated with Supersignal West Pico Chemiluminescent Substrate (Pierce

Biotechnology, Rockford, USA). Signals were visualized on Amersham Hyperfilm ECL (GE Healthcare Europe GmbH, Milan, Italy). Images of the bands were digitized and the densitometry was performed using Image-J software.

6.6 Biochemical assays

Fasting venous blood was collected in the morning and all manipulations were carried out within 2 hours. Then, the sera were aliquoted and stored at −80 °C until analysis.

6.6.1 Lactonase, Arylesterase and Paraoxonase activity of PON-1

Serum lactonase, paraoxonase, arylesterase activities of PON-1 were measured by UV–VIS spectrophotometric assays in a 96-well plate by using Tecan Infinite M200 microplate reader (Tecan Group Ltd., Switzerland). Paraoxonase activity was determined by measuring the 412 nm absorbance increase due to 4-nitrophenol formed upon addition of 5 µL of serum in 195 µL of reaction solution consisting in 1.5 mM paraoxon (Cat. No. 855790, Sigma-Aldrich, Milan, Italy), 0.9 M NaCl, and 2 mM $CaCl_2$ dissolved in 10 mM Tris–HCl, pH 8 (Charlton-Menys et al., 2006). A molar extinction coefficient of 17000 M−1 cm−1 was used for the calculation of enzyme activity, expressed in unit per liter. One unit of paraoxonase activity is defined as 1 µmol of 4-nitrophenol formed per minute under the given conditions.

Arylesterase activity was measured by assessing the rate of hydrolysis of phenylacetate by monitoring the increase of absorbance at 270 nm, after adding 10 µL of serum (diluted 24 times). The reaction mixture was composed by 1 mmol/L phenylacetate (Sigma-Aldrich) and 0.9 mmol/L $CaCl_2$ dissolved in 9 mmol/L Tris-HCl, pH=8. A molar extinction coefficient of 1310 $M^{-1}cm^{-1}$ was used for the enzyme activity calculation, expressed in kilo unit per liter. One unit of arylesterase activity accounts for 1 µmol of phenol produced in a minute under the conditions of the assay. PON-1 lactonase activity was measured by using gammathiobutyrolactone (TBL, Sigma-Aldrich) as substrate, and Ellman's procedure was used to spectrophotometrically monitor (412 nm) the accumulation of free sulfhydrylgroups via coupling with 5,5 dithiobis (2-nitrobenzoicacid) (DTNB). The reaction was started by adding 10 µL of sample to the reaction mixture containing buffer (50 mmol/L Tris, 1mM $CaCl_2$, 50 mmol NaCl, pH=8), 0.5 mmol/L DTNB, and 10.5 mmol/L TBL in each well. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used for the enzyme activity calculation, expressed in unit per liter [119], [126].

6.6.2 Lp-PLA2 activity

Lp-PLA2 activity was measured by UV–VIS spectrophotometric assays in a 96-well plate by using Tecan Infinite M200 microplate reader (Tecan Group Ltd., Switzerland).

Lp-PLA2 was assessed by using 2-thio PAF as substrate, which is hydrolyzed by the enzyme in sn-2 position. The consequent formation of free thiols was detected by Ellman's procedure. The reaction was started by adding 10 µL of sample to the reaction mixture containing buffer (100 mM Tris, 0.1 mmol/L ethylene glycol-bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA), pH=7.2), 0.5 mmol/L DTNB, and 0.2 mmol/L 2-thio PAF (Cayman Chemical, Ann Arbor, Michigan US) in each well. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used for the enzyme activity calculation, expressed in unit per liter [126].

6.7 Statistical analysis

Statistical analysis for immunoblot and immunohistochemistry was performed using unpaired two-tailed *t*-test (two groups) and one-way ANOVA. Normal distribution of data was assessed by applying Shapiro-Wilk normality test. A P-value < 0.05 was considered significant. Data are reported as mean ± SD or mean ± SEM . Exact P-values are indicated in the figure legends.

The normality of the distribution of arylesterase, paraoxonase, lactonase activities of PON-1 and Lp-PLA2 were checked by using Kolmogorov-Smirnov test. Since the distribution of paraoxonase and Lp-PLA2 was skewed, the values were log transformed in order to approximate a normal distribution before being analysed by parametric tests.

Analysis of variance (ANOVA plus Sidak post hoc test for pairwise comparisons) was employed to evaluate whether PON-1 activities were different between groups after checking for normal distribution by Kolmogorov-Smirnov test. Prevalence of categorical variables was compared by $\chi 2$ test. Analysis of covariance (ANCOVA plus Sidak post hoc test) was performed to check if the differences revealed by univariate analysis were influenced by gender. Receiver operating characteristic (ROC) analysis was performed to determine the ability of parameters examined to discriminate between RTT/ASD and control and between RTT and ASD. A P value < 0.05 was considered statistically significant.

7. RESULTS

There is a body of evidence suggesting that cholesterol metabolism is dysregulated in both periphery and brain of RTT patients. Indeed, these patients often present an altered plasma lipid profile with high levels of total, LDL- and HDL- cholesterol. It has been shown that these abnormalities could be the result of alterations in the main proteins belonging to cholesterol regulatory network, including SR-B1. In fact, SR-B1 protein expression of RTT primary fibroblasts was markedly reduced than in healthy fibroblasts, and this was due to an oxidative post-translational modification [141].
Since SR-B1 plays an important role also in the homeostasis of brain cholesterol, we investigated the expression levels of the receptor in this organ.

7.1 SR-B1 expression in the brain of Mecp2$^{-/y}$ mice

Brain cholesterol metabolism is independent from that in peripheral tissues due to the presence of the BBB. The brain possesses the highest levels of cholesterol, which is essential for neuronal physiology. The first step was to assess the expression levels of SR-B1, a receptor involved in the cholesterol uptake from HDL in our RTT model represented by cerebral cortex of Mecp2$^{-/y}$ and wt mice. As displayed in Figure 13A, by an immunostaining analysis it was discovered a strong reduction of SR-B1 (green staining) in the cortex of RTT mice compared to wild type. The Figure 13B depicted SR-B1 fluorescence intensity dropped by 50% in Mecp2$^{-/y}$ mice.

A **B**

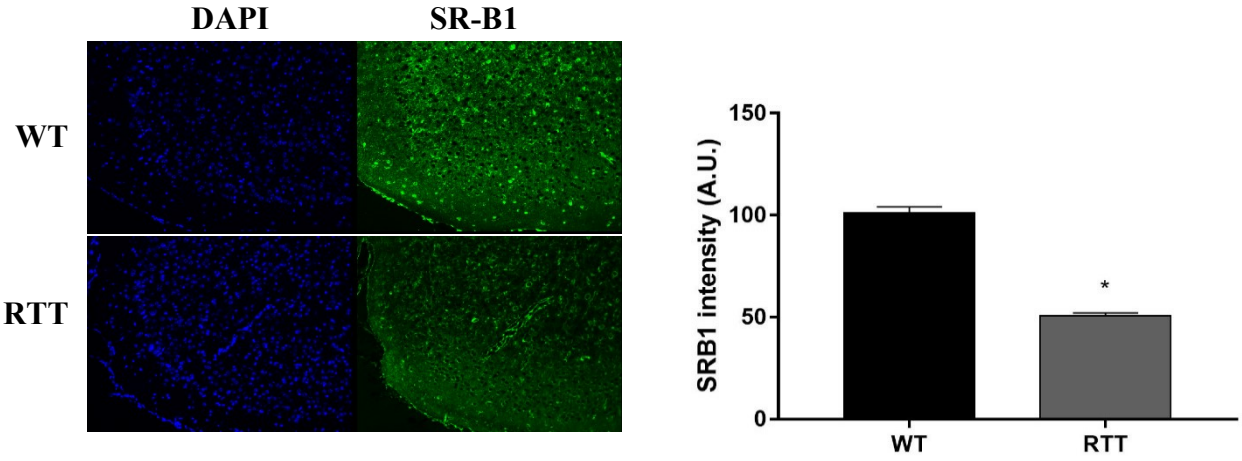


Figure 13: ***RTT mice displayed significant lower expression of SR-B1 in the cortex.***

Representative images of immunostaining of cortex sections of Mecp2$^{-/y}$ and WT mice. SR-B1 (green color) and cell nuclei (DAPI, blue color) fluorescence is shown in panel A whereas the quantification is shown in panel B (* $p<0.0001$). Data are expressed as mean ± SEM.

7.2 Reduction of SR-B1 expression in pre-symptomatic and symptomatic murine RTT cerebral cortex

Since cholesterol is very important during the development stage for a proper neuronal function, it was considered a possible relationship between SR-B1 expression and RTT clinical phenotype manifestations in murine cerebral cortex. Therefore, immunoblot analysis was performed in samples of pre-symptomatic and symptomatic Mecp2$^{-/y}$ mice. As shown in Figure 14, a 70% decrease of SR-B1 expression was observed in 5 weeks pre-symptomatic Mecp2$^{-/y}$ mice compared with wild-type. Moreover, the occurrence of the symptoms was accompanied by the complete loss of the receptor (17 weeks).

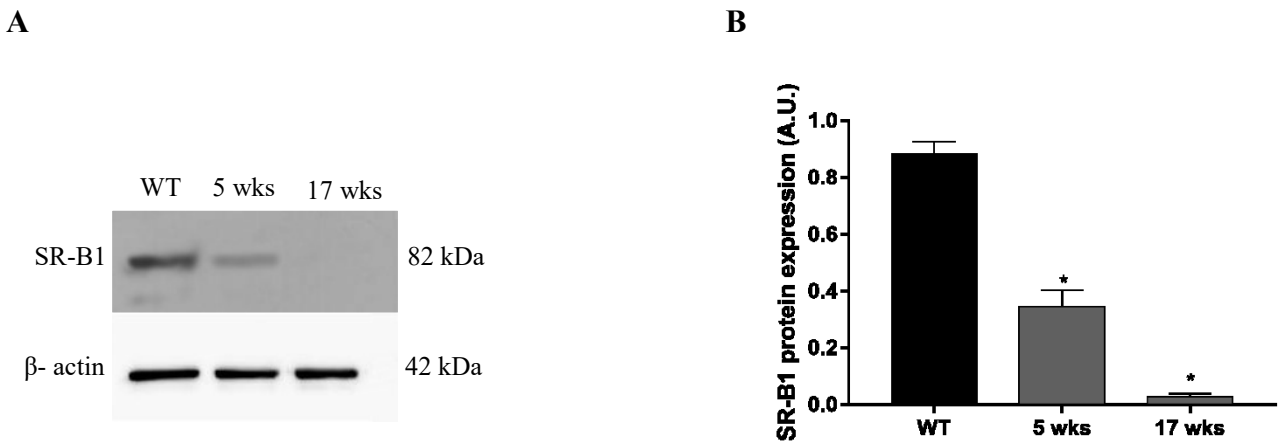


Figure 14: ***RTT symptomatic mice displayed absence of SR-B1 expression.***

Immunoblot analyses of SR-B1 in Mecp2$^{-/y}$ pre-symptomatic (5 weeks) and symptomatic (17 weeks) and WT mice. β-actin was used as loading control. Quantification of the bands is reported in panel B (* $p<0.0001$). Data are expressed as mean ± SEM. The results are representative of three independent experiments.

The interaction between SR-B1 and HDL is central in reverse cholesterol transport pathway. How SR-B1 binds HDL and then unloads cholesterol in the cells has not been fully clarified yet. It has been hypothesized that PON-1, an accessory protein of HDL, could play a role in facilitating the binding and promoting the movement of cholesterol from or to HDL, in addition to exerts antioxidant properties on the systemic level.

7.3 RTT showed absence of alterations in PON-1 and Lp-PLA2 activities

Interestingly, the reduction of SR-B1 in Mecp2$^{-/y}$ mice reflected the trend on the cellular level previously discovered [141]. It has been demonstrated that the interaction between this receptor and HDL is influenced by a protein associated to the lipoprotein itself called PON-1 [159]. This enzyme contributes to the athero-protective function of HDL, through its ability to exert antioxidant and anti-inflammatory activities as well as to mediate cholesterol efflux from peripheral tissue. The multiple evidence linking PON-1 with diseases characterized by perturbations in redox and cholesterol homeostasis both on the brain and systemic level, like RTT, gave us the idea to evaluate the serum activities of this enzyme in RTT patients. We considered also the activity of another enzyme bound up with lipoproteins and linked with lipid metabolism, Lp-PLA2.

As depicted in Figure 15, RTT sera patients showed the same trend for all the three activities of PON-1 (panel A) as well as for Lp-PLA2 (panel B) compared to controls, suggesting that these enzymes are not the main actors in protection from OxS.

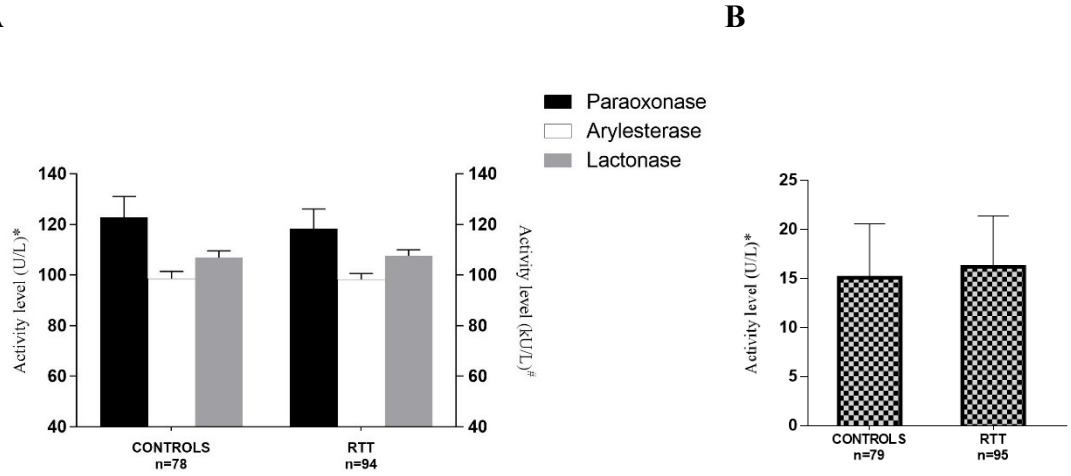


Figure 15: ***RTT patients didn't show any changes in PON-1 and Lp-PLA2 activities.***

Serum activities of paraoxonase, arylesterase and lactonase of PON-1 in RTT and control subjects are displayed in panel A, whereas activity of Lp-PLA2 in panel B. Data are represented as mean ± SD. * Lactonase and paraoxonase activities are expressed as U/L. #Arylesterase activity is expressed as kU/L.

7.4 ASD displayed lower levels of lactonase PON-1 and Lp-PLA2 activity compared to RTT and controls

Since it has been demonstrated that also ASD patients are characterized by the presence of metabolic/redox abnormalities similar to those found in RTT, we decided to extend our analyses to ASD patients.

We found that PON-1 related activities had a comparable trend of arylesterase (ANOVA: $p<0.001$) and paraoxonase (Kruskal-Wallis: $p=0.04$) activities across the sample groups. The pairwise differences were significant only for lactonase, whose levels decreased by approximately 14% in ASD ($p<0.001$ for both pairwise comparisons) compared to controls (Figure 16).

Similar to paraoxonase and lactonase activities, serum Lp-PLA2 reached the lowest level in ASD patients (Figure 16). To be noted, the decrease of Lp-PLA2 was more evident (−32% and −37%) to that observed for PON-1 activities, compared to controls and RTT patients, respectively.

A **B**

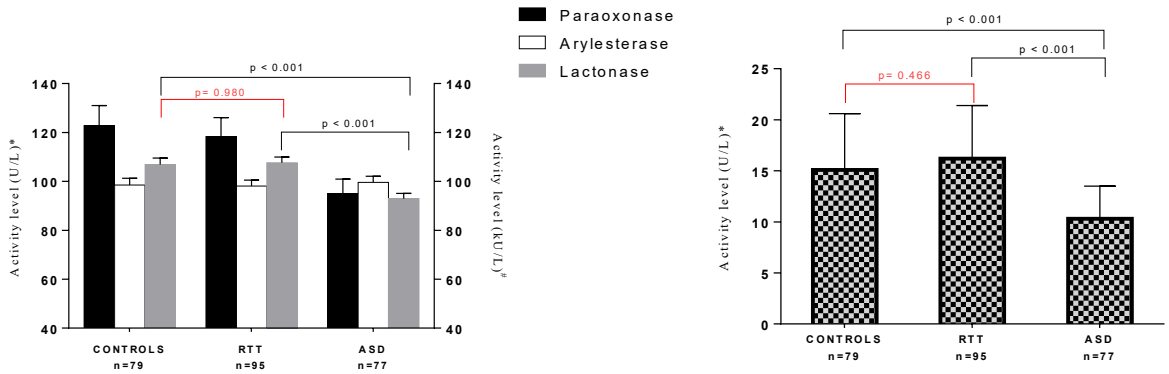


Figure 16: ***ASD patients showed lower levels of lactonase PON-1 and Lp-PLA2 activity.***

A Serum activities of paraoxonase, arylesterase and lactonase of PON-1 in RTT, ASD patients and control subjects. Data are represented as mean ± SD. * Lactonase and paraoxonase activities are expressed as U/L. #Arylesterase activity is expressed as kU/L.

B Serum activity of Lp-PLA2 in RTT, ASD patients and control subjects. Data are represented as U/L ± SD. P values are displayed in the graph.

7.5 Study of the possible effects of gender on statistical outcomes

Owing the evident differences in the female/male distribution among the groups examined (Table 5), we checked whether the changes in Lp-PLA2 and lactonase activities revealed by univariate analysis were influenced by gender. These analyses (data not shown) showed that, after adjustment for gender,

lactonase was still significantly lower in ASD compared to controls ($p<0.05$) and RTT ($p<0.001$), with the latter difference becoming even more dramatic. Alike, Lp-PLA2 remained significantly lower in ASD than in the other two sample groups ($p<0.001$ for both comparisons). Of note, the significant levels of the above-described differences in lactonase and Lp-PLA2 were not affected by age.

	Controls (n=79)	**RTT (n=95)**	**ASD (n=77)**	**Statistics**
Gender F/M (n)	38/41	95/0	26/51	Controls versus RTT, p<0,001 RTT versus ASD, p<0,001
Age (years)	12±7	16±9	13±8	Controls versus RTT, p=0,055 RTT versus ASD, p=0,940

Table 5: ***Mean age and gender prevalence across the sample groups.***

Age is expressed as mean ± SD (min–max). Difference between groups were evaluated by $\chi 2$ test (for gender) and Sidak post hoc test (for age).

7.6 ASD female patients showed lower levels of lactonase PON-1 and Lp-PLA2 activities compared to RTT and control subjects.

Since RTT group was completely composed by girls and ANOVA was not the most proper approach to check potential differences between RTT and the other two sample groups, we compared lactonase and Lp-PLA2 levels only considering the females present in ASD and control sample (n=157). As displayed in Figure 17A, lactonase activity remained significantly lower in ASD compared to RTT ($p= 0.001$), while the gap between the former and controls was reduced (when compared to total sample) and reached almost a significant threshold ($p=0.06$). The proportions were preserved also for Lp-PLA2 (Figure 17B), which resulted markedly lower among ASD compared both to controls and RTT ($p<0.001$ for both comparisons).

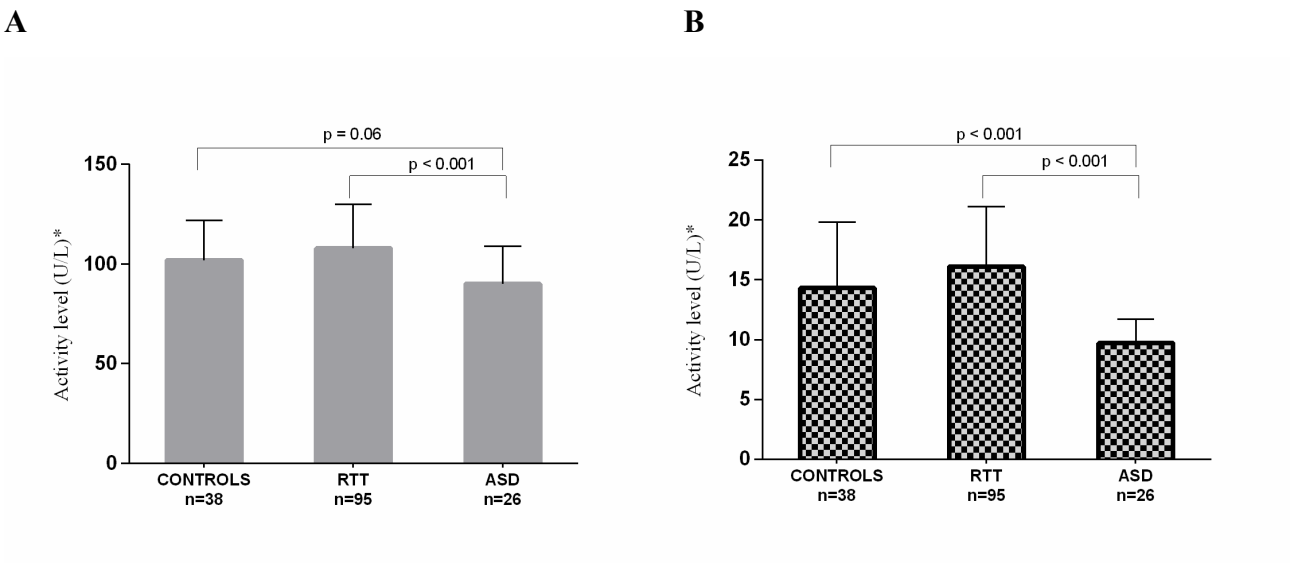


Figure 17: ***ASD female patients showed lower levels of lactonase PON-1 and Lp-PLA2 activities compared to RTT and control subjects.***

A Serum activity of lactonase PON-1 in RTT, ASD female patients and control subjects. Data are represented as U/L ± SD. p values are displayed in the graph.

B Serum activity of Lp-PLA2 in RTT, ASD female patients and control subjects. Data are represented as U/L ± SD. p values are displayed in the graph.

7.7 Lactonase PON-1 and Lp-PLA2 as possible biomarkers to discriminate between RTT and ASD patients

PON-1 and Lp-PLA2 might be implicated in the complex picture of immune dysregulation, inflammation, redox imbalance, and OxS characterizing both RTT and ASD. In addition to share some common features, these two neurodevelopmental diseases represent "biomarker-orphan" pathologies.

The results presented previously suggested a possible role of lactonase and Lp-PLA2 as diagnostic biomarkers to discriminate ASD from controls and RTT. To address this hypothesis, a ROC analysis was performed for (1) controls versus ASD (considering both males and females) and (2) RTT versus ASD (considering only females). As displayed in Figures 18 and 19 (results summarized in Table 6), Lp-PLA2 appeared to be the most efficient parameter to discriminate between ASD patients and control subjects and, in particular, girls with RTT from those with ASD (both sensitivity and specificity around 80%) (Figure 19B). On the contrary, lactonase appeared to have a lower diagnostic potential (poor sensitivity) to discriminate between ASD and controls with respect to Lp-PLA2 (Figure 18A). Similarly, as suggested by previous analyses, neither lactonase nor Lp-PLA2 showed an acceptable accuracy in distinguishing controls from RTT patients (the best sensitivity and specificity around 60%, considering only female subsample) (Figure 20).

Control versus ASD					
	AUC (95% CI)	p value	Cutoff	Sensitivity	Specificity
Lp-PLA2	0.780 (0.705-0.854)	<0.001	11.2	70	77
Lactonase	0.660 (0.576-0.746)	<0.001	96.4	59	63
ASD versus RTT					
	AUC (95% CI)	p value	Cutoff	Sensitivity	Specificity
Lp-PLA2	0.858 (0.769-0.940)	<0.001	12.0	80	83
Lactonase	0.714 (0.612-0.816)	<0.001	102	57	71

Table 6: ***Performances of lactonase and Lp-PLA2 to discriminate between controls and ASD (n=156) and between ASD and RTT females (n=121).***

Cutoff points corresponding to the best combination between specificity and sensitivity.

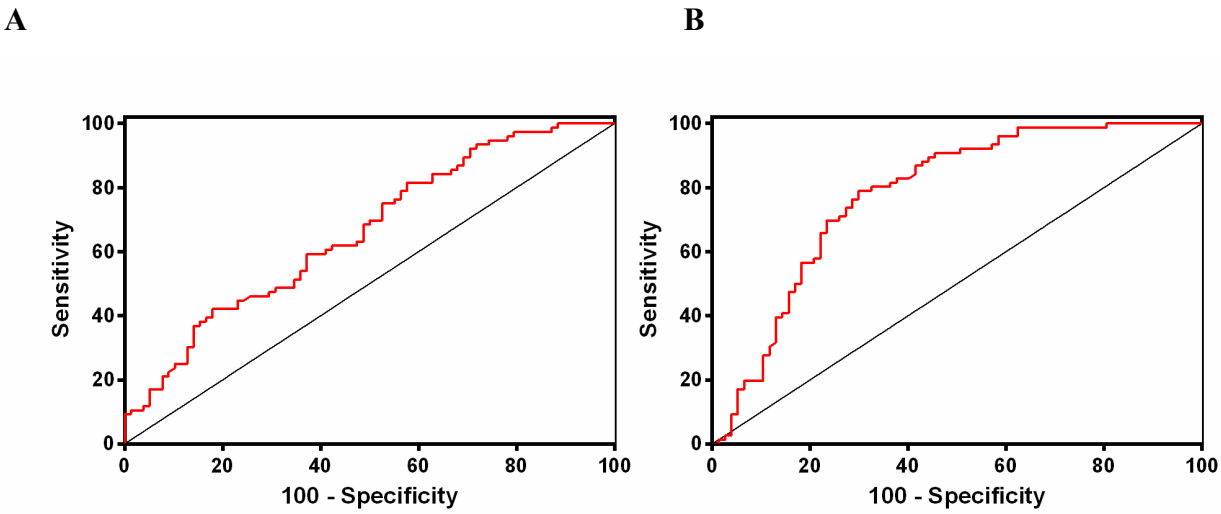


Figure 18: ***Lactonase PON-1 and Lp-PLA2 seemed represent possible biomarkers for ASD.***

A ROC curve of lactonase activity for the discrimination between controls and ASD (n=156).

B ROC curve of Lp-PLA2 activity for the discrimination between controls and ASD (n=156).

A **B**

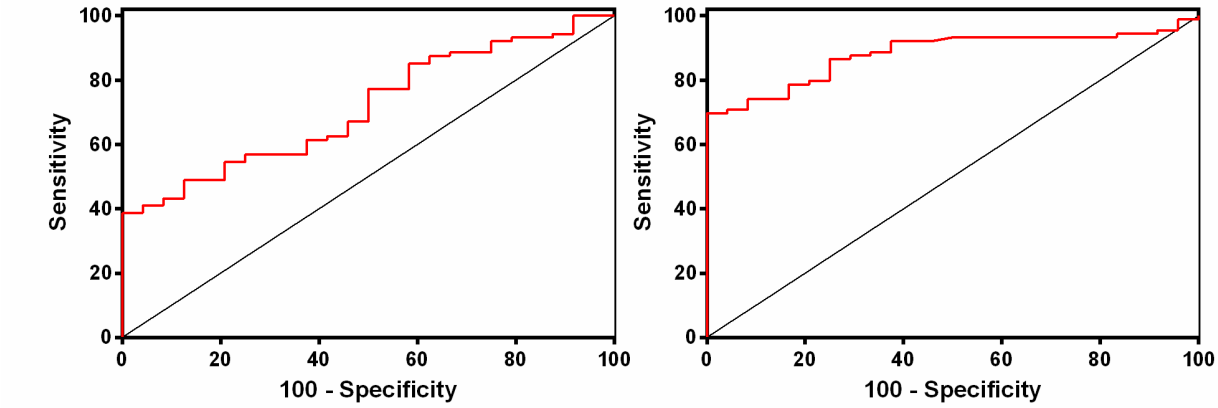


Figure 19: ***Lactonase PON-1 and Lp-PLA2 seemed represent possible biomarkers to discriminate between females ASD and RTT.***

A ROC curve of lactonase activity for the discrimination between RTT and ASD (only females, n=121).

B ROC curve of Lp-PLA2 activity for the discrimination between RTT and ASD (only females, n=121).

A **B**

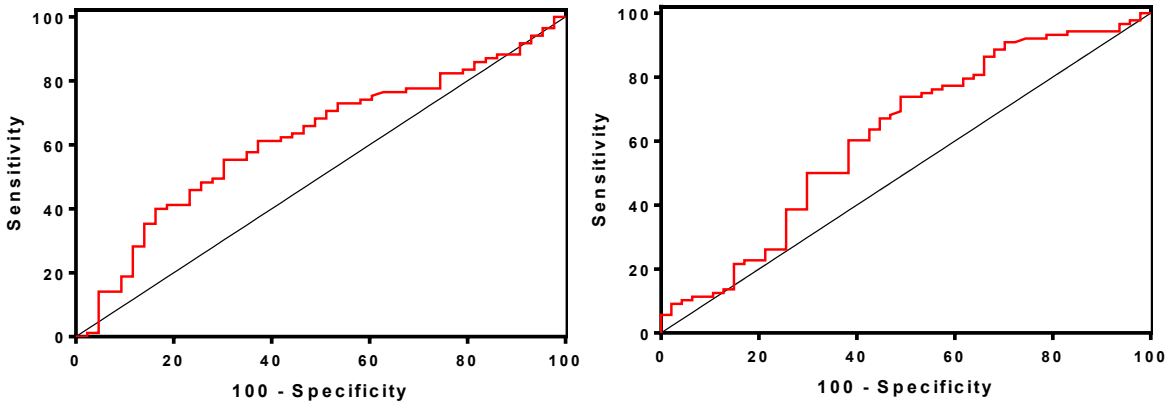


Figure 20: ***Lactonase PON-1 and Lp-PLA2 as possible biomarkers to discriminate between RTT and controls.***

A ROC curve of lactonase activity for the discrimination between RTT and ASD (only females, n=133).

B ROC curve of Lp-PLA2 activity for the discrimination between RTT and ASD (only females, n=133).

8. Discussion and conclusions

MECP2 is a gene with multifaceted roles in gene regulation and neural development and has been shown that its deletion in mice results in a host of severe metabolic defects caused by lipid accumulation [143].

A very interesting paper published by Kyle *et al.,* highlighted the importance of metabolism dysregulation in RTT, pointing out the central role of cholesterol metabolism [139]. But also several papers from our and other groups, reported increase of total cholesterol as well as impairment in the protein network responsible of cholesterol homeostasis in RTT mice and patients [70], [141], [142].

In particular, we found decrease levels of SR-B1, the physiologically functional HDL receptor involved in RCT, which underwent an oxidative modification acting as a signal for its ubiquitin-mediated degradation [141]. Since the brain is the most cholesterol enriched organ, that cholesterol is essential for neurodevelopment, and the finding that (HDL)-like particles can cross the BBB, the first purpose of this study was to assess the expression of SR-B1 also in $Mecp2^{-/y}$ mice and a possible connection of its expression with the appearance of the symptoms. The model used were cortex samples obtained from $Mecp2^{-/y}$ mice, which is suitable for the investigation manifesting many aspects of the disease being considered a good model [160].

Therefore, we started performing an immunostaining analysis for SR-B1 on $Mecp2^{-/y}$ and wild-type mice founding decrease levels of SR-B1 in RTT cortex. Interestingly, as shown by immunoblot, the decrease expression of SR-B1 was parallel to the appearance of RTT symptoms in mice. Such results validated the previous one obtained from our group, suggesting a cholesterol impairment not only localized in RTT fibroblasts but also in the brain, the main site of MECP2 expression.

A dysregulation of SR-B1 levels has been linked with many diseases; in fact, its levels were found altered in many kinds of cancer like prostate, breast, colorectal, ovarian and pancreatic [88] in addition to the finding that a partial or total loss of SR-B1 leaded to increase in atherosclerosis [161], [162]. Van Berkel and Krieger showed a significant increase in plasma HDL and total cholesterol levels in SR-B1/KO mice beside to abnormally large HDL particles developing atherosclerotic lesions of the arteries and heart with high plasma cholesterol concentrations [161], [163]. The brain possesses a highly specialized BBB which impermeability is not absolute and permits the regulated transport of various molecules, including HDL through transcytosis [164]. Recently, in a very elegant way, Fung *et al.* reported that SR-B1 mediated the HDL transcytosis in brain microvascular endothelial cells by a non-canonical signalling [165]. Therefore, taking into account the systemic OxS condition already demonstrated in RTT [152], [166], that a full rescue of brain redox homeostasis following brain-

specific MECP2 gene reactivation in Mecp2 stop/y NestinCre animals [167], it might be hypothesize that also brain SR-B1 undergo on a oxidative modification and degradation with the consequent altered cholesterol transport due to its role in HDL binding. Thus may lead to an alteration of cholesterol levels in the brain, where is fundamental for a proper neuronal functions not only during development but also in the adult life. Of note, SR-B1 is not the only one receptor involved in HDL internalization across the BBB, but also ABCG1 [168] and F0F1 ATPase [169] could participate.

HDL provides a mechanism for the transfer of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors, and, more importantly, metabolizes lipid hydroperoxides preventing their accumulation on low-density lipoproteins (LDLs) [85]. The candidates that have been suggested to be responsible of HDL's antioxidant function are several, but the most prominent seems to be paraoxonase-1 (PON1) [85]. Interestingly, it has been shown that SR-B1 represents the principal mediator of the ability of HDL to acquire PON-1 [109]. Another component of HDL that can contribute to its atheroprotective function is Lp-PLA2 [170]. Even if most Lp-PLA2 exists in complex with LDL, PLA2 on HDL is likely to have an antioxidant activity based on the same mechanism of PON-1, by hydrolysing lipid hydroperoxides [85].

There are plenty of evidence showing that PON-1 and Lp-PLA2 levels are altered in diseases apparently characterized by the detrimental crosstalk between altered redox homeostasis and chronic inflammation [119], [136], [156]–[158], but the data regarding RTT are absent. Thus, we evaluated the three serum activities of PON-1 and Lp-PLA2 in RTT patients. We discovered that RTT patients didn't show any significant alterations in both the three activities of PON-1 as well as in Lp-PLA2 compared to healthy subjects. This result could be explained considering that the systemic defensive machineries represented by PON-1 and Lp-PLA2 could play a minor role in OxS protection. It is possible that a major role in the OxInflammation condition of RTT patients is caused by a deleterious vicious cycle between dysfunctional mitochondria and an inadequate defensive enzymes activity response, in addition to the involvement of MECP2 in controlling the expression of several redox-related genes [171], [172]. Therefore, since RTT share some common characteristics with ASD, included the presence of metabolic/redox abnormalities similar to those found in RTT, we decided to continue our study performing a comparison of the activity of these enzymes between the two diseases.

Interestingly, ASD patients displayed a significant reduction of lactonase PON-1 (circa 14%) and Lp-PLA2 (37%), compared to RTT. In this regard, lactonase is considered the primary and the physiological activity of the enzyme whose natural substrates are lactones derived from food hepatic

catabolism, drugs metabolism and fatty acid oxidation [117]. The substrates of the other two activities of PON-1, paraoxonase and arylesterase, are represented by metabolites of handmade toxic chemicals such as organophosphorus compounds including insecticides and nerve agents and non-phosphorous aryl esters such as phenyl acetate [121]. Two works of different groups reported decrease PON-1 arylesterase activity in autism [173], [174], additionally to D'Amelio *et al.* that found an association between genetic polymorphisms of PON-1 and their activities just in American families [175]. These opposite results, in comparison to ours, might be due to differences in the population studied (e.g. age, sample size) but also the levels of pollutants can influence the final result leading to a still unrevealed interaction between genetic susceptibilities, toxicant exposure and ASD risk [175].

Despite the still unclear molecular mechanisms, increasing evidence support the idea that PON-1-lactonase contributes to the atheroprotective function of HDL by counteracting lipid peroxidation on LDL, HDL and immune or nonimmune cells in a variety of diseases with an inflammatory component [119], [120], [176]. Differently from PON-1, the "in vivo" action of Lp-PLA2 is suggested to be beneficial only in some physiological and pathological settings, whereas in others (e.g., atherosclerosis) seems to be detrimental [135].

Owing the evident differences in the female/male distribution among the groups examined, we checked whether the changes in Lp-PLA2 and lactonase activities were influenced by gender. These analyses showed that, after adjustment for gender, lactonase was still significantly lower in ASD compared to controls and RTT, with the latter difference becoming even more dramatic. Alike, Lp-PLA2 remained significantly lower in ASD than in the other two sample groups.

One of the key concept in the definition of an ideal biomarker is that it should be "non-invasive, easily translatable to routine clinical testing, or eventually microfluidic high-throughput population screening and expedient serial monitoring" [177]. Factors that determine the clinical usefulness of a biomarker include the ease and the cost of assessment, its performance characteristics (e.g., sensitivity, specificity, etc.) and the ability to reflect disease pathophysiology and to be helpful in the understanding the pathological process [178], [179]. In this context, the results obtained suggested a possible role of lactonase and Lp-PLA2 as diagnostic biomarkers. Therefore, we continued our study with the aim to identify a potential biomarker to discriminate between RTT and ASD, two "biomarker orphan" diseases which are almost undistinguishable during the typical regression stage of RTT, when girls display many autistic features, such as loss of communication and social skills, poor eye contact and lack of interest.

To address this hypothesis, a ROC analyses was performed. If lactonase appeared to have a low diagnostic potential (poor sensitivity) to discriminate between ASD and controls, Lp-PLA2 seemed to be the most efficient parameter to discriminate especially between girls with RTT from those with ASD, reaching levels of sensitivity and specificity around 80%.

All together, the results obtained in this study confirmed and suggested a key role of SR-B1 in cholesterol metabolism in patients affected by RTT, linking the appearance of the clinical symptoms with the total loss of the scavenger receptor. In addition, it can be proposed the novel finding of Lp-PLA2 as a possible biomarker to discriminate girls with ASD from controls and from those affected by RTT with good accuracy, leading to promising clinical application. In our opinion, children between one and two years of age could be the most appropriate targeted population for testing its diagnostic usefulness.

CHAPTER II

Mitochondria as possible key players in RTT Syndrome pathogenesis

1. Introduction

In 1990, before the identification of MECP2 as the causal gene of RTT, Olofsson and colleagues found abnormal mitochondria in muscle biopsy of six girls affected by RTT, suggesting that RTT could represent a Mitochondrial disorder (MD) [180]. Therefore, after the discovering of the gene involvement, the interest of the scientific community shifted toward the understanding of the brain functions of MECP2 and the implications of its mutations on the neurodevelopment and on the neurophysiology [181].

Recently, mitochondria became again a target of many research regarding this complex syndrome, mainly due to the increasing proofs in support of a key role exerted by OxS in the pathogenesis of RTT [181].

2. Mitochondria

The word mitochondrion derives from the Greek, *mitos* – (thread) and *chòndros* (granule) and was coined in 1898 by the microbiologist Carl Bend. The first record of this intracellular structure that possibly represented mitochondria goes back to the 1840. However, only in 1890 Altmann recognized for the first time the ubiquitous distribution of these structures calling them "bioblasts", affirming that they were "elementary organisms" living within the cells and carrying out vital functions. After almost 60 years, in 1952, it was published the first high-resolution electron micrographs of mitochondria [182].

2.1 Physiological roles of mitochondria

Mitochondria perform multiple essential functions that influence gene expression within the cell nucleus as well as the physiological regulation across the organism [183]. They represent the only organelle containing their own genome, which is of primary importance since it encodes essential genes for energy production. Although the main function ascribed to this organelle is the production of ATP leading to its consideration as the powerhouse of the cell, emerging evidence support the idea that mitochondria conduct also completely new and many fundamental roles. Among those they are involved in amino acids metabolism [184], iron-sulphur (Fe/S) protein biogenesis [185], lipid metabolism [186], ROS production [183], Ca^{2+} signalling [187] and regulation of cell death pathways [188].

2.1.2 Mitochondria as a source and a target of oxidative stress

The term OxS was first coined by Sies in 1989 to be later re-defined by Ursini *et al*. as "the consequence of the failure to maintain the physiological redox steady state, which is the self-correcting physiological response to different challenges" [189].

Mitochondria have a key role in signalling hubs and communicate with the rest of the cell through various means including ROS. In this context, through the mitochondrial respiration, they represent the major producers of ROS within the cell, acting as a signal at low levels but leading to OxS when they overcome the antioxidant defence mechanism [183].

The mitochondrial electron transport chain contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of superoxide production in most tissues. There is growing evidence that the complex I acts as the main producer of $O^{\cdot -2}$ by intact mammalian mitochondria in vitro. This $O^{\cdot -2}$ production occurs primarily on the matrix side of the IMM and was found to be markedly stimulated in the presence of succinate, the substrate of the Complex II. Also Complex III is regarded as an important site of $O^{\cdot -2}$ production, where it appears on both sides of IMM. In addition, Ubiquinone, a component of the mitochondrial respiratory chain, which links Complex I with III, and II with III, is considered the major player in the formation of $O^{\cdot -2}$ by Complex III. In particular, the oxidation of ubiquinone proceeds in a set of reactions known as the Q-cycle where the unstable semiquinone is responsible for $O^{\cdot -2}$ formation [190].

ROS are a normal side product of the respiration process, reacting with lipids, protein and DNA generating oxidative damage. Indeed, mitochondria are the major site of ROS production, but also the major targets of their detrimental effects, representing the trigger for several mitochondrial dysfunctions [191]. The wide range of mitochondrial ROS-induced damage that have been described include proteins carbonylation, lipid peroxidation or mtDNA damage. These harm, either individually or collectively, can lead to a cellular energetic catastrophe, possibly affecting the physiological balance within the organism leading to the manifestation of diseases [191].

3. The mitochondrial life cycle

Mitochondria are dynamic organelles that constantly change their number, size, shape, and distribution in response to intra- and extracellular stimuli [192], [193]. They proliferate from pre-existing ones through the process called biogenesis and enter in the constant cycles of fission and fusion that organize them into two distinct states — "individual state" and "network state" (Figure 21). When compromised from various injuries, solitary mitochondria generated via fission events are subjected to degradation through a clearance pathway, known as mitophagy [192].

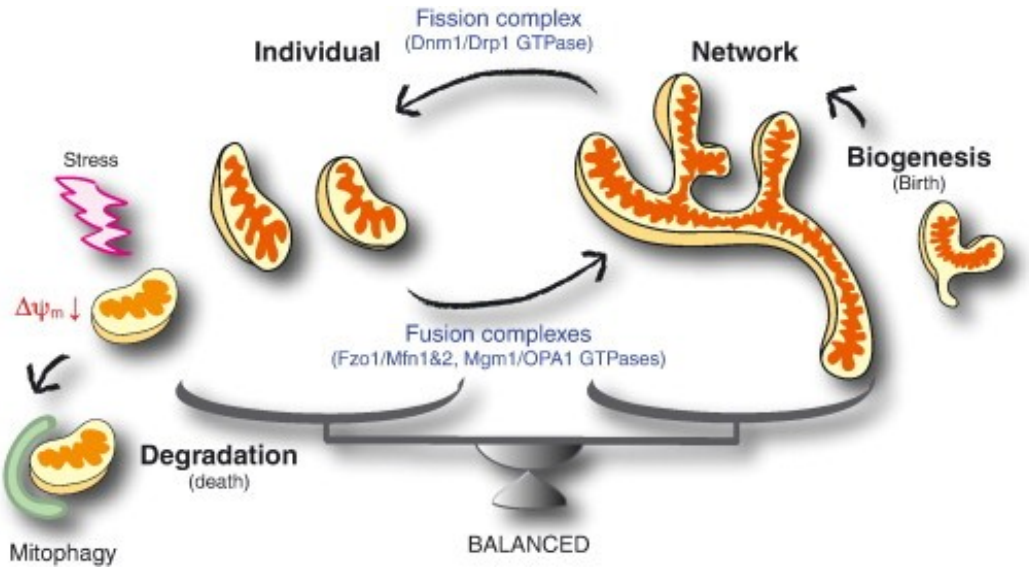


Figure 21: ***Representation of the mitochondrial life cycle.***

Reported from *Okamoto et al.* 2012 (192).

3.1 Mitochondrial fusion

Mitochondrial fusion involves multiple steps, including mitochondrial tethering and fusion of OMMs/MOMs), docking and fusion of the IMMs/MIMs and mixing of intramitochondrial components (Figure 22).

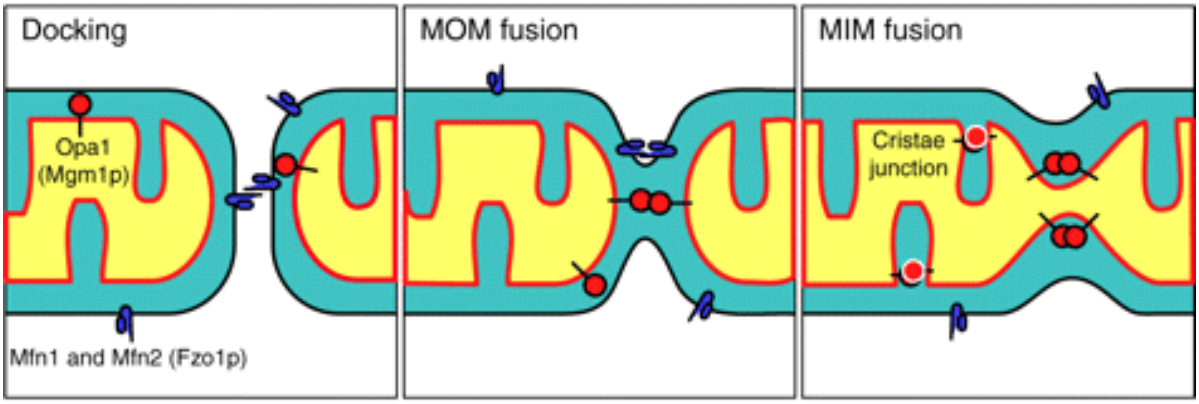


Figure 22: ***Schematic illustration depicting the core proteins of the fusion molecular machinery.***
Reported from *Seo et al.* 2010 (193).

The two dynamin-related GTPases Mfn1 and Mfn2 are the principal regulators of OMM fusion in mammals [194]. Mfn1 and Mfn2 are anchored to the OMM through their C-terminal membrane-binding domain, whereas their N-terminal GTPase domain reside in the cytoplasm. Both Mfns mediate fusion through their active GTPase domains by forming homo- and hetero-oligomers promoting tethering and fusion of the OMMs from two different mitochondria [193], [195].
Mammalian Mfn1 and Mfn2 proteins have greater than 70% sequence similarity and share much of the same functional domain. Although both proteins are widely expressed, Mfn2 is highly abundant in heart and skeletal muscle and is reported to be expressed at low levels in numerous other human tissues [193]. Despite some redundancies in their function, the two proteins possess different roles in cell physiology. Mfn2 is the more versatile protein and participates in cell metabolism, tethering the endoplasmic reticulum (ER) to mitochondria, and cell proliferation. Moreover, mutations in the Mfn2 gene are associated with the peripheral neuropathy [194].
IMM fusion is controlled by another dynamin-related GTPase, optic atrophy 1 (Opa1). It is believed to play a constitutive role in IMM fusion and remodelling of mitochondrial cristae structure by facing the inter membrane space [193]–[195].

3.2 Mitochondrial fission

The regulation of mitochondrial fission in mammalian cells is mainly controlled by two key proteins: dynamin-related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1) (Figure 23).

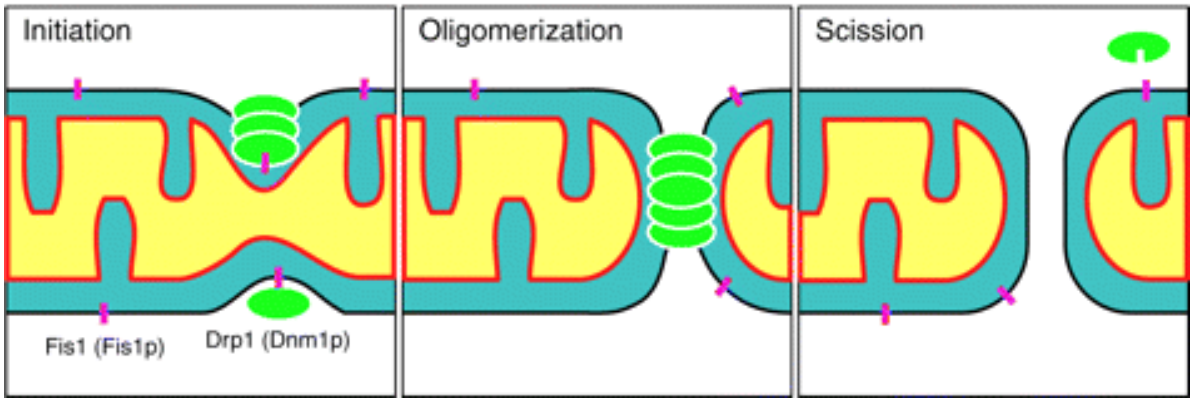


Figure 23: ***Schematic illustration depicting the core proteins of the fission molecular machinery.***
Reported from *Seo et al.* 2010 (193).

Drp1, a member of the dynamin family of large GTPases, is predominantly located in the cytosol and needs to be activated and translocate to mitochondria in order to constrict and cut the mitochondria. Drp1 activity is modulated by post-translational modifications such as phosphorylation, SUMOylation and ubiquitylation to ensure adaptation to the various cellular needs [193], [195]. Among those, S616 is a site phosphorylated by many kinases including CDK1, ERK 1/2 and PKC. Phosphorylation at this site enhances the activity of Drp1 under certain circumstances such as OxS, mitosis or high glucose, with the result of mitochondrial fragmentation [196].

The pro-fission GTPase Drp1 does not contain a membrane-localizing pleckstrin homology (PH) domain, transmembrane (TM) or any other membrane-anchoring domain; therefore, it needs to be actively recruited to the mitochondrial surface by MOM-anchored receptors in order to execute its function [197]. Furthermore, Fis1 has been proposed to acts as a Drp1 receptor, being necessary for mitochondrial fission. In contrast to Drp1, mammalian Fis1 does not contain a GTPase domain and is primarily localized to the OMM by a transmembrane domain located in its C-terminal region. However, the mechanism of action of human Fis1 is still highly controversial. In this regard, new possible Drp1 receptors have been recently identified: the OMM-anchored mitochondrial fission factor (Mff) and the OMM-bound mitochondrial elongation factor1/mitochondrial dynamics 51 (MIEF1/MiD51) [195].

3.3 Mitophagy

Mitophagy is a fundamental and physiological cell process with the main role to eliminate the excessive harmful damage represented by old or dysfunctional mitochondria and maintain the specific organismal homeostasis. It is a selective bulk degradation process in which, mainly using the macroautophagy machinery, entire mitochondria are enclosed in a double-membrane vesicle called autophagosome, and subsequent delivered to lysosomes for their hydrolytic degradation. It is accompanied by a characteristic phenotype of fragmented mitochondria [198].

Mitophagy is also a mechanism which regulates the number of mitochondria in response to developmental signals: i) the "programmed" mitophagy which occurs in case of reticulocyte differentiation and during the elimination of parental mitochondria in the fertilized oocyte; ii) the "reactionary" mitophagy which starts when mitochondria are defective [195].

3.3.1 Pathways of mitophagy

According to these two programs of mitophagy, three well-known effectors are involved. The first is Nix/BNIP3L, which is located on mitochondria and is the main player of mitophagy during reticulocyte differentiation whereas PINK1 and Parkin play a key role when mitochondria are damaged [195].

3.3.1.2 PINK1/Parkin-mediated mitophagy

The PINK1/Parkin-mediated mitophagy is considered the best described for the degradation of dysfunctional and depolarized mitochondria [195].

Recent studies indicate that this pathway ensures the integrity and the functionality of mitochondria [199].

At the beginning, Parkin was identified as a cytosolic E3 ubiquitin ligase mutated in familial forms of Parkinson's disease (PD), and mutations in this protein are now known to be the most prevalent cause of autosomal recessive monogenic PD.

It has been discovered that in other autosomal recessive cases of PD the gene encoding PTEN-induced kinase 1 (PINK1), a serine/threonine kinase, is also mutated [198]. Studies of loss-of-function mutations in Drosophila melanogaster first linked Parkin to mitochondrial maintenance [200]. It was demonstrated that in this model, the Pink1-knockout phenotype is rescued by overexpression of

Parkin, whereas the Parkin-knockout phenotype is not rescued by PINK1 overexpression, indicating that PINK1 is an upstream regulator of Parkin function [198].

Based on these reports, the finding that Parkin translocates to depolarized mitochondria and induces mitophagy [198], in 2010 several groups referred that the recruitment of Parkin to impaired mitochondria required PINK1 expression and its kinase activity [201]–[204]. Even if a predicted mitochondrial targeting signal (MTS) was identified in the N-terminus of PINK1 [205], it still to be not known how PINK1 recruits Parkin to mitochondria.

However, a very elegant paper published by Okatsu *et al*. tried to explain the molecular basis of PINK1 localization on the outer membrane of depolarized mitochondria. In mitochondria with a normal $\Delta\Psi m$, the positively charged MTS of PINK1 is imported into the mitochondrial matrix with its subsequent stepwise cleavage; in particular, it is first processed by the mitochondrial processing peptidase (MPP), and then cleaved by the intramembrane Presenilins-associated rhomboid-like protein (PARL) (Figure 24). The following exposure of the phenylalanine (Phe) residue at position 104 of the N-terminus processed PINK1 following PARL-mediated cleavage acts as a signal for the 'N-end rule pathway'-mediated degradation. Thus leads to PINK1 proteasomal degradation and the PINK1/Parkin signal get turn off [206].

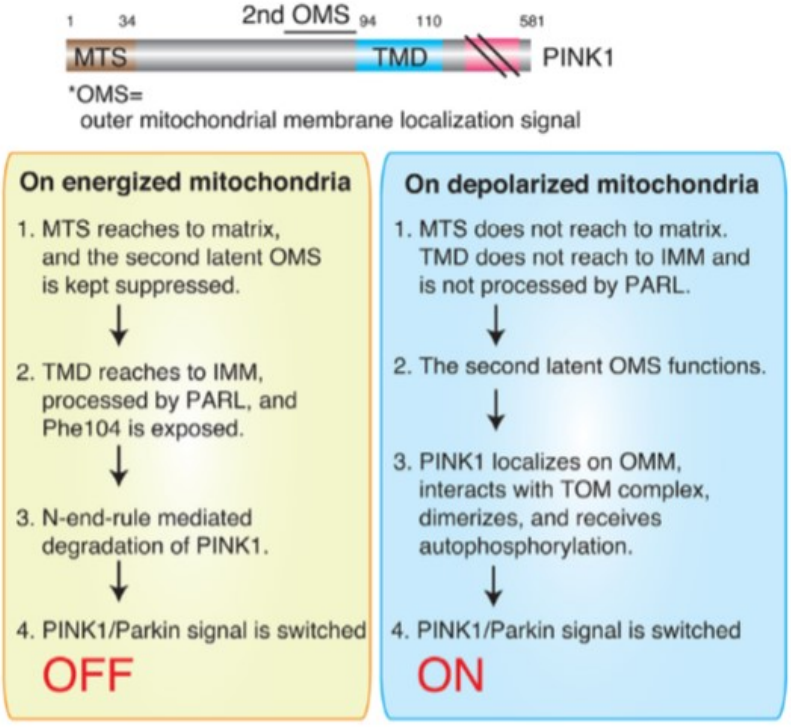


Figure 24: *Proposed model for PINK1 localization on the OMM of depolarized mitochondria.*
Reported from *Okatsu et al.* 2015 (206).

In contrast, upon a strong loss of mitochondrial membrane potential, the MTS and the transmembrane domain (TMD) don't reach the matrix and the IMM, respectively. Therefore, PINK1/Parkin mitophagy is initiated with the recruitment of PINK1 on the surface of OMM of damaged mitochondria through the translocase of outer mitochondrial membrane 20 (TOM20) complex, PINK1 dimerizes and autophosphorylates, working as a sensor of mitochondrial potential.

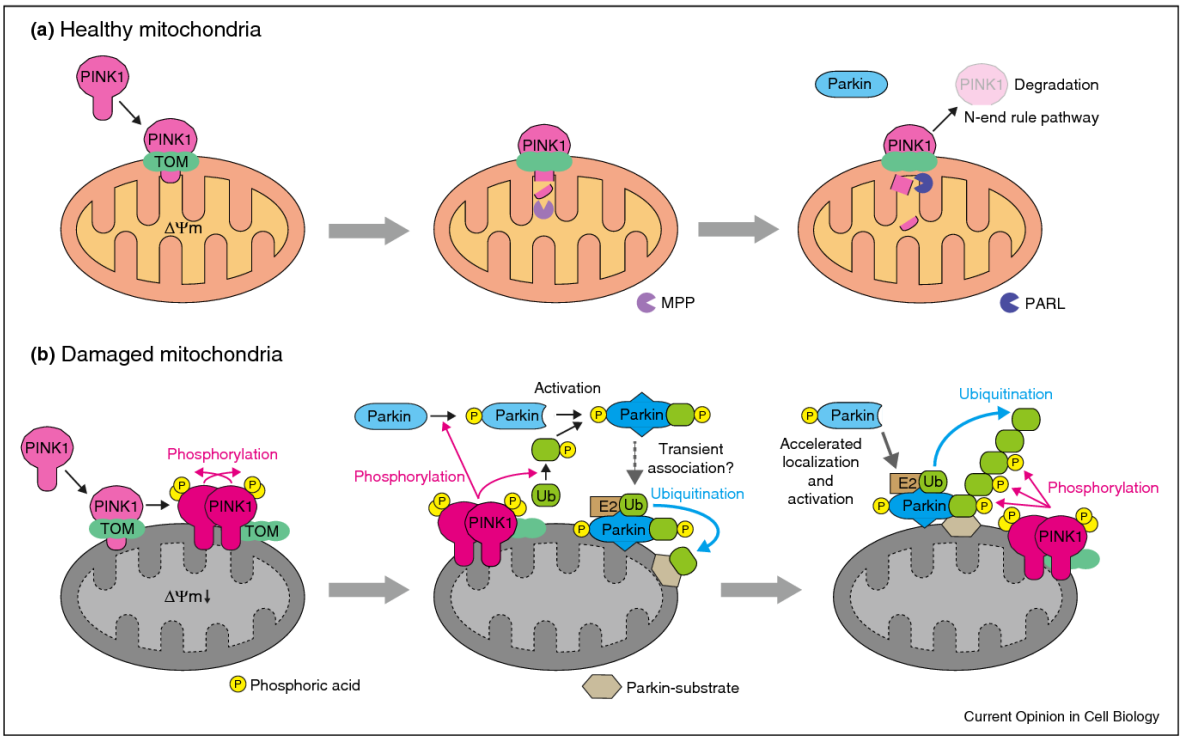


Figure 25: ***PINK1/Parkin-mediated mitophagy pathway in healthy and damaged mitochondria.***

Reported from *Okatsu et al.* (2015) (206).

The following step is represented by the recruitment of Parkin by PINK1 from the cytosol to mitochondria, its phosphorylation and the promotion of its activity. Parkin is an E3 ubiquitin ligase which ubiquitinates OMM proteins like Mitofusin-1 (MFN1), Mitofusin-2 (MFN2), voltage-dependent anion channel (VDAC), some components of the TOM complex (TOM70, TOM40, TOM20), the pro-apoptotic factor Bcl-2 homologous antagonist killer (BAK), mitochondrial Rho GTPases (MIRO) 1 and 2, mitochondrial fission 1 protein (FIS1) and activates the recruitment of ubiquitin-binding adaptor proteins, such as p62/SQSTM1 (Figure 25). The binding of mitochondria to p62 is followed by clustering of mitochondria around the nucleus. The microtubule associated protein light chain 3 (LC3) is needed as a scaffold protein recruiting the autophagic machinery which is directly linked with the classical autophagic machinery [195].

3.3.2 Physiological roles of mitophagy

Mitophagy holds a key role in mitochondrial quality control being responsible for the recognition of damaged, old or dysfunctional mitochondria and their subsequent selective removal. In this regard, by using various models it has been demonstrated that suppression or abnormalities in mitophagy result in the accumulation of mitochondria. Mitophagy is a central process not only for removing damaged mitochondria, but also in promoting biosynthesis of new mitochondria. In fact, it has been shown the presence of a crosstalk between the mitophagic pathway and the mitochondrial biosynthesis in *C. elegans* model [207]. Shin *et al.* discovered that the Parkin-interacting substrate (PARIS) acts as a transcriptional repressor in the nucleus inhibiting therefore the expression of Peroxisome proliferator-activated receptor gamma coactivator 1 - alpha (PGC-1α), an important regulator of mitochondrial biogenesis [208].

In addition to be essential in the maintenance of the mitochondrial quality control and homeostasis, recent studies suggested that mitophagy may play a more active role in controlling a high variety of cellular functions [207] (Figure 26).

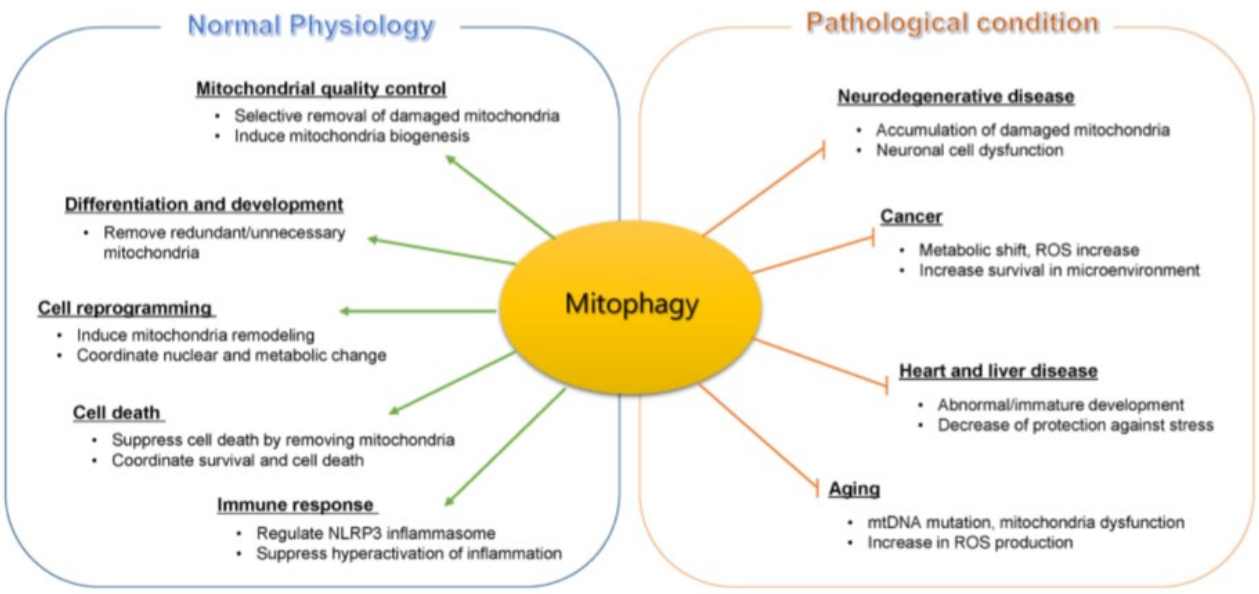


Figure 26: ***Schematic representation of the roles of mitophagy in normal physiology and human diseases*****.**
Reported from *Um et Yun*, 2017 (207).

During differentiation and development, mitophagy is involved in the removal of redundant mitochondria. For example, during erythrocyte differentiation Nix acts as a mitochondrial receptor to mediate mitochondrial removal through mitophagy; interestingly, mitochondria are not removed from

erythrocytes in Nix knockout mice, therefore anaemia may develop due to a decrease of survival [209]. In the embryo, the removal of paternal mitochondria is linked to mitophagy and the phenomena was reported in 2011 from two research in models of *C. elegans* [210], [211].

Recently, Wilson-Fritch *et al.* displayed that during cell differentiation of 3T3-L1 fibroblasts to adipocytes occur not only functional, but also quantitative changes in mitochondria [212]. Moreover, it has been reported that the quantitative changes are strictly connected with mitochondria remodelling and with alterations in the protein composition and in the metabolism of mitochondria [213]. In fact, Sin *et al.* showed that in the early phase of differentiation take place both a high clearance of mitochondria and a subsequent biogenesis of new mitochondria; this special remodelling of mitochondria is essential for the metabolic shift from glycolysis to oxidative phosphorylation and C2C12 myoblast differentiation to mature myotubes [214]. Therefore, the inhibition of the autophagic flux abrogates both mitochondria remodelling and myoblast differentiation. A very interesting and recent study highlighted that the induction of orchestrated dedifferentiation and induction of pluripotent stem cells (iPS) depend on mitochondrial restructuring and energy metabolism [215].

Despite the ability of mitophagy to suppress cell death by removing mitochondria, upon high-stress conditions the number of damaged mitochondria increases over the amount that can be removed by mitophagy, leading to the activation of the cell death pathway [216].

Moreover, it has been shown that the repression of mitophagy reduced lifespan in *C. elegans* models as well as overexpression of Parkin led to increase of mitochondrial activity and lifespan in Drosophila, suggesting a role of mitophagy also in aging [207].

3.3.3 Mitophagy impairment in diseases

As presented above, it is clear that beyond the primary and fundamental function of mitophagy in the identification and in the selective removal of damaged mitochondria, it exerts also a critical role for maintaining proper cellular functions influencing various physiological processes. Therefore, is not surprising that every kind of mitochondrial dysfunction can be associated with many pathological conditions [207], [217], [218] (Figure 2.2.2).

It is known that mitochondrial damage and dysregulation of mitophagy have been implicated in several neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease. Gene mutations in PINK1 and Parkin as well as the discovery of the

accumulation of dysfunctional mitochondria have been identified in the brain of patient's affected by hereditary Parkinson's disease [207].

Even if the role of mitophagy seems rather complex, several recent studies suggest that functional loss of mitophagy regulators is strictly linked to cancer development and progression. It has been reported that, since Parkin is frequently deleted in cancer and its absence is associated with progression of breast, colon and liver cancer [219]–[221], it could represent a potential tumour suppressor [222], [223].

Loss of PINK1, BNIP3, NIX and ATG5 leads to various heart defects in mice in addition to accumulation of dysfunctional mitochondria [224]–[226]. Also Atg7 knockout in liver is responsible of the accumulation of abnormal and swollen mitochondria in hepatocyte [227].

3.4 Mitophagy and mitochondrial shaping

Mitophagy, together with mitochondrial fusion and fission, provide a quality-maintenance mechanism that facilitates the removal of damaged mitochondria from the cell. Thus, dysfunctional regulation of mitochondrial dynamics might be one of the intrinsic causes of mitochondrial dysfunction, which contributes to OxS and cell death [193].

As previously reported in Figure 21, during the mitochondrial life cycle mitophagy selectively target to depolarized mitochondria that are generated via fission events [192]. It is therefore conceivable that the balance between the opposite fusion and fission determines the morphology of the organelle affecting also the degree of mitophagy [192], [193]. Indeed, if decreased fusion can result in mitochondrial fragmentation because of excessive fission, decreased fission can generate long and highly interconnected mitochondria, becoming an obstacle for the mitophagic degradation due to the inability of the isolation membranes to surround these abnormal mitochondria [192], [193], [195]. Interestingly, the expression of a dominant-negative variant of Drp1 in INS1 cells led to the formation of elongated mitochondrial tubules and reduction of mitophagy. Moreover, Parkin-mediated degradation of depolarized mitochondria was severely suppressed in $DRP1^{-/-}$ MEFs, indicating the close relationship existing between mitophagy and mitochondrial dynamics [192].

Notably, genetic defects in the proteins involved in mitochondrial fusion and fission lead to severely altered mitochondrial shape, loss of mtDNA integrity, increased OxS and apoptotic cell death; it has been shown that these alterations can subsequently cause developmental abnormality, neuromuscular degeneration and metabolic disorders in humans [193].

4. Apoptosis

Although required for life, paradoxically, mitochondria are often essential to initiate apoptotic cell death, in addition to undergo on mitophagy [188].

The term “apoptosis” comes from the Greek *apo* – (from) and *ptosis* – (falling off) and has been generally known as the falling off of leaves from tree.

The word apoptosis was coined by Kerr, Wyllie and Currie in 1972 to describe a morphological specific form of cell death. The investigation of the programmed cell death during the development of the nematode Caenorhabditis elegans, led to the comprehension of the mechanism involved in apoptosis in mammalian cells [228].

Apoptosis is a genetically programmed cell death involving the elimination of cells, and it is considered a vital component of various processes such as the functionality of the immune system, embryonic development, normal cell turnover and hormone dependent atrophy [228].

4.1 Morphology of apoptosis

The morphology of apoptosis has been discovered thanks to light and electron microscopy giving the idea of the various morphological changes that occur during the process [229].

The early phase of apoptosis is typically characterized by cell shrinkage, pyknosis, dense cytoplasm and the tightly packing of organelles. Among these, pyknosis is the result of the condensation of the chromatin and is the most special feature of apoptosis.

The apoptotic process involves single or small clusters of cells that appear as a round or with an oval mass; it occurs an extensive bebbling of the plasma membrane followed by the separation of the cell fragments into apoptotic bodies. These peculiar apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment; the organelle integrity is maintained as well as those of the plasma membrane. The apoptotic bodies are phagocytosed by macrophages, parenchymal or neoplastic cells in order to be degraded within phagolysosomes.

Since there is no release of cellular components in the surrounding interstitial tissue, the phagocytosis occurs quickly and the engulfing cells do not produce anti-inflammatory cytokines, the apoptotic process is not typically associated with inflammatory reaction [228].

4.2 Mechanisms of apoptosis

The mechanisms of apoptosis are very complex, refined and based on an energy-dependent cascade of molecular events. Two are the main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 27).

Evidence support the idea that the two pathways are linked and one can influence the other.

The extrinsic apoptosis is triggered by death factors of the tumour necrosis factor's (TNF) family like FasL, TNFα, TRAIL which bind specific death receptors initiating a death signalling pathway characterized by the recruitment of Fas-associated proteins with death domain (FADD). This is followed by the formation of a specific death complex called DISC, which lead to the activation of the caspase cascade.

The intrinsic pathway is regulated by members of Bcl-2 family, including proapoptotic BH3-only members, proapoptotic effector molecules (Bax and Bak) and antiapoptotic Bcl-2 family proteins. After an apoptotic stimulus, the BH3-only members are transcriptionally or posttranscriptionally upregulated, the activated BH3-only proteins act on Bak and Bax or antagonize the Bcl-2 family members. Then Bax and Bak stimulate the release of cytochrome c from mitochondria, which together with Apaf-1 forms the apoptosome promoting the activation of the caspase cascade.

An additional pathway involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. This pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic and the granzyme B pathways have the common characteristic to be mediated by the cleavage of caspase-3, which is located at the end of the caspase cascade with the final step represented by the uptake of the apoptotic bodies by phagocytic cells. Interestingly, it is known that many apoptotic features like membrane bebbling, DNA fragmentation, and PtdSer exposure are mediated by caspase 3 targets. Differently, granzyme A activates a caspase-independent cell death pathway [230],[228], [231].

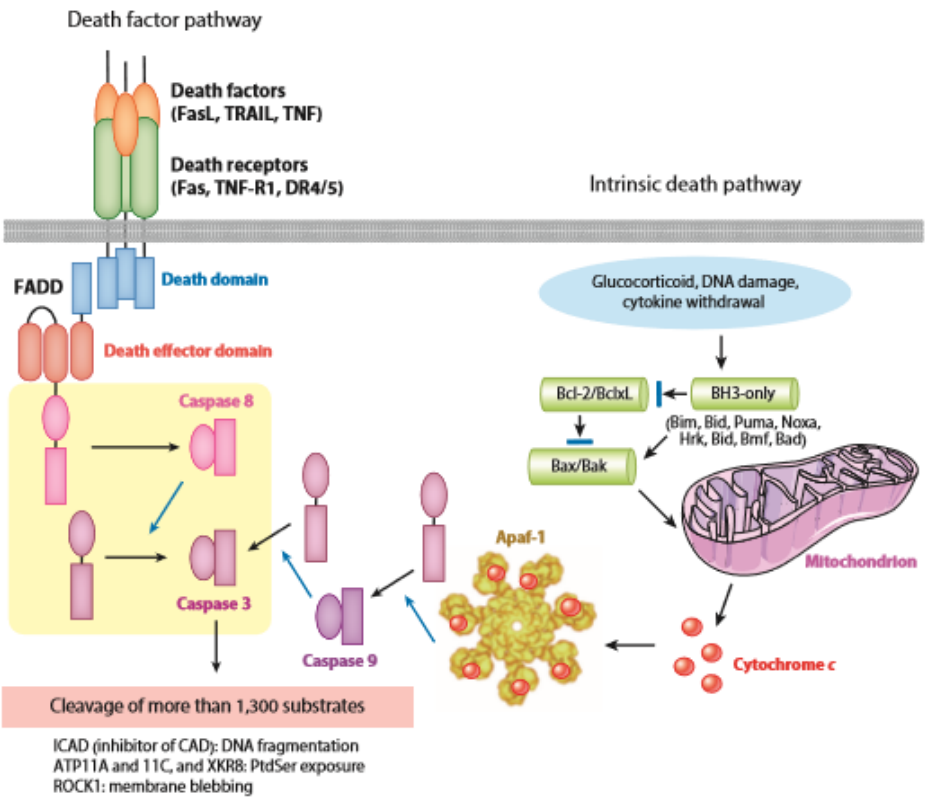


Figure 27: *Schematic representation of the two apoptotic pathways.*

Reported from *Nagata et al.* 2018 (231).

4.3 Physiological roles of apoptosis

Apoptosis plays a fundamental role in animal development and aging during which the cells undergo a physiological and necessary programmed death [228], [231]. For example, during embryogenesis the body is shaped and many cells are overproduced leading to a developmental programmed cell death in order to remove the excessive or the harmful cells [232]. Also dysfunctional nerve cells, activated lymphocytes and interdigital cells are removed by apoptosis as wells as the involution of mammary gland is mediated by this process. Each cell type possesses a special lifespan lasting from hours to few or many days; thus several hundred billion cells die daily and are replaced by newly generated cells [231]. Apoptosis represents also a defence mechanism during immune reactions or when the cells are damaged by diseases or noxious agents [233]. Of note, although there is a wide variety of physiological and pathological stimuli that can trigger apoptosis, the death or the survival may depend on the cell type [228]. Therefore, apoptosis exerts a key role to maintain homeostasis in the human body, controlling the cell population in several tissues.

4.4 Relationship between mitochondrial dynamics and apoptosis

It is well known that mitochondria play a crucial role in mediating apoptosis, being important checkpoints to initiate the intrinsic pathway of cell death described above [188]. Less than a decade ago it has been first proven that mitochondria undergoing apoptosis are drastically altered and converted from long reticular tubules to small puncta-like organelles [193]. Since then, mitochondrial fusion and fission proteins have been shown to exert a central role in apoptosis, regulating not only mitochondrial dynamics but also mitochondrion-dependent cell death [193] .

In this regard, overexpression of a dominant-negative mutant $Drp1^{K38A}$ induced mitochondrial fusion conferring resistance to apoptosis. Similarly, it was found that downregulation of Fis1 expression significantly enhanced fusion and inhibited cell death [193] as well as downregulation or Mfns overexpression, strongly delay caspase activation and cell death induced by numerous stimuli [234]. However, although many evidence highlight the close relationship between mitochondrial dynamics and apoptosis, it is rather complex and mitochondrial fragmentation is not necessarily related to apoptosis [193], [234].

4.5 Apoptosis in RTT Syndrome

Despite plenty of studies regarding the impairment of apoptosis in several pathologies [235], the role of the apoptotic process in RTT Syndrome has not been completely elucidated yet.

Since the molecular bases of RTT were still not clear, in the 1994 Anvret and colleagues hypothesized that the programmed early infantile death of neuronal cells was reprogrammed. Therefore, they analysed the sequences of Bcl-2 gene on chromosome 22 in six RTT patients, which is well known to exerts a key role in the intrinsic apoptotic pathway, but they didn't found any sequence alterations [236].

After ten years, Battisti *et al.* showed that lymphoblastoid cell lines of RTT patients treated with 2-deoxy-D-ribose (dRib), a reducing sugar that induces oxidative damage-mediated apoptosis, may have a low susceptibility or an increased resistance to the oxidative-stress induced apoptosis, which in turn, may be corrected just in the presence of a strong apoptotic stimulus [237].

In line with these findings, Squillaro *et al.* observed that bone marrow mesenchymal stem cells from a patient affected by RTT, presented a significant lower degree of apoptosis, suggesting that aberrant stem/progenitor cells can survive instead of being eliminated [238].

5. Rationale and aims

Despite years of intense research, the pathogenic mechanism that links MECP2 dysfunctions to disease manifestation is not fully understood yet. However, thanks to the effort of researchers and clinicians in the understanding of the functional role of MECP2 and RTT physiopathology, the disease is now no more regarded as a pure neurological disease, but as a multi-systemic syndrome affecting both brain and several other organs/tissues. Recent converging findings from our and other groups, clearly suggested that the systemic nature of RTT might represent the clinical outcome of two synergic adverse condition, i.e. OxS and subclinical inflammation [172].

As mentioned before, MECP2 regulates the expression of many genes involved in several different pathways acting as a transcriptional control orchestrator [181]. Given its global expression, mutations that negatively affect MECP2 functions would likely alter transcriptional control in virtually every cell in the body. In particular, even if has not been yet demonstrated that altered mitochondrial function is a consequence of MECP2 mutations, is now established that MECP2 either directly or indirectly regulates the expression of several nuclear genes encoding for mitochondrial factors [181]. In this regard, among the multitude, results from Mecp2-null mice showed an epigenetic regulation of the expression of BDNF and proline dehydrogenase (Prodh), proteins implicated in the modulation of cellular redox defensive system and ROS mitochondrial production, respectively [172]; also alterations in genes encoding proteins that regulate mitochondrial structure and organization have been found [239]. Mitochondria are double membrane organelles which are essential for many cellular processes such as ATP and lipid synthesis, protein biogenesis and ROS production. In the same time, they are also highly dynamic organelles that move within the cells and continuously fuse and divide, dynamic morphological changes that are essential for the control of cellular processes such as embryonic development, neuronal plasticity, calcium signalling and apoptosis [240] .

Impaired mitochondrial quality control leads to the accumulation of damaged mitochondria that may release more ROS and mitochondrial components into cytosol activating inflammation, producing less ATP and have a lower threshold for cytochrome c resulting in apoptosis. Thus, mitochondrial turnover is an integral aspect for quality control in which dysfunctional mitochondria are selectively eliminated through autophagy (mitophagy) and replaced through expansion of pre-existing mitochondria (biogenesis) [240].

In our previous work we have demonstrated that the systemic OxS levels in RTT fibroblasts could be a consequence of both increase endogenous oxidants as well as altered mitochondrial membrane

potential, bioenergetic profile and mitochondrial biogenesis with a decrease activity of defensive enzymes [171]. Therefore, our aim was to investigate whether these kind of alterations could reflect on mitochondrial morphology and subsequently, on the ability of mitochondria to activate the physiological process of mitophagy, focusing on the relationship with mitochondrial dynamics (e.g. fusion and fission) and also with a cell death pathway mitochondria-dependent represented by apoptosis, after FCCP-induced mitochondrial damage.

Since their implications in differentiation, development, cell reprogramming, cell death and immune response [207], [231], [233], both mitophagy and apoptosis are fundamental to maintain homeostasis and to regulate the normal turnover of organelles or cells in the human body. Altered levels of mitophagy and apoptosis have been associated with many pathologies like cancer, heart, liver and neurodegenerative diseases [207], [235].

A better comprehension of the biology of these powerful organelles in RTT patients as well as the molecular mechanisms involved under pathophysiological condition, might contribute to the explanation of the chronic pathological features that occur in this devastating neurodisorder, presenting also new possible approaches for a so needy therapy.

6. Methods

6.1 Subjects and ethic statement

The study population consisted of 3 female patients with classical RTT (mean age +/- SD: 20 ± 8) and 3 healthy female controls age-matched (mean age +/- SD: 21.8 ± 7.3). All the patients were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Siena, Italy). Diagnosis of RTT and selection criteria (inclusion/exclusion) were set in accordance with revised RTT nomenclature consensus [7]. This study was designed and performed according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Institutional Review Board of University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Siena, Italy. Informed consents were obtained in written form from either the parents or the legal tutors of the participants.

6.2 Fibroblasts isolation from skin biopsy

Control skin biopsies were obtained during routine health checks or by donations, while skin biopsies from RTT patients were carried out during the periodic clinical checks-up. Human skin fibroblasts were isolated from 3 mm skin punch biopsy (n = 3 for RTT and n = 3 for controls), as described in previous report [141].

6.3 Cell culture

Human primary fibroblasts were grown in DMEM, containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) (Lonza, Milan, Italy) in 100 mm dishes (Sarstedt) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Only fibroblasts from passage 2–5 were used for the experiments.

Cells were seeded onto 24-mm glass coverslips for morphological analysis.

6.3.1 Cell treatments

MitoTracker green (M7514) was obtained by Thermo Fisher Scientific and first dissolved in DMSO, then a 0,1 μM salt solution was prepared to stain the cells.

For Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) treatment (ab120081 abcam), RTT and control fibroblasts were treated with 10 μM FCCP for 2, 4, 8, 16, 24 h (mitophagy assay) or 20 μM FCCP for 48h (apoptosis assay) in complete medium, previously dissolved in DMSO.

For 2,4- Dinitrophenol (DNP) treatment (Merck Darmstadt 3111), RTT and control fibroblasts were treated with 2 mM DNP for 24 and 48 h in complete medium, previously dissolved in H_2O.
After the treatments, cells were collected or processed as described below.

6.4 Transmission electron microscopy (TEM) analysis

Mitochondrial morphology was evaluated by transmission electron microscopy (TEM). Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C. Then were washed with 0.1 M cacodylate buffer (pH 7.4) three times and postfixed in 1% osmium tetroxide and 0.1 M cacodylate buffer at pH 7.4 for 1 h at room temperature. The specimens were dehydrated in graded concentrations of ethanol and embedded in epoxide resin (Agar Scientific, 66 A Cambridge Road, Stanstead Essex, CM24 8DA, UK). Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60°C for 48 h. Semithin sections (0.5–1 μm thickness) were cut using an ultramicrotome (Reichard Ultracut S, Austria), stained with toluidine blue and blocks were selected for thinning. Ultrathin sections of approximately 40–60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% citrate for 30 min each and examined under a transmission electron microscope (Hitachi, H-800) at an accelerating voltage of 100 KV.

6.5 Confocal microscopy analysis

Mitochondrial morphology was assessed in basal condition. Human RTT and healthy primary fibroblasts were stained with 1 mM MitoTracker green in Krebs Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 20 mM Hepes (pH 7.4 at 37°C) for 30 minutes at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 to mark mitochondria. The experiment was performed on confocal microscope (Zeiss LSM510) using a 63 × 1.4 NA Plan-Apochromat oil-immersion objective. To obtaine the number and the volume of mitochondria, the Z-series acquisitions were deconvolved using Fiji Software.

6.6 Immunoblot analysis

For immunoblotting, cells were harvested, washed and pelleted (280 g 4 min 4°C) in phosphate-buffered saline (PBS), resuspended in RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). After 30 min of incubation in ice and centrifugation at 12000 rcf 4°C for 15 min, proteins were quantified by Bradford assay (Bio-Rad) and 10 μg of each

sample were loaded on home-made gels and transferred to nitrocellulose membranes. After incubation with TBS–Tween-20 (0.1%) supplemented with 5% non-fat powdered milk for 1 h to saturate unspecific binding sites at room temperature, membranes were incubated overnight with primary antibodies anti-caspase 3 (bs-0081R), anti-Parkin PRK8 (sc-32282 1:1000), anti-PINK1 (sc-517353 1:1000), anti-MFN2 (sc-100560 1:1000), anti-ATP5A (sc-136178 1:1000), anti-MFN1 (sc50331- 1:500), anti-DRP1 (#5391 Cell Signaling 1:1000), anti-pDRP1 (#3455 Cell Signaling 1:1000), anti-FIS1 (Calbiochem AP 1165 1:1000), anti-TOM20 (sc-17764 1:1000). Anti-GAPDH (#5174 Cell Signaling 1:5000) and anti-β-tubulin (Sigma T8328 1:3000) were included as loading controls. After over-night incubation, the nitrocellulose membranes were incubated with appropriate HRP-conjugated secondary antibodies (goat anti-mouse 1:10000 Bio-Rad; anti-rabbit #7074 Cell Signaling 1:10000). The proteins were detected by chemiluminescence, using ChemiDoc Imaging System (BioRad) or ImageQuant LAS 4000 (GE Healthcare).

6.7 Subcellular fractionation

RTT and controls fibroblasts were harvested, washed with PBS by centrifugation at 280 × *g* for 4 min, resuspended in homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 2 mM EGTA) and gently disrupted by using a tight Potter. The homogenate was centrifuged twice at 2400 × *g* for 3 min at 4°C to remove membranes and unbroken cells. Then the supernatant was centrifuged at 10000 × *g* at 4°C for 10 min to pellet crude mitochondria. The resultant pellet containing mitochondria was resuspended in the homogenization buffer and gently disrupted by using a loose Potter to better purify the mitochondrial sub-fraction. Then, it was first centrifuged at 3400 g for 4 min at 4°C and subsequent at 10000 × *g* for 10 min at 4°C. The pellet containing mitochondria was finally resuspended in lysis buffer with protease and phosphatase inhibitors. The cytosolic fraction was obtained by centrifugation a portion of the homeogenate at 16000 g for 30 min at 4°C and the supernatant was collected and added of lysis buffer with protease and phosphatase inhibitors.

6.8 Annexin V/PI staining

For Annexin V/PI staining, RTT and healthy fibroblasts were plated onto 60 mm well dishes. After treatment with 20 μM FCCP or 2 mM DNP for 48 h, cells were gently harvested, processed with buffers, and incubated with Annexin V/PI according to manufacturer's protocols (Macs Miltenyi Biotec GmbH). The cells were then analyzed with a BD FACSCanto II flow cytometer and the several parameters were collected.

6.9 ApoTox-Glo assay

For caspase 3/7 activation, RTT and healthy fibroblasts were plated onto 96 well plate (Corning). After treatment with 20 μM FCCP for 48 h, reagents included in the kit were added according to manufacturer's protocols (Promega). Then luminescence was measured by Synergy H1 microplate reader (BioTek) and data were analysed.

6.10 Statistical analysis

Statistical analysis was performed using unpaired two-tailed *t*-test (two groups) or one/two-way ANOVA followed by Bonferroni's multiple comparisons test. Normal distribution of data was assessed by applying Shapiro-Wilk normality test. A p-value < 0.05 was considered significant. All data are reported as mean ± SEM. Exact p-values are indicated in the figure legends.

7. RESULTS

7.1 Alterations of mitochondrial morphology in RTT fibroblasts

Mitochondria are fundamental organelle which exerts a central role in metabolism and energy production. The first step was to assess mitochondrial morphology by transmission electron microscopy in our cellular model, represented by human fibroblasts isolated from skin biopsies of RTT patients and healthy subjects. Left panel of Figure 28 showed a normal tubular morphology of mitochondria of control fibroblasts with well defined cristae structure. On the contrary, RTT mitochondria seemed to have an altered morphology with a dumbbell-like shape, a structural swollen cristae disarrangement and a predominantly electron dense matrix deposition, as displayed in the right panel.

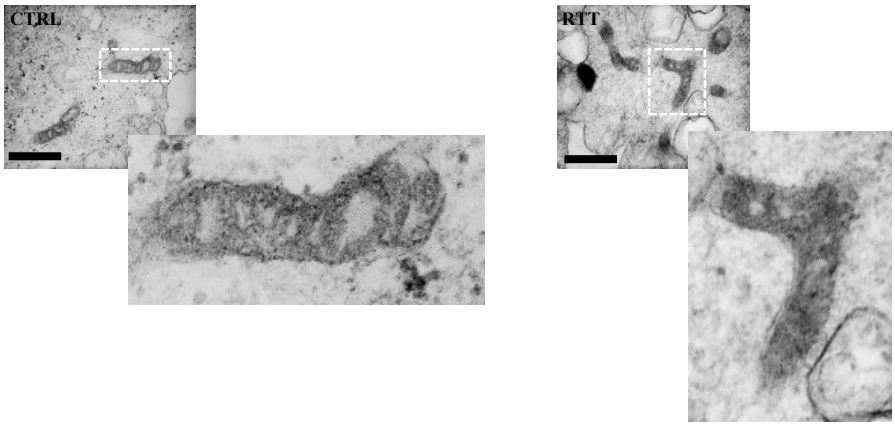


Figure 28: ***RTT fibroblast had an altered mitochondrial morphology***

Representative TEM images of mitochondria of Rett and healthy fibroblasts. Cells were detached from the dish and observed by transmission electron microscope. Magnification 40000X. Scale bar 2 μm.

7.2 Impairments of mitochondrial network in RTT fibroblasts

Mitochondria are highly dynamic organelles that fuse and divide to form constantly changing tubular network in order to maintain a proper cellular functionality. Thus, we decided to confirm the result obtained by TEM and assess changes in mitochondrial morphology in our human cell model, staining live fibroblasts with green MitoTracker, a mitochondrion specific dye which passively diffuse across the plasma membrane and accumulates in active mitochondria, and subsequently observing them by confocal microscope. As shown in Figure 29, by reconstructing 3D pictures using Fiji software, we discovered that RTT fibroblasts (right panel) were characterized by a more filamentous interconnected network of elongated and hyperfused mitochondria compared to control cells, in

which are visible short lines or dots that represent the typical mitochondrial structure (left panel).

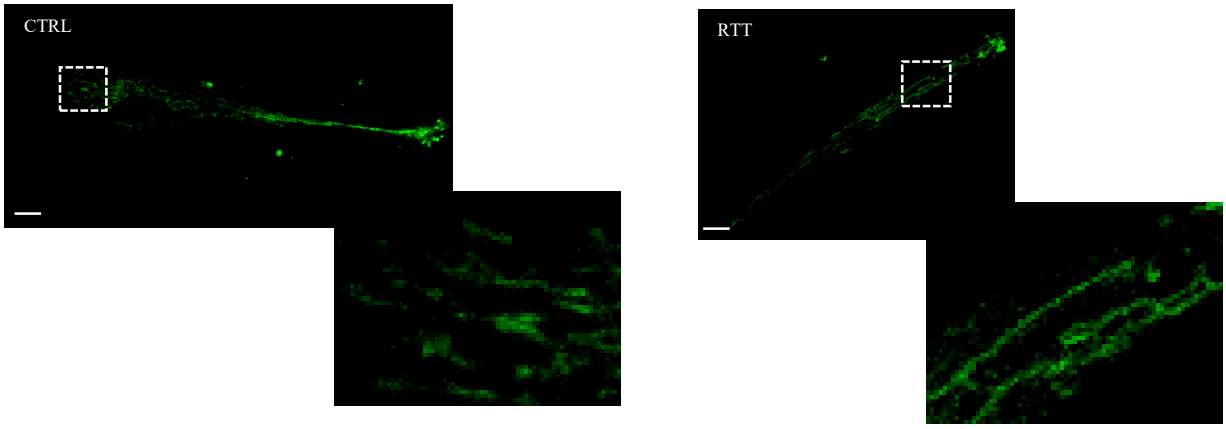


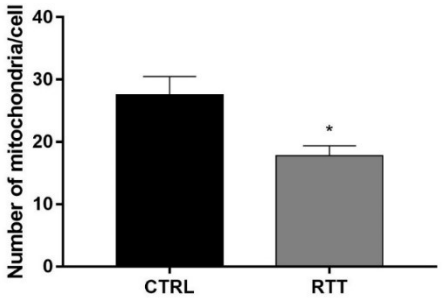
Figure 29: ***RTT mitochondria showed a more interconnected mitochondrial network.***

Representative confocal images of mitochondrial network of Rett (n=30 cells) and healthy (n=21 cells) fibroblasts. Cells were stained with Mitotracker (green fluorescence) and observed by confocal microscope. Magnification 40X. Scale bar 10 µm.

7.3 Differences in mitochondrial number and volume in RTT fibroblasts

Then, from the 3D images reconstructed previously, we calculated the number and the volume of RTT and control fibroblasts mitochondria. Confocal images revealed decreased number (almost halved) of mitochondria in RTT fibroblasts compared with those of controls (Figure 30A). Moreover, analysis of the mean and the total mitochondrial volume displayed an increase of both in RTT, suggesting a possible effective alteration in mitochondrial dynamics (Figure 30B and 30C).

A



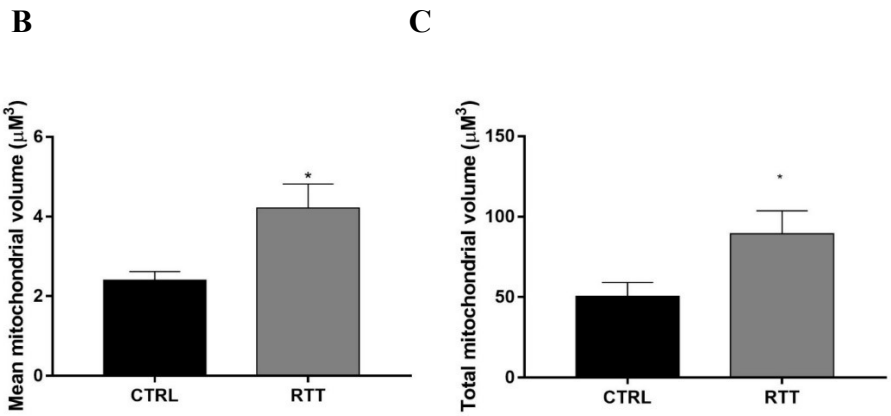
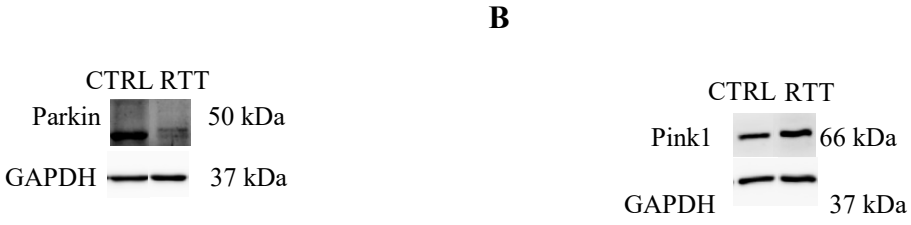


Figure 30: ***RTT fibroblasts displayed decrease number and increase volume of mitochondria***

A, B, C Quantification of mitochondrial number, mean and total volume (panel A, B and C, respectively) of Rett (n=30 cells) and healthy (n=21 cells) fibroblasts. Data are represented as mean ± SEM. * p = 0.0031 * p = 0.0174 and p = 0.0407 for panel A, B, C, respectively. Unpaired t test was performed as statistical analysis.

7.4 RTT displayed alterations in PINK1/Parkin-mediated mitophagy

Mitophagy is a mitochondria-specific autophagic process involved in mitochondrial quality control, designed for the maintenance of mitochondrial fitness by the efficient removal of dysfunctional mitochondria. Although several mechanisms regulating mitophagy have been described for mammalian cells, the best understood is the PINK1/Parkin-mediated mitophagy. Thus, since RTT mitochondria appeared more fused, we were interested in understanding possible alteration in their physiological removal by mitophagy. Therefore, immunoblot analyses reported in Figure 31, showed a significant decrease of the *ubiquitin E3 ligase Parkin protein* expression, whereas no differences were found for Parkin receptor, PINK1, in basal condition (Figure 31B and 31E). A specific mitochondrial-damage inducing agent is the potent mitochondrial oxidative phosphorylation uncoupler FCCP, which is known to activate mitophagy. Surprisingly, as shown in Figure 31E, a strong defective PINK1 translocation from cytosol to mitochondria upon mitophagy induction by FCCP was observed in the mitochondrial sub-fraction of RTT fibroblasts.



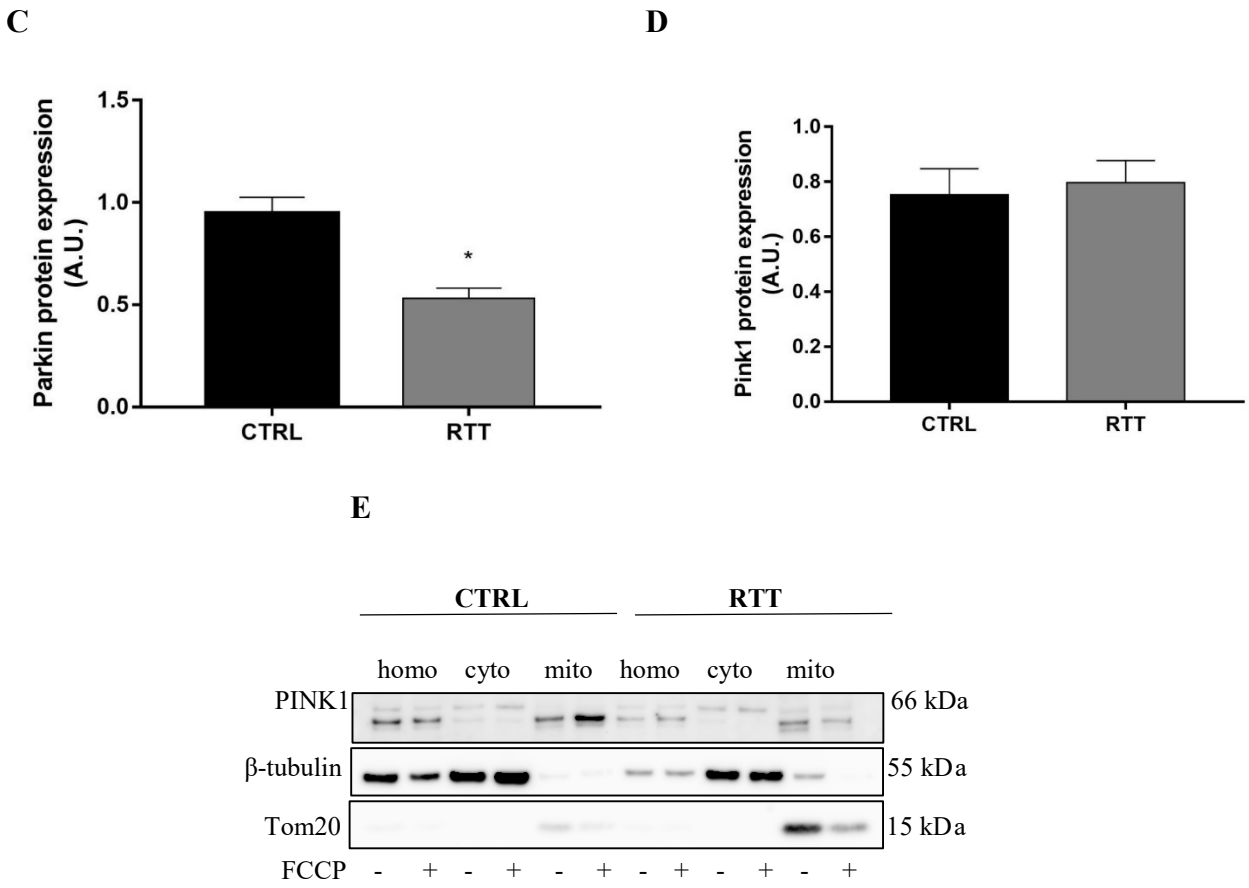


Figure 31: ***RTT fibroblasts showed decrease levels of Parkin and Pink1 protein expression.***

A, B Immunoblot for Parkin and Pink1 in Rett (n=3) and control (n=3) fibroblasts. GAPDH was used as loading control. **C, D** Quantification of Parkin and Pink1 bands. Data are represented as mean ± SEM of three independent experiments. * p = 0,0021. Unpaired t-test was performed as statistical analysis.

E Immunoblot for Pink1 in homogenate (homo), cytoplasmic (cyto) and mitochondrial (mito) fractions of Rett (n=1) and control fibroblasts (n=1). β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial sub-fractions, respectively.

7.5 FCCP induced mitophagy in healthy fibroblasts

Several are the methods to study whether the mitophagic process ends; among those it is accepted that the decrease of mitochondrial proteins means a proper degradation of mitochondria. Thus, as a proof of concept, we treated our healthy cells with FCCP for several time points evaluating the expression of some mitochondrial proteins. As depicted in Figure 32, FCCP induced a significant and time-dependent decrease of Parkin and Mfn2, a physiological relevant outer mitochondrial membrane protein, suggesting a good activity of the mitophagic machinery in healthy fibroblasts.

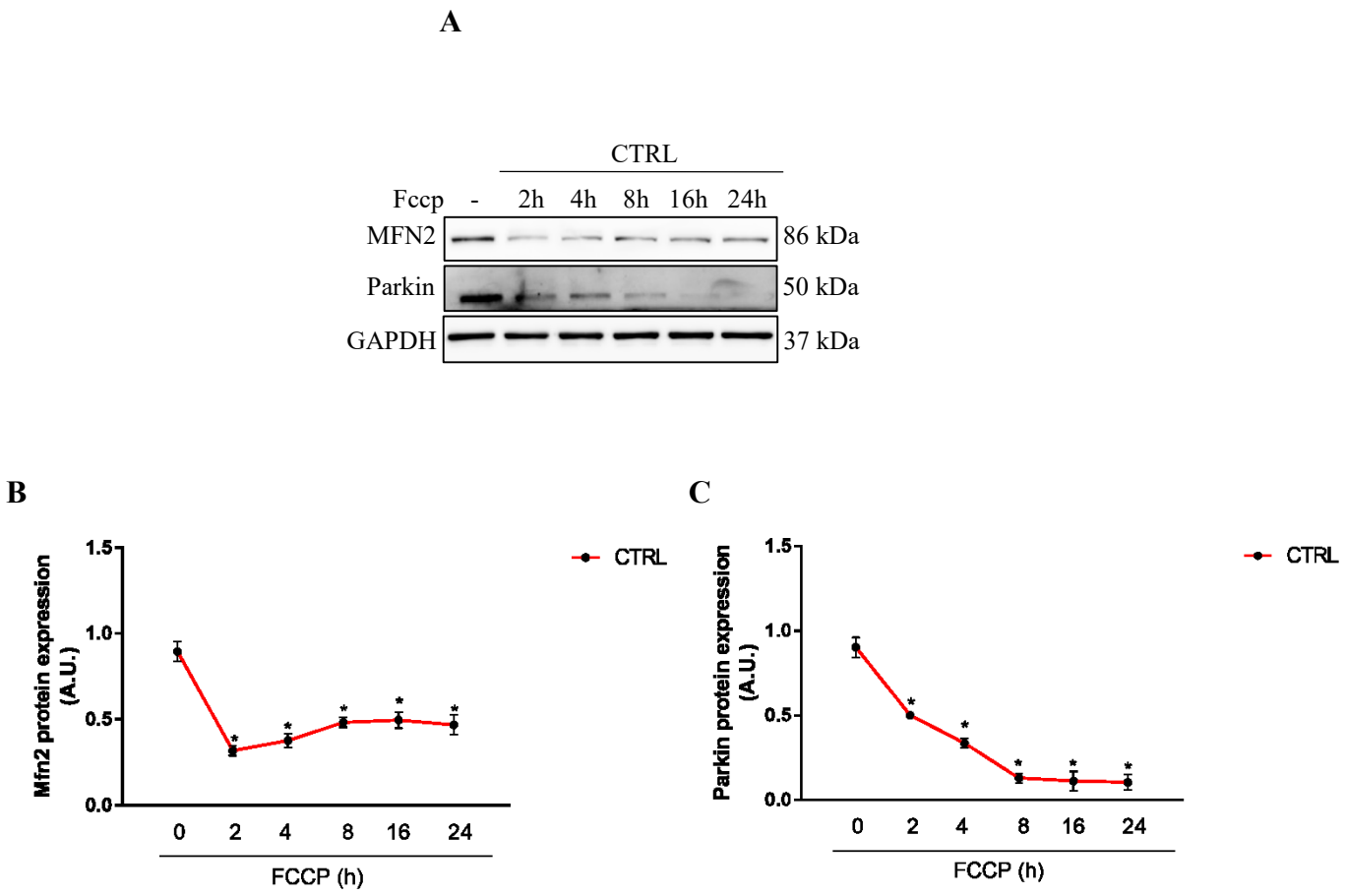


Figure 32: ***Healthy fibroblasts showed decrease of mitochondrial proteins expression after FCCP treatment.***

A Representative immunoblot for Mfn2 and Parkin in human control fibroblasts (n=3) after treatment with 10 µM FCCP for the time points indicated in the figure. GAPDH was used as loading control.

B, C Quantification of Mfn2 and Parkin bands. Data are represented as mean ± SEM of three independent experiments. * p< 0.0001. One-way ANOVA was performed as statistical analysis.

7.6 FCCP didn't induce mitophagy in RTT fibroblasts

Taking into account the previous results of increase volume of RTT mitochondria and the ability of FCCP to induce mitophagy in our cell model of healthy fibroblasts, we were interested in understanding whether also RTT cells were able to be degraded by mitophagy. Therefore, we performed the same experiment with RTT fibroblasts considering one more mitochondrial protein which is ATP5A, that correspond to the α-F1 subunit of the ATP synthase complex localized within the IMM. Our results showed that, as founded before, FCCP induced a decrease of all of the three mitochondrial proteins considered in the control, with the maximum loss 24 h after the treatment. Regarding RTT cells, interestingly, they displayed almost completely absence of Parkin protein and

any significant change was appreciable in Mfn2 and ATP5A expression after FCCP-induced mitochondrial damage (Figure 33). Indeed, the data suggested a possible impairment in PINK1/Parkin-mediated mitophagy in RTT fibroblasts.

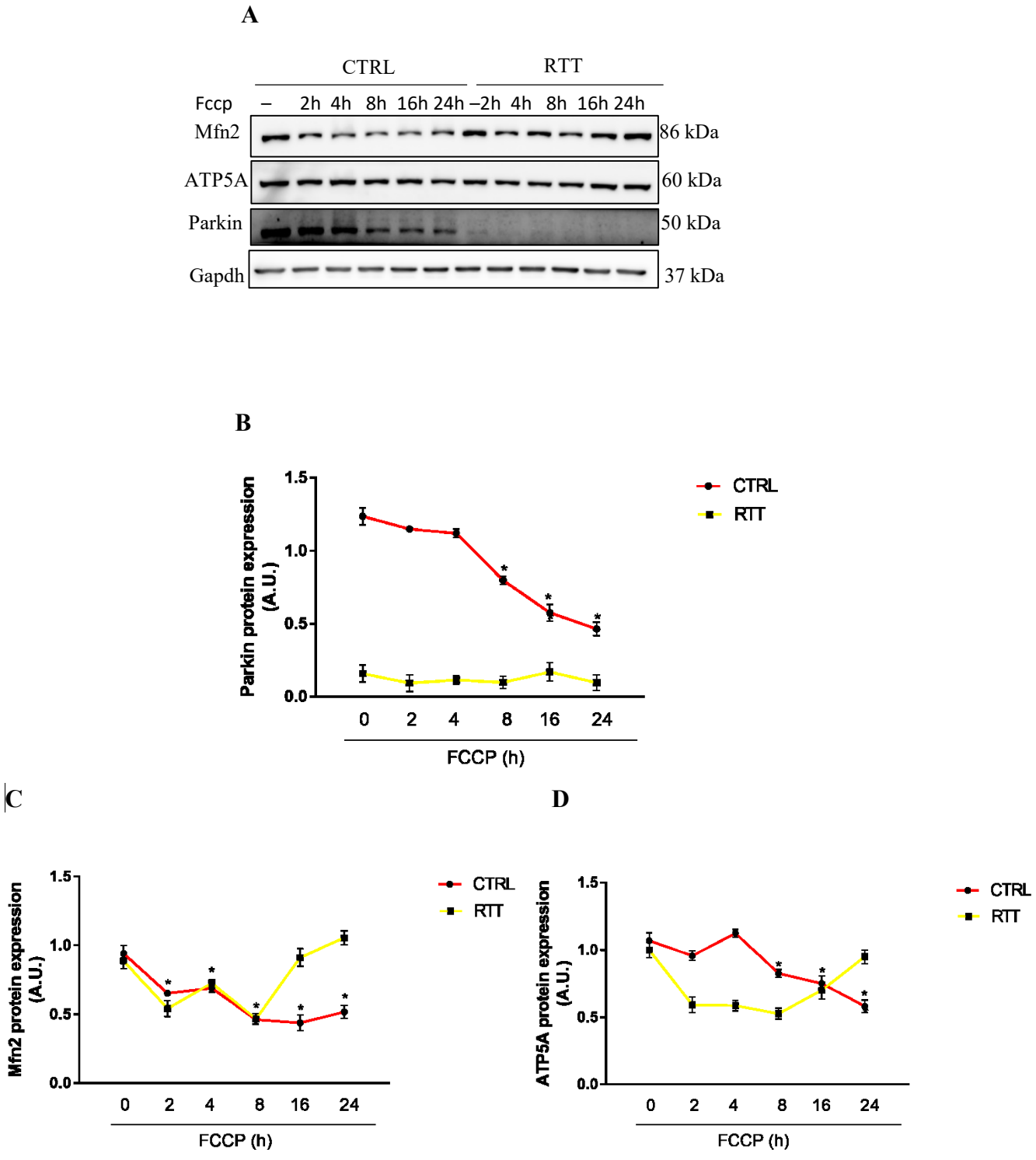


Figure 33: ***RTT fibroblasts showed impairment in mitophagy.***

A Representative immunoblot for Mfn2, ATP5A and Parkin in human control (n=3) and RTT fibroblasts (n=3) after treatment with 10 μM FCCP for the time points indicated above. GAPDH was used as loading control.

B, C, D Quantification of Parkin, Mfn2 and ATP5A bands. Data are represented as mean ± SEM of three independent experiments. * $p < 0.05$. Two way Anova followed by Bonferroni's multiple comparison test was performed as statistical analysis

7.7 Mitophagy defect could be due to impaired mitochondrial fusion

Mitochondria undergo a balanced and continuous remodelling within the mitochondrial life cycle, involving fusion and fission processes. In this regard, it has been demonstrated that mitophagy could be blocked by an excessive mitochondrial fusion. Thus, since RTT mitochondria displayed an hyperfused morphology, we investigated some possible alterations in fusion process. Interestingly, mitochondrial sub-fraction of RTT fibroblasts showed increase expression of MFN1 and MFN2 proteins (Figure 34), two main markers of mitochondrial fusion in basal condition and upon FCCP treatment, compared to control.

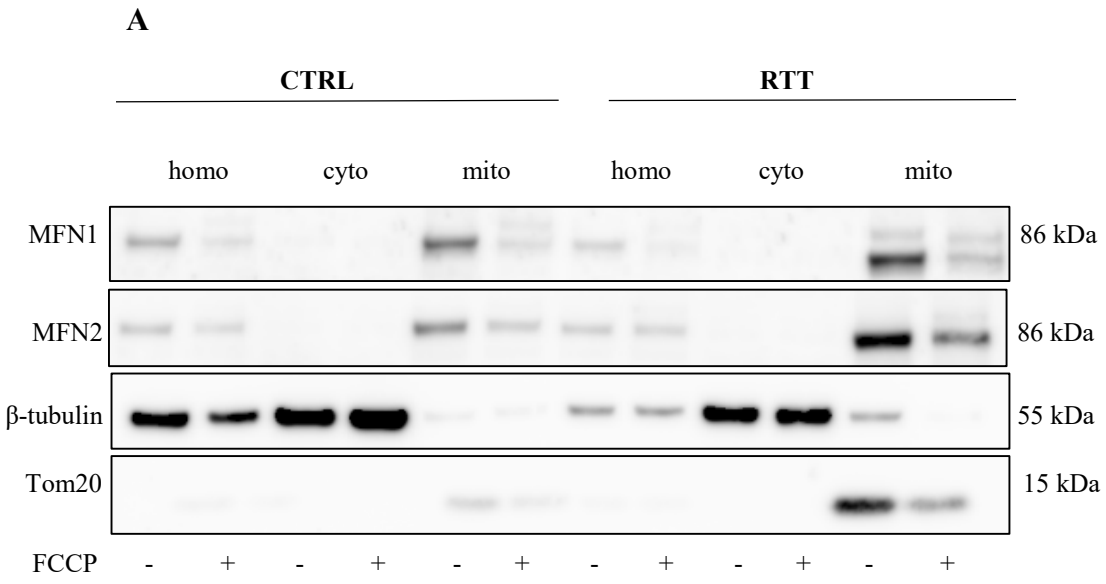
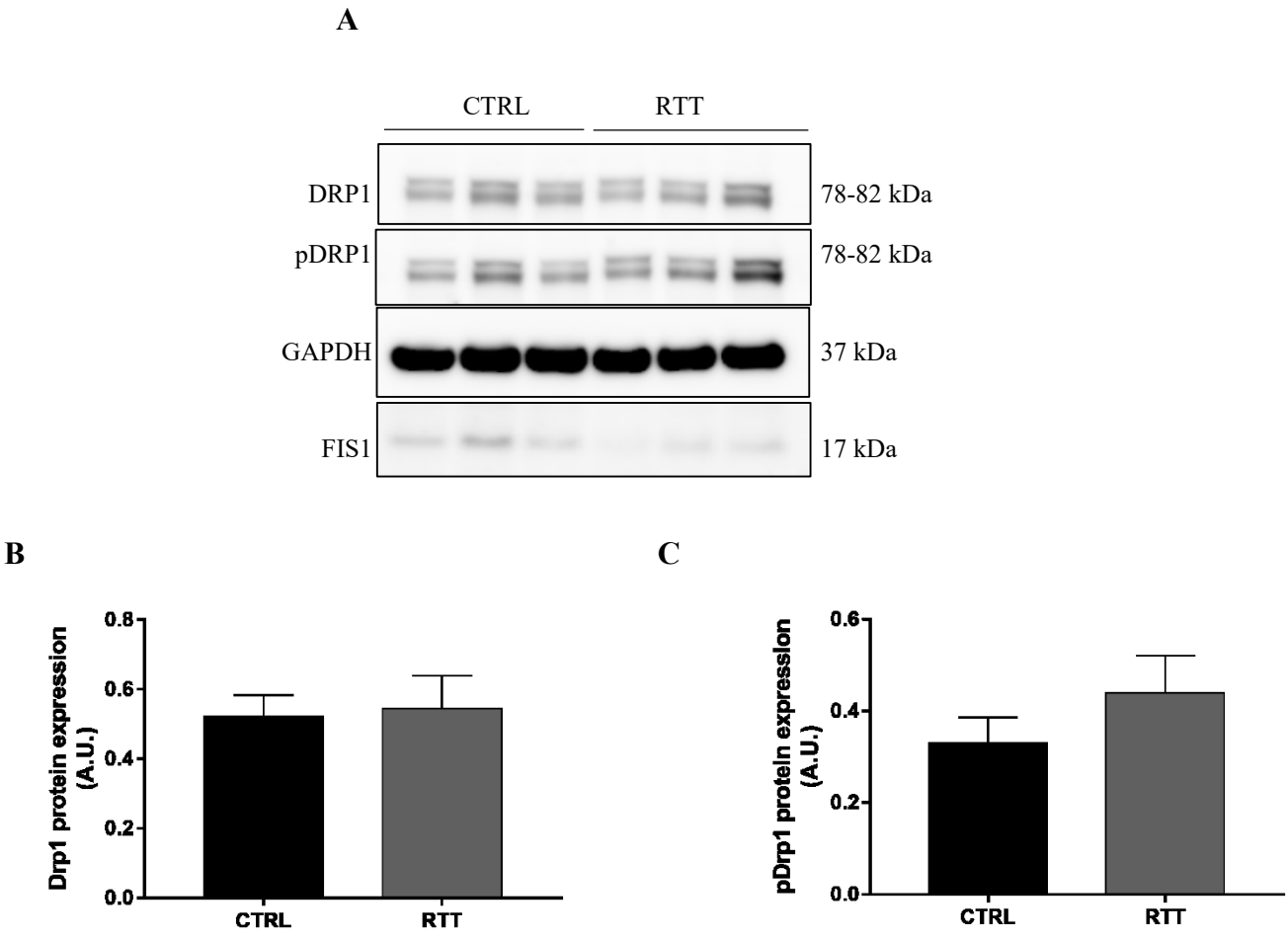


Figure 34: ***RTT fibroblasts showed impairment in mitochondrial fusion.***

A Immunoblot for MFN1 and MFN2 in homogenate (homo), cytoplasmic (cyto) and mitochondrial (mito) fractions of Rett (n=1) and control fibroblasts (n=1) after treatment with 10 μM FCCP for 16h. β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial sub-fractions, respectively.

7.8 Mitophagy defect could be due to impaired mitochondrial Drp1/Fis1-mediated fission

Mitochondrial fission is essential for the physiological degradation of damaged mitochondria, via the selective autophagic mechanism, specifically termed mitophagy. One of the most important pathway which regulates mitochondrial fission is the one Drp1/Fis1-dependent. As shown in Figure 35A, we discovered a marked decrease of the mitochondrial fission 1 protein FIS1 in RTT fibroblasts whereas no differences were found for Drp1 and its phosphorylated form pDrp1 (S616) in the total lysate. It is widely known that, upon mitophagic stimulus, Drp1 translocates from cytosol to mitochondria to activate mitochondrial fission. Surprisingly, RTT cells showed the inability to properly recruit Drp1 at mitochondria upon FCCP-dependent mitophagic stimulus compared to control fibroblasts (Figure 35E). These results highlighted that the mitophagic defect could be due to impairment in this physiological mitochondrial fission process.



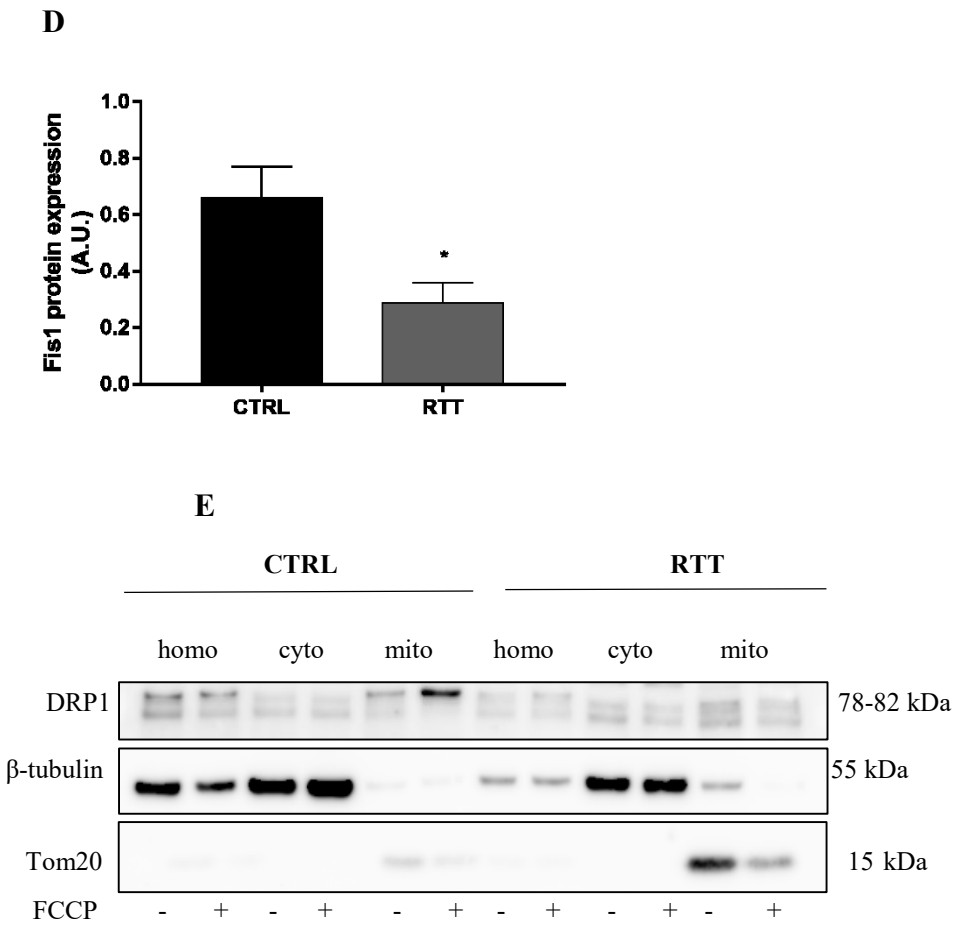


Figure 35: ***RTT fibroblasts showed impairment in mitochondrial fission.***

A Representative immunoblot for DRP1, pDRP1 (S616) and FIS1 in human control (n=3) and RTT fibroblasts (n=3). GAPDH was used as loading control.

B, C, D Quantification of Drp1, pDRP1 and FIS1 bands. Data are represented as mean ± SEM. * p = 0.0463. Two way Anova followed by Bonferroni's multiple comparison test was performed as statistical analysis

E Immunoblot for DRP1 and FIS1 in homogenate (homo), cytoplasmic (cyto) and mitochondrial (mito) fractions of Rett (n=1) and control fibroblasts (n=1) after treatment with 10 μM FCCP for 16h. β-tubulin and Tom20 were used as loading controls for the cytoplasmic and the mitochondrial sub-fractions, respectively.

7.9 RTT exhibited low FCCP and 2,4-DNP-induced apoptotic cell death

Paradoxically, mitochondria are required for life and are also often essential to initiate apoptotic cell death. Since RTT mitochondria displayed an altered morphology as well as a defect in the fission process which usually represent an early event during apoptosis, we tried to understand whether this structural impairment could affect also the functionality of mitochondria in activating apoptosis. As shown in Figure 36A, we discovered that there was a significant decrease of apoptotic RTT cells (Figure 36B) after 48h of FCCP, which in addition to mitophagy, is also known to activate apoptosis.

compared to healthy fibroblasts. This result was also confirmed treating RTT and controls fibroblasts with 2,4-DNP, another well known uncoupler of oxidative phosphorylation and inductor of apoptosis. Indeed, RTT fibroblasts showed marked lower levels of apoptotic cells after 48h of 2,4-DNP treatment, suggesting an aberrant apoptosis (Figure 36C).

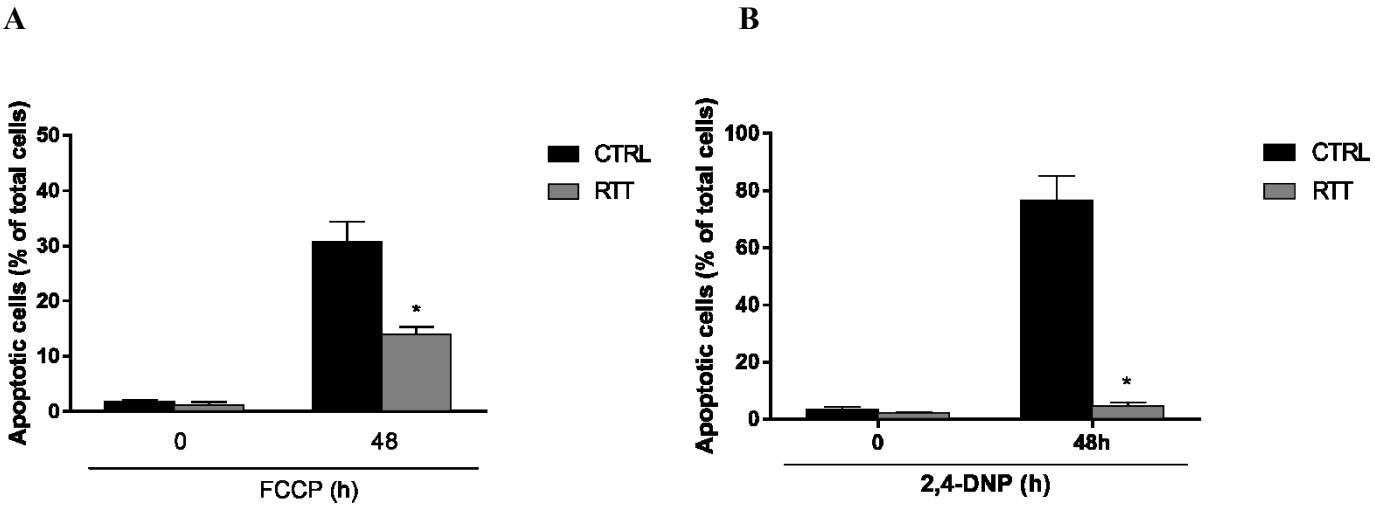


Figure 36: ***RTT fibroblasts displayed lower apoptotic cells after FCCP and 2,4 DNP treatment.***

A, B Quantification of total apoptotic cells. RTT (n=3) and healthy (n=3) fibroblasts were treated with 20 μM FCCP for 48 h (panel B) or 2 mM DNP for 24 and 48h (panel C) and then stained with Annexin V/PI. Data are represented as mean ± SEM. * $p < 0.0001$. Two way Anova followed by Bonferroni's multiple comparison test was performed as statistical analysis. The results are representative of three different experiments.

7.10 RTT fibroblasts didn't show activation of caspase3-mediated FCCP-induced apoptosis

Next, it was of our interest to investigate the molecular mechanism involved in the lower apoptosis FCCP-driven in RTT fibroblasts. The final step of the intrinsic mitochondria-dependent apoptotic caspase cascade implicates the cleavage of caspase-3. Therefore, we considered the activation of caspase 3/7 in our cellular model, in which luminescence signal is proportional to the amount of caspase activity in the sample. Interestingly, RTT fibroblasts didn't show any activation of caspase 3/7 compared to controls (Figure 37A). As a proof of concept, we performed an immunoblot, confirming that no cleavage of caspase 3 occurred in RTT fibroblasts (Figure 37B and 37C).

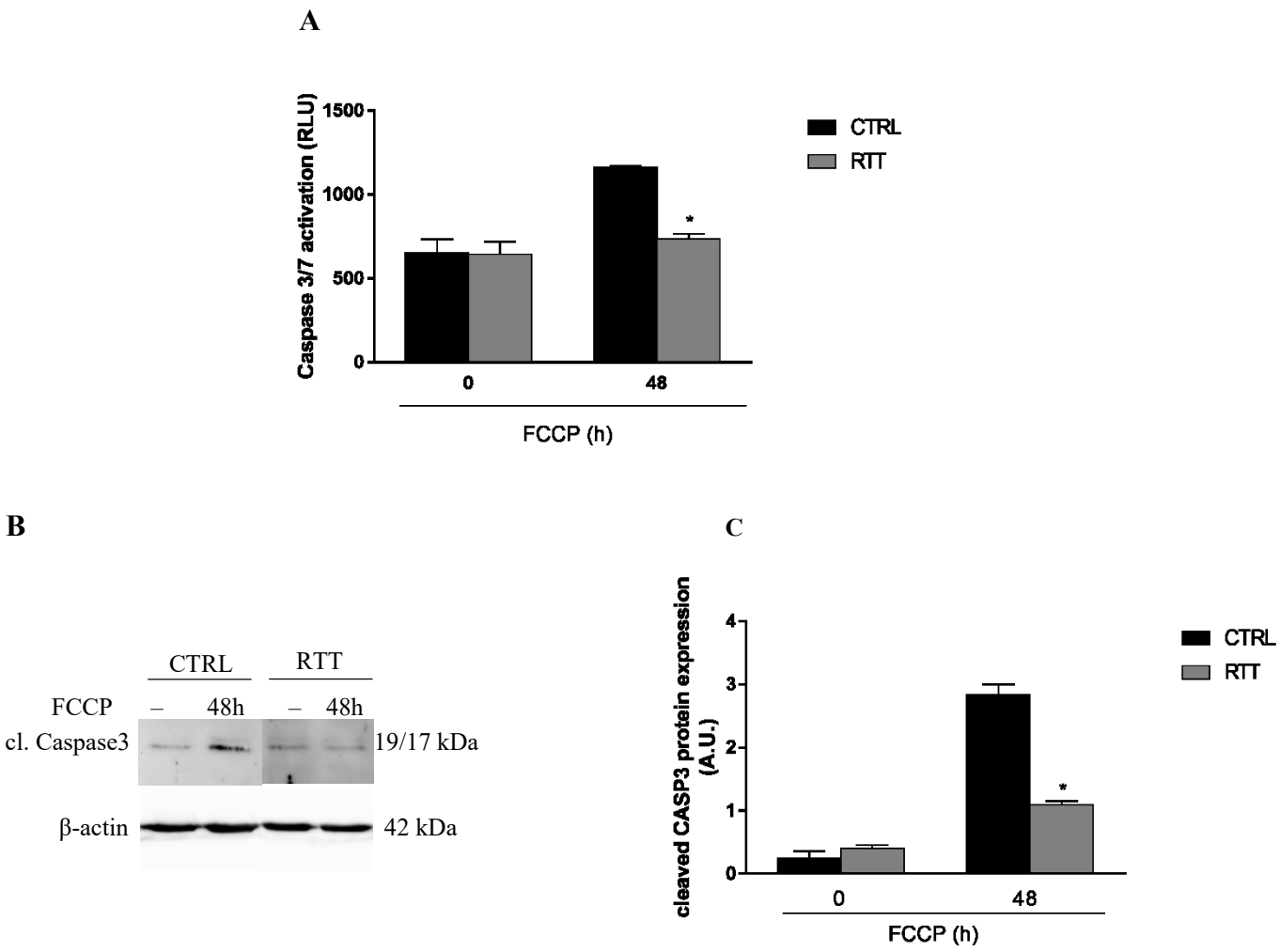


Figure 37: ***RTT fibroblasts displayed no activation of caspase 3 after FCCP treatment.***

A Caspase 3/7 activation in healthy (n=3) and RTT (n=3) fibroblasts after treatment with FCCP 20 µM measured by ApoTox-Glo Assay (Promega). Data are expressed as RLU. * p = 0.04 Two way Anova followed by Bonferroni's multiple comparison test was performed as statistical analysis.

B, C Representative immunoblot (B) and relative quantification (C) for cleaved caspase 3 in human Rett (n=3) and control (n=3) fibroblasts. β-actin was used as loading control. Data are represented as mean ± SEM of three independent experiments. * p < 0.0001. Two way Anova followed by Bonferroni's multiple comparison test was performed as statistical analysis. The results are representative of three different experiments.

8. Discussion and conclusions

Mitochondria are structurally complex, biochemically active and dynamically motile organelles which, in addition to the production of energy, perform many physiological roles [241]; in fact, they are involved in migration, senescence, inflammation [242], lipid metabolism [186], ROS production [183], Ca^{2+} signalling [187] and regulation of the cell death pathways [188]. Indeed, impairment of mitochondrial homeostasis has been strongly linked to a wide range of diseases, from neuronal pathologies to aging [242], [243]. Methyl-Cp-G binding protein 2, the main genetic hallmark of RTT, is known to be able to regulate the expression of a multitude of genes involved in mitochondrial homeostasis and functionality, among which are included genes encoding proteins related to mitochondrial structure and organization, such as HMN, TIMM, TOMM7, MRPL, MRPS, Crls1 and NR3C1 [239], suggesting a clear link between RTT and alterations at the mitochondrial level.

In a previous work of our group, it has been discovered that RTT mitochondria showed increase depolarization and superoxide next to a reduction of bioenergetic profile and biogenesis [171]. Therefore, our aim was to assess the mitochondrial morphology and whether its alterations could affect the ability of RTT cells to counteract the oxidative cell damage already demonstrated, analysing two mitochondria-dependent cell quality control pathways, mitophagy and apoptosis, taking into account also their strictly link with mitochondrial fusion and fission. The biological model used was primary human fibroblasts isolated from skin biopsies, which represent a valid system with defined mutations and the cumulative cellular damage of the patients.

The first approach was to assess mitochondrial morphology in our cell model of RTT Syndrome, by using transmission electron microscopy which remains a powerful tool for the morphological examination of mitochondria [244]. Previous studies have shown that muscle and frontal lobe biopsies of RTT patients as well as neurons from the frontal cortex, cerebellum and substantia nigra in post-mortem patient samples revealed abnormally swollen mitochondria with vacuolization, granular inclusions and membranous changes [150], [245]–[248]. Also Belichenko *et al.* confirmed these ultrastructural changes in cortical and hippocampal mitochondria of $Mecp2^{-/y}$ mice [249].

In line with the results obtained from other groups, by electron transmission microscopy we discovered that RTT mitochondria showed an altered morphology characterized by dumbbell-like shape, a structural swollen cristae disarrangement and a predominantly electron dense matrix deposition.

Mitochondria are highly dynamic organelles that fuse and divide to form constantly changing tubular networks being modulate by a complex pathway of cytosolic and mitochondrial proteins, in order to

maintain a proper cellular functionality [250]. Therefore, is very important to be able to image mitochondrial shape changes to relate to the variety of cellular functions that these organelles have to accomplish, especially under a pathological condition [251].

Thus, we stained live fibroblasts with green MitoTracker, a mitochondrion specific dye, to acquire Z stacks across the depth of the cell, which provide steady state 3D information about mitochondrial morphology [251]. Interestingly, we discovered that RTT fibroblasts were characterized by a more filamentous interconnected network of elongated and hyperfused mitochondria compared to control cells, in which are visible short lines or dots that represent the typical mitochondrial structure. Then, from the reconstructed 3D images, we calculated the number and the volume of RTT and control mitochondria.

Confocal images revealed decreased number (almost a third less) of mitochondria in RTT fibroblasts compared with those of controls. This specific result is nicely in line with our previous finding of PCG-1α downregulation in RTT as well as its downstream target NRF1 and the alteration in c-AMP mediated signal transduction pathway, with reduced cAMP level next to a decrease of p-CREB [171]. The analysis of the mean and the total mitochondrial volume displayed an increase of both in RTT, suggesting that there were possible effective alterations in mitochondrial dynamics (e.g. fusion and/or fission). In a very recent paper, Bebensee *et al*. reported that also hippocampal astrocytes of $Mecp2^{-/y}$ mice showed increased mitochondrial mass, even if they found an increase number of mitochondria; this could be explained with the different cellular model used [252].

It has been established that mitochondrial functions are intrinsically linked to their morphology and membrane ultrastructure changes of mitochondrial volume may strongly modulate mitochondrial physiology [253].

An emerging hypothesis is that swelling may trigger autophagy, a process through which aging or damaged organelles are degraded via the lysosomal pathway, called “mitophagy” when mitochondria removal is involved [250]. Mitophagy holds a key role in mitochondrial quality control being central to the health of the cell, additionally to be recently unquestionably considered fundamental for normal physiology; alterations at this level are associated with many pathological conditions [207]. We have previously reported that autophagy is impaired in RTT fibroblasts under nutrient starvation conditions and also that mature RBCs of RTT patients carrying the R255X MECP2 mutation retain mitochondria [254]. Therefore, since no other data are present in literature regarding this mechanism in RTT, we were interested in understanding whether our hyperfused RTT mitochondria were able to be degraded by mitophagy. Although several mechanisms regulating mitophagy have been described in

mammalian cells, the best understood is the PINK1/Parkin-mediated mitophagy, which ubiquitylates outer mitochondrial membrane proteins, triggering mitophagy that selectively clears damaged or depolarized mitochondria [255], [256].

Evaluating PINK1 and Parkin protein expression in RTT fibroblasts, we surprisingly found a marked decrease of the ubiquitin E3 ligase Parkin protein expression, whereas no differences were found in Pink1 protein in basal condition in the total protein lysates. It is widely reported in the literature that the localization of Pink1 varies from cytosol to mitochondria upon a mitophagic stimulus [202], [206]. Thus, we performed a subcellular fractionation in order to separate the cytoplasmic proteins from those mitochondrial, confirming no alteration of Pink1 protein expression also at mitochondrial level. A specific mitochondrial damage induced agent is the potent mitochondrial uncoupler of oxidative phosphorylation carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), which is able to activate PINK1/Parkin pathway by depolarizing mitochondria [255].

The challenge of our cells with FCCP unexpectedly revealed a defective translocation of Pink1 from cytosol to mitochondria upon mitophagy induction in RTT mitochondrial subfraction, compared to control.

Several are the methods to study whether the mitophagic process ends; among those it is accepted that the decrease of mitochondrial proteins means a proper degradation of mitochondria. Thus, we treated our cells with FCCP, evaluating the expression of some mitochondrial proteins, MFN2 and ATP5A, that are important for mitochondrial functionality. Usually, following the treatment with FCCP, Parkin shows a very robust and complete recruitment to mitochondria within several hours, with the subsequent clearance of mitochondria [255]. Parkin functions as an E3 ubiquitin ligase, ubiquinating various proteins to regulate a moltitude of cellular processes, including mitochondrial homeostasis and OxS, additionally to mitophagy [256]; its loss has been strictly associated to PD [198].

In our model, FCCP induced a significant and time-dependent decrease of Parkin, Mfn2 and ATP5A in healthy fibroblasts (maximum loss at 24h after the treatment), indicating a good activity of the mitophagic machinery. Interestingly, RTT displayed almost completely absence of Parkin protein and any significant change was appreciable in Mfn2 and ATP5A expression after FCCP-induced mitochondrial damage.

During mitophagy, entire parts of the same organelles are removed; thus, based on steric principles, damaged mitochondria are titled toward a fragmented phenotype, so as to be more disposed to segregation and removal [195]. Therefore, we analysed fusion and fission processes in our model

upon FCCP-mediated mitophagy induction. The analysis of fusion process revealed increased levels of Mfn1 and Mfn2, two main markers of mitochondrial fusion, in the mitochondrial subfraction in basal condition and after FCCP treatment, molecularly explaining the hyperfused morphology of RTT mitochondria. One of the main pathway of mitochondrial fission is the one involving the Dynamin related protein 1 (Drp1) which binds the mitochondrial fission 1 protein (Fis1). Early studies revealed that, when Drp1 activity is inhibited, wild-type mitochondria are transformed into long and interconnected organelles and, conversely, overexpression of Drp1 in cells results in mitochondrial fragmentation [193]. In parallel, Fis1 overexpression in cultured cells resulted in mitochondrial fragmentation and depletion of Fis1 leads to elongated mitochondria [193]. We discovered a marked decrease of Fis1 whereas no differences were found in Drp1 and its phosphorylated form pDrp1 (S616), which is known to enhances the activity of Drp1 under certain circumstances in the total lysate of RTT fibroblasts, possibly due to unchanged total expression levels. It is widely known that, upon mitophagic stimulus, Drp1 translocates from cytosol to mitochondria to activate fission. Surprisingly, RTT cells showed the inability to properly recruit Drp1 at mitochondria surface upon FCCP-mediated mitophagic stimulus. These results highlighted that the mitophagic defect observed in RTT could be possibly related to impairment in the physiological mitochondrial fusion and fission processes.

Furthermore, swelling is one of the fundamental features of pathological states of mitochondria, leading to the final result to activate downstream cascades, mostly life-or-death decisions including apoptosis [255]; in fact, the mitochondria peculiarity is their integral role in cellular death and survival [255].

Normally, the mitochondrial network disintegrates during apoptosis, when cytochrome *c* is released and prior to caspase activation, producing more numerous and smaller mitochondria [257].

Since RTT mitochondria displayed an altered morphology in addition to increased levels of OxS, we tried to understand whether these impairments could affect also their functionality in activating apoptosis, which is physiologically programmed to maintain homeostasis. FCCP is known to regulate various biological functions of cell proliferation, cell cycle regulation as well as apoptosis [258]; in this regard several papers reported its use to stimulate not only mitophagy but also apoptosis, being a potential trigger for the initiation of the apoptotic process, possibly via alterations of mitochondrial membrane potential and generation of ROS [258], [259]. Previous paper reported a low susceptibility to apoptosis in lymphoblastoid cell lines of RTT patients as well as in bone marrow mesenchymal stem cells from a patient affected by RTT [237], [238]. In line with these results, we founded that

RTT fibroblasts had a lower percentage of apoptotic cells after 48h of FCCP, compared to healthy fibroblasts. The induction of apoptosis with an alternative synthetic lipid-soluble uncoupler of oxidative phosphorylation, 2,4-DNP, led to same conclusion, further validating our result. Because the cleavage of caspase 3 represents the final step of the intrinsic mitochondria dependent apoptotic caspase cascade, playing an essential role as an executor in apoptosis, we determined whether caspase 3 was activated after the FCCP-mediated induction of apoptosis [258], [259]. Interestingly, by a luminescence-based assay, we found that RTT fibroblasts didn't show any activation of caspase 3/7 (which both work at the same level of the caspase cascade) compared to controls. As a proof of concept, we performed an immunoblot, obtaining the same result, thus confirming that no cleavage of caspase 3 occurred in RTT fibroblasts, suggesting a possible impairment of RTT cells to activate this crucial defensive pathway.

Altogether, the results presented in this study underline new aspects regarding impairment of mitochondrial dynamics in RTT fibroblasts. It has been shown that the altered mitochondrial morphology, observed both in fixed and in live condition, is accompanied by the decrease of mitochondrial number and the increase in their volume. Furthermore, these bigger mitochondria seemed to be not degraded, with a loss of the PINK1/Parkin mitochondrial quality control pathway and a possible consequent accumulation of impaired mitochondria, which is thought to be a source of toxic ROS. The impairment in mitophagy could be caused by an increase of mitochondrial fusion and a parallel decrease of mitochondrial Drp1/Fis1-mediated fission. These hyperfused and FCCP-mediated damaged RTT mitochondria are less susceptible to apoptotic death next to inability to activate the caspase 3 pathway. It is possible to hypothesize that the electron dense matrix observed, corresponds to a more packed protein conformation, blocking therefore the physiological regulation of the release of proteins from the intermembrane space to the cytosol. Although the expression of Parkin is linked with a pro-survival effect via mitophagy activation, a very elegant and recent paper reported its ability to sensitize cells toward apoptosis induced by mitochondrial depolarization inducing-agent [260]. Interestingly, the authors suggested that Parkin trigger apoptosis through lowering the threshold for opening of the mitochondrial Bax/Bak channel, at least in part through the selective degradation of the prosurvival Bcl-2 family member, Mcl-1. They also reported that silencing of endogenous Parkin protects against apoptosis specifically activated by mitochondrial depolarization. Therefore, the loss of Parkin here discovered in RTT, might exert a very crucial role, blocking both apoptosis and mitophagy via Parkin loss, even if more investigation is needed to better clarify the molecular mechanism. Thus, may lead to the hypothesis that dysfunctional and damaged

RTT mitochondria remains within the cells being detrimental to cellular homeostasis, possibly contributing to the vicious cycle of OxInflammation already proposed for this disease, and highlighting a new target for future studies and hopefully, for future therapies.

9. Future perspectives

Since our data highlighted an impairment in processes involving mitochondria, further investigations will be needed to better dissect the physiopathological role of mitochondria in RTT Syndrome. Indeed, it could be of interest to evaluate the implication of oxidative stress in mitophagy and apoptosis, two fundamental processes in cell quality control. In particular, future studies might clarify whether the treatment with 4-Hydroxynonenal (4-HNE), which levels were found to be altered in RTT patients, reflect in the mitochondrial alterations here observed.

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