OxInflammation in Rett Syndrome

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Abstract

Rett syndrome (RTT) is an orphan progressive neurodevelopmental disease affecting almost exclusively females (frequency 1:10,000). RTT clinical expression is typically characterized by loss of purposeful hand movements, severe mental retardation and motor impairment, breathing disorders, ataxia and increased risk of sudden death. Although the main genetic cause, i.e. mutation in the methyl-CpG binding protein 2 gene (*MECP2*), has been already identified, the molecular and pathogenic mechanisms by which MECP2 deficiency drives pathology in RTT remains not fully understood. A wealth of evidence from our and other laboratories suggests a potential causal relationship between MECP2 dysfunction and systemic redox imbalance, a condition that has been widely found in association with RTT. In turn, a "short-circuit" of redox pathways may contribute to the systemic immune dysfunction expressed as cytokines/chemokines dysregulation, a feature clearly emerged from two recent studies on RTT patients. In this light, the purpose of this review is to describe and to stimulate a new discussion on the idea that systemic subclinical inflammation and oxidative stress are crucial players of a detrimental vicious circle, driving the pathogenesis and clinical course of RTT.

Key Words:

Rett syndrome; MECP2; Oxidative stress; Inflammation; cytokines

1. Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder, caused in the "classic' form by mutation in Methyl-CpG-binding protein 2 gene (*MECP2*). Despite years of intense research, the pathogenic mechanism that links MECP2 dysfunction to disease expression is not fully understood, yet. However, these efforts enable researchers and clinicians to remarkably advance in the knowledge of the functional role of MECP2 and RTT physiopathology. Thank to these achievements, RTT is now no more regarded as a pure neurological disease, but as a multi-systemic syndrome affecting both brain and several other tissues/organs. Recent converging findings from us and other groups, clearly suggested the systemic nature of RTT might be the clinical outcomes of two synergic adverse conditions, i.e. oxidative stress (OxS) and subclinical inflammation. Indeed, both abnormalities were repeatedly found in RTT patients and appeared to be intimately related to neurological and non-neurological symptom severity and clinical presentation.

The purpose of this review is to describe and to stimulate a new discussion on the idea that systemic subclinical inflammation and OxS are crucial players of a detrimental vicious circle driving the pathogenesis and clinical course of RTT.

2. RTT syndrome: main clinical features

RTT syndrome, the second most prevalent cause of severe mental retardation in female gender (with a frequency of approximately 1: 10,000 to 1:15,000 females), is a progressive neurodevelopmental disorder (Christodoulou *et al.* 2003). Recently published guidelines of DSM-5 (Firth Edition of the Diagnostic and Statistical Manual Disorders) have removed RTT from the group of syndromes classified as autism spectrum disorders (ASD). Regardless of this formal exclusion, RTT still shares some pathogenic processes and physiopathological traits with autism (Percy 2011). Indeed, in its classical clinical picture, after a close to normal development for 6-18 months, most RTT patients show an autistic-like behaviors (Hagberg 2002). RTT progresses through a typical four-stages neurological regression characterized by loss of purposeful hand movements (replaced by stereotypic and repetitive hand movements "hand wash"), severe mental retardation and motor impairment, including ataxia, apraxia and tremors (De Felice *et al.* 2016). Further common symptoms of RTT include seizures, hyperventilation, and apnea (Cronk *et al.* 2016).

It has been clearly ascertained that, mostly de novo, mutations in the X-linked gene encoding the MECP2 represent by far the most prominent etiological factor (90% of cases with typical RTT) (Guy *et al.* 2011). Mutations in other genes can be found in association with RTT phenotype, in particular *cyclin-dependent kinase-like*, *CDKL5*, and *forkhead box G1* (*FOXG1*), which are responsible of the most severe non-classical forms of the disorder (although this classification is at the moment re-evaluated) (Ariani *et al.* 2008; Scala *et al.* 2005). Regardless of the recently growing interests of researchers on these atypical RTTs, that associated with MECP2-deficiency remains by far the most studied form. More than 200 site-mutations in this gene has been discovered so far, with the nine most frequent "hotspots" (near to 80% of total cases) leading with an ample phenotypic variability (Christodoulou *et al.* 2003).

Several years of intense research have led to an almost full functional characterization of the MECP2 protein, which finally emerged as implicated in chromatin architecture, regulation of RNA and splicing (Cronk *et al.* 2016). The protein is almost ubiquitously expressed, since it has been found in, among others, astrocytes, microglia and, mostly neurons (Cronk *et al.* 2015). This localization may account for the brain anatomical abnormalities observed in MECP2-deficient patients and animal models, consisting in reduced brain size, mostly visible in frontal and temporal lobe, caudate nucleus, thalamus, midbrain and cerebellum (Guy *et al.* 2011). The widely documented decreased number of cerebral synapsis, leading to an altered excitatory/inhibitory balance, has been described as the main underlying cause of a less efficient neuronal networks featuring RTT-like phenotypes (Guy *et al.* 2011).

However, despite this well-defined anatomical-functional correlate of MECP2 deficiency, the molecular mechanisms linking the defect in protein expression to disease expression as well as many clinical aspects of the pathogenic processes of RTT are not fully elucidated yet. Besides, to make the landscape more nebulous, although the neurological symptoms are dominant, most RTT patients suffer from complex symptoms and somatic impairments, such as osteopenia, scoliosis, Raynaud's phenomenon and gastrointestinal difficulties (Gonnelli *et al.* 2008; De Felice *et al.* 2016; Filosa *et al.* 2015; Signorini *et al.* 2014). Finding definitive answers to these open questions is the only way to build up a rationale for new pharmacological therapies for this still uncured disease.

3. RTT syndrome as a systemic disease

As mentioned in the previous paragraph, decades of investigations have still not brought an exhaustive understanding of the pathogenesis of RTT syndrome. However, these efforts significantly enriched the knowledge of many aspects of the disease and lead to redefine RTT as a multi-systemic rather than a mere neurological disorder, thereby opening promising and still unexplored therapeutic frontiers (Cronk *et al.* 2016; Filosa *et al.* 2015). The novel definition was inspired by the cumulating evidence showing that classical RTT phenotype is associated with disseminated abnormalities in multiple organs/tissues such as microvascular/endothelial system, bone, heart, lungs, skin fibroblast, the autonomic nervous system, red blood cells, gastrointestinal tract etc.(De Felice *et al.* 2016).

The recent findings of immune dysfunction characterized by a deregulation of cytokine/chemokine profile, consistent with prior hypotheses based on experimental evidence, point to chronic inflammation as the most likely culprit of the systemic nature of the disease. In the next paragraphs we will present the most highlighting data in support of the implication of subclinical inflammation in RTT pathogenesis.

4. Chronic inflammation is an underlying systemic condition in RTT

Immune system dysfunction might play a critical role in the onset and progression of the multiple somatic and neurological symptoms associated with RTT. The recent results in support of this thesis arise from observations that, as compared to healthy controls, RTT patients present: 1) higher levels of inflammatory markers and deregulation of acute phase response (APR) proteins; 2) unbalanced cytokines profile; 3)

abnormal morphology of peripheral blood mononuclear cells (PBMCs) (Table 1). Noteworthy, these data are in line with previous findings obtained in *in vitro* and animal models.

-Change in inflammation-related proteins

Plasma proteome analysis by 2-DE/MALDI-TOF identified a pattern of protein expression clearly resembling a subclinical inflammatory status in RTT patients with stage II (i.e., "pseudo-autistic"). Indeed, the work by Cortelazzo et. al. (Cortelazzo *et al.* 2014) showed upregulation of a number of APR positive proteins, including serum amyloid A-1 protein (SAA1), alpha-1 antitripsin (A1AT) and complement factor B (CFAB) as well as the downregulation of well-recognized negative APR, such as albumin and Retinol binding protein 4 (RET4). Notably, SAA1 is synthetized and released into the blood circulation upon inflammatory stimuli only as structural apoliprotein of high density lipoprotein (HDL), thus displacing the constitutive APO-A1. This change seems to affect cholesterol removal, transport and delivery, leading to the shift from the originally anti-atherogenic into a pro- atherogenic lipoprotein (Artl *et al.* 2000; Weichhart *et al.* 2012; Van Lenten *et al.* 2006; Cervellati *et al.* 2015b). Accordingly, lipid dyshomeostasis has been often described as one of the hallmarks of RTT (Sticozzi *et al.* 2013; Segatto *et al.* 2014).

More generally, both up-regulated SAA1 and A1AT play important role in modulating immunity and inflammation by inducing inflammatory cell chemiotaxis, positive regulation of cytokine secretion and platelet activation (Carrell 1986; Kisilevsky and Manley 2012).

Consistent to the presence of a subclinical persistent inflammatory status in RTT, the same study showed higher levels erythrocytes sedimentation rate (ESR) in RTT as compared to controls and a positive correlation between this standard laboratory index and clinical severity scores.

-Cytokines Dysregulation

The presence of an altered cytokine profile in RTT patients have been found in two very recent works. More specifically, Leoncini et al. showed a major cytokine dysregulation in both the most common forms of the disease, i.e. that related to MECP-2 mutation and that associated with the rarer CDKL5 mutation (Leoncini *et al.* 2015). Focusing our attention on MECP-2-RTT, the general finding of the study was that T helper Type 2- (Th2) cytokine response was up-regulated (in particular the levels of IL-5 and IL-6 were higher than in controls), while most of the Th2 related cytokines (e.g. IL1β and Interferon-γ) were either lower or unvaried in plasmatic levels. Accordingly, Pecorelli et al. found increased levels of IL-9 and IL-13, other two TH2-citokines, in RTT patients compared to controls (Pecorelli *et al.* 2016). Of interest, this apparent undisclosed dysregulation of inflammatory mediators was accompanied and, at least in part, explained by severe morphological changes with potential abnormal functioning of immunocompetent PBMC. As we will illustrate in the forthcoming paragraphs in major details, the observed Th2-shifted balance is consistent with the hypothesis of a compensatory effect of immune response related to a defective Th1 differentiation (Jiang *et al.* 2014). This imbalance could have as clinical consequence an (although not classical) autoimmune condition, supporting the clues emerged in earlier studies reporting the presence of circulating antineuronal

antibodies, autoantibodies to nerve growth factor and folate receptor antibodies (Ramaekers *et al.* 2007; De Felice *et al.* 2016).

Among the aforementioned cytokines, IL-9 is worth to be examined in more depth because of its possible link with some frequent clinical manifestations of RTT. This interleukin is produced by mast cells and many T cell subsets besides Th-2, such as TH-9, Th-17, Treg cells and Th-9. IL-9 promotes the secretion of several pro-inflammatory cytokines, contributing to allergies, inflammation and autoimmune diseases (Pan *et al.* 2013). Il-9 has been also related with cardiovascular disease (CVD), showing a close association with ischemic and non-ischemic cardiopathies similar to the subclinical biventricular myocardial dysfunction documented in RTT (Cappuzzello *et al.* 2011; Acampa and Guideri 2006). IL-9 together with IL-13 and IL-8, that were also found at increased levels (Pecorelli *et al.* 2016), is associated with inflammatory lung diseases (for instance respiratory bronchiolitis) and clinical features, as central and obstructive apneas found in RTT patients. High levels of IL-9 have been detected in inflammatory airway disorders (Goswami and Kaplan 2011) while elevated serum levels of IL-13 have been seen in association with asthma (Corren 2013; Goswami and Kaplan 2011). Finally, increase in IL-8 has been found to be specifically related with obstructive sleep apnea syndrome (McNicholas 2009) .

A further piece of the picture describing the pronounced pro-inflammatory status in RTT is represented by the altered circulating levels of macrophages-related cytokines, with increase in the pro-inflammatory tumor necrosis factor-α (TNF-α), IL-6, and decrease in the anti-inflammatory IL-10 and transforming growth factor-β (TGF-β) (Leoncini *et al.* 2015). These last data are compelling since, as we will illustrate shortly, MECP2 is an important regulator of macrophage response to inflammatory stimuli.

4.1. Putative role of MECP2 in immune system regulation

The close association between RTT and systemic inflammation suggests that MECP2 mutation can negatively affect immune function. One of the most convincing clue of the axis MECP2-immune system came from Maezawa and colleagues who showed that lack of this gene in astrocytes led to brain-derived neurotrophic factor (BDNF) dysregulation and cytokine production (Maezawa *et al.* 2009), which, in turn, might account for the decreased dendritic arborization and impaired immune regulation in RTT brain. Compellingly, another study showed that replacing by transplantation of wild-type bone marrow, MECP2 knockout microglia led to the arrest of disease development in murine model of RTT (i.e. *Mecp2* knockout mice) (Derecki *et al.* 2012). The MECP2-deficiency was found not be merely an issue of glial cells but also of several peripheral macrophage and monocyte populations (Cronk *et al.* 2015), where MECP2 might serve as a crucial epigenetic regulator of immune responsiveness. These observations suggest that the dysfunction of tissue-resident macrophages, due to a defective MECP-2 regulation, may contribute to systemic manifestations of RTT.

Other immune cells showed severe function impairment, reduced growth and defective differentiation as consequence of MECP2-deficiency. This is the case of natural killer (low number in RTT patients) and mostly, various subsets of T-lymphocytes (Fiumara *et al.* 1999; Balmer *et al.* 2002) . One the pioneering

study on this field showed that single cell cloning of T lymphocytes from RTT patients with MECP2 mutations caused an evident growth disadvantage in cultured clonal T cells, by hindering the response to mitogenic stimuli (Balmer *et al.* 2002).

A direct mechanistic link between MECP2 and immunogenic responses of T lymphocytes (specifically CD4+ type) and how the loss of MECP2 proper function led to impaired differentiation of these cells was excellently dissected by Jiang et al. (Jiang *et al.* 2014). The authors showed that MECP2 was essential for the differentiation of naïve CD4+ T cells into Th1 and Th17 cells. The complex process underlying this regulatory role of MECP2 takes place through enhancing the expression of the microRNA (miR) miR-124 in CD4+ T cells, which represses the translation of mRNA for suppressor of cytokine signaling 5 (Socs5). This downregulation is indispensable for the activation of signal transducer and activator of transcription 3 (STAT3) and STAT1, which, in turn, are necessary inflammatory signals that stimulate the differentiation of Th1 and Th17 cells, respectively. Notably, these experimental data are in accordance with the previously described reduced levels of cytokines known to be produced or related to Th1 (while Th2 immune response appeared to be upregulated) (Leoncini *et al.* 2015).

Besides a defective genetic/epigenetic control on target genes, the systemic inflammatory phenotype associated with RTT might also be explained by an aberrant redox imbalance. This hypothesis is supported by the cumulating evidence indicating that MECP2 loss-of-function mutations could lead to a significant derangement of redox homeostasis which could chiefly influence signaling cascades, including Jak/STAT3 (Kesarwani *et al.* 2013).

5. The cross-talk between oxidative stress and inflammation in RTT

The synergic relationship between inflammation and OxS has been widely described and is now unanimously accepted. OxS has been defined "the consequence of the failure to maintain the physiological redox steady state, which is the self-correcting physiological response to different challenges" (Ursini *et al.* 2016). The disruption of this physiological homeostasis caused by a permanent toxic challenge or inadequate feedback response gives rise to an inflammatory state (Ursini *et al.* 2016; Forman *et al.* 2014). The challenges that can impair this finely tuned homeostasis are exogenous stressors (smoking, pollution, nutrient overload etc.) or endogen factors, including energy crisis, diseases and inflammation itself.

5.1 Oxidants and antioxidants: sources, biological function and mechanisms of actions

Endogen and exogenous factors can trigger the generation of oxidants, by definition electrophiles, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), that take one or two electrons from a nucleophile, from various sources (Forman *et al.* 2014; Cadenas and Davies 2000). Oxidants can be generated by reactions catalyzed by free transition metals, leaks from lipoxygenases, cyclooxygenases, xanthine oxidase and phagocytic and non-phagocytic nicotinamide adenine dinucleotide phosphate oxidases (Noxs) etc (Sies 2015; Forman and Torres 2002; Bergamini *et al.* 2004; Valko *et al.* 2005). Of note, professional phagocytic NOXs represent an ideal example of the mutuality between inflammation and OxS,

since their activation during the neutrophils respiratory burst, resulting in formation of superoxide anion (O_2^-), is essential for the defense against pathogens (Forman and Torres 2002). However, the most thriving intracellular source of reactive species is mitochondrial respiratory chain, where almost 5% of oxygen flux give rise to $O_2^{\bullet-}$, the second most reactive ROS after hydroxyl radical ($^{\cdot}OH$) (Cadenas and Davies 2000). The mitochondrial production of ROS becomes markedly larger in case of impairing damage of the organelles (Valko *et al.* 2007; Cervellati *et al.* 2016). The fact that generation of the potentially cytotoxic oxidants is an inevitable phenomenon of the aerobic metabolism implies that: 1) within a given range, a moderate generation of reactive species is essential for cell biology 2) there must be an innate defensive system able to prevent excessive generation of oxidants.

Low production of ROS/RNS is a fundamental physiological process, since they play as signaling molecules in several pathways, including those regulating immune function, cell growth and proliferation (Gough and Cotter 2011; Forman *et al.* 2014). On the other hand, when these reactive substances exceed the defensive capability of a biological systems, damages to all cell constituents and the initiation of detrimental cascade reactions become an unavoidable consequence (Ursini *et al.* 2016). Lipid peroxidation, the most important example of ROS-triggered chain reaction, is a key-pathogenic factor of many OxS-related diseases since it brings about: 1) structural and functional alterations of targeted lipid-enriched cell components, in particular cell and organelle membranes 2) generation of toxic primary and secondary lipoperoxidation by-products, such as hydroperoxides (LOOH), 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Of these two reactive aldehydes, 4-HNE, a reactive electrophilic α,β-unsaturated aldehyde deriving from oxidative attack of poly-unsaturated fatty acids, is by far the most studied. In a oxidative environment, this aldehyde is able to bind, via Micheal addition, and modify nucleophilic protein residues, specifically side chains of cysteine, histidine, and lysine (Poli *et al.* 2008). This chemical interaction inevitably affects protein structure and function, and depending on the biological role of the target, modulate a number of signaling processes (Poli *et al.* 2008).

Innate defensive mechanisms against ROS/RNS insult are afforded by a series of enzymatic and nonenzymatic substances. The frontline of defense is represented by scavenging enzymes such as superoxide dismutase (SOD) isoforms, which in mitochondria, cytosol or extracellular fluids can directly scavenge $O_2^{\bullet-}$ leading to the formation of the more stable hydrogen peroxide (H_2O_2) (Valko *et al.* 2007; Forman *et al.* 2014; Cadenas 1997). The “antioxidant flux” is continued and terminated by other two enzymes, catalase (CAT) and glutathione peroxidase (Gpx) the catalyze the final neutralization of H_2O_2, thus preventing the metal catalyzed conversion of this ROS to the highly toxic hydroxyl radical. Other antioxidants involved in restoring the optimal redox balance are the enzymes such as glutathione transferase and reductase, thioredoxin (TRX), peroxiredoxin and non-enzymatic compounds of various origin (e.g. α-tocopherol, vitamin C, glutathione etc) (Cadenas 1997; Sies 2015).

5.2. The Vicious Circle of Oxidative stress and inflammatory processes in RTT

The general notion that emphasizes the central role of the mutual interplay between OxS and inflammation in diseases might also be applied to RTT. Indeed, there are abundant lines of evidence gathered by ours and other group showing that systemic redox imbalance is a hallmark feature of the genetic syndrome and that reactive species might be involved in the inflammatory processes that plausibly occur in RTT.

5.2.1. Central and peripheral redox homeostasis dysregulation in RTT: animal and human evidences

Regardless of the pronounced systemic component of RTT, it is undoubted that MECP2 deficiency mostly leads to damage to neurons. Brain cells are known to be highly vulnerable to reactive species challenge, since they have a long life, relatively low levels of endogenous antioxidants (particularly glutathione), high levels of polyunsaturated fatty acids (PUFA, the primary target of reactive species), high levels of pro-oxidant metals, and the conspicuous use of oxygen, which enhance the physiological extent of ROS derived by mitochondria (Cervellati *et al.* 2016; Cervellati *et al.* 2014b; Golden *et al.* 2002). This predisposition to oxidative injury accounts, at least partially, to the amply demonstrated implication of OxS in a number neurological disease, including Parkinson's, Alzheimer's, Down's Syndrome, vascular dementia, ataxia, multiple sclerosis, amyloid lateral sclerosis, etc. (Thanan *et al.* 2015; Cervellati *et al.* 2016; Cervellati *et al.* 2014a; Cervellati *et al.* 2013; Abramov *et al.* 2004; Olivieri *et al.* 2011).

The first body of evidence linking OxS with RTT was gathered at the end of 1980s and disclosed an impaired antioxidant defense in RTT brain (postmortem analysis on patients) with severe reduction in vitamin C and glutathione (Sofić *et al.* 1987). Depletion of enzymatic and non-enzymatic antioxidants were found to be extended to erythrocytes (Sierra *et al.* 2001), serum/plasma (Formichi *et al.* 1998; De Felice *et al.* 2014) and skin fibroblasts (Cervellati *et al.* 2015a). To cope with redox alteration, a possible feedback mechanism in RTT implies an upregulation of a group of genes related to antioxidant defensive systems, such as SOD, CAT, glutathione transferases etc. (Pecorelli *et al.* 2013), that, however, as demonstrated later on, did not appear to translate in a more efficient catalysis (Cervellati *et al.* 2015a). Indeed, we have recently found that the activity of SOD, CAT, Gpx was lower in primary skin fibroblasts isolated from RTT patients compared to those from healthy controls (Cervellati *et al.* 2015a).

Proofs of a severely compromised systemic redox balance in RTT patients also emerged from studies employing peripheral biomarkers of biomolecular oxidative damage. Indeed, as compared to healthy controls, girls affected by the disease showed increased levels of various byproducts of protein oxidation (carbonyls) and, mostly lipoperoxidation (F_2-isoprostanes, neuroisoprostanes, 4-HNE and MDA) in a wealth of studies (De Felice *et al.* 2011; Cervellati *et al.* 2015a; De Felice *et al.* 2012b; Leoncini *et al.* 2015; Pecorelli *et al.* 2011; Signorini *et al.* 2011). Notably, levels of these markers were found to change as function of time and clinical severity scales (Leoncini *et al.* 2015).

5.2.2. From MECP2 dysfunction to an imbalanced redox homeostasis and potential implication in inflammatory processes: mechanistic hypotheses

Thus far, the molecular mechanisms linking MECP2 mutations to the redox balance derangement occurred in RTT are still not well defined. However, important steps towards the understanding of these pathways have been taken in the last 10 years, thanks to the discovery of the MECP2 ability to control the expression of several redox-related genes. In this regard, it is worth it to mention the results from *Mecp2*-null mice that showed an epigenetic regulation of the expression of brain-derived neutrophic factor (BDNF) and proline dehydrogenase (Prodh), proteins implicated in the modulation of cellular redox defensive system and ROS mitochondrial production, respectively (Chang *et al.* 2006; He and Katusic 2012; Urdinguio *et al.* 2008; Servet *et al.* 2012). Details on the roles and in the implications of these, and other, redox modulators in RTT pathogenesis have been already nicely reviewed (Katz 2014; Filosa *et al.* 2015).

None of the above mentioned proteins could account for the exacerbated redox status that chiefly characterizes the typical RTT phenotypes . The culprits of this disease hallmark must be sought among those promiscuous sources of reactive species that resulted to be closely related with the disease expression, *in primis* hypoxia , mitochondrial dysfunction and NOX enzymatic machinery activation. Although the causal relationship between MECP2 mutation and the trigger of these pro-oxidant factors is still not ascertained, they deserved consideration in light of this review topic because of the well-known association with systemic inflammation.

Chronic intermittent hypoxia with impaired lung gas exchange has been described in association with OxS-related damage in typical RTT patients (De Felice *et al.* 2009). This condition can be promoted by several clinical manifestations of the syndromes such as central apneas, significant obstructive apneas, and hyperventilation, which are all attributed to autonomic dysfunction and/or brain-stem immaturity. Hypoxia can, in cooperation with OxS, activate a series of transcription factors, such as NF-kB, AP-1 and HIF-1-α, that, in turn, can induce inflammatory mediators (*in primis* IL-8) and contribute to the cytokine dysregulation observed in RTT patients (Pecorelli *et al.* 2016).

One of the adaptive attempt of the cells to limit oxygen supply is the increased number of mitochondria in order to make more efficient the yield of energy from oxidative phosphorylation process (Arismendi-Morillo 2009). This feature in combination with severe morphological and ultrastructural modifications has been repeatedly observed in mitochondria of both RTT neurons and non-neural cells (Valenti *et al.* 2014; Filosa *et al.* 2015; Cervellati *et al.* 2015a; Pecorelli *et al.* 2016). In specific, convergent evidence from muscle and frontal lobe biopsies of RTT patients and in cortex and hippocampus of *Mecp2*-null mice revealed abnormally swollen mitochondria with vacuolization, granular inclusions, and membranous changes (Eeg-Olofsson *et al.* 1988; Wakai *et al.*; Matsuoka *et al.* 1997). Other studies in the same specimens confirmed these morphological alterations that also resulted correlate with abnormalities in mitochondrial respiratory chain enzymes, including NADH cytochrome c reductase, succinate c reductase and cytochrome c reductase, and therefore with energy metabolism impairment (Dotti *et al.* 1993; Coker and Melnyk 1991). Accordingly, fibroblasts derived from RTT patients feature overall suppressed bioenergetics as indicated by reduction of both basal and maximal respiration, which also implies decreased reserve capacity, and thus a general mitochondrial dysfunction (Cervellati *et al.* 2015a).

Increased number of mitochondria as well as the cristae fragmentation and matrix swelling also characterize circulating immune competent PBMCs from RTT patients, thus reflecting a status of hyperactivation with atypical cytokine production (Pecorelli *et al.* 2016). A recent microarray on these peripheral cells showed an altered gene expression profile with significant upregulation of several mitochondria-related genes (Pecorelli *et al.* 2013). Noteworthy, the most significantly upregulated transcripts included those encoding for many subunits of respiratory chain multi-enzymatic complexes and thus linked to mitochondrial ATP production and, indirectly, to ROS generation. In turn, the increase in pro-oxidant yield gives rise to vicious cycle, with reactive species exacerbating mitochondria impairment and further enhancing the electron leaks from respiratory chain (Pecorelli *et al.* 2013; Albers and Beal 2000; Cadenas and Davies 2000).

We have recently found that OxS in RTT might also result from an increased activation of membrane bound NOXs enzymes, most likely the isoform NOX2 which is located in both phagocytic and non-phagocytic cells (Cervellati *et al.* 2015a). NOX2-derived ROS are required for microbial killing but also seems to have a paradoxical potential anti-inflammatory function. The most convincing clues in favor of this proposed immune modulation functions of NOX2 derives from the study by Whitmore et al. on systemic inflammatory response syndrome (SIRS) (Whitmore *et al.* 2014). Using a murine model of sterile generalized inflammation, the authors found a less pronounced SIRS phenotype and lower levels of systemic inflammation in wildtype compared to NOX2-deficient mice. Activation of NOX2 in RTT cells might thus be a compensatory mechanism primed for contrasting the exacerbation of chronic inflammation. According to this hypothesis, pro-inflammatory cytokines have been shown to act as signaling molecules mediating this production of ROS in erythrocytes NOX (George *et al.* 2013).

-Possible redox-mediated epigenetic control of the expression of genes involved in T-cells differentiation, maturation and cytokine secretion

To date there is no direct evidence on a MECP2 redox-mediated control the expression of genes implicated in immune function and organization. However, intriguing clues on this regard have been very recently discovered.

Several T cell subsets are highly susceptible to OxS, which plays a critical role in the determination of the fate of these cells and of the immune response outcomes in various pathologies. Kesarwani et al. have already exhaustively described the multiple pathways and transcription factors, such as Nuclear Factor Kappa B (NF-κB), p53, activator protein 1 (AP1), that are selectively activated by intracellular ROS (Kesarwani *et al.* 2013). Here we focus our attention to those pathways plausibly targeted in RTT disease.

Of note is a recent paper reporting that NF-κB signaling is up-regulated in the cortex of animals with Mecp2 loss-of-function. In addition, the authors were able to demonstrate that the lifespan of Mecp2-null mice increases once NF-κB signaling was genetically suppressed (Kishi et al., 2016). MeCP2 deficiency also enhances expression of inflammatory cytokines, such as IL-6 and TNFα, by sustaining NF-κB signaling in myeloid derived cells (O'Driscoll et al., 2015). These findings provide new insights on potential therapeutic

strategies for RTT via the modulation of NF-κB and its downstream regulated processes such as inflammation.

The redox- sensitive Jak/STAT3 pathway regulates a number of cells processes and inflammatory-related mechanisms, among which the differentiation of T-cells (Kesarwani *et al.* 2013). More specifically, the STAT3 protein appeared as a crucial determinant of whether the naïve T cell differentiates into regulatory (Treg) or an inflammatory (Th17) T cell lineage (Egwuagu 2009). Being this process of differentiation impaired in MECP2-deficient CD4+ T cells, it was tempting to explain this phenomenon as the consequence of the lack of redox-regulatory skill of MECP2 (Leoncini *et al.* 2015). Oxidative processes might also be the driving factors of the observed Th2- shifted balance (mostly suggested by the increase of IL-2 and IL-4) observed in RTT patients (Leoncini *et al.* 2015; Pecorelli *et al.* 2016). Indeed, it has been shown that oxidative microenvironment elicits an opposing effect on cytokine secretion by Th1 compared to Th2 cells (Frossi *et al.* 2008a). In particular, converging evidence suggests that increased levels of intracellular reactive species, originated from mitochondrial respiratory chain complexes and/or defective antioxidant enzymes suppresses the number of Th1 cells (Kaminski *et al.* 2010b; Frossi *et al.* 2008b). In particular, the *in vitro* experiments by Frossi et al on Th1 and Th2 cells clones from autoimmune thyroiditis revealed that low-doses of H_2O_2 reduced the INF-gamma production of the former and potentiate the IL-4 secretion of the latter. Accordingly, Kaminski et al. found that the secretion rate of IL-4 and IL-2 in activated T-cell strongly depended on the levels of mitochondria derived ROS (Kaminski *et al.* 2010a)

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It is possible that the chronic and systemic OxS well-documented in RTT will induce a "mild-subclinical chronic inflammation" that will contribute to the altered redox homeostasis, leading to a viscous circle able to chronically induce a pro-inflammatory status identified as OxInflammation (Figure 1).

This leads to the generation of electrophiles, such as reactive aldehydes (4-HNE) and F_2-isoprostanes, which are shown to induce the infiltration and activation of inflammatory cells. Reactive aldehydes derived from LPO, such as 4-HNE and MDA, can directly react with DNA to form exocyclic DNA adducts, which have been detected in a variety of inflammatory diseases (Uchida 2003; Pang *et al.* 2007). In tissues, MDA and 4-HNE can cause protein oxidation due to the close interaction between lipids and proteins and create neoantigens that can then cause immune reactions, stellate cell activation, and neutrophil chemotaxis. This condition can be the responsible for several clinical features observed in RTT patient, making these patients more susceptible to infections and to a subclinical inflammatory status.

6. Conclusion

The studies on RTT patients have confirmed the robust body of evidence coming from animal and *in vitro* studies: undisclosed dysregulation of the immune system in mutual combination with a persistent abnormal redox balance predispose the individuals affected by the disorder to a chronic and subclinical inflammatory

state. These findings open novel windows and targets for future therapy against this devastating and still drug orphan neurodevelopmental disorder.

Relevant to this regard and, more broadly, to the framework of translational medicine, recent data from our group show the beneficial effects of a selection of ω-3 polyunsaturated fatty acids (ω-3 PUFAs) (De Felice *et al.* 2012a) in terms of clinical severity (in particular, motor-related signs, nonverbal communication deficits, and breathing abnormalities) as well as a significant decrease in all the OxS markers. The proved doubled action, both as antioxidant and anti-inflammatory, of these substances might account for the promising results obtained in this short-trial.

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Figure Legend

Figure 1. OxInflammation in Rett syndrome. A chronic and subclinical inflammatory state in mutual combination with a persistent abnormal redox balance, in a kind of detrimental vicious circle, predispose RTT patients at a constant biomolecular damage, which adversely affects on multiple organs and tissues in the body.

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