1-2024

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Studying the interaction between two proteins in healthy human subjects will help us to establish better understanding of TDP-43 protein in biology of mitochondria so that we will enhance our understating of the biology of Alzheimer's disease.

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Abstract

Optic atrophy 1(OPA-1) is a GTPase protein that controls mitochondrial fusion, cristae integrity, and mtDNA maintenance. In neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), the mitochondrial network morphology is compromised. Studies on TAR-DNA binding protein 43 (TDP-43) has been the focus in our lab. OPA-1 and TDP-43 interaction may shed a light on how aberrant TDP-43 interacts with OPA-1, which will lead to mitochondrial dysfunction. The preliminary study tested the idea of whether OPA-1 and TDP-43 are physically interacting in human platelet derived mitochondria obtained from healthy human subjects.

Intention of Study

Alzheimer's disease is the most common cause of dementia in the United States. An estimated 6.2 million people in the US over the age of 65 are currently living with Alzheimer's disease. Projections estimate this number could grow to 13.8 million by 2060, hence, it is crucial to discover a way to slow the progress or cure the disease.

Our lab had identified platelet TAR DNA-binding protein (TDP-43) as a potential blood-based biomarker. We have observed the presence of TDP-43 protein in mitochondria-enriched pellets obtained from human platelets. We studied the interaction between mitochondrial Optic Atrophy-1 (OPA-1) and TDP-43. OPA-1 is involved in many functions such as oxidative phosphorylation, mitochondrial DNA maintenance, mitochondria cristae integrity, and mitochondrial fusion. In Alzheimer's disease, TDP-43 is mislocalized from the nucleus and accumulates in the cytosol and possibly in mitochondria. Our overarching hypothesis is that TDP-43 may physically interact with OPA-1 in platelet-derived mitochondria obtained from Alzheimer's disease patients, which will interfere with OPA-1 homeostasis and mitochondrial integrity. Our preliminary data obtained from immunoblotting and High-Performance Immunoprecipitation indicate that there are not only multiple OPA-1 isoforms, but also a potential interaction between TDP-43 and OPA-1 in platelet-derived mitochondrial lysate. OPA-1 and TDP-43 are both related to the mitochondria and are affected in patients with Alzheimer's disease.

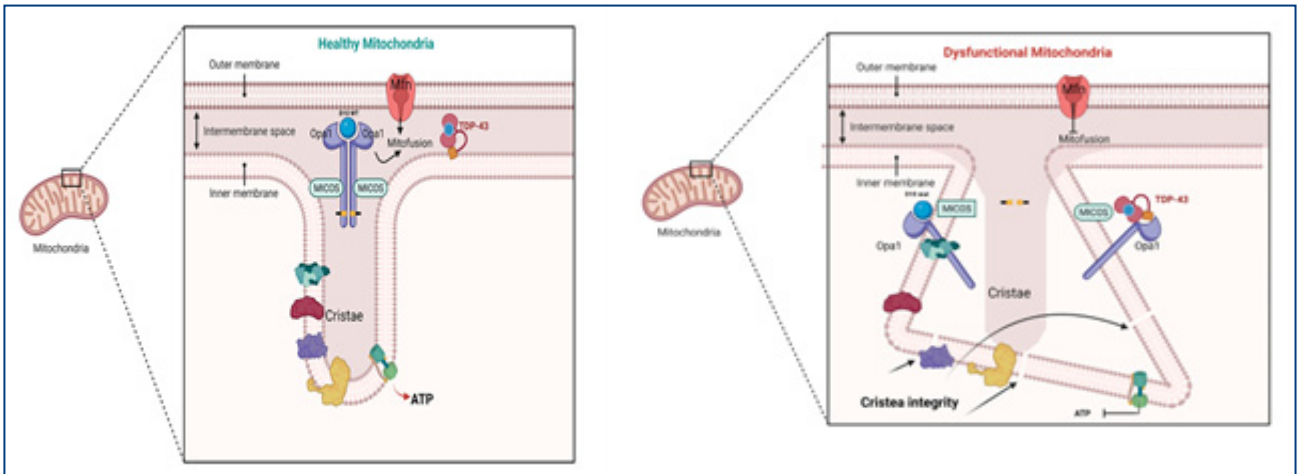

Figure 1. OPA-1 binding partners in health and disease condition. In healthy mitochondria OPA-1 protein is in homodimer (Left panel). In a disease states, TDP-43 interacts with OPA-1 protein and keeps them apart in dysfunctional mitochondria (right panel). Since OPA-1 lost its dimerization, mitochondrial cristae also lost integrity; hence mitochondria become dysfunctional organelle.
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Thus, studying the interaction between these two proteins in healthy human subjects will help us to establish better understanding of TDP-43 protein in biology of mitochondria so that we will enhance our understating of the biology of Alzheimer's disease.

Introduction

Platelets are small mitochondria-containing anucleated cells 3-5 µm in size that patrol the vasculature to maintain the homeostasis by preventing blood loss and promoting wound repair.[1] Platelets possess five to eight functional mitochondria per cell,[2] making platelets a feasible venue to study mitochondria relevant research in neurodegenerative diseases. Platelets provide easy access to human tissue with a less invasive method of obtaining human samples (requires 8-10 mL of whole venous blood). We have shown that platelet TDP-43 protein reflects chemical changes in the central nervous system in neurodegenerative diseases.[3] OPA-1 is critical for mitochondria fusion, cristae integrity, and mtDNA maintenance.[4-6] TDP-43 is a nuclear protein; however, this protein has the ability to shuttle back and forth between the nucleus and cytosol,[7] and it was detected in human platelets in our laboratory.[8] We have observed the presence of approximately 25 kDa truncated TDP-43 in mitochondria-enriched pellets obtained from human platelets.

Recent findings and knowledge of the mechanistic function of OPA-1 protein and relevant isoforms offer a starting point for development of a more precise and defined therapeutic prospect for neurodegenerative disease.[9] In this preliminary study, we aim to identify the number of isoforms of human OPA-1 that detectable in human blood-derived platelet lysate. The notion of TDP-43 induced disruption of the OPA-1 homeostasis that leads to deficits in mitochondrial fusion signifies the role of TDP-43 in mitochondria in neurodegenerative diseases (Figure 1).

Our goal was to test the hypothesis that OPA-1 and TDP-43 physically interact in platelets obtained from healthy human subjects. Therefore, it is necessary to demonstrate the feasibility of protein-protein interaction in healthy human platelets and thereafter apply the same approach to platelets obtained from patients with neurodegenerative diseases. We will test this hypothesis with two experimental approaches:

1. to demonstrate OPA-1 presence and its isoforms in platelets obtained from healthy human subjects; and

2. to demonstrate the physical interaction between OPA-1 and TDP-43. OPA-1 and TDP-43 will be immunoprecipitated by their respective antibodies. Both immunoprecipitated proteins will be cross-immunoblotted with their respective antibodies (i.e. OPA-1 immunoprecipitate will be probed with anti-TDP-43 antibody. TDP-43 immunoprecipitate will be probed with anti-OPA-1 antibody).

Material and Methods

Platelet Cytosol Preparation

Human platelets were obtained from a local blood bank. We used about 50-100 mL of clinical grade platelets already isolated and stored in the facility of the Community Blood Bank of Kansas City. The platelet suspension was subjected to centrifugation at 1,200 x g for 15 minutes. The supernatant was removed and the pellet was resuspended in citrate wash buffer. At this stage some of the platelets pellets were kept at 80°C for mitochondria isolation. Some of the platelet pellets were resuspended in citrate wash buffer (11 mM glucose,128 mM NaCl; 4.3 mM NaH2PO4; 7.5 mM Na2HPO4; 4.8 mM Na-Citrate; 2.4 mM citric acid, pH 6.5) for preparation of platelet lysate. The suspension was subjected to centrifugation at 1,200 x g for 15 minutes. The platelet pellet volume was about 3 mL. We resuspended the platelet pellet in 3 mL of lysate buffer (250mM sucrose; 1mM EDTA; 10mM Tris, pH 7.4) including 6 µL of protease inhibitor cocktails and 24 µL of phosphatase inhibitor cocktails. The platelet resuspension was incubated on an ice bucket for 20-25 minutes and the material was sonicated (setting 3, 20 seconds in continuous) in order to rupture the platelets. The platelet lysate was subjected to centrifugation at 20K x g for 30 minutes. Clear supernatant was saved for next phase of the experiment and membrane fragment containing pellet was discarded. Protein concentration was assessed by BCA assay. Platelet cytosol fractions were stored at -80°C until use.

Mitochondria Isolation from Frozen Human Platelets

An aliquoted platelet pellet (~ 3 mL) was gently resuspended in 3 mL of citrate wash buffer. The platelet suspension was subjected to centrifugation at 1,200 x g for 15 min at room temperature. The supernatant was decanted and the platelet pellet was resuspended in 25 mL of mitochondria isolation buffer, Mito-IB, (320mM sucrose; 10mM HEPES (free acid); 0.5mM EGTA, pH adjusted to 7.4 with KOH). We used 1 mL of Mito-IB for every $2x10^9$ platelets. We added about 195 µL of Proteinase K solution and gently inverted for five minutes at room temperature. The platelet suspension was gently homogenized by 30-40 passes in a Teflon pestle Potter- Elvehjem glass homogenizer placed in an ice-bucket without getting froth. We also added 1 µL of protease inhibitor cocktails (Calbiochem #539134) for every 2 mL of suspension. The platelet homogenate was subjected to centrifugation at 1,300 x g for 10 min at 4°C. The supernatant was saved and the resulting pellet was resuspended in 1 mL of Mito-IB and the suspension was subjected to centrifugation (1,300 x g for 10 min at 4°C). The supernatants were combined and the pellet was discarded. The poled supernatants were subjected to centrifugation at 13K x g for 10 min at 4°C to obtain a crude mitochondria pellet. The pellet was resuspended in 2.5 mL of Mito-IRB, pH 7.4. 250 µL of the resuspended crude mitochondria-enriched sample was layered on previously prepared 15% Percoll layer. We used 10 individual 15% Percoll density gradient for isolating a sufficient amount of mitochondria. All tubes were subjected to centrifugation at 21K x g for 8 minutes at 4°C without break so that Percoll gradient will not be disturbed at the end of the centrifugation. We harvested the mitochondria-enriched hazy band near the bottom of the tubes. Mitochondria- enriched fractions were combined and resuspended in 1 mL of Mito-IRB without EDTA, and re-centrifuged at 22K x g for 10 min at 4°C to remove the Percoll residue. The supernatant was removed, the pellet was washed one more time with 1 mL of Mito-IRB without EDTA, and final mitochondria–enriched pellet was obtained and stored in packed-ice at 4°C for subsequent immunoprecipitation process.

High Performance Immunoaffinity Chromatography

Ten µg of the primary antibodies (i.e. a-TDP-43 and a-OPA-1 antibodies) were chemically cross-linked in Protein-A coupled resin by disuccininilimidyl suberate (DSS) following the manufacturer's protocol (ThermoScience). We used tip-column manufactured by Phytips (Biotage # PTR-92-20-01) to cross-link the primary antibodies according to the manufacturer's protocol. 200 µL of tip-column was placed on a programmable electronic pipette (RAININ E4XLS) for performing the immunoprecipitation procedure. The program was fully driven by a software (PureSpeed) and pipetting error was minimized. The protocol flow-chart was illustrated in Figure 2.

The acidic eluate from the tip-column was

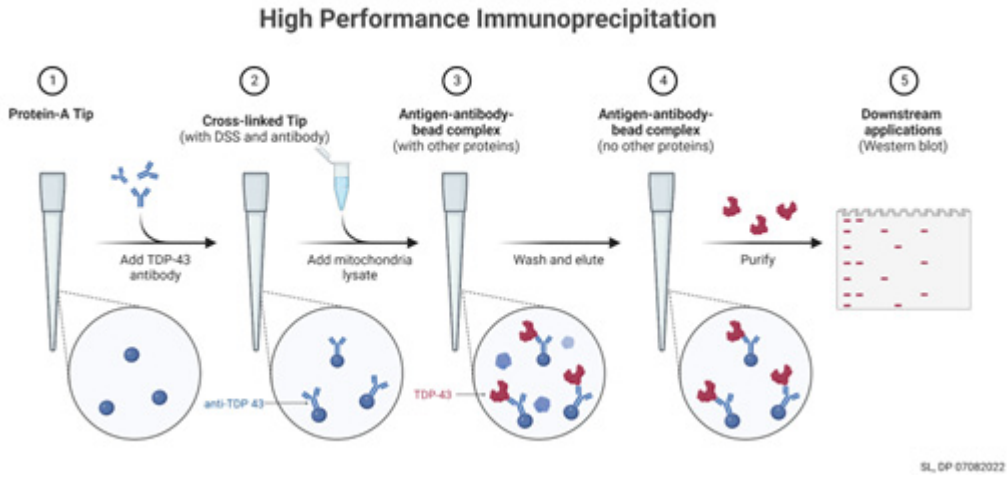

Figure 2. High performance immunoprecipitation flow-chart. Anti-OPA-1 and anti-TDP-43 antibodies were chemically cross-linked to Protein-A coupled resin. The immunoprecipitation procedure was fully automated. The eluate was analyzed by immunoprobing assay. *Figure created with BioRender.com*

automatically collected in a 1.5 mL microtube containing 5 µl of neutralizing buffer (1 M Tris-HCL, pH 9.5). The tube content was mixed well and stored in a -20°C freezer until use.

Immunoblotting

The eluates collected from High Performance Immunoaffinity Chromatography (HPIP) procedure were loaded on 4-20% gradient SDS/polyacrylamide gel and proteins were resolved by classical electrophoresis (100 V; 1h or until the dye-front reach to just above the bottom of the gel). The resolved proteins were transferred onto a PVDF membrane (75 V; 90 min without methanol). The membrane was stained for total protein to assess the transfer efficiency. The membrane was blocked in 50% fish serum blocking buffer (ThermoScientific # 37527) for one hour at room temperature (RT) and subsequently incubated in relevant primary antibody solution (1/500 a-OPA-1 antibody. 1/1000 a-TDP-43 antibody) for overnight at 4°C. The next day, the primary antibody solutions were removed, the membrane was washed 3 x 5 min and incubated with infrared dye-tagged secondary antibody solution for 1 hour at RT. The PVDF membrane was washed with a Tris Buffer Saline-Tween-20 (TBST) buffer 5 X 5 min and one final wash with TBS buffer excluding Tween-20 detergent to eliminate auto fluorescence caused by detergent. The PVDF membrane was imaged by LICOR-Odyssey system, and protein bands were analyzed by Image studio (v.5.2.)

Results

Both platelet cytosol and mitochondria lysates obtained from human platelets were resolved in 4-20% gradient SDS/PAGE. The resolved proteins were transferred onto PVDF membrane and proteins were immunoprobed by anti-OPA-1 antibody (1:1000 dilution). Figure 3A depicts the global OPA-1 profile of human platelet cytosol. Eight OPA-1 positive protein bands were identified (arrow). We have also included mouse brain cell lysate as a positive control for OPA-1 immunodetection. Figure 3B shows only two major protein bands in mitochondrial lysate isolated from human platelet. We have tested these proteins under reducing and non-reducing condition. The proteins did not resolve well and stayed on the top of the gel (~250 kDa) whereas at least two protein species were identified (79 and 55 kDa) under the reducing conditions.

To interrogate which of the OPA-1 positive proteins in mitochondrial lysate physically interact with TDP-43, we utilized a high-performance immunoprecipitation (HPIP) approach. We chemically cross-linked anti-TDP-43 antibodies and anti-OPA-1 antibodies in Protein-A conjugated tip-columns. Both TDP-43 and OPA-1 proteins were pull-down from mitochondrial lysates prepared from human platelets. The eluates were subjected to 4-20% gradient SDS/PAGE and subsequently immunoprobed with anti-TDP-43 and anti-OPA-1 antibodies. Figure-4 A shows immunoprobing of both TDP-43 and OPA-1 HPIP. A positive TDP-43 immunoblotting in OPA-1 HPIP was observed (right-hand side of the blot). TDP-43 HPIP

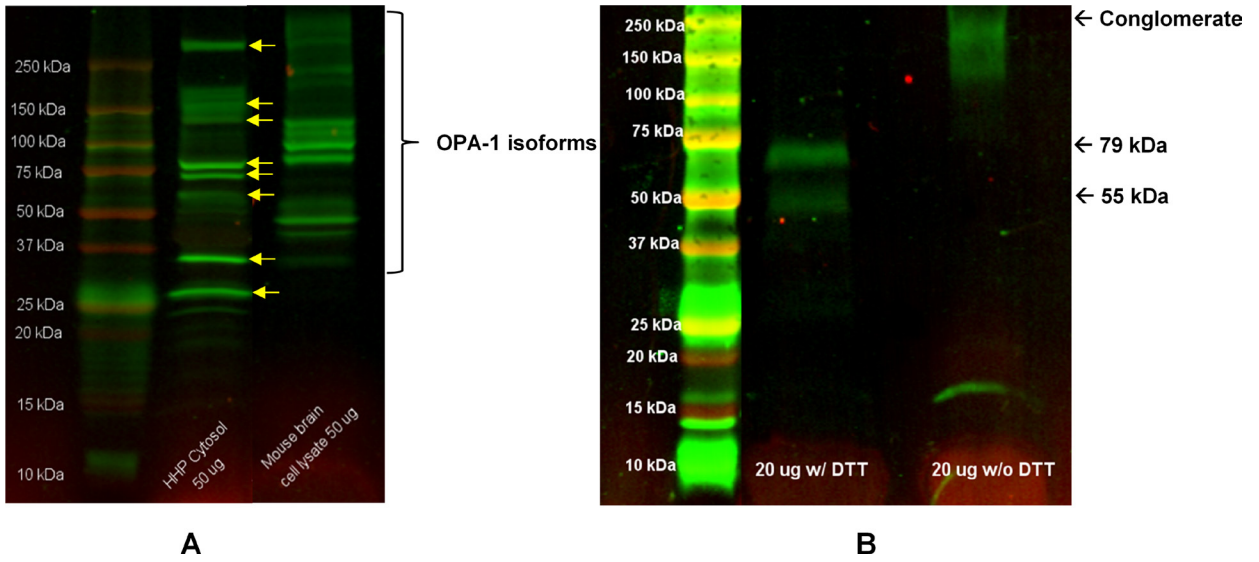

Figure 3. OPA-1 profile in human platelet cytosol and mitochondrial lysate. Human platelet cytosol and mitochondrial lysate obtained from human platelet lysate were analyzed by immunoblotting assay.
(A) Anti-OPA-1 pan antibody recognized 8 isoforms in human platelet cytosol in different protein levels (pointed with arrows). We include mouse brain lysate as positive control.
(B) Mitochondria-enriched pellet suspension was analyzed by an anti-OPA-1 pan antibody. Two isoforms were identified under reducing conditions. These proteins remained in conglomerate in the electrophoretic field (ask author) under non-reducing conditions.

(left hand side of the blot) eluate was also labeled by anti-TDP-43 antibody. Figure 4A showed that anti-OPA-1 antibody labeled about 25 kDa protein that pulldown by TDP-43 HPIP. There was an additional low molecular weight protein labeled by anti-OPA-1 Antibody at above 15 kDa marker. OPA-1 antibody did recognize two protein species that were pulldown by OPA-1 HPIP (arrow).

Discussion

We have made some interesting observations of the presence of ~25 kDa and ~ 17 kDa TDP-43 fragments pulldown by TDP-43-HPIP and immunolabeled by anti-OPA-1 antibody in mitochondria-enriched pellet prepared from human platelets. We are unclear as to the function of this fragmented TDP-43 in mitochondria-enriched pellets, as this fragment of TDP-43 is considered to be toxic to cells. Liu et al.[10] proposed a hypothesis that TDP-43 may interfere normal function of OPA1, which is maintaining cristae integrity and mitochondrial fusion. Demonstrating the interaction of OPA-1 and TDP-43 in human platelet-derived mitochondria is critical since this approach can be used for demonstrating the physical interaction that provides some explanation for our understanding how mitochondria integrity is compromised in neuro degenerative diseases.

In this preliminary study, we provided some evidence that there are several isoforms of OPA-1 in human blood serum. We were able to label them with a pan anti-OPA-1 antibody (Figure 3A). We were unable to identify isoform specific OPA-1; it is because the unavailability of specific antibodies for each isoform. Nevertheless, we attempted to demonstrate that OPA-1 proteins interact with TDP-43; but we do not know which specific isoform of OPA-1 interacts the best with TDP-43. More research is needed in this field.

Anti-TDP-43 antibody-HPIP and anti-OPA-1 antibody-HPIP methods yielded some interesting results. Anti-TDP-43 antibody labeled full size TDP-43 proteins as expected at ~48-50 kDa marker; however, anti-OPA-1 antibody did not label the full size of TDP-43, but it did label the fragmented TDP-43 at ~25 kDa marker (Figure 4B). This region is located in C-terminus of TDP-43 and highly rich in glycine, glutamine, and asparagine[11]. There was an additional distinct protein band at ~17 kDa marker in both TDP-43-HPIP and OPA-1-HPIP eluates. The same ~17 kDa protein band intensity was strong in flow through fractions. We do not know what these

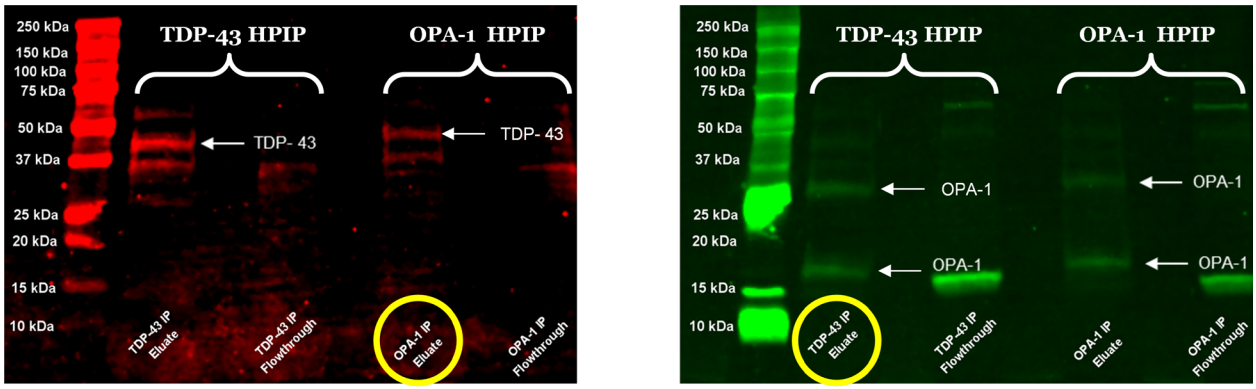

Figure 4. Co-immunoprecipitation profile of TDP-43 and OPA-1 in mitochondrial lysate.
(A) Both TDP-43 and OPA-1 HPIP eluates were resolved in 4-20% gradient SDS/PAGE under reducing conditions, and proteins were immunoblotted by anti-TDP-43 antibody. Both HPIP eluates were positively labeled with ant-TDP-43 antibody. It is important to note that OPA-1 and TDP-43 proteins were co-eluted.
(B) Both OPA-1 and TDP-43 HPIP eluates were resolved in 4-20% gradient SDS/PAGE under reducing conditions, and resolved proteins were immunoblotted with anti-OPA-1 antibody. Anti-OPA-1 antibody did not label its own protein immunoprecipitated by OPA-1 HPIP; instead, anti-OPA-1 antibody recognized two lower molecular weight proteins (25, kDa and ~ 17 kDa marker) in both HPIP eluates.

proteins are at present time. They don't seem to be fragmented TDP-43 since TDP-43 protein produces two major protein fragments, 35, and 25 kDa.[12] Anti-OPA-1 antibody did not perform well in terms of recognizing OPA-1 protein(s) pull down by OPA-1 HPIP (Figure 4B) whereas OPA-1 HPIP pulldown TDP-43 proteins (Figure 4A). We demonstrated that TDP-43 and OPA-1 physical interaction is possible under *in vitro* conditions; however, OPA-1 antibody did not label well the OPA-1 protein pulldown by OPA-1-HPIP. OPA-1 antibody, however, did recognize ~89 kDa protein in flow through fractions of both HPIP, indicating that OPA-1 is present in the sample, but not efficiently pulled down by HPIP (i.e. TDP-43 and OPA-1 HPIP). In addition, the mitochondrial lysate sample was not sufficient to perform effective HPIP. As a result, both TDP-43 and OPA-1 proteins are physically interacting in mitochondrial lysate obtained from human platelet. This interaction needs to be verified with a mammalian cell/tissue system where more mitochondria can be harvested.

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Disclosure

None reported. Artificial intelligence was not used in the study, research, preparation, or writing of this manuscript. **MM**