**ABSTRACT**

**Objectives:** Oxidative stress seems to be involved in Rett syndrome (RTT). The aim of this study was to assess the antioxidant status in RTT children with MECP2 gene mutations with respect to healthy controls, and to explore novel blood antioxidant markers for RTT severity.

**Methods:** In erythrocytes from RTT females aged 2–14 years ($n = 27$) and age-matched controls ($n = 27$), we measured the levels of malonaldehyde and the activity of two antioxidant enzymes, Cu/Zn-superoxide dismutase and catalase, by spectrophotometric assays. In leukocytes, the expression of metallothioneins, the main non-enzymatic antioxidants, was assessed by real-time RT-PCR. In nine selected RTT children, methylome analysis was also performed.

**Results:** Blood of RTT patients showed increased lipid peroxidation and a dysregulated pattern of MT expression, while enzymatic activities did not change significantly with respect to controls. Moreover, we observed no epigenetic dysregulation in CpG-enriched promoter regions of the analysed genes but significant hypomethylation in the random loci.

**Conclusions:** As the haematic level of MT-1A directly correlates with the phenotype severity, this metallothionein can represent a marker for RTT severity. Moreover, the attempt to link the level of blood oxidative stress with MECP2 mutation and specific clinical features led us to draw some interesting conclusions.

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**Key Words**

Rett syndrome; MECP2 gene mutation; oxidative stress; metallothionein; methylome analysis

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**Introduction**

Rett syndrome (RTT; OMIM 312750; Rett 1966) is an X-linked neurodevelopmental disorder that affects almost exclusively females with a frequency of approximately 1:10,000 live births (Chahrour and Zoghbi 2007). RTT is the second most common cause of mental retardation in females after Down syndrome, and is usually included among the autism spectrum disorders (ASDs). Disease is characterised by 6–18 months of apparently normal neurodevelopment followed by neurological regression (Hagberg and Romell 2002). The major clinical issues associated with RTT include growth failure, scoliosis, gastrointestinal and breathing dysfunction, stereotypic hand movements with poor fine motor skills, ambulation, and communication (Neul et al. 2010). However, the clinical presentation of RTT is rather heterogeneous: in addition to the classic pattern (C), variant forms have been described including the congenital variant (Co), the preserved speech variant (PSV) and the infantile seizure onset variant (Chahrour and Zoghbi 2007; Renieri et al. 2009; Neul et al. 2010).

In nearly 95% of cases, classical RTT is associated with mutations in the MECP2 gene (Amir et al. 1999) encoding the methyl-CpG-binding protein 2 (MeCP2) that binds methylated CpGs (SmC) in symmetrically positioned CpG dinucleotides and modifies chromatin structure (Chahrour and Zoghbi 2007). MECP2 mutation type has been proposed as a strong predictor of disease severity (Grillo et al. 2013; Cuddapah et al. 2014;
Mutations in other genes have been identified, in particular, CDKL5 mutations in the infantile seizure onset variant (Kalscheuer et al. 2003; Weaving et al. 2004), and FOXG1 mutations in the congenital variant (Ariani et al. 2008). Recently it has been suggested that MeCP2 may act as a transcriptional modulator rather than a classical transcriptional repressor (Yasui et al. 2007). Since MeCP2 is highly expressed in neurones, its loss and/or reduction might affect mainly the central nervous system (CNS). To date, despite the large amount of data on the role of MECP2 and other genes in RTT syndrome, the pathogenic mechanisms are still unclear and no effective therapy to arrest or rescue the neurological regression in disease development is available.

Oxidative stress has been proposed as one of the pathogenic mechanisms of RTT in the light of its role in several neurodevelopmental and neurodegenerative disorders (Lanza et al. 2009; Uccelli et al. 2012; Milanese et al. 2014). Condition of oxidative stress develops when the physiological balance between oxidants and antioxidants is disrupted due to depletion of antioxidants and/or excess accumulation of reactive oxygen species (ROS). Organisms have evolved a comprehensive range of proteins for ROS detoxification. Antioxidant systems include both enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT), and non-enzymatic factors such as metallothioneins (MTs), glutathione and vitamins. CNS is one of the main targets of ROS damage owing to high concentrations of unsaturated lipids, high metabolic rate, high levels of iron, and relatively low levels of antioxidants (Coyle and Puttfarcken 1993; Robb-Gaspers and Connor 1997). Blood is considered a good reporter for the oxidative status of the whole organism, of the brain in particular (Kosenko et al. 2012). A few studies investigated the alteration of antioxidant enzymes in RTT patients showing a decreased SOD activity (Sierra et al. 2001), while several reports showed an increased blood oxidative stress in RTT patients by evaluating non-enzymatic markers such as isoprostanes (Leoncini et al. 2011; De Felice et al. 2011; Signorini et al. 2011; Ciccoli et al. 2012), protein carbonyls (De Felice et al. 2009) and plasma 4HNE-protein plasma adducts (Pecorelli et al. 2011).

Many human diseases are linked to changes in the genetic and epigenetic landscape of the cell (James et al. 2004). Recently, a study on ageing underlined the role of ROS in altering epigenetic machinery mainly acting on methylation of CpG and hydroxide CpG (Cencioni et al. 2013). Moreover, a possible link between MeCP2 and oxidative stress has recently been suggested (De Felice et al. 2011; Pecorelli et al. 2011). On this basis we hypothesised that oxidative stress may alter gene methylation in RTT, and this effect could interact with the altered functionality of the mutated MeCP2 protein.

In the present study, we selected a cohort of RTT females highly homogeneous for age with demonstrated MECP2 gene mutations to assess the blood oxidative status by using both enzymatic and non-enzymatic oxidative stress markers. In nine selected RTT patients, we also investigated the possible occurrence of epigenetic dysregulations as a consequence of the imbalance of the normal redox state.

**Materials and methods**

**Chemicals**

All chemicals, unless otherwise indicated, were of analytical grade and were obtained from Sigma-Aldrich Corp. (Milano, Italy).

**Subjects**

A total of 54 female children aged 2–14 years were enrolled between 2011 and 2012, and divided into two groups. The RTT group included 27 females (mean age: 6.8 ± 2.9 years) with clinical diagnosis of RTT and demonstrated MECP2 gene mutation. The control group included 27 age-matched mentally and physically healthy children free of any medication (mean age: 6.7 ± 3.2 years).

Rett children were enlisted from a cohort of patients referred to four Italian child neuropsychiatry departments: G. Gaslini Children’s Hospital of Genoa; S. Paolo Hospital of Milan; V. Emanuele Hospital of Catania; Spedali Civili of Brescia. The diagnosis of RTT was made according to New Rett diagnostic criteria defined in 2010 (Neul et al. 2010). Patients who had a history of chronic systemic disease, inflammatory disease, or severe head injury were excluded. Healthy controls were recruited at G. Gaslini Hospital. All patients were screened for psychiatric disorders and were free of any medication except for the antiepileptic drugs in epileptic patients (17 children). The study was conducted with the approval by the Institutional Review Board and all informed consents were obtained. Blood samples in patients were collected in accordance with local ethics committee rules.

**Phenotype severity scoring**

Phenotypes of patients were categorised as follows: classic (C), congenital variant (Co) and preserved speech variant (PSV; Neul et al. 2010). Patient evaluation included a comprehensive assessment regarding clinical status and classification, presence and type of MECP2.
mutations, and clinical severity. Evaluation of clinical severity included a detailed history and physical examination, comprehensive anthropometric measurements, and two quantitative measures of global clinical status: Clinical Severity Scale (CSS; Neul et al. 2008) and Kerr scale (KS; Kerr et al. 2001). The CSS is a composite score based on the following 13 individual, ordinal categories measuring clinical features common in RTT: age of onset of regression, somatic growth, head growth, independent sitting, ambulation (independent or assisted), hand use, scoliosis, language, non-verbal communication, respiratory dysfunction, autonomic symptoms, onset of stereotypies and seizures, as previously described. All scores range from 0 to 4, or 0 to 5 with 0 representing the least severe and 4 or 5 representing the most severe finding. The KS is based on 20 individual, ordinal categories: head circumference, early development, weight and height, muscle tone, spine posture, joint contractures, gross motor function, hand stereotypy, involuntary movements, voluntary hand use, oro-motor difficulty, intellectual disability, speech, epilepsy, awake breathing rhythm, peripheral circulation, mood disturbance, sleep disturbance. All scores range from 0 to 2 with 0 representing the least severe and 2 the most severe finding. Clinical and genetic characteristics of the RTT patients are reported in Table I. The single values of all the individual clinical categories for both CSS and KSS are included as supplementary data (Table 1) available online.

**Mutation screening**

DNA was extracted from peripheral leucocytes and **MECP2** coding sequence was analysed by Sanger sequence using Automated Sequencer ABI-Prism 3005 and MLPA for **MECP2** gene deletion (MRC-Holland). Patients’ mutations were divided in truncating and missense mutations. Depending on the portion of the protein affected, truncating defects were grouped in early truncating (ET) with loss of the N-terminal region at the methyl-CpG-binding domain (MBD) and/or at the transcriptional repression domain (TRD), and late truncating (LT) with loss of the C-terminal region saving the nuclear localisation signal (NLS). Missense mutations (M) should have been considered singularly, but because there were only three patients for each single mutation we grouped them in a single group.

**Sample collection and handling**

Blood sampling in RTT patients was performed during the periodic clinical checks, while in the control group it was carried out during routine health checks. Blood was collected into heparinised tubes and all manipulations were carried out within 2 h after sample collection. Leukocytes (WBCs) and erythrocytes (RBCs) were separated by centrifugation as previously described (Grasselli et al. 2014). Then, WBCs were collected and resuspended in Trizol for RNA extraction (Vergani et al. 2007), and RBCs were collected, washed and stored at –80°C.

| Patient | Age (years) | Clinical phenotype | Protein mutation | Group of mutation | Kerr score | CSS score |
|---------|-------------|--------------------|------------------|-------------------|------------|-----------|
| 1       | 9           | Co                 | p.Val331Gly fsX34 | LT                | 25         | 22        |
| 2       | 9           | C                  | p.Arg168X        | ET                | 23         | 27        |
| 3       | 5           | Co                 | p.Arg270Glu fs288X | ET          | 31         | 28        |
| 4       | 11          | C                  | 3-4 Exon deletion | ET                | 17         | 22        |
| 5       | 6           | C                  | p.Arg255X        | ET                | 23         | 25        |
| 6       | 3           | C                  | p.Lys135Asn      | M                 | 11         | 17        |
| 7       | 14          | C                  | p.Arg294X        | LT                | 19         | 27        |
| 8       | 3           | C                  | p.Arg168X        | ET                | 21         | 25        |
| 9       | 11          | C                  | p.Leu386His fs390X | LT         | 23         | 28        |
| 10      | 6           | C                  | p.Leu386His fs390X | LT         | 17         | 23        |
| 11      | 10          | PSV                | p.Pro387Leu fs19X | LT             | 14         | 6         |
| 12      | 12          | C                  | p.Arg306Cys      | M                 | 22         | 27        |
| 13      | 7           | C                  | p.Pro389Gly fs6X  | LT                | 13         | 13        |
| 14      | 7           | C                  | p.Lys144Arg fs2X | ET                | 19         | 19        |
| 15      | 6           | C                  | p.Asp156Glu      | M                 | 18         | 22        |
| 16      | 4           | C                  | p.Arg306Cys      | M                 | 14         | 17        |
| 17      | 4           | C                  | p.Pro225Arg      | M                 | 16         | 10        |
| 18      | 4           | C                  | p.Arg255X        | ET                | 19         | 24        |
| 19      | 4           | C                  | p.Arg255X        | ET                | 13         | 29        |
| 20      | 3           | C                  | p.Glu37Xfs       | ET                | 12         | 19        |
| 21      | 6           | C                  | p.Arg168X        | ET                | 11         | 20        |
| 22      | 7           | C                  | p.Arg306Cys      | M                 | 10         | 12        |
| 23      | 3           | Co                 | p.Thr158Met      | M                 | 25         | 28        |
| 24      | 12          | PSV                | p.Arg294X        | LT                | 12         | 10        |
| 25      | 6           | Co                 | p.Thr158Met      | M                 | 16         | 36        |
| 26      | 4           | Co                 | p.Thr158Met      | M                 | 22         | 36        |
| 27      | 8           | PSV                | p.Pro389X        | LT                | 8          | 17        |

Full data are available in supplementary Table S1 available online.
RNA isolation and real-time RT-PCR

RNA was isolated using the Trizol reagent; cDNA was synthesised and quantitative real-time PCR (qPCR) was performed in quadruplicate using 1× IQ™SybrGreen SuperMix and Chromo4™ System apparatus (Biorad, Milan, Italy) (Vergani et al. 2011). The relative quantity of target mRNA was calculated by the comparative Cq method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, and expressed as fold induction with respect to controls (Pfaffl et al. 2002). The following primer pairs were used: MT-1A (Fwd 5′-ACTGGTGCCCTTCAGCTGC-3′; Rev 5′-ACACGAGCTGCACTCTCTGAT-3′), MT-2A (Fwd 5′-GGCGGTGAACCTCCTGACACCTGCG-3′; Rev 5′-GGCGAGCTGCACTTGTCCGAC-3′), MT-1E (Fwd 5′-GCTGGTCGTCCTCACATCTTG-3′; Rev 5′-CACAGGTTGAGGTGTTTCA-3′) (Grasselli et al. 2014). Data are the mean±SD of three measurements in quadruplicate.

Determination of antioxidant enzyme activities

RBCs were lysed in ice-cold milliQ water (Zoroglu et al. 2004). Haemoglobin (Hb) content was measured accordingly to Drabkin (1949). Catalase activity in haemolysates was evaluated following the consumption of H2O2 at 240 nm (Aebi 1984), and it was expressed as micromoles of decomposed H2O2 per min/mg Hb (Grasselli et al. 2008). SOD activity was measured as the inhibition of the reduction rate of cytochrome c by the superoxide radical in the haemolysate previously treated to eliminate the Hb interference (McCord and Fridovich 1969; Zoroglu et al. 2004). SOD activity was expressed as mU/mg Hb. Spectrophotometric analyses were carried out at 25°C using a Varian Cary 50 spectrophotometer (Agilent, Milan, Italy).

Determination of lipid peroxidation by TBARS assay

Lipid peroxide formation in haemolysates was determined through the Thiobarbituric Acid Reactive Substances (TBARS) assay to measure the amount of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane), a major product of lipid peroxidation (Iguchi et al. 1993; Kawamoto et al. 2005).

DNA methylation analysis

Array-based gene-specific DNA methylation analysis was performed using the Human Methylation450 BeadChip kit (Illumina, San Diego, CA, USA), including more than 485,000 methylation sites at single nucleotide resolution, distributed among all human chromosomes, with 99% coverage of RefSeq genes and 96% coverage of CpG islands (CGI). Genomic DNA samples (600 ng) were bisulphite converted by the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s instructions and processed through the Illumina Infinium 450K HD Methylation array assay procedure. Briefly, bisulphite-treated DNA was whole genome amplified and hybridised to the Illumina BeadChips. Fluorescence intensities were captured by using the fluorescent scanner Illumina HiScan SQ. The methylation status (β value) of each CpG site was extracted utilising the GenomeStudio® Methylation module (v.2010). To detect differentially methylated CpG sites, methylation data of RETT samples were compared to a control methylation profile of 112 anonymous adult healthy samples (age ranking from 25 to 60 years) previously used from an unrelated study. A group of 15 healthy aged-matched subjects (age<12 years) was loaded on the platform in parallel with RETT samples. A three-step pipeline was set. Step 1: to assess the quality of the experiment (quality control QC) and adjust differences in the distribution of methylation values due to the two different chemistries (type I and II probes) data were pre-processed using the Illumina methylation analyser (IMA) and LUMI packages, respectively, within the R statistical environment, as described previously (Du et al. 2008; Wang et al. 2012). Step 2: in the control group, for every CpG site, a range of β values was calculated as follows: upper value = Q3+(k*IQR), lower value = Q1−(k*IQR), where k = 3, Q1 = first quartile, Q3 = third quartile, IQR = Interquartile range. The β values of single cases that passed QC criteria were compared to the methylation profile of controls population; β values (outliers values) falling outside of this range were considered epimutated. Step 3: epimutated CpGs were filtered out by excluding CpGs not belonging to Island/Shore regions and not associated to transcripts. Only loci characterised by a significant epimutations number (hypergeometric distribution, P<0.05/no. epimutated loci) and not present in healthy aged-matched subjects were investigated. Moreover, by using a genome browser (IGV), a further filtering process was set to evaluate only regions characterised by adjacent epimutated CpGs. Consent to participate to this study was obtained. Sex chromosomes were excluded from the analysis.

Statistical analysis

Data are described as absolute and relative frequencies for categorical variables, while means, standard deviation (SD), medians and range are used for continuous
variables. Normality of distribution was determined using the Kolmogorov–Smirnov test. Data are represented as Box and Whisker plots depicting a five-number summary: minimum, first quartile, median, third quartile, and maximum; if present, outliers are also indicated. Parameters of RTT patients vs. healthy controls were compared using Mann–Whitney U-test for continuous variables. The Spearman rank correlation test was performed to evaluate the correlation of the oxidative-stress marker with clinical severity score. A P value less than 0.05 was considered statistically significant; all P values were based on two-tailed tests. Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA) or GraphPad Software (GraphPad Software Inc., San Diego, CA 92130, USA).

Results

Phenotype severity and MECP2 mutations

All RTT patients included in the study had MECP2 gene mutations. Diagnosis of RTT was made according to New Rett Diagnostic Criteria (Neul et al. 2010). The clinical phenotype of the 27 RTT children was classified as classical (C; N = 19), preserved speech variant (PSV; N = 3) and congenital (Co; N = 5). Clinical severity was quantified using two different scales (CSS and Kerr). For RTT patients the MECP2 gene mutation, the altered protein domain, and both the CSS and KS scores are summarised in Table I. The mean score using the CSS scale was 21.8 ± 7.5 and ranged from 6 to 36, whereas the mean score using the KS was 17.6 ± 5.6 and ranged from 8 to 31.

The genotypes of RTT patients were categorised on the basis of MECP2 mutations as ET, LT and M. In Figure 1 we report the median of CSS and Kerr score values determined for these three groups of patients. We can see, as for the ET group, the median of both Kerr and CSS scores was higher than in the LT and M groups, even though the differences are not statistically significant.

Oxidative stress in RBCs

At the level of circulating erythrocytes we evaluated both TBARS level, as a marker of lipid peroxidation, and catalase and SOD activity, as markers of antioxidant defence. The TBARS levels (Figure 2A) were significantly higher in RTT children compared to controls (about +42%; P ≤ 0.01). Also, the catalase activity showed a slight increase in RTT children compared with controls (about +18%), but the difference was not significant (Figure 2B). Interestingly, when catalase activity was analysed on the basis of MECP2 mutations higher values were observed in the patients with missense mutations compared to controls (239 ± 42 vs. 207 ± 38; P = 0.06) (Figure 2C). The SOD activity did not change in RTT cohort (Figure 2D), although a trend to increase could be appreciated (about +24% with respect to controls).

In the control group, distribution of the top-quartile cut-off values were 0.13 nmol MDA/mg Hb for TBARS, 0.241 mmol H2O2/min/mg Hb for catalase, and 0.0183 mU/mg Hb for SOD. In the RTT group, the patients with values exceeding the top-quartile threshold were 59.3% for TBARS, 46.2% for catalase and 39.2% for SOD.

Figure 1. Clinical severity and MECP2 mutations. Box plot of Kerr (A) and CSS (B) score for the three different mutation groups identified in the 27 RTT children enrolled in the study: early truncating (ET), late truncating (LT) and missense (M). Data are represented as Box and Whisker plots. Boxes enclose middle 50% (IQR) of observations. Black line indicates median. Whiskers extend to cover points within 1.5 × IQR above the 75% percentile.
Metallothionein expression in WBCs

MT-2A is the most abundant isoform in WBCs from healthy children (Vergani et al. 2011), with the following relative abundance of transcripts: MT-2A > MT-1E > MT-1A. The present results showed that the mRNA level of MT-1A (Figure 3A) was dramatically higher in RTT group than in the control one (about 14 fold compared to controls; P < 0.001). Lower differences were observed for MT-2A expression (about 1.5 fold in RTT children compared to controls; P < 0.01) (Figure 3B), whereas MT1E expression was not significantly altered (Figure 3C). Interestingly, the largest up-regulation in MT-1A expression was

Figure 2. RBC antioxidant enzyme activities in RTT and healthy children. RBC level of TBARS (A), and specific activities of catalase (B) and SOD (D) are represented as Box and Whisker plots depicting a five-number summaries of: minimum, first quartile, median, third quartile, and maximum; if present, outliers are also indicated as empty circles. Catalase specific activity in the different groups of patients classified on the basis of MECP2 mutation is also shown (C). If statistically relevant, P values were indicated in the graphs.

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observed in RTT patients, with both ET and LT truncating mutations (Figure 3D).

In the control group, distribution of the top-quartile cut-off values were 5.47, 1.29 and 2.87 fold for MT-1A, MT-2A and MT-1E, respectively. In the RTT group, the fractions exceeding the top-quartile values were about 74% for MT1A, 66.7% for MT2A and 44.4% for MT1E.

**Methylome analysis in WBCs**

Out of the cohort of 27 RTT patients, we investigated nine girls carrying “hot spot” truncating mutations for whole genome methylation: one p.Arg255X, one p.Arg270E fs288X, two p.Arg294X, three C-ter deletion and one missense mutation p.Arg306Cys. CpG

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**Figure 3.** WBC expression of MT isoforms in RTT and healthy children. Expression of the three MT isoforms in WBCs from the two cohorts of children are represented as Box and Whisker plots. Relative expression of MT-1A (A), MT-1E (B) and MT-2A (C) isoforms was quantified by q-PCR using GAPDH mRNA as a reference. Expression values are given as fold induction with respect to controls (mean±SD) of n=4 replicates. MT-1A expression in the different groups of patients classified on the basis of MECP2 mutation is also shown (C). If statistically relevant, P values were indicated in the graphs.
associated with classic coding messenger (74.4%), intergenic CpGs (24.6%), non-coding RNAs (<1%), distributed within CpG islands, shore (2kb from islands) and shelves (2–4 kb from islands) were analysed. Most of these CGI are unmethylated, apart from a small subset of methylated CpGs, randomly distributed (random loci, <1%) and representative of the effective methylation status in the genome. A paediatric control of 15 healthy children was taken into consideration in order to ponder the effect of age on methylation in specific loci. RTT and paediatric control samples were randomly loaded on microarrays. One RTT sample did not pass the first quality control test and was excluded.

The \( \beta \) values of single RTT and paediatric control samples were compared with the control methylation profile evaluating the epimutated CpGs associated to transcripts and those with a regulatory meaning (CpG island and CpG shore). No significant difference was found (Wilcoxon rank-sum tests, \( P < 0.05 \)) in the number of hyper-/hypo-methylated CpGs, outliers in RTT patients (\( n = 8 \)) vs. paediatric healthy controls (\( n = 15 \)) (data not shown). A few loci with a significant number of hyper- or hypo-methylated CpGs were identified, but any of them was recurrent in our RTT cohort, nor strongly correlated with RTT. Moreover, some of them were discarded because found in the healthy paediatric cohort.

For an additional level of analysis, the CpGs were divided into three groups: CpGs restricted to islands (30%) (Figure 4A), CpGs belonging to promoter regions (19.6%) (Figure 4B), and CpGs in random loci (0.8%) (Figure 4C). A genome wide comparison of methylation levels between RTT and controls groups was carried out. The median methylation values of the three groups were compared and the results are summarised as box-plots.

This analysis was concordant with previous results not evidencing strong differences on methylation of CpGs involved in transcriptional regulatory mechanisms. An interesting observation comes from a selective analysis of the methylation within random loci. Methylation levels of RTT and also of paediatric samples appeared to be very different from the large adult cohort confirming the observation that the global DNA methylation decreases with age. A slight, but statistically significant, decrease in global DNA methylation was observed in RTT children with respect to the paediatric controls (\( P < 0.02 \)).

**Correlations between oxidative stress, MT pattern, MECP2 mutations and phenotype severity**

The possible correlation among the erythrocyte indices of oxidative stress (TBARS levels) and of antioxidant defence (catalase and SOD activities), the leukocyte panels of MTs and the clinical phenotype severity as measured by both CSS and KS was assessed (Table II). Interesting results were obtained when expressions of the MT isoforms were tested for possible correlations with clinical severity or with some individual clinical features included in KS (Table II). For MT-1A we found a significant positive correlation with Kerr total score (\( r = 0.45; P = 0.02 \)), with intellectual disability (\( r = 0.43; P = 0.02 \)) and with sleep disturbance (\( r = 0.43; P = 0.04 \)). MT-1E showed a negative correlation with oro-motor...
difficulty ($r = 0.45; P = 0.02$). A negative correlation was also observed between the global oxidative stress measured by TBARS and the spine posture ($r = -0.48; P \leq 0.01$), as well as between the catalase activity and intellectual disability ($r = -0.43; P \leq 0.03$).

In our study we could not find any significant correlation between specific MECP2 mutations and individual clinical features or global clinical score (using both KS and CSS).

**Discussion**

Rett syndrome manifests as a large variety of phenotypes ranging from very severe to mild disease, and is commonly associated with MECP2 mutations. However, the role and the mechanisms by which impaired MeCP2 activity induces pathological abnormalities are still unclear. There are three main functional domains in MeCP2: the methyl-CpG-binding domain (MBD), the central transcriptional repression domain (TRD) and the nuclear localisation signal (NLS). Recent studies suggested that phenotype severity might be associated with specific mutations in MECP2 domains.

All RTT patients of our study carried MECP2 mutations which have been classified as ET, LT and M. We observed a more severe clinical phenotype (calculated using both CSS and Kerr scores) for the ET group. This is in accordance with previous data showing a milder disease in patients with LT mutations compared to patients with other mutations (Bienvenue and Chelly 2006; Cuddapah et al. 2014).

To date, the possible linking between the MECP2 mutations and the oxidative imbalance is still unclear. Our results show that lipid peroxidation is significantly increased in blood from RTT patients with respect to age-matched controls, in line with previous reports (De Felice et al. 2011; Leoncini et al. 2011). In the attempt to protect against oxidative stress, catalase was stimulated in RBCs of RTT children, even though the increase with respect to controls was not significant. Interestingly, the increase was significant when we focussed on RTT patients with missense mutations. No significant changes of SOD activity were instead observed. As a protection against oxidative stress, RTT children exhibited a marked induction of blood metallothioneins. Our results show a significant increase in expression of MT-1A and MT-2A in RTT patients with respect to healthy controls. Interestingly, the largest up-regulation in MT-1A expression was observed in RTT children with truncating mutations (both ET and LT). We did not evidence any significant correlation between oxidative stress markers and the type of MECP2 mutations. These data agree with a recent paper (Grillo et al. 2013) showing that RTT patients with identical MECP2 mutations, as two pairs of sisters, exhibit a different pattern of oxidative stress markers according to their clinical phenotype (concordant genotype with discordant phenotype), and that the patients with classic phenotype have a higher oxidative stress which might depend on the non-MECP2 genetic background. It has to be underlined that we could not perform statistical analyses for possible differences in stress oxidative parameters among the different variants of phenotypes because of the small patient number of some groups.

The possible correlation among the erythrocyte indices of oxidative stress (TBARS levels, catalase and SOD activities), the leukocyte panels of MTs and the clinical phenotype severity were assessed using the Spearman rank correlation test. For MT-1A we found a positive correlation with Kerr score, intellectual disability and sleep disturbance, whereas MT-1E negatively correlated with oro-motor difficulty. A negative correlation was also observed between global oxidative stress measured by TBARS and spine posture, and between catalase activity and intellectual disability. We wish to underline that oxidative stress has been hypothesised to contribute to onset and evolution of epilepsy (Torres Aguiar et al. 2012). A recent clinical study indicated that oxidative

**Table II. Erythrocyte levels of TBARS (TBARS) and activity of catalase (CAT), leukocyte expression of MTs, CSS and Kerr score, individual clinical features were analysed with Spearman correlation algorithm.**

| Oxidative stress marker | Clinical severity | Individual clinical features | $R$  |
|-------------------------|-------------------|-----------------------------|------|
| MT-1A                   | Kerr score        | Intellectual disability     | 0.45 ($P \leq 0.02$) |
|                         |                   | Sleep disturbance           | 0.40 ($P < 0.02$)   |
|                         |                   | Oro-motor difficulty        | -0.45 ($P < 0.02$)  |
|                         |                   | Spine posture               | -0.48 ($P < 0.01$)  |
|                         |                   | Scoliosis                   | -0.55 ($P < 0.005$) |
| MT1E                    |                   | Intellectual disability     | -0.43 ($P < 0.03$)  |
| TBARS                   |                   | Sleep disturbance           | -0.47 ($P < 0.02$)  |
| CAT                     | CSS score         | Scoliosis                   | -0.52 ($P < 0.01$)  |
|                         |                   | Autonomic system            | -0.53 ($P < 0.01$)  |

The $R$ values for correlation and significance of correlation ($P$) were indicated.
stress was significantly higher in patients with epilepsy compared with controls, but that antiepileptic therapy did not alter the oxidative markers (Menon et al., 2012). When we compared the 17 girls with epilepsy to the ones without epilepsy in our cohort of RTT patients, we did not find any significant correlation between epilepsy and oxidative stress.

With regard to the possible influence of respiratory dysfunction on oxidative stress, in our RTT patients we were not able to identify significant differences in patients with respiratory abnormalities (17/27) compared to the other ones. Therefore, our results confirm previous data indicating that oxidative stress biomarkers could be mainly related to the severity of the neurological symptoms (De Felice et al. 2009).

Although a recent paper showed that speech-language abilities are influenced by MECP2 mutation (Urbanowicz et al. 2014), we could not find any significant correlation between specific MECP2 mutations and individual clinical features or global clinical score (using both KS and CSS), probably due to the small number of patients (Bebbington et al. 2008; Neul et al. 2008).

It is widely accepted that the role of MeCP2 in regulating gene expression results from its binding to 5mCs which is characteristic of highly condensed chromatin regions (Skene et al. 2010). Indeed, the loss of MeCP2 in RTT alters the transcriptional silencing of coding sequences and microRNAs (Skene et al. 2010). However, the observation that more than half of MeCP2 molecules in the brain reside in low-condensed chromatin regions which have lower 5mC levels, and the recent insights about multifaceted DNA-binding properties of MeCP2 (Mellén et al. 2012; Baker et al. 2013) suggest that MeCP2 function might be more complex (Guy et al. 2011). Moreover, the recent literature on ageing shows consistent evidence for the correlation between increasing levels of ROS and global genome hypomethylation (Cencioni et al. 2013).

On this basis, we hypothesised that impairment in MeCP2 may lead to epigenome dysregulation, which could be associated with oxidative imbalance in RTT. Therefore, we investigated the methylation pattern in our cohort of RTT patients compared with both paediatric and adult controls. To our knowledge, this is the first time that methylation was analysed in a cohort of RTT patients using a genome-wide approach. Contrary to our expectations, no significant differences were identified either using single locus/single patient approach, or global median methylation evaluation in the regulatory regions. It has to be noted that in the Methylation450 BeadChip kit the majority of CpGs are representative of regulatory dynamically unmethylated regions, covering 20–30% of the CpG in the genome. Therefore, a custom array including CpGs mapping in the heterochromatin region or associated to non-coding RNAs (ncRNAs) should be a novel approach to this topic. A third set of CpGs, named random loci, was analysed and it turned out to be differently methylated in RTT respect to controls. Scant information is supplied by the literature or by Illumina concerning the exact coordinates of these randomly distributed CpGs; a small fraction of them overlaps with the other two sets and most of them appear to be methylated. In this case we observed a relevant hypomethylation in the adult control compared with the two paediatric cohorts, according to an aged population, but also the RTT appear significantly hypomethylated compared to the paediatric control. It could be interesting to replicate these results on an enriched array containing an increased number of normally methylated loci, and on a wider number of RTT patients, to validate or reject them.

In conclusion, for the first time, this study shows that in RTT children there is an up-regulation of metallothionein expression in blood. The MT-1A isoform, in particular, might represent a reliable haematic marker for clinical severity of disease, as its plasma level directly correlates with phenotype severity (Kerr score). Moreover, the grade of intellectual disability and scoliosis seems to be correlated with the level of MT-1A, whereas the clinical score is inversely related with the activity of catalase. Since a limitation of this study is the relatively low number of RTT patients, this makes it impossible to divide patients into subgroups on the basis of phenotype and/or genotype. Similar studies with a wider number of patients should be performed in order to confirm these data. Finally, no epigenetic alterations in CpG-enriched promoter regions of the analysed genes have been found associated with RTT, even though a significant hypomethylation could be appreciated in the random loci in RTT subjects compared to the paediatric controls. Therefore, our data suggest that the oxidative stress-related parameters could offer a non-invasive approach for evaluating RTT status and could be used in follow-up of RTT patients. Moreover, these results hypothesise the possible efficacy of proper anti-oxidant therapies to improve the oxidative imbalance and likely some clinical aspects in RTT children.

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Statement of interest
None to declare.

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