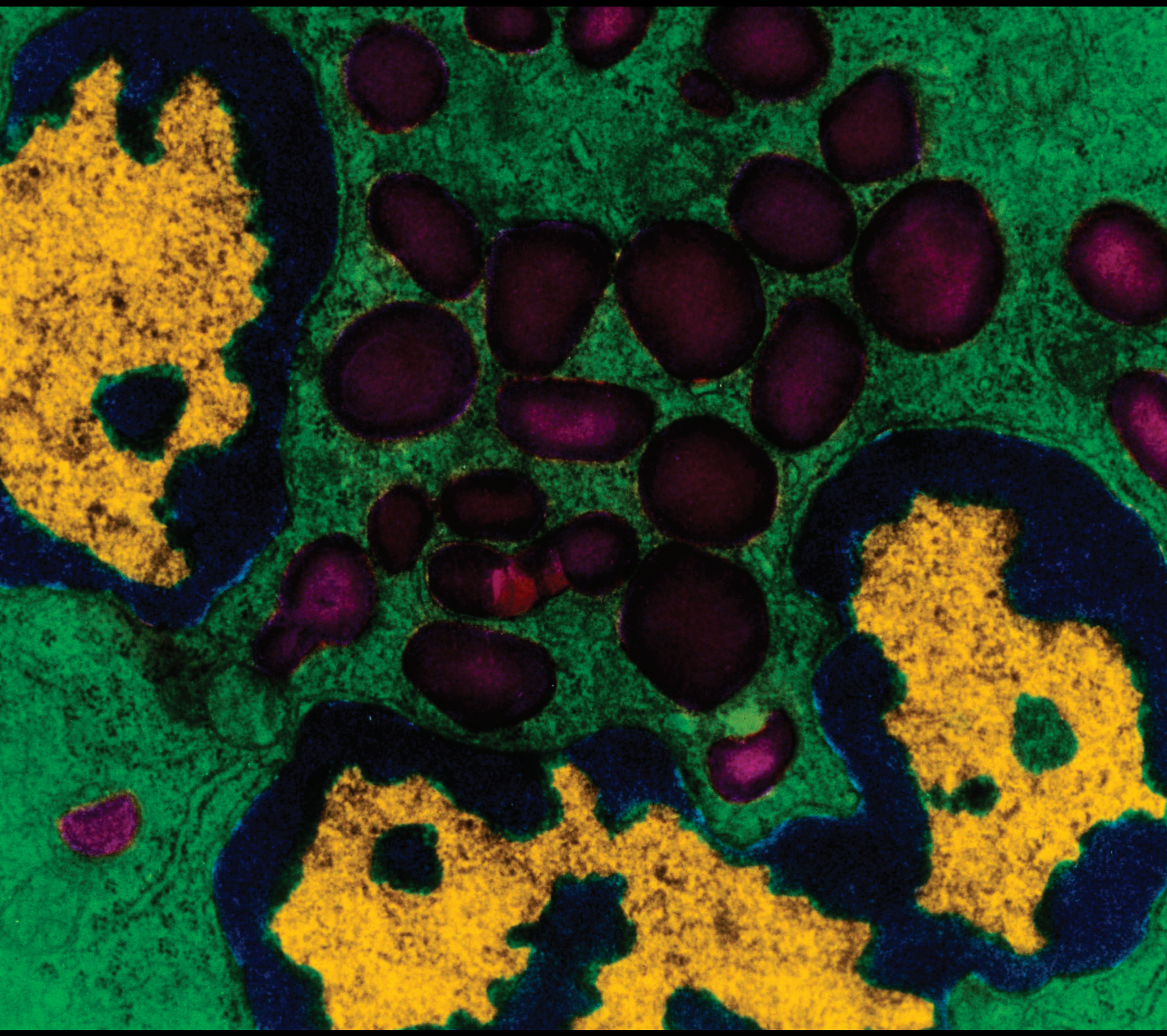
Mediators of Inflammation

# ASD: Biochemical Mechanisms behind Behavioral Disorders

Guest Editors: Giuseppe Valacchi and Paul Ashwood



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# Editorial Board

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*Editorial*

# ASD: Biochemical Mechanisms behind Behavioral Disorders

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Autism spectrum disorders (ASD) are complex neurobiological disorders of development characterized by impairments in social interaction and communication, together with restricted, repetitive, and stereotyped interests/behavior. There has been a dramatic increase in the reported rates of ASD over the last 40 years which has risen in USA from 1 in 5000 in the mid-1970s to 1 in 88 in 2012. However, little is known about the underlying pathophysiological mechanisms and there is currently a lack of reliable biological markers to help in the diagnosis or monitoring of the changes in clinical definitions over time or in response to therapies. Currently, there are few effective treatments for ASD with most medication and behavioral therapies aimed at minimizing the symptomology. Although the knowledge base of ASD is rapidly growing as research examines more and varied aspects of these disorders, their complex nature makes it difficult to determine the causation or catalysts. There is probably no singular unique cause for these neurobehavioral disorders but a combination of genetic and environmental factors may be responsible for pathological changes in brain and immune, metabolic, and endocrine systems.

The present special issue collects reports related to the possible pathological mechanisms involved in ASD with special focus on classic autism and Rett syndrome (RTT). Growing evidence suggests that immune dysfunction and the presence of autoimmune responses including autoantibodies may play a role in ASD. The article by M. Careaga et al. provides evidence that young children with ASD have an elevated production of anti-phospholipid antibodies. The authors have shown an increased level of anticardiolipin, $\beta$2-glycoprotein 1, and anti-phosphoserine antibodies and this increase was associated with more impaired behaviors of the patients. In line with Careaga's work, a paper by C. Giulivi et al. investigated the maternal immune activation (MIA) as a potential risk factor for ASD and schizophrenia in a mouse model. The authors showed that splenocytes isolated from adult offspring exposed gestationally to the viral mimic poly(I:C) (to activate MIA) had long-lasting effects in the bioenergetics with a significant reduction of ATP production as a consequence of lower mitochondrial complex I activation. The work by L. Ciccoli et al. showing an unrecognized triad combination of erythrocyte shape abnormalities, erythrocyte membrane oxidative damage, and $\beta$-actin alterations in individuals with ASD provides, therefore, a new possible biological marker for the diagnosis of ASDs.

In the second part of the special issue, the reports are related to several clinical and biochemical aspects present in Rett syndrome (RTT). Of interest is the paper by A. Pecorelli et al. where the authors have analysed the genome expression of limphomonocytes isolated from RTT patients showing altered gene expression related to mitochondrial function, cellular ubiquitination, proteasome degradation, RNA processing, and chromatin folding, suggesting, therefore, that mitochondrial-ATP-proteasome function is likely to be involved in RTT clinical features. In addition, the work by C. De Felice et al. has shown that pulmonary gas exchange abnormality (GEA) is present in RTT and that terminal bronchioles are a likely major target of the disease. There are a further 2 papers relating to the positive effect of $\omega$-3 treatment in RTT. The first by S. Maffei et al. shows that $\omega$-3 PUFAs are able to improve the biventricular myocardial systolic function in those patients and that the functional

gain is partially mediated through a regulation of the redox balance. The other, by C. De Felice et al., shows that $\omega$-3 PUFAs supplementation is able to partially rescue the acute phase response present in RTT. This last result is in line with the paper by A. Cortelazzo et al. where, through a proteomic approach, the authors were able to demonstrate the presence of a subclinical chronic inflammatory status related to the severity carried by the *MECP2* gene mutation. Finally, again utilizing a proteomic approach, A. Cortelazzo et al. were able to compare the proteasome expression of classical RTT versus speech variant RTT in 2 sisters. They suggest that unique familial cases offer the opportunity to identify new protein patterns involved in the RTT phenotype expression.

Taken together, these publications offer new light on the pathophysiology of ASD and Rett syndrome and the potential to further investigate biological markers and possible therapeutic approaches.

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*Clinical Study*

# Inflammatory Lung Disease in Rett Syndrome

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Rett syndrome (RTT) is a pervasive neurodevelopmental disorder mainly linked to mutations in the gene encoding the methyl-CpG-binding protein 2 (MeCP2). Respiratory dysfunction, historically credited to brainstem immaturity, represents a major challenge in RTT. Our aim was to characterize the relationships between pulmonary gas exchange abnormality (GEA), upper airway obstruction, and redox status in patients with typical RTT ($n = 228$) and to examine lung histology in a *Mecp2*-null mouse model of the disease. GEA was detectable in ~80% (184/228) of patients versus ~18% of healthy controls, with "high" (39.8%) and "low" (34.8%) patterns dominating over "mixed" (19.6%) and "simple mismatch" (5.9%) types. Increased plasma levels of non-protein-bound iron (NPBI), $F_2$-isoprostanes ($F_2$-IsoPs), intraerythrocyte NPBI (IE-NPBI), and reduced and oxidized glutathione (i.e., GSH and GSSG) were evidenced in RTT with consequently decreased GSH/GSSG ratios. Apnea frequency/severity was positively correlated with IE-NPBI, $F_2$-IsoPs, and GSSG and negatively with GSH/GSSG ratio. A diffuse inflammatory infiltrate of the terminal bronchioles and alveoli was evidenced in half of the examined *Mecp2*-mutant mice, well fitting with the radiological findings previously observed in RTT patients. Our findings indicate that GEA is a key feature of RTT and that terminal bronchioles are a likely major target of the disease.

## 1. Introduction

Rett syndrome (RTT), for a long time included among the Autism Spectrum Disorders (ASDs), is a nosologically distinct, genetically determined neurological entity associated in up to 95% of cases to *de novo* loss-of-function mutations in the X-chromosome-linked gene encoding the methyl-CpG-binding protein 2 (MeCP2) [1]. MeCP2, a ubiquitous protein particularly abundant in brain, is known to either activate or repress transcription [2, 3], is critical to the function of several types of cells (i.e., neurons and astroglial cells), and targets several genes essential for neuronal survival, dendritic growth, synaptogenesis, and activity dependent plasticity [4].

In its classical clinical presentation, RTT affects heterozygous females and shows a typical 4-stage neurological regression after 6 to 18 months of apparently normal development. RTT is a relatively rare disease, affecting about 1 : 10,000 female live births, although it represents the second most common cause of severe intellectual disability in the female gender [5, 6]. Preserved speech, early seizure, and congenital are well-known atypical variants often linked to mutations in genes other than *MECP2*, that is, the cyclin-dependent kinase-like 5 (*CDKL5*) in the early seizure variant and the forkhead boxG1 (*FOXG1*) in the congenital variant [6, 7].

Breathing disorders are considered a hallmark feature of RTT and represent a major clinical challenge [8]. To date, a large number of studies have been focusing on this particular characteristic of the disease, both in the clinical and experimental environments. Breathing abnormalities in RTT variably include/feature breath holdings, apneas, apneusis, hyperventilation, rapid shallow breathing, and spontaneous Valsalva maneuvers [9]. In particular, a highly irregular respiratory rhythm particularly during daytime is considered among the key symptoms of RTT [9–11]. Cumulating evidence indicates a predominantly hyperventilatory pattern with increased respiratory frequency and decreased expiratory duration, which is associated with frequent episodes of breath-holding/obstructive apnea or Valsalva breathing against closed airways during wakefulness [12–14]. However, the breath-holding/obstructive apnea phenotype of RTT is often confused in the related clinical literature with central apnea, which has fundamentally distinct neurological mechanisms [9, 15–26]. The wide spectrum of respiratory disorders detectable in RTT patients has been historically credited to brainstem immaturity and/or cardiorespiratory autonomic dysautonomia [9, 27, 28]. However, as the pathogenesis of the respiratory dysfunction in RTT appears far from being completely understood, alternative or complementary hypotheses can be formulated [29].

In particular, the potential role of oxidative stress (OS) mediators and the role of the lung itself in the pathogenesis of the respiratory dysfunction in the human disease are incompletely understood. More recently, biochemical evidence of redox imbalance and, in particular, enhanced lipid peroxidation, in blood samples from RTT patients, was further confirmed in primary skin fibroblasts cultures from patients [30–37], although the nature of the relationship, that is, whether causal or correlational, between *MECP2* gene mutation and abnormal redox homeostasis remains currently unclear. Significantly increased pulmonary alveolar-arterial gradient for $O_2$, highly suggestive for an abnormal pulmonary gas exchange, has been previously described by our group in the majority of the examined RTT patients [29] and was found to be related in about a half of the cases to a pulmonary radiological picture partially mimicking that of the respiratory bronchiolitis-associated interstitial lung disease (RB-ILD) [38], one of the three lung conditions showing the stronger epidemiological association with tobacco smoke [39, 40].

However, to date, no information exists regarding the lung pathological lesions underlying the radiological changes observed in RTT patients.

Aims of the present study were to characterize the possible role of pulmonary gas exchange abnormality (GEA) in the pathogenesis of redox imbalance and respiratory dysfunction in RTT and to evaluate lung histology in an experimental mouse model of MeCP2 deficiency.

## 2. Methods

*2.1. Subjects.* In the present study, a total of $n = 228$ female patients with a clinical diagnosis of typical RTT and demonstrated *MECP2* gene mutation were recruited (mean age, 12.9 ± 7.9 years; range, 1.5–32 years). RTT diagnosis and inclusion/exclusion criteria were based on a revised nomenclature consensus [6]. Clinical severity was assessed by the use of the clinical severity score (CSS), a specifically validated clinical rating system based on 13 individual ordinal categories measuring clinical features common in the disease [7]. Respiratory dysfunction on a clinical basis was categorized based on the corresponding Percy scale item (+ as minimal hyperventilation and/or apnea; ++ as intermittent hyperventilation and/or apnea; and +++ as hyperventilation and/or apnea with cyanosis) [41].

The corresponding $z$-scores for body weight, height, head circumference, and body mass index were calculated on the basis of validated RTT-specific growth charts [42]. Clinical stages distribution was: stage I ($n = 4$), stage II ($n = 69$), stage III ($n = 92$), and stage IV ($n = 63$). All the patients were admitted to the Rett Syndrome National Reference Centre of the University Hospital of the Azienda Ospedaliera Universitaria Senese. A total of 114 healthy and typically developed female subjects of comparable age (mean age, 12.9 ± 7.8 years; range, 1.6–32 years) were also enrolled in the study as a control population. Blood samplings from the control group were performed during routine health checks, sports, or blood donations obtained during the periodic checks. All the examined subjects were on a typical Mediterranean diet. The study was conducted with the approval by the Institutional Review Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

*2.2. Oxidative Stress (OS) Markers and Antioxidant Defence Evaluations*

*2.2.1. Blood Sampling.* Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. An aliquot (90 $\mu$L) of each sample was used for reduced and oxidized glutathione assay. Blood samples were centrifuged at 2400 ×g for 15 min at 4°C; the platelet poor plasma was saved and the buffy coat was removed by aspiration. RBCs were washed twice with physiologic solution (150 mM NaCl). An aliquot of packed erythrocytes was resuspended in Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$ , 32 mM N-2 hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM glucose, and 1 mM $CaCl_2$), pH 7.4 as a 50% (vol/vol) suspension for the determination of intraerythrocyte NPBI. Plasma was used for the NPBI assay.

*2.2.2. Intraerythrocyte and Plasma Non-Protein-Bound Iron (IE-NPBI).* Generally, NPBI is considered not only an OS marker but a prooxidant factor. In particular, IE-NPBI is a critical marker of hypoxia. IE-NPBI (nmol/mL erythrocyte suspension) was determined as a desferrioxamine- (DFO) iron complex (ferrioxamine) as previously reported [29] Plasma NPBI (nmol/ml) was determined as above reported for IE-NPBI [29].

*2.2.3. Plasma $F_2$-Isoprostanes ($F_2$-IsoPs).* $F_2$-IsoPs, generated by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid, are considered specific and reliable OS markers *in vivo*. $F_2$-IsoPs were determined by a gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis after solid phase extraction and derivatization steps [43]. For $F_2$-IsoPs the measured ions were the product ions at *m/z* 299 and *m/z* 303 derived from the $[M-181]^-$ precursor ions (*m/z* 569 and *m/z* 573) produced from 15-$F_{2t}$-IsoPs and $PGF_{2\alpha}$-d4, respectively [43].

*2.2.4. Blood Reduced and Oxidized Glutathione.* Glutathione ($\gamma$-L-glutamyl-L-cysteinyl-glycine) is a tripeptide that plays an important role in protecting cells and tissues against OS [44]. Under nonoxidative and nitrosative stress conditions, over 98% of the glutathione is considered to be in the reduced form (GSH) [45], whereas under oxidative conditions GSH is converted to glutathione disulfide (GSSG), its oxidized form, with a resulting decrease in the GSH/GSSG ratio. As blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both GSH and GSSG in blood has been considered essential as an index of whole-body glutathione status and a useful indicator of antioxidant defence [46]. Specifically, the GSH/GSSG ratio reflects the cellular redox status. Blood GSH and GSSG levels were determined by an enzymatic recycling procedure according to Tietze [47] and Baker et al. [48].

*2.3. Cardiorespiratory Monitoring.* In order to analyze the occurrence of apnoeas and hypopneas, breathing monitoring was carried out in RTT patients during wakefulness and sleep state by using portable polygraphic screening devices (SOMNOwatchTM plus, SOMNOmedics, Randersacker, Germany; importer for Italy Linde Medicale srl) for a mean recording time of 13 ± 0.5 h for each state. Monitoring included nasal airflow, arterial oxygen saturation by pulse oximetry, and respiratory efforts by abdominal and thoracic bands. Breathing patterns were analyzed for the presence of apnoeas and hypopnoeas according to the standardized definitions by the American Academy of Sleep Medicine [49] and the American Academy of Pediatrics [50]. Apnoeas were defined as a >90% airflow decrease for 10 sec, while hypopnoeas were defined as a >50% airflow reduction for ~10 sec associated with a decrease of 3% in oxygen saturation [49]. Apnoeas were categorized as obstructive (i.e., cessation of airflow for 10 sec with persistent respiratory effort), central (i.e., cessation of airflow for 10 sec with no respiratory effort), and mixed (an apnea that begins as a central apnea and ends up as an obstructive apnea). Apnoeas were further categorized as mild (10 to 15 sec), moderate (15 to 30 sec), and severe (>30 sec) on the basis of their recorded duration. The apnea-hypopnea index (AHI) was defined as the number of obstructive and central apnoeas and hypopnoeas per hour of sleep and calculated by dividing the total number of events by the total sleep time. An AHI > 15 during sleep was considered to be indicative of obstructive sleep apnea/hypopnea syndrome (OSAHS). All records were reviewed by a pneumologist with a longstanding expertise in OSAHS (i.e., coauthor M.R.).

*2.4. Pulmonary Gas Exchange Analysis.* Pulmonary gas exchange was evaluated from direct measurements of total volume ($V_{tot}$), respiratory rate, and expiratory fractions of $CO_2$ and $O_2$ by using a portable, commercially available gas analyzer (Hanky Hapy, version 1.2; Ambra Sistemi; Pianezza, Turin, Italy), as previously described [29]. The method to evaluate pulmonary gas exchange works essentially as a multicompartment model (Figure 1) and is essentially based on the classical West function [51]. Air gas sampling was obtained by applying a facial mask of appropriate size connected to the gas analyzer. Low invasivity and the easy-to-use features of the method allowed us to evaluate a relatively large population size of patients. Actually, the methodology does not require patient's cooperation and is therefore easily applicable to RTT patients and has been proven to be sufficiently simple, noninvasive, accurate, and precise in determining alveolar-arterial gradient lung exchange for $O_2$ and ventilation/perfusion ratio ($V/Q$) inequalities. Respiratory rate, total ventilation, and expired gas composition were measured during either a 60-sec or 120-sec time period. $V/Q$ distribution parameters were calculated by a minimizing mathematical function in order to reset to zero the differences between measured and calculated $PaO_2$ and $PaCO_2$. All respiratory measurements were carried out in duplicate, and the averages used for data analysis. Arterial blood for gas analyses was sampled from either the humeral or the radial artery, and $PaO_2$, $PaCO_2$, and pH values were determined using a commercially available blood gas analyzer (ABL520 Radiometer; Radiometer Medical A/S; Copenhagen, Denmark). Ventilation-perfusion ($V/Q$) inequalities (i.e., GEA) were classified as low, high, mixed, and simple mismatch. A "low" pattern indicates the presence of perfusion in poorly ventilated pulmonary areas; a "high" pattern points out the existence of high ventilation in poorly perfused pulmonary areas; a mixed pattern indicates a combination of the former two patterns; a simple "mismatch" was defined as a $V/Q$ uncoupling showing a modest fraction of low $V/Q$ ratios (1 to 0.1) and a modest fraction of high $V/Q$ ratios (1 to 10). In order to account for the low $PaCO_2$ values often encountered in RTT patients, standard $PaO_2$ was calculated with the formula $PaO_2 = 1.66 \times PaCO_2 + PaO_2 - 66.4$, according to Sorbini et al. [52].

*2.5. RTT Mouse Model: Murine Lung Histology.* A total of ($n = 4$) *Mecp2* null mice and ($n = 4$) wild-type matched mice were examined. Experimental subjects were derived from heterozygous B6.129SF1-*Mecp2tm1Jae* knockout

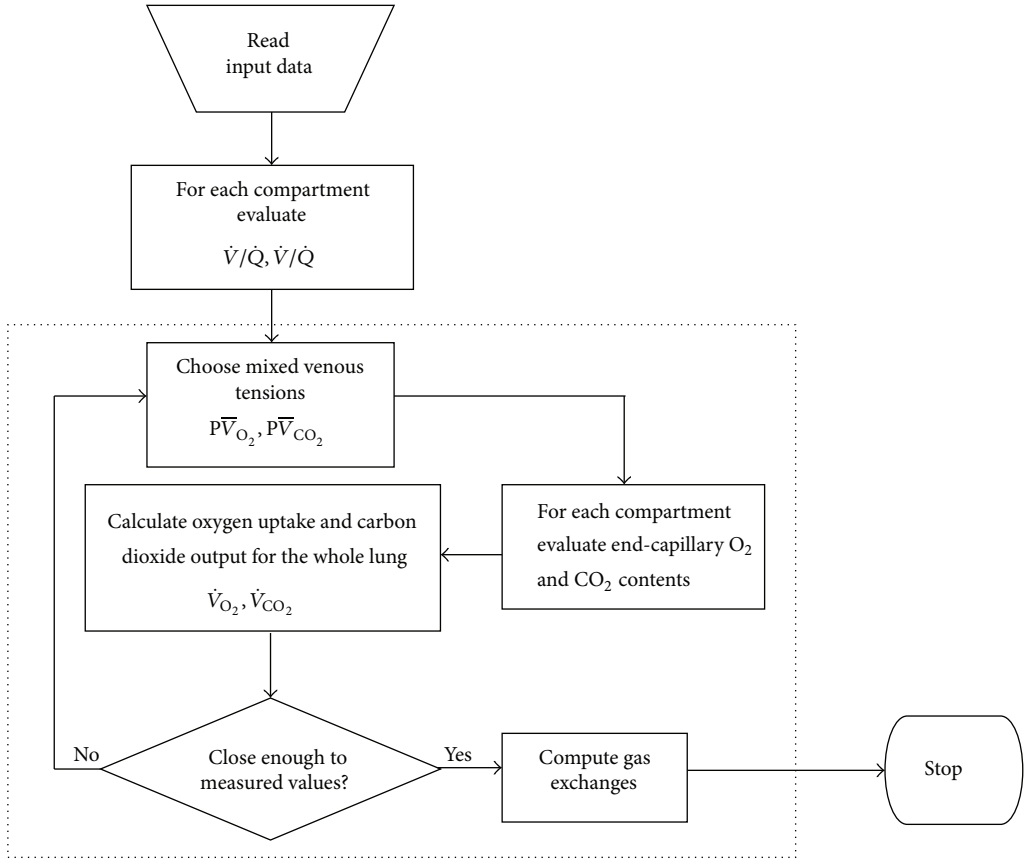


Figure 1: Algorithm for the noninvasive assessment of pulmonary gas exchange (Hanky Hapy gas analyzer version 1.2).

females (Mecp2+/−) [53]. Females were originally crossed to C57BL6/J for one generation, followed by breeding amongst offspring of the same generation with breeder changes, and were maintained on a mixed background. Mixed background reduced mortality and was necessary to obtain the high numbers of mice required by extensive analysis. Age-matched littermates were used in all experiments to control for possible effects of genetic background unrelated to the *Mecp2* mutation [54]. Mice were killed by decapitation at the thirty-eighth day of life; their lungs were removed rapidly and immediately frozen on liquid nitrogen. National and institutional guidelines were used for the care and use of animals, and approval for the experiments was obtained. Lungs were inflated with neutral buffered 10% formalin solution for about 24 h until adequate fixation. Each lung was dissected and sections were embedded in paraffin. Several 5 micrometres sections from each inclusion were stained with a standard hematoxylin and eosin staining protocol.

*2.6. Statistical Data Analysis.* All variables were tested for normal distribution (D'Agostino-Pearson test). Data were presented as means ± standard deviation or medians and interquartile range for normally distributed and non-Gaussian continuous variables, respectively. Differences between RTT and control groups were evaluated using independent-sample $t$-test (continuous normally distributed data), Mann-Whitney rank sum test (continuous nonnormally distributed data), chi-square statistics (categorical variables with minimum number of cases per cell ≥5) or Fisher's exact test (categorical variables with minimum number of cases per cell <5), one-way analysis of variance (ANOVA), Student-Newman-Keuls post hoc test, or Kruskal-Wallis test, as appropriate. Associations between variables were tested by either parametric (Pearson's coefficients) or nonparametric univariate (Spearman's rho) regression analysis. Predictive accuracy of apneas frequency/severity in identifying enhanced OS markers in RTT patients was calculated using a receiver operating characteristic curve (ROC) analysis, and an area under the curve value >0.5 was accepted to indicate good discrimination. The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used for data analysis and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

## 3. Results

*3.1. Clinical Respiratory Dysfunction.* Relevant demographic clinical characteristics for the examined RTT population are shown in Table 1. According to the specifically related items in the severity scoring system, all patients showed clinical signs for a respiratory dysfunction at different degrees, with moderate or severe dysfunction being detectable on a clinical basis in 81.6% (186/228) of the RTT patients.

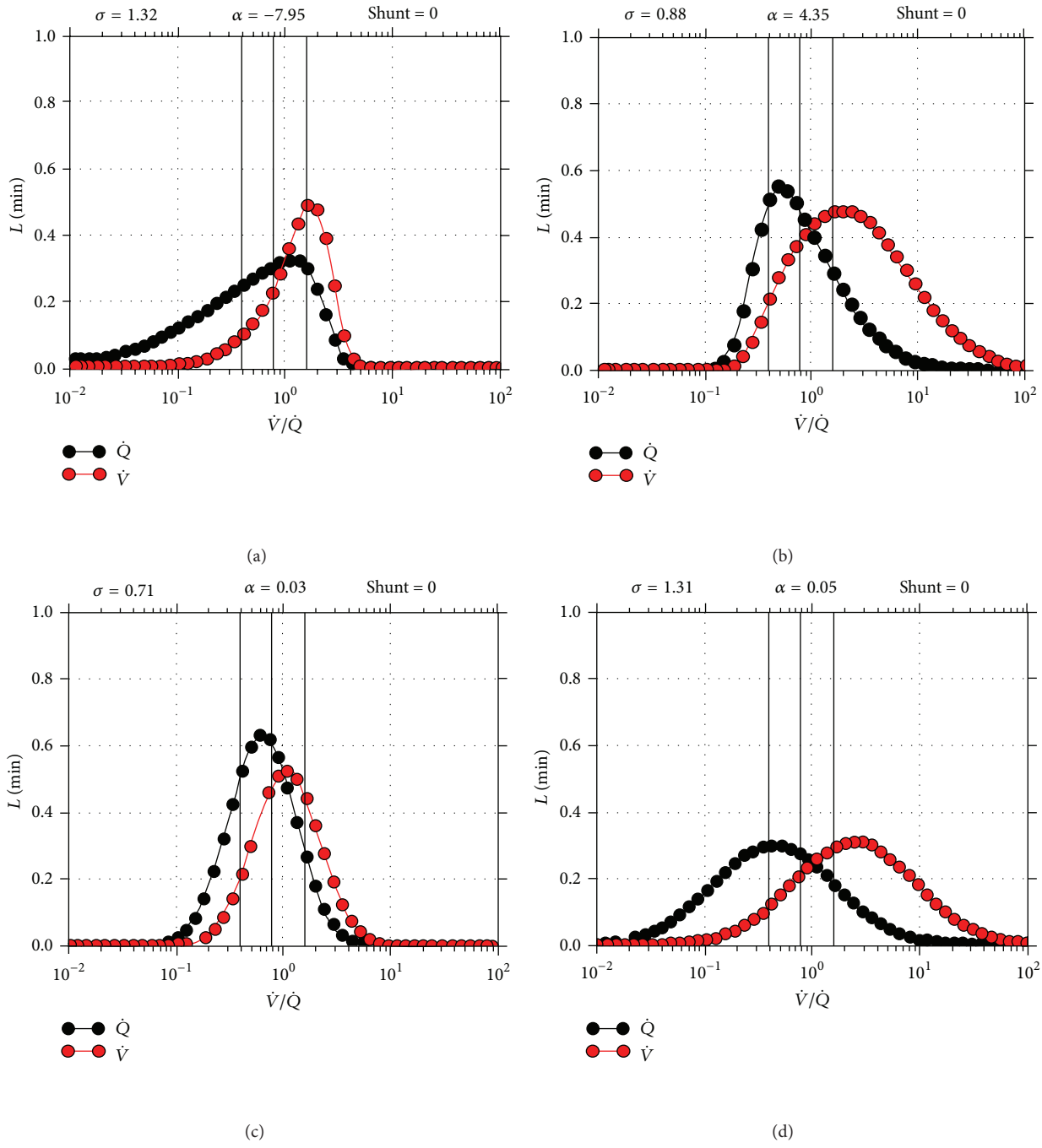


Figure 2: Representative pulmonary gas exchange abnormalities (GEA) patterns in patients with typical RTT and *MeCP2* gene mutation: (a) "low pattern" abnormality; (b) "high pattern" abnormality; (c) "simple $V/Q$ mismatch"; and (d) "mixed pattern" abnormality. Ventilation-perfusion ($V/Q$) inequalities (i.e., GEA) were detectable in 80.7% of the whole RTT population, whereas only 19.3% of the patients showed a normal gas exchange. A "low" pattern (i.e., 34.8 of all GEA types in RTT) indicates the presence of perfusion in poorly ventilated pulmonary areas; a "high" pattern (i.e., 39.8% of all GEA types) points out the existence of high ventilation in poorly perfused pulmonary areas; a mixed pattern (i.e, 19.6% of all GEA types) is a combination of the former two patterns, while a "simple mismatch" (i.e., 5.9% of GEA types) is a $V/Q$ uncoupling, showing a modest fraction of low $V/Q$ ratios (1 to 0.1) and a modest fraction of high $V/Q$ ratios (1 to 10).

*3.2. Pulmonary Gas Exchanges.* Gas pulmonary exchange investigations demonstrated the existence of a variety of ventilation-perfusion inequalities (Figure 2 and Table 2) in more than 3/4 (i.e., 80.7%) of the whole RTT population; a "low" pattern (i.e., presence of perfusion in poorly ventilated pulmonary areas) was observed in 64 patients (28.1% of the examined whole RTT population), a "high" pattern (i.e., high ventilation in poorly perfused pulmonary areas) in 73 cases (32%), and a simple "mismatch" in 11 cases (4.8%), while a "mixed" pattern was present in 36 patients (15.8%).

Table 1: Relevant demographic and clinical characteristics of female subjects with Rett syndrome.

| Variables | |
|---|---|
| Patients ($N$) | 228 |
| Age (years) | 12.9 ± 7.9[§] |
| Body weight (RTT $z$-score for age)[1] | 0.025 ± 1.12 |
| Body height (RTT $z$-score for age)[1] | −0.05 ± 1.13 |
| Head circumference (RTT $z$-score)[1] | −0.22 ± 1.11 |
| Body mass index (BMI) (RTT $z$-score for age)[1] | −0.36 ± 1.45 |
| Clinical severity score (CSS)[2] | 17.4 ± 7.3 |
| Tachypnea[a] | 59 (25.9 %) |
| Respiratory dysfunction on a clinical basis[b] | |
| + | 42 (18.4 %) |
| ++ | 127 (55.7 %) |
| +++ | 59 (25.9 %) |
| Additional clinical features | |
| Air-S[c] | 64 (28.1 %) |
| Severe GERD[d] | 27 (11.8 %) |

[1]Calculated $z$-scores for age are referred to a validated Rett syndrome-specific growth charts [42]. [2]Clinical severity score was defined according to Neul et al., 2008 [7]. [a]Tachypnea was defined as a respiratory rate >1.8 times (i.e., above the upper quartile) of the expected respiratory rate for age and gender; [b]respiratory dysfunction was categorized based on the corresponding Percy's clinical severity scale item [41]; [§]mean ± SD; [c]Air-S: abnormal air swallowing; [d]GERD: gastroesophageal reflux disease.

Overall, only 19.3% (44/228) of the RTT population showed a physiological (i.e., coupled $V/Q$) gas exchange pattern (RTT versus controls, chi-square: 138.472, DF = 4, $P < 0.0001$; chi-square for trend: 56.154, DF = 1, $P < 0.0001$).

Pulmonary gas exchanges parameters (Table 2) detected a general trend toward hyperventilation in the RTT patients, with mean total ventilation rates ($V_{tot}$) of 6.3 ± 3.6 L/min (95% C.I. for the mean 5.8 to 6.7) versus 5.2 ± 2.1 L/min in the control subjects ($P = 0.0028$). Hyperventilation was absent in the "low" pattern of GEA, while being extreme in the "high" GEA pattern. Likewise, alveolar ventilation was the largest in the "high" pattern subpopulation of patients, with alveolar ventilation values usually >3 L/min for all GEA subcategories that is volumetrically consistent ventilation.

Blood gas analyses in RTT patients confirmed the presence of a relative hypoxia ($PaO_2$: 87.5 ± 18.1 versus 98.7 ± 6.5 mmHg, difference ± SE: 11.2 ± 1.75, 95% C.I.: 7.76 to 14.6, $P < 0.0001$) and hypocapnia ($PaCO_2$: 35.2 ± 7.5 versus 42.8 ± 5.8 mmHg, difference ± SE: 7.6 ± 0.08, 95% C.I.: 6.02–9.18, $P < 0.0001$), whereas blood pH was comparable between RTT and healthy controls (7.417 ± 0.043 versus 7.413 ± 0.045; difference ± SE: −0.004 ± 0.005854, 95% C.I.: −0.0138 to 0.005854, $P = 0.4252$). When hypocapnia was accounted for, standard $PaO_2$ values in patients were found to be on average 17.6 ± 1.5% lower than those of their healthy control counterparts ($PaO_2$: 81.1 ± 15.3 versus 98.7 ± 6.5, difference ± SE: 17.6 ± 1.5, 95% C.I.: 14.66 to 20.54, $P < 0.0001$) despite a normal-to-increased total volume ($V_{tot}$) 5.8 ± 2.97 versus 5.2 ± 2.3 L/min, difference ± SE: −0.6 ± 0.317, 95% C.I.: −1.224 to 0.0239, $P = 0.0594$.

Remarkably, larger differences in hyperventilation were associated with consistently smaller intergroup differences (1-way ANOVA, $P = 0.025$) in $PaO_2$ and even smaller differences when hypocapnia was accounted for (standard $PaO_2$, $P = 0.082$), thus indicating a reduced efficiency of pulmonary exchange despite normal pH values. However, the physiological dead space, as calculated by the Bohr equation, was found to be at the upper physiological limits (i.e., 30 to 45% of the $V_t$ in the healthy control population) in the "no mismatch," "simple mismatch," and "low" patterns (41.2 to 45.0 $V_t$%), whereas it appears to be increased up to 55.4 ± 10.8 $V_t$% and 54.7 ± 15.5 $V_t$% in "high" and "mixed" patterns, respectively. These findings confirm the occurrence of a reduced efficiency of pulmonary exchanges in the RTT population, with a statistically significant relationship between respiratory rate and Bohr's physiological dead space (rho = 0.144, $P = 0.0303$). Overall, oxygen uptake ($V_{O_2}$) and carbon dioxide production ($V_{CO_2}$) values appear to be lower than those of healthy controls subjects ($V_{O_2}$: 250 to 300 mL/min and $V_{CO_2}$: 200 to 250 mL/min, resp.). Likewise, respiratory exchange ratios (i.e., $V_{CO_2}/V_{O_2}$) in the RTT patients were accordingly higher than those observed in healthy controls (1.56 ± 1.23 versus 0.81 ± 0.32, $P < 0.0001$).

*3.3. Redox and Antioxidant Status.* The results of the redox and antioxidant markers in RTT patients showed significantly increased plasma levels of non-protein-bound iron (NPBI) (~2-fold), $F_2$-isoprostanes ($F_2$-IsoPs) (~2.9-fold), reduced glutathione (GSH) (~1.4-fold), oxidized glutathione (GSSG) (~50-fold), and intraerythrocyte NPBI (IE-NPBI) (~1.5-fold) as compared to healthy control subjects (Table 3). Consequently, a significantly decreased GSH/GSSG ratio (~ −15-fold) in patients was evidenced.

*3.4. Cardiorespiratory Monitoring.* Cardiorespiratory monitoring showed a significant prevalence of obstructive apnoeas both during the sleep and the wakefulness states in RTT patients, with median rates of obstructive apnoeas of 17.7/h and 6.2/h, respectively (Table 4). Of note, obstructive episodes were more prevalent as compared to central events by 25.3- and 15.5-fold during the wakefulness and sleep state, respectively. The lowest recorded SpO2 values during the apnoeic events were 78.8 ± 13.1%. Apneas during the sleep phase were detectable in 63.6% (145/228) of patients, with a mean AHI of 15.9 ± 4.69. Positive criteria for OSAHS (AHI > 15) were present in 27.2% (62/228) of the whole RTT patients population.

*3.5. Relationship between Redox Imbalance and Apnea Frequency/Severity.* Statistically significant positive correlations were observed between recording of apneas, independently of the degree of severity, and IE-NPBI (rho coefficients, range: 0.324 to 0.358; $P$ values, range: 0.0024 to 0.0089) or GSSG (rho coefficients range: 0.258 to 0.267; $P$ values, range: 0.0392 to 0.0156) (Table 5). On the other hand, positive relationships between apneas and p-NPBI (rho: 0.265, $P = 0.0346$) or $F_2$-IsoPs (rho: 0.305, $P = 0.0142$) were also observed but limited

Table 2: Pulmonary gas exchange abnormality (GEA) in patients with typical Rett syndrome ($n$ = 228): relationships between lung ventilation-perfusion ($V/Q$) inequalities patterns and respiratory variables.

| Variables | Pulmonary ventilation/perfusion ($V/Q$) patterns in typical Rett syndrome | | | | | $P$ value (ANOVA) |
|---|---|---|---|---|---|---|
| | No mismatch ($N$ = 44) | "Low" ($N$ = 64) | "High" ($N$ = 73) | "Mixed" ($N$ = 36) | "Simple" Mismatch ($N$ = 11) | |
| $V_{tot}$ (L/min) | **6.74 ± 2.87**[a] | **5.26 ± 2.08**[a,b] | **9.71 ± 5.60**[b,c,d] | **6.07 ± 2.46**[c] | **6.3 ± 1.46**[d] | **<0.001** |
| Respiratory rate (breaths/min) | 27.4 ± 6.9 | 25.9 ± 9.1 | 30.4 ± 8.0 | 26.7 ± 8.3 | 27.1 ± 9.0 | 0.162 |
| Alveolar vent. (L/min) | **4.22 ± 2.60**[a] | **3.14 ± 1.80**[a,b] | **6.93 ± 4.80**[a,b,c,d] | **4.01 ± 2.2**[c] | **4.23 ± 1.30**[d] | **<0.001** |
| $PaO_2$ (mmHg) | **95.4 ± 15.2**[a,b] | **85.7 ± 15.7**[a,d] | **92.2 ± 12.4**[c,d] | **83.9 ± 16.9**[b,c] | **89.8 ± 3.6** | **0.025** |
| Std. $PaO_2$ (mmHg)* | 87.6 ± 14.4 | 81.2 ± 14.9 | 87.3 ± 11.2 | 78.7 ± 20.3 | 88.8 ± 6.5 | 0.082 |
| $PaCO_2$ (mmHg) | 35.3 ± 8.5 | 36.3 ± 7.9 | 37.0 ± 6.6 | 36.9 ± 6.9 | 39.4 ± 3.7 | 0.753 |
| Blood pH | 7.429 ± 0.05 | 7.436 ± 0.05 | 7.417 ± 0.04 | 7.422 ± 0.04 | 7.413 ± 0.01 | 0.462 |
| (A-a) $O_2$ (mmHg) | **14.1 ± 7.9**[a,b,c,d,e] | **25.1 ± 11.3**[a,b,d] | **27.8 ± 10.8**[a,c,d] | **36.9 ± 10.8**[a,b,c,d,e] | **19.4 ± 2.6**[b,c,d,e] | **<0.001** |
| Bohr's DS % $V_t$ | **45.0 ± 19.4**[a,c,d] | **41.2 ± 15.7**[b,c,d] | **55.4 ± 10.8**[c] | **54.7 ± 15.5**[a,b,d] | **41.8 ± 9.5**[a,c,d] | **<0.001** |
| $Q_s/Q_t$ (%) | 6.3 ± 2.9 | 24.3 ± 2.9 | 8.3 ± 7.2 | 19.4 ± 8.9 | 21.6 ± 15.1 | 0.224 |
| $V_{O_2}$ (mL/min) | **137.8 ± 63.5**[a] | **89.5 ± 58.3**[a,b,c,d] | **181 ± 132**[b,c,d] | **88.6 ± 29.7**[d,e] | **164.8 ± 20.8**[e] | **<0.001** |
| $V_{CO_2}$ (mL/min) | **148 ± 78** | **126 ± 58**[a,e] | **193 ± 146**[a,b] | **117 ± 55**[b,e] | **174 ± 59**[e] | **0.015** |
| Respiratory ratio | 1.12 ± 0.43[a] | 1.72 ± 0.77[a,b,c,d] | 1.09 ± 0.30[b] | 1.37 ± 0.59[c] | 1.04 ± 0.25[d] | **<0.001** |

Data are expressed as means ± SD. Bold characters indicate statistical significant differences; superscript letters indicate significant pairwise post hoc differences; $V_{tot}$: total ventilation; (A-a) $O_2$: Bohr's DS: physiological dead space, as calculated by the Bohr equation, which, by quantifying the ratio of physiological dead space to the total tidal volume ($V_d/V_t = PaCO_2 - PaCO_2/PaCO_2$), gives an indication of the extent of wasted ventilation; (A-a) $O_2$: $O_2$ alveolar-arterial gradient; $V_t$: tidal volume; $Q_s/Q_t$: pulmonary functional shunting; $V_{O_2}$: oxygen uptake; $V_{CO_2}$: carbon dioxide production; *values were calculated according to the formula by Sorbini et al. accounting for hypocapnia: standard $PaO_2 = 1.66 \times PaCO_2 + PaO_2 - 66.4$ [52]; respiratory ratio: respiratory exchange ratio, that is, $V_{CO_2}/V_{O_2}$. See text for further methodology details.

Table 3: Redox/antioxidant status in patients with typical Rett syndrome: systemic oxidative stress with decreased GSH/GSSG ratio.

| Redox and antioxidant markers | Rett syndrome ($n = 228$) | Healthy controls ($n = 114$) | $P$ value |
|---|---|---|---|
| P-NPBI (nmol/mL) | 0.90 ± 0.18 | 0.43 ± 0.25 | <0.0001 |
| IE-NPBI (nmol/mL) | 1.20 ± 0.30 | 0.78 ± 0.17 | <0.0001 |
| $F_2$-IsoPs (pg/mL) | 70.1 ± 20.5 | 24.2 ± 11.5 | <0.0001 |
| GSH ($\mu$mol/L) | 1673.0 ± 591.0 | 1165.0 ± 132.0 | <0.0001 |
| GSSG ($\mu$mol/L) | 179.0 ± 73.9 | 3.55 ± 1.90 | <0.0001 |
| GSH to GSSG ratio | 10.9 ± 5.5 | 160.0 ± 61.0 | <0.0001 |

P-NPBI: plasma non-protein-bound iron; IE-NPBI: intraerythrocyte non-protein-bound iron; $F_2$-IsoPs: plasma $F_2$-isoprostanes; GSH: reduced glutathione; GSSG: oxidized glutathione.

Table 4: Results of cardiorespiratory monitoring in patients with typical Rett syndrome ($n = 228$) confirming a high frequency of apneas and hypopneas either during wakefulness or sleep states.

| Recorded events | Median events/h | Interquartile range |
|---|---|---|
| Sleep | | |
| Obstructive apneas | 6.2 | 3.4–58 |
| Central apneas | 0.4 | 0.15–0.92 |
| Mixed apneas | 1.5 | 0.4–2.5 |
| Hypopneas | 25.6 | 20.1–34.7 |
| Wakefulness | | |
| Obstructive apneas | 17.7 | 4.9–11.38 |
| Central apneas | 0.7 | 0.08–1.07 |
| Mixed apneas | 1.7 | 0.92–2.4 |
| Hypopneas | 22 | 12.7–26 |

Apnoeas were defined as a >90% airflow decrease for ≥10 sec; hypopnoeas were defined as a >50% airflow reduction for ≥10 sec associated with a decrease of ≥3% in oxygen saturation [51]. Obstructive apneas refer to recorded events with cessation of airflow for ≥10 sec associated with persistent respiratory effort; central apneas refer to events characterized by cessation of airflow for ≥10 sec without associated respiratory effort; mixed apneas refer to respiratory events that begin as central apneas and end up as obstructive apneas.

Table 5: Correlation matrix between redox/antioxidant status and severity of recorded apnoeas, either during wakefulness or sleep, in patients with typical Rett syndrome ($N = 228$).

| Redox and antioxidant markers | Apnoeas | | |
|---|---|---|---|
| | Mild | Moderate | Severe |
| P-NPBI | 0.185 (0.1431) | 0.176 (0.1631) | **0.265 (0.0346)** |
| IE-NPBI | **0.324 (0.0089)** | **0.373 (0.0024)** | **0.358 (0.0037)** |
| $F_2$-IsoPs | 0.2220 (0.0800) | 0.225 (0.0744) | **0.305 (0.0142)** |
| GSH | **0.252 (0.0449)** | 0.101 (0.4262) | 0.183 (0.1485) |
| GSSG | **0.258 (0.0392)** | **0.260 (0.0378)** | **0.267 (0.033)** |
| GSH/GSSG ratio | −0.210 (0.0961) | **−0.247 (0.0488)** | −0.241 (0.0552) |

Data are expressed as rank correlation rho coefficients with $P$ values in brackets. Bold characters indicate statistically significant associations. Apnoeas were defined as a >90% airflow decrease for ~10 sec; hypopneas were defined as a >50% airflow reduction for ≥10 sec associated with a decrease of ≥3% in oxygen saturation [50]. Apnoeas were further categorized as mild (10 to 15 sec), moderate (15 to 30 sec), and severe (>30 sec) on the basis of their recorded duration. Legends: P-NPBI: plasma non-protein-bound iron; IE-NPBI: intraerythrocyte non-protein-bound iron; $F_2$-IsoPs: plasma $F_2$-isoprostanes; GSH: reduced glutathione; GSSG: oxidized glutathione.

to the most severe events only. A significant inverse relationship between moderate apneas and GSH to GSSG ratio was present (rho: −0.247, $P = 0.0488$). An average number of >7.4/h for total apneas, >0.8/h for moderate apneas, and >2 for severe apneas (either recorded during wakefulness or sleep states) were found to be predictive for increased IE-NPBI plasma levels in patients, with 50% to 80.7% sensitivity, 55.3% to 82.02% specificity, 55.3% to 65% positive predictive value, and 62.7% to 80.8% negative predictive value ($P$ values for the AUC, range: 0.0044 to 0.0163) (Table 6). In contrast, frequency/severity of the recorded apneas was not predictive for plasma levels of $F_2$-IsoPs in RTT patients ($P$ values for the AUCs, range: 0.3149 to 0.9487).

*3.6. Relationship between Redox Imbalance and Pulmonary Gas Exchange Abnormality.* When evaluated as a function of GEA in RTT, striking differences in the redox/antioxidant markers levels were detectable among the different categories of pulmonary $V/Q$ inequality, ranging from values comparable to those of the control group in the "no mismatch" group to significant redox/antioxidant imbalance in the various GEA patterns (Table 7). In particular, $F_2$-IsoPs plasma levels were approximately proportional to the degree of severity for $V/Q$ abnormality, with increase of ~1.7-fold for "simple mismatch," ~2.4-fold for "low" patterns, ~2.8-fold for "high" patterns, and ~3.7-fold for "mixed" patterns, as compared to patients without detectable GEA.

*3.7. Mutant Mecp2 Murine Lung Histology.* The results of lung histology in the *Mecp2* null RTT mouse models showed a picture of nonspecific lymphocytic bronchiolitis associated with lymphocytic vasculitis (Figures 3(b) and 3(d)) and desquamative alveolitis in a half of the examined mutant mice, whereas no significant histological abnormalities were observed in the wt animals.

Table 6: Frequency/severity of apneas, recorded during either the wakefulness or sleep, identifies Rett patients with increased intraerythrocyte non-protein-bound iron (IE-NPBI) levels: receiver operating characteristic (ROC) curves analyses.

| Variable | AUC ± SE | 95% C.I. | *P*-value | Criterion | Sens.% | Spec.% | +LR | −LR | +PV | −PV |
|---|---|---|---|---|---|---|---|---|---|---|
| Total apneas/h | **0.690 ± 0.0669** | **0.563–0.799** | **0.0044** | **>7.4** | **53.8** | **82.05** | **1.5** | **0.89** | **56.2** | **62.7** |
| Mild apneas/h | 0.634 ± 0.0714 | 0.504–0.751 | 0.0605 | >1 | 30.7 | 89.5 | 1.54 | 0.51 | 51.4 | 74.1 |
| Moderate apneas/h | **0.664 ± 0.0687** | **0.563–0.778** | **0.0163** | **>0.8** | **80.7** | **55.3** | **1.81** | **0.35** | **55.3** | **80.8** |
| Severe apneas/h | **0.670 ± 0.0693** | **0.541–0.782** | **0.0142** | **>2** | **50** | **81.6** | **2.71** | **0.61** | **65** | **70.5** |

AUC: area under the curve; SE: standard error; Sens.: sensitivity; Spec: specificity; +LR: positive likelihood ratio; −LR: negative likelihood ratio; +PV: positive predictive value; −PV: negative predictive value. Bold characters indicate statistically significant items.

Table 7: Relationships between lung ventilation/perfusion ($V/Q$) patterns and the redox/antioxidant status in patients with typical Rett syndrome ($n = 228$).

| Redox and antioxidant markers | Pulmonary ventilation/perfusion ($V/Q$) patterns in typical Rett syndrome | | | | | *P* value (ANOVA) |
|---|---|---|---|---|---|---|
| | No mismatch ($N = 44$) | "Low" ($N = 64$) | "High" ($N = 73$) | "Mixed" ($N = 36$) | "Simple" mismatch ($N = 11$) | |
| P-NPBI (nmol/mL) | **0.50 ± 0.32$^{a}$** | **0.86 ± 0.07$^{a,b,c}$** | **0.91 ± 0.15$^{a}$** | **1.02 ± 0.22$^{a,b,c}$** | **0.71 ± 0.05$^{b,c}$** | **<0.001** |
| IE-NPBI (nmol/mL) | **0.80 ± 0.24$^{a}$** | **1.04 ± 0.05$^{a,b}$** | **1.20 ± 0.21$^{a,b}$** | **1.30 ± 0.49$^{a,b}$** | **0.96 ± 0.13$^{b}$** | **<0.001** |
| $F_2$-IsoPs (pg/mL) | **27.3 ± 11.1$^{a}$** | **65.2 ± 14.4$^{a,b}$** | **76.5 ± 13.2$^{a,b}$** | **100.8 ± 11.4$^{a,b}$** | **46.2 ± 7.9$^{b}$** | **<0.001** |
| GSH ($\mu$mol/L ) | **1206 ± 140$^{a}$** | **1867 ± 759$^{a,b}$** | **1794 ± 507$^{a,b}$** | **1442 ± 373$^{a,b}$** | **1419 ± 523$^{b}$** | **<0.001** |
| GSSG ($\mu$mol/L ) | **8.0 ± 3.4$^{a}$** | **193.6 ± 85.3$^{a,b}$** | **222.5 ± 61.5$^{a,b}$** | **132.0 ± 25.9$^{a}$** | **144.3 ± 71.4$^{b}$** | **<0.001** |
| GSH/GSSG ratio | **175 ± 83$^{a}$** | **12.2 ± 7.8$^{a}$** | **8.2 ± 1.9$^{a}$** | **11.6 ± 5.1$^{a}$** | **11.1 ± 5.2$^{a}$** | **<0.001** |

Data are expressed as means ± SD. Bold characters indicate statistical significant differences; superscript letters indicate significant pairwise post hoc differences; P-NPBI: plasma non-protein-bound iron; IE-NPBI: intraerythrocyte non-protein-bound iron; $F_2$-IsoPs: plasma $F_2$-isoprostanes; GSH: reduced glutathione; GSSG: oxidized glutathione.

## 4. Discussion

Respiratory dysfunction in RTT appears to be far more complex than previously thought. Specifically, the findings of the present study indicate that, besides brainstem dysfunction, several intertwined critical factors, either directly or indirectly related to the disease, appear to concur to adversely affect respiratory function in RTT patients. In particular, our findings strongly support the hypothesis that the respiratory behavior in RTT, historically credited to neurological dysfunction, can be considered as the result of a previously unrecognized inflammatory process and/or abnormal immune response [55].

Abnormal pulmonary gas exchange, as the result of the imbalance between $V$ and $Q$, is a main cause of hypoxemia [56]. While confirming the coexistence of a relative hypoxia (−17.6 ± 1.5% as compared to a cohort of healthy controls; 95% C.I.: −14.66 to −20.54) and abnormal pulmonary gas exchange in over 3/4 of the patients with typical RTT, we identified for the first time the relative distribution for the different GEA patterns. GEA was detectable in ~80% of the RTT patients, with "high" and "low" patterns dominating over "mixed" and "simple mismatch" types of pulmonary $V/Q$ inequalities. This $V/Q$ behavior here observed could be linked to either the presence of unventilated pulmonary areas in the low patterns (i.e., unrecognized pulmonary dysventilation or microatelectasis) or unperfused areas in the high patterns (i.e., unrecognized pulmonary microembolism). Therefore, our findings strongly suggest that GEA is a key feature of respiratory dysfunction in RTT that brainstem immaturity [9, 12] and/or cardiorespiratory autonomic dysautonomia [13, 14], both repeatedly evoked in the disease, are *per se* evidently unable to explain. It is interesting to observe that several features of the respiratory behaviour observed in RTT patients, chiefly hyperventilation, can be interpreted as compensatory mechanisms rather than the effects of dysfunctional brainstem activity and/or cardiorespiratory autonomic dysautonomia. However, hyperventilation, as a likely compensatory mechanism in order to overcome the negative effects of GEA, can lead to an adverse increase of the physiological dead space, as thus reducing the fraction of ventilation that effectively participates in alveolar gas exchanges. In addition, hyperventilation can lead to severe hypocapnia, a very frequent finding in RTT, thus further decreasing the effective (i.e., normalized to $PaCO_2$) $PaO_2$ levels. The observed decrease in $V_{O_2}$ and $V_{CO_2}$ could also be potentially regarded as possible effects of compensatory mechanisms, although more investigation is needed to understand this unexpected feature of the RTT lung pathophysiology.

OSAHS is described as repetitive obstructions of the upper airways during sleep, causing concomitant episodes of systemic hypoxia and associated cardiovascular and metabolic pathologies, with an estimated prevalence of 0.7% to 1.8% in the general paediatric population [57–62] and of 2% for adult women and 4% for adult men [63–65]. The condition can be difficult to diagnose clinically, although even mild-to-moderate obstructive sleep apnoea can result in adverse neurobehavioral consequences and negatively affect quality of life [58, 59]. Our cardiorespiratory monitoring data in a relatively large cohort of RTT patients indicate a significantly increased prevalence of OSAHS in this patient population (i.e., 27.2%) and confirm the presence of

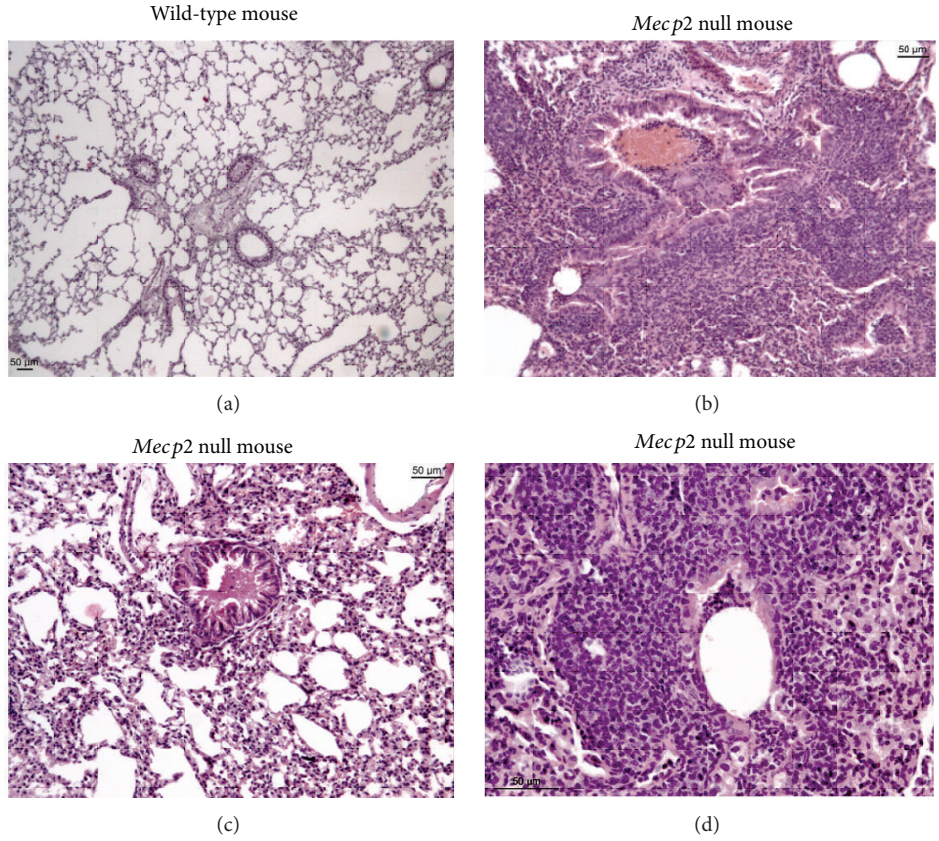


Figure 3: Lymphocytic bronchiolitis and desquamative alveolitis in *Mecp2* null mice. (a) Wild-type mouse lung: normal histological features (magnification 25x), (b) *Mecp2* null mouse: peribronchiolar lymphocyticinfiltrate (magnification 50x), (c) *Mecp2* null mouse lung: desquamative alveolitis with mild amount of alveolar exudate (50x), and (d) *Mecp2* null mouse lung: terminal bronchiolitis at higher magnification (200x) lymphocytes and histiocytes infiltrates outside and inside thickened terminal bronchioles, with a resulting picture of lymphocytic bronchiolitis.

clinically significant apneas also during wakefulness. These findings indicate that RTT should be added to the list of the already known heterogeneous pediatric conditions carrying an increased risk for OSAHS, including Down's syndrome, neuromuscular disease, craniofacial abnormalities, achondroplasia, mucopolysaccharidoses, and Prader-Willi syndrome. Although the overwhelming majority of previous polysomnographic studies in RTT patients have reported a prevalence of apnoeas of central origin [15–26], our findings indicate that obstructive apnoeas are far more common in typical RTT than previously reported.

Human and experimental evidence indicate that OSAHS and intermittent hypoxia can be associated with enhanced OS, although conflicting reports exist [66–76]. In the present study, we confirm the coexistence of a significant redox abnormality in RTT patients. However, the relationship between upper airways obstruction/intermittent hypoxia and OS status in RTT appears to be limited to the generation of a prooxidant status, as indicated by the link here observed between IE-NPBI, but not $F_2$-IsoPs, and apneas. Iron is a major player in redox reactions, as it has been known for a long time that redox-active iron is one of the most active sources of OS [77]. Furthermore, iron release is much higher under hypoxic conditions than under normoxia [78, 79], so that hypoxia paradoxically represents a condition of OS that is consistent with a condition of ischemia-reperfusion injury. Released iron can diffuse out of the erythrocytes and the diffusion is higher with hypoxic erythrocytes [79]. Intermittent hypoxia can affect the stability of the bond of iron to the tetrapyrrole ring of protoporphyrin, thus releasing iron inside the erythrocytes, along with hemoglobin autoxidation [80]. However, it becomes clear that mechanisms other than intermittent hypoxia should be the major sources of enhanced OS in RTT. Actually, in the present study, we observed a very intimate relationship between redox abnormality and GEA in RTT. These results suggest that chronic, rather than intermittent, hypoxia resulting from a pulmonary $V/Q$ inequality is likely the main source of systemic OS in RTT. To this regard, it is of relevance that we detected a dramatic increase of GSSG in RTT patients, with a parallel dramatic decrease of the GSH/GSSG ratio, thus strongly suggesting the coexistence of a chronic OS status.

While future studies are obviously needed to address this major topic, several other factors apparently concur to

adversely affect the respiratory function and, either directly or indirectly, contribute to pulmonary gas exchanges impairment in RTT patients. These factors likely include abnormal erythrocyte shape with oxidative membrane damage [81], microvascular dysplasia [82], alterations in the vascular/endothelial system [83], mitochondrial dysfunction [84–87], subclinical biventricular myocardial dysfunction [88], systemic oxidative stress [29, 35], and, chiefly, subclinical inflammatory processes [38, 55].

Over the last decade, several experimental animal models have been developed in which the *Mecp2* allele has been modified to prevent production of a fully functional Mecp2 protein. In particular, several experimental mouse models of Mecp2 deficiency have been established in mice, ranging from null-*Mecp2* mutations to specific point mutations mimicking those observed in humans, phenocopying several motor and cognitive features of RTT patients. In particular, the irregular breathing pattern observed in human RTT has been replicated in several mutant mouse models to varying degrees of fidelity, although the corresponding respiratory phenotype varies among different mouse strains [89–91], with mutations in the *Mecp2* gene leading to disparate respiratory phenotypes. For instance, in *Mecp2*tm1.1Jae null (hemizygous) mice on a mixed-strain background [92], the principal phenotype is tachypnea along with hyperventilation similar to human RTT [93], whereas in *Mecp2*tm1.1Bird null or heterozygous mice on a pure C57BL/6J background [94] the principal phenotype is repetitive spontaneous central apnea [95–97], whereas the *Mecp2*tm1.1Bird male mice provide an excellent animal model of spontaneous central apnea and possibly obstructive apnea [28]. A more recent study indicates that a clinically relevant RTT endophenotype, that is, tachypnea with a shortened expiratory time, appears to be more faithfully reproduced in Mecp2tm1.1Jae female mice [98]. Despite the fact that *MeCp2* mutant mouse models cannot model all aspects of the human RTT, certainly they do recapitulate many aspects of the disease and are generally accepted as excellent tools to study MeCP2 function. In this context, our findings of a lymphocytic bronchiolitis in a half of the examined *Mecp2*-null mice are highly suggestive of a previously unrecognized inflammatory lung disease in RTT patients and are well fitting with our prior observations of radiological features at high-resolution computed tomography partially overlapping with those of RB-ILD [38] and including micronodules (i.e., inflammatory infiltrates in the smaller airways, such as terminal bronchioles and/or alveoli), "ground glass opacities" (i.e., radiological signs of alveolar inflammation), and, remarkably, thickening of bronchiolar walls (a radiological sign of inflammatory infiltrates in the terminal bronchioles). Further studies are obviously needed in different *Mecp2*-mutant animal models in order to ascertain prevalence and possible differences related to different mouse strains.

Cumulating evidence indicates that RTT is a multisystemic disease, which, besides the brain, is known to affect several organs and systems, including the autonomic nervous system [12–14], microvascular/endothelial system [12, 13], bone [99], heart [88, 100], red blood cells [81], the gastrointestinal tract [101], and the immune system [102, 103]. Our study strongly supports the concept that the lung is a previously unrecognized major target organ in this genetically determined neurodevelopmental disease and that pulmonary GEA is likely a key feature within the multisystemic characteristics of the disease.

## 5. Conclusions

The findings of the present clinical study confirm the emerging concept according to which no single putative mechanism can account for all the complexity of the respiratory behaviour exhibited by RTT patients. The present study indicates that (1) pulmonary GEA, not brainstem immaturity, is likely the key feature of respiratory dysfunction in RTT; (2) the RTT-related GEA is likely the result of several contributing factors, involving OS and chronic subclinical inflammation; and (3) terminal bronchioles and alveoli are likely a major, under-recognized, inflammatory target of the disease. Moreover, these data confirm the clinical relevance of respiratory dysfunction in this rare neurological disease as a valuable pathophysiological model for a better understanding of the complex involvement of the lung in a multisystemic disease. Our findings strongly support the hypothesis that the respiratory behavior in RTT, historically credited to neurological dysfunction, is rather the result of an inflammatory process and/or abnormal immune response.

## Abbreviations

(A-a) $O_2$: $O_2$ alveolar-arterial pulmonary gradient
AHI: Apnea-hypopnea index
ASDs: Autism Spectrum Disorders
AUC: Area under the curve
Bohr's DS: Physiological dead space as calculated by the Bohr equation
*CDKL5*: Cyclin-dependent kinase-like 5
$F_2$-IsoPs: $F_2$-isoprostanes
*FOXG1*: Forkhead box G1
GEA: Lung gas exchange abnormality
GSH: Reduced glutathione
GSSG: Oxidized glutathione
IE-NPBI: Intraerythrocyte non-protein-bound iron
MeCP2: Methyl-CpG-binding protein 2
OS: Oxidative stress
OSAHS: Obstructive sleep apnea-hypopnea syndrome
$PaCO_2$: Partial arterial pressure of $CO_2$
$PaO_2$: Partial arterial pressure of $O_2$
P-NPBI: Plasma non-protein-bound iron
RB-ILD: Respiratory bronchiolitis-associated interstitial lung disease
$Q_s/Q_t$: Pulmonary functional shunting (i.e., venous admixture)
RTT: Rett syndrome
$V/Q$: Lung ventilation-to-perfusion ratio
$V_t$: Tidal volume
$V_{tot}$: Total ventilation

$V_{CO_2}$: $CO_2$ production
$V_{O_2}$: $O_2$ uptake.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Claudio De Felice and Marcello Rossi contributed equally to this work, and Tommaso Pizzorusso and Joussef Hayek equally supervised this work.

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*Research Article*

# Effects of $\omega$-3 PUFAs Supplementation on Myocardial Function and Oxidative Stress Markers in Typical Rett Syndrome

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Rett syndrome (RTT) is a devastating neurodevelopmental disorder with a 300-fold increased risk rate for sudden cardiac death. A subclinical myocardial biventricular dysfunction has been recently reported in RTT by our group and found to be associated with an enhanced oxidative stress (OS) status. Here, we tested the effects of the naturally occurring antioxidants $\omega$-3 polyunsaturated fatty acids ($\omega$-3 PUFAs) on echocardiographic parameters and systemic OS markers in a population of RTT patients with the typical clinical form. A total of 66 RTT girls were evaluated, half of whom being treated for 12 months with a dietary supplementation of $\omega$-3 PUFAs at high dosage (docosahexaenoic acid ~71.9 ± 13.9 mg/kg b.w./day plus eicosapentaenoic acid ~115.5 ± 22.4 mg/kg b.w./day) versus the remaining half untreated population. Echocardiographic systolic longitudinal parameters of both ventricles, but not biventricular diastolic measures, improved following $\omega$-3 PUFAs supplementation, with a parallel decrease in the OS markers levels. No significant changes in the examined echocardiographic parameters nor in the OS markers were detectable in the untreated RTT population. Our data indicate that $\omega$-3 PUFAs are able to improve the biventricular myocardial systolic function in RTT and that this functional gain is partially mediated through a regulation of the redox balance.

## 1. Introduction

Rett syndrome (RTT) is a genetically determined, neurodevelopmental disorder with autistic features [1, 2]. Although relatively rare, RTT represents the second most common cause of severe intellective disability in the female gender. To date, the disease has been classified into a typical form and three main atypical variants, that is, preserved speech, early seizure, and congenital.

In up to 95% of cases, RTT is caused by *de novo* mutation in the X-linked gene encoding MeCP2, a protein known to either activate or repress several transcriptional genes [3, 4].

Cumulating evidence indicates that RTT, for a long time considered exclusively a disease of the brain, is actually a systemic disease with involvement of several organs besides the brain, including autonomic nervous system, lung, bone, and heart [5–8]. Girls affected by typical RTT show a 300-fold increased risk for sudden cardiac death as compared to general population (about 26% of all deaths are sudden and of unknown cause [9]), although a satisfactory explanation for the association is still missing. In the lack of evidence for an increased prevalence of congenital heart defects, the attention of several authors has been focused on the presence of cardiac dysautonomia and rhythm abnormalities. In particular, a

Table 1: Phenotypical severity, biometrics, bone densitometry estimates, and 25-hydroxy vitamin D serum levels were comparable between the $\omega$-3 PUFAs-supplemented and the untreated Rett patients subgroups.

| Variables | Rett syndrome population | | $P$ value |
|---|---|---|---|
| | $\omega$-3 PUFAs supplemented ($n = 33$) | Untreated ($n = 33$) | |
| Age (years) | 13.0 ± 8.6 | 12.4 ± 9.3 | 0.7864 |
| Clinical severity score (CSS) | 26.2 ± 11.2 | 26.0 ± 11.1 | 0.9421 |
| Height (RTT $z$-score for age)[1] | 0.078 ± 0.924 | −0.025 ± 1.47 | 0.7344 |
| Body weight (RTT $z$-score for age)[1] | −0.027 ± 1.026 | −0.02 ± 1.2 | 0.9798 |
| Body mass index (RTT $z$-score for age)[1] | −0.30 ± 1.55 | −0.40 ± 1.6 | 0.7973 |
| Head circumference (RTT $z$-score for age)[1] | −0.07 ± 1.23 | 0.01 ± 0.99 | 0.7719 |
| Heart rate (bpm) | 91 ± 17 | 93 ± 15 | 0.6141 |
| Systolic blood pressure (mmHg) | 107.8 ± 8.2 | 107.1 ± 9.3 | 0.7468 |
| Diastolic blood pressure (mmHg) | 70.2 ± 11.6 | 69.9 ± 8.9 | 0.9065 |
| Bone densitometry | | | |
| AD-SoS ($z$-score for age) | −2.86 ± 1.75 | −2.74 ± 1.81 | 0.7951 |
| BTT ($z$-score for age) | −1.87 ± 1.93 | −1.9 ± 1.85 | 0.9488 |
| Serum 25-OH vitamin D (ng/mL) | 45.1 ± 26.1 | 46.5 ± 25.8 | 0.8307 |

[1] $z$-scores are referred to validated Rett syndrome-specific growth charts [27].
AD-SoS: amplitude-dependent speed of sound; BTT: bone transmission time.
AD-SoS and BTT were evaluated by quantitative ultrasound (QUS) of the distal end of the first phalanx diaphysis of the last four fingers of the hand.

prolonged QT interval, an indicator of a repolarization abnormality and a well-established risk factor for sudden cardiac death [10], is reported in nearly 20% of patients with Rett syndrome [9, 11–13]. Abnormally persistent sodium currents have been reported in cardiomyocytes from *Mecp2*$^{\text{Null/y}}$ mice and found to be normalized by the sodium channel-blocking antiepileptic drug phenytoin, which strongly suggests a brain-heart link as a possible explanation for the increased risk of sudden death in RTT [13]. However, our recent observation of a subclinical myocardial biventricular dysfunction in a large series of typical and atypical RTT patients may add new perspectives to the heart involvement in this neurodevelopmental disease [14].

Evidence of enhanced oxidative stress (OS) and, in particular, lipid peroxidation has been well established by our group in blood samples from patients with RTT and recently confirmed in primary skin fibroblasts cultures [15–21]. However, the molecular pathways linking the *MeCP2* gene mutation to the OS derangement remain to be explored and, in particular, whether the nature of the relationship between *MeCP2* gene mutation and abnormal redox homeostasis is causal or correlational remains currently unclear [22].

At the same time, experimental models have shown that OS is detrimental for myocardial function [23, 24]. Therefore, we speculate that OS may play a role in the myocardial dysfunction of RTT patients.

Omega-3 polyunsaturated fatty acids ($\omega$-3 PUFAs) are natural molecules with a wide range of physiological functions on multiple tissues including the heart. In particular, $\omega$-3 PUFAs are able to affect a myriad of molecular pathways, including alteration of physical and chemical properties of cellular membranes, direct interaction with and modulation of membrane channels and proteins, regulation of gene expression via nuclear receptors and transcription factors, changes in eicosanoid profiles, and conversion of $\omega$-3 PUFAs to bioactive metabolites [25].

$\omega$-3 PUFAs have gained increasing attention in the prevention of cardiovascular disease, although their biological effects and molecular mechanisms are highly debated [25].

In previous studies, we have demonstrated that supplementation of $\omega$-3 PUFAs moderately reduces clinical severity and significantly reduces the levels of several OS biomarkers in the blood of RTT patients [17, 19, 26].

The aim of the present study was to assess the effects of 12 months of dietary supplementation with high-dose $\omega$-3 PUFAs on the RTT-related subclinical myocardial dysfunction.

## 2. Methods

*2.1. Patients.* In this study, a total of 66 RTT patients (mean age 12.7 ± 9.1 years) with typical presentation and demonstrated *MeCP2* mutation were enrolled (Table 1) [27]. RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [28, 29]. RTT clinical severity was assessed using the clinical severity score (CSS), a validated clinical rating specifically designed for RTT, based on 13 individual ordinal categories measuring clinical features common in RTT [28]. All the patients were admitted to Child Neuropsychiatric Unit, University Hospital Azienda Ospedaliera Universitaria Senese (Head Dr. Joussef Hayek). Blood samplings in the patients' group were performed during the routine follow-up study at hospital admission. Sampling from the control group was carried out during routine health checks, sports, or blood donations obtained during the periodic clinical checks. The study was conducted with the approval of the Institutional Review

Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

*2.2. Study Design.* The experimental design was single centre, single blind, and randomized. Patients were randomized at admission ($n = 33$ treated, mean age at supplementation time zero: 13.0 ± 8.6 years; $n = 33$ untreated, mean age at time zero: 12.4 ± 9.3 years) to either oral supplementation with $\omega$-3 PUFAs oil for twelve months or no treatment.

Administered $\omega$-3 PUFAs were in the form of fish oil (Norwegian Fish Oil AS, Trondheim, Norway, Product Number HO320-6; Italian importer: Transforma AS Italia, Forlì Italy; Italian Ministry Registration Code: 10 43863-Y) at a dose of 5 mL twice daily, corresponding to docosahexaenoic acid (DHA) 71.9 ± 13.9 mg/kg b.w./day and eicosapentaenoic acid (EPA) 115.5 ± 22.4 mg/kg b.w./day, with a total $\omega$-3 PUFAs 242.4 ± 47.1 mg/kg b.w./day. Use of EPA plus DHA in RTT was approved by the AOUS Ethical Committee.

All the subjects, included patients, examined in this study were following a standard Mediterranean diet.

*2.3. Echocardiography.* The study was performed using a commercially available echocardiography equipment (Philips IE 33 Vision 2009, qLAB 7.0 software; 5 and 8 MHz transducers) as previously reported [14]. Briefly, two-dimensional right and left chambers quantification (areas and volumes), left ventricle ejection fraction (Simpson's method), and pulmonary arterial systolic pressure (PASP) were estimated. Mitral flow velocities ($E$ wave, $A$ wave, and $E/A$ ratio) were recorded using pulsed wave (PW) Doppler on the mitral valve. The evaluation of left and right ventricular longitudinal systolic function was performed by (a) mitral annular plane systolic excursion (MAPSE) and tricuspid annular plane systolic excursion (TAPSE), using M-mode, and (b) systolic ($S'$) and early diastolic ($E'$) peak velocities, using PW tissue Doppler imaging (TDI) of the lateral (lat) and septal (sep) mitral annulus for left ventricle (LV) and of tricuspidal annulus for right ventricle (RV) in four-chamber apical view. The $E/E'_{\text{lat}}$ ratios were determined as surrogate of LV filling pressures.

In order to reduce operator-dependent bias, all measures were performed by two operators, blinded for clinical and therapeutical data of RTT group.

*2.4. Blood Sampling.* Blood sampling was carried out in all subjects at around 8.00 am after the overnight fast. For the $\omega$-3 PUFAs treated group, blood sampling was performed the day before starting the supplementation and the day after the end of the selected 12-month period.

Blood was collected in heparinized tubes, and all manipulations were carried out within 2 h after collection. Blood samples were centrifuged at 2,400 g for 15 min at room temperature. The platelet poor plasma was saved, and the buffy coat was removed by aspiration. The erythrocytes were washed twice with physiological solution, resuspended in ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$), pH 7.4 as a 50% (vol/vol) suspension, and then used for the determination of erythrocyte non protein-bound iron (NPBI).

Plasma was used for free isoprostanes ($F_2$-isoprostanes, $F_2$-IsoPs, and $F_4$-neuroprostanes, $F_4$-NeuroPs), 4-hydroxnonenal protein adducts (4-HNE PAs), and NPBI determinations. For all isoprostane determinations, butylated hydroxytoluene (BHT) (90 $\mu$M) was added to plasma as an antioxidant and stored under nitrogen at −70°C until analysis.

*2.5. Intraerythrocyte and Plasma NPBI.* NPBI is a pro-oxidant factor, associated with hypoxia, hemoglobin oxidation, and subsequent heme iron release [30]. Intraerythrocyte and plasma NPBI were determined as a desferrioxamine (DFO)-iron complex by high-performance liquid chromatography, as previously reported [15].

*2.6. Plasma Isoprostanes.* Isoprostanes are considered the gold standard for the OS *in vivo* evaluation [31, 32]. Specifically, $F_2$-IsoPs are the end products of arachidonic acid oxidation, a polyunsaturated fatty acid which is abundant in both brain grey and white matter. $F_4$-NeuroPs are the end products of docosahexaenoic acid, abundant in neuronal membranes. Plasma $F_2$-IsoPs and $F_4$-NeuroPs were determined by a gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis after solid-phase extraction and derivatization steps [33, 34].

For $F_2$-IsoPs, the measured ions were the product ions at m/z 299 and m/z 303 derived from the $[M\text{-}181]^-$ precursor ions (m/z 569 and m/z 573) produced from 15-$F_{2t}$-IsoPs and $PGF_{2\alpha}$-$d_4$, respectively [34]. For $F_4$-NeuroPs, the measured ions were the product ions at m/z 323 and m/z 303 derived from the $[M\text{-}181]^-$ precursor ions (m/z 593 and m/z 573) pro duced from oxidized DHA and the $PGF_{2\alpha}$-$d_4$, respectively [19].

*2.7. Plasma 4-HNE PAs.* Plasma 4-hydroxynonenal protein adducts (4-HNE PAs) are markers of protein oxidation due to aldehyde binding from lipid peroxidation sources [35]. Western blot protocols were performed as previously described [18].

Plasma proteins (30 $\mu$g protein) were resolved on 4–20% SDS-PAGE gels (Lonza Group Ltd., Switzerland) and transferred onto a hybond ECL nitrocellulose membrane (GE Healthcare Europe GmbH, Milan, Italy). After blocking in 3% nonfat milk (BioRad, Hercules, CA, USA), the membranes were incubated overnight at 4°C with goat polyclonal anti-4-HNE adduct antibody (code AB5605; Millipore Corporation, Billerica, MA, USA). Following washes in TBS Tween and incubation with specific secondary antibody (mouse anti-goat horseradish peroxidase-conjugated, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at RT, the membranes were incubated with ECL reagents (BioRad, Hercules, CA, USA) for 1 min. The bands were visualized by autoradiography.

Quantification of the significant bands was performed by digitally scanning the amersham hyperfilm ECL (GE Healthcare Europe GmbH, Milan, Italy) and measuring immunoblotting image densities with ImageJ software.

*2.8. Statistical Analysis.* All variables were tested for normal distribution (D'Agostino-Pearson test). Differences between

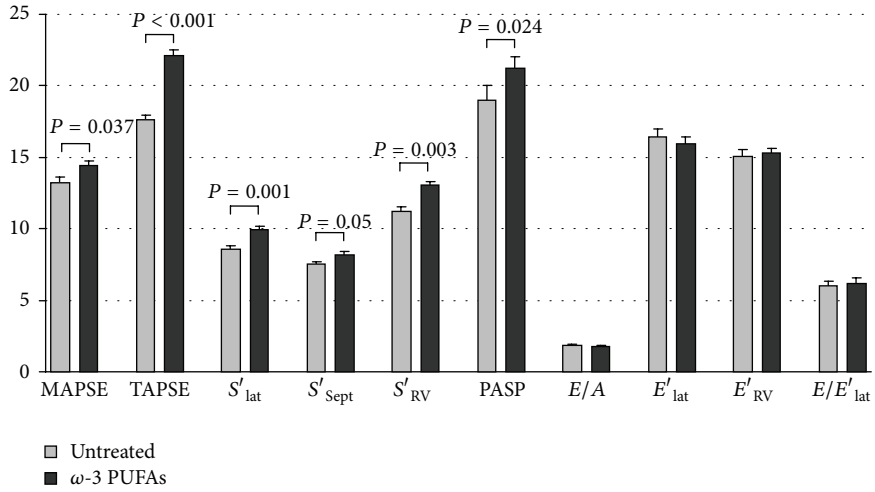


Figure 1: Dietary $\omega$-3 PUFAs supplementation for 12 months significantly improves biventricular systolic longitudinal parameters in girls with typical Rett syndrome. MAPSE: mitral annular plane systolic excursion; TAPSE: tricuspid annular plane systolic excursion; $S'_{\text{lat}}$: peak systolic velocity of lateral mitral annulus; $S'_{\text{sep}}$: peak systolic velocity of septal mitral annulus; $S'_{\text{RV}}$: peak systolic velocity of tricuspid annulus of right ventricle; PASP: pulmonary arterial systolic pressure; $E/A$: ratio between peak early diastolic mitral flow ($E$) and peak late diastolic mitral flow ($A$); $E'_{\text{lat}}$: peak early diastolic velocity of lateral mitral annulus; $E'_{\text{RV}}$: peak early diastolic velocity of tricuspid annulus of right ventricle; $E/E'_{\text{lat}}$: this ratio indirectly estimates left ventricle end-diastolic filling pressure.

groups were evaluated using independent-sample $t$-test (continuous normally distributed data), Mann-Whitney rank sum test (continuous nonnormally distributed data), and Kruskal-Wallis test. Associations between variables were tested by nonparametric univariate regression analysis. Two-tailed $P$ values of less than 0.05 were considered significant. The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

## 3. Results

*3.1. Effect of 12-Month $\omega$-3 PUFA Supplementation on Myocardial Function.* All patients of the $\omega$-3 PUFAs arm of the study completed the 12-month supplementation and no side effects were observed. Phenotypical severity, biometric data, and bone densitometry estimates as well as serum 25-OH vitamin D levels were found to be comparable between the $\omega$-3 PUFAs-supplemented and unsupplemented RTT subpopulations (Table 1) [27].

Following $\omega$-3 PUFAs (EPA plus DHA) supplementation, significant improvements in systolic longitudinal parameters of both ventricles were observed (Figure 1), along with increased PASP. On the other hand, no significant changes in the echocardiographic parameters were detectable in the untreated RTT patients.

*3.2. OS Markers.* Following 12 months of $\omega$-3 PUFAs supplementation, NPBI, plasma $F_2$-IsoPs, and $F_4$-NeuroPs were significantly reduced as compared to time $0'$ values (Figures 2(a)–2(d)). No significant changes were observed for 4-HNE PAs values (Figure 2(e)). Significant differences were already observed in the treated group at time of 6 months for plasma NPBI, intraerythrocyte NPBI, and plasma $F_2$-IsoPs.

The correlation matrix for OS markers and myocardial function variables in Rett syndrome following $\omega$-3 PUFAs supplementation is reported in Table 2. Plasma $F_2$-IsoPs and $F_4$-NeuroPs and 4-HNE PAs were found to be inversely related to the left ventricular systolic function parameters.

On the other hand, no significant changes in OS markers were detectable in the untreated RTT patients (data not shown).

In the $\omega$-3 PUFAs-supplemented group, clinical severity decreased to 25.5 and 30.1% at 6 and 12 months, respectively (CSS at time zero: $26.2 \pm 11.2$; CSS at time 6 months: $19.52 \pm 8.7$; CSS at time 12 months: $18.3 \pm 7.8$; ANOVA $P < 0.005$; pairwise comparisons time 0 > time 6 months = time 12 months). Conversely, no significant differences in clinical severity were observed in the unsupplemented group of patients (CSS at time zero: $26.0 \pm 11.1$; CSS at time 12 months: $26.5 \pm 10.9$; $P = 0.911$). Significant improvements were observed in the areas of attention, breathing abnormalities, muscular tone, ambulation, autonomic dysfunction, and somatic growth.

## 4. Discussion

Our findings indicate, for the first time, that the subclinical myocardial dysfunction observed in typical RTT can be, at least partially, rescued by 1-year high-dose $\omega$-3 PUFAs dietary supplementation. Specifically, $\omega$-3 PUFAs appear to reverse all the examined longitudinal systolic parameters (MAPSE, $S'_{\text{lat}}$, $S'_{\text{sep}}$ and TAPSE, $S'_{\text{RV}}$) of the left and right ventricles. Moreover, the improvement in the systolic myocardial function was found to be associated with a marked decrease of OS markers as determined in plasma, whereas no significant changes in the diastolic function were detectable in $\omega$-3 PUFAs-treated patients. Taken as a whole, these findings suggest that OS may play a key role in the systolic performance of the RTT myocardium and that it can be at least partially rescued by $\omega$-3 PUFAs dietary supplementation.

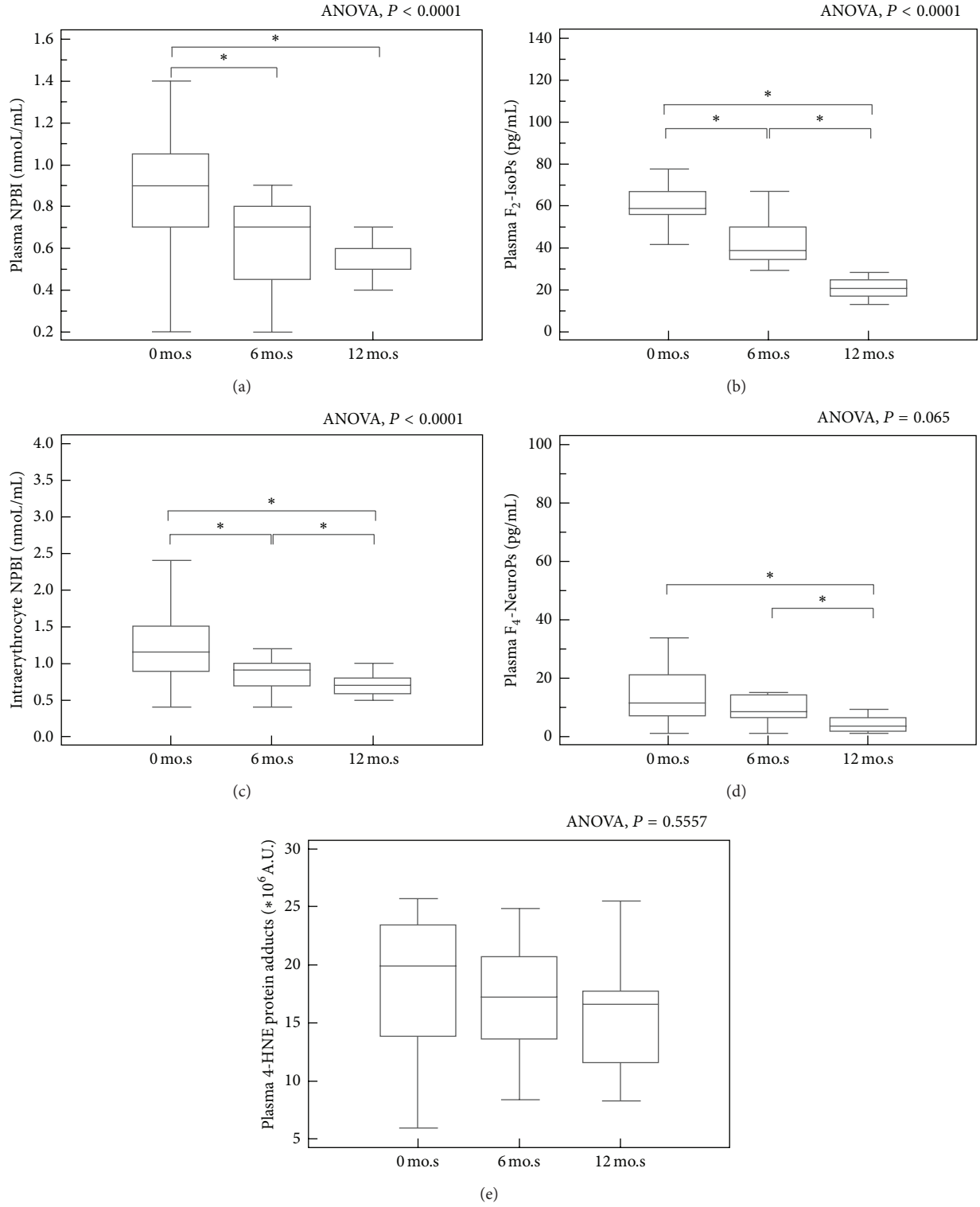


Figure 2: Oxidative stress markers levels (NPBI, plasma $F_2$-IsoPs, and $F_4$-NeuroPs) are significantly reduced in the $\omega$-3 PUFAs supplemented Rett population, as compared to basal values (Panels (a)–(d)). Conversely, no significant changes were observed for 4-HNE PAs values (Panel (e)). *denotes $P$ value < 0.05. NPBI: non protein-bound iron; $F_2$-IsoPs: plasma free $F_2$-isoprostanes; $F_4$-NeuroPs: plasma free $F_4$-neuroprostanes; 4-HNE protein adducts: 4-hydroxynonenal protein adducts; mo.s: months.

Table 2: Correlation matrix for OS markers and myocardial function variables in RTT patients following $\omega$-3 PUFAs supplementation.

| Echocardiographic variables | Plasma NPBI | Intraerythrocyte NPBI | Plasma $F_2$-IsoPs | Plasma $F_4$-NeuroPs | Plasma 4-HNE PAs |
|---|---|---|---|---|---|
| MAPSE | −0.0363 (0.7654) | 0.0707 (0.5608) | **−0.313 (0.0117)** | −0.902 (0.5466) | −0.175 (0.2552) |
| TAPSE | −0.0045 (0.9710) | −0.0809 (0.5121) | −0.194 (0.1238) | −0.176 (0.2374) | −0.0614 (0.6959) |
| $S'_{lat}$ | −0.215[§] (0.0764) | −0.155 (0.2045) | **−0.258 (0.0392)** | −0.273[§] (0.0601) | **−0.394 (0.0108)** |
| $S'_{sept}$ | −0.0884 (0.4840) | −0.0366 (0.7722) | −0.222 (0.0907) | **−0.340 (0.0240)** | −0.0433 (0.7963) |
| $S'_{RV}$ | 0.211 (0.1087) | 0.126 (0.3434) | 0.0292 (0.8338) | −0.0485 (0.7663) | 0.0941 (0.5907) |
| PASP | −0.133 (0.3086) | −0.212 (0.1007) | −0.218 (0.1073) | −0.0481 (0.7712) | 0.069 (0.6984) |

Data are Spearman's rho correlation coefficients with in brackets $P$ values ($N = 33$). Bold characters indicate statistically significant correlations. [§]indicates statistically non-significant trend. Legend: NPBI: non protein-bound iron; $F_2$-IsoPs: free $F_2$-isoprostanes; $F_4$-NeuroPs: free $F_4$-neuroprostanes; 4-HNE PAs: 4-hydroxynonenal protein adducts; MAPSE: mitral annular plane systolic excursion; TAPSE: tricuspid annular plane systolic excursion; $S'_{lat}$: peak systolic velocity of lateral mitral annulus; $S'_{sept}$: peak systolic velocity of septal mitral annulus; $S'_{RV}$: peak systolic velocity of tricuspid annulus of right ventricle; PASP: pulmonary arterial systolic pressure.

To date, among the molecular mechanisms potentially underlying the $\omega$-3 PUFAs action there are changes in membrane structures and gene expression, direct interactions with ion channels, and alterations in eicosanoid biosynthesis [36]. In particular, EPA and DHA have been reported to compete with arachidonic acid for the conversion by cytochrome P450 enzymes, thus resulting in the formation of alternative, physiologically active, metabolites [37] which could likely mediate some of their beneficial effects [38].

Our current working hypothesis on the beneficial effects of $\omega$-3 PUFAs in RTT is that the increased isoprostanes levels in RTT are not simply the effect of the peroxidation of the PUFAs precursors following the attack by radical oxygen species (ROS), but rather the effect of a potential dysregulation of the molecular targets of $\omega$-3 PUFAs. Contrary to expectations, the assumed fatty acids are not further oxidized, while the actual endogenous IsoPs production is reduced (the "fatty acid paradox") together with amelioration of the clinical disease severity [39].

Conceivably, an excess of peroxidation end products from $\omega$-6 and $\omega$-3 PUFAs would actually imply an excessive consumption of these PUFAs in the cell membranes, thus paving the way for a new perspective on the nutritional horizons in RTT. As RTT girls appear to chronically suffer from oxidation of PUFAs, either $\omega$-3 (i.e., DHA/EPA) or $\omega$-6 (i.e., AA, AdA), but, at the same time, benefit from $\omega$-3 PUFAs supplementation, it can be inferred that these patients would need $\omega$-3 PUFAs replacement as a consequence of a persistent PUFAs oxidation within the chronic OS context. On the other hand, it is also possible that, in RTT, the endogenous PUFAs are, for their own nature, more susceptible to the OS as compared to the exogenous ones. Therefore, administered PUFAs may be seen as counteracting this intrinsic defect.

Further research is needed to explore this point, although a very interesting recent report indicates that $\omega$-3 PUFAs supplementation, as fish oil, in mice with nonalcoholic fatty liver disease is able to prevent hepatic lipid accumulation and improve lipid metabolism without causing oxidative stress [40]. This report lends further support to our "fatty acids paradox" theory by generalizing it to different abnormal lipid metabolism conditions, either genetic or environmental.

A further critical new piece of research indicates that cholesterol synthesis is impaired in a mutant mouse model in RTT [40]. This latter work strongly indicates that a congenital lipid metabolism error may play a role in the RTT pathogenesis and suggests the use of statins as a potentially valuable alternative treatment for the human disease. In line with this hypothesis, we have previously described an unrecognized hypercholesterolemia in girls affected by the syndrome [41] and pointed out the possibility of an abnormal cholesterol synthesis with a likely partial block in the squalene catabolism due to coexistence of heterozygous mutations in CYP24A1 (OMIM∗126065) or TM7SF2 (OMIM∗603414), which encode the proteins CP24A and ERG24, respectively [18].

Moreover, a promising recent line of research suggests that the beneficial actions of $\omega$-3 PUFAs (or their secondary metabolites) could be related to the modulation of an unrecognized subclinical inflammatory status in RTT, a point certainly in need of further exploration, but well fitting with the known anti-inflammatory properties of $\omega$-3 PUFAs [26, 42].

On the other hand, a possible explanation for the incomplete rescue of the myocardial dysfunction in RTT could reside in the fact that MeCP2 appears to be involved in myocytes differentiation and maturation [43]. Therefore, the relationship between the MeCP2 protein and heart needs to be further evaluated in the experimental models of the disease.

## 5. Conclusion

Our data indicate that $\omega$-3 PUFAs are able to improve the subclinical biventricular myocardial systolic function observed in typical RTT and that this functional gain is partially mediated through a regulation of the redox balance.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Research Article*

# Subclinical Inflammatory Status in Rett Syndrome

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Inflammation has been advocated as a possible common central mechanism for developmental cognitive impairment. Rett syndrome (RTT) is a devastating neurodevelopmental disorder, mainly caused by *de novo* loss-of-function mutations in the gene encoding MeCP2. Here, we investigated plasma acute phase response (APR) in stage II (i.e., "pseudo-autistic") RTT patients by routine haematology/clinical chemistry and proteomic 2-DE/MALDI-TOF analyses as a function of four major *MECP2* gene mutation types (R306C, T158M, R168X, and large deletions). Elevated erythrocyte sedimentation rate values (median 33.0 mm/h versus 8.0 mm/h, $P < 0.0001$) were detectable in RTT, whereas C-reactive protein levels were unchanged ($P = 0.63$). The 2-DE analysis identified significant changes for a total of 17 proteins, the majority of which were categorized as APR proteins, either positive ($n = 6$ spots) or negative ($n = 9$ spots), and to a lesser extent as proteins involved in the immune system ($n = 2$ spots), with some proteins having overlapping functions on metabolism ($n = 7$ spots). The number of protein changes was proportional to the severity of the mutation. Our findings reveal for the first time the presence of a subclinical chronic inflammatory status related to the "pseudo-autistic" phase of RTT, which is related to the severity carried by the *MECP2* gene mutation.

## 1. Introduction

RTT (OMIM ID: 312750) occurs with a frequency of up to 1 : 10,000 live female births. Causative mutations in the X linked methyl-CpG binding protein 2 gene (*MECP2*) are detectable in up to 95% of cases, although a wide genetical and phenotypical heterogeneity is well established [1]. Approximately 80% of RTT clinical cases show the so-called "typical" clinical picture; after an apparently normal development for 6–18 months, RTT girls lose their acquired cognitive, social, and motor skills in a typical 4-stage neurological regression [2]. It has become apparent that there is a spectrum of severity in RTT, as some patients may present with atypical features, sometimes overlapping with those suggestive of autism spectrum disorders (ASDs) [3–5]. Autistic features are typically transient in RTT, although this condition has long been considered as a genetic/epigenetic model of ASDs [6, 7], RTT has been recently distinct from the ASDs group [8, 9]. Recently, the gene sequence analysis indicates that several hundreds of gene mutations appear to be associated with the *MECP2* gene mutation and, therefore, are to be considered as potential disease modifiers [10], although the genetic mechanisms of RTT have been explored to an extraordinary extent, to date the details of the biological mechanisms linking the

*MECP2* gene mutation to protein expression as a function of clinical phenotype, and yet to be clarified. In particular, with the single exception of a proteomic study on a mouse model [11], very little information exists on possible RTT-related protein changes. Although the neuropathology of RTT is well understood, the cellular and molecular mechanisms, leading to the disease initiation and progression, have yet to be elucidated.

Several lines of evidence indicate the presence of an early immune activation in ASDs with an associated peripheral and central chronic inflammation [12–16], with a particular focus on mast cells dysfunction and cytokines dysregulation [12, 14].

To date, experimental and clinical evidence has generated the idea that several serum proteins, considered as biomarkers, are strictly correlated with the pathophysiology of the autistic disorder [17–20]. In particular, significant changes in inflammation-related proteins suggested that at least some autistic children display a subclinical inflammatory state [21]. During inflammation, particularly during the APR, there is a known reduction in several proteins potentially affecting cholesterol transport and inhibiting oxidation phenomena. This protein list includes cholesterol ester transfer protein, hepatic lipase, and apolipoproteins. It is thought that reduction in these proteins, associated with an increase in positive APR proteins, may change the high density lipoprotein from anti-inflammatory into proinflammatory particles [22]. In the present study, we investigated the occurrence of a plasma APR in stage II (i.e., "pseudo-autistic") RTT patients by using routine haematology/clinical chemistry and proteomic 2-DE/MALDI-TOF analyses.

## 2. Materials and Methods

*2.1. Subjects.* The study included 25 female patients with clinical diagnosis of typical RTT (median age: 5.0 years inter-quartile range 3–6, values range 3–10 years) with demonstrated *MECP2* gene mutation (R306C ($n = 5$), T158M ($n = 5$), R168X ($n = 8$), and large deletions (deletions of exons 3 and 4, $n = 7$)) carrying different phenotype severity, and 40 age-matched healthy controls (median age: 5.0 years inter-quartile range 3–5.5, values range 3–10 years). RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [23]. Given the specific aims of the study, subjects with clinically evident inflammatory conditions either acute or chronic were excluded, as well as individuals on anti-inflammatory drugs, or undergoing supplementation with known antioxidants, such as $\omega$-3 polyunsaturated fatty acids. All the patients were consecutively admitted to the Rett Syndrome National Reference Centre of the University Hospital of the Azienda Ospedaliera Universitaria Senese (AOUS). The subjects examined in this study were on a typical Mediterranean diet. RTT clinical severity was assessed using either the total clinical severity score (CSS), a validated clinical rating specifically designed for RTT, or the "compressed" CSS both based on 13 individual ordinal categories measuring clinical features common in RTT [24], and phenotypical severity was also measured by the use of the Rett Syndrome Behaviour Questionnaire (RSBQ) [25]. Blood samplings in the patients' group were performed during the routine follow-up study at hospital admission, while the samples from the control group were carried out during routine health checks, sports, or blood donations, obtained during the periodic clinical checks. The healthy control subjects were gender (given that over 98% of RTT patients are females, we selected a female control group) and age-matched. The study was conducted with the approval by the Institutional Review Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

*2.2. Sample Collection and Preparation.* All samplings from RTT patients and healthy controls were carried out around 8 a.m. after overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection.

The blood samples were centrifuged at 2400 g for 15 min at 4°C; the platelet poor plasma was saved and the buffy coat was removed by aspiration. Plasma samples were stored at −70°C until assay.

*2.3. Erythrocyte Sedimentation Rate (ESR).* The TEST 1 analyzer, a closed automated analyzer, determines the length of sedimentation reaction in blood in a standard-size primary tube with a perforating stopper. The principle of measurement is the study of the aggregation capacity of red blood cells (RBCs) by telemetry. The tubes are placed in appropriate racks, and their contents are rotated slowly for about 2 min. The sample loader simultaneously accepts 4 racks containing 15 tubes each. By using a closed aspiration needle, the blood is directly drawn from the collection tube, distributed in a capillary, and centrifuged at about 20 g. The sensing area temperature is maintained at 37°C. The system uses an infrared ray microphotometer with a light wavelength of 950 nm and performs 1,000 readings during 20 seconds. The electrical impulses, collected using a photodiode detector, are directly correlated to the aggregation of RBCs present at each capillary level. For each sample, an aggregation and sedimentation curve is obtained. A mathematical algorithm converts the raw data obtained from evaluation of optical density signals into ESR results, which are transformed to comparable Westergren values. The system operates at a rate of 180 specimens per hour in continuous loading, providing a result every 20 seconds, and requires 150 $\mu$L of blood for each sample [26, 27].

*2.4. C-Reactive Protein (CRP).* A Modular analytics P module (Roche, Hitachi) was used to determine serum CRP by immunoturbidimetry using 3rd generation CRP Tinaquant reagent (Roche, Hitachi). The functional sensitivity for CRP testing was 0.042 mg/dL. The interval reference was <0.5 mg/dL for CRP [28].

*2.5. Two-Dimensional Gel Electrophoresis (2-DE).* 2-DE is a protein separation technique that combines two different electrophoretic methods: isoelectric focusing (IEF) in the

first dimension, in which proteins are separated according to their isoelectric points (pI), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, in which proteins are separated according to their molecular weights (MW) [29]. Samples containing 60 $\mu$g of protein as determined by Bradford [30] were denatured with 10 mL of a solution containing 10% of SDS and 2.3% of dithiothreitol (DTT). Afterwards, samples were combined with 350 mL of solubilizing buffer containing 8 M urea, 2% of 3-[(3-cholamidopropyl)-dimethylammonium]-1-propane sulfonate (CHAPS), 0.3% DTT, and 2% of immobilized pH gradient (IPG) buffer, and loaded into 18 cm IPG strips 3–10 nonlinear on an Ettan IPGphor (GE Healthcare) apparatus system, and rehydrated for 7 h. IEF was carried out for a total of 32 kV h. After focusing, the strips were equilibrated with the buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min. Subsequently, strips were equilibrated again with the same equilibration buffer described above, except that it contained 4% w/v iodoacetamide instead of DTT and a trace of bromophenol blue. The second dimension was performed on an EttanDalt Six Electrophoresis system (GE Healthcare). IPG strips and a MW standard were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8–16% T) using 0.5% w/v agarose and run at a constant current of 40 mA/gel at 20°C. Each sample was carried out in triplicate under the same conditions.

*2.6. Image Analysis.* Images of gels were analyzed using ImageMaster 2D Platinum v7.0 software (GE Healthcare). The reference gel for each group (i.e., healthy controls, RTT, R306C, T158M, R168X, and large deletions in exons 3 and 4) was defined and used for the comparative analyses. The background was subtracted from all gels using the average-on-boundary method. Spot volume was expressed as a ratio of the total protein percentage volume (%V) detected from the entire gel to minimize differences between gels (gel normalization), for pooling them. Only spots appearing in all gels of the same group were matched with those of the reference gel.

*2.7. Trypsin Digestion and Proteins Identification by Mass Spectrometry.* 2-DE/MALDI-TOF is a complex and advanced technique to identify and characterize proteins from biological fluids and/or tissues whose results are considered to be reliable and reproducible when fitting a series of different parameters, such as peptide matches, sequence coverage (%), MOWSE score, and pI/relative molecular mass (Mr, kDa). A 2-DE/MALDI-TOF approach is helpful in order to reveal global protein pattern changes in a given tissue/body fluid for a given condition. However, the technique has assay sensitivity limitations when applied to either less abundant or small proteins (i.e., cytokines) [22, 31].

After mass spectrometry compatible silver staining, the preparative gel was matched to the master gel in the analytical gel match set [32]. A spot-picking list was generated and exported to Ettan Spot Picker (GE Healthcare). The spots were excised and delivered into 96-well microplates where they were destained and dehydrated with acetonitrile (ACN) for subsequent rehydration with trypsin solution. Tryptic digestion was carried out overnight at 37°C. Each protein spot digest (0.75 mL) was spotted into the MALDI instrument target and allowed to dry. Then 0.75 mL of the instrument matrix solution (saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in 50% ACN and 0.5% v/v trifluoroacetic acid) was applied to dried samples and dried again. Mass spectra were obtained, as described in [33], using an ultrafleXtreme MALDI-ToF/ToF (Bruker Corporation, Billerica, MA, USA). After tryptic peptide mass acquisition, mass fingerprint searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT (Matrix Science, London, UK, http://www.matrixscience.com). A mass tolerance of 100 ppm was allowed and only one missed cleavage site was accepted. Alkylation of cysteine by carbamidomethylation was assumed as a fixed modification, whereas oxidation of methionine was considered a possible modification. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides, and probabilistic score.

*2.8. STRING 9.0 Network Analysis.* Possible connections among identified plasma proteins with significant variations as compared to healthy controls expression levels were analyzed by a protein and gene network software. For each protein, UniProtKB entry numbers and related gene names were acquired in UniProtKB and used for network generation by the use of STRING 9.0 (http://www.string-db.org/) [34]. The UniProtKB entry numbers were inserted into the input form as "multiple proteins" and "Homo sapiens" was selected as the reference organism.

*2.9. Statistical Data Analysis.* All variables were tested for normal distribution (D'Agostino-Pearson test) and data were presented as means ± SD for normally distributed variables. Statistical analysis for protein expressed differently in the groups was carried out using GraphPad Prism software using Student's $t$-test and one-way ANOVA test. Bonferroni-corrected significance levels were used for multiple $t$-tests. Data were expressed as median and interquartile range unless otherwise. Unmatched spots or spots with significantly different %V were considered "differently expressed" in the groups. Comparisons between differently expressed proteins as a function of *MECP2* mutation type were evaluated using either Mann-Whitney rank sum test or Kruskal-Wallis test. The effects of small population sizes on possible type I ($\alpha$)/type II ($\beta$) errors in the data interpretation were examined using a sampling size algorithm. A two-sided $P < 0.05$ was considered to indicate statistical significance. The MedCalc version 12.1.4 statistical software (MedCalc Software, Mariakerke, Belgium) was used.

## 3. Results

*3.1. Clinical Severity and MECP2 Mutation Types.* Median clinical severity score for the whole Rett population was 17 (inter-quartile range 9–21.2, values range 7–31).

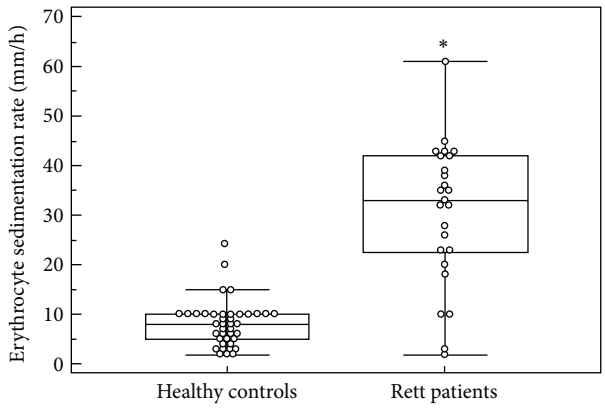


Figure 1: Erythrocyte sedimentation rate (ESR) measurements by TEST 1 in healthy controls and RTT patients. $^{*}P < 0.001$.

*3.2. Routine Haematology/Clinical Chemistry.* Elevated ESR values (median 33.0 mm/h versus 8.0 mm/h, $P < 0.0001$) were detectable in RTT (Figure 1), whereas CRP levels were within the reference range (RTT median 0.04 mg/dL inter-quartile range 0.02–0.08 mg/Dl, values range 0.01–0.18 versus Control median 0.04 mg/dL inter-quartile range 0.001–0.11 mg/dL, values range 0.01–0.86, $P = 0.6343$). The existence and relevance of an unrecognized subclinical inflammatory status in Rett syndrome are strongly suggested by the evidence of relationships between ESR values and phenotypical severity of the disease (Figure 4).

*3.3. Protein Expression Profile Differences Between RTT and Healthy Control Subjects.* Changes for a total of 17 different proteins were identified and expressed as folds changes relative to controls (Figures 2(a)–2(e) and see Supplementary Table in Supplementary Material available online at http://dx.doi.org/10.1155/2014/480980) and shown in 2-DE maps (Figures 3(a)–3(e)). Proteins were subsequently identified by mass spectrometry. Protein identification as well as peptide matches, sequence coverage, and the probabilistic score were obtained using the MASCOT software. The biological functions and their role in APR are summarized in Table 1. Actually, the majority of the proteins were categorized as either positive APR proteins ($n = 6$ spots, such as complement factor B (CFAB, spot 1), alpha-1-antitrypsin (A1AT, spot 5), fibrinogen gamma chain (FIBG, spot 6), haptoglobin (HPT, spots 8 and 15), and serum amyloid A-1 protein (SAA1, spot 17)) or negative APR proteins ($n = 9$ spots, such as serum transferrin (TRFE spot 2), albumin (ALBU, spot 3 as whole protein while spots 7, 11, and 14 as C terminal fragments), transthyretin (TTHY, spots 10 and 16), apolipoprotein A1 (APOA1, spot 12), and retinol-binding protein 4 (RET4, spot 13)) and, to a lesser extent, as proteins involved in the immune system ($n = 2$ spots, such as alpha-2-HS-glycoprotein (FETUA, spot 4) and Ig gamma-2 chain C region (IGHG2, spot 9)). Some proteins have known overlapping functions on metabolism and signal transduction ($n = 10$ spots, such as TRFE (spot 2), ALBU (spots 3, 7, 11, and 14), FIBG (spot 6), TTHY (spots 10 and 16), APOA1 (spot 12), and RET4 (spot 13)). The number of protein changes was found to be proportional to the severity of the *MECP2* gene mutation.

SAA1, AIAT, and CFAB were all overexpressed in the examined *MECP2* mutation types. The behaviour of HPT appears to be discrepant among different *MECP2* mutations RTT, with the 40 kDa spot being overexpressed in T158M and large deletions, while underexpressed in the R306C and normally expressed in the R168X mutation type. The HTP 18 kDa spot appears to be normally expressed in R306C and T158M while being overexpressed in the plasma samples from patients with R168X and large deletions. A1AT (spot 5) was significantly overexpressed ($P < 0.001$) in all *MECP2* mutation types and varied from +2.96 in R306C to +5.15 in large deletions when compared to controls (Figure 2). Furthermore, our data indicate that FETUA is overexpressed in all the *MECP2* mutations examined. On the other hand, RET4 and APOA1 were significantly underexpressed in three of the four examined *MECP2* gene mutation classes, that is, T158M, R168X, and large deletions. The TTHY 15 kDa spot (very close to the theoretical MW of 15,900 Da) was found to be underexpressed in all the examined mutation types and the TRFE spot appeared to be underexpressed in the R168X mutation type. A detailed analysis of protein expression patterns as a function of *MECP2* gene mutation types is reported as Supplementary Material (*Protein Expression Patterns in the Different MECP2 Mutation Types: Detailed Analysis).*

*3.4. Protein-Protein Interaction Analysis.* In the RTT population identified proteins undergoing significant changes, potentially classifiable as positive APR proteins, negative APR proteins and immune response proteins, appear to be closely connected. STRING software created a prediction of protein-protein interaction networks (PPI) based on confidence (Figure 5(a)), evidence (Figure 5(b)) and actions (Figure 5(c)). In the confidence PPI, the thickness of the lines shows how strong the interactions are (threshold: 0.4, medium confidence). The strongest interactions were between TTHY and RET4 (combined association score: 0.997), APOA1 and ALBU (combined association score: 0.994), and TTHY and APOA1 (combined association score: 0.994) (Figure 5(a)). Positive APR proteins, negative APR proteins, and immune response proteins are highlighted in three different ellipses. In the evidence PPI, the colours of the lines represent the types of evidence which characterized the protein-protein association. More evidence was between TTHY and RET4 (combined association score: 0.997), ALBU and APOA1 (combined association score: 0.994), and TTHY and APOA1 (combined association score: 0.993) (Figure 5(b)). In the actions PPI, the different colours of the lines represent the mode of action of proteins. Code lines: black is reaction between TTHY and APOA1 (combined association score: 0.993), A1AT and ALBU (combined association score: 0.951), and APOA1 and TRFE (combined association score: 0.982) while blue is binding between TTHY and RET4 (combined association score: 0.997), TRFE and ALBU (combined association score: 0.988), HPT and APOA1 (combined association score: 0.975), and TTHY and TRFE (combined association score: 0.970) (Figure 5(c)).

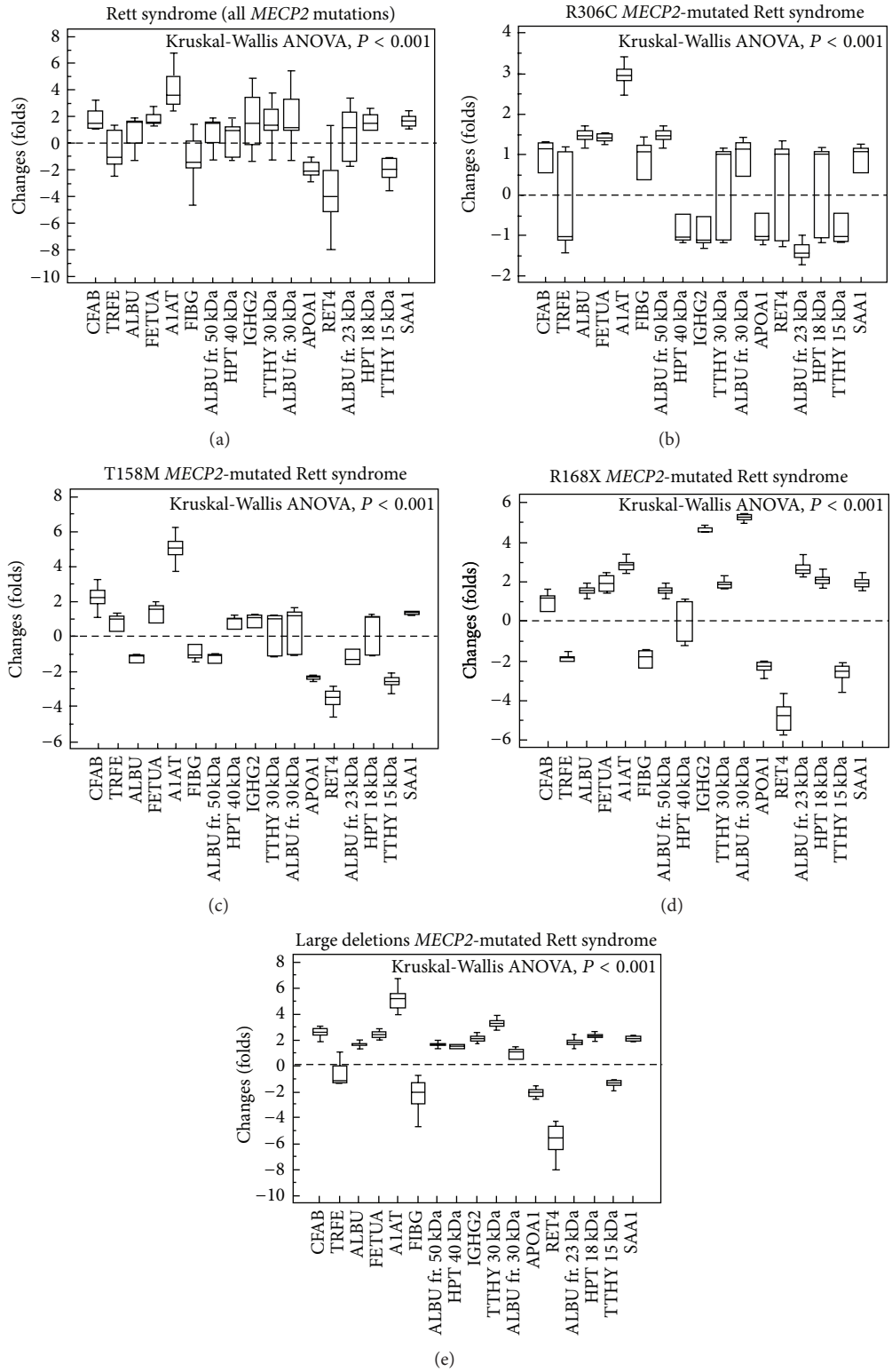


Figure 2: Plasma proteins expression as a function of *MECP2* mutations in girls with classical Rett syndrome. (a) All *MECP2* mutations, (b) R306C mutation (milder form), (c) T158M mutation (intermediate severity), (d) and (e) correspond to R168X and large deletions (severe forms), respectively. Data are compared to matched healthy controls and expressed as box-and-whiskers plots. Results of Kruskal-Wallis ANOVA are indicated.

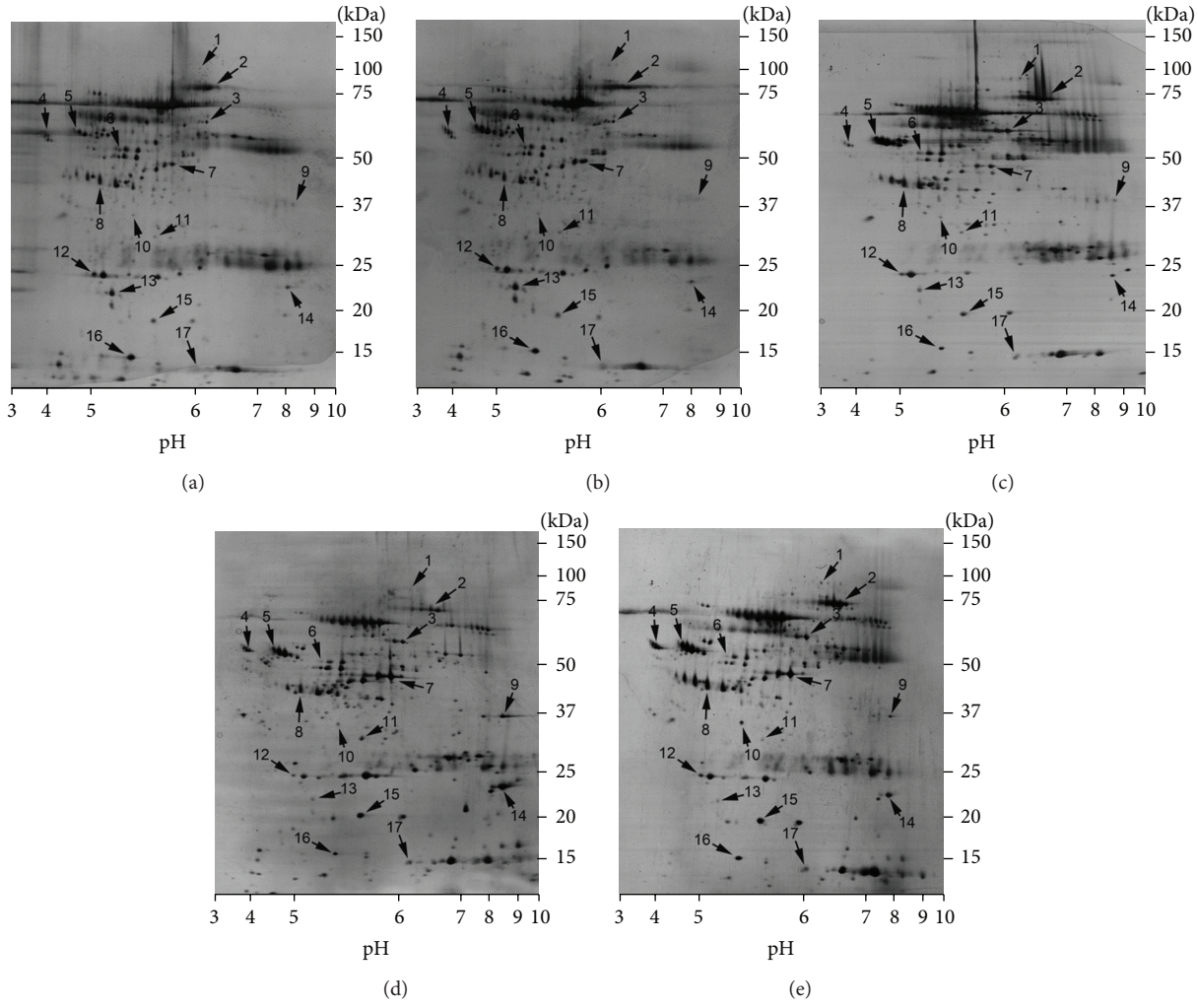


Figure 3: Silver-stained 2-DE gel of proteins from a typical healthy control (a), R306C (b), T158M (c), R168X (d), and large deletions (exons 3 and 4) (e). 60 $\mu$g of total protein was subjected with nonlinear IPG strips, with a pH range of 3 to 10, followed by SDS-polyacrylamide gradient gel (8–16% T) electrophoresis. Numbers denote the identified proteins by mass spectrometry and are listed in Table 1 and Supplementary Table. Molecular mass (kDa) and pI markers are indicated along the gels.

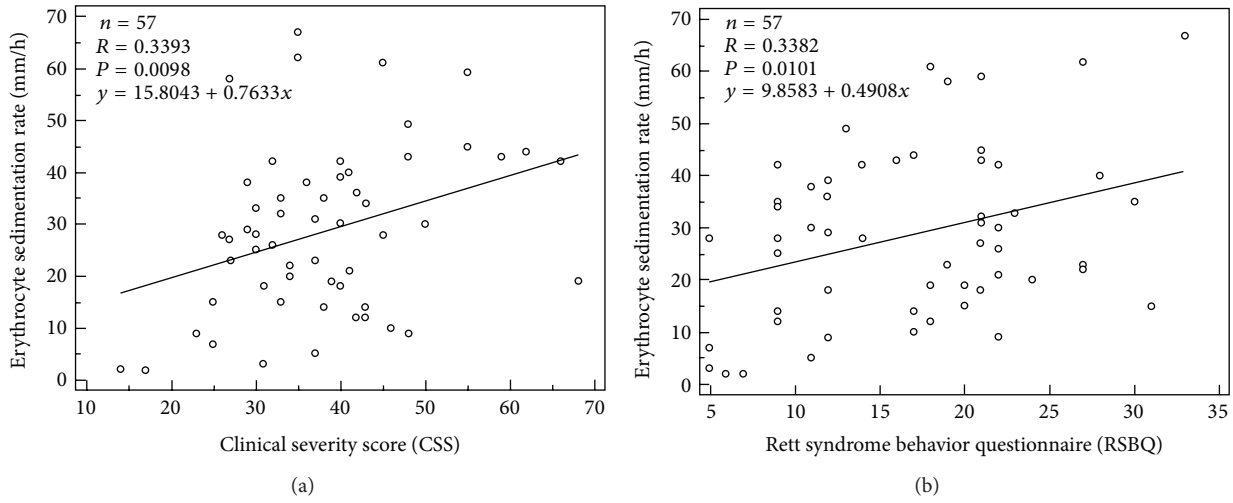


Figure 4: Statistically significant positive correlations were observed between erythrocytes sedimentation rate (ESR) and the clinical severity of the disease, as measured by (a) Clinical Severity Score (CSS) and (b) Rett Syndrome Behaviour Questionnaire (RSBQ).

Table 1: Summary of the proteins identified as differently expressed using the proteomics approach.

| Spot | SwissProt code | Protein name | Short name | Theoretical pI/Mr (kDa) | Peptides matches | Sequence coverage (%) | MOWSE score | Biological functions | APR proteins |
|---|---|---|---|---|---|---|---|---|---|
| 1[a] | P00751 | Complement Factor B | CFAB | 6.67/86.8 | 22/39 | 37 | 219 | Immune system, complement system regulation | (+) |
| 2 | P02787 | Serum transferrin | TRFE | 6.81/79.2 | 40/80 | 50 | 340 | Iron binding and transport | (−) |
| 3 | P02768 | Albumin | ALBU | 5.92/71.3 | 30/62 | 55 | 268 | Transport, regulation of colloidal osmotic pressure, platelet activation | (−) |
| 4 | P02765 | Alpha-2-HS-glycoprotein | FETUA | 5.43/40.09 | 10/22 | 38 | 110 | Endocitosis, opsonization | N.A.[b] |
| 5 | P01009 | Alpha-1-antitrypsin | A1AT | 5.37/46.8 | 16/37 | 46 | 164 | Acute phase response, coagulation, proteases inhibition | (+) |
| 6 | P02679 | Fibrinogen gamma chain | FIBG | 5.37/52.1 | 13/43 | 41 | 122 | Coagulation, signal transduction | (+) |
| 7 | P02768 | Albumin (C terminal fragment) | ALBU | 5.92/71.3 | 16/29 | 28 | 138 | Transport, regulation of colloidal osmotic pressure, platelet activation | (−) |
| 8 | P00738 | Haptoglobin | HPT | 6.13/45.8 | 6/10 | 14 | 51 | Acute phase response, hemoglobin binding | (+) |
| 9 | P01859 | Ig gamma-2 chain C region | IGHG2 | 7.66/36.5 | 5/8 | 13 | 71 | Innate immunity | N.A.[b] |
| 10 | P02766 | Transthyretin | TTHY | 5.52/15.9 | 7/18 | 68 | 115 | Thyroid hormone binding and transport | (−) |
| 11 | P02768 | Albumin (C terminal fragment) | ALBU | 5.92/71.3 | 18/27 | 28 | 188 | Transport, regulation of colloidal osmotic pressure, platelet activation | (−) |
| 12 | P02647 | Apolipoprotein A1 | APOA1 | 5.56/30.7 | 26/87 | 65 | 213 | Lipid transport and metabolism | (−) |
| 13 | P02753 | Retinol-binding protein 4 | RET4 | 5.76/23.3 | 8/18 | 57 | 104 | Retinol transport and metabolism | (−) |
| 14 | P02768 | Albumin (C terminal fragment) | ALBU | 5.92/71.3 | 6/12 | 12 | 66 | Transport, regulation of colloidal osmotic pressure, platelet activation | (−) |
| 15 | P00738 | Haptoglobin | HPT | 6.13/45.8 | 8/24 | 20 | 86 | Acute phase response, hemoglobin binding | (+) |
| 16 | P02766 | Transthyretin | TTHY | 5.52/15.9 | 6/13 | 68 | 104 | Thyroid hormone binding and transport | (−) |

Table 1: Continued.

| Spot | SwissProt code | Protein name | Short name | Theoretical pI/Mr (kDa) | Peptides matches | Sequence coverage (%) | MOWSE score | Biological functions | APR proteins |
|---|---|---|---|---|---|---|---|---|---|
| 17 | P0DJI8 | Serum amyloid A-1 protein | SAA1 | 6.28/13.5 | 5/12 | 51 | 62 | Acute phase response, apolipoprotein of the HDL complex | (+) |

[a]Spot numbers match those reported in the representative 2-DE images shown in Figure 3. N.A.: not applicable. (+) and (−) indicate positive and negative APR proteins, respectively. [b]Identified proteins showing variations in RTT for which a possible involvement in the inflammatory response is unknown.

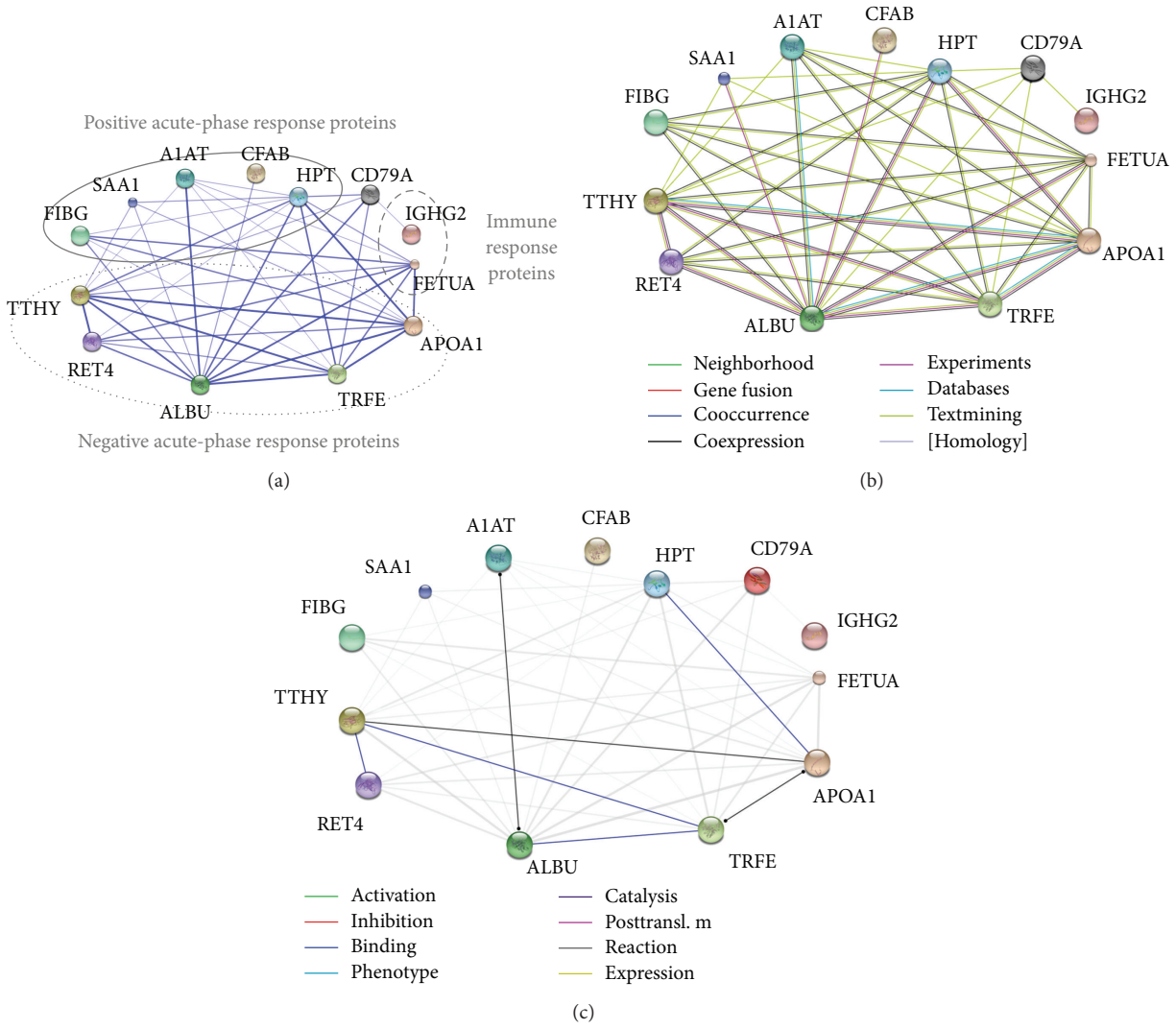


Figure 5: Predicted protein-protein interaction networks (PPI) created by STRING 9.05. Each circle represents an individual protein with the recognized abbreviated name. (a) Confidence PPI in which the thickness of the lines shows how strong the interactions are (threshold: 0.4, medium confidence). Positive, negative APR proteins and immune response proteins are highlighted in three different ellipsis. CD79A is represented with grey colour to indicate an unidentified protein by MS but helpful for linking IGHG2 with HPT. (b) Evidence PPI in which the colour of the lines represent the types of evidence which characterized the protein-protein association. Code lines: green: neighborhood, brown: coexpression, pink: experiments, sky-blue: databases, olive green: textmining. (c) Actions PPI of all proteins in which the different colour of the lines represent the mode of protein actions. Code lines: black: reaction, blue: binding.

## 4. Discussion

Etiology of autism is still unknown although several explanations have been proposed [35], including environmental factors combined to particular genotype [36], vaccines [37], and phthalates [38]. Experimental and clinical evidence accumulated in the last decades indicates the presence of an early immune activation in ASDs with an associated peripheral and central chronic inflammation [12–16]. In particular, a subclinical chronic inflammatory condition has been previously suggested to occur in autism [35].

Inflammation, as the first nonspecific defensive response in mammalians, is a component of the innate immunity, a self-protective process whose aim is to remove harmful stimuli in order to maintain the homeostasis [39]. Chronic inflammation differs from the acute inflammatory processes because of the duration, types of involved cells and primary mediators (i.e., cytokines, reactive oxygen species, hydrolytic enzymes, and growth factors versus eicosanoids and vasoactive amines), site (tissue versus vascular), and outcome [40]. Besides a few isolated studies regarding complement factors, TRFE and A1AT [41–43], not consistent information regarding a correlation between our individual identified plasma protein and ASDs or RTT exist in literature. Cumulating evidence indicates a link between astrocytes abnormalities and Rett syndrome [44–47]. In particular, a recent report analyzing the response of astrocytes during activation by proinflammatory stimulation [48] proposes that the protective phenotype against iron-mediated oxidative stress in cell death involves a complex change in the expression and activity of several genes involved in the control of the cellular redox state. Therefore, it is plausible that abnormal redox status and unrecognized pro-inflammatory stimuli would converge in RTT patients to functionally damage astrocytes.

The present study indicates, for the first time, the occurrence of a subclinical persistent inflammatory status in RTT patients with stage II (i.e., "pseudo-autistic"). In addition, our findings show a direct relationship between the number of the plasma protein quantitatively modified and the clinical severity of the disease.

A moderately increased ESR value was the only standard laboratory clue for an underlying inflammatory process, whereas, interestingly, other standard routine tests (i.e., CRP) appear to be unchanged. At the molecular level, production of CRP is induced by proinflammatory cytokines IL-1, IL-6, and IL-17 [49]. It is plausible that a cytokine dysregulation may exist in Rett syndrome, although no clear demonstration has been so far brought in this sense. A cytokine dysregulation can be inferred by an interesting parallel between RTT and APR protein variations in perinatal human plasma [50].

Plasma proteome analysis by 2-DE/MALDI-TOF is known to be able to identify even subtle changes with high specificity in protein identification and recognition of structural changes and posttranslational modifications [51]. This approach has allowed in the present study to unveil the upregulation of several positive APR proteins, such as SAA1 and A1AT [52, 53], as well as the downregulation of known negative APR proteins, such as APOA1 and RET4 [54, 55]. SAA1, A1AT, and CFAB, known as positive APR proteins, are all overexpressed in the examined *MECP2* mutation types. SAA1 is a major acute phase reactant and an apolipoprotein belonging to the HDL complex. Inflammatory cells chemotaxis, positive regulation of cytokines secretion, and platelet activation are some of its functions [52]. A1AT is a multifunctional protein involved in anti-inflammatory and tissue protective properties by protecting tissues from enzymes of inflammatory cells. More broadly, A1AT plays an important role in modulating immunity, inflammation, apoptosis, and possibly cellular senescence programs [53, 56]. By comparing R168X to T158M it is interesting to note that A1AT (spot 5) is underexpressed, whereas one C-terminal fragment of ALBU (spot 11) appears to be overexpressed, thus supporting the hypothesis that in the most severe RTT phenotypes more proteolysis occurs along with a lower activity by protease inhibitors, including A1AT. CFAB, a component of the alternate pathway of the complement system which contributes to generate C3 or C5 convertase and plays a role in the hemolytic uremia complex [57], appears to be overexpressed in T158M and large *MECP2* gene deletions. Nonetheless, none of the features of the hemolyticuremia complex is present in RTT.

Hence, we can suppose that the "pseudo-autistic" phase of RTT is characterized by a tissue damage to which an adaptive/defensive response ensues. To this regard, the mounting evidence of a persistent redox imbalance in RTT [6, 58] appears to be related to the wider context of a chronic inflammatory process whose fine mechanisms remain to be elucidated.

As predicted by the STRING software (Figure 5) the most strong interaction was represented by TTHY and RET4 (combined association score: 0.997) and demonstrated by the binding of RET4 to TTHY when circulating in plasma (in a 1 to 1 stoichiometry). In vitro one tetramer of TTHY can bind two molecules of RET4 [59].

TTHY has been suggested to be a transporter for thyroxin from the bloodstream to the brain [60]. To this regard, subtle changes in the levels of the thyroid hormones have been reported in RTT [61], although no evidence of clinical hypothyroidism is present in the affected patients.

TTHY shows in the proteomic maps, a contrasting behavior, with the 15 kDa spot being uniformly underexpressed in all the examined RTT patients, while the 30 kDa spot appears to be normally expressed in the R306C and T158M patients, but overexpressed in the R168X and *MECP2* large deletion mutation types. In the light of our findings, the observed underexpression of the TTHY 15 kDa spot could be interpreted as mirroring the behavior of a negative APR protein. The TTHY spot which appears to be corresponding to a higher MW (exactly 34,400 Da) already reported in the literature whose biological meaning is still to be clarified [62]. A likely source for the MW discrepancy could reside in the reducing experimental condition of 2-DE which could lead to splitting of the whole original protein into two subunits.

Another TTHY interaction was with APOA1 (combined association score: 0.994), demonstrated as relevant not only in physiological condition but also in amyloidosis (Reactome Pathways as of October 2012, visit http://www.reactome.org/ for the latest updates).

APOA1 and RET4 were found to be significantly underexpressed, as compared to controls, in three out of four examined *MECP2* gene mutations (i.e., T158 M, R168X, and large deletions). Both proteins are involved in the lipid metabolism and have been suggested to be involved in the dyslipidemia of children with metabolic syndrome [54, 55, 63]. On the other hand, their involvement in RTT appears to support the concept of an altered lipid metabolism in this condition featuring hyperleptinemia [64] and hypercholesterolemia [65] and for which the coexistence of a "fatty acids paradox" has been suggested, given that an excessive endogenous fatty acids oxidation is paradoxically ameliorated by administration of the same exogenous fatty acids [66, 67].

Interestingly, also the interaction of APOA1 with ALBU (combined association score: 0.994) takes place in the lipid metabolism particularly in the pathway of HDL-mediated lipid transport.

In contrast, the interaction of APOA1 with TRFE (combined association score: 0.993) is involved in the release of platelet secretory granule components (Reactome Pathways as of October 2012, visit http://www.reactome.org/ for the latest updates).

In our findings, TRFE was underexpressed in the R168X mutation type, a variation which is in accordance with the classical negative APR protein changes.

In another biochemical pathway, HPT can bind APOA1 and impairs its stimulation of lecithin:cholesterol acyltransferase (LCAT). LCAT plays a major role in reverse cholesterol transport [68]. HPT is a protein which captures and combines with free plasma hemoglobin to allow hepatic recycling of heme iron in order to prevent kidney damage [69, 70]. Its behaviour appears to be discrepant among different *MECP2* mutations RTT, with the 40 kDa spot being overexpressed in T158M and large deletions, while underexpressed in the R306C and normally expressed in the R168X mutation type. The HTP 18 kDa spot appears to be normally expressed in R306C and T158M while being overexpressed in the most severe examined mutations (i.e., R168X and large deletions).

Significant changes in RTT plasma were evidenced in FETUA and IGHG2 both involved in the immune response. In particular, FETUA is directly connected with APOA1 (combined association score: 0.874). Our data indicate that FETUA is overexpressed in all the *MECP2* mutations examined. FETUA is synthesized in the liver and subsequently concentrated in bone matrix. This protein is known to promote endocytosis, possess opsonic properties, and influence the mineral phase of bone. FETUA shows high affinity for calcium and barium ions [71]. To this regard, cumulating evidence indicates that osteoporosis is a phenotypic feature of RTT [72, 73].

Of course, several unsolved questions arise from the present study, including the causes of APR in the autistic phase of RTT. A stimulating parallel is the recent demonstration of a subclinical inflammatory process in autism [35]. Therefore, inflammation seems to be a previously unrecognized feature in autistic and cognitive disorders, which could play a role in the evolution of the pathology and modulations of phenotypical severity.

## 5. Conclusion

Cumulating evidence indicates that RTT is a multisystemic disorder, with the involvement of lung [53], bone [72, 73], heart [74], and gastrointestinal apparatus [75, 76], besides the central nervous system. For the first time, we evidenced a subclinical chronic inflammatory status related to the severity carried by *MECP2* gene mutations in the "pseudo-autistic" (stage II) phase of RTT. Our detection of a persistent inflammatory status is compatible with a systemic disease and adds a new perspective in the pathogenesis and future therapeutic strategies for RTT [67] and ASDs.

## Abbreviations

CFAB: Complement factor B
TRFE: Serum transferrin
ALBU: Albumin
FETUA: Alpha-2-HS glycoprotein
A1AT: Alpha-1-antitrypsin
FIBG: Fibrinogen gamma chain
HPT: Haptoglobin
IGHG2: Ig gamma-2 chain C region
TTHY: Transthyretin
APOA1: Apolipoprotein A1
RET4: Retinol-binding protein 4
SAA1: Serum amyloid A-1 protein.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Alessio Cortelazzo, Claudio De Felice, and Roberto Guerranti contributed equally to this work, and Luca Bini and Joussef Hayek equally supervised this work.

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illustrations of a book in progress dedicated to the Rett syndrome girls and families. They heartily thank the professional singer Matteo Setti (http://www.matteosetti.com/) for having serendipitously triggered the scientific studies on hypoxia-related oxidative stress in Rett girls and autistic children, as well as his many charity concerts and continued interest in the scientific aspects of our research. Finally, they sincerely thank the Rett girls and their families.

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*Research Article*

# A Plasma Proteomic Approach in Rett Syndrome: Classical versus Preserved Speech Variant

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Rett syndrome (RTT) is a progressive neurodevelopmental disorder mainly caused by mutations in the gene encoding the methyl-CpG-binding protein 2 (MeCP2). Although over 200 mutations types have been identified so far, nine of which the most frequent ones. A wide phenotypical heterogeneity is a well-known feature of the disease, with different clinical presentations, including the classical form and the preserved speech variant (PSV). Aim of the study was to unveil possible relationships between plasma proteome and phenotypic expression in two cases of familial RTT represented by two pairs of sisters, harbor the same *MECP2* gene mutation while being dramatically discrepant in phenotype, that is, classical RTT versus PSV. Plasma proteome was analysed by 2-DE/MALDI-TOF MS. A significant overexpression of six proteins in the classical sisters was detected as compared to the PSV siblings. A total of five out of six (i.e., 83.3%) of the overexpressed proteins were well-known acute phase response (APR) proteins, including alpha-1-microglobulin, haptoglobin, fibrinogen beta chain, alpha-1-antitrypsin, and complement C3. Therefore, the examined RTT siblings pairs proved to be an important benchmark model to test the molecular basis of phenotypical expression variability and to identify potential therapeutic targets of the disease.

## 1. Introduction

Rett syndrome (RTT; OMIM no. 312750), with a frequency of ~1 : 10000–1 : 15000 females, is a severe and complex neurodevelopmental disorder, as well as the second most common cause of severe mental retardation in the female gender [1]. RTT presents in about 74% of cases in a classical form (typical presentation); after 6–18 months of an apparently normal development girls lose their acquired cognitive, social, and motor skills in a typical 4-stage neurological regression. A wide phenotypical heterogeneity is a well-known feature of the disease, which includes at least four major different clinical presentations, that is, classical, preserved speech (PSV), early seizure (ESV), and congenital variants [2]. Studies have implicated *de novo* mutations of the X-linked methyl-CpG-binding protein 2 (*MECP2*) gene (OMIM∗300005) in the majority of the RTT cases, while mutations in cyclin-dependent kinase-like 5 (*CDKL5*) and forkhead box G1 (*FOXG1*) have been more rarely reported [3–5]. Typical RTT has been described worldwide, whereas PSV

is more rarely reported. Girls affected by PSV have been often misreported with various diagnoses ranging from autism to mental retardation [6, 7].

While the available RTT literature is mainly focusing on the molecular genetics aspects, very little is known about possible disease-related protein changes, with the single exception of a proteomic study on a mouse model [8]. Among the several hundred RTT sporadic patients examined in the Child Neuropsychiatry Unit of the University Hospital of Siena, Italy, we have encountered two rare familial cases consisting of two pairs of sisters with RTT that are phenotypically discordant as previously reported [9]; that is, individuals in each pair demonstrate extremes of the RTT spectrum, that is, classical RTT and PSV-RTT. X chromosome inactivation (XCI) status is able to modulate X-linked disorders [10]. However, all four mentioned individuals show a balanced XCI, indicating that other factors beyond XCI may contribute to the phenotypic outcome [7, 11, 12]. Aim of the study was to unveil possible relationships between plasma proteome and phenotypic expression in two cases of familial RTT represented by two pair of sisters, harbor the same *MECP2* gene mutation while being dramatically discrepant in phenotype, that is, classical RTT versus PSV.

## 2. Materials and Methods

*2.1. Subjects.* Two pairs of sisters with discordant phenotype and identical mutation for each pair (pair 1: c.1157del32; pair 2: *de novo MECP2* deletion including exon 3 and part of exon 4) were enrolled in the present study [12]. Siblings no. 1 (42 years old) and no. 2 (33 years old) exhibits classical RTT and PSV-RTT, respectively. Both sisters showed a balanced XCI and inherited the same mutation from their unaffected mother, who had a completely skewed XCI [7]. Siblings no. 3 (34 years old) and no. 4 (40 years old) exhibits classical RTT and PSV-RTT, respectively. XCI status analysis in this couple of sisters revealed balanced XCI in both [12]. The unrelated classical RTT individuals no. 1 and no. 3 could not speak and walk and had a profound intellectual deficit, while the PSV individuals no. 2 and no. 4 could speak and walk and had a moderate intellectual disability (PSV-RTT). Striking differences in somatic, neurodevelopmental, and neurovegetative features between the sisters were present. A full clinical description of the affected siblings has been already reported by Grillo et al. [9]. The diagnostic criteria for the PSV form of RTT have been previously reported [13]. Mean classical RTT and PSV scores were, respectively, 27.5 ± 5.3 and 13.8 ± 5.9 (see the two pedigrees in Figure 1).

Gender and age-matched controls were also enrolled in the study. Blood samplings in the control group ($n = 10$) were carried out, during routine health checks or blood donations, always followed by written informed consent. This study was approved by the institutional review board of AOUS, Siena, Italy.

*2.2. Blood Sampling.* All samplings from RTT patients and healthy controls were carried out around 8 a.m. after overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. The blood samples were centrifuged at 2400 g for 15 min at 4°C; the platelet poor plasma was saved and the buffy coat was removed by aspiration. Plasma samples were stored at −70°C until use.

*2.3. 2-DE Analysis.* 2-DE was performed according to Görg et al. [14] with slight modifications. Samples containing 60 $\mu$g of protein as determined by Bradford [15] were denatured with 10 mL of a solution containing 10% of sodium dodecyl sulfate (SDS), 2.3% of dithiothreitol (DTT) heated to 95°C for 5 min. The sample was then combined with 350 mL of solubilizing buffer containing 8 M urea, 2% of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 0.3% DTT, 2% of immobilized pH gradient (IPG) buffer, and a trace of bromophenol blue and loaded into 18 cm IPG strips 3–10 NL on an Ettan IPGphor (GE Healthcare) apparatus system and rehydrated for 7 h. Isoelectric focusing (IEF) was carried out for a total of 32 kV h. After focusing, the strips were first equilibrated with equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min; then they were equilibrated again with the same equilibration buffer described above, except that it contained 4% w/v iodoacetamide instead of DTT and a trace of bromophenol blue. The strips were washed further for 10 min with Tris-glycine buffer. The second dimension was performed on an EttanDalt Six Electrophoresis system (GE Healthcare). IPG strips and a molecular weight standard were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8–16% T) using 0.5% w/v agarose and run at a constant current of 40 mA/gel at 20°C. Each sample was carried out in triplicate under the same conditions. The exposure time for silver staining was also optimized to avoid overexposure of some gels with respect to others.

*2.4. Tryptic Digestion and MALDI-TOF MS.* After mass spectrometry compatible silver staining [28], the preparative gel was matched to the master gel in the analytical gel match set. A spot-picking list was generated and exported to Ettan Spot Picker (GE Healthcare). The spots were excised and delivered into 96-well microplates where they were destained and dehydrated with acetonitrile (ACN) for subsequent rehydration with trypsin solution. Tryptic digestion was carried out overnight at 37°C. Each protein spot digest (0.75 mL) was spotted into the MALDI instrument target and allowed to dry. Then 0.75 mL of the instrument matrix solution (saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in 50% ACN and 0.5% v/v trifluoroacetic acid) was applied to dried samples and dried again. Mass spectra were obtained, as described [29], using an ultrafleXtreme MALDI-ToF/ToF (Bruker Corporation, Billerica, MA, USA).

*2.5. Protein Identification by MS.* After tryptic peptide mass acquisition, mass fingerprint searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT (Matrix Science, London, UK, http://www.matrixscience.com/). A mass tolerance of 100 ppm was allowed and only one missed cleavage site was accepted. Alkylation of cysteine

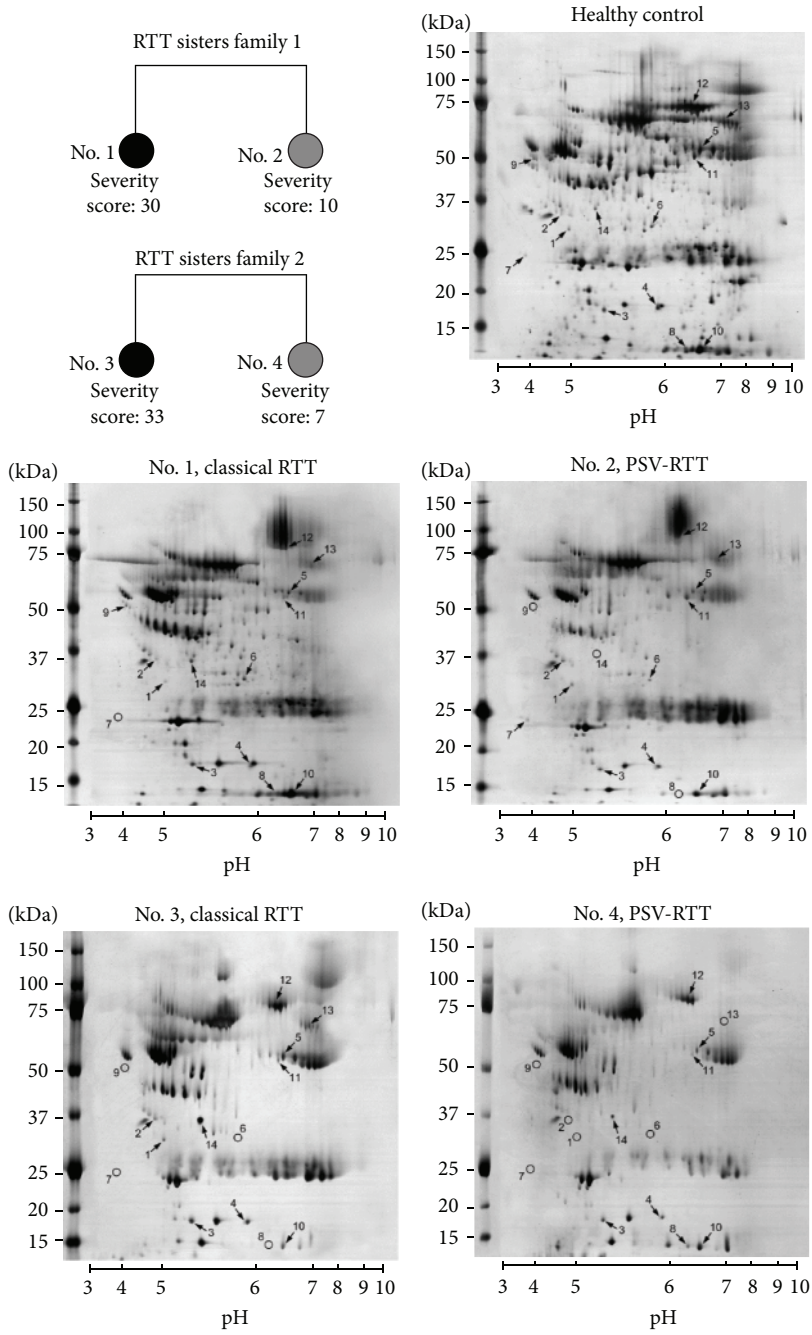


Figure 1: In the pedigrees the two RTT sisters families are represented by grey circles (milder variant = preserved speech variant, PSV-RTT) or black circles (more severe phenotype = classical RTT) with their respective clinical scores as derived by the approbation of phenotypical severity scale [9]. In the 2-DE maps typical control plasma proteome (healthy control), RTT sisters Family 1 (no. 1, no. 2) and RTT sisters Family 2 (no. 3, no. 4) are shown. Arrows indicate the protein spots with significant variations in their major or minor relative volume; circles are used to indicate the absence of the spots (i.e., qualitative variations).

by carbamidomethylation was assumed as a fixed modification, whereas oxidation of methionine was considered a possible modification. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides, and probabilistic score.

*2.6. Image and Statistical Analysis.* Images of gels were analyzed using ImageMaster 2D Platinum v7.0 software (GE Healthcare). The reference gel for each group (i.e., RTT, controls, classical RTT, PSV-RTT, RTT sisters Family 1, RTT sisters Family 2, and cases no. 1, no. 2, no. 3, and no. 4) was defined and used for the comparative analyses. Statistical analysis for protein differently expressed in the groups was carried out using GraphPad Prism software and the MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used. All variables were tested

Table 1: Identification of plasma proteins in RTT patients and healthy controls by MS.

| ID | AC[a] | Protein name | Short name | pI/Mr (kDa) predicted | pI/Mr (kDa) experimental | Peptide matches | Sequence coverage (%) | MOWSE score | Biological process involved; molecular function; references |
|---|---|---|---|---|---|---|---|---|---|
| 1 | P02760 | Alpha-1-microglobulin | AMBP | 5.07/30.9 | 5/31.1 | 9/15 | 25 | 77 | Host-virus interaction; trypsin and plasmin inhibitor; [16] |
| 2 | P10909 | Clusterin | CLUS | 4.9/36.9 | 4.8/36.4 | 12/18 | 25 | 146 | Chaperone; prevents stress-induced aggregation of blood plasma proteins; [17] |
| 3 | P00738 | Haptoglobin | HPT | 5.4/16.8 | 5.2/17 | 6/14 | 19 | 75 | Immunity; captures hemoglobin, antimicrobial, and antioxidant; [18] |
| 4 | P00738 | Haptoglobin | HPT | 6.07/16.8 | 5.9/17 | 6/13 | 19 | 73 | Immunity; captures hemoglobin, antimicrobial, and antioxidant; [18] |
| 5 | P02675 | Fibrinogen beta chain | FIBB | 6.4/55.2 | 6.6/55.2 | 37/88 | 60 | 231 | Blood coagulation and hemostasis; [19] |
| 6 | P02768 | Serum albumin | ALBU | 5.6/67.7 | 5.8/68 | 5/7 | 8 | 56 | Regulation of the osmotic blood pressure; binds ions, hormones, and fatty acids; [20] |
| 7 | P01591 | Immunoglobulin J chain | IGJ | 4.5/23.4 | 4.5/24 | 5/18 | 32 | 61 | Immunity; links two monomer units of either IgM or IgA; [21] |
| 8 | P68871 | Hemoglobin subunit beta | HBB | 6.8/10.5 | 6.4/12.5 | 6/9 | 53 | 110 | Oxygen transport; [22] |
| 9 | P01009 | Alpha-1-antitrypsin | A1AT | 4.8/50.3 | 4.8/50.2 | 10/14 | 32 | 141 | Serine proteases inhibitor; [23] |
| 10 | P68871 | Hemoglobin subunit beta | HBB | 7.05/10.5 | 6.8/12.5 | 15/31 | 95 | 220 | Oxygen transport; [22] |
| 11 | P01859 | Ig gamma-2 chain C region | IGHG2 | 6.1/24.4 | 6/24.6 | 7/40 | 17 | 44 | Immunity; antigen binding; [24] |
| 12 | P02787 | Serum transferrin | TRFE | 6.3/80.7 | 6.3/79.3 | 36/71 | 45 | 311 | Iron binding transport proteins which can bind two $Fe^{3+}$ ions; [25] |
| 13 | P01024 | Complement C3 | CO3 | 6.6/70.6 | 6.8/69.7 | 16/21 | 14 | 144 | Immunity; central role in the activation of the complement system; [26] |
| 14 | P02766 | Transthyretin | TTHY | 5.5/35.3 | 5.4/34.4 | 9/27 | 77 | 136 | Thyroid hormone-binding protein; [27] |

Spot ID refers to that shown in 2-DE maps (Figure 1). [a]Accession numbers of Swiss-Prot or GenBanK databases.

for normal distribution (D'Agostino-Pearson test). Data were expressed as median values and interquartile range, unless otherwise stated. Unmatched spots or spots with significantly different percentage volume (%V) were considered as "differently expressed". Differences between groups were tested by the nonparametric Mann-Whitney rank sum test or Kruskal-Wallis analysis of variance, as appropriate. A two-sided $P < 0.05$ was considered to indicate statistical significance.

## 3. Results

To better characterize the RTT plasma protein pattern, we carried out a proteomic analysis based on 5 different analytical groups: (1) classical RTT versus PSV-RTT, (2) RTT versus controls, (3) RTT sisters Family 1 versus RTT sisters Family 2, (4) no. 1 classical RTT versus no. 3 classical RTT, and (5) no. 2 PSV-RTT versus no. 4 PSV-RTT. Among these groups there were significant quantitative and qualitative variations in 14 protein spots subsequently identified by mass spectrometry. Protein name as well as peptide matches, sequence coverage, and the probabilistic score obtained using the MASCOT software are summarized (Table 1). All the identified proteins are known to be involved in specific biological processes [16–27]. Proteomic plasma maps of healthy control, RTT sisters Family 1, and RTT sisters Family 2 with the protein spots are represented (Figure 1). Black arrows indicate the spots with quantitative variations while all the identified qualitative variations are reported with black circles.

As shown in Figure 2, significant changes appeared in alpha-1-microglobulin (AMBP), haptoglobin (HPT/Hp, spots 3 and 4), fibrinogen beta chain (FIBB), complement C3 (CO3), and transthyretin (TTHY) in classical RTT siblings as compared to PSV-RTT sisters. In addition, quantitative and qualitative protein variations values as derived from the examined RTT sister pairs and healthy controls comparative analyses were reported (Tables 2 and 3).

RTT patients, when compared to control group, showed 6 underexpressed protein spots including FIBB, hemoglobin subunit beta (HBB), serum transferrin (TRFE), HPT, Ig gamma-2 chain C region (IGHG2), and CO3, while 1 spot of clusterin (CLUS) is overexpressed (Table 3).

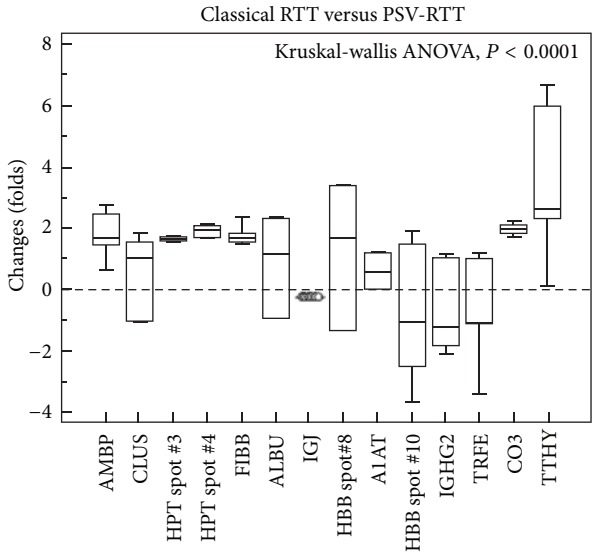


Figure 2: Plasma proteins expression in sisters with classical Rett syndrome as protein expression ratios of classical RTT versus PSV-RTT plasma proteome. Data are expressed as box-and-whiskers plots. Results of the Kruskal-Wallis ANOVA are shown.

Family 1 versus Family 2 (third group in Table 3) showed a significant underexpression of TTHY, a significant overexpression of HBB, and the appearance of one protein spot of albumin (ALBU). The comparison between the two classical forms showed underexpression of 4 protein spots (AMBP, CLUS, IGHG2, and TTHY) and appearance of 3 protein spots (ALBU, HBB, and A1AT) in no. 1 as compared to no. 3. Significant qualitative variations are most evident in the comparison between the two PSV variants in which 5 protein spots appeared (AMBP, CLUS, ALBU, immunoglobulin J chain, IGJ and CO3) while 2 proteins (HBB and TTHY) disappeared in no. 2 as compared to no. 4. Moreover, in the same comparative group, an overexpression of HPT was observed (Table 3). In addition, another comparative analysis of each RTT patient versus healthy controls has resulted in several significant quantitative and qualitative variations in plasma proteome (see Table 4 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/438653) and a number of protein spots changes likely due to the size effect originating from the healthy control group were detected but considered to be not significant (data not shown) and not comparable (*n.c.*, Table 2).

## 4. Discussion

Proteomic analysis has proven effective in identifying variations in proteins with biological and/or clinical significance [30]. The examined RTT siblings pairs represented an interesting benchmark model to test the molecular basis of phenotypical expression variability and to identify potential therapeutic targets of the disease.

Rett syndrome is the result of a monogenic mutation, that is, the X-linked *MECP2* gene in the overwhelming majority of cases. As RTT is an X-linked trait and the *MECP2* locus is subject to X inactivation, different patterns of X inactivation may lead to different phenotypes within a group of patients who carry the same mutation. Based on these data some authors speculate that there might be a group of RTT patients with milder phenotypes owing to skewed X inactivation, who have not so far been identified because of their atypical phenotypes. Nonetheless, variations in XCI are known to explain only 1/5 of the variance in severity of the disease [31], thus not fully accounting for the phenotype severity range typically seen in RTT [32].

We can safely state that in our patients, as well as in the majority of Rett syndrome patients reported in the literature, X inactivation was found to be balanced. Thus, it is reasonable to assume that the clinical phenotype of our pairs of sisters appears to be determined mainly by the type and location of the *MECP2* mutations.

Statistical analysis, represented by the fold changes, revealed in the classical RTT versus PSV-RTT comparison a significant overexpression of proteins involved in APR including AMBP, HPT, FIBB, A1AT, and CO3 [16, 18, 23, 26]. Although little is known about the APR and the frequency of infections in RTT [33], our findings evidenced a lack of some key APR components. Possible explanations for these findings may include a continuous stimulation of cytokine-mediated liver protein synthesis, an accelerated turnover of APR proteins, or a combination of both. The evidence that the RTT patients present chronic terminal bronchiolitis and an increase in intestinal microbiome due to constipation suggest the coexistence of recurrent infections [34, 35]. Evidence of the involvement of inflammatory events in RTT, was mainly represented by the significant variations of AMBP and A1AT (fifth and sixth comparative groups in Table 3), two serine protease inhibitors linked to the acute phase reaction, which limit the damage caused by activated neutrophils and their enzyme elastase [16, 23]. A major role for the immune system in RTT pathogenesis has been previously documented by the fact that transplantation of wild-type bone marrow restores wild-type microglia and arrests pathology in a mouse model of RTT [36].

The other finding on a partial deficit in an oxygen transport HBB [22] could be compatible with our prior finding of a subclinical hypoxia with an altered redox status in RTT patients with the classical phenotype [37].

Interestingly, TRFE was significantly underexpressed in RTT as compared to healthy controls, confirming the association previously reported in autism [38]. Alterations in the TRFE levels may lead to abnormal iron metabolism in RTT; it has been suggested for autism [38]. On the other hand, TRFE is also a negative APR protein whose expression levels decrease during inflammation [25]. Thus the underexpression of TRFE in RTT suggests once again that inflammatory process may play a key role in the pathogenesis of the disease.

More intriguing is the finding of an overexpressed CLUS, which may reflect a counterbalanced response to excessive proteins accumulation, namely, the unfolded protein response [17]. Abundant evidences demonstrated that CLUS expression is increased during cellular stress [17]. The chaperone action of CLUS could be cytoprotective in either or

Table 2: Quantitative and qualitative protein variations values.

| ID | AC[a] | Short name | Classical versus PSV | | RTT versus healthy controls | | Family 1 (*F1*) versus Family 2 (*F2*) | | No. 1 Classical versus no. 3 Classical | | No. 2 PSV versus no. 4 PSV | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | No. 2, no. 4 | No. 1, no. 3 | Controls | *F1, F2* | *F2* | *F1* | No. 3 | No. 1 | No. 4 | No. 2 |
| 1 | P02760 | AMBP | 0.63 ± 0.69 | 1.29 ± 0.35 | 1.92 ± 0.36 | 0.96 ± 0.63 | 0.80 ± 0.88 | 1.12 ± 0.18 | 1.59 ± 0.12 | 0.99 ± 0.12$^{*}$ | n.d. | 1.26 ± 0.11 |
| 2 | P10909 | CLUS | 0.93 ± 1.02 | 1.21 ± 0.34 | 0.51 ± 0.23 | 1.07 ± 0.74$^{**}$ | 0.75 ± 0.83 | 1.39 ± 0.52 | 1.50 ± 0.17 | 0.92 ± 0.05$^{*}$ | n.d. | 1.87 ± 0.08 |
| 3 | P00738 | HPT | 2.14 ± 0.31 | 3.56 ± 0.19$^{**}$ | 3.56 ± 1.04 | 2.85 ± 0.78 | 2.98 ± 0.65 | 2.72 ± 0.94 | 3.56 ± 0.16 | 3.57 ± 0.25 | 2.40 ± 0.09 | 1.88 ± 0.18 |
| 4 | P00738 | HPT | 2.53 ± 0.80 | 4.88 ± 0.62$^{**}$ | 5.74 ± 1.49 | 3.70 ± 1.40$^{**}$ | 3.08 ± 1.41 | 4.33 ± 1.18 | 4.35 ± 0.34 | 5.40 ± 0.08 | 1.81 ± 0.03 | 3.26 ± 0.11$^{**}$ |
| 5 | P02675 | FIBB | 2.02 ± 0.26 | 3.65 ± 0.63$^{**}$ | 7.35 ± 3.10 | 2.83 ± 0.97$^{**}$ | 3.01 ± 1.26 | 2.66 ± 0.62 | 4.11 ± 0.56 | 3.18 ± 0.20 | 1.90 ± 0.16 | 2.13 ± 0.33 |
| 6 | P02768 | ALBU | 0.94 ± 1.02 | 1.08 ± 1.19 | 3.49 ± 1.34 | 1.01 ± 1.06 | n.d. | 2.02 ± 0.17 | n.d. | 2.17 ± 0.09 | n.d. | 1.87 ± 0.04 |
| 7 | P01591 | IGJ | 0.22 ± 0.24 | n.d. | 0.55 ± 0.16 | 0.11 ± 0.19 | n.d. | 0.22 ± 0.24 | n.d. | n.d. | n.d. | 0.43 ± 0.02 |
| 8 | P68871 | HBB | 1.34 ± 1.47 | 2.25 ± 2.46 | 4.81 ± 2.37 | 1.80 ± 1.99 | 1.34 ± 1.47 | 2.25 ± 2.46 | n.d. | 4.50 ± 0.04 | 2.68 ± 0.07 | n.d. |
| 9 | P01009 | A1AT | n.d. | 0.60 ± 0.26 | 2.57 ± 1.24 | 0.30 ± 0.54 | n.d. | 0.60 ± 0.26 | n.d. | 1.20 ± 0.03 | n.d. | n.d. |
| 10 | P68871 | HBB | 6.91 ± 2.61 | 6.59 ± 4.53 | 10.38 ± 4.30 | 6.75 ± 3.53$^{*}$ | 3.85 ± 1.50 | 9.65 ± 2.25$^{**}$ | 2.93 ± 1.07 | 10.26 ± 3.15 | 4.78 ± 1.37 | 9.03 ± 1.25 |
| 11 | P01859 | IGHG2 | 1.97 ± 1.56 | 1.61 ± 0.57 | 4.16 ± 1.57 | 1.79 ± 1.14$^{**}$ | 1.34 ± 0.86 | 2.25 ± 1.27 | 2.12 ± 0.15 | 1.11 ± 0.18$^{**}$ | 0.56 ± 0.09 | 3.39 ± 0.35 |
| 12 | P02787 | TRFE | 3.21 ± 0.44 | 3.11 ± 0.50 | 10.51 ± 3.90 | 3.16 ± 0.45$^{**}$ | 3.30 ± 0.61 | 3.03 ± 0.18 | 3.26 ± 0.72 | 2.96 ± 0.22 | 3.33 ± 0.65 | 3.09 ± 0.15 |
| 13 | P01024 | CO3 | 0.99 ± 1.08 | 2.04 ± 0.31 | 4.64 ± 1.32 | 1.51 ± 0.94$^{**}$ | 1.12 ± 1.24 | 1.91 ± 0.12 | 2.23 ± 0.34 | 1.85 ± 0.15 | n.d. | 1.97 ± 0.06 |
| 14 | P02766 | TTHY | 1.34 ± 1.47 | 5.86 ± 2.91$^{**}$ | 2.61 ± 0.83 | 3.60 ± 3.20 | 5.59 ± 3.20 | 1.61 ± 1.78$^{**}$ | 8.49 ± 0.56 | 3.23 ± 0.30$^{**}$ | 2.69 ± 0.04 | n.d. |

Spot ID refers to that shown in 2-DE maps (Figure 1). [a]Accession numbers of Swiss-Prot or GenBanK databases. Proteins values are expressed as relative %V (mean ± SD). For proteins significantly decreased or increased: $^{*}P < 0.05$, $^{**}P < 0.01$. n.d.: not detected.

Table 3: Protein variations as derived from the examined RTT sister pairs and healthy controls comparative analyses.

| Analytical groups | Plasma proteome differences | | | |
|---|---|---|---|---|
| | Quantitative variations | | Qualitative variations | |
| | Underexpressed | Overexpressed | Disappearance | Appearance |
| Classical RTT[(2)] versus PSV-RTT[(2)] | N.D. | ↑ AMBP*, ↑ HPT*, ↑ FIBB*, ↑ CO3*, ↑ TTHY* | N.D. | A1AT |
| RTT[(4)] versus controls[(10)] | ↓ HPT, ↓ FIBB, ↓ HBB, ↓ IGHG2, ↓ TRFE, ↓ CO3 | ↑ CLUS | N.D. | N.D. |
| RTT sisters Family 1[(2)] versus RTT sisters Family 2[(2)] | ↓ TTHY | ↑ HBB | N.D. | +ALBU |
| No. 1 classical RTT[(1)] versus no. 3 classical RTT[(1)] | ↓ AMBP, ↓ CLUS, ↓ IGHG2, ↓ TTHY | N.D. | N.D. | +ALBU, +HBB, +A1AT |
| No. 2 PSV-RTT[(1)] versus no. 4 PSV-RTT[(1)] | N.D. | ↑ HPT | −HBB, −TTHY | +AMBP, +CLU, +ALBU, +IGJ, +CO3 |

↓: protein spot underexpressed; ↑: protein spot overexpressed; −: protein spot disappearance; +: protein spot appearance; N.D.: not detectable; * changes referred to relative variations between classical RTT and PSV-RTT siblings.
A1AT: alpha-1-antitrypsin; AMBP: alpha-1-microglobulin; ALBU: albumin; CLUS: clusterin; CO3: complement C3; FIBB: fibrinogen beta chain; HBB: hemoglobin subunit beta; HPT: haptoglobin; IGHG2: immunoglobulin gamma-2 chain C region; IGJ, immunoglobulin J chain; TRFE, serum transferrin; TTHY: transthyretin. Numbers in the parentheses indicate the number of patients or subjects compared.

both the intra- or extracellular environments. In particular, extracellular CLUS binds to and prevents aggregation of partly unfolded proteins such as receptors on the surface of stressed cells [17]. This action may promote cell survival by minimizing stress-induced aberrant signaling.

Of course, our results have to be confirmed in a larger patients population. In the present study, we focused on the comparison between the two RTT variants by exposing the differences in protein patterns. Both siblings with classical RTT had a significant overexpression of HPT, one of the APR proteins induced in response to infection, tissue injury, and malignancy. HPT was originally described as functioning by the absorption of free hemoglobin and prevention of the consequent kidney damage [18]. However, it has subsequently became apparent that the physiological role of HPT is not limited to the trapping of free hemoglobin. Bacteriostatic and angiogenic effects, antibody-like and antioxidative properties have also been reported [18]. Evidence of an association between the Hp 2-2 phenotype and neurological disorders, like epilepsy and autism, has been reported [39, 40]. In support of this, we have distinguished Hp 2-2 phenotype in the 2-DE plasma maps corresponding to the two PSV-RTT (no. 2 and no. 4) and to a no. 3 classical RTT, referring to the previous 2-DE reference plasma maps reported in the literature [41]. Hp 2-2 phenotype is associated with a higher immune reactivity and ability to form antibodies. Moreover, the possession of this particular phenotype has been associated with the prevalence and clinical evolution of many inflammatory diseases including infections as tuberculosis, vaccination, viral hepatitis, atherosclerosis, and cardiovascular and autoimmune diseases [18, 39, 42]. Furthermore, Hp 2-2 shows lower binding of hemoglobin and antioxidant capabilities than Hp 1-1 phenotype [18].

Both classical RTT subjects also likely had a significant overexpression of FIBB which has a double function: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation [19]. Moreover, FIBB expression level can be greatly increased as key component of the APR following tissue injury and infection/inflammation [19].

Our results also suggest that classical RTT subjects, given the significant variation observed in TTHY, may be likely more prone to have a dysfunction in thyroid hormone binding and transport proteins [27]. Physiologically, TTHY is responsible for thyroid hormone and retinol transport, through the binding of retinol binding protein [27]. Evidence of underexpression of TTHY and thyroid dysfunction has been reported in patients with RTT [43, 44].

Underexpression and/or disappearance of TTHY in the third, fourth, and fifth analytical groups (see Table 3) may suggest a possible relationship of this protein with oxidative stress (OS), as its changes could be related to low plasma retinol levels, in turn contributing to the production of reactive oxygen species [45]. The already solid evidence of enhanced OS and lipid peroxidation in RTT patients at different stages and with different gene mutations [37] seems to be in line with this interpretation.

We used plasma samples in order to embrace all the protein components of the blood soluble as the plasma proteome represents the largest and deepest version of the human proteome present in any sample [30].

Interestingly, in our findings plasma APR proteins (i.e., AMBP, HPT, FIBB, A1AT, and CO3) are five out of six (i.e., 83.3%) of the overexpressed proteins found in the classical sisters as compared with the PSV ones. TTHY might be the result of an adaptive endocrinological response to yet be clarified inflammatory stimuli. Therefore, inflammation could represent a potential novel target for the disease and inflammation-modulating drugs might be tested for the reduction of phenotype severity. To this regard, the naturally occurring and less aggressive anti-inflammatory molecules $\omega$-3 polyunsaturated fatty acids (PUFAs) have been suggested to reduce phenotype severity in RTT [46–49]. Therefore, a proteomic analysis of plasma samples from RTT patients could provide a personalized pharmacological intervention.

The study of sibling pairs, that is, partially genetically related subjects, further stresses the importance of personalizing the treatment. This kind of studies may contribute to a better understanding of the biological mechanisms for the observed benefits of $\omega$-3 PUFAs supplementation in classical RTT patients. Actually, our unpublished data seem to suggest that $\omega$-3 PUFAs supplementation is less efficient for PSV-RTT patients (J. Hayek, unpublished data).

Of note, our study may have identified novel targets for personalized RTT pharmacological intervention. To date there are no specific treatments to counterbalance protein expression and to reduce some of the clinical outcomes of RTT patients. Recently, a partial rescue of some of the neurological defects in RTT by $\omega$-3 PUFAs has been reported [48]. Future plasma proteomics investigation on classical RTT and PSV-RTT patients treated with PUFAs would be an innovative strategy. This proteomic approach could be applied on patients presenting other clinical variants (ESV and congenital variant), in different tissues, cells, and biological fluids (i.e., cerebrospinal fluid, urine) and using experimental mouse and rat RTT models. Taken together, our results also suggest that (i) independently of the *MECP2* mutation type, some still unknown posttranscriptional modulating factors can be able to influence the clinical phenotype; (ii) these factors, combined with specific comutations [9], can determine alterations in the amount of plasma proteins (i.e., the significant increase of proteins involved in the inflammatory process, evident in the more severe phenotype) and/or hypothetically the functionality of some plasma proteins; (iii) there is a complexity degree high than previously thought as based on the exclusive effects of the *MECP2* gene mutation.

## 5. Conclusion

In summary, our results demonstrate that variations observed in RTT plasma proteome relate to proteins involved in several relevant biological processes other than those confined to the central nervous system. In particular APR/inflammation, blood coagulation, and OS response associated pathways appear to be involved. Our findings indicate that the study of unique familial cases offers the opportunity to identify new protein patterns involved in the RTT phenotype expression.

## Conflict of Interests

All authors declare that they have no conflict of interests related to the present study.

## Authors' Contribution

Alessio Cortelazzo and Roberto Guerranti contributed equally to this work.

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*Research Article*

# Erythrocyte Shape Abnormalities, Membrane Oxidative Damage, and $\beta$-Actin Alterations: An Unrecognized Triad in Classical Autism

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Autism spectrum disorders (ASDs) are a complex group of neurodevelopment disorders steadily rising in frequency and treatment refractory, where the search for biological markers is of paramount importance. Although red blood cells (RBCs) membrane lipidomics and rheological variables have been reported to be altered, with some suggestions indicating an increased lipid peroxidation in the erythrocyte membrane, to date no information exists on how the oxidative membrane damage may affect cytoskeletal membrane proteins and, ultimately, RBCs shape in autism. Here, we investigated RBC morphology by scanning electron microscopy in patients with classical autism, that is, the predominant ASDs phenotype (age range: 6–26 years), nonautistic neurodevelopmental disorders (i.e., "positive controls"), and healthy controls (i.e., "negative controls"). A high percentage of altered RBCs shapes, predominantly elliptocytes, was observed in autistic patients, but not in both control groups. The RBCs altered morphology in autistic subjects was related to increased erythrocyte membrane $F_2$-isoprostanes and 4-hydroxynonenal protein adducts. In addition, an oxidative damage of the erythrocyte membrane $\beta$-actin protein was evidenced. Therefore, the combination of erythrocyte shape abnormalities, erythrocyte membrane oxidative damage, and $\beta$-actin alterations constitutes a previously unrecognized triad in classical autism and provides new biological markers in the diagnostic workup of ASDs.

## 1. Introduction

Autism spectrum disorders (ASDs) are considered to be the result of a complex interaction between a genetic background and environmental factors [1, 2]. Autism is a heterogeneous, behaviorally defined neurodevelopmental disorder affecting four times more males than females [3], with a clinical onset usually within the 2nd year of life and mainly consisting of social impairment; communication difficulties; and restricted, repetitive, and stereotyped patterns of behavior.

Classical autism is the overwhelmingly predominant ASDs phenotype, which also include other four disorders, that is, Asperger syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS), and childhood disintegrative disorder. Rett syndrome (RTT) is a genetically determined neurodevelopmental disorder with autistic features, and has been recently separated by the ASDs as a distinct nosological entity (Diagnostic and Statistical Manual of mental disorders V-DSMV).

The search for specific and reliable biomarkers is of paramount importance in autism, given its dramatically rising prevalence in the general population over the last two decades [4] from 1 in 5000 in the mid-1970s to 1 in 88 in 2008 [4, 5].

A redox imbalance has been repeatedly reported in autism [6–8] although its role in the pathogenesis is still under question. In particular, it is unclear whether oxidative stress (OS) is a cause or consequence of autism [9]. Recent studies indicate that autism is associated with deficits in glutathione antioxidant defense in selective regions of the brain, thus potentially contributing to OS, immune dysfunction, and apoptosis, particularly in the cerebellum and temporal lobe [10].

More recently, OS and erythrocyte membrane alterations have been described in autistic children, including elevated erythrocyte thiobarbituric acid reactive substances, urinary isoprostane, and hexanoyl-lysine levels, associated with a significant reduction of $Na^+/K^+$-ATPase activity, a reduction of the erythrocyte membrane fluidity and an alteration in the erythrocyte fatty acid membrane profile linked to an increase in monounsaturated fatty acids, a decrease in eicosapentaenoic acid and docosahexaenoic acid, and a consequently increased $\omega$-6/$\omega$-3 ratio [11].

The peculiar triad of OS imbalance, mild chronic hypoxia, and an abnormally high frequency of leptocytes in the peripheral blood has been reported by our group in girls RTT [12], a relatively rare neurodevelopmental disorder almost affecting females and mainly due to *de novo* mutations of the X-linked methyl-CpG-binding protein 2 (*MeCP2*) gene [13]. These data indicate that redox imbalance and oxygen exchange could be key players in the pathogenesis of this particular human model of autism.

The shape is critical to red blood cells (RBCs) function and blood rheological properties, and emerging evidence indicates that OS is a key factors in modulating erythrocyte shape [14–16]. Although RBCs membrane lipidomics [11] and rheological variables [17] have been reported to be altered, and some suggestions of an increased lipid peroxidation in the erythrocyte membrane exist [11], to date no information is available on how the RBCs oxidative membrane damage may affect cytoskeletal membrane proteins and, ultimately, erythrocyte shape in autism.

In the present study, we investigated RBC morphology, *in situ* membrane oxidative damage, and cytoskeletal proteins in patients with classic autistic disorder.

## 2. Methods

*2.1. Subjects Population.* A total of $n = 15$ patients (male: 9; female: 6), with classic autistic disorder (mean age at examination: 15.9 ± 5.9 years, range 6–26), as well as $n = 15$ healthy controls of comparable age and a typical neurodevelopment (mean age: 16.3 ± 6.2 years, range 5–30; male: 8; female: 7) participated in the study. Furthermore, a third group of 15 patients (male: 7; female: 8) with nonautistic neurodevelopmental disorders (NA-NDDs) (mean age at examination: 15.8 ± 6.0 years, range 5–28) was enrolled as a "positive control" population, including idiopathic mental retardation ($n = 6$), cerebral palsy ($n = 3$), Attention-Deficit/Hyperactivity Disorder (ADHD) ($n = 4$), and language disorders ($n = 2$). Childhood Autism Rating Scale scores (CARS) [18] for the examined autistic patients and the NA-NDDs groups were estimated. The populations of patients and healthy subjects were recruited by the medical staff of the Child Neuropsychiatry Unit of the Azienda Ospedaliera Senese (Siena, Italy). All the enrolled patients and healthy subjects were genetically unrelated. The autistic patients were diagnosed by DSMV and evaluated using Autism Diagnostic Observation Schedule (ADOS), and Autism Behaviour Checklist (ABC). Patients with RTT, X-fragile syndrome, or tuberous sclerosis, as well as subjects with clinical evident sideropenic anemia, perinatal adverse events, and/or brain abnormalities on magnetic resonance imaging, were excluded for the present study. All subjects were on a typical Mediterranean diet.

The study was conducted after the approval by the Institutional Review Board and all written informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

*2.2. Routine Hematological Analyses.* For these particular laboratory determinations, samples collected in tubes with $K_2$EDTA were analyzed by an automated hematology system Sysmex XE-2100 (Sysmex corporation, Japan) in the automated aspiration (i.e., closed) sampling mode, using 200 $\mu$L sample volume. The instruments are in routine use for count blood cells and automated differential counts analyses and underwent periodic quality assessment in internal and external control programs. A 5-part differential count was performed by lysing erythrocytes and analyzing the light scatter/fluorescence [19]. Blood smears were stained with standard May-Grünwald Giemsa within 6 hours after blood sampling (SP1000 instrument) and visualized by an automated image recognition system CellaVision DM96, an automated microscope with software showing digitalized images of the blood smears.

*2.3. Blood Sampling for Erythrocyte Oxidative and Shape Analysis Studies.* For these aims, an aliquot of blood was collected in heparinized tubes, and manipulations were carried out within 2 hrs after sample collection. Blood samples were centrifuged at 2400 ×g for 15 min at 4°C, whereas the platelet poor plasma and the buffy coat were removed by aspiration. RBCs were washed twice with physiological solution (150 mM NaCl). An aliquot of packed erythrocytes was resuspended in Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM glucose, 1 mM $CaCl_2$), pH 7.4 as a 50% (vol/vol) suspension for the determination

of intraerythrocyte nonprotein-bound-iron (NPBI). The remaining volume of packed RBCs was used for erythrocyte membranes preparations (i.e., hemoglobin-free ghosts) for 4-hydroxynonenal protein adducts (4-HNE PAs) determinations.

An aliquot of each blood sample (1 mL) was centrifuged at 800 ×g for 10 minutes at 4°C for scanning electron microscopy (SEM) analysis of erythrocytes.

*2.4. Scanning Electron Microscopy (SEM).* As previously described [12], erythrocytes were plated on poly-l-lysine coated slides and fixed in Karnowsky (2.5% glutaraldehyde—4% paraformaldehyde in 0.1 M sodium-cacodylate buffer, pH 7.2) for 2 h at 4°C, rinsed twice for 10 min with 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer for 1 h at 4°C. Specimens were then dehydrated through a graded ethanol series, dried in a $CO_2$ critical point dryer (CPD010, Balzers Union, Liechtenstein), mounted on specimen stub, sputter coated with gold (Sputter Coater S150B, Edwards, England), and examined in a XL 20 SEM (Philips, Eindhoven, Netherlands). Altered RBCs shapes were evaluated by counting ≥800 cells (50 erythrocytes for each different SEM field at a magnification of ×3000) from all groups of subjects. All countings were carried out in triplicate and averages were taken for data analysis.

*2.5. Intraerythrocyte NPBI.* Intraerythrocyte NPBI (nmol/mL) was determined as a desferrioxamine (DFO)-iron complex (ferrioxamine), as previously reported [20]. Briefly, 25 $\mu$M DFO was added to the samples (1 mL erythrocyte suspension), the erythrocytes were then lysed by adding water (1 vol) and freezing (−70°C) and thawing. The hemolysate was ultrafiltered at 3373 ×g for 30 min in centrifugal filters with a 30 kDa molecular weight cutoff (VIVASPIN 4, Sartorius Stedim Biotech GmbH, Goettingen Germany) and the ultrafiltrate was stored at −20°C until analysis. The DFO excess was removed by silica (Silicagel; 25–40 $\mu$m) column chromatography. The DFO-iron complex was determined by HPLC and the detection wavelength was 229 nm. The calibration curve correlation for intraerythrocyte NPBI was adequate ($r^2$ = 0.994009), the minimum detection limit was 0.1 nmol/mL, and mean intra- and inter-observer coefficients of variation were ≤2.5% and ≤5%, respectively.

*2.6. Erythrocyte Membrane Preparation.* An aliquot (600 $\mu$L) of packed RBCs was lysed in Dodge buffer, and erythrocyte membranes were prepared, according to Dodge et al. [21], by repeated washing until the "ghosts" were pearly white. Samples were kept frozen at −70°C until used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation (IP), and esterified $F_2$-isoprostanes ($F_2$-IsoPs).

*2.7. Immunoprecipitation of Erythrocyte β-Actin.* Erythrocyte membrane (ghosts) proteins (200 $\mu$g) were incubated with 5 $\mu$g $\beta$-actin antibody (Millipore Corporation, Billerica, MA, USA) overnight at 4°C on a rotator. Then, immune complex was incubated with 50 $\mu$L of Protein A-Sepharose (Sigma-Aldrich, Milan, Italy) and rotated at 4°C for 2 h. Samples were centrifuged at 10,000 g for 5 min and washed three times with 1 mL ice-cold PBS. The pellet was mixed with 2X reducing sample buffer, boiled, and loaded on SDS-PAGE gels for silver staining or western blotting analysis.

*2.8. Silver Staining of Erythrocyte Membrane Proteins and Immunoprecipitated β-Actin.* Erythrocyte membrane (i.e., "ghosts") proteins (20 $\mu$g protein, determined using BioRad protein assay; BioRad, Hercules, CA) or immunoprecipitated $\beta$-actin were separated by the polyacrylamide gel electrophoresis (one-dimensional) method for discontinuous SDS-PAGE on 10% polyacrylamide gels in denaturing conditions, according to Laemmli [22]. At the end of the electrophoretic run, gels were stained with silver nitrate for protein visualization (Sigma-Aldrich, Milan, Italy). Gel image was acquired using image scanner and the bands were automatically detected and analyzed using TotalLab software (nonlinear dynamics, version 1.0). Band volume was expressed as a ratio of the total protein volume detected from the entire gel to minimize differences between band (band normalization) and to compare band measurements in different lanes.

*2.9. Western Blot for 4-HNE Protein Adducts in Erythrocyte Membrane Proteins and Immunoprecipitated β-Actin.* Western blot protocols were performed as previously described [12]. Erythrocyte membrane proteins (40 $\mu$g protein, determined using BioRad protein assay; BioRad, Hercules, CA) or the immunoprecipitated $\beta$-actin were resolved on 10% SDS-PAGE gels and transferred onto a hybond ECL nitrocellulose membrane (GE Healthcare Europe GmbH, Milan, Italy). After blocking in 3% nonfat milk (BioRad, Hercules, CA, USA), the membranes were incubated overnight at 4°C with goat polyclonal anti-4-HNE adduct antibody (cod. AB5605; Millipore Corporation, Billerica, MA, USA). Following washes in TBS Tween and incubation with specific secondary antibody (mouse anti-goat horseradish peroxidase-conjugated, Santa Cruz Biotechnology, Inc., CA, USA) for 1 h at RT, the membranes were incubated with ECL reagents (BioRad, Hercules, CA, USA) for 1 min. Images were digitized (ChemiDoc XRS, BioRad, Hercules, CA) and band optical densities were quantified using a computerized imaging system (Quantity One Imaging system).

*2.10. Erythrocyte Membrane Esterified $F_2$-Isoprostanes ($F_2$-IsoPs).* A 100 $\mu$L aliquot of the erythrocyte membrane samples was resuspended with $H_2O$ (0.9 mL) and 1 N KOH (500 $\mu$L) was added for the basic hydrolysis. After incubation at 45°C for 45 min, the sample was acidified to pH 3 with HCl 1 N (500 $\mu$L), spiked with tetradeuterated Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$-$d_4$) (500 pg in 50 $\mu$L of ethanol; Cayman, Ann Arbor, MI, USA), as internal standard, and extracted with 10 mL of ethyl acetate. The upper organic layer, obtained after centrifugation at 1000 ×g for 5 min, was applied onto an aminopropyl ($NH_2$) cartridge (Waters, Milford, MA, U.S.A.) preconditioned with 10 mL of hexane. After derivatization the determination of $F_2$-IsoPs was accomplished by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis [12].

Esterified $F_2$-IsoPs were normalized for membrane proteins and quantified by BioRad protein assay (BioRad, Hercules, CA) using 0.2% Triton X-100 to dissolve the membranes. The measured ions were the product ions at *m/z* 299 and *m/z* 303 derived from the $[M\text{-}181]^-$ precursor ions (*m/z* 569 and *m/z* 573) produced from 15-$F_2$t-IsoPs (the most represented $F_2$-IsoP isomer) and $PGF_{2\alpha}$-$d_4$, respectively [12].

*2.11. Statistical Data Analysis.* All variables were tested for normal distribution (D'Agostino-Pearson test) and data were presented as means ± SD for normally distributed variables. Differences between groups were evaluated using independent-sample *t*-test (continuous normally distributed data), Mann-Whitney rank sum test (continuous nonnormally distributed data), chi-square statistics (categorical variables with minimum number of cases per cell ≥5) or Fisher's exact test (categorical variables with minimum number of cases per cell <5), one-way analysis of variance (ANOVA), Student-Newman-Keuls post hoc test or Kruskal-Wallis test. Associations between variables were tested by univariate regression analysis (Pearson's coefficients or Spearman's rho, as appropriate). The effects of small population sizes on possible type I ($\alpha$)/type II ($\beta$) errors in the data interpretation were examined using a sampling size algorithm. A two-sided $P < 0.05$ was considered to indicate statistical significance, and the Bonferroni-corrected significance levels were used for multiple *t*-tests. The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

## 3. Results

*3.1. IQ and CARS Estimates.* Estimated IQ and CARS values for the autistic group were 40.6 ± 12.80 (range 20–60), and 51.9 ± 7.0 (range 41–60), respectively, versus 63.0 ± 8.54 (values range 55–72) and 26.0 ± 2.2 (values range: 24–29), respectively, (*t*-test statistics 5.638 and −13.671, respectively; *P* value $s < 0.0001$).

*3.2. Red Blood Cell Counts.* Blood cell counts in patients were not significantly different from those of the control groups, with the single exception of a nonsignificant trend for MCHC, slightly lower in the autistic group ($^*P \geq 0.0669$) (Table 1). In particular, no laboratory signs of anemia in any of the three groups were evidenced. Nevertheless, statistical differences regarding Hb and MCV were detectable for the NA-NDDs group (Table 1). These findings would suggest a "relative microcytic anemia" in this latter population.

At the SEM analysis, significantly higher percentages of altered RBCs shapes were present in the patients' groups, as compared to healthy control subjects (Figures 1 and 2). In particular, abnormally shaped erythrocytes in the blood samples from autistic subjects predominantly featured elliptocytes (33.2 ± 11.2% of all erythrocytes, range: 15.4–55.5). Besides elliptocytes, various abnormal RBC shape were observed, including echinocytes, leptocytes, knizocytes, codocytes, and stomatocytes.

The NA-NDDs group showed mixed abnormally shaped RBCs, without an overwhelmingly predominant erythrocyte shape phenotype, although a slight prevalence of leptocytes (~25%) was detectable.

Table 1: Routine RBC variables in subjects with classical autism and nonautistic neurodevelopmental disorders (NA-NDDs) versus healthy controls.

| RBC variables | Healthy controls | NA-NDDs | Classical autism | *P* value (ANOVA) |
|---|---|---|---|---|
| Hb (g/dL) | 14.5 ± 0.9 | 13.6 ± 1.0* | 14.2 ± 1.6 | **0.001** |
| MCV (fL) | 85.1 ± 6.6 | 83.3 ± 4.4* | 86.1 ± 4.0 | **0.004** |
| MCH (pg/cell) | 28.8 ± 2.4 | 27.9 ± 1.6 | 28.6 ± 1.9 | 0.362 |
| MCHC (g/dL) | 33.9 ± 1.0 | 33.6 ± 0.9 | 33.2 ± 0.9 | 0.490 |
| RDW (%) | 13.4 ± 1.5 | 14.0 ± 2.1 | 13.1 ± 0.7 | 0.188 |

Data are expressed as means ± SD. $^*P$ value < 0.05 (post hoc analysis). Bold character indicates statistically significant differences.
Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width.

Significant positive relationships were observed for the percentages of discocytes, leptocytes, or stomatocytes and RDW ($\rho$ correlation coefficients range: 0.7245 to 0.8915). In addition, significant positive relationship were evidenced for the percentages of knizocytes and stomatocytes with Hb ($\rho$ = 0.6907 and $\rho$ = 0.5855, resp.), and the percentage of stomatocytes versus MCHC ($\rho$ = 0.6070). Significant inverse relationships were found between percentages of codocytes and RDW ($\rho$ = −0.8181), percentage of echinocytes and MCHC ($\rho$ = −0.8379), and echinocytes versus Hb ($\rho$ = −0.7776) (Table 2).

*3.3. Erythrocyte Oxidative Status.* Intraerythrocyte NPBI, together with erythrocyte membrane esterified $F_2$-IsoPs and 4-HNE PAs, were significantly increased in autistic subjects, as well as patients with NA-NDDs (*F*-ratio range: 6.41 to 64.31; *P* value range: 0.001 to 0.004), as compared to healthy controls (Figure 3). A distinct 4-HNE PAs pattern was detectable (i.e., autism > NA-NDDs > healthy controls), thus indicating that classical autism and NA-NDDs are sharing erythrocyte oxidative membrane damage, although membrane proteins likely undergo different degrees of oxidation.

*3.4. Erythrocyte Membrane Proteins.* Typical SDS-PAGE analysis are shown in Figure 4(a). Alterations in the whole electrophoretic pattern of autistic patients were observed. In particular, a quantitative decrease in $\beta$-actin (−16 to −18.8%; *F*-ratio = 314.5; $P < 0.001$) was evidenced and further confirmed in the western blot analysis carried out after actin-IP by using a specific antibody (Figure 4(b)). In autistic patients the blotting study indicated detectable protein bands showing increased binding with 4-HNE, that is, the major aldehyde product of lipid peroxidation, as compared to both negative and positive control subjects (Figure 4(c)). $\beta$-actin was found to be a major target for 4-HNE binding, as indicated by densitometric analysis (+61.9 to +78.8%; *F*-ratio = 2622.8; $P < 0.001$) following $\beta$-actin IP (Figure 4(d)).

*3.5. Correlations between Erythrocyte Shape, Membrane Oxidative Markers and β-Actin.* The percentage of elliptocytes

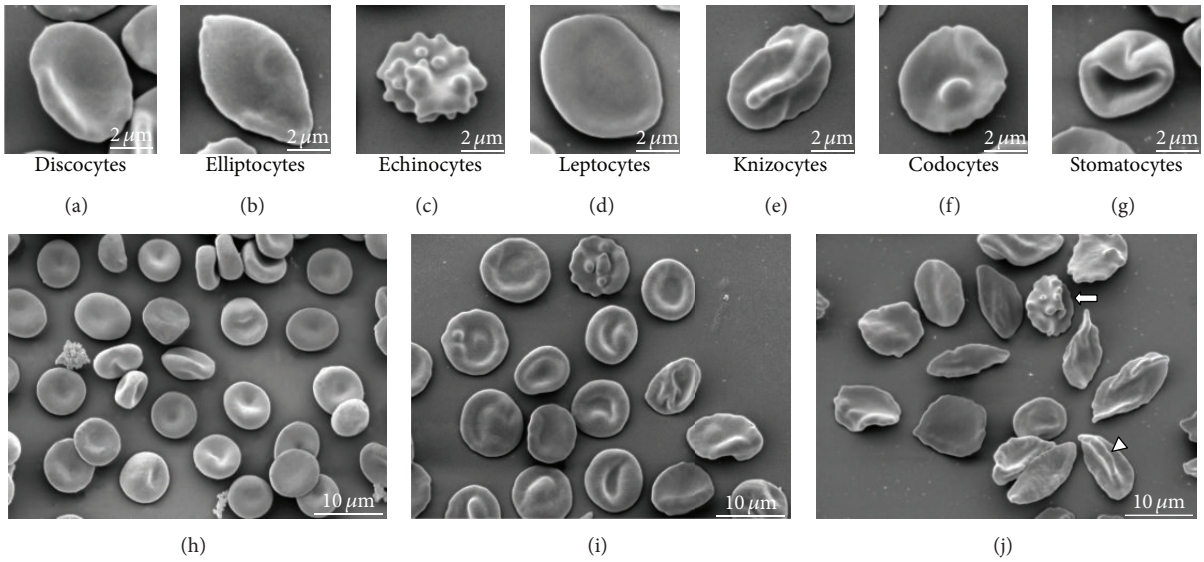


Figure 1: Abnormal erythrocyte shapes in classical autism at the scanning electron microscopy (SEM). (a): normal discocyte shape; (b) to (g): main shape-altered RBCs observed in autistic patients; (h): healthy controls; (i): a typical morphological pattern in nonautistic neurodevelopmental disorders (NA-NDDs); (j): typical picture in an autistic patient with predominant elliptocytosis. Symbols indicate intermediate-shaped RBCs: the arrow indicates a disco-echinocyte shape, while the arrowhead indicates the presence of a knizo-echinocyte shape in autistic patients, bars correspond to 2 $\mu$m in (a) to (g) upper panels and to 10 $\mu$m in the (h), (i), and (j) lower panels.

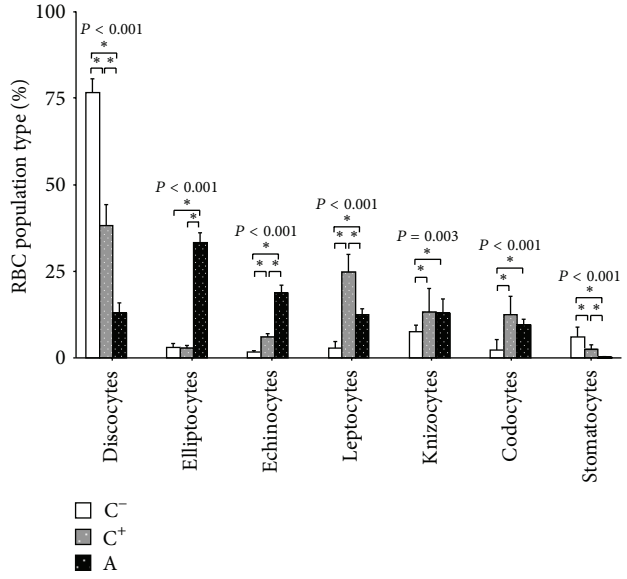


Figure 2: RBC morphology distribution in autistic patients, subjects with nonautistic neurodevelopmental disorders (NA-NDDs) and healthy controls. Statistically significant differences are denoted by single asterisk, $P > 0.05$. $P$ values above each erythrocyte shape classification refer to one-way ANOVA.

was positively correlated with IE-NPBI, erythrocyte membrane 4-HNE PAs, and esterified $F_2$-IsoPs, as well as membrane 4-HNE $\beta$-actin adducts (4-HNE $\beta$-AAs) levels. The examined OS markers showed positive associations between them, while elliptocytes were inversely related to $\beta$-actin membrane content, and a remarkably high inverse relationship between amount of $\beta$-actin in the membrane and 4-HNE $\beta$-AAs was observed ($r = -0.960$; $P < 0.001$) (Table 3).

## 4. Discussion

Our findings show the coexistence of a peculiar combination of erythrocyte shape abnormalities, erythrocyte membrane oxidative damage, and $\beta$-actin alterations, which represents a previously unrecognized triad in classical autism.

*4.1. RBCs Morphology.* Several conditions, either congenital or acquired, are known to lead to abnormal erythrocyte shape [23]. The maintenance of the discoid shape is of paramount importance for the RBC main physiological role (i.e., transport of respiratory gases to and from tissues), while the deformability of the circulating cells has critical effects on the rheological properties of blood [24].

Our observations indicate that elliptocytes are the predominant abnormal erythrocyte shape occurring in the peripheral blood from patients with classical autism, and, to the best of our knowledge, this is the first time that an abnormal erythrocyte shape is reported in ASDs other than RTT. The presence of abnormal RBC shapes appears to be associated with an altered redox status in erythrocytes and a decrease and/or oxidization for some of the main cytoskeletal proteins known to be critical for the maintenance of the horizontal interactions in the erythrocyte membrane architecture. Although, abnormal RBC shape are reported in a series of associated conditions [23], this is likely the first time that an elliptocytosis is described outside of the known conditions of hereditary elliptocytosis and thalassemias [25]. Hereditary elliptocytosis is caused by weakened horizontal

Table 2: Correlation matrix for RBC shape as a function of RBC parameters in autistic patients.

| RBC variables | Erythrocyte shape class | | | | | | |
|---|---|---|---|---|---|---|---|
| | Disco- | Ellipto- | Echino- | Lepto- | Knizo- | Codo- | Stomato- |
| Hb | 0.0159 | 0.1831 | **−0.7776*** | 0.1151 | **0.6907*** | −0.0336 | **0.5855*** |
| | (0.5452) | (0.9729) | (**0.0011**) | (0.4698) | (**0.0062**) | (0.9864) | (**0.0278**) |
| MCV | −0.2616 | 0.2063 | −0.0556 | −0.2841 | 0.2308 | 0.1960 | 0.4090 |
| | (0.1479) | (0.2834) | (0.8471) | (0.2442) | (0.3414) | (0.4069) | (0.1580) |
| MCH | −0.0771 | 0.2980 | −0.3959 | −0.1329 | 0.2868 | 0.0847 | 0.4720 |
| | (0.6423) | (0.3586) | (0.1732) | (0.4530) | (0.1144) | (0.8030) | (0.1099) |
| MCHC | 0.2571 | 0.3688 | **−0.8379*** | −0.0232 | 0.2720 | −0.1233 | **0.6070*** |
| | (0.3502) | (0.1553) | (**0.0002**) | (0.8842) | (0.2860) | (0.3714) | (**0.0214**) |
| RDW | **0.8915*** | −0.0476 | −0.4498 | **0.7245*** | 0.0357 | **−0.8181*** | **0.8278*** |
| | (**0.0050**) | (0.8806) | (0.0672) | (**0.0078**) | (0.9516) | (**0.0038**) | (**0.0031**) |

Data are Spearman's rho correlation coefficients with in brackets $P$ values ($N = 15$). Bold characters with asterisks indicate statistically significant correlations. Disco-: discocytes; Ellipto-: elliptocytes; Echino-: echinocytes; Lepto-: leptocytes; Knizo-: knizocytes; Codo-: codocytes; Stomato-: stomatocytes; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width.

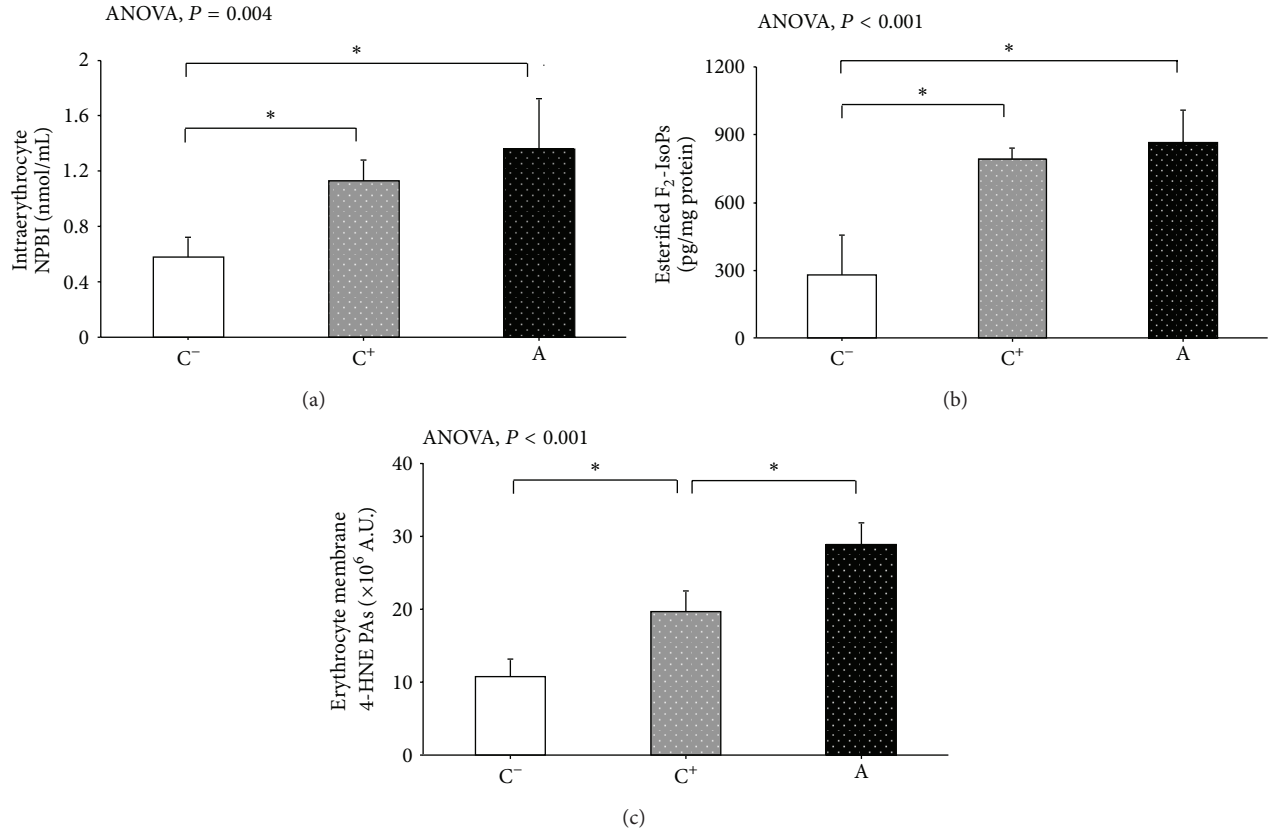


Figure 3: Intraerythrocyte NPBI, along with erythrocyte membrane esterified $F_2$-IsoPs and 4-HNE protein adducts in autistic patients, subjects with nonautistic neurodevelopmental disorders (NA-NDDs), and healthy controls. NPBI was reported as nmol/mL erythrocyte suspension, esterified $F_2$-IsoPs as pg/mg of erythrocyte membrane proteins, and 4-HNE protein adducts as arbitrary units. The data are expressed as means ± SD. Statistically significant differences are denoted by asterisks, $P < 0.05$. NPBI: non protein-bound-iron; $F_2$-IsoPs: $F_2$-isoprostanes; 4-HNE PAs: 4-HNE protein adducts.

linkages in membrane skeleton due either to a defective spectrin dimer-dimer interaction or a defective spectrin-actin-protein 4.1R junctional complex. The severity of the disease is related to extent of decrease in membrane mechanical stability and resultant loss of membrane surface area. In contrast to the healthy population, in which elliptical RBCs shapes are up to 15% of erythrocytes, a diagnosis of elliptocytosis requires that at least 25% of erythrocytes in the specimen are abnormally elliptical in shape [25]. Therefore, it can be hypothesized that autism and hereditary elliptocytosis,

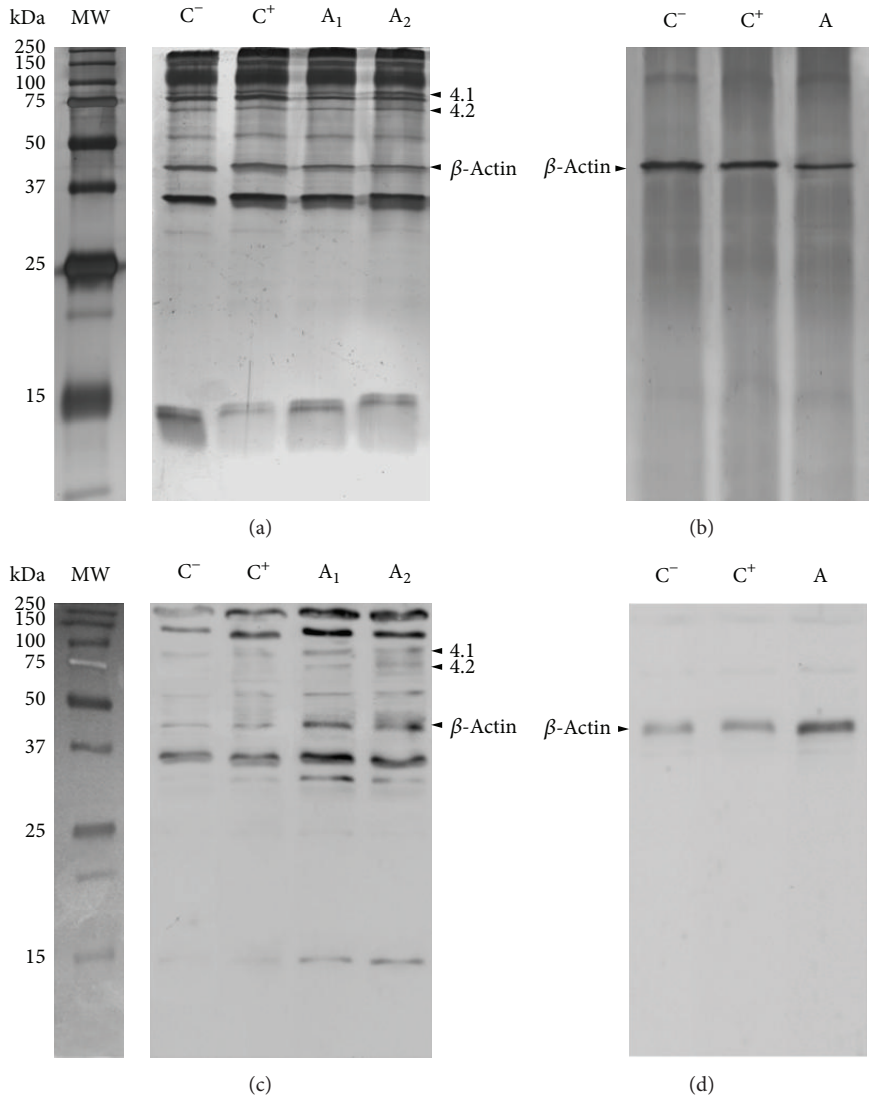


Figure 4: (a) Representative SDS-PAGE analyses of RBC ghosts (silver staining) in a healthy control subject (lane $C^-$), a positive control subject (see text for definition; lane $C^+$), and two autistic patients (lanes $A_1$ and $A_2$): visible reduction of intensity for $\beta$-actin in autistic patients (lanes $A_1$ and $A_2$) is evidenced; the electrophoretic position of bands 4.1 and 4.2 is indicated by arrowheads; (b) SDS-PAGE of immunoprecipitated $\beta$-actin (silver staining) from RBC ghosts of a healthy control subject (lane $C^-$), a positive control individual (lane $C^+$), and an autistic patient (lane A): visible reduction of intensity for $\beta$-actin in autistic patients (lane A); (c) immunochemical detection in the 4-HNE protein adducts in RBC ghosts: representative western blot from a healthy control subject (lane $C^-$), a positive control individual (lane $C^+$) and from an autistic patients (lanes $A_1$ and $A_2$). An increase in the 4-HNE PAs signal is evident in RBC ghosts from autistic patients (lanes $A_1$ and $A_2$), in particular as it concerns the $\beta$-actin band; the 4-HNE binding to bands 4.1 and 4.2 is indicated by arrowheads; and (d) immunochemical detection in the 4-HNE protein adducts in immunoprecipitated $\beta$-actin from RBC ghosts: representative western blot from a healthy control subject (lane $C^-$), positive control individual (lane $C^+$), and from an autistic patient (lane A): a visible increase in the 4-HNE PAs signal in $\beta$-actin from autistic patient (lane A) is evident. Molecular weight marks are indicated on the right side.

an inherited blood disorder with an estimated incidence of 3 and 5 per 10,000 in the US population, may share common physiopathological mechanisms, although neither clinical signs of hemolytic anemia nor evidence of a shortened RBCs lifespan are known in autistic patients.

The NA-NDDs group, on the other hand, shows a heterogenous variety of abnormal RBC shape changes, with only ~40% of discocytes and a slightly dominance of leptocytes which appears to be just below 25% of all erythrocyte shapes in the blood samples. In this patient group, the SEM data were associated with a lower hemoglobin content and a higher degree of anisocytosis. Interestingly, we have previously described a marked (~55%) leptocytosis in $\omega$-3 PUFAs unsupplemented girls with RTT [12], a neurodevelopmental disorder known to be the result of a single gene mutation [13]. In the near future, in our opinion, possible membrane cytoskeletal changes are to be carefully explored in this particular cause of cognitive impairment with autistic features.

Table 3: Correlation matrix for elliptic erythrocyte shape, oxidative stress markers, and $\beta$-actin erythrocyte membrane content.

| | Elliptocytes | 4-HNE PAs | IE-NPBI | $F_2$-IsoPs | $\beta$-Actin content | 4-HNE $\beta$-Actin adducts |
|---|---|---|---|---|---|---|
| Elliptocytes | | 0.779<br>[0.630–0.873]<br>$P < 0.0001$ | 0.334<br>[0.045–0.572]<br>$P = 0.00246$ | 0.588<br>[0.356–0.752]<br>$P < 0.0001$ | −0.860<br>[−0.921 to −0.758]<br>$P < 0.0001$ | 0.887<br>[0.803–0.937]<br>$P < 0.0001$ |
| 4-HNE PAs | | | 0.397<br>[0.117–0.618]<br>$P = 0.0068$ | 0.774<br>[0.622–0.869]<br>$P < 0.0001$ | −0.886<br>[−0.936 to −0.801]<br>$P < 0.0001$ | 0.889<br>[0.806–0.938]<br>$P < 0.0001$ |
| IE-NPBI | | | | 0.415<br>[0.139–0.632]<br>$P = 0.0045$ | −0.414<br>[−0.631 to −0.138]<br>$P = 0.0046$ | 0.340<br>[0.052–0.576]<br>$P = 0.0223$ |
| $F_2$-IsoPs | | | | | −0.628<br>[−0.778 to −0.410]<br>$P < 0.0001$ | 0.579<br>[0.344–0.746]<br>$P < 0.0001$ |
| $\beta$-Actin content | | | | | | −0.960<br>[−0.978 to −0.929]<br>$P < 0.0001$ |
| 4-HNE $\beta$-Actin Adducts | | | | | | |

Data are Spearman's rho correlation coefficients (in square brackets are the 95% confidence intervals for regression).
IE-NPBI: intraerythrocyte nonprotein-bound-iron; $F_2$-IsoPs: $F_2$-isoprostanes; 4-HNE Pas: 4-HNE protein adducts.

Although iron deficiency in about a quarter of autistic children has been recently reported, resulting in iron deficiency anemia in about 15% of the cases and related to detective iron intake [26], none of our examined patients had clinically evident anemia. Interestingly, in a series of conditions, collectively termed "neuroacanthocytoses," a distinctly abnormal erythrocyte shape (i.e., acanthocytosis) is reported to be associated with neurodegeneration and specific RBCs protein defects [27].

Genetic analyses over the past two decades have linked a full host of rare mutations to increased risk for ASDs, with a list of hundreds of autism risk loci in the human genome [28], whereas also common genetic variants are reported to affect the ASDs risk, but their individual effects appear to be modest [29]. Nevertheless, to the best of our knowledge, no genetic mutations involving the erythrocyte cytoskeleton have been previously described in autism.

*4.2. Preliminary Assessment of the Relationship between RBC Biology and Autistic Behavior.* Estimated IQ was significantly different between the two neurodevelopmental groups (i.e., autistic versus NA-NDDs), thus suggesting that the NA-NDDs group was actually composed by nonautistic patients with a demonstrated cognitive impairment, although of a less severe entity, as compared with that observed in the autistic population. Therefore, it is possible that stepwise changes in red blood cell phenotype between the autistic, positive, and negative control groups may be more closely linked to IQ than autism-specific differences. As a consequence, caution is needed before stating that an elliptic RBC shape is synonymous of autism, as we indicate that the triad "RBC shape + membrane OS damage + $\beta$-actin alteration" is associated with autism rather than elliptocytosis *per se*.

Furthermore, during our preliminary studies, we found that patients not currently included in the NA-NDDS nor in the autistic group showing mild "autistic like features" (i.e., a few patients with anorexia nervosa (nervous anorexia), currently classified as a psychiatric illness in the group of eating disorders and two patients with juvenile schizophrenia) showed an intermediate percentage of elliptocytes between the values here reported for the NA-NDDs and the control population (our unpublished data). Therefore, a proportionality between percentage of elliptocytes in the peripheral blood and autistic features in the behavior might be present, although, at the present time, we have no definite proof for it.

*4.3. RBCs Cytoskeletal Proteins: β-Actin.* Erythrocyte membrane integrity is critical for maintaining the erythrocyte characteristic shape and is based on both vertical and horizontal interactions among the cytoskeletal proteins, the integral membrane proteins, and the phospholipid bilayer. Vertical interactions are based either on spectrin, ankyrin, and band 3 protein or spectrin, 4.1 protein, and glycophorin, while horizontal interactions are mainly based on spectrin, 4.1 protein and actin [30–32]. In particular, $\beta$-actin is a globular protein composed of filaments, weakly binding to the tail end of $\alpha$ and $\beta$ spectrins [33]. Our present findings evidence a deficiency in $\beta$-actin in the RBCs membranes from patients affected by classical autism, thus suggesting the coexistence of defective horizontal interaction forces in the cytoskeletal proteins of the erythrocyte membranes of these patients.

Besides the potential effects of $\beta$-actin deficiency on the membrane cytoskeletal structure of classical autistic patients, the detected $\beta$-actin alterations might be even more far-ranging given that emerging evidence indicated a role for $\beta$-actin in motor neuron function and axonal regeneration [34]. In particular, it has been suggested that distinct dynamics of $Ca^{2+}$-$\beta$-actin could be a critical player in mediating the localized actin polymerization required for cellular

constriction events mediating tissue bending, synaptic plasticity, and behavior [34, 35].

*4.4. Potential Role of Exogenous or Endogenous Factor on the Erythrocyte Changes.* Emerging evidence indicates that the role of environmental factors acting on genome by epigenetic regulation is relevant in autism and other neurodevelopmental diseases [36]. Moreover, a large number of toxic substances are known to cause erythrocyte damage in both experimental (i.e., phenylhydrazine and dapsone) [14, 37] and human settings (i.e., lead, penicillins, methyl-DOPA, and antiarrhythmics) [38], often leading to haemolytic anemias through different mechanisms. In our patients no clues exist for a specific exogenous molecule potentially causing an increased prevalence of elliptocytosis, and no evidence of haemolytic anemias was detectable. To date, no specific gene mutations involving the cytoskeletal components of the erythrocyte membrane have been demonstrate. Therefore, the demonstrated alteration of $\beta$-actin in our autistic patients appears to be the effect of a posttranslational modification, rather than the result of a specific gene mutation in the progenitor erythrocyte cells.

*4.5. Oxidative Stress of the Erythrocyte Membrane.* A link between oxidative stress and ASD has been previously reported by several authors [39–41]. Over the last few years, our team has demonstrated that OS is a key player in modulating the genotype-phenotype expression in RTT [20, 42–46].

Moreover, in previous reports, a correlation between OS and ASD has been widely explored by measuring different molecules, possibly coming from oxidative pathway as metabolic biomarkers of OS, in biological fluids [10, 39].

In autistic patients, the immunochemical detection of 4-HNE PAs indicate that oxidative events are ongoing in the RBCs of autistic patients and individuals with several neurodevelopmental disorders without autistic features. The reduction in membrane $\beta$-actin appears to be inversely related to the levels of 4-HNE $\beta$-AAs, thus indicating that the apparently reduced protein expression in the erythrocyte membranes from autistic patients is rather the consequence of a peculiar posttranscriptional modification linked to lipid peroxidation than the results of a reduced protein synthesis. This event, triggered by NPBI as a prooxidant factor [47], produces several compounds of degradation, 4-HNE among them. This highly reactive aldehyde can covalently bind proteins, phospholipids, and DNA; in particular, 4-HNE reacts readily with nucleophilic groups of amino acidic side chains, and its covalent attachment to proteins lead to alteration in their structure and biological activity [48]. Depending on its concentration and location, 4-HNE may be therefore considered as a "second toxic messenger," which disseminates and augments initial free radical events.

Although a protective physiological role for OS—for instance oxidative shielding—has been recently underlined [49], there is little doubt that an oxidized protein is a damaged molecule with a likely reduced function. Thus, within the red cell membrane environment, oxidized proteins contribute to alter the phospholipid bilayer integrity and weaken the membrane mechanical properties, including a loss of the membrane fluidity as already reported in autism [6]. The report of significant alterations in the fatty acid profiles in individuals with ASDs in erythrocyte membrane [50] appears to be in line with our morphological and biochemical observations. Although an increased lipid peroxidation in the erythrocyte membrane from autistic patients has been suggested by using a thiobarbituric acid reactive substances (TBARs) assay kit [11], to date no further information on the *in situ* lipid and protein membrane damage is available. Our findings of increased erythrocyte membrane $F_2$-IsoPs, as measured by a specific spectrometric method, and increased membrane 4-HNE-PAs reveal an increased arachidonic acid peroxidation, with a subsequent protein posttranslational modification in the erythrocyte membrane of patients with classical autism and NA-NDDs.

## 5. Conclusions

Our findings indicate the presence of an unrecognized triad combination of erythrocyte shape abnormalities, erythrocyte membrane oxidative damage, and $\beta$-actin alterations in classical autism and provides new biological markers in the diagnostic workup of ASDs. At least two unsolved questions are generated by our observations. Firstly, the specificity of our findings to autistic disorders is unknown to date and needs to be further explored. Secondly, the relationship between the abnormal erythrocyte shape and the neurological development in autistic children needs to be further investigated. The reported alteration in erythrocyte shape for classical autism could be theoretically translatable into a routine technology, such as fluorescence-activated cell sorting (FACS) either testing the volume or the morphological complexity of cells, in addition to the membrane fluidity.

In conclusion, our data shed new light on the concept of OS as a key factor in the pathogenesis of neurodevelopmental disorders. Interestingly, our data indicate that erythrocyte shape, either due to a defective RBC cytoskeletal scaffold or being the consequence of an oxidative cell damage, could be considered as a new potential physical biomarker for neurodevelopmental disorders.

## Conflict of Interests

The authors declare that they have no conflict of interest.

## Authors' Contribution

Lucia Ciccoli and Claudio De Felice contributed equally to this work.

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*Research Article*

# Genes Related to Mitochondrial Functions, Protein Degradation, and Chromatin Folding Are Differentially Expressed in Lymphomonocytes of Rett Syndrome Patients

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Rett syndrome (RTT) is mainly caused by mutations in the X-linked methyl-CpG binding protein (*MeCP2*) gene. By binding to methylated promoters on CpG islands, MeCP2 protein is able to modulate several genes and important cellular pathways. Therefore, mutations in *MeCP2* can seriously affect the cellular phenotype. Today, the pathways that *MeCP2* mutations are able to affect in RTT are not clear yet. The aim of our study was to investigate the gene expression profiles in peripheral blood lymphomonocytes (PBMC) isolated from RTT patients to try to evidence new genes and new pathways that are involved in RTT pathophysiology. LIMMA (Linear Models for MicroArray) and SAM (Significance Analysis of Microarrays) analyses on microarray data from 12 RTT patients and 7 control subjects identified 482 genes modulated in RTT, of which 430 were upregulated and 52 were downregulated. Functional clustering of a total of 146 genes in RTT identified key biological pathways related to mitochondrial function and organization, cellular ubiquitination and proteosome degradation, RNA processing, and chromatin folding. Our microarray data reveal an overexpression of genes involved in ATP synthesis suggesting altered energy requirement that parallels with increased activities of protein degradation. In conclusion, these findings suggest that mitochondrial-ATP-proteasome functions are likely to be involved in RTT clinical features.

## 1. Introduction

Rett syndrome (RTT) is a rare form of autism spectrum disorder (ASD), which mostly affects girls with worldwide prevalence rate ranges from 1 : 10,000 to 1 : 20,000 live births [1–5]. RTT is a clinically defined condition with a large spectrum of phenotypes associated with a wide genotypic variability [6, 7]. Classic or typical RTT, the most common form of the condition, is caused in about 90–95% of cases by *de novo* mutations in the *MeCP2*, a gene mapped on chromosome X and encoding methyl-CpG binding protein 2 [7, 8]. The clinical picture of classical form progresses through 4 stages and is characterized by normal development for the first 6 to 18 months, followed by loss of purposeful hand movements, failure of speech development, autistic-like behavior, slowed brain and head growth, and mental retardation [9].

To date, it is not known how *MeCP2* mutations lead to RTT phenotypes; therefore the identification of the pathways

that are affected by *MeCP2* functions could bring new insight in the RTT pathogenetic mechanisms. MeCP2 was originally thought to function as a transcription repressor by binding to methylated CpG dinucleotides, but recent studies have individuated more functions related to MeCP2 [10, 11]. In fact MeCP2 is now considered a multifunctional protein, since it is implicated not only in genome transcriptional silencing, but also in transcriptional activation, by regulating chromatin and nuclear architecture [11]; therefore, its malfunction or mutation can lead to severe cellular function alterations.

Hence, it is very difficult to understand the link between *MeCP2* mutation and the clinical feature present in RTT. One of the most common approaches used to better understand the molecular pathways involved in genetic disorders has been the determination of gene expression profiling, since it provides the opportunity to evaluate possible transcriptome alterations at both gene and gene-network levels. This approach should not be considered an end point but a magnifying lent where new aspects involved in the diseases can be discovered and then studied.

So far, only a handful of studies have investigated the gene expression profiles of RTT children in tissues, that is, postmortem brain samples [12], or in cells, such as clones of fibroblasts isolated from skin biopsies [13, 14] and immortalized lymphoblastoid cell lines [14–16]. Moreover, several studies have performed microarray gene expression analysis using *in vitro* cellular models representing MeCP2 deficiency induced by siRNAs [17] or cells and tissues from RTT mouse models [18], but to our knowledge there are no data on microarray analysis from "*ex vivo*" fresh samples. For this reason, the aim of this study was to evaluate the gene expression patterns in PBMC isolated from RTT patients. This approach lets us bypass some of the limitations/variables of the previous gene arrays studies on RTT, such as the use of postmortem samples, gene-modified cells and murine tissues that do not always reflect all features of the human disease. In fact, studying *ex vivo* samples, such as PBMC, provides some advantage that can be summarized by the fact PBMC are the only readily available cells in humans; various studies showed disease-characteristic gene expression patterns in PBMC that can be easily obtained.

Our results identified a clear difference in gene expression profile between control and RTT patients, with almost 500 genes being deregulated, suggesting several new pathways involved in this disorder.

## 2. Subjects and Methods

*2.1. Subjects Population.* The study included 12 female patients with clinical diagnosis of typical RTT (mean age: 10.9 ± 4.9 years, range: 6–22) with demonstrated *MeCP2* gene mutation and 7 sex- and age-matched healthy controls (mean age: 15.1 ± 9.03 years, range: 4–32). RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [4]. All the patients were consecutively admitted to the Rett Syndrome National Reference Centre of the University Hospital of the Azienda Ospedaliera Universitaria Senese (AOUS). Table 1 presents the demographic and genetic characteristics of the enrolled patients subjected to microarray analysis. Blood sampling in the control group was carried out during routine health checks, sports, or blood donations, while blood sample in patients were obtained during the periodic clinical checks. The study was conducted with the approval of the Institutional Review Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

Table 1: Demographic and genetic data for RTT patients enrolled in study.

| No. | Age | Stage | Mutation type | Nucleotide change | Amino acid change |
|---|---|---|---|---|---|
| #1 | 7 | 3 | ETMs | | |
| #2 | 10 | 3 | missense | c.403A>G | p.K135E |
| #3 | 9 | 4 | missense | c.403A>G | p.K135E |
| #4 | 9 | 4 | missense | c.455C>G | p.P152R |
| #5 | 12 | 3 | missense | c.473C>T | p.T158M |
| #6 | 19 | 3 | nonsense | c.763C>T | p.R255X |
| #7 | 22 | 3 | frameshift insertion or deletion | c.806_807delG | p.G269fs |
| #8 | 7 | 4 | nonsense | c.808C>T | p.R270X |
| #9 | 7 | 3 | nonsense | c.808C>T | p.R270X |
| #10 | 12 | 4 | nonsense | c.880C>T | p.R294X |
| #11 | 6 | 3 | nonsense | c.880C>T | p.R294X |
| #12 | 11 | 3 | missense | c.916C>T | p.R306C |

ETMs: early truncating mutations.

*2.2. Blood Specimen Collection, Peripheral Blood Lymphomonocytes Isolation, and RNA Extraction.* Blood was collected in heparinized tubes and all manipulations were carried out within 30 minutes after sample collection. PBMC were separated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Europe GmbH, Milan, Italy). After PBMC isolation, total RNA was extracted from cells using RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The total nucleic acid concentration and purity were estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality of RNA was checked on Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA). The isolated RNA samples were stored at −80°C until the analysis.

*2.3. Microarray Processing.* For the microarray processing, RNA was amplified and labeled using the Affymetrix Whole-Transcript (WT) Sense Target Labeling Protocol. Affymetrix GeneChip Human Gene 1.0 ST arrays were hybridized with labeled sense DNA, washed, stained, and scanned according to the protocol described in WT Sense Target Labeling Assay Manual.

Briefly, 100 ng of total RNA was reverse transcribed into double-stranded cDNA with random hexamers tagged with

a T7 promoter sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7 RNA polymerase, producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. dUTP was incorporated in the DNA during the second-cycle, first-strand reverse transcription reaction. This single-stranded DNA sample was then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that specifically recognized the unnatural dUTP residues and broke the DNA strand. DNA was labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix proprietary DNA Labeling Reagent that is covalently linked to biotin. 5 $\mu$g of labeled cDNA was hybridized to the Human Gene 1.0 ST Array at 45°C for 17 hours. The arrays were washed and stained in Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000.

*2.4. Microarray Data Analysis.* The microarray experiments were analyzed with the oneChannelGUI package available in R software. The signal intensities from each chip were preliminarily normalized with RMA method and filtered by IQR filter choosing as threshold a value of 0.25. This specific filter removes the probesets that do not present changes in their expression across the analyzed microarray. The threshold of 0.25 is an intermediate value that retains the probesets that show significative changes of their signal at least in the 25% of analyzed microarray. The microarray data were analyzed by SAM and LIMMA analyses. SAM approach uses permutation based statistics and is a valid method to analyze data that may not follow a normal distribution, outperforming other techniques (e.g., ANOVA and classical $t$-test), which assume equal variance and/or independence of genes. LIMMA fit each gene expression to a linear regression model testing the significativity of the distance from the model with a $t$-test robust against nonnormality and inequality of variances. In order to characterize the biological processes enriched in the list of modulated genes, a statistical analysis of overrepresented Gene Ontology (GO) terms (BP level 5) was performed with DAVID (Database for Annotation, Visualization, and Integrated Discovery) web server (DAVID Bioinformatics Resources) [19]. Only the GO terms enriched with a $P$ value $< 0.05$ corrected with the Benjamini and Hochberg method [20] were selected and hierarchically clusterized (Ward's method) according to their "semantic" similarity estimated with the GOSIM R package with default parameters. After clusterization the best number of clusters was identified according to the analysis of the silhouette scores and the medoid term in each cluster was selected as the representative member of the cluster. Each cluster represents a group of biological processes with similar e/o related functions.

*2.5. Validation of Microarray Data by RT-qPCR (Reverse Transcription Quantitative Real-Time PCR).* For confirmation of Affymetrix expression microarray results, RT-qPCR analysis was performed as previously described [21]. For validation, six of the differentially expressed genes, 3 upregulated—*GSTO1*, *PSMB10* and *COX8A*—and 3 downregulated—*HIST1H1B*, *MMP9* and *ARHGAP11B*—by microarray, were chosen. Validation was done in a randomly selected subset of the original samples (submitted for microarray analysis) that included 3 healthy controls and 3 RTT patients. Primer pairs were obtained from the Real-time PCR GenBank Primer to hybridise unique regions of the appropriate gene sequence: *GSTO1* (Fw: 5′-AGA GTT GTT TTC TAA GGT TCT GAC T-3′) and (RW: 5′-ACT TCA TTG CTT CCA GCC GT-3′), product length 116 bp; *PSMB10* (Fw: 5′-ACA GAC GTG AAG CCT AGC AG-3′) and (Rw: 5′-ACC GAA TCG TTA GTG GCT CG-3′), product length 294 bp; *COX8A* (Fw: 5′-GCC AAG ATC CAT TCG TTG CC-3′) and (RW: 5′-TCT GGC CTC CTG TAG GTC TC-3′), product length 137 bp; *HIST1H1B* (Fw: 5′-CCC GGC TAA GAA GAA GGC AA-3′) and (RW: 5′-ACA GCC TTG GTG ATC AGC TC-3′), product length 99 bp; *MMP9* (Fw: 5′-GTC CGT GAG GGT GTT GAG TG-3′) and (RW: 5′-ACT GCT CAA AGC CTC CAC AA-3′), product length 145 bp; *ARHGAP11B* (Fw: 5′-AAC TGC CAG AGC CCA TTC TC-3′) and (RW: 5′-GTC TGG TAC ACG CCC TTC TT-3′), product length 295 bp. All reactions were run in triplicate. *GAPDH* (FW: 5′-TGA CGC TGG GGC TGG CAT TG-3′ and RW: 5′-GGC TGG TGG TCC AGG GGT CT-3′, 134 pb) was used in our experiments as internal standard. As previously described, samples were compared using the relative cycle threshold (CT) method (Livak and Schmittgen 2001). After normalization to more stable mRNA GAPDH, the fold increase or decrease was determined with respect to control, using the formula $2^{-\Delta\Delta CT}$, where $\Delta$CT is (gene of interest CT)−(reference gene CT) and $\Delta\Delta$CT is ($\Delta$CT experimental)−($\Delta$CT control). Results are the means ± SEM of three independent experiments, each analysed in triplicate. $^{*}P < 0.001$ versus control (one-way ANOVA followed by Bonferroni's posttest).

## 3. Results and Discussion

*3.1. Differentially Regulated Genes in RTT Patients.* Given that RTT results from dysfunction of the transcriptional modulator MeCP2, several strategies have been developed to identify its target genes in order to gain insights into the disease pathogenesis [12–18]. In our study, to identify gene expression changes associated with and potentially related to *MeCP2* mutations and to delineate alterations of pathways associated with the disease, we evaluated and compared transcriptomic profiles in PBMC from RTT patients and control subjects.

This work showed for the first time, to our knowledge, altered expression of a large set of genes that may help elucidating and explaining the link between MeCP2 and some of the molecular and cellular aspects observed in RTT patients. In our previous studies on RTT we were able to show increased levels of oxidative stress (OS) markers, such as isoprostanes (IsoPs) and 4-hydroxynonenal protein adducts (4-HNE PAs) [22, 23], and increased ubiquitination and degradation of oxidatively modified proteins [24], but how

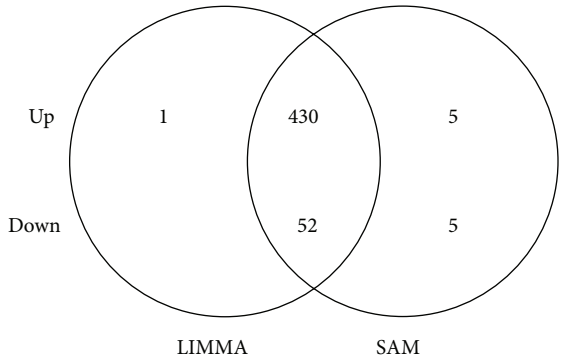



Figure 1: Comparison of two distinct approaches for screening of differentially expressed genes in RTT PBMC. Venn diagram representing numbers of common and exclusively up- and downregulated genes for LIMMA (left) and SAM (right) analyses (FC ± 1; adj. $P$ value ≤ 0.05). The 11 genes indicated by only one method were excluded by further analysis.

*MeCP2* mutation is able to affect cellular redox balance and proteins turn over still needs to be defined.

Using the SAM and LIMMA methods, we have defined a set of genes differentially expressed in RTT patients with respect to controls (healthy subjects) and a significant overlap was found by comparing results from two approaches. A cut-off level based on a minimum of 1-fold change in expression resulted in a list of 482 common deregulated genes, while 10 genes were suggested only by SAM and only 1 gene was indicated by LIMMA. Among the shared genes, 430 showed significant upregulation, while 52 were downregulated in RTT compared to controls (Figure 1 and Supplemental Tables 1 and 2). The 11 genes indicated by only one method were excluded for further analysis. Genes with the strongest changes in expression, both upregulated (FC ≥ 2) and downregulated (FC ≤ −1.2), are listed in Tables 2 and 3, respectively. The complete list of differentially expressed genes, both upregulated and downregulated (FC ± 1), is shown in Supplemental Tables 1 and 2.

It is evident from this first set of data that mutations in *MeCP2* influence more the genes upregulation with respect to the downregulation. This is in part in line with the first functions that have been attributed to MeCP2, such as a gene expression repressor [10, 11]. In addition, it is worth it to underline that both screening approaches used in this study (LIMMA and SAM) almost overlap between them making the results more reliable.

Next, using the DAVID databases, we performed the functional annotation of the significant genes and identification of the biochemical pathways in which they are involved. A comparison of the differentially regulated mRNA transcripts in RTT PBMC compared to control group shows significant changes in cellular pathways. In particular, we identify 10 major clusters corresponding to 62 biological processes enriched by 146 genes (Table 4). These clusters highlight key biological pathways related to mitochondrial function and organization (i.e., mitochondrial ATP synthesis coupled to electron transport, inner mitochondrial membrane organization such as: *ATP5A1*, *COX6C*, *ETFA*, *UQCRQ*, *TIMM10*, and *TSPO*), cellular protein metabolic process (i.e., regulation of protein ubiquitination, regulation of ubiquitin-protein ligase activity, and proteasomal ubiquitin-dependent protein catabolic process such as *PSMA2*, *PSMD6*, *UBE2E3*, and *UFC*), RNA processing (i.e., nuclear mRNA splicing and spliceosome assembly and RNA elongation from RNA polymerase II promoter such as *RPL15*), DNA organization in chromatin, and cellular macromolecular complex assembly (i.e., nucleosome assembly, DNA packaging, and protein complex biogenesis such as *HIST1H4L*, *H2AFZ*, *TOP2A*, and *HMGB2*).

*3.2. Mitochondrial Related Genes in RTT Patients.* Among these clusters, the most significantly regulated transcripts include those encoding several subunits of mitochondrial respiratory chain complexes and thus linked directly to mitochondrial ATP production and, indirectly, to potential reactive oxygen species (ROS) generation. In particular, *NDUFA1*, *NDUFAB1*, *NDUFA2*, and *NDUFB6*, all components of mitochondrial complex I (NADH: ubiquinone oxidoreductase), showed the greater changes with a FC of more than 2. Moreover, other subunits of complex I (*NDUFV2*, *NDUFS4*, *NDUFA9*, *NDUFS6*, *NDUFB10*, *NDUFB4*, *NDUFC2*, *NDUFB2*, *NDUFS5*, *NDUFC1*, *NDUFB9*, and *NDUFA8*) were clearly upregulated in RTT group.

Complex I plays a vital role in cellular ATP production, the primary source of energy for many crucial processes in living cells. It removes electrons from NADH and passes them by a series of different protein coupled redox centers to the electron acceptor ubiquinone. Because complex I is central to energy production in the cell, it is reported that its malfunction results in a wide range of neuromuscular diseases [25]. Some of them are due to mutations in the mitochondrial genome, but others, which result from a decrease in the activity of complex I or an increase in the production of ROS, are not well understood. The production of ROS by complex I is linked to Parkinson's disease and to ageing [26, 27] and this is in line with RTT since it is now well documented as an increased OS condition in this pathology [22, 23].

Another gene involved in complex I function is *NDUFV2* that was also clearly upregulated in our study. Mutations in this gene are implicated in Parkinson's disease, bipolar disorder, and schizophrenia and have been found in one case of early onset hypertrophic cardiomyopathy and encephalopathy; also it has been shown for *NDUFA2*, a subunit of the hydrophobic protein fraction of the complex I. Mutations in this gene are associated with Leigh syndrome, an early onset progressive neurodegenerative disorder. Of note is *NDUFAB1*, which is a carrier of the growing fatty acid chain in fatty acid biosynthesis in mitochondria and alteration in fatty acid levels has been noted in ASD [28, 29].

Not only complex I subunits were upregulated in RTT, but also we have detected an upregulation of genes involved in all the five complexes of the electron transport chain. In fact, also *SDHB* (succinate dehydrogenase complex, subunit

Table 2: Genes upregulated in RTT patients by LIMMA and SAM analyses.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_004541.3 | NDUFA1 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5 kDa | NADH dehydrogenase (ubiquinone) activity | Mitochondrial electron transport, NADH to ubiquinone | 3.1 |
| NM_006498.2 | LGALS2 | Lectin, galactoside-binding, soluble, 2 | Carbohydrate binding | — | 2.8 |
| NM_014302.3 | SEC61G | Sec61 gamma subunit | Protein transporter activity | Protein targeting to ER; antigen processing and presentation of exogenous peptide antigen via MHC class I | 2.8 |
| NM_001040437.1 | C6orf48 | Chromosome 6 open reading frame 48 | — | — | 2.7 |
| NM_001098577.2 | RPL31 | Ribosomal protein L31 | RNA binding; structural constituent of ribosome | Translational elongation; translational initiation; translational termination | 2.7 |
| NM_006989.5 | RASA4 | RAS p21 protein activator 4 | GTPase activator activity; phospholipid binding | Intracellular signal transduction; positive regulation of GTPase activity; regulation of small GTPase mediated signal transduction | 2.6 |
| NM_000983.3 | RPL22 | Ribosomal protein L22 | RNA binding; heparin binding; structural constituent of ribosome | Alpha-beta T cell differentiation; translational elongation; translational initiation; translational termination | 2.6 |
| NM_019059.3 | TOMM7 | Translocase of outer mitochondrial membrane 7 homolog (yeast) | Protein transmembrane transporter activity | Cellular protein metabolic process; protein import into mitochondrial matrix; protein targeting to mitochondrion | 2.6 |
| NM_031157.2 | HNRNPA1 | Heterogeneous nuclear ribonucleoprotein A1 | Nucleotide binding; single-stranded DNA binding; single-stranded RNA binding | RNA export from nucleus; mRNA splicing, via spliceosome; mRNA transport; nuclear import | 2.5 |
| NM_001828.5 | CLC | Charcot-Leyden crystal galectin | Carbohydrate binding; carboxylesterase activity; lysophospholipase activity | Lipid catabolic process; multicellular organismal development | 2.4 |
| NM_032901.3 | COX14 | Cytochrome c oxidase assembly homolog 14 (S. cerevisiae) | Plays a role in the assembly or stability of the cytochrome c oxidase complex (COX) | Mitochondrial respiratory chain complex IV assembly | 2.3 |
| NM_024960.4 | PANK2 | Pantothenate kinase 2 | ATP binding; pantothenate kinase activity | Cell death; coenzyme A biosynthetic process; coenzyme biosynthetic process; pantothenate metabolic process | 2.3 |
| NR_002309.1 | RPS26P11 | Ribosomal protein S26 pseudogene 11 | Structural constituent of ribosome | Translation | 2.3 |
| NM_005003.2 | NDUFAB1 | NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8 kDa | NADH dehydrogenase (ubiquinone) activity; ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process; fatty acid binding; calcium ion binding | Cellular metabolic process; protein lipoylation; small molecule metabolic process; respiratory electron transport chain; fatty acid biosynthetic process; mitochondrial electron transport, NADH to ubiquinone | 2.3 |

Table 2: Continued.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_152851.2 | MS4A6A | Membrane-spanning 4-domains, subfamily A, member 6A | May be involved in signal transduction as a component of a multimeric receptor complex | — | 2.3 |
| NM_004269.3 | MED27 | Mediator complex subunit 27 | Transcription coactivator activity | Regulation of transcription from RNA polymerase II promoter; stem cell maintenance; transcription initiation from RNA polymerase II promoter | 2.2 |
| NR_015404.1 | C12orf47 | MAPKAPK5 antisense RNA 1 | — | — | 2.2 |
| NM_001865.3 | COX7A2 | Cytochrome c oxidase subunit VIIa polypeptide 2 (liver) | Cytochrome-c oxidase activity; electron carrier activity | Oxidative phosphorylation | 2.2 |
| NM_004374.3 | COX6C | Cytochrome c oxidase subunit VIc | Cytochrome-c oxidase activity | Respiratory electron transport chain; small molecule metabolic process | 2.2 |
| NM_002984.2 | CCL4 | Chemokine (C-C motif) ligand 4 | Chemokine activity | Cell adhesion; cell-cell signaling; chemotaxis; immune response; inflammatory response; positive regulation of calcium ion transport; positive regulation of calcium-mediated signaling; positive regulation of natural killer cell chemotaxis; response to toxic substance; response to virus; signal transduction | 2.2 |
| NM_014060.2 | MCTS1 | Malignant T cell amplified sequence 1 | RNA binding | Cell cycle; positive regulation of cell proliferation; regulation of growth; regulation of transcription, DNA-dependent; response to DNA damage stimulus transcription, DNA-dependent | 2.1 |
| NM_004832.2 | GSTO1 | Glutathione S-transferase omega 1 | Glutathione dehydrogenase (ascorbate) activity; glutathione transferase activity; methylarsonate reductase activity | L-ascorbic acid metabolic process; glutathione derivative biosynthetic process; negative regulation of ryanodine-sensitive calcium-release channel activity; positive regulation of ryanodine-sensitive calcium-release channel activity; positive regulation of skeletal muscle contraction by regulation of release of sequestered calcium ion; regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion; xenobiotic catabolic process | 2.1 |
| NM_001867.2 | COX7C | Cytochrome c oxidase subunit VIIc | Cytochrome-c oxidase activity | Respiratory electron transport chain; small molecule metabolic process | 2.1 |

Table 2: Continued.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_014206.3 | C11orf10 | Transmembrane protein 258 | — | — | 2.1 |
| NM_002413.4 | MGST2 | Microsomal glutathione S-transferase 2 | Enzyme activator activity; glutathione peroxidase activity; glutathione transferase activity; leukotriene-C4 synthase activity | Glutathione biosynthetic process; glutathione derivative biosynthetic process; leukotriene biosynthetic process; positive regulation of catalytic activity; xenobiotic metabolic process | 2.1 |
| NM_001124767.1 | C3orf78 | Small integral membrane protein 4 | — | — | 2.1 |
| NM_152398.2 | OCIAD2 | OCIA domain containing 2 | — | — | 2.1 |
| NM_002488.4 | NDUFA2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8 kDa | NADH dehydrogenase (ubiquinone) activity | Mitochondrial electron transport, NADH to ubiquinone small molecule metabolic process | 2.1 |
| NM_004528.3 | MGST3 | Microsomal glutathione S-transferase 3 | Glutathione peroxidase activity; glutathione transferase activity | Glutathione derivative biosynthetic process; lipid metabolic process; signal transduction; small molecule metabolic process xenobiotic metabolic process | 2.0 |
| NM_003095.2 | SNRPF | Small nuclear ribonucleoprotein polypeptide F | RNA binding | Histone mRNA metabolic process; mRNA 3′-end processing ncRNA metabolic process; spliceosomal snRNP assembly; termination of RNA polymerase II transcription | 2.0 |
| NM_005213.3 | CSTA | Cystatin A (stefin A) | Cysteine-type endopeptidase inhibitor activity; protein binding, bridging structural molecule activity | Cell-cell adhesion; keratinocyte differentiation; peptide cross-linking | 2.0 |
| NM_033318.4 | C22orf32 | Single-pass membrane protein with aspartate-rich tail 1 | — | — | 2.0 |
| NM_053035.2 | MRPS33 | Mitochondrial ribosomal protein S33 | Structural constituent of ribosome | Translation | 2.0 |
| BC014670.1 | LOC147727 | Hypothetical protein LOC147727, mRNA (cDNA clone IMAGE: 4864993), partial cds | — | — | 2.0 |
| NM_001014.4 | RPS10 | Ribosomal protein S10 | Protein binding | Translational elongation; translational initiation; translational termination; viral transcription | 2.0 |
| NM_001130710.1 | LSM5 | LSM5 homolog, associated U6 small nuclear RNA (S. cerevisiae) | RNA binding | RNA splicing; exonucleolytic nuclear-transcribed mRNA catabolic process involved in deadenylation-dependent decay; mRNA processing | 2.0 |

Table 2: Continued.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_002801.3 | PSMB10 | Proteasome (prosome, macropain) subunit, beta type, 10 | Threonine-type endopeptidase activity | DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest; G1/S transition of mitotic cell cycle; T cell proliferation; anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process; apoptotic process; cell morphogenesis; gene expression; humoral immune response; mRNA metabolic process; negative regulation of apoptotic process; negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle; positive regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle; protein polyubiquitination; regulation of cellular amino acid metabolic process; small molecule metabolic process | 2.0 |
| NM_014044.5 | UNC50 | Unc-50 homolog (C. elegans) | RNA binding | Cell surface receptor signaling pathway; protein transport | 2.0 |
| NM_032747.3 | USMG5 | Up-regulated during skeletal muscle growth 5 homolog (mouse) | Plays a critical role in maintaining the ATP synthase population in mitochondria | — | 2.0 |
| NM_001001330.2 | REEP3 | Receptor accessory protein 3 | May enhance the cell surface expression of odorant receptors | — | 2.0 |
| NM_004074.2 | COX8A | Cytochrome c oxidase subunit VIIIA (ubiquitous) | Cytochrome-c oxidase activity | Respiratory electron transport chain; small molecule metabolic process | 2.0 |
| NM_002493.4 | NDUFB6 | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17 kDa | NADH dehydrogenase (ubiquinone) activity | Mitochondrial electron transport, NADH to ubiquinone; small molecule metabolic process | 2.0 |
| NM_032273.3 | TMEM126A | Transmembrane protein 126A | — | Optic nerve development | 2.0 |

"—": lacking item.

B, and iron sulfur (Ip)) gene encoding for a subunit of mitochondrial complex II (succinate: ubiquinone oxidoreductase) was significantly upregulated. This subunit is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q). Complex II of the respiratory chain, which is specifically involved in the oxidation of succinate, carries electrons from FADH to CoQ. Of note, also 3 genes, *UQCRQ*, *UQCRFS1,* and *UQCRH*, encoding for subunits of complex III (ubiquinol-cytochrome c oxidoreductase), were upregulated with a mean FC = 1.60. This complex plays a critical role in biochemical generation of ATP, contributing to the generation of electrochemical potential by catalyzing the electron transfer reaction from ubiquinol to cytochrome c coupled with proton translocation across the membrane. Lines of evidence report that in mouse models some of the promoters of ubiquinol-cytochrome c reductase subunit are able to be targeted by MECP2 protein, contributing to the development of the pathology [30].

Furthermore, the cytochrome c gene (*CYCS*) together with genes encoding subunits of mitochondrial complex IV (cytochrome c oxidase) (*COX14*, *COX7A2*, *COX6C*, *COX7C*, and *COX8A*) was upregulated with a mean FC of circa 1.5. Of note is the upregulation of cytochrome c gene. The encoded protein accepts electrons from cytochrome b and transfers them to the cytochrome oxidase complex. This protein is also involved in initiation of apoptosis and this would be in line

Table 3: Genes down-regulated in RTT patients by LIMMA and SAM analyses.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_170601.4 | SIAE | Sialic acid acetylesterase | Sialate O-acetylesterase activity | — | −2.3 |
| NR_002312.1 | RPPH1 | Ribonuclease P RNA component H1 | — | — | −2.2 |
| NM_005322.2 | HIST1H1B | Histone cluster 1, H1b | DNA binding | Nucleosome assembly | −1.8 |
| NM_000902.3 | MME | Membrane metalloendopeptidase | Metalloendopeptidase activity | Proteolysis | −1.8 |
| NM_032047.4 | B3GNT5 | UDP-GlcNAc: betaGal beta-1,3-N-acetylglucosaminyltransferase 5 | Galactosyltransferase activity | Glycolipid biosynthetic process; posttranslational protein modification | −1.7 |
| NM_003513.2 | HIST1H2AB | Histone cluster 1, H2ab | DNA binding | Nucleosome assembly | −1.7 |
| NR_002562.1 | SNORD28 | Small nucleolar RNA, C/D box 28 | — | — | −1.5 |
| NM_004668.2 | MGAM | Maltase-glucoamylase (alpha-glucosidase) | Alpha-glucosidase activity; amylase activity | Carbohydrate metabolic process | −1.5 |
| NM_012081.5 | ELL2 | Elongation factor, RNA polymerase II, 2 | — | Regulation of transcription, DNA-dependent | −1.4 |
| NM_021066.2 | HIST1H2AJ | Histone cluster 1, H2aj | DNA binding | nucleosome assembly | −1.4 |
| NM_002424.2 | MMP8 | Matrix metallopeptidase 8 (neutrophil collagenase) | Metalloendopeptidase activity; zinc ion binding | Proteolysis | −1.4 |
| NM_004994.2 | MMP9 | Matrix metallopeptidase 9 (gelatinase B, 92 kDa, gelatinase, 92 kDa, type IV collagenase) | Collagen binding; metalloendopeptidase activity; zinc ion binding | Collagen catabolic process; extracellular matrix disassembly; positive regulation of apoptotic process; proteolysis | −1.4 |
| NM_003533.2 | HIST1H3I | Histone cluster 1, H3i | DNA binding | Nucleosome assembly; regulation of gene silencing | −1.3 |
| NG_000861.4 | GK3P | Glycerol kinase 3 pseudogene | ATP binding; glycerol kinase activity | Catabolic process; glycerol metabolic process | −1.3 |
| NR_033423.1 | LOC1720 | Dihydrofolate reductase pseudogene | — | — | −1.3 |
| NM_003521.2 | HIST1H2BM | Histone cluster 1, H2bm | DNA binding | Nucleosome assembly | −13 |
| NM_002417.4 | MKI67 | Antigen identified by monoclonal antibody Ki-67 | ATP binding | DNA metabolic process; cell proliferation; cellular response to heat; meiosis; organ regeneration | −1.2 |
| NM_020406.2 | CD177 | CD177 molecule | — | Blood coagulation; leukocyte migration | −1.2 |
| NM_001039841.1 | ARHGAP11B | Rho GTPase activating protein 11B | GTPase activator activity | Positive regulation of GTPase activity; regulation of small GTPase mediated signal transduction; small GTPase mediated signal transduction | −1.2 |
| NM_001004690.1 | OR2M5 | Olfactory receptor, family 2, subfamily M, member 5 | G-protein coupled receptor activity; olfactory receptor activity | Detection of chemical stimulus involved in sensory perception of smell | −1.2 |
| NM_052966.3 | FAM129A | Family with sequence similarity 129, member A | — | Negative regulation of protein phosphorylation; positive regulation of protein phosphorylation; positive regulation of translation; response to endoplasmic reticulum stress | −1.2 |

Table 3: Continued.

| NCBI reference sequence | Gene symbol | Gene name | Molecular function | Biological process | Fold change |
|---|---|---|---|---|---|
| NM_001067.3 | TOP2A | Topoisomerase (DNA) II alpha 170 kDa | ATP binding; DNA binding, bending; chromatin binding; drug binding; magnesium ion binding; ubiquitin binding | DNA ligation; DNA repair; DNA topological change; DNA-dependent DNA replication; apoptotic chromosome condensation; mitotic cell cycle; phosphatidylinositol-mediated signaling; positive regulation of apoptotic process; positive regulation of retroviral genome replication; positive regulation of transcription from RNA polymerase II promoter; sister chromatid segregation | –1.2 |
| NM_021018.2 | HIST1H3F | Histone cluster 1, H3f | DNA binding | S phase; blood coagulation; nucleosome assembly; regulation of gene silencing | –1.2 |
| NM_182707.2 | PSG8 | Pregnancy specific beta-1-glycoprotein 8 | The human pregnancy-specific glycoproteins (PSGs) are a group of molecules that are mainly produced by the placental syncytiotrophoblasts during pregnancy. PSGs comprise a subgroup of the carcinoembryonic antigen (CEA) family, which belongs to the immunoglobulin superfamily. | Female pregnancy | –1.2 |
| NM_003535.2 | HIST1H3J | Histone cluster 1, H3j | DNA binding | S phase; blood coagulation; nucleosome assembly; regulation of gene silencing | –1.2 |
| NM_004566.3 | PFKFB3 | 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 | 6-Phosphofructo-2-kinase activity; ATP binding; fructose-2,6-bisphosphate 2-phosphatase activity | Fructose metabolic process; glycolysis; small molecule metabolic process | –1.2 |
| NM_016448.2 | DTL | Denticleless E3 ubiquitin-protein ligase homolog (Drosophila) | — | DNA replication; G2 DNA damage checkpoint; protein monoubiquitination; protein polyubiquitination; ubiquitin-dependent protein catabolic process | –1.2 |
| NG_001019.5 | IGHM | Immunoglobulin heavy constant mu | Antigen binding | Immune response | –1.2 |
| NR_002907.2 | SNORA73A | Small nucleolar RNA, H/ACA box 73A | — | — | –1.2 |

"—": lacking item.

with previous studies that have shown a possible involvement of apoptosis in RTT [31, 32], although only few studies have investigated the role of apoptosis in RTT and the current literature is still controversial. In addition, several subunits of complex IV were upregulated. It receives an electron from each of the four cytochrome c molecules and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. In the process, via a protons translocation,

Table 4: GO analysis of genes reported to be deregulated in PBMC of RTT patients.

| Term | Description | *P* value | Fold enrichment |
|---|---|---|---|
| | Cluster 1 | | |
| GO:0022900 | Electron transport chain | $2.0 \times 10^{-12}$ | 9.01 |
| GO:0006091 | Generation of precursor metabolites and energy | $1.7 \times 10^{-12}$ | 5.06 |
| GO:0045333 | Cellular respiration | $2.7 \times 10^{-12}$ | 9.71 |
| GO:0006120 | Mitochondrial electron transport, NADH to ubiquinone | $4.9 \times 10^{-12}$ | 16.32 |
| GO:0022904 | Respiratory electron transport chain | $1.8 \times 10^{-11}$ | 12.04 |
| GO:0006119 | Oxidative phosphorylation | $2.0 \times 10^{-11}$ | 9.17 |
| GO:0042775 | Mitochondrial ATP synthesis coupled electron transport | $2.0 \times 10^{-11}$ | 12.99 |
| GO:0042773 | ATP synthesis coupled electron transport | $2.0 \times 10^{-11}$ | 12.99 |
| GO:0015980 | Energy derivation by oxidation of organic compounds | $3.4 \times 10^{-9}$ | 6.54 |
| GO:0055114 | Oxidation reduction | $1.5 \times 10^{-8}$ | 3.01 |
| | Cluster 2 | | |
| GO:0006626 | Protein targeting to mitochondrion | $4.3 \times 10^{-3}$ | 8.81 |
| GO:0070585 | Protein localization in mitochondrion | $4.3 \times 10^{-3}$ | 8.81 |
| GO:0045039 | Protein import into mitochondrial inner membrane | $7.5 \times 10^{-3}$ | 28.54 |
| GO:0007007 | Inner mitochondrial membrane organization | $2.8 \times 10^{-2}$ | 19.03 |
| GO:0006839 | Mitochondrial transport | $3.1 \times 10^{-2}$ | 4.96 |
| | Cluster 3 | | |
| GO:0051436 | Negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle | $1.8 \times 10^{-4}$ | 7.24 |
| GO:0051444 | Negative regulation of ubiquitin-protein ligase activity | $2.1 \times 10^{-4}$ | 7.03 |
| GO:0051352 | Negative regulation of ligase activity | $2.1 \times 10^{-4}$ | 7.03 |
| GO:0051437 | Positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle | $2.4 \times 10^{-4}$ | 6.92 |
| GO:0051443 | Positive regulation of ubiquitin-protein ligase activity | $2.8 \times 10^{-4}$ | 6.73 |
| GO:0051439 | Regulation of ubiquitin-protein ligase activity during mitotic cell cycle | $3.0 \times 10^{-4}$ | 6.63 |
| GO:0051351 | Positive regulation of ligase activity | $3.7 \times 10^{-4}$ | 6.45 |
| GO:0051438 | Regulation of ubiquitin-protein ligase activity | $6.2 \times 10^{-4}$ | 6.04 |
| GO:0051340 | Regulation of ligase activity | $8.4 \times 10^{-4}$ | 5.81 |
| GO:0043086 | Negative regulation of catalytic activity | $8.4 \times 10^{-3}$ | 2.78 |
| GO:0044092 | Negative regulation of molecular function | $9.6 \times 10^{-3}$ | 2.56 |
| | Cluster 4 | | |
| GO:0031145 | Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process | $1.8 \times 10^{-4}$ | 7.24 |
| GO:0010498 | Proteasomal protein catabolic process | $4.4 \times 10^{-3}$ | 4.62 |
| GO:0043161 | Proteasomal ubiquitin-dependent protein catabolic process | $4.4 \times 10^{-3}$ | 4.62 |
| | Cluster 5 | | |
| GO:0031397 | Negative regulation of protein ubiquitination | $4.1 \times 10^{-4}$ | 6.36 |

Table 4: Continued.

| Term | Description | $P$ value | Fold enrichment |
|---|---|---|---|
| GO:0031400 | Negative regulation of protein modification process | $9.2 \times 10^{-4}$ | 4.68 |
| GO:0031398 | Positive regulation of protein ubiquitination | $1.0 \times 10^{-3}$ | 5.61 |
| GO:0032269 | Negative regulation of cellular protein metabolic process | $3.2 \times 10^{-3}$ | 3.58 |
| GO:0031396 | Regulation of protein ubiquitination | $4.2 \times 10^{-3}$ | 4.71 |
| GO:0051248 | Negative regulation of protein metabolic process | $4.4 \times 10^{-3}$ | 3.43 |
| GO:0031401 | Positive regulation of protein modification process | $4.1 \times 10^{-2}$ | 2.98 |
| GO:0032268 | Regulation of cellular protein metabolic process | $4.7 \times 10^{-2}$ | 2.08 |
| | Cluster 6 | | |
| GO:0065003 | Macromolecular complex assembly | $1.4 \times 10^{-8}$ | 2.96 |
| GO:0043933 | Macromolecular complex subunit organization | $9.9 \times 10^{-8}$ | 2.77 |
| GO:0034622 | Cellular macromolecular complex assembly | $3.2 \times 10^{-6}$ | 3.63 |
| GO:0034621 | Cellular macromolecular complex subunit organization | $2.6 \times 10^{-5}$ | 3.24 |
| GO:0065004 | Protein-DNA complex assembly | $1.1 \times 10^{-4}$ | 6.12 |
| GO:0034728 | Nucleosome organization | $5.2 \times 10^{-4}$ | 5.52 |
| GO:0006461 | Protein complex assembly | $2.1 \times 10^{-3}$ | 2.37 |
| GO:0070271 | Protein complex biogenesis | $2.1 \times 10^{-3}$ | 2.37 |
| | Cluster 7 | | |
| GO:0006334 | Nucleosome assembly | $2.4 \times 10^{-4}$ | 6.12 |
| GO:0031497 | Chromatin assembly | $3.1 \times 10^{-4}$ | 5.90 |
| GO:0006323 | DNA packaging | $8.2 \times 10^{-4}$ | 4.76 |
| GO:0006333 | Chromatin assembly or disassembly | $6.0 \times 10^{-3}$ | 4.04 |
| | Cluster 8 | | |
| GO:0008380 | RNA splicing | $5.6 \times 10^{-6}$ | 3.77 |
| GO:0006396 | RNA processing | $5.7 \times 10^{-6}$ | 2.82 |
| GO:0006397 | mRNA processing | $1.5 \times 10^{-4}$ | 3.20 |
| GO:0000398 | Nuclear mRNA splicing, via spliceosome | $2.0 \times 10^{-4}$ | 4.48 |
| GO:0000377 | RNA splicing, via transesterification reactions with bulged adenosine as nucleophile | $2.0 \times 10^{-4}$ | 4.48 |
| GO:0000375 | RNA splicing, via transesterification reactions | $2.0 \times 10^{-4}$ | 4.48 |
| GO:0016071 | mRNA metabolic process | $8.5 \times 10^{-4}$ | 2.78 |
| | Cluster 9 | | |
| GO:0006412 | Translation | $2.6 \times 10^{-8}$ | 4.01 |
| GO:0006414 | Translational elongation | $5.4 \times 10^{-5}$ | 5.93 |
| | Cluster 10 | | |
| GO:0006367 | Transcription initiation from RNA polymerase II promoter | $1.1 \times 10^{-3}$ | 6.30 |
| GO:0006352 | Transcription initiation | $4.5 \times 10^{-3}$ | 5.16 |
| | Cluster 11 | | |
| GO:0006368 | RNA elongation from RNA polymerase II promoter | $1.1 \times 10^{-3}$ | 7.13 |
| GO:0006354 | RNA elongation | $4.5 \times 10^{-3}$ | 6.71 |

it is able to generate ATP. This data would suggest that RTT patients are in continuous new ATP synthesis and this could be a consequence of new protein synthesis.

Our data are in line with a previous work by Kriaucionis where the authors have analyzed the gene profile in the brain of RTT animal model [30]. They have shown that there were several mitochondrial abnormalities and an upregulation of both complexes I and III subunits. In particular they were also able to show a correlation between upregulation of complexes I and III and the animal symptoms severity with a significant increase in mitochondrial respiratory capacity and a reduction in respiratory efficiency. The defect appears to be associated with respiratory complex III, which is also upregulated in our study, and that containing the Uqcrc1 protein. In addition it has been shown that MeCP2 binds to the promoter of the Uqcr gene *in vivo* and that Uqcr mRNA expression is elevated in brains of *Mecp2*-null mice that have acquired neurological symptoms and this is in line with our results.

Finally, we also observed in RTT PBMC an upregulation of mitochondrial complex V (ATP synthase) subunits (*ATP5A1*, *ATP5EP2*, *ATP5J2*, and *ATP5O*) together with ATPase inhibitory factor 1 gene (*ATPIF1*). Mitochondrial membrane ATP synthase is a master regulator of energy metabolism and cell fate; therefore, a misregulation of this gene can be associated with altered ATP production and cell metabolism. It is interesting to note that also the ATPase inhibitory factor 1 (*ATPIF1*) that inhibits the activity of the mitochondrial $H^+$-ATP synthase was upregulated, and this lets us speculate the existence of an aberrant loop between making new ATP and inhibiting its production. In addition, recent findings indicate that *ATPIF1* has additional functions by promoting adaptive responses of cell to ROS [33], a condition (OS) that has been well documented to be present in RTT [22, 23].

Similarly, other genes related to the ATP synthesis showed significant expression changes (i.e., *CYB5A*, *CYB561D2*, *ETFA*, *LDHB*, *PDHB,* and *SURF1*). For instance, *ETFA* (electron transfer flavoprotein, alpha polypeptide) serves as a specific electron acceptor for several dehydrogenases and in mitochondria it shuttles electrons between primary flavoprotein dehydrogenases and the membrane-bound electron transfer flavoprotein ubiquinone oxidoreductase. In addition, *LDHB* encodes lactate dehydrogenase B, an enzyme that catalyzes the reversible conversion of lactate and pyruvate and NAD and NADH, in the glycolytic pathway, being therefore correlated with ATP generation. Of note is the upregulation of *SURF1* (surfeit 1) gene that encodes a protein localized to the inner mitochondrial membrane and thought to be involved in the biogenesis of the cytochrome c oxidase complex.

Related to mitochondrial structure/organization, we found upregulation of 7 translocase genes (*TIMM10*, *TSPO*, *TIMM9*, *TIMM17A*, *TOMM7*, *TIMM13,* and *TIMM8B*) involved in proteins import into mitochondrion (mean FC of 1.34) and of several mitochondrial ribosomal proteins (*MRPL13*, *MRPL20*, *MRPL21*, *MRPL33*, *MRPL51*, *MRPL52*, *MRPS25*, *MRPS30*, *MRPS33*, *MRPS36*, *RPL10A*, *RPL13*, *RPL15*, *RPL22*, *RPL22L1*, *RPL26*, *RPL31*, *RPL32*, *RPL39L*, *RPS10*, *RPS25*, *RPS26*, *RPS26P11*, *RPS29*, *RPS5,* and *RPS7*) with a mean FC of 1.5.

All together these lines of evidence seem to suggest an increased mitochondrial activity that might be linked to the observation of the pathologic phenotype. Anyways at this stage of the study, it is not possible to determine whether or not there is an increase in ATP levels. It is possible to speculate that increased genes related to mitochondrial subunits could be a consequence of increased cells request of energy (ATP). This hypothesis is in line with recent study where the authors have shown decreased levels of ATP in brain mouse RTT [34].

Recent discussions regarding a possible connection between RTT and mitochondrial dysfunction have generated significant interest. The basis for these discussions is related in part to the common features of RTT on the one hand and disorders of mitochondrial function on the other. Of interest is the fact that a patient with symptoms normally associated with mitochondrial disorders harbored mutations in the *MeCP2* gene [35]. This overlap between symptoms of RTT and mitochondrial disorders recalls early reports of structural abnormalities [36, 37] and defects in the electron transport chain [37, 38] in mitochondria from skin and muscle biopsies of RTT patients. Moreover, about half of RTT patients were reported to have elevated levels of circulatory lactic or pyruvic acid, which might be caused by defects in the efficiency of the respiratory chain and urea cycle complexes, both of which are mitochondrial [39–41]. Several disorders related to the brain are a consequence of mitochondrial alteration with the resultant increase of OS and in certain cases the cell apoptosis. As RTT is not a neurodegenerative disorder [42], any contribution of mitochondrial dysfunction to RTT symptoms may take the form of chronic mitochondrial underperformance, rather than catastrophic failure leading to neuronal death.

To date, no systematic study of mitochondrial function in individuals with RTT has presented whether these findings represent a primary or secondary effect; that is, are they involved directly in the clinical features of RTT or do they reflect effects of these clinical features on mitochondrial function? Prior to identification of mutations in *MeCP2* in 1999, several reports appeared to be related to mitochondrial structure and function [36–54]. However, since 2001, publications on a possible role of mitochondria in the pathogenesis of RTT have been very few [36, 54]. In a recent work, investigators in Australia reported gene expression results from postmortem brain tissue of individuals with RTT and normal controls [55]. One gene related to a mitochondrial enzyme, cytochrome c oxidase subunit 1, had reduced expression in RTT tissue raising the possibility that loss of MeCP2 function could be responsible. However, whether this is a primary or secondary finding remains to be established and provides an important target for further investigation.

In summary, while mitochondrial abnormalities related to structure and functions have been reported, sufficient information is lacking as to the precise role of such abnormalities in RTT. As mentioned, alteration of mitochondrial functions is often correlated with OS and, in particular, the mitochondrial sites that are often invoked as the most important mitochondrial superoxide producers are in respiratory complexes I and III [56, 57] and this can explain the increased OS levels found in RTT patients.

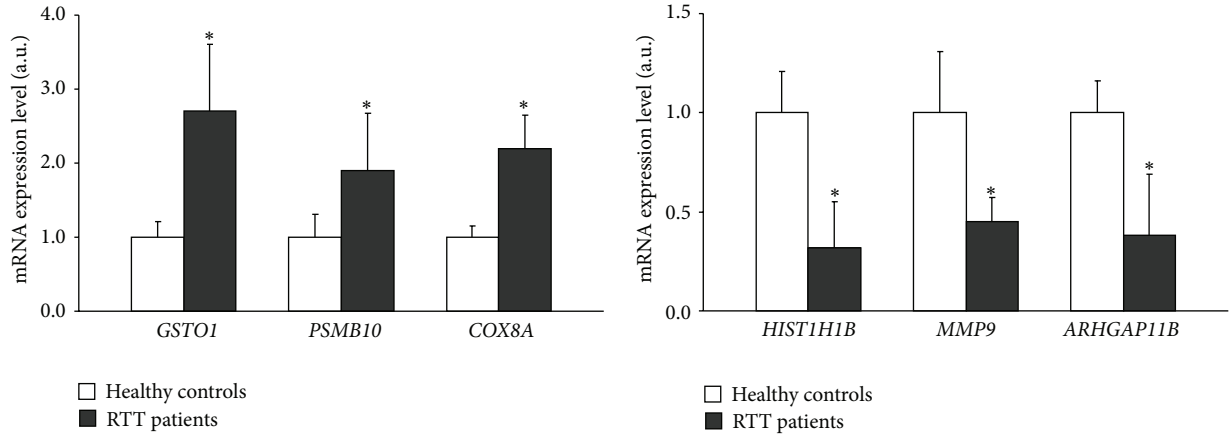


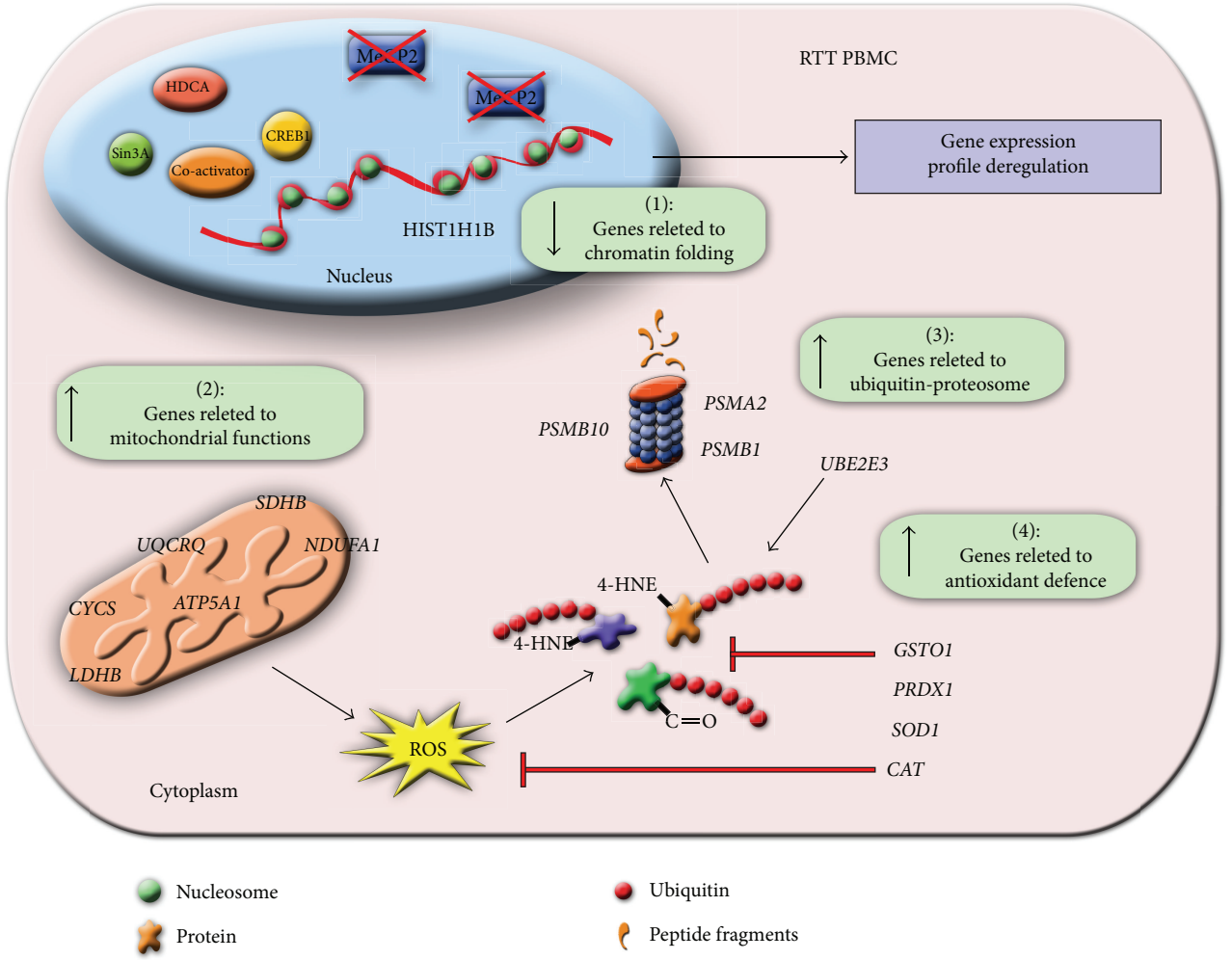

Figure 2: Validation of relative gene expression levels for selected genes using RT-qPCRin PBMC from 12 RTT patients and 7 controls. Results are the means ± SEM of three independent experiments, each analysed in triplicate. * $P$ value < 0.001 versus control (one-way ANOVA followed by Bonferroni's post-test).

*3.3. Oxidative Stress Related Genes in RTT Patients.* The presence of a redox unbalance in RTT is confirmed by the upregulations of several genes involved in redox homeostasis such as superoxide dismutase 1, catalase, and peroxiredoxin 1 (*SOD1*, *CAT*, and *PRDX1*) with a 1.6, 1.14, and 1.12 FC, respectively. Moreover, glutathione S-transferase omega 1, microsomal glutathione S-transferase 2, and microsomal glutathione S-transferase 3 (*GSTO1*, *MGST2*, and *MGST3*) are also overexpressed in RTT with a FC = 2.1. For instance, *SOD1* upregulation could be a consequence of increased superoxide production by aberrant activation of complex I and III and the dismutation of superoxide in $H_2O_2$ can explain the increased expression of *CAT* and *PRDX1*. It is likely to believe that the compensatory antioxidant system is not quite sufficient to quench ROS production and this could explain the high OS level present in RTT [22, 23]. For this reason the induction of glutathione S-transferase omega-1 (*GSTO1*) is not surprising. This enzyme is involved in the detoxification mechanisms via conjugation of reduced glutathione (GSH) to oxidatively modified proteins (carbonyls and 4-HNE PAs). In fact, the GST genes are upregulated in response to OS. In addition, our results showed the upregulation of *MGST2* and *MGST3* which are the microsomal glutathione S-transferases; this data correlates very well with our previous findings where RTT patients showed an increased level of 4-HNE PAs, as *MGST2* is able to also conjugate 4-HNE with GSH [58]. We also observed an increased expression of mRNA for alcohol dehydrogenase 5 (*ADH5*), aldo-keto reductase family 1, member A1 (*AKR1A1*) and aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1*), all enzymes involved in lipid peroxidation products detoxification [59].

*3.4. Ubiquitin-Proteasome Related Genes in RTT Patients.* Our results evidenced also the upregulation of genes related to protein degradation and ubiquitination. In fact, RTT PBMC microarray data revealed increased expression levels of genes associated with protein turnover, such as genes encoding proteasome subunits (*PSMA2*, *PSMA3*, *PSMA5*, *PSMA7*, *PSMB1*, *PSMB10*, *PSMC5*, *PSMC6*, *PSMD6*, and *PSMD9*); furthermore, proteasome maturation protein (*POMP*), involved in proteasome assembly, and genes regulating the activity of the ubiquity ligases (*RBX1*, *UFC1*, *CCNB1IP1*, and *DAXX*) are also up regulated (Table 2), suggesting an increase in cell and protein degradation processes. This could also be a consequence of oxidized proteins and the presence of 4-HNE PAs. This evidence is supported by the observed upregulation of the ubiquitin-conjugating enzyme E2E3 (*UBE2E3*, FC = 1.19) that accepts ubiquitin from the E1 complex and catalyzes its covalent attachment to other proteins. However, this is in contrast with some lines of evidence that link RTT to the downregulation of ubiquitin conjugating enzymes (*UBE3A*) by MeCP2 [60]. Overall the effect of MeCP2 on *UBE3A* regulation is still controversial. In fact, there is even a recent work that did not find any difference in *UBE3A* expression between wild type and a RTT mouse with the mutation R168X [61]. In general, the levels of protein ubiquitination, that is one of the steps to degrade modified proteins, are increased in RTT [24].

*3.5. Chromatin Folding Related Genes in RTT Patients.* In addition several histone related genes (*HIST1H1B*, *HIST1H2AB*, *HIST1H2AI*, *HIST1H2AJ*, *HIST1H2AL*, *HIST1H2BB*, *HIST1H2BH*, *HIST1H2BM*, *HIST1H3B*, *HIST1H3F*, *HIST1H3I*, *HIST1H3J*, *HIST1H4D*, *HIST1H4F*, and *HIST1H4*) were downregulated with a mean FC of –1.28 suggesting a reduced production of proteins necessary to the DNA chromatin assembly.

In general, histone modifications are very dynamic and include acetylation, methylation, isomerization, phosphorylation, and ubiquitination [62]. The combination of such modifications confers enormous variability of cellular signals to environmental stimuli. It is easy to understand that modifications such as histone methylation can display additional complexity since the degree of methylation is very variable (mono-, di-, or trimethylation) [63]. Furthermore, combinations or sequential additions of different histone marks can




Scheme 1: Schematic summary related to the altered gene expression observed in RTT PBMC. MeCP2 in a normal situation binds to several cofactors (Sin3A, CREB1, etc.) to regulate gene transcription. Mutation of MeCP2 (crossed boxes) will affect gene expression leading to a gene profile deregulation. There are mainly 4 gene clusters significantly affected in RTT PBMC, that is, those related to chromatin folding (1), mitochondrial functions (2), ubiquitin-proteasome (3), and antioxidant defence (4) (green boxes). The overexpression of the genes involved in ATP synthesis processes (2) can be interpreted as a possible energy requirement for an increment of cellular protein degradation (3), consequent to increased mitochondrial ROS production and protein oxidation. The increased expression of the "antioxidant cellular defence" genes (4) is the possible compensatory mechanism activated by the cells to quench ROS production and protein oxidation (red arrows).

affect the chromatin organization and subsequently alter the expression of the corresponding target genes.

In our case, several genes related to histone expression were downregulated and this can dramatically affect gene expression. For instance, histone H1 protein binds to linker DNA between nucleosomes forming the macromolecular structure known as the chromatin fiber. Histones H1 are necessary for the condensation of nucleosome chains into higher-order structured fibers. Acts also as a regulator of individual gene transcription through chromatin remodeling, nucleosome spacing, and DNA methylation. We have detected a down-regulation of several H1 subunits ranging from 2- to 3-fold in RTT patients.

*3.6. Validation of Selected PBMC mRNAs by qPCR Analyses.* Next, we wanted to confirm the differential expression observed for selected mRNAs, on an individual basis, by RT-qPCR. Six genes were selected based on their different patterns of expression (3 up- and 3 downregulated). Assessment of their mRNA expression levels by RT-qPCR accurately reflected those obtained by microarray profiling (Figure 2), thereby confirming the validity of our microarray results. The levels of three mRNAs encoding for glutathione S-transferase omega 1 (*GSTO1*), proteasome (prosome, macropain) subunit, beta type-10 (*PSMB10*), and cytochrome c oxidase subunit VIIIA (*COX8A*) were upregulated in RTT patients by 2.5-, 2- and 2.2-fold, respectively (Figure 2), very similarly to the levels measured by gene array. In contrast, histone cluster 1, H1b (*HIST1H1B*), matrix metallopeptidase 9 (*MMP9*), and Rho GTPase activating protein 11B (*ARHGAP11B*) were downregulated by ~50% in RTT patients, as compared to healthy subjects (Figure 2) and also in this case similar expression was detected by gene array analysis.

*3.7. Conclusion.* Our microarray data reveal an altered gene expression profile in RTT lymphomonocytes with the upregulation of genes related to mitochondrial biology and ubiquitin-proteasome proteolytic pathway. In particular, the overexpression of the genes involved in ATP synthesis processes means the tendency of cells to show an altered energy requirement, perhaps to cope with the increased activities of protein degradation. On the other hand, it should be noted that mitochondrion plays essential roles in mediating the production of ROS and these in turn cause damage to proteins, as well as lipids and nucleic acids. To remove damaged molecules, in a kind of vicious circle, increased cellular proteolytic activity requires an extra mitochondrial ATP production with a further ROS generation. This picture is consistent with our previous reports [24], indicating the alteration of redox status in RTT patients, coupled with the increased ubiquitination and degradation of oxidatively modified proteins (Scheme 1).

In conclusion, these findings on transcriptional profiling in RTT patients reveal new molecular mechanisms underlying RTT phenotype, suggesting that mitochondrial-ATP-proteosome are likely to have direct actions on redox balance in RTT syndrome. Furthermore, it confirmed a possible indirect role of OS in pathogenesis and progression of disorder. Thus, RTT should be considered as possible mitochondriopathy.

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*Research Article*

# Effects of $\omega$-3 Polyunsaturated Fatty Acids on Plasma Proteome in Rett Syndrome

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The mechanism of action of omega-3 polyunsaturated fatty acids ($\omega$-3 PUFAs) is only partially known. Prior reports suggest a partial rescue of clinical symptoms and oxidative stress (OS) alterations following $\omega$-3 PUFAs supplementation in patients with Rett syndrome (RTT), a devastating neurodevelopmental disorder with transient autistic features, affecting almost exclusively females and mainly caused by sporadic mutations in the gene encoding the methyl CpG binding protein 2 (MeCP2) protein. Here, we tested the hypothesis that $\omega$-3 PUFAs may modify the plasma proteome profile in typical RTT patients with *MECP2* mutations and classic phenotype. A total of 24 RTT girls at different clinical stages were supplemented with $\omega$-3 PUFAs as fish oil for 12 months and compared to matched healthy controls. The expression of 16 proteins, mainly related to acute phase response (APR), was changed at the baseline in the untreated patients. Following $\omega$-3 PUFAs supplementation, the detected APR was partially rescued, with the expression of 10 out of 16 (62%) proteins being normalized. $\omega$-3 PUFAs have a major impact on the modulation of the APR in RTT, thus providing new insights into the role of inflammation in autistic disorders and paving the way for novel therapeutic strategies.

## 1. Introduction

Omega-3 polyunsaturated fatty acids ($\omega$-3 PUFAs) have received an increasing attention by the scientific community and the society as well due to their status of natural molecules with a number of claimed positive effects on a large variety of conditions, in particular in the prevention of cardiovascular disease. Most common $\omega$-3 PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in fish oil and $\alpha$-linolenic acid C18:3 n-3, derived from plants. The hypotriglyceridemic effect is the best defined metabolic action of $\omega$-3 PUFAs [1], with a mechanism likely to be related to activation of peroxisome proliferator-activated receptors [2]. A host of other potential beneficial effects of $\omega$-3 PUFAs has been suggested. Besides the demonstrated or claimed beneficial cardiovascular effects (reduction of susceptibility

to ventricular arrhythmia [3]; antithrombogenic and antioxidant effect [4]; retardation of the atherosclerotic plaque growth by reduced expression of adhesion molecules and platelet-derived growth factor [4]; promotion of endothelial relaxation by induction of nitric-oxide production; and mild hypotensive effect [5]), a more general anti-inflammatory effect, either direct or indirect has been reported [6–8]. However, the molecular mechanisms underlying the $\omega$-3 PUFAs effects in the regulation of the inflammatory process are still poorly understood and an emerging major field of research [9–11]. Several factors may indeed contribute to limit our understanding in this specific area of the lipid metabolism, including the type of PUFAs, formulation, dose, duration, age and underlying condition of the target subjects, and—last but not least—lack of objective measurement of effects. However, a relatively recent report in adult smoker subjects [12] demonstrates that $\omega$-3 PUFAs are able to affect plasma protein expression by regulating acute phase response (APR).

Rett syndrome (RTT) is a devastating neurodevelopmental disorder mainly caused by sporadic mutations in the gene encoding the methyl-CpG binding protein 2 (MeCP2) protein. RTT affects almost exclusively females with an average frequency of 1 : 10,000 female live births and is considered to be the second commonest cause of severe cognitive impairment in this gender [13]. RTT has been formerly included in the autism spectrum disorders (ASDs), although evident differences with autism exist [14] and is no longer classified in the ASDs group. Nevertheless, transient autistic features are always present in the RTT natural history. Therefore, this relatively rare disease actually offers a good opportunity to test the effects of $\omega$-3 PUFAs in an objective way in an autistic condition, given that (1) a persistent redox imbalance has been demonstrated [15]; (2) $\omega$-3 PUFAs have been suggested to reduce phenotypical severity and improve the redox balance in supplemented patients at several stages of their clinical history [15]; (3) an impaired cholesterol metabolism has been very recently demonstrated in a *Mecp2*-null mouse model of RTT, with statin treatment leading to improvement of motor symptoms and conferring increased longevity [16]; and (4) an unexplained hypercholesterolemia has been reported by our group in RTT patients [17].

Since only one study has examined the plasma proteome of RTT [18], here we hypothesized that $\omega$-3 PUFAs may have an impact on the RTT plasma proteome by modulating the APR.

## 2. Materials and Methods

*2.1. Subjects.* The study included a total of 24 female patients with a clinical diagnosis of typical RTT (mean age: 14.4 ± 8.0 years, range 4–33 years) with demonstrated *MECP2* gene mutations [i.e., T158 M ($n$ = 5), C-terminal deletions ($n$ = 4), R255X ($n$ = 4), R270X ($n$ = 3), R133C ($n$ = 2), early truncating mutations ($n$ = 1), large deletions ($n$ = 1) other mutations ($n$ = 4)]. Clinical stages distribution was stage I ($n$ = 4), stage II ($n$ = 6), stage III ($n$ = 7), and stage IV ($n$ = 7). RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [19, 20]. All the patients were admitted to the Rett Syndrome National Reference Centre of the University Hospital of the Azienda Ospedaliera Universitaria Senese (Head: Professor J. Hayek). Blood samplings in the patients' group were performed during the routine follow-up study at hospital admission, while the samples from the control group were carried out during routine health checks, sports, or blood donations, obtained during the periodic clinical checks. The healthy control subjects ($n$ = 24) were gender (given that over 98% of RTT patients are females, we selected a female control group) and age matched (mean age: 14.4 ± 8.2 years, range 4.1–33 years). All the examined subjects were on a typical Mediterranean diet. The study was conducted with the approval by the Institutional Review Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

*2.2. Study Design.* The aim of the present study was to assess the effects on the plasma proteome of a supplementation already tested to be effective in the clinical setting [21–23]. Therefore, as a consequence we did not include a placebo arm for ethical reasons and limited the study to three subjects population, that is, healthy controls, "untreated" Rett syndrome, and $\omega$-3 PUFAs supplemented Rett syndrome.

*2.3. ω-3 PUFAs Supplementation.* Administered $\omega$-3 PUFAs were in the form of fish oil (Norwegian Fish Oil AS, Trondheim, Norway, product number HO320-6; Italian importer: Transforma AS Italia, Forlì, Italy; Italian Ministry registration code: 10 43863-Y) at a dose corresponding to DHA, 71.9 ± 13.9 mg/kg b.w./day and EPA, 115.5 ± 22.4 mg/kg b.w./day, with a total $\omega$-3 PUFAs of 242.4 ± 47.1 mg/kg b.w./day. Use of EPA plus DHA in RTT was approved by the AOUS Ethical Committee.

The dose used in this specific RTT girls cohort is likely to be 5 to 6 times higher than the standard one, which is typically 2 g per day in adult subjects. The rationale for it is contained in a prior paper [21], in which we proposed a very high dose in Rett syndrome. After several attempts, the final dose per kg/day was found empirically in the clinical setting.

*2.4. Sample Collection.* All samplings from RTT patients and healthy controls were carried out around 8 AM after overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection.

*2.5. Sample Preparation.* The blood samples were centrifuged at 2400 g for 15 min at 4°C; the platelet poor plasma was saved; and the buffy coat was removed by aspiration. Plasma samples were stored at −70°C until assay.

*2.6. Two-Dimensional (2D) Gel Electrophoresis.* 2-DE was performed according to Görg et al. [24], and samples containing 60 $\mu$g of protein as determined by Bradford [25] were denatured with a solution containing 10% of sodium dodecyl sulfate (SDS) and 2.3% of dithiothreitol (DTT) heated to 95°C for 5 min. The sample was then combined solubilizing buffer composed by 8 M urea, 2% of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS),

0.3% DTT, and 2% of immobilized pH gradient (IPG) buffer and loaded into 18 cm IPG strips 3–10 non linear on an Ettan IPGphor (GE Healthcare) apparatus system and rehydrated for 7 h. Isoelectric focusing (IEF) was carried out for a total of 32 kV h. The strips were first equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min; then they were equilibrated again with the same buffer described above, except it contained 4% w/v iodoacetamide instead of DTT. The second dimension was performed on an Ettan Daltsix Electrophoresis system (GE Healthcare). IPG strips were embedded at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8–16% T) using 0.5% w/v agarose and run at a constant current of 40 mA/gel at 20°C. Each sample was carried out in triplicate under the same conditions.

*2.7. Image Analysis.* Images of gels were analyzed using ImageMaster 2D Platinum v7.0 software (GE Healthcare). The reference gel for each group (i.e., healthy controls, untreated RTT, and RTT after $\omega$-3 supplementation) was defined and used for the comparative analyses. The background was subtracted from all gels using the average-on-boundary method. Spot volume was expressed as a ratio of the total protein percentage volume (%V) detected from the entire gel to minimize differences between gels (gel normalization), for pooling them. Only spots appearing in all gels of the same group were matched with those of the reference gel.

*2.8. Proteins Identification.* After mass spectrometry compatible silver staining [26], a spot-picking list was generated and exported to Ettan Spot Picker (GE Healthcare). The spots were excised and delivered into 96-well microplates where they were destained and dehydrated with acetonitrile (ACN) for subsequent rehydration with trypsin solution. Tryptic digestion was carried out overnight at 37°C. Each protein spot digest (0.75 mL) was spotted into the MALDI instrument target and allowed to dry. Then, 0.75 mL of the instrument matrix solution (saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in 50% ACN and 0.5% v/v trifluoroacetic acid) was applied to dried samples and dried again. Mass spectra were obtained, as described [27], using an ultrafleXtreme MALDI-ToF/ToF (Bruker Corporation, Billerica, MA, USA). After tryptic peptide mass acquisition, mass fingerprint searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using MASCOT (Matrix Science, London, UK, http://www.matrixscience.com/).

*2.9. Data Analysis.* All variables were tested for normal distribution (D'Agostino-Pearson test) and data were presented as median and interquartile range unless otherwise. Statistical analysis for protein expressed differently in the groups was carried out using Student's $t$-test and one-way ANOVA test. Bonferroni-corrected significance levels were used for multiple $t$-tests. Unmatched spots or spots with significantly different %V were considered "differently expressed" in the groups. Comparisons between differently expressed proteins of untreated RTT and RTT after $\omega$-3 supplementation were evaluated using either Mann-Whitney rank sum test or Kruskal-Wallis test. The effects of small population sizes on possible type I ($\alpha$)/type II ($\beta$) errors in the data interpretation were examined using a sampling size algorithm. A two-sided $P < 0.05$ was considered to indicate statistical significance, The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

## 3. Results

The expression of 16 proteins, mainly related to the APR, was found to be changed at the baseline level in the untreated RTT patients.

As compared to healthy controls, the whole RTT group showed significant increase in 10 protein spots [i.e., complement factor B (CFAB), fibrinogen alpha chain (FIBA), serum albumin (ALBU, spots number 3, number 7 C terminal fragment and number 14 N terminal fragment) alpha-1-antitrypsin (A1AT, spots number 4 and number 5), haptoglobin (HPT, spots number 9 and number 15), and transthyretin (TTHY spot number 11)] and decrease in 6 protein spots [i.e., vitamin D-binding protein (VTDB), apolipoprotein A4 (APOA4), clusterin (CLUS), apolipoprotein A1 (APOA1), retinol-binding protein 4 (RET4), and transthyretin (TTHY spot number 16)] (Table 1 and Figures 1(a), 1(b), and 2(a)). A full list of the known biological functions for the identified plasma proteins is shown in Table 2.

Following $\omega$-3 PUFAs supplementation, the expression of 10 out of 16 (62%) proteins was found to be comparable to that of control subjects (Figure 2(c)). In particular, after $\omega$-3 PUFAs, plasma proteins expression levels were comparable to those of the control population, with the exception of persistent overexpression of A1AT (spot number 4), VTDB (spot number 6), ALBU C Terminal fragment (spot number 7), and HPT (spot number 15) and the persistent underexpression of FIBA (spot number 2) and ALBU (spot number 3).

By comparing the plasma protein profile of treated RTT with the one before treatment, significant decreases in 9 protein spots previously overexpressed before treatment, including CFAB, FIBA, ALBU (spots number 3 and number 14), A1AT, HPT, and TTHY (spot number 11) were reported, whereas significant increases were observed for 5 protein spots that were underexpressed before the $\omega$-3 PUFAs supplementation and included VTDB, APOA4, CLUS, APOA1, and RET4. After $\omega$-3 PUFAs supplementation, the levels ALBU (spot number 7) and TTHY (spot number 16) remained unchanged compared to untreated RTT (Table 1 and Figures 1(a), 1(c), and 2(b)).

## 4. Discussion

The mechanism of action of $\omega$-3 PUFAs is a major area of research, which has led in the last two decades to the discovery of protectins, resolvins and maresins, and all lipid mediators involved in the active resolution of the inflammatory process [28].

Our results indicate that $\omega$-3 PUFAs are able to modulate plasma protein expression in RTT, having a major impact on the modulation of the APR, with a partial (approximately 62%) rescue of the protein changes observed at baseline.

Table 1: Identification results of proteins differentially expressed in basal and in RTT before and after $\omega$-3 PUFAs supplementation.

| Spot | Protein name | Experimental | | | | | | Data bank exploration | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Untreated RTT | | | RTT after $\omega$-3 supplementation | | | UniProt ID | Entry name | Mascot score | Sequence coverage (%) | Peptides matches | Theoretical pI/Mr (kDa) |
| | | ANOVA ($P < 0.05$) | Fold change* | Protein expression | ANOVA ($P < 0.05$) | Fold change** | Protein expression | | | | | | |
| 1 | Complement Factor B | 0.042 | +1.58 | Upregulated | 0.030 | −1.40 | Downregulated | P00751 | CFAB | 102 | 21 | 11/24 | 6.67/86.4 |
| 2 | Fibrinogen alpha chain | 0.009 | +2.07 | Upregulated | 0.008 | −2.04 | Downregulated | P02671 | FIBA | 120 | 30 | 25/86 | 5.70/95.6 |
| 3 | Albumin | 0.016 | +1.67 | Upregulated | 0.012 | −1.66 | Downregulated | P02768 | ALBU | 242 | 38 | 22/33 | 5.92/71.3 |
| 4 | Alpha-1-antitrypsin | 0.001 | +1.97 | Upregulated | 0.019 | −1.33 | Downregulated | P01009 | A1AT | 235 | 60 | 26/69 | 5.37/46.8 |
| 5 | Alpha-1-antitrypsin | 0.003 | +1.73 | Upregulated | 0.005 | −1.47 | Downregulated | P01009 | A1AT | 106 | 25 | 10/18 | 5.37/46.8 |
| 6 | Vitamin D-binding protein | 0.002 | −1.52 | Downregulated | 0.001 | +1.85 | Upregulated | P02774 | VTDB | 123 | 35 | 14/40 | 5.40/54.5 |
| 7 | Albumin (C terminal fragment) | 0.027 | +1.46 | Upregulated | — | — | — | P02768 | ALBU | 79 | 18 | 9/25 | 5.92/71.3 |
| 8 | Apolipoprotein A-IV | 0.038 | −1.45 | Downregulated | 0.029 | +1.46 | Upregulated | P06727 | APOA4 | 107 | 36 | 13/48 | 5.28/45.3 |
| 9 | Haptoglobin | 0.010 | +1.17 | Upregulated | 0.027 | −1.15 | Downregulated | P00738 | HPT | 58 | 16 | 9/23 | 6.13/45.8 |
| 10 | Clusterin | 0.013 | −1.34 | Downregulated | 0.009 | +1.50 | Upregulated | P10909 | CLUS | 131 | 24 | 8/8 | 5.89/53.0 |
| 11 | Transthyretin | 0.012 | +1.91 | Upregulated | 0.015 | −1.79 | Downregulated | P02766 | TTHY | 113 | 59 | 6/10 | 5.52/15.9 |
| 12 | Apolipoprotein A-I | 0.043 | −1.50 | Downregulated | 0.024 | +1.52 | Upregulated | P02647 | APOA1 | 253 | 70 | 27/69 | 5.56/30.7 |
| 13 | Retinol-binding protein 4 | 0.001 | −2.56 | Downregulated | 0.001 | +2.59 | Upregulated | P02753 | RET4 | 112 | 46 | 8/18 | 5.76/23.3 |
| 14 | Albumin (N terminal fragment) | 0.021 | +1.73 | Upregulated | 0.014 | −1.78 | Downregulated | P02768 | ALBU | 143 | 24 | 14/23 | 5.92/71.3 |
| 15 | Haptoglobin | 0.001 | +1.81 | Upregulated | 0.003 | −1.55 | Downregulated | P00738 | HPT | 72 | 21 | 10/31 | 6.13/45.8 |
| 16 | Transthyretin | 0.044 | −1.32 | Downregulated | — | — | — | P02766 | TTHY | 120 | 68 | 7/15 | 5.52/15.9 |

Experimental section includes Spot ID, protein name, and the significant threshold level ($P < 0.05$). Variation in spot intensities represented by fold is expressed as ratio of RTT to control and as a ratio of RTT after $\omega$-3 supplementation to RTT. Positive fold changes denote proteins upregulated, while negative fold changes denote decrease of protein expression levels. — denotes the absence of significant changes. Data bank exploration of proteins includes SwissProt data bank accession numbers and entry name, the Mascot score, the sequence coverage, the number of peptides matching the protein sequence, and theoretical pI/Mr $\times 10^{-3}$. *Comparisons versus healthy controls; **Comparisons versus untreated RTT.

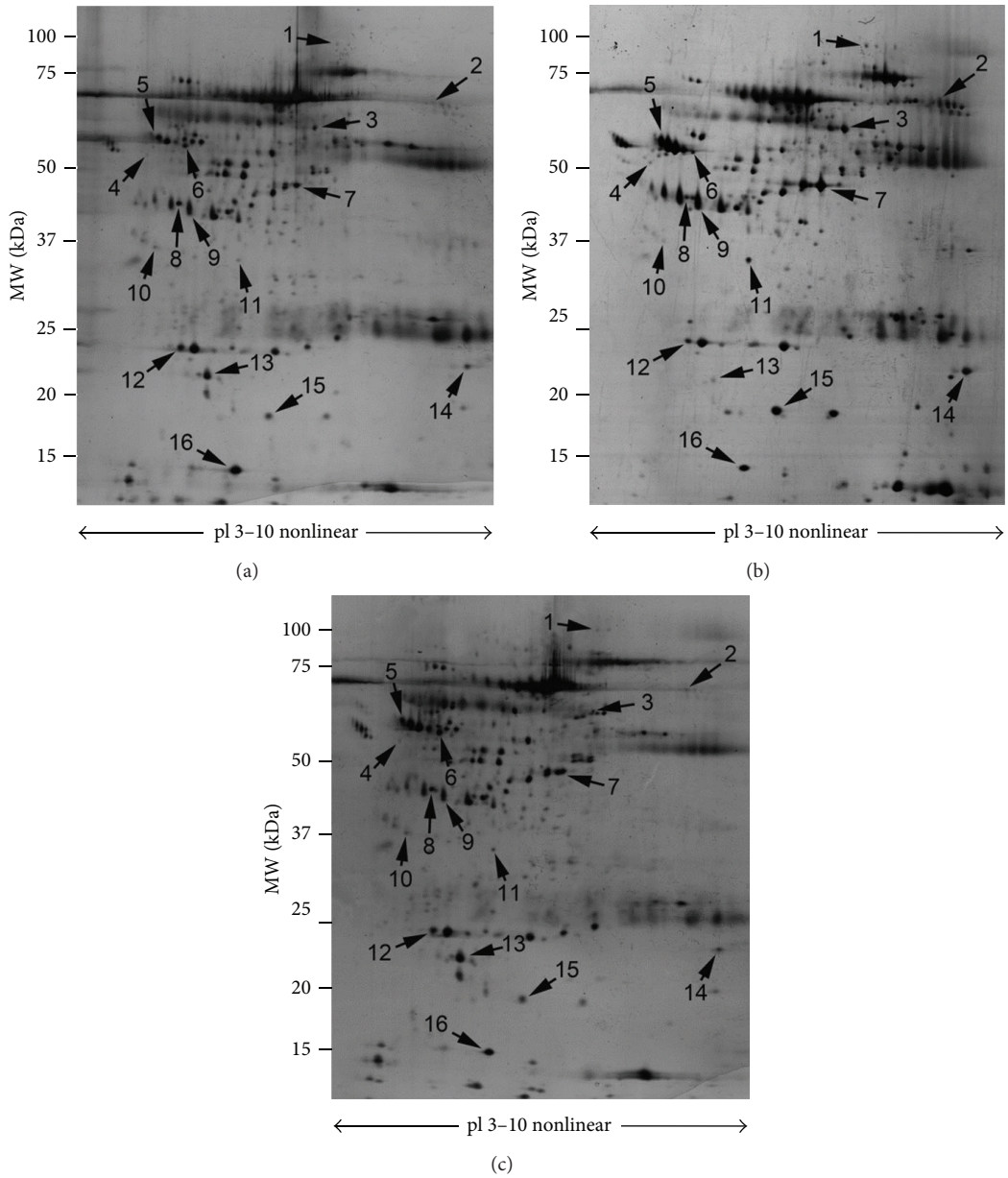


Figure 1: Silver-stained 2-DE gel of proteins from healthy control (a), untreated RTT (b), and RTT after $\omega$-3 PUFAs supplementation (c). 60 $\mu$g of total protein was subjected to nonlinear IPG strips, with a pH range from 3 to 10, followed by SDS-polyacrylamide gradient gel (8–16% T) electrophoresis. Molecular mass and pI markers are indicated along the gels. Numbers denote the mass spectrometry-identified protein spots which are listed in Tables 1 and 2. The same protein spots are reported in 3 representative gels from (a) healthy controls, (b) untreated RTT patients; and (c) $\omega$-3 PUFAs supplemented RTT patients.

These findings well fit with the known anti-inflammatory properties of this fatty acids family [29].

The APR is a highly conserved adaptive mechanism [30] and is a core part of the innate immune response. Profound changes occur in the plasma proteome as the consequence of APR, reflecting a highly regulated process as part of a more generalized reprogramming of signaling events under the influence of cytokines. The involved proteins (i.e., APR proteins) are known to be predominantly synthesized in the liver, and the signaling events result in either an upregulation or downregulation of APR proteins. More than 200 plasma proteins are known to vary in the APR, some of which may control tissue damage and participate in tissue repair, although their role still remains speculative [31].

Omega-3 PUFAs have multiple health benefits mediated at least in part by their anti-inflammatory actions. A recent paper [29] demonstrated that EPA and DHA are competitors for arachidonic acid (AA) in binding to the 5-lipooxygenase enzyme, since $\omega$-3 PUFAs displace AA in membrane phospholipids, reducing the production of AA-derived

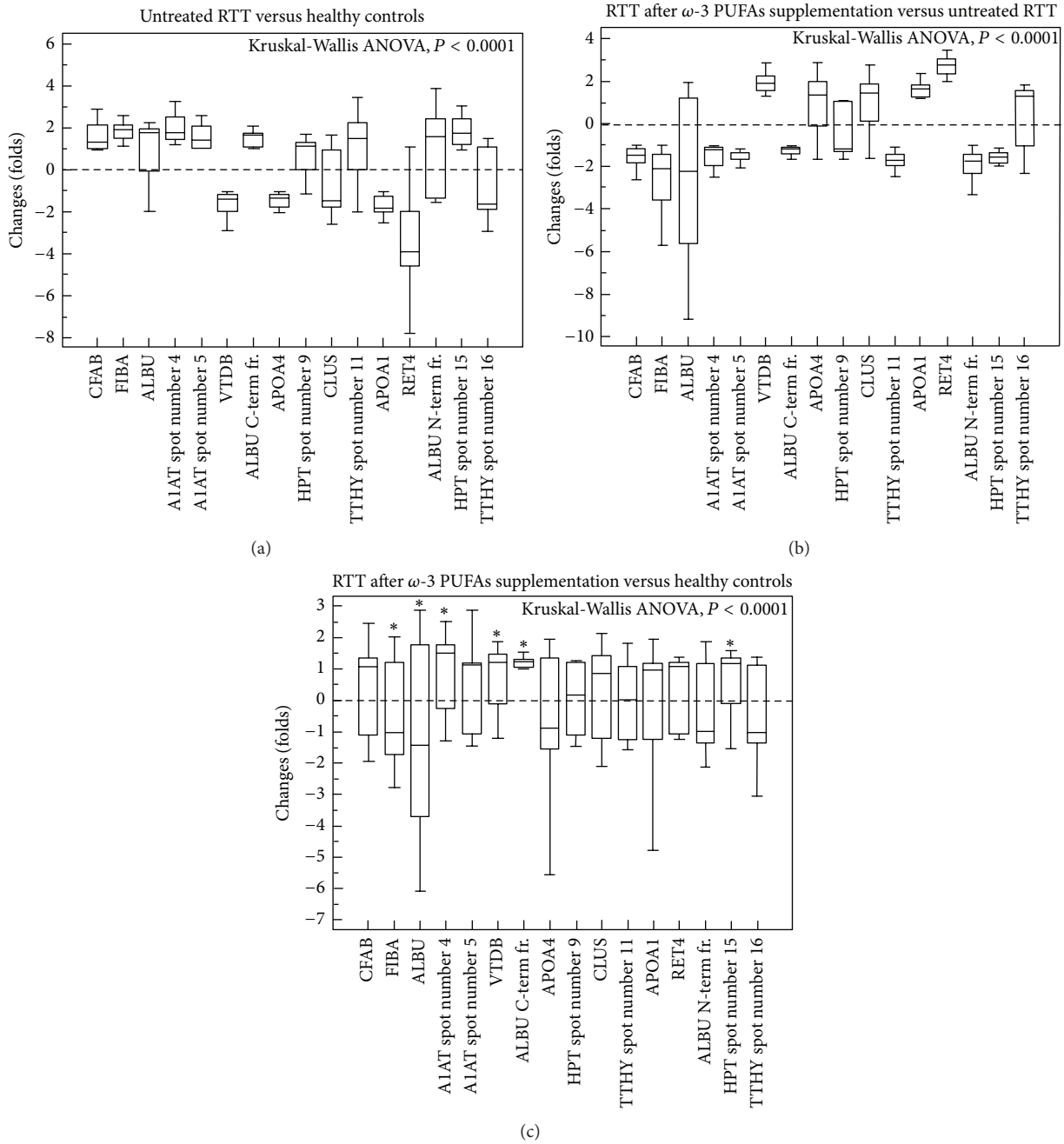


Figure 2: Plasma proteins expression as a function of $\omega$-3 PUFAs supplementation in *MECP2*-mutated girls with classical RTT. (a) RTT patients before supplementation: expression levels are compared to healthy controls; (b) RTT patients after supplementation: expression levels are compared to untreated RTT; (c) RTT patients after supplementation: expression levels are compared to healthy controls. Data are expressed as box-and-whiskers plots. Box-and-whisker plots representation was used in a quite unconventional fashion. Our aim was to try to visually display relative changes in the expression of individual protein as compared to healthy controls. The variables represented correspond to relative changes: a value of 0 represents no changes in expression as compared to controls; positive values indicate protein overexpression, while negative values indicate protein underexpression. Significance of the changes were detected at ImageMaster analysis; therefore, this figure corresponds to a graphical device in order to visually detect the observed protein changes. Results of Kruskal-Wallis ANOVA are indicated. Asterisks in panel (c) indicates persistently overexpressed (top symbols) and persistently underexpressed proteins following $\omega$-3 PUFAs supplementation as compared to control levels of expression.

eicosanoids (i.e., prostaglandin $E_2$) while increasing those generated from $\omega$-3 PUFAs activation. As reported in several trials, $\omega$-3 PUFAs supplementation is able to reduce plasma and urine levels of eicosanoids such as leukotriene $E_4$ [32–35]. Besides the anti-inflammatory effects based on the interruption of the AA cascade, $\omega$-3 PUFAs impart their anti-inflammatory effects via a decreased activation of nuclear factor-kappa B (NF-KB), a potent inducer of the production

Table 2: Biological functions for the identified proteins.

| Proteins overexpressed in untreated RTT | |
|---|---|
| Protein | Biological function |
| Complement factor B | Immune system and complement system regulation |
| Fibrinogen alpha chain | Coagulation and signal transduction |
| Serum albumin | Transport, regulation of colloidal osmotic pressure, and platelet activation |
| Alpha-1-antitrypsin (spot number 4) | Acute phase response, coagulation, and proteases inhibition |
| Alpha-1-antitrypsin (spot number 5) | Acute phase response, coagulation, and proteases inhibition |
| Serum albumin (C terminal fragment) | Transport, regulation of colloidal osmotic pressure, and platelet activation |
| Haptoglobin (spot number 9) | Acute phase response and hemoglobin binding |
| Transthyretin (spot number 11) | Thyroid hormone binding and transport |
| Serum albumin (N terminal fragment) | Transport, regulation of colloidal osmotic pressure, and platelet activation |
| Haptoglobin (spot number 15) | Acute phase response and hemoglobin binding |
| Proteins underexpressed in untreated RTT | |
| Protein | Biological function |
| Vitamin D-binding protein | Vitamin D sterols carrier |
| Apolipoprotein A-IV | Lipid metabolism |
| Clusterin | Apoptosis, complement system regulation, and innate immunity |
| Apolipoprotein A-I | Lipid transport and metabolism |
| Retinol-binding protein 4 | Retinol transport and metabolism |
| Transthyretin (spot number 16) | Thyroid hormone binding and transport |

of proinflammatory cytokines, including interleukin-6 and tumor necrosis factor-$\alpha$. Overall, enrichment of cellular membranes with $\omega$-3 PUFAs disrupts dimerization and recruitment of toll-like receptor-4, which might contribute to anti-inflammatory effects by downregulation of NF-KB activation [29]. Further evidence demonstrates that $\omega$-3 PUFAs can repress lipogenesis and increase generation of resolvins and protectins, ultimately leading to reduced inflammation. Finally, the effects of EPA and DHA include their ability to increase secretion of adiponectin, an anti-inflammatory adipokine [36]. When considering the effects of $\omega$-3 PUFAs on the cellular function, their direct modulation of G-protein-coupled receptor is noteworthy and might contribute to the anti-inflammatory properties. At the same time, $\omega$-3 PUFAs show a direct regulation on gene expression vianuclear receptors and transcription factors, which are in turn modulated by cytoplasmic lipid binding proteins transporting these fatty acids into the nucleus. Regulation of $\omega$-3 PUFAs on gene expression could explain the altered protein expression that we reported on the RTT plasma proteome and is in line with previous findings in adult smokers following a short course (i.e., 5 weeks) of $\omega$-3 PUFAs enriched diet [12]. In this latter study, proteins related to the antioxidant, anti-inflammatory, and antiatherosclerotic properties of HDL were upregulated, in contrast with down-regulation of complement activation and APR proteins.

In particular, in our study major changes for proteins involved in APR (CFAB, FIBA, ALBU (spot number 3 C terminal fragment and spot number 14 N terminal fragment), A1AT (spots number 4 and number 5) and HPT (spots number 9 and number 15)), immunity, unfolded protein response (CLUS), blood coagulation (FIBA), transport pathways (TTHY (spots number 11 and number 16), RET4 and VTDB), and lipid metabolism (APOA4 and APOA 1) were evidenced at the proteome analysis of plasma samples from a population of patients with typical RTT at different stages and harboring a variety of *MECP2* gene mutations. Overexpressed plasma proteins in the unsupplemented RTT patients were mainly related to APR and underexpressed spots corresponded to negative APR proteins, unfolded protein response, and proteins involved in the lipid metabolism. Our data indicated that $\omega$-3 PUFAs almost completely rescued the APR detected at the baseline.

The molecular mechanisms of the $\omega$-3 PUFAs action remain only partially understood and include changes in membrane structures and gene expression, direct interactions with ion channels, and alterations in eicosanoid biosynthesis [28]. EPA and DHA, key $\omega$-3 PUFAs, have been reported to compete with AA for the conversion by cytochrome P450 enzymes, thus resulting in the formation of alternative, physiologically active, metabolites, given that cytochrome P450 enzymes are known to efficiently convert EPA and DHA to epoxy and hydroxy metabolites (17,18-epoxyeicosatetraenoic, and 19,20-epoxydocosapentaenoic acid, resp.) [37], which could likely mediate some of their beneficial effects [38].

The present study strongly suggests that the main beneficial action of $\omega$-3 PUFAs (or their secondary metabolites) in RTT is the modulation of an unrecognized subclinical inflammatory status and fits well with the known anti-inflammatory properties of this fatty acids family and suggesting that the super-family of the multiple actions attributed to $\omega$-3 PUFAs could be attributable to the APR modulation.

## 5. Conclusion

A subclinical inflammatory state has been previously reported in autistic subjects with significant changes in inflammation-related proteins [39]. Overall, our findings of a subclinical APR in RTT that can be modulated by a dietary supplementation of $\omega$-3 PUFAs provide new insights into the role of inflammation in autistic disorders and support the role of $\omega$-3 PUFAs as key nutraceuticals [40].

## Abbreviations

CFAB: Complement factor B
FIBA: Fibrinogen alpha chain
ALBU: Serum albumin
A1AT: Alpha-1-antitrypsin
VTDB: Vitamin D-binding protein
APOA4: Apolipoprotein A4
HPT: Haptoglobin
CLUS: Clusterin
TTHY: Transthyretin
APOA1: Apolipoprotein A1
RET4: Retinol-binding protein 4.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Claudio De Felice, Alessio Cortelazzo, and Cinzia Signorini contributed equally to the work.

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*Research Article*

# Increased Anti-Phospholipid Antibodies in Autism Spectrum Disorders

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Autism spectrum disorders (ASD) are characterized by impairments in communication, social interactions, and repetitive behaviors. While the etiology of ASD is complex and likely involves the interplay of genetic and environmental factors, growing evidence suggests that immune dysfunction and the presence of autoimmune responses including autoantibodies may play a role in ASD. Anti-phospholipid antibodies are believed to occur from both genetic and environmental factors and have been linked to a number of neuropsychiatric symptoms such as cognitive impairments, anxiety, and repetitive behaviors. In the current study, we investigated whether there were elevated levels of anti-phospholipid antibodies in a cross-sectional analysis of plasma of young children with ASD compared to age-matched typically developing (TD) controls and children with developmental delays (DD) other than ASD. We found that levels of anti-cardiolipin, $\beta$2-glycoprotein 1, and anti-phosphoserine antibodies were elevated in children with ASD compared with age-matched TD and DD controls. Further, the increase in antibody levels was associated with more impaired behaviors reported by parents. This study provides the first evidence for elevated production of anti-phospholipid antibodies in young children with ASD and provides a unique avenue for future research into determining possible pathogenic mechanisms that may underlie some cases of ASD.

## 1. Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by stereotyped interests, repetitive behaviors, and impairments in communication and social interaction. Currently 1 in 88 children has been identified as having ASD [1]. Despite the high incidence of ASD, etiology and pathogenesis remain poorly understood. Current research suggests a significant role for immunodysregulation and autoimmune processes in the pathophysiology of ASD, with the existence of autoantibodies directed against neuronal proteins repeatedly demonstrable in a substantial number of children with ASD (reviewed in [2, 3]).

The reported targets of autoantibodies exhibited by children and adults with ASD are diverse and range from neurotransmitter receptors such as serotonin receptors, markers of astroglial activation such as glial fibrillary acidic protein (GFAP), and myelin sheath cellular products such as myelin basic protein (MBP), as well as yet unidentified neuronal protein targets (reviewed in [4]). Moreover, the presence of neuronal protein-specific autoantibodies are associated with increased behavioral impairments and more severity in children with ASD [5], suggesting a link between the autoimmune processes and behavioral dysfunction. For example, autoantibodies directed against a 45 kDa protein present in the cerebellum were not only found more frequently in children with ASD but were also associated with lower adaptive and cognitive function, as well as increased aberrant behaviors [6, 7]. However, replication studies of antibody-specific antigen targets, such as MBP

and GFAP, have been inconsistent, suggesting that further studies are needed to identify the target or targets and/or associated autoimmune phenomena [8, 9]. Recent studies have highlighted a role for anti-phospholipid antibodies in altering function and behaviors such as cognition, anxiety, and hyperactivity.

Anti-phospholipid antibodies recognize a number of diverse targets including cardiolipin, phosphoserine, and $\beta$2-glycoprotein 1. They are found in roughly ten percent of the population [10, 11] but are thought to cause pathology in only a small segment of those with antibodies. Elevated levels of anti-phospholipid antibodies have been found in the blood and cerebral spinal fluid of psychiatric patients having hallucinatory and/or delusionary episodes [12]. In individuals with neuropsychiatric systemic lupus erythematosus, elevated titers of anti-cardiolipin antibodies are reported most often in patients with cognitive impairment, psychosis, depression, seizures, chorea, and migraines [13]. Moreover, in animal models, administration of anti-phospholipid antibodies to rodents can induce a number of psychological side effects, including increased anxiety and decreased cognition learning and memory [14, 15].

When anti-phospholipid antibodies are present in an individual consistently over time, along with arterial or venous thrombosis or pregnancy morbidity, this is termed antiphospholipid syndrome (APS). Although rare in children, APS does occur and is thought to be underdiagnosed likely due to lack of testing in this population, as these autoantibodies are generally related to fertility and cardiovascular events [16]. It is still unclear what the presence of elevated levels of anti-phospholipid antibodies signify in the pediatric population in terms of comorbidities [17].

Based on the links between anti-phospholipid antibodies and altered behaviors and cognition, we evaluated a panel of autoantibodies associated with APS including anticardiolipin, antiphosphoserine, and anti-$\beta$2-glycoprotein 1 in plasma from a large cohort of well-characterized children enrolled in a population-based case-control study. To better define the immune status of children with ASD, autoantibody profiles were assessed in children 24–82 months of age who had ASD and compared with typically developing children and children with developmental delays other than ASD who were of the same age and lived in the same geographical area. In addition, antibody levels were investigated for associations with clinical behavioral outcomes.

## 2. Methods

*2.1. Subjects.* One hundred and nine participants who were enrolled through the population-based case controlled Childhood Autism Risks from Genetics and Environment (CHARGE) study conducted at UC Davis MIND Institute were recruited to this study [18]. The study protocols including recruitment and behavioral assessments have been described in detail [18–20]. In brief, following clinical evaluation for diagnostic confirmation, participants were placed in one of three groups: (1) children with a confirmed diagnosis of ASD [$N$ = 54, median age 44.8 months (interquartile range 32.0–57.7), 45 males]; (2) children diagnosed with developmental delay but not ASD [$N$ = 22. median age 41.7 months (IQR 25.7–57.8), 18 males.]; or (3) children who were confirmed as typically developing controls [$N$ = 33, median age 40.6 months (IQR 27.7–53.6), 27 males]. Final diagnosis of ASD was confirmed by the Autism Diagnostic Interview-Revised (ADI-R) [21] and the Autism Diagnostic Observation Schedule (ADOS) [22]. The ADOS and ADI-R consist of a standardized, semistructured interview and a diagnostic algorithm from the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Text Revision (DSM-IVTR) [23], with definitions of autism from the International Classification of Diseases, Tenth Revision (ICD-10) [24]. The administration of all diagnostic instruments was carried out by experienced clinicians at the UC Davis MIND Institute.

Additional behavior testing included the Aberrant Behavior Checklist (ABC), Mullen Scales of Early Learning (MSEL), and Vineland Adaptive Behavior Scales (VABS). The ABC was taken by parents of children in the study and consists of questions designed to measure the severity of autism-associated behaviors, including irritability, lethargy, stereotypy, hyperactivity, and inappropriate speech. Assessment scores for the ABC range from 0 to 174, with higher scores indicating more severely affected behavior. In addition to the ABC, children enrolled in the study were assessed for cognitive function using MSEL. The MSEL has components for visual reception, fine motor, receptive language, and expressive language, each of which yields a $T$ score with mean = 50 and SD = 10. Adaptive function was assessed through parental interview using the VABS. The VABS has components for communication, daily living, socialization, and motor skills. These components each component yields a score from 20 to 160 with a mean among typically developing children of 100.

Participants did not differ for age or sex ratios. All children were medication-free and in good health and without diagnosis of autoimmune conditions at time of the blood draw. This study was approved by the institutional review boards in the University of California, Davis. Informed consent was obtained prior to participation.

*2.2. Antibody Analysis.* For each subject peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences; San Jose, CA), centrifuged at 2300 rpm for 10 min and the plasma harvested. Plasma was aliquoted and stored at −80°C until antibody levels were measured. The IgG antibody levels of anticardiolipin, antiphosphoserine, and anti-$\beta$2-glycoprotein 1 were assessed by commercial ELISA (Orgentec, Mainz, Germany) using the manufactures protocol. In brief, plasma samples were diluted 1 : 100 in assay buffer, and 100 uL of diluted plasma was loaded on plate in duplicate along with calibrators and controls. Samples were incubated for 30 minutes at room temperature. Samples were visualized using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and read at 450 nm. All results are reported in IgG phospholipid units per milliliter (GPL-U/mL).

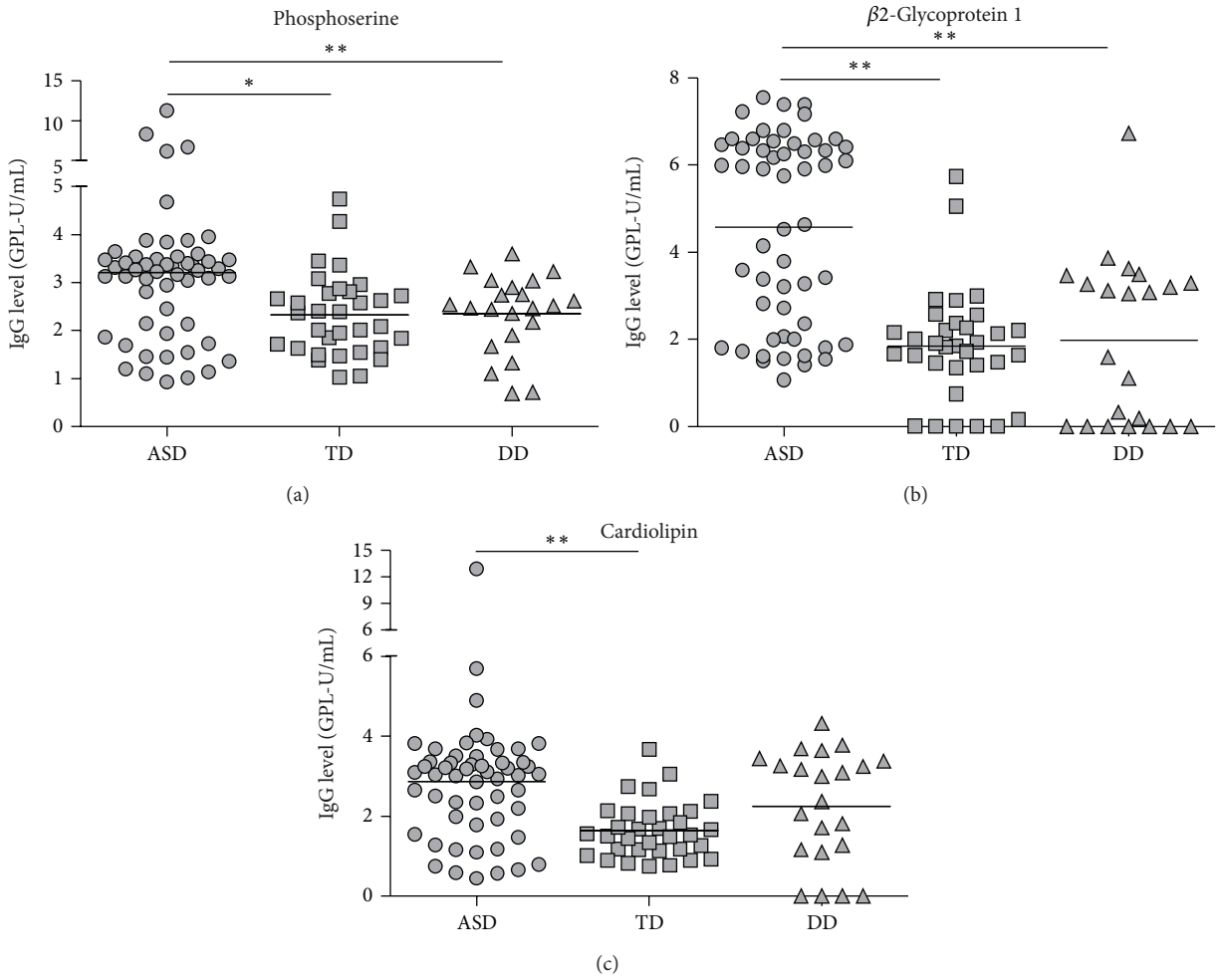


Figure 1: Anti-phospholipid antibody levels. (a) ASD subjects were found to have significant ($P < 0.01$) levels of anti-phosphoserine and (b) anti-$\beta$2-glycoprotein 1 antibodies compared with TD and DD controls. Levels of (c) anti-cardiolipin were found to be significantly ($P < 0.001$) higher in ASD compared with TD control, but differences to DD control did not reach significance. $^{*}P < 0.01$, and $^{**}P < 0.001$.

*2.3. Statistical Analysis.* Data analysis was performed using STATA 12 software. Data was determined as nonparametric using Shapiro-Wilks test for normality. Wilcoxon rank-sum tests were used to compare antibody levels between subject groups. Spearman correlations were used to determine the association between anti-phospholipid antibodies and variations in scores on behavioral, cognitive, and adaptive assessments. A probability value ($P$) of less than 0.05 was considered to be significant.

## 3. Results

*3.1. Anti-Phospholipid Antibody Levels in ASD.* An approximate 38% increase in anti-phosphoserine antibody levels were observed in children with ASD compared with TD controls (mean 3.209 ± SEM 0.204 versus mean 2.324 ± SEM 0.149; $P < 0.01$) and a 37% increase compared with children with DD (mean 3.209 ± SEM 0.238 versus mean 2.344 ± SEM 0.172; $P < 0.01$) (Figure 1). There was also a 149% increase in anti-$\beta$2-glycoprotein 1 antibody levels in children with ASD compared with age-matched TD controls (mean 4.584 ± SEM 0.294 versus mean 1.845 ± SEM 0.224; $P < 0.001$) and a 132% increase over children with DD (mean 4.584 ± SEM 0.294 versus mean 1.975 ± SEM 0.406; $P < 0.001$). Antibody levels of anticardiolipin were increased approximately 75% higher in children with ASD compared with TD controls (mean 2.873 ± SEM 0.245 versus mean 1.642 ± SEM 0.121; $P < 0.001$), and there was a trend toward elevated levels in children with ASD compared with DD controls, although this did not reach statistical significance after multiple comparison correction (Figure 1).

*3.2. Association of Anti-Phospholipid Antibody Levels and Behaviors.* We next examined whether anti-phospholipid antibody levels were associated with impairments in behavior. Significant associations were found between all three anti-phospholipid antibodies assessed and increased severity of behaviors, such as lethargy, irritability, and stereotypic behaviors as assessed by the ABC. Impairments in cognitive and adaptive behaviors as measured by MSEL and VABS

Table 1: Association analysis of anticardiolipin, $\beta$2-glycoprotein 1, and antiphosphoserine with behavioral outcome measures as assessed by the Aberrant Behavior Checklist (ABC), Mullen Scales of Early Learning (MSEL), and Vineland Adaptive Behavior Scales (VABS) using Spearman's rank correlations demonstrated that there were significant correlations between anti-phospholipid antibody levels and the severity of impairments in behavior in participants enrolled in this study. For the ABC, a higher score corresponds to more behavioral impairments. For the MSEL and VABS, a lower score corresponds to increased cognitive and adaptive impairments.

| Autoantibody profile | Cardiolipin | | $\beta$2-Glycoprotein 1 | | Phosphoserine | |
|---|---|---|---|---|---|---|
| | $P$ value | $r$ | $P$ value | $r$ | $P$ value | $r$ |
| Aberrant Behavior Checklist | | | | | | |
| Subscale I: irritability | <0.001 | 0.368 | 0.001 | 0.343 | 0.002 | 0.312 |
| Subscale II: lethargy | 0.001 | 0.334 | <0.001 | 0.406 | 0.001 | 0.317 |
| Subscale III: stereotypy | 0.002 | 0.309 | <0.001 | 0.421 | 0.002 | 0.309 |
| Subscale IV: hyperactivity | 0.010 | 0.257 | 0.001 | 0.343 | 0.040 | 0.206 |
| Subscale V: inappropriate speech | 0.409 | 0.084 | 0.010 | 0.258 | 0.865 | 0.017 |
| Subscale VI: moods | <0.001 | 0.345 | <0.001 | 0.346 | 0.002 | 0.303 |
| Mullen Scales of Early Learning | | | | | | |
| Visual reception | 0.031 | −0.206 | 0.002 | −0.291 | 0.042 | −0.195 |
| Fine motor | 0.004 | −0.272 | 0.002 | −0.292 | 0.002 | −0.300 |
| Receptive language | 0.009 | −0.249 | <0.001 | −0.356 | 0.003 | −0.286 |
| Expressive language | 0.004 | −0.271 | <0.001 | −0.350 | 0.007 | −0.257 |
| Vineland Adaptive Behavior Scales | | | | | | |
| Communication | 0.011 | −0.244 | 0.003 | −0.284 | 0.001 | −0.324 |
| Daily living skills | 0.047 | −0.190 | 0.007 | −0.255 | 0.014 | −0.234 |
| Socialization | 0.022 | −0.219 | 0.002 | −0.289 | 0.002 | −0.287 |
| Motor skills | 0.088 | −0.164 | 0.127 | −0.147 | 0.014 | −0.236 |

were also associated with increased antibody levels. These impairments included deficits in functional communication on the VABS and receptive and expressive language domains measured by the MSEL (Table 1). Although there were strong correlations observed in the pediatric population as a whole, there were no significant differences found when analyzing within the individual groups based on diagnosis.

## 4. Discussion

In this study we demonstrate that the levels of anti-phospholipid antibodies in children with ASD are significantly elevated when compared with typically developing children and children with developmental delays other than ASD. Furthermore, elevated anti-phospholipid antibodies are associated with increased impairments in a number of clinical cognitive and behavioral measures such as stereotypy, hyperactivity, and communication. Together with previous studies in the field demonstrating the increased presence of autoantibodies in children with ASD, this study adds further support for a possible role for autoimmune phenomena in the pathogenesis of ASD [25]. Several previous studies have shown that increased anti-phospholipid antibodies are present in a number of neuropsychiatric conditions; however, it is currently unclear what, if any, pathologic significance these anti-phospholipid antibodies have in behavioral disorders including ASD. In addition, it is noteworthy that while this study found elevated anti-phospholipid antibodies in children with ASD, the levels are below what is considered clinically significant levels for APS. These current data highlight the importance for further research to investigate the role of anti-phospholipid antibodies in a variety of childhood behavioral disorders.

APS in children is thought to be rare; however, current assessments are biased away from recognizing the syndrome in prepubescent individuals [17]. One study demonstrated that anti-phospholipid autoantibody levels are elevated in a significant number of children, suggesting that elevated levels of anti-phospholipid antibodies may have importance in children even in the absence of the defining clinical features of APS, such as arterial or venous thrombosis or pregnancy morbidity that is observed in adults [26]. The additional symptoms of APS vary greatly in adults, with cognitive [27], neuropsychiatric [12, 13], and neuromotor [28] symptoms having been observed. Animal models of APS, in which antibodies isolated from individuals with APS are transferred into mice, demonstrate that the effects of the antibodies are associated with neuropsychiatric symptoms such as anxiety, hyperactivity, and impairments in cognition [15, 29, 30].

Anti-phospholipid antibodies have been associated with numerous central nervous system involvements, with many symptoms such as stroke and optic neuropathy thought to result from thrombic events. However, the exact mechanism of how these antibodies cause pathology is unknown (reviewed in [31, 32]). It is unlikely that the thrombotic events are responsible for all APS-related symptoms; in many subjects with chorea, lesions are not apparent on CT scans, suggesting that a more direct cellular mechanism could be involved [25, 33, 34]. Direct binding of anti-phospholipid

antibodies to neurons has been demonstrated in human neurons *ex vivo,* and these antibodies have been shown to permeabilize and depolarize brain synaptoneurosomes [35, 36]. In mice, when anti-phospholipid antibodies derived from human subjects are administered they recognize neuronal targets and have been shown to decrease astroglia proliferation [37]. It is not clear how this could translate to pathology in ASD, but many studies have shown the increased presence of autoantibodies that interact with neuronal targets. The exact targets are generally not known in ASD, but it is possible that these antibodies, or at least a fraction, could be anti-phospholipid antibodies [7, 25, 34]. In addition, anti-phospholipids may be related to other previous findings such as MBP-specific antibodies [5]. In subjects with SLE increased, elevated anti-cardiolipin antibodies titers are associated with increased myelin binding antibodies [38].

Alternatively, anti-phospholipid antibodies may represent a biomarker for nonspecific neuronal damage or inflammation. In a study looking at anti-neuronal antibodies in 129 young children with and without ASD, 43% of children showed some positive staining for brain reactive antibodies [38]. Although a differential pattern of staining was not readily apparent between those children with ASD compared with controls, those children who did show positive autoantibody staining displayed more severe score on the Child Behavior Checklist (CBCL) [39]. This suggests that non-specific anti-brain antibodies may hearken a more general developmental impairment. In fact, in ASD numerous antibodies directed against brain or central nervous system tissue have been identified (reviewed in [2]). The targets of these antibodies are quite diverse and include serotonin receptors [40], MBP [41], nucleus [42], and GFAP [43], as well as numerous unidentified protein targets. Moreover, unique autoantibody targets seem to be found only in subsets of children with ASD, and their detection has been difficult to replicate across studies; primary examples are antibodies against such targets as MBP and GFAP [8, 9]. Additionally, anti-phospholipid antibodies have been associated with a number of infectious agents, such as syphilis or HIV [44, 45]. However, there is no evidence of increased rates of infection in children with ASD at the ages reported in this study, and all participants in the study were screened for illness at the time of the blood draw [46, 47]. Given the apparent ability of anti-phospholipid antibodies to discriminate children with ASD compared with controls as seen in this study, these antibodies may offer additional novel biomarkers for evaluating pathogenic mechanisms and possible targeted treatments for children with ASD.

While this study is limited due to its cross-sectional nature and further longitudinal testing is warranted, it is among the first to attempt to measure anti-phospholipid antibodies in a pediatric population with ASD with age-matched controls. The associations between anti-phospholipid antibody levels and impairments in behaviors may be of significance to young children beyond those with ASD. In particular, we demonstrate that these autoantibodies are associated with impairments in behaviors similar to previous studies looking at unidentified neuronal targets [7]. These observations warrant further study.

## 5. Conclusion

In summary, the findings of increased anti-phospholipid antibody levels in young children with ASD, and the association between antibody levels and impaired behaviors in the pediatric population as a whole, offer potential new targets for understanding the mechanisms involved in the pathogenicity of ASD. Our novel preliminary findings support the importance of further study of the biological impact of autoantibodies and their association with behavioral and cognitive impairments in children with ASD.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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*Research Article*

# Gestational Exposure to a Viral Mimetic Poly(I:C) Results in Long-Lasting Changes in Mitochondrial Function by Leucocytes in the Adult Offspring

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Maternal immune activation (MIA) is a potential risk factor for autism spectrum disorder (ASD) and schizophrenia (SZ). In rodents, MIA results in changes in cytokine profiles and abnormal behaviors in the offspring that model these neuropsychiatric conditions. Given the central role that mitochondria have in immunity and other metabolic pathways, we hypothesized that MIA will result in a fetal imprinting that leads to postnatal deficits in the bioenergetics of immune cells. To this end, splenocytes from adult offspring exposed gestationally to the viral mimic poly(I:C) were evaluated for mitochondrial outcomes. A significant decrease in mitochondrial ATP production was observed in poly(I:C)-treated mice (45% of controls) mainly attributed to a lower complex I activity. No differences were observed between the two groups in the coupling of electron transport to ATP synthesis, or the oxygen uptake under uncoupling conditions. Concanavalin A- (ConA-) stimulated splenocytes from poly(I:C) animals showed no statistically significant changes in cytokine levels compared to controls. The present study reports for the first time that MIA activation by poly(I:C) at early gestation, which can lead to behavioral impairments in the offspring similar to SZ and ASD, leads to long-lasting effects in the bioenergetics of splenocytes of adult offspring.

## 1. Introduction

The most recent estimates indicate that the prevalence of autism spectrum disorders (ASD) in the United States has raised to 1 in 54 boys and 1 in 252 girls [1]. Although increased awareness and changes in diagnostic criteria have been proposed as the major contributors to this increased prevalence [2], as of today, the etiopathology of disorders like ASD and schizophrenia (SZ) remains largely unknown.

Several studies have suggested that impaired mitochondrial function and altered energy metabolism in individuals with ASD may contribute to their social and cognitive deficits [3–5], and recent reports indicate the presence of mitochondrial dysfunction (MD) in brain, skeletal muscle, and peripheral blood mononuclear cells (PBMC) from children with ASD. The MD in ASD is generally characterized by lower complex I activity accompanied, in a subset of cases, by deficits in other complexes [6–8].

Beside their critical role in a number of pathways, spanning from ATP production (via oxidative phosphorylation), one-carbon metabolism regulation, heme biosynthesis, fatty acid catabolism, and branched chain amino acid metabolism [9], mitochondria may also impact the immune response and vice versa [10–12]. For example, human neutrophil mitochondria are involved in several functions such as chemotaxis, respiratory burst activity, maintenance of cell shape, and apoptosis [13–17]. Furthermore, neutrophil phagocytosis may involve the incorporation of some mitochondrial proteins into the phagosome [18]. In addition, mitochondria can be involved in the immune response by providing part of the metabolic pathway for Gln, in a process named "glutaminolysis" [19, 20]. Interestingly, Gln is implicated in the expression

of NADPH oxidase components, cytokine production in lymphocytes, and macrophage, and as a provider for substrates required for nucleic acid synthesis [21–25]. Taken together, these lines of evidence unveil a link between mitochondria and immune response [10–12]. Indeed, deficits in bioenergetics have been reported in lymphocytes from children with ASD enrolled in the case-control population-based Childhood Autism Risk Genetics and Environment (CHARGE) Study [26, 27]. Children with ASD in this study display a number of immune dysfunctions including abnormalities in monocytes, T cells and NK cell responses [28]. These observations suggest the presence of a genetic background that results in a distinct immune profile in responses to a variety of triggers, among them psychological stressors, exposure to chemical triggers, and infectious agents [29, 30].

Considering that (i) mitochondria are inherited maternally via oocyte, (ii) maternal diet or immune activation during pregnancy has an impact on fetal metabolic and immune programming [31–33], and (iii) offspring born to pregnant mice injected with poly(inosinic:polycytidylic acid) (poly(I:C)), a synthetic double-stranded RNA that mimics viral infection via activation of Toll-like receptor-3 (TLR3), at embryonic day 12.5 (E12.5), display core behavioral symptoms of ASD [34, 35] and SZ [34], it is hypothesized that prenatal exposure of mothers to an immunogenic response, that is, poly(I:C) elicits changes in mitochondrial function in splenocytes from progeny lasting into adulthood. Exposure to TLR ligands can lead to maternal hypertension, vascular dysfunction, and proteinuria in pregnant animals but not in nonpregnant animals [36–38] suggesting the occurrence of a differential immune response/pathway during pregnancy. Differences are also evident between pregnant individuals with human placentas and patients with preeclampsia showing greater expression of TLR3, along with TLR2, TLR4, and TLR9, compared to nonpreeclampsia mothers [39, 40]. These data suggest that TLR signaling may be involved in placental deficiencies/abnormalities that may provide a framework for altered fetal programming. Of note, trophoblastic inclusions, which are also observed in preeclampsia and other placental defects, were reported to be increased in placenta from mothers of children with ASD compared to controls [41]. Furthermore, maternal exposure to various pathogens, including viruses, significantly increases the risk for ASD and SZ [42–48]. Considering that maternal exposure to various pathogens is associated with ASD and SZ, the critical link between prenatal maternal infection and postnatal brain and behavioral pathology seem to be the maternal immune response, including cytokine production [47, 49–54], which may contribute to the fetal imprinting of the neuroimmune response and, possibly, mitochondria-mediated metabolic responses. Although it is already known that upon poly(I:C) injection, the induction of maternal cytokines alters the expression of several cytokines in the fetal brain (with only IL-1$\beta$ remaining elevated at 24 h [53] with only a few changes during adulthood), it is unknown if maternal immune activation (MIA) also causes chronic changes in the bioenergetics of immune cells (such as splenocytes) of adult offspring.

In this study, we sought to determine whether MIA in pregnant dams alters mitochondrial function in splenocytes from affected offspring. To test this, dams were exposed to poly(I:C) on gestational day 12.5 to induce MIA. This stage of gestation correlates with the late first trimester in humans [55], coincidental with the time that infections are most closely linked to increased incidence of ASD and SZ [47, 48]. The present study reports for the first time that MIA activation by poly(I:C) at early gestation, which can lead to impairments in multiple psychological domains, is associated with mitochondrial changes in immune the cells of adult offspring.

## 2. Materials and Methods

*2.1. Animals.* Male and female C57BL/6J (Jackson Laboratory, Sacramento, CA, USA) mice were bred and maintained by the Center for Laboratory Animal Research, at University of California, Davis, and maintained at ambient room temperature on a 12 h light/dark cycle (lights on at 06:00 h). Food and water were provided *ad libitum*. All procedures were performed with approval by the Institutional Animal Care and Use Committee, University of California, Davis, and in accordance with the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

*2.2. Treatment and Behavioral Assessment.* Mice were mated overnight and females were checked daily for the presence of seminal plugs, noted on gestational day 0.5 (G0.5). On G12.5, pregnant female mice were weighed and injected with a single dose (20 mg/kg; i.p.) of poly(I:C) (Sigma Aldrich, St Louis, MO, USA) or saline vehicle (SHAM) as previously described [35]. Each dam was returned to its cage and left undisturbed until the birth of its litter. All mice pups remained with the mother until weaning on postnatal day 21, at which time mice were group-housed 3-4 per cage with same-sex littermates. Mice born from poly(I:C)-treated dams exhibited autism-like behavioral deficits including reduced social approach, increased ultrasonic vocalizations, and repetitive marble burying behaviors [35].

*2.3. Splenocyte Isolation.* One week following behavioral testing, 12 wk old mice were sacrificed by cervical dislocation and spleens were collected for tissue processing. While spleen is constituted by a variety of cells relevant to the immune response (including T- and B-lymphocytes, dendritic cells, and macrophages), it has recently been shown that spleens from the offspring of MIA mice elicited by poly(I:C) provide a more homogeneous preparation enriched in granulocytes compared to the preparation obtained from whole blood [56]. Briefly, spleens were homogenized into single cell suspensions by gently pushing them through a 100 $\mu$m nylon mesh filter (Fisher Sci) into PBS at 4°C. Cells were then pelleted, and RBCs were lysed using ACK lysis buffer according to the manufacturer's instructions (Gibco). Cell suspensions were kept on ice until analyzed for mitochondrial activity. Cell viability was determined by trypan blue staining and found to be about 90%.

*2.4. Mitochondrial Activities.* The oxygen uptake of intact cell suspensions ($10^6$ cells/mL) obtained as described above was measured by using a Clark-type $O_2$ electrode from Hansatech (King's Lynn, UK) at 22°C. Cells were incubated in the presence of 5 mM glucose in calcium and magnesium-supplemented HBSS buffer without phenol red at 20–22°C. NADH, succinate, and cytochrome oxidase activities were evaluated under phosphorylating conditions as described before [6, 30]. To this end, cells were permeabilized with a controlled treatment with digitonin [57] by adding 60 $\mu$g/mL 2x recrystallized digitonin for 2 min. The solubilization was stopped by the addition of 1 mg/mL BSA. Oxygen consumption rates were evaluated in the presence of 1 mM ADP plus 1 mM malate-10 mM glutamate followed by the addition of 5 $\mu$M rotenone; 10 mM succinate followed by the addition of 3.6 $\mu$M antimycin A, and 10 mM ascorbate and 0.2 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine followed by the addition of 1 mM KCN.

*2.5. Statistical Analyses.* All mitochondrial experiments were run in triplicates. Mitochondrial data were expressed as mean ± standard error. Student's two-tailed *t*-test was used to evaluate the differences between offspring of poly(I:C)-treated and SHAM-treated dams.

## 3. Results and Discussion

*3.1. Deficits in Complex I in Splenocytes from Mice Gestationally Exposed to Poly(I:C).* Splenocytes from adult mice born to either SHAM- or poly(I:C)-treated dams were isolated for mitochondrial function testing. Given that most of the oxygen uptake by cells is linked to ATP production via oxidative phosphorylation, this parameter was evaluated in intact cells in the presence of glucose (Figure 1(a)). The rate of oxygen uptake by intact cells from SHAM-treated dams was 0.22 ± 0.03 nmol oxygen × (min × $10^6$ cells)$^{-1}$. Under the same conditions, this rate was decreased by 36% in poly(I:C)-treated dams (Figure 1(a)). Addition of oligomycin, an inhibitor of ATPase, was used to stop the fraction of oxygen utilized to synthesize ATP via mitochondria. In both groups, more than 90% of the total oxygen uptake was inhibited by oligomycin, supporting the previous assumption that most—if not all—oxygen uptake by these cells was derived from oxidative phosphorylation. The oxygen uptake resistant to oligomycin, considered somewhat equivalent to State 4 (non-phosphorylating mitochondria), was not different between groups. This result indicated that the proton leak across the inner mitochondrial membrane was similar in both groups, suggesting no major mitochondrial membrane damage by either treatment. Addition of FCCP, an uncoupler of electron transport and ATP synthesis, increased significantly the basal oxygen uptake to a similar extent in both groups (2.5- to 3-fold) with no changes between treatments, suggesting that the maximum respiratory capacity was similar in both groups.

Coupling between oxygen uptake and ATP production was evaluated by the respiratory control ratio in intact cells (RCR). Mitochondria from either treatment showed a significant coupling with glucose as a substrate (with malate-glutamate, RCR = 3.5 ± 0.4 and 2.7 ± 0.3; with succinate, RCR = 4 ± 1 and 6.2 ± 0.5, for saline and poly(I:C), resp.) with no statistical differences between treatment groups. This result indicated that mitochondria were highly coupled and provided a means of support for their integrity during the testing process.

Phosphorylating mitochondria from splenocytes of SHAM animals in the presence of an NAD-linked substrate (such as malate) consumed oxygen at a rate of 0.31 ± 0.04 nmol oxygen × (min × $10^6$ cells)$^{-1}$. Phosphorylating mitochondria from offspring of poly(I:C)-treated dams showed a significant decrease in oxygen consumption (by 55%; $P < 0.01$; Figure 1(b)). By adding rotenone, an inhibitor of Complex I, and succinate, a substrate for complex II, the segment comprising from complex II to complex V was evaluated. No differences in terms of oxygen uptake were observed between controls and poly(I:C) suggesting that the deficit in offspring of poly(I:C)-treated dams was located at the level of complex I. Confirming this result, complex IV activity was not different between treatments (Figure 1(b)) suggesting that mitochondrial mass was equivalent between groups. However, attempts to directly evaluate complex I activity were unsuccessful due to the limited amount of biological material.

The ratios among complexes need to be preserved to provide suitable oxidation of substrates [58]. To this end, the ratios of electron transport chain activities indicated that both treatments allow oxidizing FAD-linked substrates (such as fatty acids) similarly, whereas a significantly lower oxidation of NAD-linked substrates (such as glucose) was evident in the poly(I:C)-treated condition compared to controls (Figure 1(c)). This imbalance in the complexes' ratios suggests that splenocytes from offspring of dams exposed to poly(I:C) use preferentially fatty acids over glucose as their main substrate for mitochondrial oxidative phosphorylation.

These results are consistent with the MD observed in lymphocytes from ASD children characterized by lower complex I activity and accompanied, in some cases, by deficits in other complexes and/or pyruvate dehydrogenase [6–8].

The cytokine production (IL-1$\beta$, IL-6, IL-10, IL-17, and TNF-$\alpha$) from ConA-activated splenocytes obtained from adult offspring of poly(I:C)-treated animals was not different from that of SHAM-treated animals (see Supplementary Material available online on http://dx.doi.org/10.1155/2013/609602). This is consistent with the findings of others utilizing a similar MIA model in which only a handful of cytokines was still increased in early adulthood (frontal corteces IL-1$\alpha$, IL-6, IL-10, and IL-9; cingulate corteces IL-10 and IFN-$\gamma$; none in hippocampus or serum [33]).

## 4. Conclusions

The aim of this study was to evaluate mitochondrial function in splenocytes from offspring gestationally exposed to an acute viral mimetic, that is, poly(I:C), to induce MIA. Our results indicate that the exposure of dams to a single dose of poly(I:C) at gestational day 12.5 likely triggers a TLR3-mediated response in the mother that is transmitted transplacentally to the offspring. In particular, the proinflammatory cytokine IL-6 has been proved to be a key intermediary in

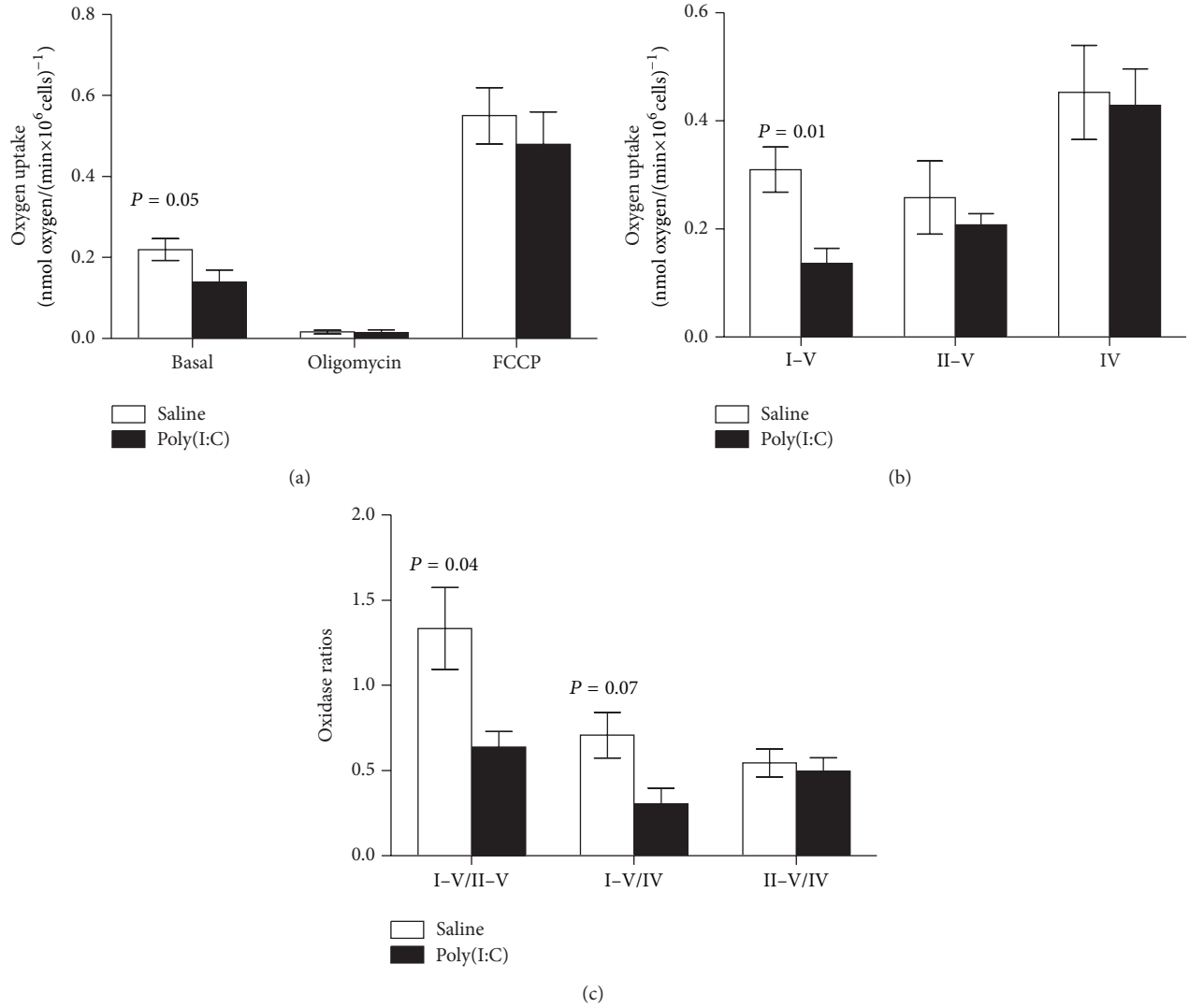


Figure 1: Mitochondrial outcomes in splenocytes. Splenocytes from offspring (10–12 weeks old) poly(I:C)-($n$ = 8) or saline-($n$ = 8) treated dams at 12.5 gestational day were isolated, and mitochondrial outcomes were tested as described in detail in Section 2. (a) Oxygen uptake of intact cells was determined using a Clark-type electrode in Hanks balanced salt solution (HBSS) supplemented with 6 mM glucose as substrate. Basal (only glucose), oligomycin (0.2 $\mu$M), and FCCP (20 $\mu$M) were added, and the initial rates of oxygen uptake were calculated and normalized by million cells. (b) Permeabilized cells were tested for their capability to consume oxygen coupled to ATP production by supplementing the media with malate-glutamate (or NADH oxidase comprised by complexes I–V), succinate with rotenone (or succinate oxidase comprised by complex II–V), and TMP-ascorbate for cytochrome $c$ oxidase activity (or complex IV). (c) ratio of rates of oxygen consumption in the presence of various substrates derived from (b).

the behavioral changes observed in the offspring of dams treated with poly(I:C) [59]. Moreover, blocking IL-6 with antibodies prevents behavioral changes in the offspring [60], and poly(I:C)-induced MIA in IL-6 KO does not result in behavioral changes in the offspring [60]. These data seem to suggest a role for IL-6 in MIA-induced behavioral changes, although we cannot exclude other inflammatory agents such as type 1 interferons which have also been shown to take part in the response to poly(I:C) [61]. This MIA imprints a fetal programming that can still be detected during adulthood characterized by abnormal behaviors resembling those of ASD [34, 35] and SZ [34] and, biochemically, by a lower oxidative phosphorylation capacity in mitochondria within intact cells and isolated mitochondria. This suggests that prenatal immune changes ensuing the maternal poly(I:C) administration are likely to imprint the long-lasting changes in the bioenergetics of the adult offspring splenocytes. While, at the normal murine fetomaternal interface, immune cells such as neutrophils, macrophages, and NK cells are assumed to be excluded from the placenta and localized only in the decidua [62], treatment with poly(I:C) disrupts this normal distribution and induces a significant increase in the levels of proinflammatory cytokines in the placenta and a large migration of immune cells, primarily NK cells from the decidua towards the placenta, invading the spongiotrophoblast and then the labyrinth [63]. Trophoblasts, which express TLR3

[63], play a role in coordinating the maternal innate immune response to infection at the fetomaternal interface [62–64] and, especially in this case, in response to viral infection.

These results beget the question, what is the link between lower complex I activity (or lower oxidative phosphorylation) in the offspring and an acute maternal immune response? A growing body of evidence is placing mitochondria at the center of bioenergetics and immune response/inflammation. Immunity to infection is also dependent on mitochondria function by regulating the synthesis of both pro- and anti-inflammatory cytokines [65–69]. More recently, the view that mitochondria act as a platform facilitating innate immune responses adds to our understanding of the molecular complexity of sensor and adaptor interactions that promote effective host defense [11, 70].

Therefore, an emerging concept is that innate immune signaling is regulated by basic host metabolic functions. For instance, Toll-like receptor signaling activates mitochondrial biogenesis during critical illness [71–73], perhaps in response to increased oxidative damage in host cells [71, 74, 75]. Acute inflammation is accompanied by increases in inflammatory cytokines sustained by glycolysis, whereas chronic inflammation is sustained by less inflammatory cytokines, with more reparative features fueled mainly by mitochondria-derived ATP [76]. Thus, in this study, MIA seems to imprint the immune cells of adult offspring with this more glycolytic stage resembling the influence of an acute inflammation, without switching back to the less inflammatory response. Without pointing at cause or consequence, it is interesting to note that the changes in bioenergetics in the immune cells (and not their immune response or the immune response in brain or serum [33]) segregate with the abnormal behaviors observed in this MIA model [35]. However, a number of studies have shown differential MIA induction and behavioral responses depending on the gestational exposure stage [77–79]. This would suggest the existence of a window of vulnerability to infection during gestation for the onset of different behavioral defects, which may be reflected also on the mitochondrial function of the offspring.

The above effects can be explained by (i) the transfer of immune cells and/or cytokines from mother to the fetus at the maternal-fetal interface and (ii) a genetic predisposition/susceptibility of the offspring that, in association with maternal viral or bacterial infections, might increase the risk of long-lasting behavioral and immune changes [35]. Furthermore, we cannot exclude the possibility that the relatively high doses of poly(I:C) used in this study could have affected the well-being of the mother and therefore that of the fetus. For instance, poly(I:C) inhibits the development of diabetes in the NOD mouse [80] whereas the development of diabetes in diabetes-prone BB rats is poly(I:C)-dose dependent [81–84]. Indeed, a recent report indicated that at least some nongenetic risk factors are shared between ASD and SZ, in particular, diabetes, exposure to drugs, nutritional deficiencies, and infectious agents among others [85].

Considering the mechanisms described above, studies are now needed to clearly identify the key players affected in this acute viral response in order to evaluate the increase in risk of either ASD or SZ that is associated with these (and other [30, 86, 87]) modifiable environmental factors to elicit public health interventions.

## Authors' Contribution

Cecilia Giulivi and Eleonora Napoli contributed equally to this work.

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