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Delay of gCJD aggravation in sick TgMHu2ME199K mice by combining NPC transplantation and Nano-PSO administration

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ABSTRACT

gCJD is a fatal late-onset neurodegenerative disease linked to mutations in the PRNP gene. We have previously shown that transplantation of neural precursor cells (NPCs), or administration of a nano-formulation of pomegranate seed oil (Nano-PSO, GranaGard), into newborn asymptomatic TgMHu2-ME199K mice modeling for E200K gCJD significantly delayed the advance of clinical disease. In the present study, we tested the individual and combined effects of both treatments in older and sick TgMHu2ME199K mice. We show that while transplantation of NPCs at both initial (140 days) and advance clinical states (230 days) arrested disease progression for about 30 days, after which scores rapidly climbed to those of untreated Tgs, administration of Nano-PSO to transplanted TgMHu2ME199K mice resulted in detention of disease advance for 60-80 days, followed by a slower disease progression thereafter. Pathological examinations demonstrated the combined treatment extended the survival of the transplanted NPCs, and also increased the generation of endogenous stem cells. Our results suggest that administration of Nano-PSO may increase the beneficial effects of NPCs transplantation.

1. Introduction

Creutzfeldt-Jakob disease (CJD), a human prion disease, may present as a transmissible, sporadic (sCJD) or a genetic disease (gCJD) (Brown and Mastrianni, 2010). The different etiological presentations share important features, such as late-onset appearance/long incubation times, and most important, the accumulation of disease related forms of the PrP protein in the central nervous system (CNS) followed by a fatal outcome (Kovacs and Budka, 2008). In addition, there are specific features for genetic prion diseases, which are linked to mutations in the *PRNP* gene. Individuals carrying such mutations are born healthy, then at some point in their adult life are affected with the fatal disease (Meiner et al., 2011; Tee et al., 2018). This indicates a very long "incubation" period in which metabolic (Keller et al., 2019) and may be other unknown events leading to disease outbreak occur. It also allows for years of preventive treatments once such reagents are identified.

Candidate treatments for prion diseases are divided into those associated with the reduction of PrP expression and/or accumulation to those related to general brain neuroprotection. In the first category investigators developed antibodies against different forms of PrP (Campana et al., 2009; Heppner and Aguzzi, 2004; Sakaguchi and Arakawa, 2007; Sakaguchi et al., 2009), or molecules that can either inhibit the PrP^{C} to PrP^{Sc} conformational change (Prusiner et al., 1987) or affect PrP binding to other molecules (Bardelli et al., 2018; Gunther et al., 2019). Also, antisense oligonucleotides that can reduce the expression of all PrP molecules (Raymond et al., 2019; Reidenbach et al., 2019) were recently shown to delay prion infection. In the second category, we found reagents designated to reduce cell damage and subsequently cell death even in the presence of pathological insults such as PrP aggregates (Halliday et al., 2017; Mizrahi et al., 2014). Indeed, we have shown in our lab that Nano-PSO, a brain targeted nano-formulation of PSO comprising high levels of punicic acid (PA), delayed disease onset and increase survival when administered to young and asymptomatic TgMHu2ME199K mice (Tgs), a transgenic mouse model mimicking for genetic CJD linked to the E200K PrP mutation (Binyamin et al., 2017; Friedman-Levi et al., 2011; Keller et al., 2019). These mice are born healthy, start to present neurological abnormalities at 5–6 months of age and deteriorate to a terminal stage between 12 and 15 months of age (Friedman-Levi et al., 2011). Nano-PSO administration also exerts beneficial clinical effects on mice models of Alzheimer's disease (AD) and multiple sclerosis (MS)

Data availability: The authors confirm that the data supporting the findings of this study are available.

Disclaimer: Gabizon is the founder and scientific director of Granalix BioTechnologies.

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(Binyamin et al., 2015, 2019). As opposed to natural PSO (Pereira de Melo et al., 2018; Yuan et al., 2009), Nano-PSO administration did target conjugated linoleic acid, the main metabolite of PA, to the brain, where it exerts its activity as a calpain inhibitor (Binyamin et al., 2019; Lee et al., 2013). Similarly to Nano-PSO administration to newborn/asymptomatic TgMHu2ME199K mice, transplantation of NPCs, also designated as a neuroprotective treatment (Ben-Hur and Goldman, 2008; Koutsoudaki et al., 2016; Martino and Pluchino, 2006), delayed disease aggravation when administered to 2-day-old TgMHu2ME199K mice (Frid et al., 2018). Although in some cases NSCs may exert their therapeutic effects by directly replacing missing cells (Kumamaru et al., 2018), the beneficial effect of NSCs in disease models may be also attributable to alternative biologic properties such as a bystander neuroprotective effect (Ding et al., 2013; Einstein and Ben-Hur, 2008). Transplantation into the CNS has shown beneficial effects in several models of disease (De Feo et al., 2012; Einstein et al., 2006; Harrower et al., 2006), probably by inducing bystander therapeutic effects that ameliorate neuroinflammation, protect neighboring brain cells from injury, and facilitation of endogenous repair processes (Nishri et al., 2019; Zuo et al., 2015). Such treatment was also shown to have a beneficial effect on infectious prion disease manifestation (Relano-Gines et al., 2009, 2011).

In this work, we tested the effects of NPC transplantation alone or in combination with Nano-PSO administration to TgMHu2ME199K mice in symptomatic stages, which may represent human PrP mutation carriers at the stage of disease diagnosis. To this effect, NPCs were transplanted into brains of these Tgs at either 140 or 235 days (minimal and significant disease signs, respectively). Concomitantly, Nano-PSO was administered continuously in the drinking water to untransplanted as well as to transplanted TgMHu2ME199K mice from 140 days onward. Our results show that NPC transplantation to these mice at the clinical stages resulted in arrest of disease advance for about 30–40 days, after which scores rapidly climbed to the levels of untreated mice. When Nano-PSO was administered to TgMHu2ME199K mice simultaneously with NPC transplantation, the period of arrest doubled, concomitant with a longer survival of the transplanted cells. Subsequently, the disease scores of mice subjected to the combined treatment climbed gradually and reached the levels of Nano-PSO treated TgMHu2ME199K mice (Binyamin et al., 2017), demonstrating a synergistic effect for the combined treatments. Regardless of the treatment, there was no disease-related PrP accumulation in the transplanted NPCs, indicating as shown before (Frid et al., 2018), that there is no transmission of infections prions from the TgMHu2ME199K brains to the transplanted stem cells.

2. Materials and methods

2.1. Ethical statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were conducted under the guidelines and supervision of the Hebrew University Ethical Committee, which approved the methods employed in this project (Permit Number: MD-15 14,462-5).

2.2. Generation of TgMHu2ME199K mice

TgMHu2ME199K mice (harboring the equivalent of the pathogenic human E200K PrP mutation) have been maintained as a breeding colony in our laboratory continuously since they were generated (Friedman-Levi et al., 2011). For this project, TgMHu2ME199K mice were crossed with PrP ablated mice. Both male and female mice were used, maintaining equal distribution between experimental groups. No systematic randomization protocol was employed.

2.3. TgMHu2ME199K mice scoring system for disease signs

Mice were followed twice a week for the appearance of spontaneous neurological disease. Each mouse was tested by 2 different investigators, one measuring scores and the other assigning the score to the designated groups, constituting blinded conditions. Mice were scored for disease severity and progression according to the next scale: no clinical score = 0; initial hind limb weakness presented by smaller legs spread, lower body position, and gentle assembly of hind limbs while walking = 1; partial hind limb weakness = 1.5; significant hind limb/s weakness = 2; significant hind limb/s weakness or partial paralysis with significant legs clasping = 2.5; full paralysis in one limb = 3; full paralysis in one limb and weakness at the other hind foot = 3.5; full paralysis in both limbs = 4. Any other sign of illness such as hunchback or glued fur added 0.5 point to the score. Mice were sacrificed at designated time points or when they were too sick or paralyzed to reach food and water, or after losing 20% body weight, according to the ethical requirements of the Hebrew University Animal Authorities (Binyamin et al., 2017; Frid et al., 2018).

2.4. Isolation and growth of mouse NPCs

NPCs were isolated from the forebrain of C57BL/6JOlaHsd embryos on day 13.5 of pregnancy and grown as free-floating neurospheres (Cohen et al., 2014; Fainstein et al., 2013a). Briefly, the tissue was dissociated using Earle's Balanced Salt Solution containing 0.25 mg/mL trypsin and 10 mg/mL DNase I (5 minutes at 37 °C). Tissue was further mechanically dissociated by aspiration and expulsion with a 5-mL Falcon pipette. Dissociated cells were transferred to T-75 flasks and grown as suspended neurospheres in a serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium containing B27 supplement. Cells were supplemented daily with basic fibroblast growth factor 2 (10 ng/mL; R&D systems) and epidermal growth factor (20 ng/mL; PeproTech, Rocky Hill, NJ).

2.5. Intracerebroventricular transplantation

Ten thousand cells in 2 μL DMEM/F-12 medium were injected per mouse using a Hamilton syringe (catalog #702) to symptomatic female and male TgMHu2ME199K mice (140 days old) as well as to sick TgMHu2ME199K mice (230 days old) into each lateral ventricle (intracerebroventricular transplantation), using a stereotaxic device (coordinates, bregma 0; 1 mm lateral; 2.3 mm depth). All mice were scored for neurological symptoms several times a week and sacrificed at designated time points as required by the experimental protocol. Brains were processed for pathological and biochemical experiments (Cohen et al., 2014; Fainstein et al., 2013a).

2.6. Administration of Nano-PSO to TgMHu2ME199K NPC transplanted/TgMHu2ME199K mice

Nano-PSO was administered to designated groups of TgMHu2ME199K mice in their drinking water. Concentrated Nano-PSO (16.5 mL) self-emulsion formulation was diluted in 300 mL of water to form a white emulsion with a final concentration of 1.6% oil, as previously described (Binyamin et al., 2017) and as defined in patent no. 14/523,408. All mice were scored for neurological symptoms and sacrificed at designated time points when required by the experimental protocol. Brains of sacrificed mice were processed for pathological and biochemical experiments.

2.7. Western blotting

Brain extracts form wild-type (wt), TgMHu2ME199K and TgMHu2ME199K treated mice at different time points (230, 380 days old) were homogenized at 10% (W/V) in 10 mM Tris–HCl, pH 7.4, and 0.3 M sucrose. Samples normalized by pierce BCA protein assay kit (Thermo Fisher Scientific) to 200 μg proteins in each brain sample for WB detection. Each sample of 200 μg brain homogenate was mixed with 2% sarcosyl on ice. For Proteinase K digestions, samples were incubated with 20 mg/mL Proteinase K for 30 minutes at 37 °C. All samples were subsequently boiled in the presence of SDS, subjected to 12% SDS PAGE and transferred to nitrocellulose membrane for 1.5 hours, 300 mA. Membranes were blocked with 3% milk for 1 hour and immunoblotted with α-PrP pAb RTC (Canello et al., 2010) over night, and developed with α-rabbit horse radish peroxoidase (Jackson Immune Research Laboratories, Inc) at a dilution of 1:10,000. Protein signals were obtained using an enhanced chemiluminescent western blotting detection method and developed using chemiluminescent substrates (solution A: 100 mM Tris–HCl, pH 8.5. and H_2O_2 30%; solution B: 100 mM Tris–HCl, pH 8.5, Luminol 250 mM [sigma A8511, Israel], and *p*-Coumaric acid 90 mM [sigma C9008, Israel]).

2.8. Pathological examinations and immunocytochemistry

Histological evaluations were performed on paraffin-embedded sections of brain samples from wt, TgMHu2ME199K and TgMHu2-ME199K treated mice at 3 different time points (180, 230, 380 days old). Sections were stained by immunofluorescence with an array of designated antibodies.

The antibodies used were mouse α-Nestin (ab11306) rabbit α-DCX (doublecortin) (ab18723), rabbit α-glial fibrillary acidic protein (GFAP) (Dako 70334), and rabbit α-PrP pAb RTC (Canello et al., 2010). Mice were evaluated for the presence of disease-related PrP as previously described (Glatzel et al., 2003; Kovacs et al., 2011; Muramoto et al., 1993). This method includes a harsh antigen-retrieval step that was shown to destroy PrP^C recognition and reveal only disease-related forms such as proteinase K resistant and/or aggregated PrP isoforms. Secondary antibodies (α-rabbit or α-mouse) coupled to Alexa Fluor 488 and 568 were used (Abcam). Nuclei were labeled with DAPI Fluoromount (Vector Laboratories) Confocal analysis was performed with Nikon A1R Confocal Laser Microscope System using the NIS-Elements C control software.

2.9. TUNEL

The In Situ Cell Detection Kit TMR red (Roche) was used to compare cell death rate on Paraffin-embedded sections of brain samples to form wt, TgMHu2ME199K, and TgMHu2ME199K treated mice at 3 different time points (180, 230, 380 days old).

2.10. Statistical studies

Statistical analysis was performed using IBM SPSS Statistics V.23. Data were analyzed using one-way analysis of variance (ANOVA) for

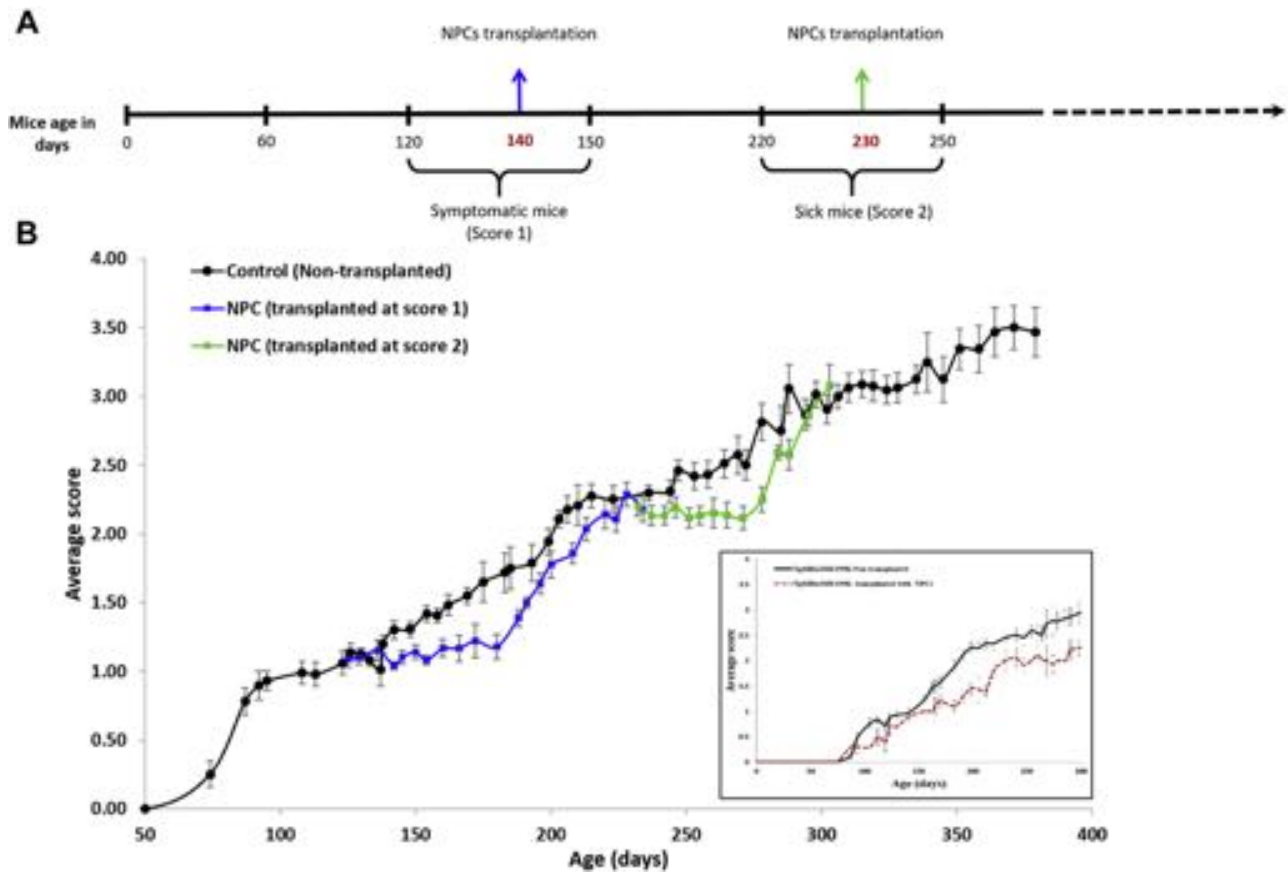

Fig. 1. Transplantation of NPCs into sick TgMHu2ME199K mice arrests disease advance. (A) Outline of experiment. (B) NPC spheres were transplanted into symptomatic 140-day-old ($n = 7$) and sick 230-day-old ($n = 13$) TgMHu2ME199K mice. Subsequently, mice were followed closely for disease scores until scores of transplanted groups reached those of untreated mice. The inserted graph in panel B represents the results of NPC transplantation into 2-day-old TgMHu2ME199K mice (Frid et al., 2018). Data represent means SD ($p < 0.05$). Abbreviations: NPC, neural precursor cell; SD, standard deviation.

the results of multiple groups presenting clinical score, selected periods of time were compared. The differences between the control group and the NPC transplanted groups were assessed by 1-way ANOVA followed by the paired 2-tailed Student's *t*-test. The data of all clinical score graphs are presented as mean ± standard error of the mean. The survival curves were compared using the Kaplan–Meier analysis with log-rank test calculating χ^2 on 3 degrees of freedom. Statistical analysis for additional experiments (quantification of immunohistochemistry) was done using ImageJ and analyzed by using one-way ANOVA for the results of multiple groups and the Tukey's post hoc test.

3. Results

3.1. Transplantation of NPCs into sick TgMHu2ME199K mice arrests disease advance

NPCs were transplanted into the brains of symptomatic TgMHu2ME199K mice either at 140 (symptomatic) or at 230 days (sick), as described in the methods. Transplanted and control TgMHu2ME199K mice were followed several times a week for advance of disease signs as described (Frid et al., 2018; Friedman-Levi et al., 2011). The outline of these experiments is depicted in Fig. 1A and the results in Fig. 1B. Our results show that as opposed to NPC transplantation into asymptomatic 2-day-old TgMHu2ME199K mice (see insert in Fig. 1B; Frid et al., 2018), which presents a slower rate of disease advance as compared to untreated mice throughout the treatment, transplantation of NPCs to symptomatic mice resulted in the arrest of disease advance at both time points of initiation for 30–40 days. After the arrest period, disease scores rapidly climbed to those of the untreated mice, indicating NPCs transplantation cannot change the long-term fate of these mice. Two main differences exist between transplantation to newborn as compared to adult TgMHu2ME199K mice. The first is the asymptomatic state of the recipient mice, which may allow to delay disease presentation before significant accumulation of disease-related PrP is in place (Keller et al., 2019), and the second is the newborn state of the recipient brain, which may change the fate and survival of transplanted NPCs (Giannakopoulou et al., 2011; Karkkainen et al., 2012).

Concomitant administration of Nano-PSO to NPCs transplanted mice increased the length of disease arrest as well as the period of survival.

Long-term administration of Nano-PSO to newborn, as well as to 3- or 8-month-old TgMHu2ME199K resulted in a significant delay of disease advance (Binyamin et al., 2017; Mizrahi et al., 2014). To establish whether there may be a synergistic beneficial clinical effect between Nano-PSO administration and NPC transplantation to TgMHu2ME199K mice, Nano-PSO was added to the drinking water of naïve 140-day-old TgMHu2ME199K mice as well as to similar ones transplanted with NPCs at this same time point. An outline of these experiments is depicted in Fig. 2A. Disease scores for all groups were recorded several times a week and are presented in Fig. 2B. Our results show that while NPC transplantation at 140 days resulted in arrest of disease advance for about 30–40 days, the combined Nano-PSO/NPC treatment doubled the length of the arrest to about 70–80 days. After that, disease scores of the TgMHu2ME199K (Reubinoff et al., 2001) mice treated with the combination slowly elevated to those of the TgMHu2ME199K mice

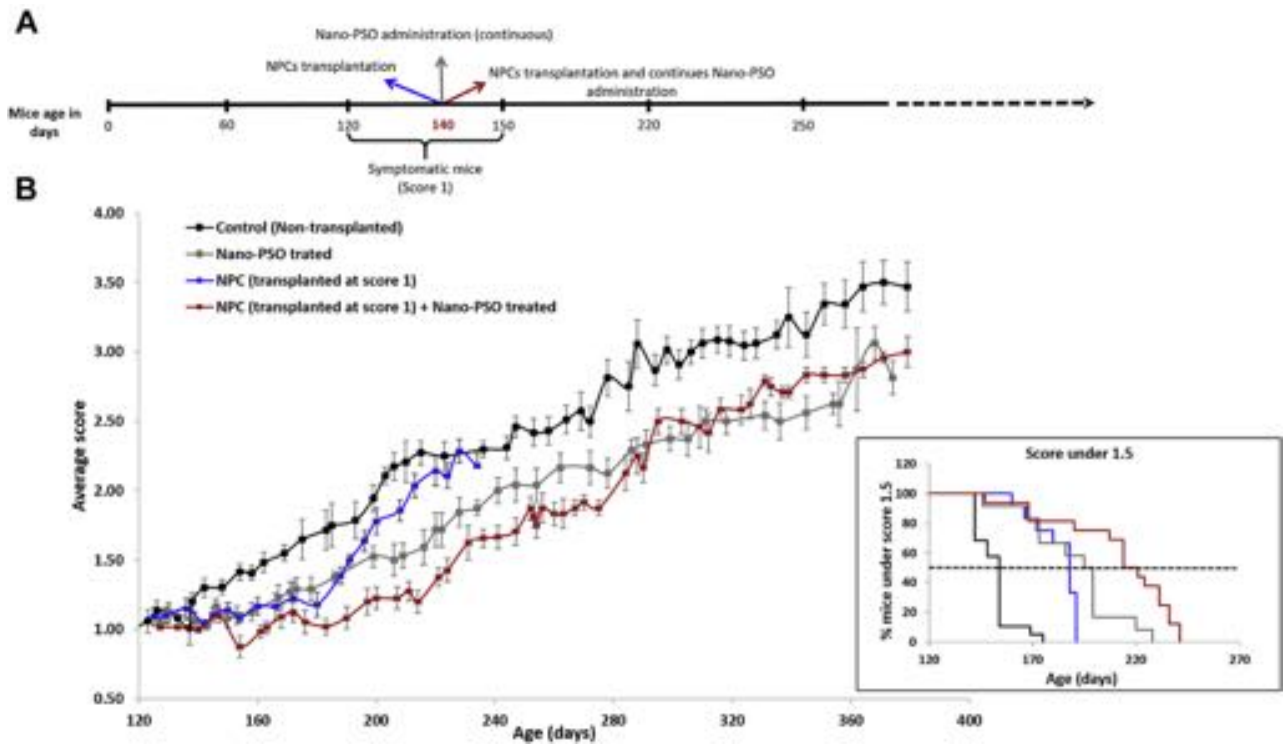

Fig. 2. Administration of Nano-PSO to NPC transplanted mice increased both the length of disease arrest as well as mice survival. (A) Outline of experiment. (B) TgMHu2ME199K mice transplanted with NPCs at 140 days were either left untreated ($n = 7$) or concomitantly treated with Nano-PSO ($n = 8$). These groups of TgMHu2ME199K mice, in addition to naïve TgMHu2ME199K mice and to a group only treated with Nano-PSO ($n = 13$), were followed for disease signs until 380 days. Mice in the NPC-only group were sacrificed when they reached the scores of naïve TgMHu2ME199K mice. One-way analysis of variance (Tukey's post hoc analysis) presents significant difference between TgMHu2ME199K untreated ($n = 13$) compared to all the treated groups in the period of 142–188 days of age ($p < 0.001$). In the period of 191–234 days of age, there was a difference between untreated Tgs ($n = 13$) to TgMHu2ME199K mice treated with Nano-PSO ($n = 8$) and those getting the combined treatments ($n = 10$) ($p < 0.001$). Inserted graph of Kaplan-Meier plot of TgMHu2-ME199K mice under score 1.5. Data represent means SD ($p < 0.05$). Abbreviations: NPC, neural precursor cell; Nano-PSO, Nano-Pomegranate Seed Oil; SD, standard deviation; Tgs, non-transplanted TgMHu2ME199K.

to which Nano-PSO was administered from 140 days, which by themselves were significantly lower than the scores of untreated TgMHu2ME199K mice. One-way ANOVA (as described in Section 2) presents significant differences between TgMHu2ME199K untreated ($n = 13$) compared to all the treated groups in the period of 142–188 days of age ($^{***}p < 0.001$). After that, and until day 234, there was a significant difference between naïve Tgs ($n = 13$) and TgMHu2ME199K mice treated either with Nano-PSO ($n = 8$) or Nano-PSO/NPCs ($n = 10$) ($^{***}p < 0.001$), but not with NPC alone. Most interestingly, there was a significant difference in the scores of TgMHu2ME199K mice treated with Nano-PSO and those getting the Nano-PSO/NPC combination until 284 days. Indeed, and as shown previously (Binyamin et al., 2017; Mizrahi et al., 2014), Nano-PSO administration to TgMHu2ME199K mice caused a slower rate of disease aggravation resulting in increased survival. We may conclude therefore that the combined treatment comprising both NPC transplantation and Nano-PSO administration in a symptomatic stage of TgMHu2ME199K mice resulted in a synergistic beneficial clinical effect, allowing for both an extensive period of disease arrest concomitant with longer survival.

3.2. Nano-PSO administration increased the survival of transplanted NPCs

Although transplantation of NPCs into newborn mice may result in the incorporation of these cells into the brain (Reubinoff et al., 2001), this is not the case for NPC transplantation into adult brains, after which these cells remain mostly in their spheres and are believed to induce a bystander neuroprotective effect (Fainstein et al., 2013b). Their beneficial effect lasts for a period of time that correlates with either their survival or their stem cell status, as determined by appropriate markers (Encinas and Fitzsimons, 2017). To investigate whether there is a correlation between NPC survival and the clinical effect of the diverse treatments described above, we immunostained relevant brain slices with designated antibodies and reagents. Brain sections comprising NPC spheres were tested for apoptosis of the transplanted cells by the terminal deoxynucleotidyl transferase (TUNEL) assay (Kyrylkova et al., 2012), and for their properties as stem cells by GFAP (stem cells and astrocytes), Nestin (all NPCs), and DCX (migratory neuroblasts) staining (Encinas and Fitzsimons, 2017; Zhang and Jiao, 2015). In addition, spheres and their surrounding brain tissue were immunostained for disease-related PrP, to explore the possibility of transmission of infectious prions from the sick TgMHu2ME199K brains to the wt spheres (Frid et al., 2018). The brain samples shown in Fig. 3 are from TgMHu2ME199K mice sacrificed at 180 and 230 days for NPC transplanted mice (end of the arrest period and end of experiment, respectively), and from 180 and 380 days for TgMHu2ME199K mice treated with the combined reagents (middle of arrest period and end of experiment). Fig. 3 shows that at 180 days, cells in the spheres present in the brains of mice treated by NPC transplantation alone were positive for TUNEL, GFAP, and Nestin, but negative for DCX. Samples from the same experiment at 230 days present fewer cells positive for Nestin and GFAP, suggesting most of the transplanted cells were no longer alive. In contrast, brain samples

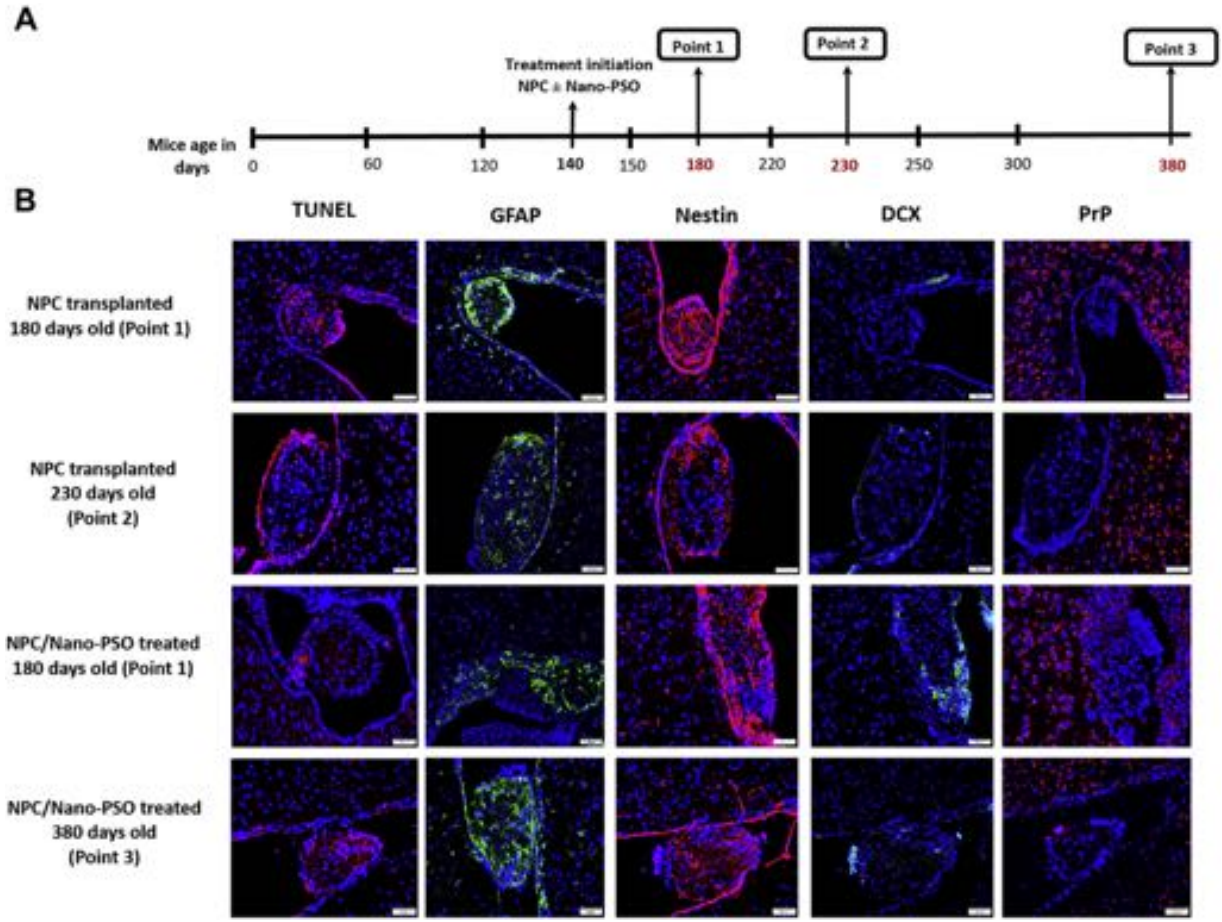

Fig. 3. Nano-PSO administration increased the survival of transplanted NPCs. (A) Outline of experiment. (B) Immunostaining of transplanted NPC spheres in coronal brain sections of mice treated in the presence or absence of Nano-PSO at several time points (180,230,380 days old). Sections were stained for TUNEL, GFAP, Nestin, doublecortin (DCX), and disease-related PrP (α PrP pAb RTC) [magnification ×20, scale bar 50 μm]. Abbreviations: Nano-PSO, Nano-Pomegranate Seed Oil; NPC, neural precursor cell.

from mice receiving the combined treatment present only traces of TUNEL staining at the 180-day time point, indicating a lower number of apoptotic cells. Most interesting, a significant number of cells in these spheres were positive for DCX, a marker of migratory oriented neuroblasts which indicates these cells were mature enough to differentiate in this direction (Encinas and Fitzsimons, 2017; Gleeson et al., 1999; Zhang and Jiao, 2015). Only significantly later, at the end of the combined treatment experiment (380 days), spheres became positive for TUNEL and negative for DCX, as was the case for the NPC transplantation only group at 180 days, when the arrest period was mostly terminated. These results are consistent with the possibility that a longer disease arrest in TgMHu2ME199K mice getting the combined treatment may result from a significantly longer survival period of NPCs conferred by Nano-PSO administration to the transplanted mice. Looking back at Fig. 2, we can see that for the combined group, the 380-day time point still represents a point in which disease scores are significantly lower than in the untreated TgMHu2-ME199K mice.

3.3. The combined NPC/Nano-PSO treatment increases the number of endogenous stem cells

Numerous studies demonstrate that during normal aging, certain areas of the brain retain pluripotent precursors with the capacity of self-renewal (Eriksson et al., 1998; Maslov et al., 2004). This feature, also known as adult neurogenesis, is partially impaired in neurodegenerative diseases such as in CJD and in AD (Fainstein et al., 2018, 2016; Mizrahi et al., 2014), but was shown to be corrected both by Nano-PSO administration as by NPC transplantation (Frid et al., 2018). Although in rare cases increased neurogenesis was found in brains of CJD or AD patients, this may constitute a brain effort to counteract the effects of chronic neurodegeneration (Gomez-Nicola et al., 2014). For mice modeling for AD, it was suggested that failure of resident NPC which provides tissue support may promote neurodegeneration (Fainstein et al., 2018; Scopa et al., 2019). To study whether the combined NPCs/Nano-PSO treatment increased neurogenesis, we subjected brain slices of wt, untreated TgMHu2ME199K mice as well as Tgs treated with Nano-PSO, NPCs, or with Nano-PSO/NPC, at different ages, to immunostaining with antibodies against both Nestin and DCX. We then looked into the subventricular zone and subepidermal zone areas, which are the sites in which adult stem cells are generated (Alvarez-Buylla and Lois, 1995; Jankovski et al., 1998). Results were quantified and analyzed by the ANOVA and Tukey's post hoc test. As expected (Fig. 4A, C, and D), brains of wt mice at both 180 and 380 days, present a significant higher number of Nestin positive cells than untreated TgMHu2ME199K mice of the same ages, indicating as before that neurogenesis is impaired in this mice model (Fainstein et al., 2016) ($p \leq 0.001$). Although both Nano-PSO administration and NPC transplantation correct neurogenesis to some extent in 180-day-old TgMHu2ME199K mice, only the combined treatments elevated neurogenesis significantly at this time point ($p \leq 0.005$). As for DCX staining (Fig. A, B, and D), there was a significant difference between wt and untreated TgMHu2ME199K mice only at 380 days ($p \leq 0.005$), which was significantly corrected by the combined treatment ($p \leq 0.01$). These results are consistent with the notion that restoring neurogenesis may be an important pathway in the treatment of neurodegenerative diseases.

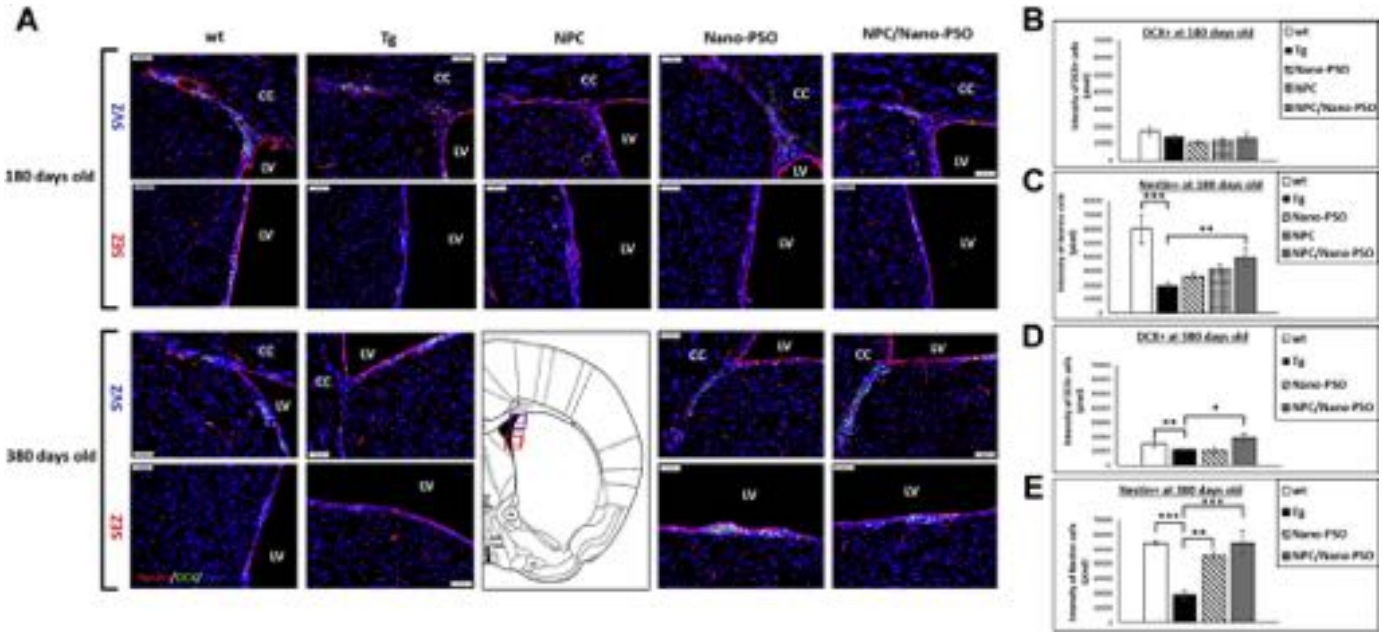

Fig. 4. The combined NPC/Nano-PSO treatment increases the generation of endogenous stem cells. (A) Coronal sections through the subventricular zone and subepidermal zone of brains from wt and naïve TgMHu2ME199K mice, as well as from Nano-PSO, NPC, and Nano-PSO/NPCs treated Tgs, at both 180 and 380 days were immunostained for Nestin (red) and DCX (green) [magnification ×20, scale bar 50 μm]. Quantitative assessment of Nestin and DCX immunostaining for all sections (bregma 0.38 mm) at 2 time points was quantified using one-way analysis of variance (Tukey's post hoc analysis) and presented in graphs B–E. (B) No difference was detected in DCX expression at 180 days between all groups when compared to wt ($n = 7$) or untreated Tgs ($n = 7$). (C) At 180 days, untreated Tgs ($n = 7$) compared to wt ($n = 7$) present significant difference in Nestin expression (***$p \leq 0.001$). When all treated groups were compared to untreated Tgs, only the combined treatment ($n = 8$) present significant elevation in Nestin levels (**$p \leq 0.005$). (D) At 380 days, there was a significant difference in DCX expression between untreated Tgs ($n = 6$) and wt mice ($n = 8$) (***$p \leq 0.001$). When all treated groups were compared to untreated Tgs, only the combined treatment ($n = 7$) presented significant difference in DCX expression (*$p \leq 0.01$). (E) At 380 days, the difference in Nestin expression between untreated Tgs ($n = 6$) and wt mice ($n = 8$) was statistically significant (***$p \leq 0.001$). Also, TgMHu2ME199K mice treated with Nano-PSO ($n = 7$) (**$p \leq 0.005$) and those getting the combined treatment ($n = 8$) (***$p \leq 0.001$) present a significant difference in Nestin expression when compared to untreated Tgs ($n = 6$). Abbreviations: CC, corpus callosum; DCX, doublecortin; LV, lateral ventricle; Nano-PSO, Nano-Pomegranate Seed Oil; NPC, neural precursor cell; SEZ, subepidermal zone; SVZ, subventricular zone; Tgs, non-transplanted TgMHu2ME199K; wt, wild-type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Disease-related PrP expression and accumulation in TgMHu2ME199K treated mice

We have shown previously that Nano-PSO administration does not result in reduction of disease-related PrP, at least when PrP levels of treated and untreated mice were compared at older ages (9 months or older) (Binyamin et al., 2017; Mizrahi et al., 2014). Also, transplantation of NPCs to newborn mice did not result in reduction of disease-related PrP accumulation when tested at 300 days (Frid et al., 2018). In Fig. 5A, we show a slight reduction in the levels of proteinase (PK) resistant PrP (by immunoblot) at 230 days that disappeared at older ages. It is unknown at this point whether these marginal results are important or coincidental, in particular since there were no apparent differences in the levels of aggregated PrP, as observed by immunocytochemistry (Fig. 5B). Indeed, we have shown previously that aggregated mutant PrP and PK resistant PrP have a different kinetics of formation (Friedman-Levi et al., 2013; Keller et al., 2019). Whether such differences in the levels of PK resistant PrP in NPC treated TgMHu2ME199K mice at early stages of disease contribute to disease arrest is unknown at this stage.

4. Discussion

We have shown here that transplantation of NPCs into brains of TgMHu2ME199K mice at symptomatic stages of disease arrested disease advance for a short period of time, but subsequently mice rapidly deteriorated to the clinical state of untreated Tgs. The period of disease arrest, 30–40 days, seems to terminate at the time in which the neuronal precursor cells in the transplanted spheres start to die (positive for TUNEL), suggesting that arrest of disease advance may require active NPCs. As opposed to NPC transplantation, Nano-PSO administration did not arrest disease advance but rather reduced the rate of disease progress from the moment treatment was initiated until the end of the experiment, resulting in a longer survival over all (Binyamin et al., 2017). Interestingly, when both treatments were combined, the period of disease arrest doubled in time, after which the rate of clinical deterioration gradually reached that observed for TgMHu2ME199K mice treated only with Nano-PSO. In general, the significant difference in the scores of TgMHu2ME199K mice receiving the combined treatments and those to which only Nano-PSO was administered lasted until the model mice were about 280 days old. Subsequently, the clinical scores for these mice joined the ones of the Nano-PSO alone treatment, leading to a significant longer survival than untreated TgMHu2ME199K mice (Binyamin et al., 2017). Pathological examinations shown in Figs. 3 and 4 indicate Nano-PSO administration prolongs the life of the transplanted cells and in addition, as suggested before, the combined treatment induced the generation of endogenous stem cells stronger than each of these treatments by themselves (Madhavan and Collier, 2010).

Transplantation of stem cells for the treatment of neurological and neurodegenerative diseases has been investigated in diverse models, such as experimental autoimmune encephalomyelitis modeling for MS, amyotrophic lateral sclerosis (ALS), PD, and even AD, and is today in advanced clinical experiments in humans suffering from some of these diseases (Abdul Wahid et al., 2019; Glat and Offen, 2013; Gugliandolo et al., 2016; Kang et al., 2014; Karussis et al., 2010; Kolagar et al., 2019; Lee et al., 2016; Lo Furno et al., 2018; Scolding et al., 2017). Stem cells have powerful immunomodulatory and neuroprotective properties (Ben-Hur and Goldman, 2008; Martino and Pluchino, 2006) and may reduce the inflammatory process in the CNS caused by the diverse diseases (Einstein et al., 2003). Therefore it was suggested that direct delivery of NPCs into the CNS might induce local immunomodulatory effects that would protect the brain from ongoing autoimmune-mediated injury in MS (Ben-Hur, 2011; Pluchino et al., 2005). Strategies were developed also for stem cell treatments in infectious prion disease (Relano-

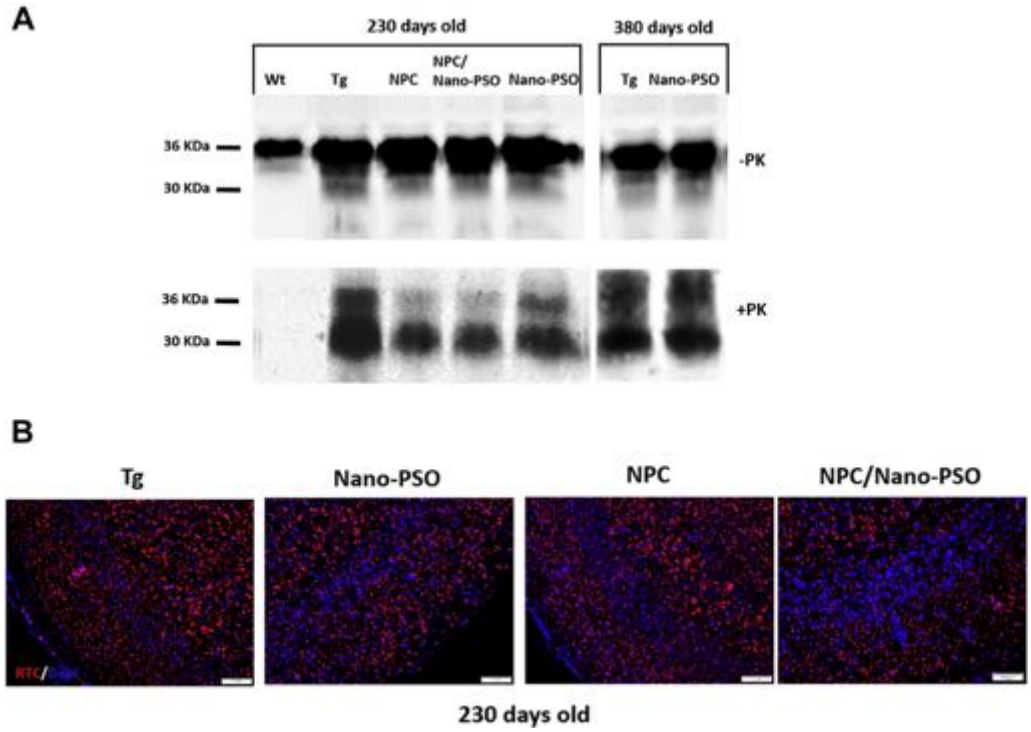

Fig. 5. Disease-related PrP expression and accumulation in TgMHu2ME199K treated mice. (A) Brain homogenates from 230-day-old to 380-day-old of wt, Tgs, Nano-PSO treated Tgs, NPC transplanted, combined treatment of NPC/Nano-PSO and scrapie infected mice were immunoblotted with α-PrP pAb RTC in the presence and absence of PK digestion. (B) Brain slices from 230 day old the same groups as described above except scrapie infected mice were immunostained for disease-related PrP (α PrP pAb RTC) [magnification ×10, scale bar 100 μm]. Abbreviations: Nano-PSO, Nano-Pomegranate Seed Oil; NPC, neural precursor cell; Tgs, non-transplanted TgMHu2ME199K; wt, wild-type.

Gines et al., 2009, 2011), as well as in Alzheimer's and Parkinson diseases (Gugliandolo et al., 2016; Kang et al., 2014; Lee et al., 2016).

The clinical benefits of stem cell transplantation are limited to the time frame of cell survival or functioning as stem cells (Fainstein et al., 2013a, b; Pluchino et al., 2009). As shown here, once these cells were no longer alive, their clinical effect, as well as their activity as resident stem cell activators, was no longer effective. This indicates that for increasing the clinical activity of stem cell transplantation, a complicated and invasive treatment (Henriques et al., 2019), we need to increase as much as possible the period these cells are alive and active in the recipient brain.

In this work, we show that in addition to its beneficial effects as a brain targeted antioxidant and as a calpain inhibitor (Binyamin et al., 2019; Keller et al., 2019), Nano-PSO administration can extend the survival of transplanted stem cells and thereby increase significantly the beneficial effect of this treatment. In addition, the combined Nano-PSO/NPC treatment helped to generate more endogenous stem cells and thereby maximize the effect of each of these treatments, as previously suggested (Madhavan et al., 2008).

Neurodegenerative diseases such as CJD and AD are multifactorial disorders (Padmakumar et al., 2020), each of which suggest possible lines of research leading to putative treatments. Thereby, it stands to reason that effective treatments will be "cocktails" of several reagents, each of them related to a specific pathological factor. Indeed, in addition to treatment approaches for neuroprotection, as presented here, there is at least one other important factor in neurodegenerative diseases in general (Jaunmuktane and Brandner, 2019) and in genetic CJD in particular, which is the accumulation of aberrant proteins specific for each disease (Jaunmuktane and Brandner, 2019; Singh et al., 2020). This is specifically true for genetic cases in which the mutant protein presents with an aberrant conformation at a very early time in life, as is the case for PrP in our TgMHu2ME199K mice (Friedman-Levi et al., 2013). We therefore speculate that, in addition to our neuroprotective approach, a methodology that can reduce accumulation of aberrant proteins, such as PrP for prion diseases, beta-amyloid for AD, or α-synuclein for Parkinson disease may be important components of the ultimate "cocktail" required for each patient's treatment.

5. Conclusion

We believe that administration of neuroprotective reagents such as Nano-PSO to subjects treated by stem cell transplantation may increase the survival of the transplanted cells as well as that of endogenous stem cells, thereby increasing the beneficial effects of the treatment. This may be true not only for CJD, but also for other conditions in which such treatments are in use (MS, ALS, stroke).

Disclosure statement

The main source of funding for this work is a grant from Granalix BioTechnologies, which commercializes GranaGard, a self-emulsion formulation of Nano-PSO. Gabizon is one of the founders of Granalix, the company developing this product.

CRediT authorship contribution statement

Kati Frid: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - review & editing. **Orli Binyamin:** Investigation. **Areen Usman:** Investigation. **Ruth Gabizon:** Conceptualization, Methodology, Writing - review & editing.

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