1. Introduction

Multiple sclerosis (MS) is a primary inflammatory demyelinating autoimmune disorder of the central nervous system (CNS) affecting mainly young people aged between 20 and 40 years at disease onset. In early stages, the disease is characterized by infiltration and activation of T cells and accumulation of monocyte-derived macrophages, which promote destruction of the myelin sheath leading to the formation of focal demyelinated lesions [1]. The disease involves a life-long, unpredictable course generally categorized as relapsing-remitting, secondary progressive, and primary progressive, though all these courses entail a progressive destruction of myelin [2]. Previous studies demonstrated that MS induces alteration in energy metabolism and in oxidants/antioxidants balance that can be monitored in serum of MS patients [3]. Moreover, recent evidence suggests that mitochondrial dysfunctions contribute to neurological disorders [4,5], supporting the role of mitochondria as a potential therapeutic target in MS [6]. Interestingly, also CD4+ T lymphocytes, the main T cell population involved in the pathogenesis of MS, present metabolic alterations. This is an intriguing aspect, because these cells could be used as bioenergetic markers. In particular, previous studies carried out by our group demonstrated that CD4+ T cells of MS patients have a reduction in oxygen consumption in association with an increase in the activity of glycolytic enzymes respect to the cells of control ones [7]. Thus, these findings lead us to propose CD4+ T cell bio-energetic status as a marker for diagnosis and follow up for MS [7]. These findings suggest that GA is able to reduce CD4+ T lymphocytes’ dysfunctions by increasing mitochondrial activity and their response to oxidative stress.

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toward regulatory Th2 phenotypes secreting IL-4 and 10 [13] and affects innate immune cells including macrophages and dendritic cells [14,15]. GA probably also increases the frequency of FoxP3-expressing regulatory T cells, effects that are at least partly mediated by the generation of anti-inflammatory antigen-presenting cells, allowing the differentiation of naïve T cells into Th2 or Th3 and regulatory T cells [16,17]. Particularly, it was shown that GA influence monocyte/macrophage polarization by shifting the balance from pathological M1 toward the M2 regulatory phenotypes [15]. The majority of studies focusing on the mechanism of action of GA were conducted in vitro and no information are available about GA effect on CD4+ T cell metabolism. Ruggieri et al. demonstrated that GA is able to restore a correct balance in the process of apoptosis of cultured PBMCs from MS patients [18]. In order to provide more insight into the effect of GA treatment on CD4+ T cell metabolism, in the present study we investigated the metabolic characteristics of this cell subset in association with response to oxidative stress in GA treated MS patients in a 12 months follow up study.

2. Materials and methods

2.1. Ethical permission

The study was approved by the Vito Fazzi Hospital Ethics Committee (Lecce, Italy) and informed consent was obtained from each patient prior to entry into the study, according to the declaration of Helsinki.

2.2. Participants/study population

20 patients diagnosed with RRMS in Vito Fazzi Hospital with an age range of 19–45 years were included into the study. Patients had to be without any immune-modulatory treatment at least 6 months prior to study entry. For each patient, blood samples were obtained at baseline (untreated) and every 6 months during GA therapy for a period of 12 months. Written informed consent was obtained from each individual before the start of the study. Blood samples were also collected from 20 sex and age matched healthy controls (HCs).

2.3. CD4+ T cell isolation

PBMC fractions were isolated from whole blood using Ficoll-Paque density-gradient centrifugation. CD4+ T cells were purified by negative selection using an indirect magnetic cell sorting kit (MiltenyiBiotec, Bergisch Gladbach, Germany). In summary, human CD4+ T cells were isolated by depletion of non-CD4+ T cells. Non-CD4+ T cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monocolonal antibodies, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labelling reagent. The magnetically labelled non-CD4+ T cells were depleted by retaining them on MACS® Column in the magnetic field of a MACS Separator, while the unlabeled cells passed through the column.

2.4. Polarographic measurement of respiratory rate

1·10^6 cells CD4+ T cells were suspended in buffer containing 10 mM HEPES, 143 mM NaCl, 4 mM KCl, pH 7.4. Oxygen consumption was measured at 36 °C by a Clark-type oxygen probe (Oxygraph, Hansatech Instruments, King’s Lynn, UK), in the presence of mitochondrial respiratory substrates (5 mM pyruvate and 5 mM malate) and 0.5 mM ADP. The rate of oxygen uptake (V) by CD4+ T cell mitochondria was expressed as nmol O_2·min⁻¹·mg⁻¹. The respiratory control ratio (RCR) was calculated by dividing V_3 (rate of oxygen uptake measured in the presence of respiratory substrates + ADP) by V_4 (rate of oxygen uptake measured with respiratory substrates alone).

2.5. ΔΨ measurement

CD4+ T cells were incubated with 2 μM JC-1 (Molecular Probes, Eugene, CA) for 20 min and then fluorescence of J-aggregates and J-monomers was measured using excitation/emission wavelengths of 535/595 nm and 485/535 nm, respectively.

2.6. Respiratory complexes activity

CD4+ T lymphocytes were resuspended in Mito buffer (2 mM HEPES, 0.1 mM EGTA, 250 mM sucrose, pH 7.4) supplemented with a protease inhibitor mixture, subjected to three freeze-thawing cycles and, after the addition of 10 mM triethanolamine and 0.1 mg/ml digitonin, were incubated for 10 min on ice, homogenised and centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was saved and the pellet resuspended in the same volume of Mito buffer supplemented with 0.1 mg/ml digitonin, homogenised and centrifuged once again. Supernatants were mixed and centrifuged at 12,000 × g for 15 min at 4 °C, and the mitochondria-rich pellet was resuspended in Mito buffer. A total of 10–40 μg of proteins were used to determine the activity of each complex. The assays were performed at 37 °C (except for citrate synthase activity that was assayed at 30 °C) using microcuvettes (volume 100 μl).

Activities of complex I, II and III were determined according to the method described by Frazier and Thorburn [19]. Complex IV activity was determined using a COX assay kit (CYTOCOX1) from Sigma-Aldrich. All activities were expressed as μU/mg of proteins.

Citrate synthase [CS], a ubiquitous mitochondrial matrix enzyme, serving as a mitochondrial marker, was measured in the presence of acetyl-CoA and oxaloacetate by monitoring the liberation of CoA-SH coupled to dithiobis[2-nitrobenzoic] acid spectrophotometrically at 412 nm. Protein concentration was determined by the Bradford method and calculated according to bovine serum albumin (BSA) standard curve.

2.7. Antioxidant enzyme activities

The superoxide dismutase (SOD) activity was measured using the Sigma SOD assay kit-WST (Sigma-Aldrich, Switzerland) following the manufacturer’s instructions.

Catalase (CAT) activity was assayed by the method of Luck [20]. The assay mixture consisted of H_2O_2-phosphate buffer (12.5 mM H_2O_2, 0.067 M phosphate buffer, pH 7.0) and cell homogenate. Absorbance changes were recorded at 240 nm for 3 min. Results were expressed as U/mg protein using molar extinction coefficient of H_2O_2 (71× M⁻¹ cm⁻¹).

Glutathione peroxidase (GPx) activity was determined according to Lawrence and Burk [21] with modifications. The activity was measured as a decrease in absorbance at 340 nm for 5 min at 25 °C based on a coupled reaction with glutathione reductase (GR) in the presence of GSH and H_2O_2. Cell homogenate was added to each well containing 100 μl of 25 mM KH_2PO_4, 0.5 mM EDTA pH 7.4, 0.5 mM NaNO_3, 0.3 mM NADPH, 0.84 units of GR, and 1 mM GSH. The reaction was initiated by adding 0.1 mM H_2O_2. Values were corrected for nonenzymatic oxidation of GSH and NADPH by H_2O_2. GPx activity was expressed as U/mg of protein using 6.22 M⁻¹ cm⁻¹ as the extinction coefficient.

2.8. Hexokinase and phosphofructokinase activity assays

A total of 30–50 μg of cellular proteins were used. The assays were performed at 37 °C using microcuvettes (volume 100 μl). Hexokinase activity was quantified following the method described by Tielens et al. [22]. Briefly, 5 μl of sample dissolved in 35 μl of water was added to a mix containing 40 mM Tris, 22 mM Mg-acetate, 10 mM β mercaptoethanol, NADP⁺ 0.75 mM, 1 U/ml G6PDH, 10 mM ATP. After
waiting until a constant reading, the reaction was started by adding 5 mM glucose and absorbance was monitored for 3 min at 340 nm.

Phosphofructokinase activity was detected as NADH oxidation by monitoring absorbance at 340 nm in a buffer containing 4 mM fructose-6-phosphate, 1 mM ATP, 0.5 mM NADH, 3 U/ml aldolase, 50 U/ml TPI, 8 U/ml GPD1.

2.9. Lactate production

Lactate production measurement was carried out following the method described by Merlo-Pich et al. [23] with some modifications. Briefly, CD4+ T cells (1 x 10^6 cells) were incubated for 1 h at 37 °C in the presence and absence of 9 μM of antimycin A; after incubation, the samples were centrifuged at 1500 g for 10 min and lactate was assayed spectrophotometrically. From the lactate data, it was possible to calculate basal lactate as glycolytic ATP (in absence of antimycin), Δ-lactate (the difference of lactate production in presence and absence of antimycin A) as mitochondrial ATP and the ratio Δ-lactate/basal lactate as the ratio of mitochondrial ATP and glycolytic ATP.

The lactate concentration is then calculated from a standard curve of known lactate concentrations.

2.10. Western blot analysis

CD4+ T cell protein extracts (~30 μg) were boiled in Laemli sample buffer (Sigma-Aldrich) for 5 min, resolved on 10% SDS-polyacrylamide gels and transferred onto PVDF filters (Amersham). Membranes were blocked for 1 h in Tris-buffered saline (TBS), 0.05% Tween-20, 5% non-fat dry milk, followed by overnight incubation with specific primary antibodies diluted in the same buffer. The primary antibodies used are listed as follows: anti-GLUT1 (1:1000, Novus Biologicals), anti-MCT1 (1:1000, Novus Biologicals), anti-p-actin (1:8000, Sigma-Aldrich). Mitochondria-rich pellet (15 μg) was separated on a SDS-PAGE and, after blocking, membranes were incubated with MitoProfile total OXPHOS human WB antibody cocktail (1:1000, Abcam) and anti-Porin (1:1000, Santa Cruz). After washing with 0.1% Tween in TBS, membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h, washed and developed using the ECL chemiluminescent detection system (ClarityTM Western ECL Substrate Biorad). The densitometric analyses of blots were performed by a computerized image processing system (Image J, 1.0 version).

2.11. Statistics

All data were analysed using the statistical software GraphPad Prism (5.0 version). Statistical differences were assessed by Student’s t-test. Comparison between MS patients and healthy controls was evaluated using an unpaired Student’s t-test (two tailed), while comparison between time point 0 (patients at diagnosis), 1 (after 6 months of treatment) and 2 (after 12 months of GA treatment) was evaluate using a paired Student’s t-test. Values at p < 0.05 were considered statistically significant.

3. Results

3.1. Respiratory rate and OXPHOS activity

Fig. 1 shows the mitochondrial respiratory efficiency by CD4+ T cells samples from control and MS subjects at baseline and during GA (Copaxone®) treatment (20 mg s.c./day) after 6 and 12 months.

In the presence of metabolic substrates, a strong decrease in V3 values (also known as the rate of oxygen consumption in the active state of respiration) was observed in the MS subjects. A slight yet significant decrease of V4 was also observed in these patients. As a consequence, the RCR values calculated by dividing V3 by V4, were profoundly and significantly lower in MS patients in comparison to the control subjects (Fig. 1).

After 6 months of GA administration, V3, V4 and RCR values did not particularly differ from those observed at baseline. Interestingly, after 12 month of GA treatment RCR values indicated a better coupling between respiration and a well-preserved integrity of the organelles in CD4+ T cells isolated from MS patients.

To further characterize the mitochondrial dysfunction, we used the JC1 fluorescent probe to measure mitochondrial membrane potential (Δψm), which is an indicator of mitochondrial activity.

The respiratory defects detected in CD4+ T cells of MS untreated patients result in an increased Δψm, as found with JC1 analysis: the ratio between 595/530 was 2.81 ± 0.64 in control subjects, 4.14 ± 0.33 in MS baseline patients, 3.46 ± 0.24 in 6 months treated and 2.36 ± 0.63 in 12 months treated subjects. These results suggest the ability of GA to completely reverse mitochondrial dysfunction (Fig. 2).

In a more selective approach for investigating the functionality of the mitochondrial oxidative phosphorylation, we assayed the activity of single components of the respiratory chain (Fig. 3). Complex activities were normalized against both the protein content and activity of citrate synthase (CS), in order to consider any possible variations in cell density and mitochondrial content. The latter did not display any differences between CD4+ T cells before and after drug treatment (data not shown), suggesting that GA treatment is not able to alter mitochondrial biogenesis, but only OXPHOS activity.

We found a decrease in the enzymatic activity of complex I and IV, respectively, of 21% and 39% in baseline subjects in comparison to the control group. A reversal in the activity of these complexes was clearly observed after 12 months of GA treatment. Complex III activity showed a significant increase in MS baseline patients, which was more pronounced during GA treatment. No significant differences were found in the activity of complex II (Fig. 3).
3.2. Antioxidant enzymes activities in CD4+ T cells from MS subjects

Mitochondria are the major ROS generator, as they convert 0.2–2% of the oxygen taken up by the cells to ROS. Therefore mitochondrial dysfunction is often associated with increased ROS production by the organelle itself.

The activity of antioxidant enzymes such as superoxide dismutases (SODs), glutathione peroxidases (GPx) and catalase (CAT) were assayed in MS treated patients and healthy subjects (Fig. 4A). SOD activity, which was significantly lower in MS baseline in comparison to control group, was restored after GA treatment (Ctrl: 100% ± 15.9, baseline: 70.04 ± 8.92, 6 months: 92.28 ± 11.04, 12 months: 92.03 ± 6.87). An increase in catalase activity was observed only after 6 month of GA administration (Fig. 4C). No differences between baseline and GA treated subjects were observed in glutathione peroxidase (GPx) activity (Fig. 4B).

3.3. Glycolytic flux in CD4+ T cells

Inhibition and/or a decrease of mitochondrial respiration could stimulate glycolysis, hexokinase and phosphofructokinase are key enzymes involved in this pathway. We measured hexokinase and phosphofructokinase activities in CD4+ T cells from control and MS treated subjects. We found an increase in the activity of hexokinase and phosphofructokinase in baseline subjects (36% and 94% respectively), that was partially restored after 12 months of GA treatment (Fig. 5A). Because lactate was the predominant metabolite which is released from cells as a result of glycolysis, we next examined amounts of extracellular lactate. Extracellular lactate release strongly decreased in CD4+ T cells from 12 months treated patients (Fig. 5B). The observed differences in glucose utilization were associated to a decrease in the expression levels of GLUT-1 during GA treatment (Fig. 5C) in lymphocytes from MS patients.
A parallel increase in MCT1 lactate transporter was observed in MS patients, although no statistically significant differences were found between baseline subjects, 6 and 12 months treated (Fig. 5C).

Finally, the ratio of mitochondrial ATP over glycolytic ATP, significantly reduced in MS baseline subjects, was gradually increased in 6 and 12 months treated patients (Fig. 5D).

4. Discussion

Multiple Sclerosis is a chronic autoimmune inflammatory disease of the CNS that affects young adults, mainly female, leading to a growing disability. CD4+ and CD8+ are the inflammatory cells that, together with microglia and macrophages, play a pivotal role in the onset of MS [24]. The animal model of MS, experimental autoimmune encephalomyelitis (EAE), the same used to synthesize the encephalitogenic components of myelin basic protein (MBP), was useful to understand that CD4+ T cell subset plays a central role in MS pathogenesis [25], having these cells the ability to cross the blood–brain barrier with axonal damage and neuronal death [26]. In the past 30 years many therapies have been developed in order to decrease clinical relapses, halting the progression of disease. Among others, Glatiramer Acetate (Copaxone, Teva Pharmaceuticals) is a first-line therapy for relapsing-remitting form of MS and Clinical Isolated Syndrome. The usual dose of GA is 20 mg subcutaneously once a day. Although the mechanism of action of GA is not completely understood, it may be similar to the process of a vaccine in which antigen-presenting cells incorporate peptides of GA and present them to lymphocytes, developing a population responsive to GA. This process is able to inhibit the number of lymphocytes that react against MBP.

Recent attention to the role of mitochondria in the etiology of MS suggests that mitochondrial defects and organelle structural and functional changes may contribute to the disease. Given the key role of mitochondria in many important cellular functions including energy production, it is reasonable that their dysfunction could contribute to neurodegenerative process of this disease [32]. In particular, during the progression of MS, inflammatory mediators, such as cytokines, oxidants, and nitric oxide, are released by microglia or are generated by hypoxia, perturb mitochondrial function causing defects in mitochondrial DNA and its replication, along with defective mitochondrial enzyme activities. Therefore, mitochondrial abnormalities and mitochondrial energy failure may impact other cellular pathways, including increased demyelination and inflammation in neurons and tissues that are affected by MS [33,34,35,36,32,37,38].

Among peripheral cells, T cells, especially CD4+, play a pivotal role in MS pathogenesis and, for this reason and for their easy accessibility, investigating their bioenergetics status could represent a follow up marker for MS. Activated lymphocytes, such as cancer cells, usually rely more on glycolysis than glucose oxidative phosphorylation (OXPHOS) in energy production: this reprogrammed metabolism is known as the Warburg effect. It provides cells advantages in energy production, biosynthesis, redox control and inhibition of apoptosis [39,40].

During glycolysis, each glucose molecule is converted to pyruvate with a net production of two ATP molecules. Non-proliferating and certain T cells, such as memory and naïve, completely oxidize pyruvate through Krebs cycle producing 36 molecules of ATP. Once activated, T cells transform pyruvate into lactate: this process is less efficient, but faster than OXPHOS in generating ATP and produces metabolic intermediates that are used in anabolic pathways able to sustain cell growth and proliferation [41].

In activated T cells, the upregulation of glycolysis is promoted by increased activity of enzymes and proteins such as Glut-1 overexpression associated with the increase in glucose uptake [42,43]. mTOR is essential to maintain aerobic glycolysis in effector T cells, transcription factors such as Hif1α (HIF1), and c-Myc are involved in glycolytic reprogramming of T cells directly binding the promoters of a variety of genes, notably those of glycolytic enzymes and glucose

![Fig. 5. Bioenergetics of CD4+ T cells in control and MS subjects. CD4+ T cells from control and MS patients were assayed for (A) enzymatic glycolytic activities of hexokinase and phosphofructokinase, expressed as percent of control, (B) lactate production, (C) GLUT-1 and MCT-1 expression and for (D) ratio of mitochondrial over glycolytic ATP production (ratio Δ-lactate/basal lactate), where Δ-lactate is the difference of lactate production in presence and absence of antimycin A and basal lactate is lactate production in absence of antimycin. Cell lysates [30 μg] were separated on a SDS-PAGE gel followed by western blot analysis using β-actin as a loading control. Representative immunoblot is shown. Histograms show the mean values, expressed as percent of n = 10 controls and n = 10 MS subjects, each performed in triplicate; bars indicate S.E.M. Asterisks indicate values that are significantly different from those obtained in control cells; *p < 0.05, **p < 0.01, Student’s t-test.](image-url)
transmitters [44,45]. Indeed, when lymphocytes are prevented from engaging aerobic glycolysis, cellular differentiation and function is compromised. This has been demonstrated using strategies to disrupt the signalling pathways that promote aerobic glycolysis [46,47]. Metabolic changes in T cells influence immune functions in human diseases: in HIV patients, it was shown that high levels of immune activation and markers of inflammation, correlated with Glut-1 overexpression and the increased glycolytic metabolism in CD4+ T cells [48]. In the same cells of Multiple Sclerosis first diagnosis patients, we found an increase in glycolytic pathway associated with a decrease in OXPHOS activity: in particular, we found a decrease in the activities of mitochondrial complex I and IV and a parallel increase in the activity of complex III, suggesting a defect in mitochondrial function [7]. The impairment of mitochondrial respiration efficiency resulted also in decreased RCR values, which are a measure of the tightness of coupling between electron transport and oxidative phosphorylation.

Apart from that study, little is known about CD4+ T cell metabolic phenotype in MS subjects, especially related to its response to pharmacological treatment. In order to depeen this issue, we investigated mitochondrial function and we demonstrated, for the first time, that GA administration is able to restore mitochondrial function, as suggested by RCR and mitochondrial membrane potential values, along with enzymatic activities of respiratory complexes. In particular, our results showed an increase in RCR values and in the activities of complex I and IV and a decrease in the values of Δψm. In this context, it is important to underline that mitochondrial hyperpolarization observed in MS patients may be due to T cell receptor stimulation, associated with the transient inhibition of Fo-Ф1 ATP synthase and the consequent ATP depletion [49]. In various cell lines, it has been proposed that the residual activity of OXPHOS complexes may trigger mitochondrial membrane alterations [50, 51]. Experiments carried out in T cells isolated from SLE patients revealed persistent mitochondrial hyperpolarization [52]; in addition, in comparison to control monocytes, lupus monocytes induced MHP of normal T cells after co-culture, mainly due to an increased nitric oxide production [53]. Accordingly, in CD4+ T cells from MS baseline subjects we detected an increased Δψm in comparison to the control group while, during GA treatment, we found a gradual reduction of Δψm, suggesting a restored OXPHOS activity.

Defects in mitochondrial activity are often associated with greatly increased ROS and the mitochondrial respiratory chain is one of the major sources of endogenous ROS, together with other oxidative enzymes, such as plasma membrane oxidases [54]. In order to counteract an excess of ROS, cells have evolved several antioxidant defences, including enzymes, and small molecules, such as glutathione. The intracellular ROS-scavenging system is represented by glutathione peroxidase, peroxiredoxins, glutaredoxins, thioredoxins, catalases and superoxide dismutases, the latter located in both cytosol and mitochondria.

Previous studies on patients with MS have shown increased free radical activity and/or reduction in antioxidant enzymes in comparison to control subjects [55,56,57,58,59,60]. A recent study carried out by our group showed a significant reduction of SOD activity in CD4+ T cells of MS patients [7]. In the present study we found that GA treatment was able to increase SOD activity, which was significantly reduced in MS baseline. On the other hand, glutathione peroxidase activity showed no differences between baseline and GA treated subjects whereas catalase activity was significantly reduced in 12 months treated patients. This last result may be due to the decrease in H2O2 rate.

In MS first diagnosed patients no subjected to a pharmacological therapy defects in mitochondrial respiration and the increase in ROS production were accompanied by a stimulated glycolytic rate. Accordingly, we found an increase in HK and PFK-1 activities, both significantly reduced in 6 and 12 months treated subjects. HK and PFK-1 are among the main controlling steps of the glycolytic flux, the first catalyzed the ATP-dependent phosphorylation of glucose to form glucose-6-phosphate, the second involved in the phosphorylation of fructose 6-phosphate [61,62].

T cell activation is dependent on high rates of glycolysis and, therefore, dependent on a rapid efflux of lactate from T cells [63]; interestingly, extracellular lactate release was strongly decreased in CD4+ T cells of 12 months treated patients respect to baseline subjects and this process was accompanied by an increase in the ratio of mitochondrial ATP over glycolytic ATP. Co-stimulation of T cells receptor lead to an upregulation of glucose transporter 1 (GLUT-1) gene expression and glucose uptake [64], MCT, also known as monocarboxylate transporters (MCTs), belonging to the SLC16 gene family export of lactate, is essential to the maintenance of the hyper-glycolytic cell phenotype. In CD4+ T cells of MS patients, GLUT-1 expression showed a significantly reduction in 12 months treated subjects respect to baseline; a parallel increase in MCT1 lactate transporter was observed in MS patients, although no statistically significant differences were found between baseline subjects, 6 and 12 months treated.

5. Conclusions

These results, on the whole, provide new insights into the therapeutic effects of GA on CD4+ T cells, as we demonstrated an additional way of action of this treatment, able to restore OXPHOS activity and, consequently, oxidative stress response. Since metabolic alteration represent an important variable in MS, it could act as a possible targets of intervention able to modify MS pathology.

Author contributions

Conceived and designed the experiments: LDR, AF, MM. Performed the experiments: LDR, AF. Analysed the data: AD, VZ, MM. Contributed reagents/materials/bioinformatics tools: FDR, GT, VZ, MM. Wrote the paper: LDR, AF, MM.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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