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MECP2 GENE MUTATIONS IN RETT SYNDROME: PROTEOMIC APPROACH INVESTIGATION ON MOLECULAR MECHANISMS INVOLVED IN OXIDATIVE STRESS

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1. Abstract

Rett Syndrome (RTT; OMIM 312750) is a neurodevelopmental disorder mainly caused by mutations present in the X-linked methyl-CpG-binding 2 (MECP2) gene. Clinical manifestations of this syndrome are complex and with distinct severity's degree; for instance, patients can have autistic-like behavior, loss of acquired speech with impaired motor skills and cardiac problems. Moreover, the correlation between specific MECP2 mutations and clinical phenotypes are not clear yet. Interesting, different genotypes can cause patient's phenotype differently. Omics approaches, and among them proteomics, can play a fundamental role in exploring molecular mechanisms and shed light in the correlation genotype - phenotype from a molecular point of view.

The aim of this work of thesis was the investigation on oxidative stress (OS) response pathways in RTT linked to specific MECP2 mutations with the use of proteomics and bioinformatic approaches.

Primary fibroblasts were collected from RTT patients having R133C and R255X mutations and from healthy donors (HC). Thus, once it was performed a clustering of primary dermal fibroblasts based on their MECP2 gene mutations, different experiments were performed to investigate difference in OS, such as ROS and 4-HNE assay. Data showed distinct behavior of RTT fibroblasts in comparison to HC, but no significant differences were observed between the two diverse RTT fibroblasts bearing different mutations. The mutations R133C and R255X are reported to exhibited distinct clinical severity score, for this reason, a shotgun proteomics analysis, in particular a label free quantification (LFQ) mass spectrometry (MS) based, was applied in order to investigate the proteome profiles of RTT and HC fibroblasts. The bioinformatics elaboration allowed to obtain information on altered molecular pathways from a qualitative and quantitative point of view. Therefore, it was possible to evidence a preliminary correlation between RTT genotype and phenotype, focusing on those proteins involved in OS molecular mechanisms. Proteomics data were confirmed by molecular biology assays.

In conclusion, proteomics, bioinformatics, and molecular biology assays were employed, enabling to study uncover phenotypic consequences linked to distinct MECP2 gene mutations. These findings confirm the high heterogeneity among RTT patients and contribute to a better understanding of this syndrome.

2. Introduction

Rare diseases, also known as orphan diseases, are a group of disorders that affect a relatively small number of people within a population. The list of this kind of pathologies is quite long and they are widely distributed all over the world. These diseases are often serious, chronic, and life-threatening. Because of the limited number of affected individuals, there may be limited research and funding available for the development of treatments.

One of the most important challenges is that clinical manifestations are very different for patients affected by the same pathology, and in some cases the applied clinical protocol is ineffective [1, 2]. There are several reasons why this can occur:

Genetic Variability: In the case of genetic diseases, even within the same disorder, different mutations in the same gene or variations in multiple genes can lead to distinct severity degrees of symptoms and clinical signs [3].

Environmental factors: Environmental factors play a role in the expression of many diseases. Two individuals with the same genetic predisposition may experience different outcomes due to differences in their environment, lifestyle, and exposure to various factors [4].

Individual differences: Each person's unique biology, including their immune system, metabolism, and other physiological factors, can contribute to differences in how diseases manifest and progress.

In many cases, the underlying mechanisms of a disease may not be fully understood. This lack of understanding can hinder the development of effective treatments and contribute to the variability in responses to standard protocols.

Addressing these challenges requires a multi-faceted approach. Improved understanding of the genetic and molecular basis of diseases are crucial [2].

For these reasons cutting edge studies deals with investigations to pave the way to precision medicine approach. But what is precision medicine? Which are the scientific approaches to reach a customized protocol of cure? Which investigations can be combined to pave the way for clinical application?

Precision medicine, also known as personalized medicine, is an innovative approach to medical treatment and healthcare that takes into account individual differences in patients' genes, environments, and lifestyles. The goal is to tailor medical care and interventions to the characteristics of each patient [5].

The scientific approaches to achieve customized protocols of cure in precision medicine are the so called “omics” technologies.

Omics is a modern term that covers a large variety of methods applied to biology’s field. Traditional omics fields include genomics, transcriptomics, proteomics, metabolomics, just to mentioned some of them [1].

In details:

-Genomics involves the comprehensive study of an individual's entire set of genes, known as the genome. By analyzing genetic information, researchers can identify variations and mutations that may contribute to disease susceptibility, progression, or treatment response [6].

-Proteomics focuses on the study of proteins present in a biological system. Understanding the complete set of proteins (proteome) can provide insights into the functional aspects of cells and tissues, aiding in the identification of disease-related proteins and potential therapeutic targets [7].

-Metabolomics examines the complete set of small molecules, or metabolites, within a biological sample. This approach helps to comprehend the metabolic pathways and processes occurring in the body, contributing to understand disease mechanisms and aiding the identification of biomarkers for various conditions [8].

-Transcriptomics explores the entire set of RNA molecules (transcriptome) within a cell or tissue. By analyzing gene expression patterns, researchers can gain insights into which genes are active or inactive, providing valuable information about the underlying molecular processes in health and disease [9].

These "omics" approaches collectively contribute to a more comprehensive understanding of biological systems, allowing for the identification of molecular signatures, biomarkers, and therapeutic targets that can be crucial in the development of personalized and precision medicine approaches [1]. Thus, as said before, precision medicine, also known as personalized medicine, is an innovative approach to medical treatment and healthcare that considers individual differences in patients' genes, environments, and lifestyles and the goal is to achieve a tailored medical care and treatment plans to the specific characteristics of each patient [1]. This approach is called patients’ stratification, and it consists in classifying patients into subgroups based on genetic, molecular, or clinical characteristics.

In this contest, in the ambit of this work of thesis, a research study was performed on samples derived from patients affected by RTT syndrome (RTT, OMIM #312750), a rare disease, trying to find a correlation between their unique genetic profile and their proteome profile to investigate the consequences from a molecular point of view by a proteomics approach and molecular biological assays.

2.1 Rett syndrome (RTT)

RTT is a rare genetic disease and a devasting neurodevelopmental disorder. RTT is mainly caused by the X-linked methyl-CpG binding protein 2 (*MECP2*) in the 95% of cases [10, 11, 12]. *MECP2* is an X-linked gene, thus X-chromosome inactivation (XCI) can occur and consequently impact RTT pathology and clinical severity, discussed and showed by several groups [13]. *MECP2* gene is an X-linked gene located in Xq28 chromosome and between the Intereukin-1 receptor associated kinase gene (IRAK1) and the Red Opsin gene (RCP). MECP2/Mecp2 gene includes four exons (exon 1-4) and three introns (intron 1-3). Regarding MeCP2 protein structure, it is composed by five major domains: N-terminal Domain (NTD), Methyl Binding Domain (MBD), Inter-Domain (ID), Transcription Repression Domain (TRD) and C-terminal Domain (CTD). MeCP2 is an intrinsically disordered protein, with 4% α-helices, 21% β-sheets and 13% β-turns. Moreover, nonetheless MeCP2 molecular weight should be 53kDa, by western blot analysis it is usually detected around 75kDa [14].Mecp2 is a key epigenetic modulator in the brain, going from gene expression controlling, DNA methylation to histone posttranslational modifications (PTMs) and noncoding RNAs [15]. Moreover, these epigenetic mechanisms have a central role in embryonic development control, differentiation of stem cell and thus having crucial impact in human diseases [16]. Other mutations appear in CDKL5and FOXG1 genes in 10% of RTT cases [17].

RTT patients start to exhibit clinical signs after 6-18 months of life and they rapidly regress after 1-5 years, until the phase during which they manifest a late motor deterioration (around 10 years of life). These patients are often misdiagnosed as autistic patients [18]. However, nonetheless RTT is a neurodevelopmental disorder, in fact brain is not the only organ with abnormalities consequences in these patients. Actually, RTT patients can suffer of breathing abnormalities [19], cardiac problems (which can cause reduced life span and higher rates of sudden deaths in RTT patients) [20] and many bone impairments and compliances, going from osteopenia to scoliosis [14].

Moreover, RTT patients' common clinical signs include loss of speech and motor skills, repetitive hand movements and seizures presence, gastrointestinal problems, hypoplasia, osteoporosis and bruxism [14]. Thus, as summarized in Fig. 1, RTT is a neurodevelopmental disorder primarily attributed to mutations in the *MECP2* gene, accounting for approximately 95% of cases. This

condition is characterized by a range of symptoms and for this reason it is considered a multisystemic disorder. Among the notable features associated with RTT, there is evidence of mitochondrial dysfunction and oxidative inflammation (OxInflammation). The interplay of these factors contributes to the complex pathophysiology of the syndrome, influencing various aspects of neurological and physiological functioning in affected individuals.

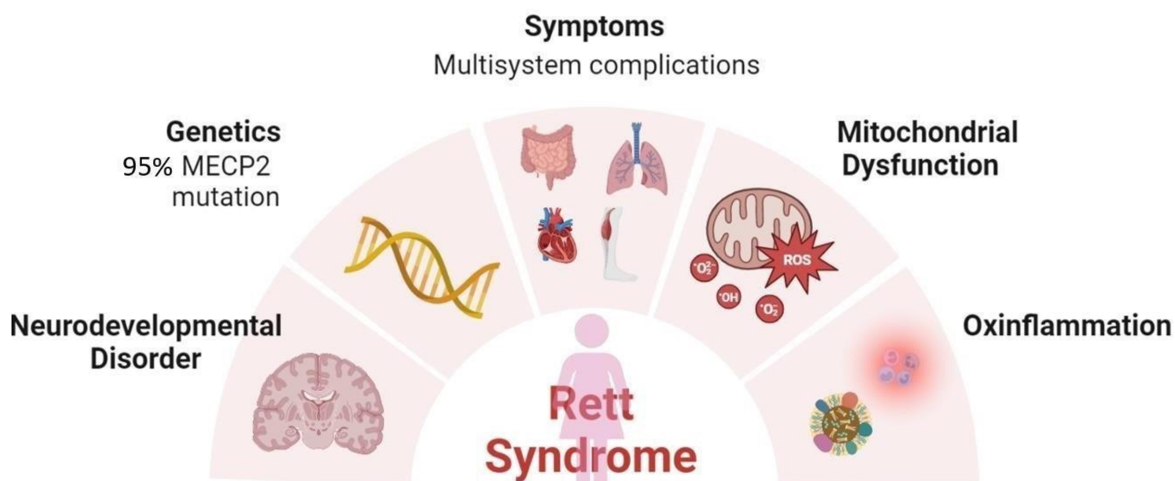

Fig.1 RTT as a multisystemic disorder with several molecular activation mechanisms [21].

Regarding the high complexity of *MECP2* gene mutations, more than 600 cause RTT; however, eight of them, account for more than 60% of RTT individuals (Fig. 2).

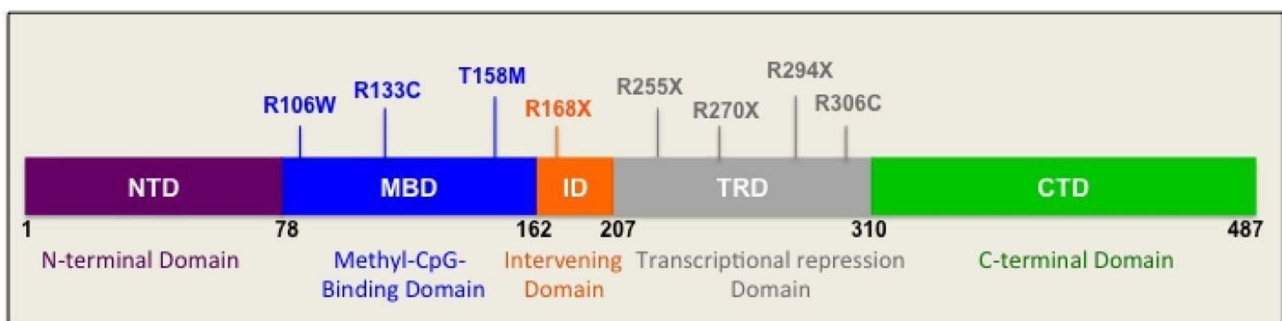

Fig.2 Protein domains representation of MeCP2 with most common mutations [17].

Interestingly, some studies showed that is present a correlation between genotype (*MECP2* mutations) and phenotypes in terms of clinical signs [22]. For instance, *MECP2* truncation mutations showed a correlation with more severe RTT phenotypes [14].

As a matter of fact, some patients with RTT may present walking problems. This phenomenon could be due to non-random X-chromosome inactivation (XCI) of one of the two X-chromosome in each female cell which exhibits a random distribution of active chromosomes in the adult usually.

However, this explanation cannot totally justify clinical severity variance in RTT patients. Thus, do specific *MECP2* mutations cause different clinical features in RTT? Actually, patients leading R133C *MECP2* mutation result with less severe clinical symptoms and phenotypes. This is the reason why, understanding correlation between clinical features in RTT and *MECP2* gene mutations, could be crucial for future therapeutic interventions [23]. A team of researchers studied 245 girls affected by RTT syndrome with different *MECP2* gene mutations, with the aim to evaluate whether a correlation among distinct clinical features and specific *MECP2* mutations could be found. In particular, taking in to account the clinical severity score (based on common clinical signs and phenotypes associated to RTT), they showed that those patients with the missense R133C mutation exhibited the mildest severity degree compared to other specific *MECP2* gene mutations like R255X and R168X [23]. Confirming that, Leonard et al., 2003, showed that patients with R133C *MECP2* gene mutation had less probability to develop severe clinical behavior including the compromised hands use (voluntary and finger feeding), speech, motor function and ambulation, losing social interaction, sleeping disturbances, respiratory dysfunction, breathing rhythm and scoliosis [24]. Thus, clinical severity degree can be linked to specific *MECP2* mutations [23].

2.2 RTT and oxidative stress (OS)

OS is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to act against them. From literature, under a molecular point of view, it is more and more evident that OS molecular mechanisms and mitochondrial responses consequences are correlated with RTT, but their link is not fully understood [25]. Some studies suggest that individuals affected by RTT are characterized by mitochondrial dysfunction, which can lead to an increase in ROS production. Mitochondria are the cellular organelles responsible for energy production, and when they do not correctly work, overproduction of ROS can occur [26], causing unbalance in OS production and response and thus inflammation [27].

The crosstalk between redox imbalance and inflammation, namely the OxInflammation phenomenon, appears to be central in the development and progression of RTT, with pathophysiological implications [23, 28, 29]. One of the main OS molecular mechanisms deals with the nuclear factor erythroid 2-related factor 2 (NRF2) pathway, which has been shown to play a crucial role in tissue defense again OS in RTT [30, 31]. For instance, Crivellari et. al, showed an increased translocation of NRF2 protein into the cell nuclei of RTT fibroblasts, in particular after an oxidant insult [30]. Another study suggests a deficiency in proteasome biogenesis (strongly linked to OS) [32], in fibroblast cells isolated from individuals with RTT [33].

The C-Jun N-terminal kinase (JNK) is another molecular signaling activated in response to OS [34]. Intriguingly, recent studies showed that The JNK stress pathway was found to be activated in *MECP2*-knockout and *MECP2*-heterozygous mice, as well as in human neurons derived from induced pluripotent stem cells (iPSCs) with mutations in the *MECP2* gene [35].

Thus, alterations and abnormalities explain the complex nature of RTT, and nowadays it is a challenge to find a possible correlation between *MECP2* mutations and RTT symptoms [36, 37]. For this reason, we focused our studies on investigation of OS molecular pathways in order to get information about correlations between phenotype and genotype, analyzing RTT fibroblasts patients with distinct *MECP2* gene mutations.

2.3 Proteomics

In this work of thesis, among the plethora of omics, a proteomics approach was applied.
What is proteomics? Proteomics is the study of the proteome, which is the total amount of proteins present in an organism (it is estimated that the human genome codes for about 26000-31000 proteins), and it represents a critical focal point in biology's field. Through the application of proteomics, it is possible to identify proteins present in certain samples, quantify them, to obtain insight into the functions and roles of these proteins in various biological processes [38]. Functions and structure of proteins are key subjects in proteomics field. On the other hand, it is challenging discovering potential novel biomarkers because of their complexity and dynamicity. In general, "Omics" approaches, together with the innovative bioinformatics tools, can contribute to a better understanding regarding information about biological systems [39].
Among "Omics", proteomics plays a crucial role in the field of precision medicine by providing valuable insights into an individual's proteome, which can help to tailor medical treatments and interventions to specific patient needs. For example, the identification of specific proteins up or down regulated in certain clinical conditions, or the discover of alteration of biological pathways associated with a specific disease, can contribute to biomarkers discovery for early-stage detection, diagnosis or in the process of monitoring of diseases and it can be used also for developing investigations that could shed light on molecular mechanisms able to explain why there are responders and non-responders to specific treatments, saving time, costs and finding in a short time the most effective therapy for a single patient [40].
This information is particularly precious if they are combined with other data coming from genomics, transcriptomics, metabolomics, and clinical observation. The whole plethora of data can be a very powerful tool to stratify patients into subgroups, paving the way tailored treatments, that is the basic principle of precision medicine, moving away from a one-size-fits-all approach the latter one [41].
As said at the beginning of this paragraph, in this work of thesis, a proteomics approach mass spectrometry based, in particular liquid chromatography-tandem mass spectrometry (LC-MS/MS), was used. The latter one is a powerful and widely used technique in proteomics for the identification and quantification of proteins in complex biological samples. LC-MS/MS involves different methods. The most common used is the "shotgun" proteomics approach, in which proteins' digestion happen without previous fractionation. The complex peptide mixture is then analyzed by LC that separates the single peptides which are successively analyzed by MS and MS/MS [42].
The general LC-MS/MS workflow is composed by several steps listed below. [43]:

- Sample preparation: process that involves protein extraction, enzymatic digestion into peptides (typically using trypsin), and peptides' cleanup. Such steps are crucial for the removal of interfering compounds like lipids, salt, buffers that can interferes with mass spectrometry analysis. As a matter of fact, optimal sample preparation plays a key role in obtaining accurate and reproducible results [44].
- Liquid Chromatography: the digestion resulting peptide mixture is then analyzed by liquid chromatography, generally using reverse phase columns in order to resolve and separate the peptides based on their hydrophobicity.
- Mass Spectrometry: The peptides eluting from the column are introduced into a mass spectrometer. Full (MS) and fragmentation mass spectra (MS/MS) are acquired in a Data Dependent Analysis (DDA) or Data Indipendent Analysis (DIA).
- Database search: The data acquired from the MS and MS/MS analysis are then submitted to bioinformatic analysis based on protein sequence databases. In particular, search algorithms compare the experimental spectra with theoretical ones generated from the protein sequence database to identify the unique peptides and, consequently, the proteins present in the sample [42, 43].

The shotgun proteomics approach can be used for both qualitative, that means identify proteins, and quantitative that means measure their relative abundances, analyses. For measuring the relative abundance there are essentially two methods: label-free and label-based quantification methods, such as spectral counting or peak intensity-based approaches, can be applied to obtain quantitative data [45].

In this work a label-free approach was used. In particular, Label-Free-Quantification (LFQ), allows to identify proteins and measure their expressions in each sample by mass spectral peak intensities for each peptide [46]. LFQ principle allows to link proteins amount with peak intensities of unique peptides to a particular protein. Peak intensities are incorporated across retention times with a defined mass window: the area under the curve (AUC) or extracted ion chromatography (XIC) [47].

Summarizing, proteomics approach LC-MS/MS based with bioinformatics elaboration allows proteins identification in complex biological samples and it is crucial for protein expression level quantification among different samples, providing a powerful tool for biomarkers identification, understanding protein-protein interactions and for the investigation of the proteome of a specific tissue or cell type.

3. The aim of this work

The central objective of this thesis was to conduct an in-depth investigation into oxidative stress (OS) pathways underlying RTT syndrome by analyzing fibroblasts obtained from patients with distinct genetic mutations. Different RTT genotypes can variously affect the patient's phenotype and clinical status. Specifically, patients characterized by R133C *MECP2* mutation present less severe clinical symptoms in comparison with R255X *MECP2* mutation. To explore the molecular mechanisms involved in OS in two opposite genotypes, a comprehensive analysis applying shotgun label-free proteomics investigation was performed.
Following this, bioinformatics analyses were employed to interpret the complex datasets generated. To strengthen the validity of the findings, biological assays were conducted, ensuring the robustness of the data. Finally, the main goal of this study was to uncover phenotypic responses linked to specific *MECP2* gene mutations by detecting potential correlations between the specific genetic mutation (genotype) and the identified differentially expressed proteins (phenotype), thereby contributing valuable insights to the understanding of RTT. In figure 3 a scheme of the thesis workflow is reported.

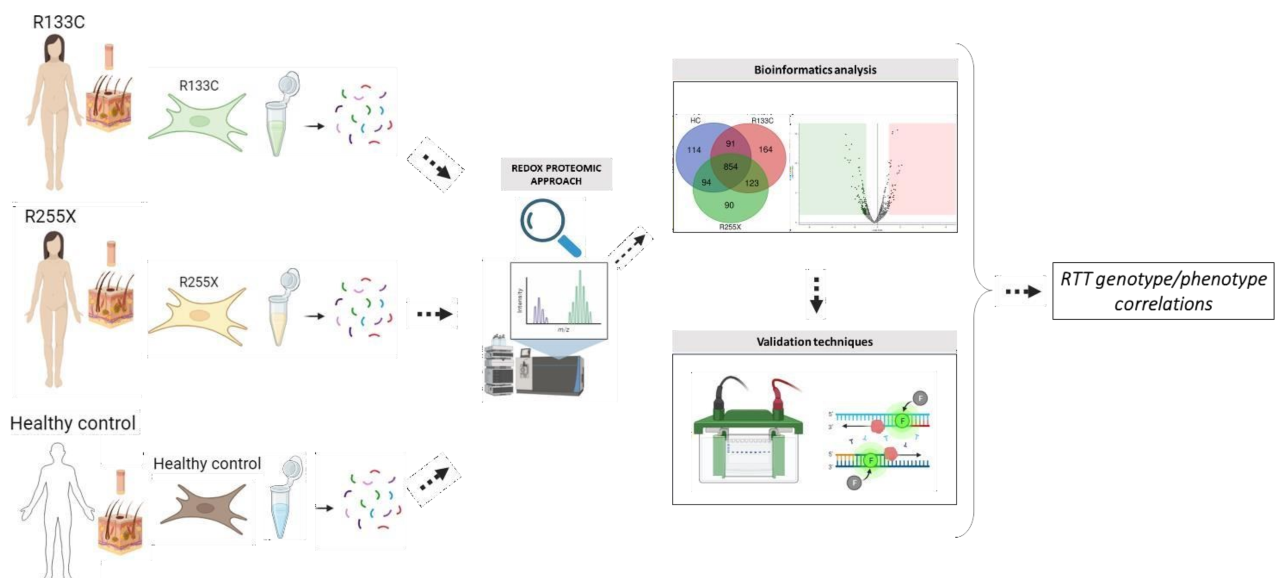

Fig. 3: workflow of RTT investigation using a proteomic approach.

4. Materials and methods

4.1 Samples

The study was conducted on primary dermal fibroblasts from female patients with classic RTT and *MECP2* gene mutation and healthy female age-matched HC (N=2) (Table 1). All the patients were admitted to the Child Neuropsychiatry Unit of the "Azienda Ospedaliera Universitaria Senese" (AOUS, Siena, Italy). Diagnosis and inclusion/exclusion criteria of RTT were set in agreement with revised RTT nomenclature consensus [48]. The study was following the Declaration of Helsinki and was conducted with the approval of the Ethics Committee of Institutional Review Board of AOUS, Siena, Italy. Written informed consents were obtained from either the parents or the legal tutors of the participants.

Table 1. Four RTT samples bearing two classic *MECP2* mutations.

Cohort	*Sex*	*Age Range (mean), y*	*Patient*	*Type of Mutation*	*Sex*	*Age, y*	*Nucleotide and Amino Acid Change*	*Affected Domain*
Classic RTT	All female	6-28 (16.7)	#1	Missense	F	21	c.397C>T; p.Arg133Cys	MBD
			#2	Missense	F	6	c.397C>T; p.Arg133Cys	MBD
			#3	Nonsense	F	28	c.763C>T; p.Arg255*	TRD
			#4	Nonsense	F	20	c.763C>T; p.Arg255*	TRD
HC	All female				F	18		
					F	23		

Skin biopsies from the HC group were taken by donation or during routine health checks, while skin biopsies from RTT patients were obtained during periodic clinical check-up. The isolation of primary dermal fibroblasts was performed as described in literature [28]. Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (cat. 10-014-CV, Thermo Fisher Scientific Inc., Waltham, MA, USA), containing 10% fetal bovine serum (FBS; cat. MT35010CV, Thermo Fisher Scientific Inc.) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, Thermo Fisher Scientific Inc.). Cells were incubated at 37 °C and 5% CO_2. Fibroblasts from passage 3 to 5 were used for the experiments. Cells were tested for mycoplasma contamination (data not shown) before the experimental procedures.

4.2 ROS Assay

The assay for the estimation of the reactive oxygen species (ROS) levels was based on the probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Thermo Fisher Scientific, USA) [49]. H_2DCFDA passively diffuses into cells, and it is deacetylated by esterases to form nonfluorescent 2′, 7′-dichlorofluorescein (DCFH) which in the presence of ROS forms the fluorescent product DCF, which is trapped inside the cells. Dermal fibroblasts were seeded at a density of 5×10^3/well in 96-well plate (Corning, USA) as previously described [50]. Twenty-four hours after seeding, culture medium was first removed, and cells were washed three times with phosphate-buffered saline (PBS). DCFH-DA, diluted to a final concentration of 10 μM with a serum free DMEM, was added to cultures and incubated for 30 min at 37°C. The fluorescence was measured at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (SpectraMax M2, Molecular Devices). Results were presented as arbitrary units.

4.3 Sample preparation for proteomics

Cells lysis was performed by using RIPA buffer. Cell lysates were reduced with Dithiothreitol (DTT) and consequently alkylated with iodoacetamide (IAA). 8 M urea (UA) was added to the cell lysates. BCA assay was used for protein quantification, following manufacturers' instructions. Approximately 30-70 µg of extracted proteins was processed. Trypsin was added and incubated overnight at 37°C. The resulting peptide mixtures were desalted and used for proteomics analysis.

4.4 Proteomics analysis, bioinformatics analysis and statistics

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) based proteomics analyses in a large-scale and high-throughput format with Q-Exactive HF-X mass spectrometer coupled with a Nano UPLC RLSC Ultimate 3000 (Thermo Scientific) was performed for each sample in triplicate. The peptide separation was carried out at 37°C using a peptide PepMap RSLC C18 column, 75µm × 15 mm, 2µm, 100 Å (Thermo Scientific) at a flow rate of 0.300 µl/min. The mobile phases A and B used for the analysis were 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile and 20% water, respectively. The gradient started with 5% of B and then it was increased up to 90% in 106 min. These experiments were performed using a Data Dependent Analysis (DDA) setting to select the "top twelve" most-abundant ions for MS/MS analysis. Protein identification was performedusing Proteome Discoverer 2.5 (Thermo Scientific) and Sequest algorithm. To analyze the technical

triplicates' reproducibility, a quality control filtering of mass spectrometry measurements was performed. The triplicates in each sample were measured with the calculation of LFQ intensity and only proteins derived from common overlap of the triplicates were taken into account. All the analyses were performed with FDR <0.001. Protein identification was based on at least one unique peptide with a minimum length of seven amino acids and a false discovery rate of 0.05 applied to both peptide and protein level. FDR of 0.05 was set as a threshold for peptide and proteinidentifications. To study biological pathways and meaning from large protein lists, UniprotKB [51] and FunRich [52] was used. The selected identifier name was "UNIPROT ACCESSION". The inputprotein list completely mapped to the internal IDs and belonged to *Homo sapiens*. For a functional interpretation of the results, the protein identifiers are associated to its related Gene Ontology (GO) terms [53], overcoming the redundancy in terminology for "biological process" and "cellular component" [54]. The LFQ intensities of proteins from the Proteome Discoverer 2.5 analysis were imported and transformed to logarithmic scale with base two. Moreover, volcano plots were used to show a summary distribution of differentially expressed proteins between RTT samples subgroups and between them and HC fibroblasts samples. The volcano plot is an easy-to-interpret scatter plot that arranges values along dimensions of biological and statistical (log10 p-value) significance. The proteins located on the upper left region and the upper right region with False discovery rate (FDR<0.05) are differentially expressed.

4.5 Samples preparation for Western Blot assays

RTT and HC fibroblasts were seeded in 100-mm dishes and, at 80%-90% confluence, treated with hydrogen peroxide (H_2O_2) (100 μM) (cat. H1009, Sigma-Aldrich, St. Louis, MO, USA) for different time points. Total protein extracts were obtained as previously described [31]. Briefly, fibroblasts were lysed in RIPA buffer (cat. AAJ62524AD, Alfa Aesar, Tewksbury, MA, USA) supplemented with 1% of protease inhibitor cocktail and 1% of phosphatase inhibitor cocktail (cat. 78430 and cat. 78420, respectively, Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates were centrifuged at 15,000 g for 20 minutes at 4°C and protein concentration in the supernatants was determined by using Bradford assay, following manufacturers' instructions (Quick Start Bradford Protein Assay Kit;cat. 5000201, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.6 Western Blot assay

Protein lysates (15 µg) were analyzed by immunoblotting analysis, as reported in literature [54]. Briefly, denatured protein samples were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked for 5 min at room temperature with Everyblot Blocking Buffer (Bio-Rad Laboratories, Inc.). Then, membranes were incubated overnight at 4°C with primary antibodies diluted in Everyblot Blocking Buffer: NQO1 (1:1,000; cat. MCA2880GA, Bio-Rad Laboratories, Inc.); 4HNE (1:3,000; cat. AB5605, MilliporeSigma, Burlington, MA, USA). Following incubation for 1h with appropriate secondary antibodies, protein bands were detected by using Clarity™ Western ECL Substrate Kit (cat. 1705060, Bio-Rad Laboratories, Inc.) and ChemiDoc™ MP Imaging System hardware and software (Bio-Rad Laboratories, Inc.). The blots were then stripped and re-probed with β-actin-peroxidase conjugate (1:100,000; cat. A3854, MilliporeSigma), as the loading control. Intensity of bands were analyzed by using ImageJ software and data normalized against β-actin. Results were given as arbitrary units. Statistical analyses were performed by using GraphPad Prism 8 software (GraphPad Software, San Diego, California USA). One-way analysis of variance (ANOVA) and Student's t test for independent groups were used. The null hypothesis was rejected with a p value of less than 0.05. All experiments were done in triplicate and data are presented as means ± standard error of mean (SEM) and as standard deviation (SD).

4.7 RNA extraction and real-time RT-PCR analysis

As previously reported [55], the extraction of total RNA from control and RTT fibroblasts was performed by using RNeasy Plus Kit, following manufacturers' instructions (cat. 74134, QIAGEN LLC, Germantown, MD, USA). Reverse transcription of RNA (1 µg) into complementary DNA (cDNA) was performed by using iScript Reverse Transcription kit (cat. 1708841, Bio-Rad Laboratories, Inc.). The cDNA (diluted 1:10) was used for real-time PCR analysis with SsoAdvanced Universal SYBR Green Supermix (cat. 1725271, Bio-Rad Laboratories, Inc.) in a LightCycler® 480 Instrument (Roche, Indianapolis, IN, USA). Primers were synthetized by Sigma-Aldrich (St. Louis, MO, USA): NQO1 (forward, 5'-GGATTGGACCGAGCTGGAA-3'; reverse, 5'-AATTGCAGTGAAGATGAA-3'); HO-1 (forward, 5′ - TCCGATGGGTCCTTACACTC -3'; reverse, 5′ - TAAGGAAGCCAGCCAAGAGA -3′) and GAPDH (forward, 5' - TGACGCTGGGGCTGGCATTG –3'; reverse, 5'- GGCTGGTGGTCCAGGGGTCT - 3').

The following amplification conditions were applied: polymerase activation and initial denaturation at 95°C for 2 min, and 40 cycles of 95°C for 5 s and 60°C for 30 s. The possible co-amplification of

non-specific targets was checked through the melting curves for all primer pairs (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [57], using GAPDH as reference, and one of the controls as internal calibrator. Each sample was assayed in triplicate.

5. Results and discussions

Since the aim of this work was the correlation between the *MECP2* mutations and molecular mechanisms related to Oxinflammation, oxidative stress assays were carried out. The first investigation dealt with the investigation of ROS levels in all the available samples (see Table 1).

5.1 Reactive oxygen species (ROS) assay

In literature OS in RTT patients is widely described, for example ROS levels have been found to be much higher in patients compared to the HC, confirming recent studies conducted in OS's field [57]. It is also known that the excess of reactive oxygen species (ROS) in the body, leading to damage to cellular components and potentially causing cell death. ROS, such as singlet oxygen, superoxide anions, and hydrogen peroxide, can harm lipids, proteins, DNA, and other molecules, and they are associated with various diseases and aging. To counteract oxidative stress, the body has defense mechanisms that include antioxidant enzymes and compounds [59].

Considering this peculiar aspect of the syndrome, to gain a deeper understanding of the diverse phenotypic consequences associated with different *MECP2* mutations, we first analyzed the levels of ROS in RTT fibroblasts, clustered by *MECP2* mutation type, compared with HC cells. Thus, intracellular ROS assay was performed among patients and in the healthy control, in order to test whether RTT patients exhibited higher levels of oxidative stress compared to healthy controls. As shown in Fig.4, RTT fibroblasts, regardless of their specific *MECP2* mutation, exhibited significantly higher ROS levels than HC fibroblasts. On the other hand, no statistical differences were observed within the two *MECP2* mutations analyzed.

Aberrant redox homeostasis is correlated to a subclinical inflammation, named "OxInflammation", which is a condition well studied in RTT [60, 61, 62, 63, 64].

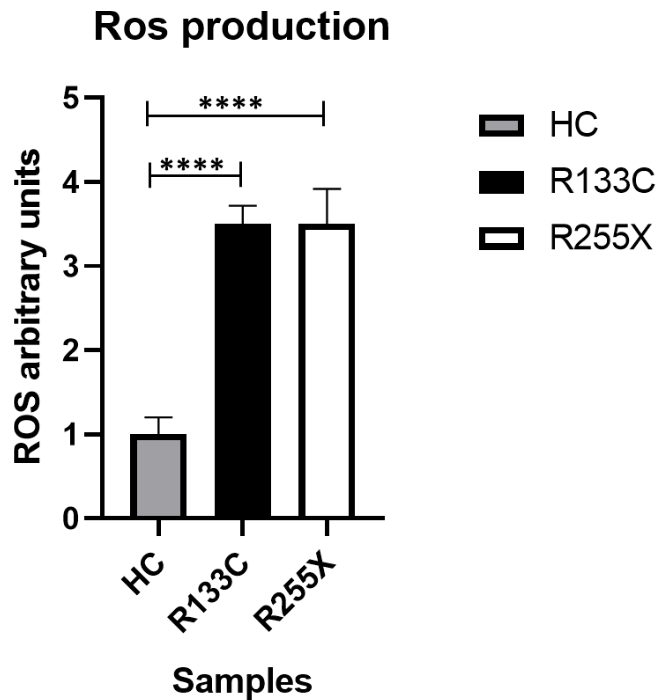

Fig.4. ROS levels in RTT fibroblasts, clustered for *MECP2* gene mutations and HC cells. Tukey's multiple comparisons test was reported: **** p-Value <0,0001.

Increased levels of OS cause peroxidation, and as a consequence, 4-hydroxynonenal (4HNE) is one of the most reactive byproducts generated [65, 66].

Thus, 4HNE protein adduct levels were investigated in the different *MECP2* mutations by immunoblotting. As depicted in Fig.5, 4HNE protein adduct levels were higher in *MECP2* mutant fibroblasts than in HC samples.

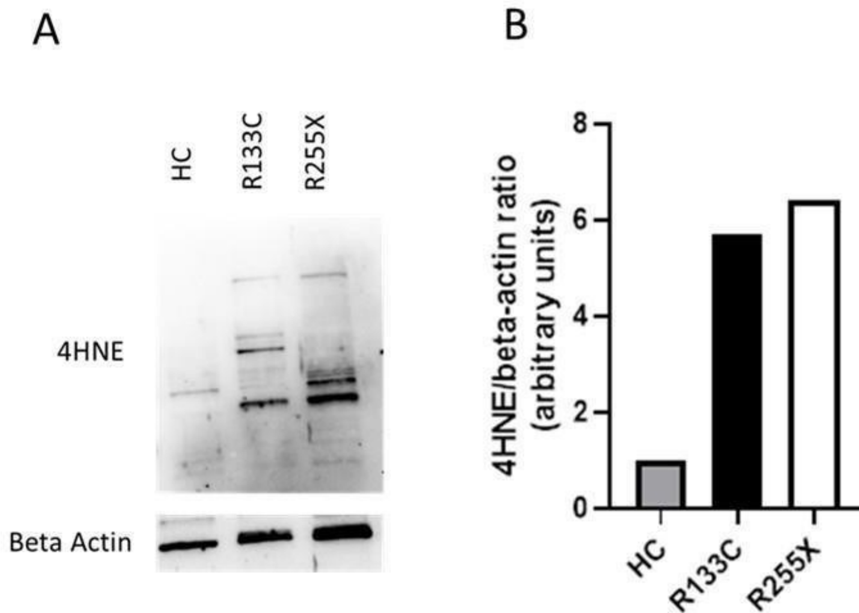

Fig.5 4HNE levels in HC and in RTT patients. Right panel (A) shows a representative Western blot image. The signals of 4HNE protein adducts levels were determined by densitometric analysis of the scanned images (B). Averages of the values from all the samples are expressed in arbitrary units, left panel (B).

Summarizing, ROS and 4HNE assays showed differences among RTT and HC, but no differences statistically significative were detected among samples with different mutations.

5.2 Proteomics and bioinformatics analysis

Given the confirmed higher level of OS in RTT patients compared to the HC group, our investigation focused on the possible categorization of patients based on their *MECP2* mutations and studying the molecular pathways that might vary among individuals with different mutations. To achieve this, we utilized a proteomics approach coupled with bioinformatic tools to determine relevant proteins associated with RTT. This analysis aimed to offer an insight into the potential correlation between the genotype and phenotype of RTT patients.

The discovery of these proteins and the subsequent exploration of molecular pathways can contribute significantly to investigating the observed heterogeneity in RTT patients. By associating specific *MECP2* mutations with distinct molecular profiles, we might potentially unveil molecular subtypes within RTT. The integration of proteomics, bioinformatics, and the exploration of molecular pathways presents a thorough and methodical approach to unravel the complexities of RTT, leading the way to comprehend OS molecular mechanisms that may underlie the diverse genotypes/phenotypes in RTT patients.

Fibroblast samples derived from both RTT and HC patients were collected, subjected to lysis, reduction, alkylation, and subsequent trypsin digestion in accordance with the procedures outlined in the materials and methods section. The resultant peptide mixtures underwent LC-MS/MS analysis to get insights into the expression of various proteins, and the data were processed using specific bioinformatics methodologies, as described in the material and methods section.

The proteomics analysis encompassed all six samples (2 HC and 4 RTT, as detailed in the methods section). Thanks to the high dimensionality of the generated proteomics datasets, we successfully identified approximately 1,200 proteins in common from the triplicate of each sample, as delineated in Table 2. In HC fibroblasts, 1,153 unique proteins were found, while 1,232 and 1,161 unique proteins were identified in R133C RTT and R255X RTT, respectively. Consequently, we qualitatively and quantitatively determined differentiated proteins within the primary dermal fibroblasts of HC and RTT, as well as variations among RTT samples, studying and elucidating their functions and characteristics.

Samples	*Number of identified proteins*
HC	1153
R133C	1232
R255X	1161

Table 2. Qualitative analysis. Common proteins from triplicates of each sample.

For qualitative analysis a Venn Diagram representation was used. By performing Venn Diagram, different proteins between HC and RTT patients' subgroups were detected. As we can see by Fig.6, a comprehensive visual representation of proteins present in each biological triplicate was performed. Intersections of unique proteins sets were depicted, finding common and distinct proteomics profiles among primary fibroblasts samples. In total, 7 intersections were achieved. Of which 854 unique proteins were common to all the samples analyzed, while 114 were uniquely present in HC, 164 in R133C and 90 in R255X. 91 and 94 unique proteins were respectively shared to HC-R133C and to HC-R255X. Finally, 123 unique proteins were found in R133C-R255X set.

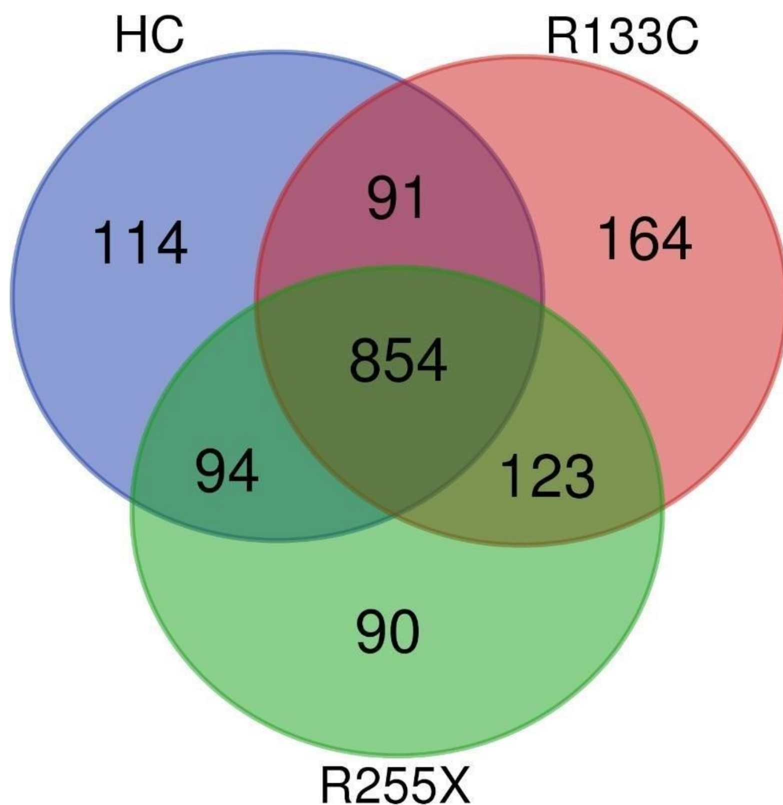

Fig.6. Venn diagram representing the number of different proteins which are present or absent among samples fibroblasts' subgroups.

Thus, starting from the distinct unique subsets from Venn diagram, a consequent analysis was performed, focusing on proteins linked to OS response.

Fig.7 shows the percentage of OS proteins identified in each fibroblast subgroup. The highest percentage was found in the intersection R133C-R255X (5.7%), immediately followed by R255X unique subset (5.5%). While in the sets HC-R133C-R255X, HC-R133C and HC-R255X were present respectively 3%, 3.3% and 2.1% of proteins included in OS biological molecular mechanisms. The lowest percentages were in R133C (1.8 %) and in HC (0.9 %).

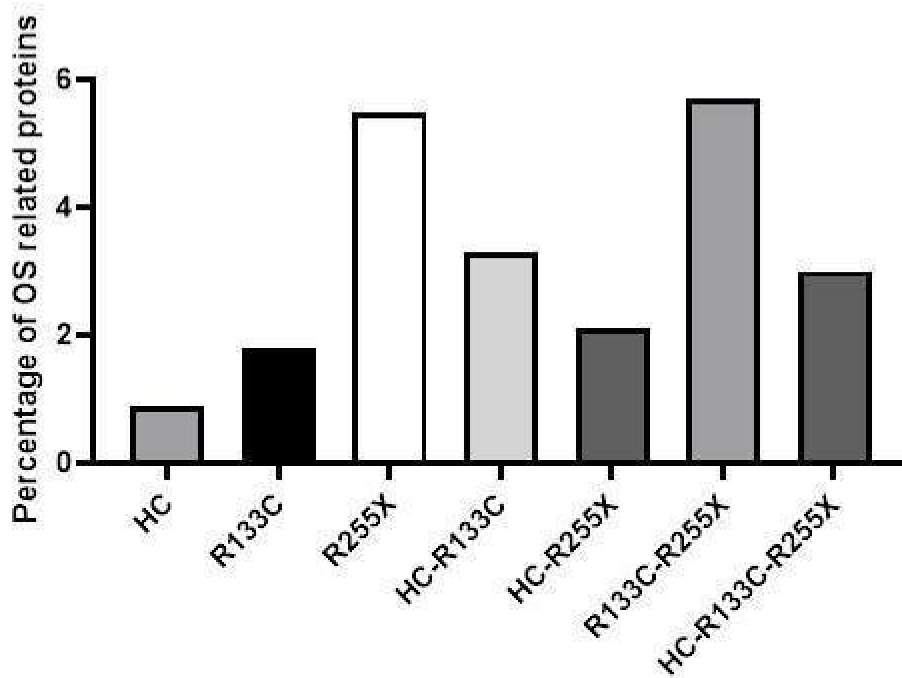

Fig.7 Percentage of OS proteins present in each fibroblast's subgroup.

Due to distinct responses in proteins associated with OS among subgroups, we initiated an investigation upon differential protein expressions. First, we compared proteome profiles between HC and each fibroblast sample from individuals with RTT.

Notably, an important finding emerged: Superoxide dismutase SOD1 (SOD1 UniProt P00441) was notably absent in all RTT fibroblasts. SOD1, a key player in maintaining cellular redox homeostasis, has been reported to be implicated in various diseases [57]. The trend in SOD1 protein levels aligned with previously described results on ROS and 4HNE (Fig.1 and Fig.2).

The presence/absence discrepancy of SOD1 in HC samples compared with RTTs suggests a distinct molecular defense mechanism against OS. This prompted us to examine deeper and investigating differentially expressed OS-related proteins between R133C and R255X RTT fibroblasts, aiming to correlate RTT genotype with OS phenotype.

Among pathways implicated in OS, NRF2 has been identified as a key player in tissue defense against OS in various pathologies, including RTT [67, 68]. However, little is known about the correlation between RTT and NRF2 downstream target genes such as NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1 UniProt P15559), a crucial player in OS responses. Interestingly, bioinformatics analysis revealed the presence of NQO1 only in the intersection of HC-R133C. Conversely, in the unique subset R255X, RAC-alpha serine/threonine-protein kinase AKT1 (AKT1 Uniprot P31749) was present, attesting a first difference between the samples bearing different mutations.

Another protein found exclusively in the R255X subset is Communication network factor 1 CCN1 (CCN1 Uniprot O00622). CCN1, induced under oxidative exposure, has been linked to increased ROS levels and the C-Jun/CCN1 axis in human dermal fibroblasts [46]. Moreover, CCN1 is involved in reducing extracellular matrix (ECM) deposition in fibroblasts during wound healing, suggesting a contrasting correlation where elevated CCN1 levels are associated with increased senescence and a simultaneous decrease in NRF2 activation [69]. Given that CCN1 was exclusively present in RTT fibroblasts with nonsense *MECP2* mutation, it suggests the activation of different pathways compared to the R133C missense mutation. Table 3 summarized the proteins present/absent in the different samples, evidenced from the qualitative proteomic analysis.

UniProt Accession	***UniProt ID/NAME***	***Gene name***	***R133C presence***	***R255X presence***	***HC presence***
P00441	SODC_HUMAN Superoxide dismutase [Cu-Zn]	SOD1			X
P15559	NQO1_HUMAN NAD(P)H dehydrogenase [quinone] 1	NQO1	X		X
O00622	CCN1_HUMAN CCN family member 1	CCN1		X	
P31749	AKT1_HUMAN RAC-alpha serine/threonine-protein kinase	AKT1		X	

Table 3. Qualitative analysis of proteins extrapolated by presence/absence of Venn diagram, among HC, R133C and R255X fibroblasts' subgroups.

Regarding common proteins between HC and RTT patient subgroups, a comprehensive investigation using statistical analyses, including volcano plots, was performed to elucidate similarities and differences in protein expression. A relative quantitative differential analysis among triplicates of each sample was executed, focusing on up/down regulation between each RTT subgroup. Before studying differences among RTT fibroblast proteins, a volcano plot analysis was initially conducted to show the distribution of differentially expressed proteins among RTT fibroblast subgroups and HCsamples (Fig.8).

Remarkably, a consistent trend was observed for the protein Plasminogen activator inhibitor 1 PAI-1 (UniProt P05121), which showed up-regulation in all RTT fibroblasts compared to HC. PAI-1, is another protein involved in the ECM complex and NRF2 activation, and it plays crucial roles in ECM remodeling. Recent studies have indicated that in fibroblasts, NRF2 activation is implicated in the

production and deposition of ECM, affecting the expression of proteins like PAI-1 [70, 71] (See Fig.8 and 9 and Table 4).

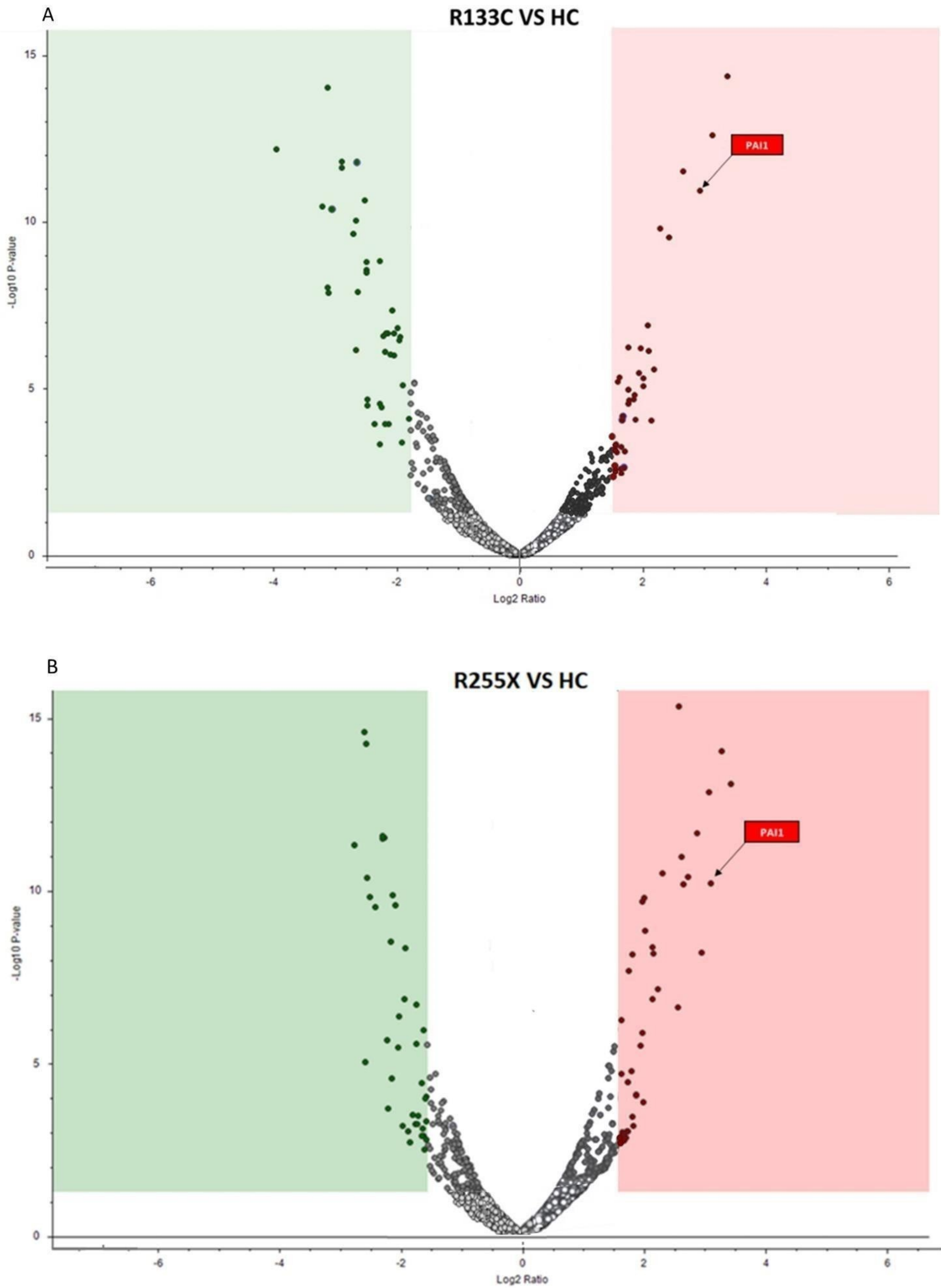

Fig.8. Graphical representation of Volcano plots analysis. Proteins that were differentially expressed among RTT patients' subgroups and between them and HC common dataset. P value <0.05. The green colored proteins were found

down-regulated in all RTT in comparison with the HC, while the red colored ones were up-regulated proteins (like PAI-1) in all RTT samples in each Volcano Plot graph.

Among proteins implicated in OS that exhibited varying regulation within the RTT subgroup, it was observed that the expression level of Quinone oxidoreductase-like protein 1 (QOH-1 UniProtO95825) was higher in R255X compared to R133C. QOH-1 is crucial in oxidoreductase activity [71],analogous to the NQO1 protein. However, despite the similar role of NQO1 and QOH-1 in oxidoreductase molecular function [72], they demonstrated opposing trends between the two distinct*MECP2* gene mutations.

Another protein mirroring the trend observed in QOH-1 was Microsomal Glutathione S-transferase 1 MGST1 protein (MGST1 UniProt P10620), which exhibited up-regulation in R255X fibroblasts and down-regulation in the R133C cells (see Fig.9 and Table 4). Recent studies have highlighted MGST1 as one of the antioxidant transcription targets regulated by NRF2 [73]. MGST1 is also involved in reduced glutathione conjugation [72] and contributes to cellular defense against OS damage [74].

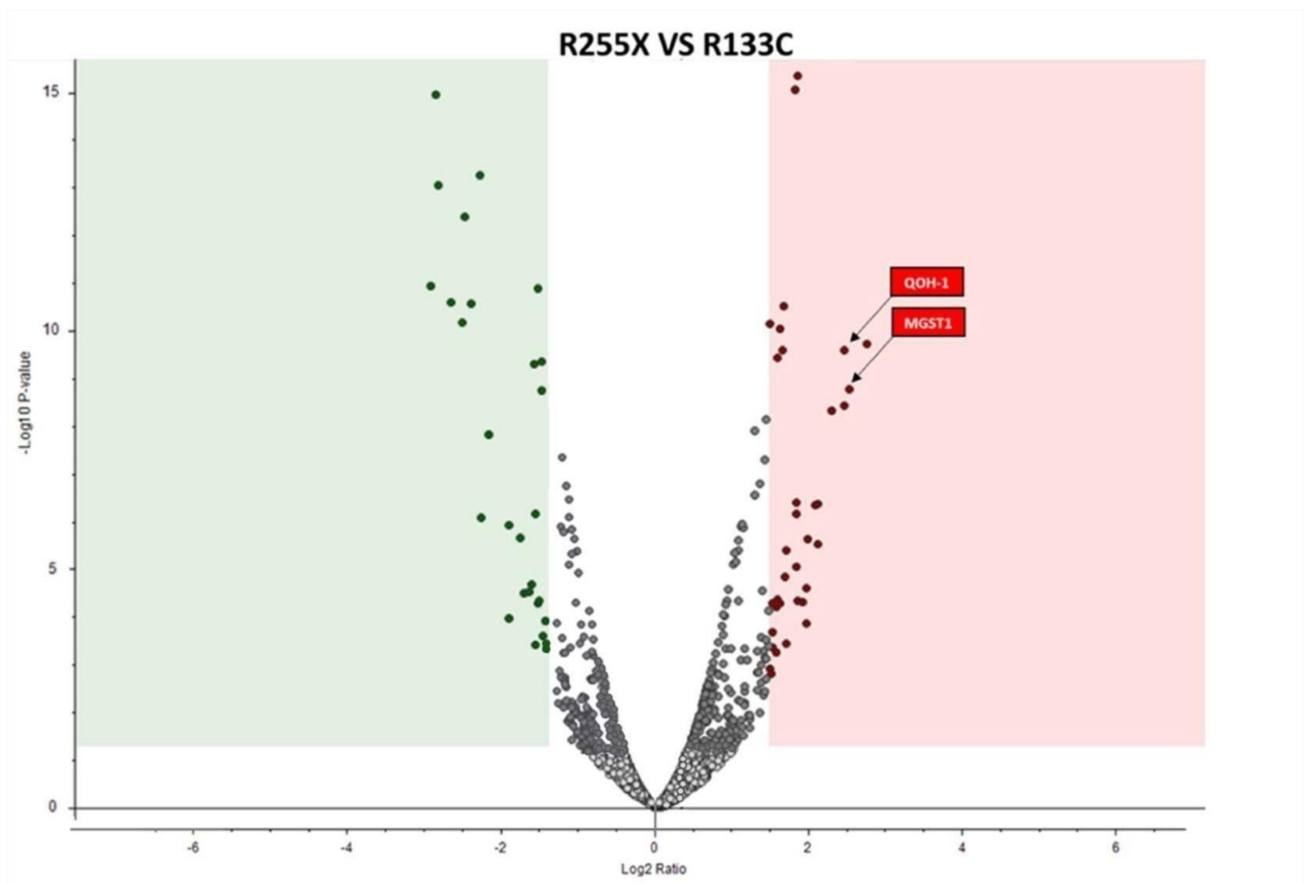

Fig.9. Graphical representation of Volcano plot analysis. Proteins that were differentially expressed among RTT patients' subgroups and between them and HC common dataset. P value <0.05. The green colored proteins were found down-

regulated in R255X in comparison with R133C, while the red colored ones were up regulated in R255X in comparison with R133C (like QOH-1 and MGST1).

UniProt accession	*FDR*	*UniProt ID/Name*	*Gene*	*Fibroblasts' subgroups*	*Up/Down regulation*
P05121	<0.05	PAI1_HUMAN Plasminogen activator inhibitor 1	SERPINE 1	R133C VS HC	Down in HC
P05121	<0.05	PAI1_HUMAN Plasminogen activator inhibitor 1	SERPINE 1	R255X VS HC	Down in HC
O95825	<0.05	QOH-1_HUMAN Quinone oxidoreductase-like protein 1	CRYZL1	R255X VS R133C	Up in R255X
P10620	<0.05	MGST1_HUMAN Microsomal glutathione S-transferase 1	MGST1	R255X VS R133C	Up in R255X

Table 4. List of proteins extrapolated by volcano plot and resulted to be up or down regulated among HC, R133C and R255X samples.

Summarizing, the proteomics analysis revealed the absence of SOD1 in all RTT fibroblast samples, indicating an abnormal molecular response to OS. Conversely, PAI-1 was up-regulated in all RTT samples compared to HC, suggesting NRF2 activation with modified ECM. Notably, each RTT subgroup exhibited distinct protein expressions related to downstream NRF2 activation and its consequences.

NQO1 was exclusively present in R133C, while CCN1 and AKT1 were detected only in R255X fibroblasts. Additionally, MGST1 and QOH-1 proteins were up-regulated in R255X. The varied regulation of these proteins across RTT fibroblasts with different *MECP2* gene mutations indicates the activation of diverse defense mechanisms against OS.

5.3 Other OS pathways involved in RTT from Proteomics results.

From proteomics studies, other OS pathways were found to have possible aberrant activation in RTT. Actually, among RTT fibroblasts with distinct *MECP2* mutations, differentially expressed proteins involved in mitochondria structures and functions, ubiquitin proteasomal and C-Jun molecular pathways were found.

5.4 Structural and mitochondrial proteins

The existing literature provides valuable insights into the connection with autism spectrum disorders (ASDs) such as RTT and mitochondrial dysfunction, which classifies them as part of "mitochondrial diseases" [63, 75].

The role of mitochondria in biochemical processes is pivotal, as they are involved in ATP production, amino acid biosynthesis, and ROS production regulation [75].

Moreover, respiratory chain dysfunctions, which can occur due to genetic mutations or environmental factors, can cause the reduction of ATP synthesis. As a result, the electron leakage from the electron transport chain increases, leading to elevated ROS and reactive nitrogen species (RNS) production. The excessive accumulation of these reactive species overwhelms the cellular antioxidant defense mechanisms, resulting in OS, triggering to specific responses from antioxidant enzymes [75].

These findings emphasize the critical role of mitochondria in the context of disorders like RTT. The interplay between mitochondrial dysfunction, impaired ATP synthesis, increased ROS production, and subsequent OS provides a potential step for further research and therapeutic interventions. Understanding these complex mechanisms can shed light on the pathophysiology of RTT, leading to improved diagnostic approaches and targeted treatments for affected individuals.

Investigating proteomics data regarding proteins involved in mitochondrial functions, we obtained a list of proteins present only in RTT samples and not in HC, that is reported in Table 5.

Uniprot Accession	*Uniprot ID/Name*	*Gene*	*R133C presence*	*R255X presence*	*HC presence*	*Up/Down regulation*
A6NCE7	MP3B2_HUMAN Microtubule-associated proteins 1A/1B light chain 3 beta 2	MAP1LC3B2		X		
P04179	SODM_HUMAN Superoxide dismutase [Mn], mitochondrial	SOD2				Up in R255X
P10620	MGST1_HUMAN Microsomal glutathione S-transferase 1	MGST1				Up in R255X
Q8TED1	GPX8_HUMAN Probable glutathione peroxidase 8	GPX8	X	X		
Q92626	PXDN_HUMAN Peroxidasin homolog	PXDN	X	X		
Q15067	ACOX1_HUMAN	ACOX1	X	X		

	Peroxisomal acyl-coenzyme A oxidase 1				
O43920	NDUS5_HUMAN NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	NDUFS5	X		
O43674	NDUB5_HUMAN NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	NDUFB5	X		
P17568	NDUB7_HUMAN NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	NDUFB7	X	X	
Q02218	ODO1_HUMAN 2-oxoglutarate dehydrogenase complex component E1	OGDH	X	X	
O14949	QCR8_HUMAN Cytochrome b-c1 complex subunit 8	UQCRQ	X		
P09669	COX6C_HUMAN Cytochrome c oxidase subunit 6C	COX6C	X		
Q9NUQ8	ABCF3_HUMAN ATP-binding cassette sub-family F member 3	ABCF3	X		
Q9H2U1	DHX36_HUMAN ATP-dependent DNA/RNA helicase DHX36	DHX36	X		
Q86WA8	LONP2_HUMAN Lon protease homolog 2, peroxisomal	LONP	X	X	
Q8WVF1	OSCP1_HUMAN Protein OSCP1	OSCP	X	X	
P05141	ADT2_HUMAN ADP/ATP translocase 2	ANT2	X	X	

Table 5: Mitochondrial proteins in RTT genotype/phenotype correlation

Some of these proteins have been reported also in the current literature. Intriguingly, in Cicaloni et Al. 2020, several proteins located in mitochondrial subunits and/or which have functional roles regarding mitochondrial bioactivities were found in RTT samples Here, we reported the same subunits and functions present only in all RTT samples, able to reduce ROS accumulation inside thecells, protecting them from OS damage [76]: probable glutathione peroxidase 8 (GPX8 UniProt

Q8TED1), peroxidasin homolog (PXDN UniProt Q92626) and peroxisomal acyl-coenzyme A oxidase 1 (ACOX1 UniProt Q15067). Another important aspect is the mitochondrial energy transduction, which is mainly controlled by NAD+. ATP synthesis level is modulated by NAD+/NADH ratio, going to activate NAD+ target enzymes modifying proteins at post-translational level [77].

Interestingly, we found two proteins placed at the inner mitochondrial membrane subunit and NADH functional role: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7 (NDUFB7 UniProt P17568) and 2-oxoglutarate dehydrogenase complex component E1 (OGDH UniProt Q02218) both present only in RTT samples.

About the inner mitochondrial membrane, it functions like a barrier for small molecules between the cytosol and the matrix, controlling proton gradient which is fundamental to oxidative phosphorylation [78]. NADH functional role is focused on energy reactions. NADH coenzymes are involved in electron carrying properties of pyridine nucleotides, modulating energy production [77].

Contextually, mitochondrial ATP-dependent protease Lon (LONP UniProt Q86WA8), ATP synthase subunit O, mitochondrial (OSCP UniProt Q8WVF1) and ADP/ATP translocase 2 (ANT2 UniProt P05141) were found in all RTT samples. LONP2 is an ATP-dependent serine protease and modulate gene expression and genome integrity in mitochondria [72]. OSCP is a Mitochondrial membrane ATP synthase, and its function is focused on ATP generation starting from ADP, especially when in the membrane there is a proton gradient which is produced by electron transport complexes of the respiratory chain [72]. The last, but not the least, is ANT2, an ADP: ATP antiporter that controls ADP import for ATP synthesis into the mitochondrial matrix and ATP export outside of the cell [72]. All the proteins mentioned above seems to confirm the aberrant response in RTT when compared to HC.

In this work of thesis, we are mainly interested in discovering proteins differentially expressed among RTT patients having different *MECP2* mutations. One example is Superoxide dismutase [Mn] (SOD2) that is upregulated in R255X rather than R133C. SOD2 or MnSOD is a mitochondrial protein placed in the mitochondrial matrix [72] and it catalyzes O_2 (produced by electron transport chain) into H_2O_2, leading to O_2 production in mitochondrial matrix and in the intermembrane space, detoxifying mtROS, [79] and controlling ROS expression [80].

In literature, it is reported that, when SOD2 has aberrant activity and expression, it could be a consequence of several diseases [80], and that enhanced level of ROS can be facilitated by SOD2, in turn increasing mitochondrial hydrogen peroxide (mtH_2O_2) [81].

Qualitative proteomics analysis showed that the proteins NADH dehydrogenase [ubiquinone] iron-sulfur protein 5 NDUFS5, and NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5 NDUFB5 were found only in the intersection R133C. Those proteins are reported to have a function

like accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) [72] and they are involved in both inner mitochondrial membrane and NADH subunits' functions.

Moreover, in RTT fibroblasts samples were present several proteins involved in structure and regulation of cytochrome C (Cytc) (see Table 5).
Cytc is a radical scavenger, taking place in the inner-membrane space, playing fundamental roles in mitochondrial electron transport, functioning like a hydrogen peroxide scavenger [82].
Specifically, the Cytochrome b-c1 complex subunit 6 UQCRQ and Cytochrome c oxidase subunit 6C COX6C were found only in R133C *MECP2* gene mutation. The first one is a ubiquitinol-cytochrome c oxidoreductase in transmembrane complex that takes part to the mitochondrial electron transport chain, fundamental for oxidative phosphorylation [72]. The latter one performs oxidative phosphorylation too: it is the last enzyme in the mitochondrial electron transport, and it is a component of the cytochrome c oxidase [72].
On the other hand, the microtubule-associated proteins 1A/1B light chain 3 beta 2 MAP1LC3B2 (or LCRB) was present only in R255X cells. MAP1LC3B2 is a ubiquitin-like modifier involved in autophagosomal vacuoles formation and in mitophagy, involved in regulation of mitochondrial quantity and quality, ameliorating cellular energy requirements, and preventing the excess of ROS production [72].
Examining proteins associated with mitochondrial function provides insights into the molecular mechanisms underlying RTT. The differences in protein expression among distinct RTT fibroblasts can indicate dysregulation or alterations in specific pathways, offering indications about the molecular pathophysiology of the disorder. Actually, the aim of this study was to detect differences between R133C and R255X cells. By identifying specific proteins associated with different *MECP2* mutations, it was performed the investigation on how these genetic variations contribute to molecularmechanisms consequences.

Proteins involved in mitochondrial functions, energy transduction, and oxidative stress responses are crucial for maintaining mitochondrial health. Analyzing these proteins helps to assess the impact of *MECP2* mutations on mitochondrial functionality in different RTT subtypes, such as R133C and R255X. As a matter of fact, RTT cells differ among them in terms of presence and up/down regulation of differentially expressed proteins involved in mitochondrial structures and functions. In particular, is present an elevated number of proteins found only in R133C (six proteins) compared to R255X cells, in which it was found only one (MAP1LC3B2) as mentioned before. These findings suggest a maintained mitochondrial activity in R133C compared to R255X, maybe due to the more severity degree of those patients with non-sense *MECP2* mutation. Moreover, among the common proteins between the two RTT subtypes, SOD2 and MGST1 were both up-regulated in R255X cells. Therefore, all these results indicate that a different activation and response in mitochondrial structure and functions in RTT cells with distinct *MECP2* mutations could be present, confirming the intricatenature of such syndrome and the importance to correlate RTT genotype to phenotype to shed light onpossible molecular mechanisms of response.

5.5 Ubiquitin-proteasome pathway (UPP)

Among the proteins differentially expressed, there are some of them that are related to the Ubiquitin Proteasome Pathways (UPP). UPP is a fundamental cellular mechanism responsible for the degradation of proteins, and the regulation of various cellular processes. It involves the covalent attachment of ubiquitin molecules to specific proteins, marking them for subsequent degradation by the proteasome, a large and complex multi-subunit structure [82].

Intriguingly, investigations into the UPP have revealed a remarkable connection between its activity and the cellular redox status. High OS levels, characterized by an excessive accumulation of ROS, can significantly impair UPP function, leading to its inactivation. This disruption has far-reaching implications for cellular health and may contribute to various diseases and conditions.

In recent years, researchers have turned their attention to the fascinating crosstalk between the ubiquitin-proteasome system (UPS) and numerous mitochondrial diseases, including RTT [63, 31].

Actually, reduced ATP mitochondrial levels compromise the energy supply for protein ubiquitination and proteasome activity. Consequently, the interconnection between the UPP and mitochondria becomes apparent, as disruptions in one system can have effects on the other [31].

Among our data, it is interesting to note the presence of Ubiquitin-like modifier-activating enzyme 5 UBA5, Small ubiquitin-related modifier 2 SUMO2, and Ubiquitin-conjugating enzyme E2 L3 UBE2L3 (see Table 6) for R133C.

The presence of these proteins suggests a significant defensive response against OS by the UPP in R133C mutation, indicating the complex nature of genotype and molecular mechanismsconsequences (phenotype). In agreement with these results, Sbardella et al., 2020, showed that in RTT fibroblasts patients having non-sense *MECP2* gene mutations such as R255X, is present a reduced and defective proteasome biogenesis [32].

UniProt Accession	*UniProt ID/Name*	*Gene*	*R133C presence*	*R255X presence*	*HC presence*	*Up/Down regulation*
Q9GZZ9	UBA5_HUMAN Ubiquitin-like modifier-activating enzyme 5	UBA5	X			
P61956	SUMO2_HUMAN Small ubiquitin-related modifier 2	SUMO2	X			
P68036	UB2L3_HUMAN Ubiquitin-conjugating enzyme E2 L3	UBE2L3	X			
P25685	DNJB1_HUMAN DnaJ homolog subfamily B member 1	DNAJB1	X			

Table 6: proteins belonging to UPP in RTT samples bearing different mutations.

5.6 C-Jun pathway response against OS.

Regarding molecular mechanisms involved in response against OS and found to be present only in RTT cells OS, the C-Jun N-terminal kinase (JNK) pathway is one of them [83, 84, 85].
This pathway plays a crucial role in cellular responses to ROS-induced stress, inducing stress-responsive genes, which participate in cellular repair and survival mechanisms [84]. Moreover, recent findings showed that JNK activation is present in RTT mouse samples. Deeply, the specific JNK inhibitor, D-JNKI1 is able to recover the synaptic dysfunction [86].
As reported by data analysis, it is interesting to note that only in R255X cells are present proteins particularly involved in C-Jun pathway: cellular communication network factor-1 (CCN1 UniProt 0OO622), lyox protein-lysisn 6 oxidase (LOX UniProt P28300) and COP9 signalosome complex subunit 5 (COPS5 UniProt Q92905) (See Table 6) [87]. The first one is a cellular communication network factor involved in cell proliferation, chemotaxis, angiogenesis, and cell adhesion. Especially

in skin fibroblasts, it can up-regulate genes' expression implicated in angiogenesis and in inflammation [72].

In details, in Qin et al. they demonstrated that in skin fibroblasts ROS enhanced C-Jun, consequently increasing C-Jun site of the CCN1 promoter [88, 89]. Regarding the Protein-lysine 6-oxidase LOX, it is involved in post-translational oxidative deamination of peptidyl lysine residues, and it is increased by C-Jun pathway (as shown by Laczko and Csiszar et al.) [89]. COPS5 is involved in various cellular and developmental processes [86] and it regulates the phosphorylation of several pathways such as C-Jun/JUN [87].

UniProt Accession	***UniProt ID/Name***	***Gene***	***R133C presence***	***R255X presence***	***HC presence***	***Up/Down regulation***
O00622	CCN1_HUMAN CCN family member 1	CCN1		X		
P28300	LYOX_HUMAN Protein-lysine 6-oxidase	LOX		X		
Q92905	CSN5_HUMAN COP9 signalosome complex subunit 5	COPS5		X		

Table 6: C-Jun and redox proteins involved in RTT genotype/phenotype correlation.

6. Molecular biology assays

Among all the proteomic data, described above, we chose some of them and we performed molecular biologyassay, such as Western blot and RT-PCR analysis, in order to confirm proteomics observation.

6.1 Western Blot Analysis: NQO1 in HC and in RTT fibroblasts

Since NQO1 protein was absent in non-sense *MECP2* gene mutations, Western blot assay was conducted to confirm the different expression of NQO1 between the two different mutations: R255X and R133C.

As shown in Fig.10, NQO1 trend was confirmed in a significant manner.

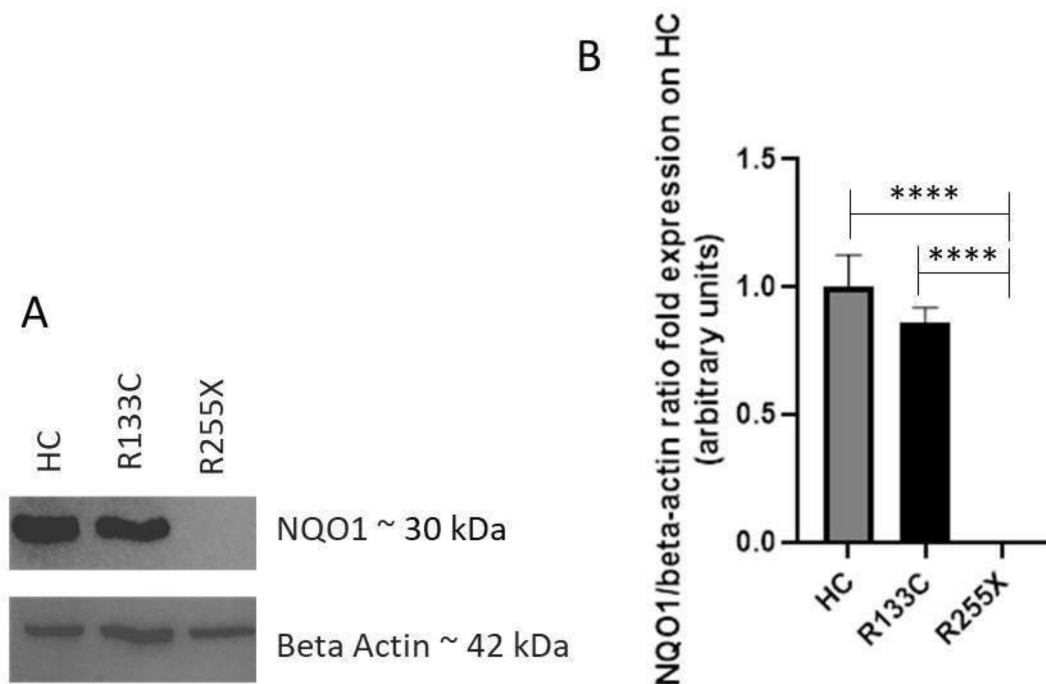

Fig.10 NQO1 levels in HC and in RTT patients. Top panel (A) shows Western blot. The signals of NQO1 protein level were determined by densitometric analysis of the scanned images. All samples were normalized per protein (15 µg). Averages of the values from all the samples are expressed in arbitrary units, bottom panel (B). Tukey's multiple comparisons test. ****P Value<0,0001.

6.2 RT-PCR: NQO1 and HO1 in HC and in RTT fibroblasts

With the aim to further study and better understand the presence or absence of the NQO1 protein and the molecular mechanisms in distinct RTT patients, we conducted a real-time RT-PCR analysis. This analysis enabled us to scrutinize the transcriptional regulation of the *NQO1* gene both with and without H_2O_2 treatment at varying time points (1 hour and 24 hours).

Notably, at the 24-hour time point, the *NQO1* gene expression in R133C RTT fibroblast samples was statistically significantly higher compared to both HC and R255X RTT fibroblast samples (no differences were discerned at 1 hour). Moreover, this trend persisted following H_2O_2 treatment, demonstrating a significant increase in *NQO1* gene expression in R133C as opposed to R255X and HC fibroblasts (Fig.11).

These findings suggest that the regulation of the *NQO1* gene is influenced by the specific *MECP2* mutation present in RTT patients, suggesting potential differences in oxidative molecular mechanisms responses based on the specific mutation. These results underline the difficulty of cellular responses to oxidative insults and highlight the potential role of NQO1 in changing the OS response in RTT.

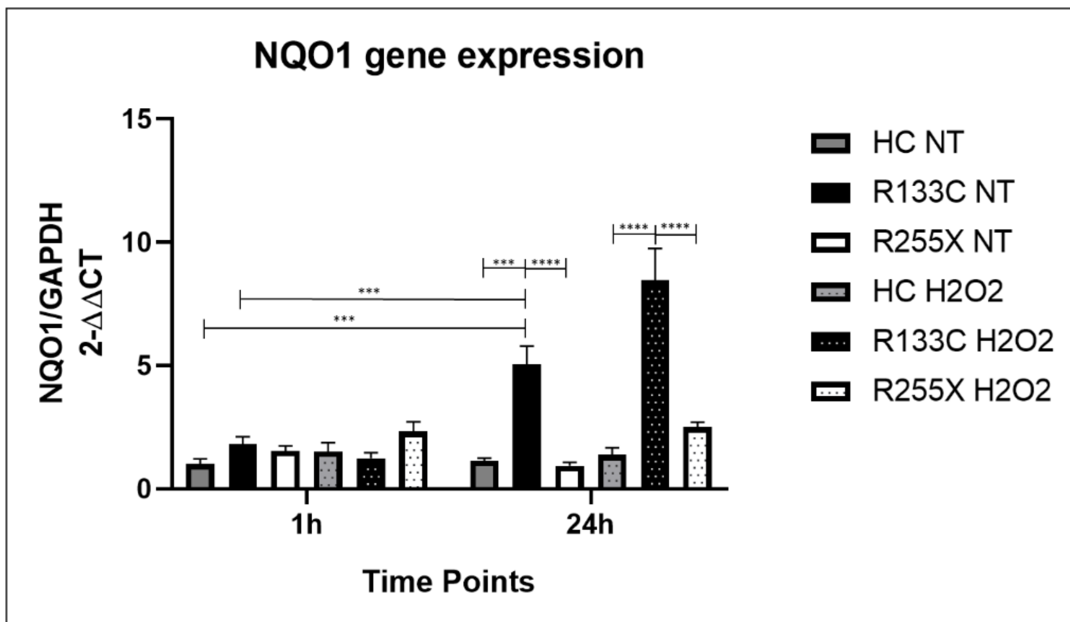

Fig.11. Transcriptional expression of NQO1 in HC and in RTT fibroblasts. Data were given as 2− ΔΔCt. Tukey's multiple comparisons test. *** P Value =0,0001. **** P Value <0,0001.

Heme oxygenase 1 (HO-1), as it is NQO1, is regulated in NRF2 pathway [89]. As shown in Fig.12, at 1 hour time point, in R133C there was a statistically significative increase in *HO-1* gene expression rather than R255X cells, regardless of the oxidant insult. In addition, this effect was more evident at 24 hours' time point.

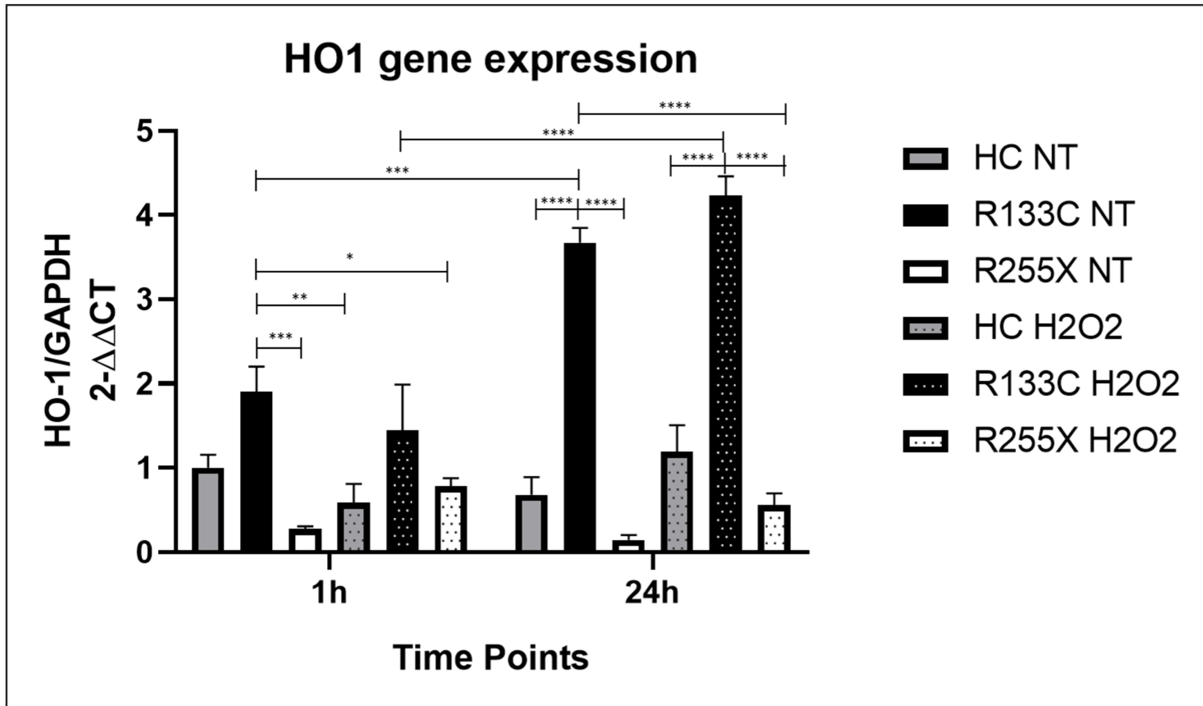

Fig.12. Transcriptional expression of HO-1 in HC and in RTT fibroblasts. Data were given as 2− ΔΔCt. Data were represented as mean ± SD. HC, control; RTT, RTT. Tukey's multiple comparisons test. * P Value =0,0186. ** P Value =0,053. *** P Value =0,0004. **** P Value <0,0001.

In summary, ss depicted in Fig. 13, OS represents an imbalance between the production of ROS and the body's ability to counteract their harmful effects. In conditions like RTT, this imbalance plays a crucial role inthe disease pathology.

The application of redox proteomic approaches enables researchers to go deeper into the identification of potential novel biomarkers associated with OS. This method allows for a more comprehensive investigation of the molecular mechanisms involved, shedding light on specific proteins and pathways that may serve as valuable indicators of OS in diseases such as RTT.

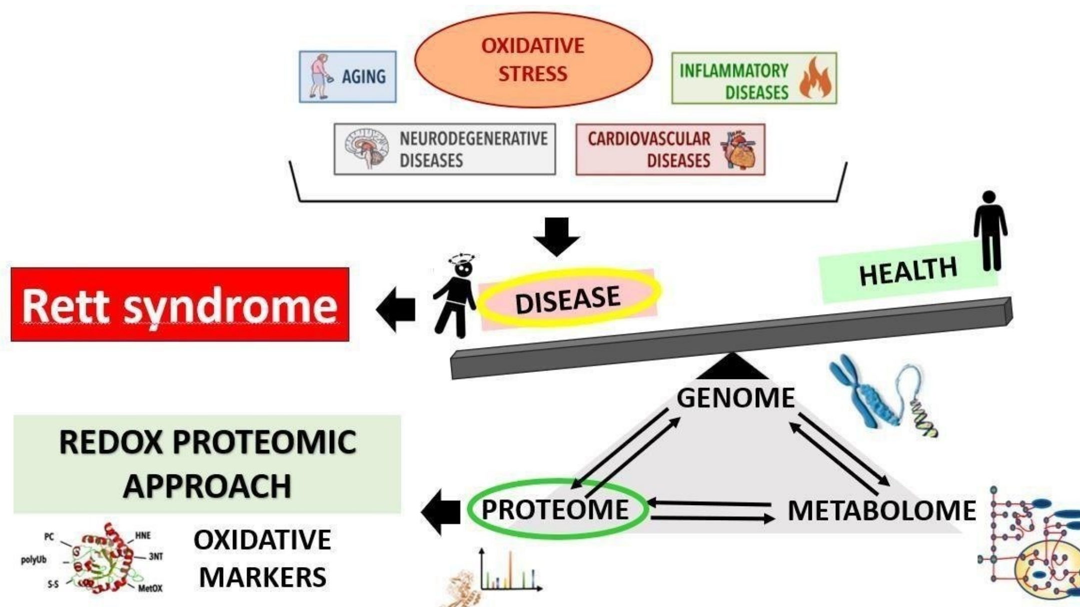

Fig.13 Redox proteomic approach in potential novel biomarker discovery.

As a matter of fact, RTT molecular mechanisms are associated to OS, mitochondrial damage, inflammation, and immune response [55, 56, 91]. However, it is not understood yet how all these molecular mechanisms and consequences of RTT are linked to specific *MECP2* mutations like R133C and R255X, (which account for about 70% of all mutations), leading to several phenotypic consequences with distinct severity's degree [54, 55, 91, 92].

Actually, clinical manifestations of this syndrome are very inconstant, due to mutation's type in the *MECP2* gene which display several clinical phenotypes with various severity's degrees, thus, it couldbe interesting to understand RTT phenotype and genotype connection [55, 56, 91, 93]. Studies performed by J. L. Neul et al., [22] and of Kyle, S. M. et al., [14] showed that the missense R133C mutation exhibited less severe phenotypes and clinical signs when compared to non-sense mutationssuch as R255X. In fact, R133C *MECP2* mutation usually causes the least severe clinical presentation,while the nonsense as R255X causes the most severe phenotypes [14] characterized by a compromised use of their hands, speech, motor functions and ambulation, respiratory dysfunction, breathing disturbances and scoliosis.

It is well known that different genotypes can significantly influence the consequences of disease. New 'omics' approaches like proteomics allow us to better understand molecular characterization of disorders. Thus, with the use of proteomics approach, we focused our analysis on OS response pathways, focusing on different protein expressions between RTT patients with distinct *MECP2* mutations, investigating on their molecular mechanisms' consequences, discovering potential novel biomarkers and paving the way for precision medicine.

In the first part of this study, our experiments confirmed the presence of increased OS damage as shown by ROS and 4HNE western blot assays. While altered redox homeostasis difference was foundamong RTT samples and HC, no significative OS differences were found among RTTs. An in-depthanalysis based on proteomics approach was performed in order to investigate OS molecular mechanisms underlying differences between RTT genotypes, through a differential protein expressions analysis.
Regarding differentially expressed proteins among RTT cells, the obtained results suggest a connection between the mutations and the proteins' expression, providing valuable insights into the underlying molecular mechanisms of this disorder, such as: NRF2 pathway, mitochondrial structuresand functions, C-Jun and UPS molecular mechanisms.

The results from the analysis of differentially expressed proteins among RTT cells reveal a compelling association between specific mutations and the observed alterations in protein expression.These findings offer valuable insights into the intricate molecular mechanisms underlying this disorder. Notably, the differential protein expressions indicate a remarkable involvement of the NRF2 pathway, of which the transcription factor NRF2 is well studied in RTT.
Actually, this pathway, which is associated with cellular defense against OS, may play a pivotal role in the response to molecular aberrations in RTT cells. The oxidoreductase enzyme NQO1 (a target gene of NRF2) was present only in R133C and inHC cells (confirmed by western blot assay), while AKT1 was found only in R255X. A possible mechanism that could explain such proteins' trends could be due to AKT1 protein involved in the mechanism of NQO1 degradation as demonstrated by the study conducted in another neurodegenerative disorder: Parkinson Disease (PD). In this study Luo et Al., 2019 demonstrated thatNQO1 was present in the early and intermediate stages in which patients exhibited less severe clinicalsigns compared to the end-stage, in which it was absent. They showed that AKT phosphorylates NQO1 at T128 residues, thus triggering its polyubiquitination and proteasomal degradation, inhibiting its anti-oxidative effects in PD [94]. Therefore, the presence of AKT1 could maybe accountfor the absence of the NQO1 protein in R255X. Moreover, QOH-1 (with the same oxidoreductase function as NQO1) was up-regulated in R255X. The opposite expression trend of NQO1 and QOH- 1 proteins having the same molecular function could be due to a different defensive mechanism between the different RTT mutation or a possible compensatory effect among the two

enzymes. Another protein present only in R255X samples was the matricellular protein CCN1, which is involved in ECM deposition and can induce senescence in fibroblasts during wound healing, leading to a decrease in NRF2 activity [95]. In addition, under oxidative challenge, CCN1 plays a key role in increasing ROS levels [96] and C-Jun expression, which in turn enhances the expression of CCN1 itself [96]. About ECM regulation and NRF2 activity, PAI-1 was found to be up-regulated in all RTT samples rather than HC. This protein is involved in ECM deposition and NRF2 activation in fibroblasts, modulating ECM production and deposition [95]. On the contrary, MGST1 protein exhibited distinct patterns among RTT fibroblasts: it was up regulated in R255X cells. MGST1 can reduce glutathione conjugation. Moreover, recent studies have further revealed that MGST1 is one ofthe antioxidant transcription targets of NRF2 [97].

Therefore, among RTT fibroblasts with distinct *MECP2* mutations exhibited different proteins expressions involved in NRF2 pathway.

Summarizing, proteomics data revealed that SOD1 was not detected in all RTT samples, and it was present only in HC, suggesting a different redox defense mechanism. From literature it is well known that SOD1 is crucial in cellular redox homeostasis, and its dysregulation can lead to several diseases [24, 98]. On the other hand, PAI-1 was up-regulated in all RTT samples rather than HC indicating an NRF2 activation with modified ECM.

Intriguingly, each RTT subgroup presented different proteins expressions belonging to NRF2 downstream activation and consequences.

NQO1 was found only in R133C and in HC, while CCN1 and AKT1 were present only in R255X fibroblasts while MGST1 and QOH-1 proteins were up-regulated in R255X.

In Fig 14 the proteins trend among RTT samples are summarized.

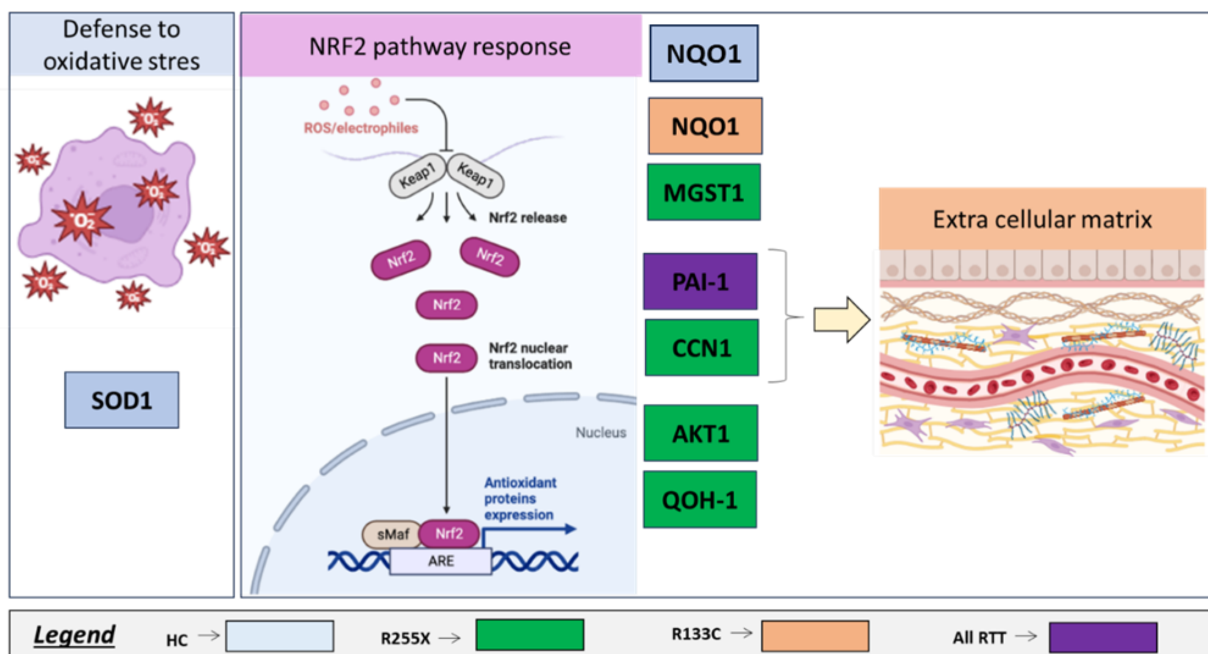

Fig. 14 NRF2 pathway response and ECM consequences in RTT genotype/phenotype correlation.

Moreover, the differential regulation of the *HO-1* gene in response to OS among the R133C and R255X *MECP2* gene mutations could help us to better understand the absence of NQO1 protein in the R133C mutation, since there might be differences in the activation or regulation of the NRF2 pathway in these specific RTT patient subgroups, leading to NQO1 and HO-1 cytoprotective enzymes variations.

To investigate whether protein expression of NQO1 could be modulated in response to an oxidant insult, RTT and HC fibroblasts were treated with H_2O_2. By western blot assay, we confirmed the previous proteomic analysis: NQO1 protein was present only in R133C *MECP2* gene mutation RTT patients and in HC and not in the nonsense R255X one, regardless of H_2O_2 stimulus.

To gain a deeper understanding of the mechanisms underlying NQO1 protein absence and presence in different RTT cells, a real-time RT-qPCR analysis was conducted. This analysis allowed us to examine the transcriptional regulation of the *NQO1* gene and determine whether the differences in protein expression were due to alterations at the mRNA level.

Interestingly, at the 24-hour time point, the R133C cells displayed higher *NQO1* expression when compared to the R255X mutation, suggesting a different response against OS between these two specific RTT cells groups over extended exposure to H_2O_2.

Additionally, these findings underline the dynamic nature of NQO1 in different *MECP2* gene mutations, highlighting cellular response complexity against oxidant insults and the potential role of this protein in modulating the OS in RTT.

Another target gene downstream of NRF2 is HO-1 [90]. To comprehend if the absence of NQO1 protein in certain *MECP2* gene mutations was influenced by aberrant regulation of the NRF2 pathway, it was performed gene expression analysis of *HO-1* after exposure to an oxidant insult (hydrogen peroxide) for different time periods (1 hour and 24 hours).

The results revealed that the *HO-1* gene was upregulated in the R133C cells exhibiting a more robust response to OS through the NRF2 pathway. It implies that there might be differences in the activation or regulation of the NRF2 pathway in these specific RTT fibroblasts groups, leading to NQO1 and HO-1 changes.

Concerning mitochondrial functions and structures, from literature it is evident the link between RTT and mitochondrial dysfunction, classifying it as part of "mitochondrial diseases" [63, 75]. As a matter of fact, our results suggest an unbalance of mitochondrial proteins present in all RTT samples, confirming that mitochondrial structures and functions are affected in RTT. Deeply, Cicaloni et al., 2020 demonstrated that in RTT patients was present a higher percentage of genes related to

mitochondria. In our study it is possible to note that some proteins are present only in R133C cells. On the other hand, the proteins SOD2, MGST1 and MAP1LC3B respectively up regulated and present in R255X cells. The mitochondrial protein SOD2 is in the mitochondrial matrix [72] and it leads to O_2 production, detoxifying [100] and controlling mtROS and ROS expressions [101].
When SOD2 shows activity dysregulation, it can be a consequence of several diseases [81, 99]; because enhanced level of ROS lead to increase mitochondrial hydrogen peroxide (mtH_2O_2) [102].
Regarding MGST1, it is in the endoplasmic reticulum and in the outer mitochondrial membrane, providing cellular defense against OS damage [72]; while MAP1LC3B2 is a ubiquitin-like modifier involved in mitophagy and in regulation of mitochondrial quantity and quality, enriching cellular energy requirements and preventing the excess of ROS production [72].
Regarding the UPS, it is responsible for the elimination of damaged or unwanted proteins. Dysregulation of this pathway can lead to abnormal proteins accumulation in cells. Interesting, proteins involved in this molecular mechanism were found to be present only in R133C cells and never in the R255X, suggesting a different defense mechanism based on RTT genotype.

Another pathway differentially regulated among RTT cells is the C-Jun. It is involved in several stress signals, including ROS, and its activation leads to stress-responsive genes transcription, able to participate in cellular repair and survival mechanisms. Moreover, this pathway is involved in cell proliferation, differentiation, and apoptosis, and alterations can impact cell signaling and functions [85]. The three proteins CCN1, LOX and COPS5, which have fundamental roles in C-Jun, were found to be present only in R255X cells, confirming a different molecular mechanism activation among distinct RTT genotype.

7.Conclusions

In conclusion, the present study offers significant insights into the intricate interplay between molecular mechanisms responding to OS and the correlation with different *MECP2* gene mutations. This investigation enhances our comprehension of the complex heterogeneity inherent in RTT.

The identified connections between OS responses and specific *MECP2* mutations not only contribute to unraveling the molecular intricacies of RTT but also hold promise for potential clinical applications.

The implications of these findings extend to the prospect of developing targeted therapies in the future. By understanding the unique molecular signatures associated with distinct *MECP2* gene mutations, there is potential for patient stratification, allowing for more precise and personalized approaches to treatment. These insights open avenues for the advancement of precision medicine strategies, offering hope for improved therapeutic outcomes and enhanced quality of life for individuals affected by RTT.

The thesis was an integral part of the work: **PASQUI**, Arianna, et al. *"A proteomic approach to investigate the role of the MECP2 gene mutation in Rett syndrome redox regulatory pathways."* Archives of Biochemistry and Biophysics, 2023, 109860.

8. References

1. NAITHANI, Nardeep, et al. Precision medicine: Concept and tools. medical journal armed forces india, 2021, 77.3: 249-257.
2. DANESE, Elisa; LIPPI, Giuseppe. Rare diseases: the paradox of an emerging challenge. Annals of Translational Medicine, 2018, 6.17.
3. GENIN, Emmanuelle; FEINGOLD, Josué; CLERGET-DARPOUX, Françoise. Identifying modifier genes of monogenic disease: strategies and difficulties. Human genetics, 2008, 124: 357-368.
4. TUKKER, Anke M., et al. The impact of environmental factors on monogenic mendelian diseases. Toxicological Sciences, 2021, 181.1: 3-12.
5. AKHOON, Neha. Precision medicine: a new paradigm in therapeutics. International journal of preventive medicine, 2021, 12.
6. BLAZER, Dan G., et al. (ed.). Genes, behavior, and the social environment: Moving beyond the nature/nurture debate. 2006.
7. AL-AMRANI, Safa, et al. Proteomics: Concepts and applications in human medicine. World Journal of Biological Chemistry, 2021, 12.5: 57.
8. CLISH, Clary B. Metabolomics: an emerging but powerful tool for precision medicine. Molecular Case Studies, 2015, 1.1: a000588.
9. SUPPLITT, Stanislaw, et al. Current achievements and applications of transcriptomics in personalized cancer medicine. International Journal of Molecular Sciences, 2021, 22.3: 1422.
10. RETT, Andreas. "Uber ein cerebral-atrophisches Syndrom bei Hyperammonamie." Wien Med Wochenschr 116 (1966): 723-726.
11. HAGBERG, Bengt, et al. "A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases." Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society 14.4 (1983): 471-479.
12. AMIR, Ruthie E., et al. "Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2." Nature genetics 23.2 (1999): 185-188..
13. KYLE, Stephanie M.; VASHI, Neeti; JUSTICE, Monica J. Rett syndrome: a neurological disorder with metabolic components. Open biology, 2018, 8.2: 170216. 2005; Xinhua et al. 2008.

14. STEPHANIE M. KYLE. ”Rett syndrome: a neurological disorder with metabolic components”.
15. DELCUVE, Geneviève P.; KHAN, Dilshad H.; DAVIE, James R. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clinical epigenetics, 2012, 4: 1-13.
16. OLYNIK, Brendan M.; RASTEGAR, Mojgan. The genetic and epigenetic journey of embryonic stem cells into mature neural cells. Frontiers in genetics, 2012, 3: 81.
17. LIYANAGE, Vichithra RB; RASTEGAR, Mojgan. Rett syndrome and MeCP2. Neuromolecular medicine, 2014, 16: 231-264.
18. YOUNG, Deidra J., et al. The diagnosis of autism in a female: could it be Rett syndrome?. European journal of pediatrics, 2008, 167: 661-669.
19. KATZ, David M., et al. Rett syndrome: crossing the threshold to clinical translation. Trends in neurosciences, 2016, 39.2: 100-113.
20. LOTAN, Meir, et al. Rett syndrome. A review with emphasis on clinical characteristics and intervention. The Scientific World Journal, 2006, 6: 1517-1541.
21. CICALONI, Vittoria, et al. Proteomic profiling reveals mitochondrial alterations in Rett syndrome. Free Radical Biology and Medicine, 2020, 155: 37-48.
22. AMIR, Ruthie E.; ZOGHBI, Huda Y. Rett syndrome: Methyl-CpG-binding protein 2 mutations and phenotype–genotype correlations. American journal of medical genetics, 2000, 97.2: 147-152.
23. NEUL, J. L., et al. Specific mutations in methyl-CpG-binding protein 2 confer different severity in Rett syndrome. Neurology, 2008, 70.16: 1313-1321.
24. LEONARD, Helen, et al. Patients with the R133C mutation: is their phenotype different from patients with Rett syndrome with other mutations?. Journal of Medical Genetics, 2003, 40.5: e52-e52.
25. DE FELICE, Claudio, et al. The role of oxidative stress in Rett syndrome: an overview. Annals of the new York Academy of Sciences, 2012, 1259.1: 121-135.
26. SHULYAKOVA, Natalya, et al. Mitochondrial dysfunction in the pathogenesis of Rett syndrome: implications for mitochondria-targeted therapies. Frontiers in cellular neuroscience, 2017, 11: 58.
27. MITTAL, Manish, et al. Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling, 2014, 20.7: 1126-1167.

28. Sticozzi, Claudia, et al. "Scavenger receptor B1 post-translational modifications in Rett syndrome." FEBS letters 587.14 (2013): 2199-2204.
29. FERRARA, Francesca, et al. Redox regulation of cutaneous inflammasome by ozone exposure. Free Radical Biology and Medicine, 2020, 152: 561-570.
30. CRIVELLARI, Ilaria, et al. Nrf2-pathway alteration in RTT syndrome. Free Radical Biology and Medicine, 2017, 108: S63.
31. SISLIYAN, Christina, et al. Recruitment of Nrf2-dependent adaptive homeostatic redox responses in Rett syndrome. Free Radical Biology and Medicine, 2018, 128: S134.
32. KODROŃ, Agata, et al. The ubiquitin-proteasome system and its crosstalk with mitochondria as therapeutic targets in medicine. Pharmacological Research, 2021, 163: 105248.
33. SBARDELLA, Diego, et al. Defective proteasome biogenesis into skin fibroblasts isolated from Rett syndrome subjects with MeCP2 non-sense mutations. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2020, 1866.7: 165793.
34. ZHANG, Yuxing, et al. Redox control of the survival of healthy and diseased cells. Antioxidants & redox signaling, 2011, 15.11: 2867-2908.
35. MUSI, Clara Alice, et al. JNK signaling provides a novel therapeutic target for Rett syndrome. BMC biology, 2021, 19.1: 1-20.
36. GONZALES, Michael L.; LASALLE, Janine M. The role of MeCP2 in brain development and neurodevelopmental disorders. Current psychiatry reports, 2010, 12: 127-134.
37. FU, Cary, et al. Multisystem comorbidities in classic Rett syndrome: a scoping review. BMJ paediatrics open, 2020, 4.1.
38. ZUBAIR, Muhammad, et al. Proteomics approaches: A review regarding an importance of proteome analyses in understanding the pathogens and diseases. Frontiers in Veterinary Science, 2022, 9: 1079359.
39. AL-AMRANI, Safa, et al. Proteomics: Concepts and applications in human medicine. World Journal of Biological Chemistry, 2021, 12.5: 57.
40. DUARTE, Trevor T.; SPENCER, Charles T. Personalized proteomics: the future of precision medicine. Proteomes, 2016, 4.4: 29.
41. KATSENOU, Angeliki, et al. Using Proteomics Data to Identify Personalized Treatments in Multiple Myeloma: A Machine Learning Approach. International Journal of Molecular Sciences, 2023, 24.21: 15570.
42. AL-AMRANI, Safa, et al. Proteomics: Concepts and applications in human medicine. World Journal of Biological Chemistry, 2021, 12.5: 57.

43. KARPIEVITCH, Yuliya V., et al. Liquid chromatography mass spectrometry-based proteomics: biological and technological aspects. The annals of applied statistics, 2010, 4.4: 1797.
44. WIŚNIEWSKI, Jacek R. Filter-aided sample preparation for proteome analysis. Microbial proteomics: methods and protocols, 2018, 3-10.
45. ABDALLAH, Cosette, et al. Gel-based and gel-free quantitative proteomics approaches at a glance. International journal of plant genomics, 2012, 2012.
46. YU, Fang; QIU, F.; MEZA, J. Design and statistical analysis of mass-spectrometry-based quantitative proteomics data. In: Proteomic profiling and analytical chemistry. Elsevier, 2016. p. 211-237.
47. LARANCE, Mark; LAMOND, Angus I. Multidimensional proteomics for cell biology. Nature reviews Molecular cell biology, 2015, 16.5: 269-280.
48. NEUL, JEFFREY L., et al. "Rett syndrome: revised diagnostic criteria and nomenclature." Annals of neurology 68.6 (2010): 944-950.
49. HERNÁNDEZ-GARCÍA, David, et al. Reactive oxygen species: a radical role in development?. Free Radical Biology and Medicine, 2010, 49.2: 130-143.
50. MILENKOVIC, Jelena, et al. Mechanisms of plasminogen activator inhibitor 1 action in stromal remodeling and related diseases. Biomedical Papers of the Medical Faculty of Palacky University in Olomouc, 2017, 161.4.
51. COUDERT, Elisabeth, et al. Annotation of biologically relevant ligands in UniProtKB using ChEBI. Bioinformatics, 2023, 39.1: btac793.
52. FONSEKA, Pamali, et al. "FunRich enables enrichment analysis of OMICs datasets." Journal of molecular biology 433.11 (2021): 166747.
53. ASHBURNER, Michael, et al. "Gene ontology: tool for the unification of biology." Nature genetics 25.1 (2000): 25-29. Ashburner, Michael, et al. "Gene ontology: tool for the unification of biology." Nature genetics 25.1 (2000): 25-29.
54. YON RHEE, Seung, et al. "Use and misuse of the gene ontology annotations." Nature Reviews Genetics 9.7 (2008): 509-515.
55. PECORELLI, Alessandra, et al. "Proteomic analysis of 4-hydroxynonenal and nitrotyrosine modified proteins in RTT fibroblasts." The international journal of biochemistry & cell biology 81 (2016): 236-245.
56. PECORELLI Alessandra, et al. "Alteration of serum lipid profile, SRB1 loss, and impaired Nrf2 activation in CDKL5 disorder." Free Radical Biology and Medicine 86 (2015): 156-165.

57. PECORELLI, Alessandra, et al. "Altered bone status in Rett syndrome." Life 11.6 (2021):
58. DE FELICE, Claudio, et al. The role of oxidative stress in Rett syndrome: an overview. Annals of the New York Academy of Sciences, 2012, 1259.1: 121-135.
59. MIN HO HAN. Cytoprotective effects of esculetin against oxidative stress are associated with the upregulation of Nrf2-mediated NQO1 expression via the activation of the ERK pathway
60. PECORELLI, Alessandra, et al. "OxInflammation in Rett syndrome." The International Journal of Biochemistry & Cell Biology 81 (2016): 246-253.
61. VALACCHI, Giuseppe, et al. "4-hydroxynonenal protein adducts: key mediator in Rett syndrome oxinflammation." Free Radical Biology and Medicine 111 (2017): 270-280.
62. PECORELLI, Alessandra, et al. "13-HODE, 9-HODE and ALOX15 as potential players in Rett syndrome OxInflammation." Free Radical Biology and Medicine 134 (2019): 598-603.
63. PECORELLI, Alessandra, et al. "Altered inflammasome machinery as a key player in the perpetuation of Rett syndrome oxinflammation." Redox Biology 28 (2020): 101334.
64. CORDONE, Valeria, et al. "The constitutive activation of TLR4-IRAK1-NFκB axis is involved in the early NLRP3 inflammasome response in peripheral blood mononuclear cells of Rett syndrome patients." Free Radical Biology and Medicine 181 (2022): 1-13.
65. VALACCHI, Giuseppe, et al. "4-hydroxynonenal protein adducts: key mediator in Rett syndrome oxinflammation." Free Radical Biology and Medicine 111 (2017): 270-280.
66. ZHANG, Hongqiao, and Henry Jay Forman. "4-hydroxynonenal-mediated signaling and aging." Free Radical Biology and Medicine 111 (2017): 219-225.
67. CRIVELLARI, Ilaria, et al. "Nrf2-pathway alteration in RTT syndrome." Free Radical Biology and Medicine 108 (2017): S63.
68. SISLIYAN, Christina, et al. "Recruitment of Nrf2-dependent adaptive homeostatic redox responses in Rett syndrome." Free Radical Biology and Medicine 128 (2018): S134.
69. HIEBERT, Paul. "The Nrf2 transcription factor: a multifaceted regulator of the extracellular matrix." Matrix Biology Plus 10 (2021): 100057.
70. HIEBERT, Paul. "The Nrf2 transcription factor: a multifaceted regulator of the extracellular matrix." Matrix Biology Plus 10 (2021): 100057.
71. MILENKOVIC, Jelena, et al. "Mechanisms of plasminogen activator inhibitor 1 action in stromal remodeling and related diseases." Biomedical Papers of the Medical Faculty of Palacky University in Olomouc 161.4 (2017). DOI: 10.5507/bp.2017.046.
72. UniProt: the Universal Protein Knowledgebase in 2023.

73. WANG, Liqun, et al. "Nicotinamide Mononucleotide Ameliorates Silica-Induced Lung Injury through the Nrf2-Regulated Glutathione Metabolism Pathway in Mice." Nutrients 15.1 (2022): 143.
74. YAN, Jianing, Guoliang Ye, and Yongfu Shao. "High expression of the ferroptosis-associated MGST1 gene in relation to poor outcome and maladjusted immune cell infiltration in uterine corpus endometrial carcinoma." Journal of clinical laboratory analysis 36.4 (2022): e24317.
75. VALENTI, Daniela, et al. Mitochondrial dysfunction as a central actor in intellectual disability-related diseases: an overview of Down syndrome, autism, Fragile X and Rett syndrome. Neuroscience & Biobehavioral Reviews, 2014, 46: 202-217.
76. KIM, Yosup; JANG, Ho Hee. The role of peroxiredoxin family in cancer signaling. Journal of cancer prevention, 2019, 24.2: 65.
77. WALKER, Matthew A.; TIAN, Rong. NAD (H) in mitochondrial energy transduction: implications for health and disease. Current opinion in physiology, 2018, 3: 101-109.
78. COOPER GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Mitochondria. Available from:
79. GARLID, Keith D. Physiology of mitochondria. In: Cell Physiology Source Book. Academic Press, 2001. p. 139-151.
80. ANSENBERGER-FRICANO, Kristine, et al. The peroxidase activity of mitochondrial superoxide dismutase. Free Radical Biology and Medicine, 2013, 54: 116-124.
81. HE, Chenxia, et al. SOD2 and the mitochondrial UPR: partners regulating cellular phenotypic transitions. Trends in biochemical sciences, 2016, 41.7: 568-577.
82. HÜTTEMANN, Maik, et al. The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian cell: From respiration to apoptosis. Mitochondrion, 2011, 11.3: 369-381.
83. ZHANG, Yadong; CHEN, Fei. Reactive oxygen species (ROS), troublemakers between nuclear factor-κB (NF-κB) and c-Jun NH2-terminal kinase (JNK). Cancer research, 2004, 64.6: 1902-1905.
84. TEMKIN, Vladislav; KARIN, Michael. From death receptor to reactive oxygen species and c-Jun N-terminal protein kinase: the receptor-interacting protein 1 odyssey. Immunological reviews, 2007, 220.1: 8-21.
85. ZHANG, Yadong; CHEN, Fei. Reactive oxygen species (ROS), troublemakers between nuclear factor-κB (NF-κB) and c-Jun NH2-terminal kinase (JNK). Cancer research, 2004, 64.6: 1902-1905.

86. MUSI, Clara Alice, et al. JNK signaling provides a novel therapeutic target for Rett syndrome. BMC biology, 2021, 19.1: 1-20.
87. https://string-db.org/.
88. QIN, Zhaoping, et al. Oxidant exposure induces cysteine-rich protein 61 (CCN1) via c-Jun/AP-1 to reduce collagen expression in human dermal fibroblasts. PLoS One, 2014, 9.12: e115402.
89. TU, Yidong; QUAN, Taihao. Oxidative stress and human skin connective tissue aging. Cosmetics, 2016, 3.3: 28.
90. VASILEVA, Liliya V., et al. "Obesity and NRF2-mediated cytoprotection: Where is the missing link?." Pharmacological research 156 (2020): 104760.
91. CICALONI, Vittoria, et al. "A proteomics approach to further highlight the altered inflammatory condition in Rett syndrome." Archives of Biochemistry and Biophysics 696 (2020): 108660.
92. KYLE, Stephanie M., Neeti Vashi, and Monica J. Justice. "Rett syndrome: a neurological disorder with metabolic components." Open biology 8.2 (2018): 170216.
93. MARANO, Domenico, et al. "Transcriptomic and epigenomic landscape in rett syndrome." Biomolecules 11.7 (2021): 967. https://doi.org/10.3390/biom11070967.
94. LUO, Shilin, et al. "Akt phosphorylates NQO1 and triggers its degradation, abolishing its antioxidative activities in Parkinson's disease." Journal of Neuroscience 39.37 (2019): 7291-7305.
95. HIEBERT, Paul. "The Nrf2 transcription factor: a multifaceted regulator of the extracellular matrix." Matrix Biology Plus 10 (2021): 100057.
96. QIN, Zhaoping, et al. "Oxidant exposure induces cysteine-rich protein 61 (CCN1) via c-Jun/AP-1 to reduce collagen expression in human dermal fibroblasts." PLoS One 9.12 (2014): e115402.
97. WANG, Liqun, et al. "Nicotinamide Mononucleotide Ameliorates Silica-Induced Lung Injury through the Nrf2-Regulated Glutathione Metabolism Pathway in Mice." Nutrients 15.1 (2022): 143.
98. XU, Joyce, et al. "Nuclear SOD1 in growth control, oxidative stress response, amyotrophic lateral sclerosis, and cancer." Antioxidants 11.2 (2022): 427.
99. VASILEVA LV, Savova MS, Amirova KM, Dinkova-Kostova AT, Georgiev MI. Obesity and NRF2-mediated cytoprotection: Where is the missing link?. Pharmacological research. 2020 Jun 1;156:104760.

100. GNAIGER, Erich, et al. Mitochondrial physiology. Bioenergetics communications, 2020, BEC2020. 1.

101. PALMA, Flavio R., et al. Mitochondrial superoxide dismutase: what the established, the intriguing, and the novel reveal about a key cellular redox switch. Antioxidants & Redox Signaling, 2020, 32.10: 701-714.

102. HE, Chenxia, et al. SOD2 and the mitochondrial UPR: partners regulating cellular phenotypic transitions. Trends in biochemical sciences, 2016, 41.7: 568-577.

9. Other publications during my PhD and awards and recognitions

i. PAMBIANCHI, Erika, et al. Tension as a key factor in skin responses to pollution. Scientific Reports, 2023, 13.1: 16013.

ii. The abstract: PASQUI, Arianna, et al. Multi-omics study in Rett syndrome to investigate the roleof the MECP2 gene mutation in redox regulatory pathways. Free Radical Biology and Medicine, 2023, 201: 30.

iii. CICALONI, Vittoria, et al. A bioinformatics approach to investigate structural and non-structural proteins in human coronaviruses. Frontiers in Genetics, 2022, 13: 891418.

iv. Winner 2nd place for an outstanding poster presentation at the 2023 North Carolina Research Campus (NCRC) Catalyst Spring Symposium in North Carolina State University (March 2023).

v. Travel grant released by Society for Free Radical Biology and Medicine (SFRR-Europe) (June 2023).

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