Neurobiology of Aging 110 (2022) 77–87

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging.org

Comparing anti–aging hallmark activities of Metformin and Nano-PSO in a mouse model of genetic Creutzfeldt-Jakob Disease

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ARTICLE INFO

Article history:
Received 27 June 2021
Revised 20 October 2021
Accepted 4 November 2021
Available online 11 November 2021

Keywords:
Metformin
Genetic CJD
Aging
Neurodegeneration
Granagard
Nano-PSO
AMPK
Prion

ABSTRACT

Advanced age is the main risk factor for the manifestation of late onset neurodegenerative diseases. Metformin, an anti–diabetic drug, was shown to extend longevity, and to ameliorate the activity of recognized aging hallmarks. Here, we compared the clinical, pathologic and biochemical effects of Metformin to those of Nano-PSO (Granagard), a brain targeted anti–oxidant shown by us to delay disease advance in transgenic mice mimicking for genetic Creutzfeldt Jacob disease (CJD) linked to the E200KPrP mutation. We demonstrate that both Metformin and Nano-PSO reduced aging hallmarks activities such as activated AMPK, the main energy sensor of cells as well as Nrf2 and COX IV1, regulators of oxidation, and mitochondrial activity. Both compounds reduced inflammation and increased stem cells production, however did not decrease PrP accumulation. As opposed to Nano-PSO, Metformin neither delayed clinical disease advance in these mice nor reduced the accumulation of sulfated glycosaminoglycans, a pathologic feature of prion disease. We conclude that elevation of anti–aging markers may not be sufficient to delay the fatal advance of genetic CJD.

1. Introduction

Several pathologic features characterize late onset neurodegenerative diseases, such as Alzheimer's (AD), Parkinson (PD) Creutzfeldt Jacob disease (CJD) diseases, and others. One is the accumulation over time and disease advance of aberrantly folded key disease proteins (Kovacs and Budka, 2008). Another is the oxidation of key proteins and lipids, which may be linked to the impairment of mitochondrial activity and of the proteasomal and lysosomal pathways (Di Carlo et al., 2012) (Faris et al., 2017) (Redmann et al., 2016). In genetic conditions, linked to pathogenic variants in the genes of the designated key disease proteins, as is the case for PrP mutations in CJD, spontaneous misfolding of the mutant proteins may result in their accumulation in nerve cells (Brown and Mastrianni, 2010; Scheckel and Aguzzi, 2018). This process starts at an early asymptomatic age, as we have previously shown for the TgMHu2ME199K model of genetic CJD, while clinical manifestations present at middle age or latter, indicating there may be a protective threshold which is present at young age but no longer active at older age (Binyamin et al., 2017; Keller et al., 2019).

Since advanced age is the obvious risk factor for the clinical manifestation of late onset neurodegenerative diseases, we decided to test whether a compound with anti–aging properties can delay the presentation, and/or aggravation of these devastating disorders. A significant number of drugs have been rigorously demonstrated to extend life span in laboratory rodents (Newman et al., 2016). One of them is the biguanide Metformin (Hu et al., 2021), an established anti–diabetic drug performing its activity by inhibition of gluconeogenesis in the liver (Agius et al., 2020). Metformin was shown to increase lifespan in various model organisms (Anisimov et al., 2008; Cabreiro et al., 2013) and is the first drug to be tested for its age-targeting effects in a large clinical trial- TAME (Targeting Aging by Metformin) (Kulkarni et al., 2020).

When testing a candidate reagent for its anti–aging effects, a since waiting years to measure life spans is not always applicable, researchers in this field measure its activity toward a line of recognized aging hallmarks (Lopez-Otin et al., 2013; Sharma and Ramanathan, 2020). These include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem

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https://doi.org/10.1016/j.neurobiolaging.2021.11.001

cell exhaustion, and altered intercellular communication (Lopez-Otin et al., 2013). Activation of AMPK, a biological sensor for systemic energy balance, can counter act a significant part of the aging hallmarks (Piskovatska et al., 2020). AMPK phosphorylates key enzymes in metabolic pathways as well as transcriptional factors and cofactors generally promoting catabolism. Activation of AMPK therefore plays an important role in cell survival and organismal longevity through modulation of energy homeostasis and autophagy, also resulting in reduction of oxidative damage, and chronic inflammation (Chen et al., 2021).

Metformin also activates additional pathways as is the case for Nrf2, a sensor of oxidative damage that also contributes to increased formation of endogenous stem cells (Fang et al., 2018). It also reduces the expression of IGF-1, resulting in restoration of proteostasis, the dynamic regulation of a functional proteome (Moll et al., 2014). Loss of proteostasis results in protein misfolding and aggregation, a well-known feature of late onset neurodegenerative disorders (Yu and Hyun, 2021). Moreover, inflammatory pathways, as is the case for increased gliosis or expression of pro-inflammatory cytokines are reduced by Metformin (Zhang et al., 2020).

To assess whether reduced activity of aging hallmarks (Barzilai et al., 2016), can prevent and/or delay the advance of genetic Creutzfeldt-Jacob disease (CJD), we examined the effect of short and long term administration of Metformin on TgMHu2ME199K mice, modeling for 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, begin to present neurologic difficulties at 5–6 months of age and subsequently deteriorate to a terminal stage between 12 and 15 months of age (Friedman-Levi et al., 2011). Since TgMHu2ME199K mice present severe symptoms already at 1 year of age, treatment with Metformin was terminated at this age for all experimental groups. Indeed, and since 1 year of age constitutes mature but not really old age in mice, testing aging hallmarks as opposed to life span is the only possibility. A similar approach was used when testing the effect of Metformin in other models of neurodegenerative diseases (Ou et al., 2018).

Concomitantly, we compared the effects of Metformin to those of Nano-PSO (Granagard), a nano formulation of pomegranate seed oil which can significantly delay disease advance and increase survival in TgMHu2ME199K mice (Binyamin et al., 2017; Mizrahi et al., 2014). Notably, Nano-PSO administration exerts beneficial clinical and pathologic effects on mice models of Alzheimer's diseases (AD) and Multiple sclerosis (MS) (Binyamin et al., 2015; Binyamin et al., 2019). In this work, we present evidence that administration of Nano-PSO and Metformin exert similar effects on aging hallmarks activities in TgMHu2ME199K mice. This includes activation of AMPK and Nrf2, increased generation of stem cells as well as reduction of IGF-1 and pro-inflammatory cytokines expression. However, and as opposed to Nano-PSO, administration of Metformin did not delay advance of clinical disease in these mice, suggesting advance of clinical signs in genetic CJD clinical disease may be independent from the activity of aging hallmarks.

2. Materials and methods

2.1. Animal experiments

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-19-15854-5).

2.2. Treatment of TgMHu2ME199K mice

Metformin (Sigma-Aldrich), 100 mg/kg/day and Nano-PSO: 64 µL PSO per day in nano emulsion as described (Binyamin et al., 2017), both of them in the drinking water, were administrated for short (11 months old mice for 2 weeks) or long (4–5 to 11–12 months) term treatments. Mice were sacrificed at designated time points. Brains, lungs and livers were processed for pathologic, molecular and biochemical measurements. Blood samples were collected for glucose test (no difference between the groups, data not shown). Figs. 2 and 3 represent samples from the short experiments, while all others are from the long-term experiments. For the quantitation and statistics, results from all experiments, and mice were included.

2.3. Mice scoring system for disease signs

TgMHu2ME199K mice were followed twice a week for the appearance of spontaneous neurologic disease. 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 or paralysis = 2; Significant hind limb/s weakness or paralysis with significant legs clasping = 2.5; Full paralysis in 1 limb = 3; Full paralysis in 1 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 according to the ethical requirements of the Hebrew University Animal Authorities.

2.4. Immunofluorescence studies

Histologic evaluations were performed on 5 µm paraffin-embedded of brain section. Sections were treated by 0.25% Tritone-X100 for 5 minutes and thereafter boiled in citrate- buffer for 40 minutes before blocking (1h, 5% BSA, 3% NGS, 0.1% Triton-X100). The antibodies that used were rabbit α - pAMPK (#2535, cell signaling), rabbit α -AMPK (#2532, cell signaling), mouse a-HIF-1(NB100-123, novus), rabbit α -COXIV-1(ab202554, abcam), mouse α -Nrf-2 (ab89443, abcam), mouse α -Nestin (ab6142, abcam), rabbit α -GFAP (#70334, Dako), and rabbit α -PrP pAb RTC (Canello et al., 2010). Secondary antibodies (a-rabbit or a-mouse) coupled to Alexa Fluor 488 and 568 were used (Abcam). Nuclei were labeled with Dapi (#4083, cell signaling).

2.5. Pathologic examinations and immunohistochemistry

Paraffin-embedded brain sections were stained with Alcian blue and/or Periodic Acid Schiff (EMD Millipore, Billerica, MA) to assess sugar polymers detection (GAGs). For co-staining with a-PrP pAb RTC, the sections were boiled in citrate- buffer for 40 minutes before blocking, then were treated with a-PrP antibody RTC and developed with DAB on the secondary antibody, α -rabbit horse radish peroxidase (Jackson Immune Research Laboratories, Inc). Alcian blue and/or Periodic Acid Schiff was applied after DAB development.

2.6. Western blot analysis

Brains, livers and lungs were homogenized in RIPA buffer (NaCl 150 mM, 0.5% sodium deoxycholate, 0.1% SDS, 1% triton x-100, 50 mM tris PH = 8, 1mM PMSF) and protease and phosphatase inhibitors cocktail (abcam). Protein levels were measured by pierce

BCA protein assay kit (Thermo Fisher Scientific) and then normalized to 100 µg proteins in each sample for pAMPK and PrP detection. For Proteinase K digestions,400 µg protein was used from each sample. Samples extracted with 2% sarcosyl on ice was incubated with 20 mg/mL Proteinase K for 15 minutes at 37°C. All samples were subsequently boiled in the presence of SDS, subjected to SDS PAGE, and transferred to nitrocellulose membrane for 1.5 hours, 300mA.

For pAMPK and AMPK detection, membranes were blocked with 5% BSA, 0.1% tween 20 in 1xTBST for 1 hour. For α-PrP pAb RTC, α -Tubulin (2148S, cell signaling), and α -β actin (ab49900, Abcam) membranes were blocked with 3% milk. The membranes were probed overnight at 4°C with the primary antibodies (AMPK 1:1000, pAMPK 1:1000, RTC 1:1000, Tubulin 1:1000, β actin 1:10,000), and developed with:a-rabbit and/or mouse horse radish peroxidase (Jackson Immune Research Laboratories, Inc) at a dilution of 1:10,000. Protein signals were obtained using an enhanced chemiluminescent western blotting detection method. Immunoreactive bands were analyzed using the ImageJ software.

2.7. Real-time PCR

RNA samples (4–6 subjects of each group) were isolated from brain samples using standard procedures. cDNA was produced from 200ng RNA with a qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD), according to the manufacturer's instructions. Real-time PCR amplification and relative quantification were analyzed with StepOne real time RT PCR (Life Technologies). The reaction mixes included 1 µL cDNA, and 300 nmol/L of each primers (Agentek and Biosearch Technologies Inc) and 5 µL of SYBR green mix (Perfecta Syber Green Fast Mix ROX, Quanta Biosciences) in a total 10 µL volume. Measurements were performed in triplicates and housekeeping gene transcript levels were used to normalize between samples. The fold changes of each mRNA were calculated based on the ratio between the analyzed TgMHu2ME199K and/or wild type tissues, as indicated.

The primers used were:

UBC. 5′ – CAG CCG TAT ATC TTC CCA GAC – 3′ (forward) 5′ – CTC AGA GGG ATG CCA GTA ATC TA - 3′ (reverse) *GAPDH*. 5′ – TCA ACA GCA ACT CCC ACT CTT C - 3′ (forward) 5′ – ACC CTG TTG CTG TAG CCG TAT T – 3′ (reverse), *IGF-1* 5′ – CAC AGC TGG ACC AGA GAC C - 3′ (forward) 5′ – CCA TAG CCT GTG GGC TTG TTG – 3′ (reverse), *IL-6* 5′-AGA AGG AGT GGC TAA GGA CCA A-3′ (forward) 5′– GGC ATA ACG CAC TAG GTT TGC– 3′ (reverse), *TNF-a* 5′– AAG GAC TCA AAT GGG CTT TCC– 3′ (forward) 5′– CCT CAT TCT GAG ACA GAG GCA AC– 3′ (reverse) *Hexosaminidase* 5′– GTC GAC TTC ACC TGC TGG AAG– 3′ (forward) 5′–CTC CAG CTG CTT GAA GTC AGT A– 3′ (reverse), *a-L-Iduronidase* 5′–AGC GTG TGG GCT CCA AGT– 3′ (forward) 5′–GTA GAC GGC CTC CTG TTG ATT G– 3′ (reverse).

2.8. Measurement of GAGs in urine

Urinary GAG content was determined spectrophotometrically by the 1,9 Dimethyl-Methylene Blue (DMB) method, as previously described (Whitley et al., 1989). The sample was compared to a chondroitin sulfate (Sigma-Aldrich St. Louis, MO) standard curve and calculated according to creatinine.

2.9. Statistical studies

The statistical significance between the studied groups versus the wt control group for immunoblot and real-time analyses was calculated according to Student's 2-tailed *t* test. Quantification of immunofluorescence was performed by measuring the stain-positive cells and the difference between the experimental groups were assessed by 1-way analysis of variance (ANOVA) using IBM SPSS Statistics V.23. The data for the clinical score graph is presented as average ± standard error. Statistical analysis for additional experiments (quantification of immunohistochemistry) was done using ImageJ and analyzed by using 1-way ANOVA for the results of multiple groups and the Tukey's post hoc test.

3. Results

To test the effect of Nano-PSO or Metformin administration on disease advance and activity of aging hallmarks in TgMHu2ME199K mice, groups of these and of C57BL mice were treated for either 2 weeks or 7 months with each of these compounds (see methods for details), so that treatments are terminated when mice were about 12 months old. In the long-term experiments treated and untreated TgMHu2ME199K mice were followed for their CJD neurologic score described in the methods (Friedman-Levi et al., 2011). The doses used for Nano-PSO or Metformin were those found most suitable from previous experiments (Abdelaziz et al., 2020; Binyamin et al., 2017; Howell et al., 2017). These dosages (for both compounds) did not have any significant effect on glucose blood levels (data not shown). Subsequently, mice were sacrificed and samples collected for evaluation of biochemical, molecular and pathologic markers of aging hallmarks and prion related neurodegeneration.

3.1. Activation of AMPK by both metformin and Nano-PSO

Samples of lung, liver and brain homogenates from the mice described above were tested by immunoblotting for the levels of unphosphorylated (AMPK) and phosphorylated AMPK (pAMPK), representing the activated enzyme (Fig. 1A). Cortex brain samples were also tested for pAMPK by immunofluorescence (Fig. 1B). Samples in the immunoblots represent mostly 2 brain homogenates from the short treatment but the quantitative graphs and statistics next to them represent the additional tested samples from long and short treatments. As stated above, AMPK activation is a major factor in counteracting the effects of aging (Muzammil et al., 2021). The liver was chosen because the antidiabetic activity of Metformin, inhibition of gluconeogenesis, requires activation of AMPK (Kim et al., 2008). Lungs were tested because Metformin was shown to reverse lung fibrosis via AMPK activation (Rangarajan et al., 2018) and more recently to protect lungs from Covid 19 damage (Sharma et al., 2020). AMPK activation in brains may relate to the long-term neuroprotective effects proposed for Metformin (Rahimi et al., 2020) (Chen et al., 2021). As depicted in Fig. 1A, basal pAMPK levels in both lungs and livers of wt and TgMHu2ME199K mice were low in the untreated mice, then elevated similarly and significantly following treatment of the mice with both Metformin and Nano-PSO. Levels of pAMPK were normalized for AMPK (the non–activated enzyme) and for Actin or Tubulin (representing total protein). A different and intriguing picture could be observed for brains. While pAMPK levels were high and similar in untreated and treated wt mice, the levels of pAMPK in untreated TgMHu2ME199K mice was significantly lower than in wt mice but subsequently elevated significantly after both Nano-PSO or Metformin treatments. Similar results could be seen when brain slices (cortex) from these mice were immunostained for pAMPK (Fig. 1B shows 2 magnifications and quantification of results). Reduced brain levels of activated AMPK in disease, as compared to wt mice, and elevation following Metformin treatment were observed also in stroke and AD mice models (Grissi et al., 2021; Ou et al., 2018).

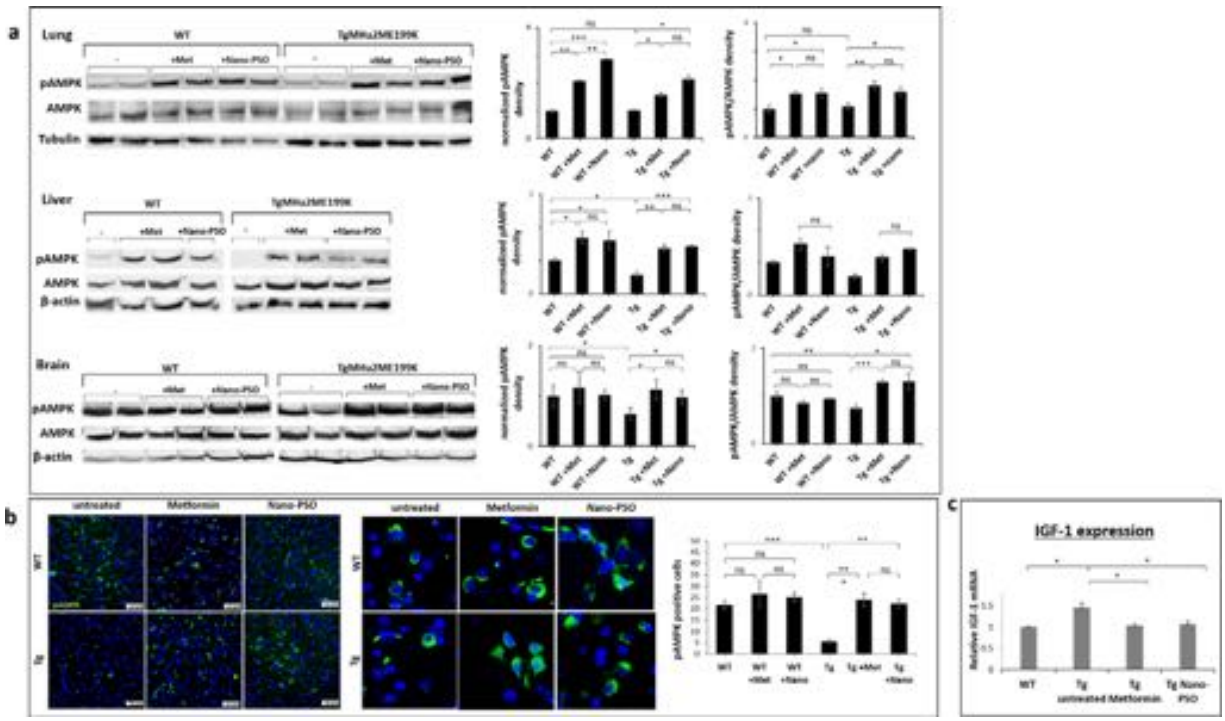

Fig. 1. Activation of AMPK and reduction of IGF1 expression by administration of Metformin and Nano-PSO to TgMHu2ME199K mice. (A) Western blot analysis of lung, liver and brain extracts of wt and TgMHu2ME199K mice at 12 months of age, untreated or after treatment with Metformin or Nano-PSO. The membranes were immunoblotted for AMPK, pAMPK and Tubulin (lung) or actin (brain and liver). These and additional blots were quantified by Image J. In each case, the pAMPK signal was normalized against the control protein and against AMPK. ns= no statistical difference, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (B) Immunofluorescence evaluation of pAMPK levels from paraffin embedded brain slices (cortex) of Metformin and Nano-PSO treated mice (magnifications x20, x40). Quantification was performed by Image J (see methods). Positive cells were counted in the cortex of mice in each group (n = 3–6/ group, 3 section of each brain). F = 10.134; ns = no statistical difference, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Relative IGF-1 mRNA levels from brains of WT, Tg untreated, and long term treated Tg metformin and Tg nano-PSO groups (n = 4/ group), as measured by Real-Time PCR analysis. Relative expression levels were normalized in reference to UBC (* $p < 0.05$).

3.2. Metformin and nano-PSO reduce IGF-1 levels in TgMHu2ME199K brains

In addition to AMPK, also the insulin-like growth factor 1 (IGF-1) signaling pathway is believed to be a major regulator of some aging hallmarks, as is the case for proteostasis (Moll et al., 2016). While at young age IGF-1 is essential for the normal development of the central nervous system, reduction in IGF-1 signaling in older age protects the brain from aging and neurodegenerative damage, by reducing loss of proteostasis (El-Ami et al., 2014). Treatment with Metformin was shown to reduce IGF-1 levels in different settings (Sunjaya and Sunjaya, 2021). In this work, we measured the levels of IGF-1 mRNA in brains of untreated TgMHu2ME199K mice as well as in those treated with Metformin or Nano-PSO. As seen in Fig. 1C, the levels of IGF-1 mRNA were elevated in TgMHu2ME199K brains as compared to wt brains, but then reduced to normal levels in brains of Tg mice treated with either Metformin of Nano-PSO.

3.3. Both metformin and nano-PSO can normalize brain mitochondrial function

Mitochondrial dysfunction is an important hallmark of aging (Majd and Power, 2018). We have shown previously that in TgMHu2ME199K mice brains, the levels of COX IV-1, the main regulatory component of complex IV (cytochrome *c* oxidase) are reduced (Keller et al., 2019) and replaced by COX V-2, an isoform that can function under higher levels of oxidative stress (Arnold, 2012). Treatment of these mice with Nano-PSO restored the normal levels of COX IV-1. Indeed, at high ROS (reactive oxygen species) levels, as those that can be caused by the accumulation of disease related PrP (Shah et al., 2018), the COX IV-1 to COX IV-2 switch constitute a self-protective mechanism of energy production in the brain. Reduced COX IV-1 and elevated levels of COX4-2 levels also result from HIF-1α nuclear localization, associated with high levels of hypoxia (Douiev et al., 2021). Chronic hypoxia as controlled by HIF-1α, the master oxygen sensor within cells, reduced mitochondrial mass and/or metabolism (Burtscher et al., 2021). In this experiment we stained with antibodies against COX IV-1 and Hif-1α brain samples from wt and TgMHu2ME199K mice, as well as in brains from TgMHu2ME199K mice treated with either Nano-PSO or Metformin (Fig. 2A). Quantification of the immunostaining can be observed in Figs. B & C. As shown before (Keller et al., 2019), COX IV-1 expression was significantly reduced in TgMHu2ME199K brains as compared to wt mice, but was restored to normal levels following treatment with either Nano-PSO or Metformin. TgMHu2ME199K brains presented mostly the nuclear staining of HIF-1α, indicating hypoxia, and high levels of ROS (Merelli et al., 2018). Again, both Metformin, and Nano-PSO treatment normalizes the levels of cytosolic HIF to those observed in wt brains. These results indicate that Metformin, as is the case for Nano-PSO can regulate mitochondrial function and hypoxia in the brains of TgMHu2ME199K mice.

3.4. Increased levels of Nrf2 in brains of treated TgMHu2ME199K mice

Metformin also activates Nrf2, a transcription factor which controls the expression of a variety of antioxidant and detoxifying enzymes (Ishii et al., 2000; McMahon et al., 2001), and also plays

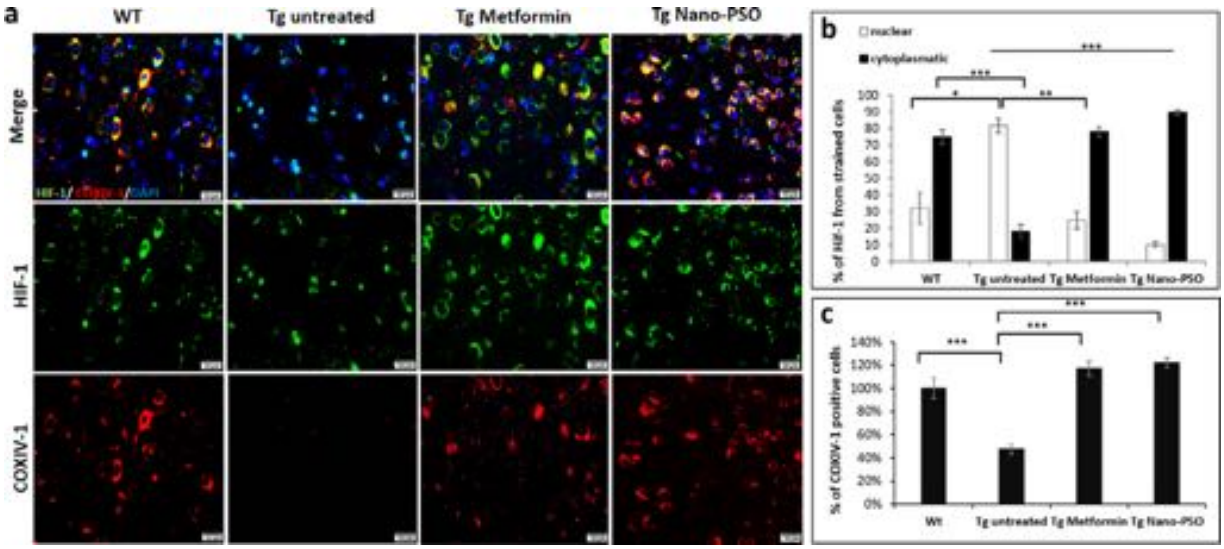

Fig. 2. Metformin and Nano-PSO administration restore HIF-1 and COX IV-1 expression in TgMHu2ME199K brains. (A) Brain sections of WT, Tg untreated, Tg metformin and Tg nano-PSO treated mice were co-immunostained for HIF-1 (green)and COX IV-1(red), and counterstained with DAPI (blue). Magnification of cortex area, X40 lens. (B) Percentage of positive cells for nuclear or cytoplasmic HIF-1. Positive cells were counted in the cortex of mice in each group (n = 3–4/ group, 3 section of each brain). For nuclear: F = 21.508, for cytoplasmic: F = 50.905 (C) Percentage of COX IV-1 positive cells. Positive cells were counted in the cortex of mice in each group (n = 4–7/ group, 3 section of each brain). F = 35.323; $^{*}p < 0.01$, $^{**}p < 0.001$,$^{***}p < 0.0001$ (Color version of the figure is available online.)

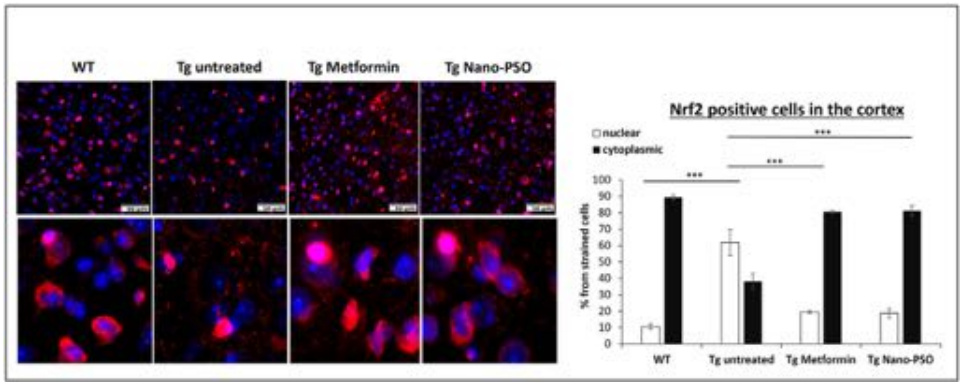

Fig. 3. Increased levels of Nrf2 in brains of treated TgMHu2ME199K mice. Immunofluorescence detection of Nrf2 in the cortex of WT, Tg untreated, Tg metformin and Tg nano-PSO mice (magnifications X20, and x40). Positive nuclear and cytoplasmic Nrf2 cells were counted in the cortex of mice in each group (n = 4/ group, 3 section of each brain). For nuclear: F = 51.941, for cytoplasmic: F = 48.623; $^{***}p < 0.001$ for both nuclear and cytoplasmic positive cells.

a role in the generation of endogenous stem cells (Kahroba et al., 2021). Non activated Nrf2 is accumulated in cell cytoplasm, while oxidative stress activated Nrf2, after its dissociation from Keap-1, is accumulated as a strong signal in the cell nucleus (Esteras et al., 2016). We have previously shown that total levels of Nrf2 were reduced in TgMHu2ME199K brains, and that the number of cells presenting a nuclear activated signal was significantly higher than in wt brains. Nano-PSO treated TgMHu2ME199K brains presented higher levels of cytosolic Nrf2, both in young, and in adult Tg mice (Keller et al., 2019). Fig. 3 shows a similar effect for brains of mice treated with Metformin. Administration of both Metformin and Nano-PSO elevated cytoplasmic and reduced the nuclear accumulation of Nrf2 in TgMHu2ME199K mice, indicating a significant reduction in ROS accumulation (Yu and Xiao, 2021).

3.5. Nano-PSO and metformin induce the generation of endogenous stem cells

We have shown previously that brain neurogenesis is reduced in TgMHu2ME199K mice (Fainstein et al., 2016), but can be restored to normal levels by Nano-PSO administration (Frid et al., 2018). Also Metformin was shown in some models to increase neurogenesis (Zhu et al., 2020). To establish if this is also the case for Metformin when administrated to TgMHu2ME199K mice, we immunostained the brain SVZ (sab-ventricular zone) area of TgMHu2ME199K mice treated with either Nano-PSO or Metformin for Nestin, a well-established marker of stem cells (von Bohlen Und Halbach, 2007). Fig. 4 shows that while untreated TgMHu2ME199K mice (12 months) presented low levels of Nestin stained cells as compared to wt mice, neurogenesis as represented by the number of Nestin positive cells was similarly restored in brains of TgMHu2ME199K mice treated either with Nano-PSO or with Metformin (see statistical study next to picture).

3.6. Inhibition of the inflammatory response by metformin and nano-PSO

Inflammation is an important feature of both aging and neurodegeneration (Prasad, 2017; Toricelli et al., 2021). Increased expression of GFAP in astrocytes and elevated expression of pro-inflammatory cytokines are both aging hallmarks as well as features of neurodegeneration (Green et al., 2011; Yin et al., 2016). To

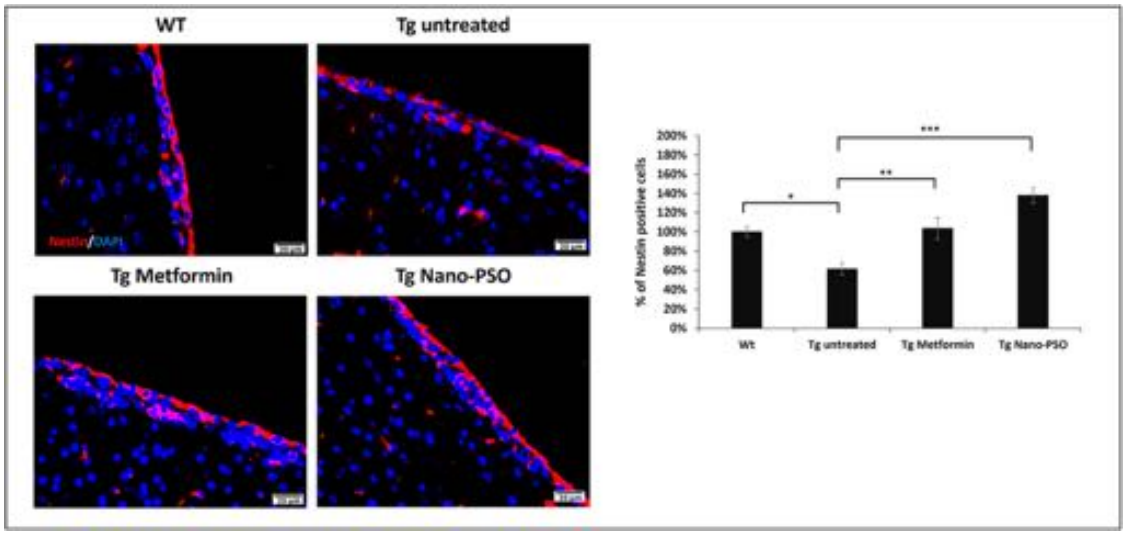

Fig. 4. Increased generation of endogenous stem cells in brains of treated TgMHu2ME199K mice. Coronal sections through the subventricular zone of WT, untreated TgMHu2ME199K mice, as well as Tg mice treated with Metformin and Nano-PSO, were immunostained for Nestin, an endogenous stem cells marker. (magnification x40). F = 15.624; $^{*}p < 0.05$, $^{**}p < 0.005$,$^{***}p < 0.001$.

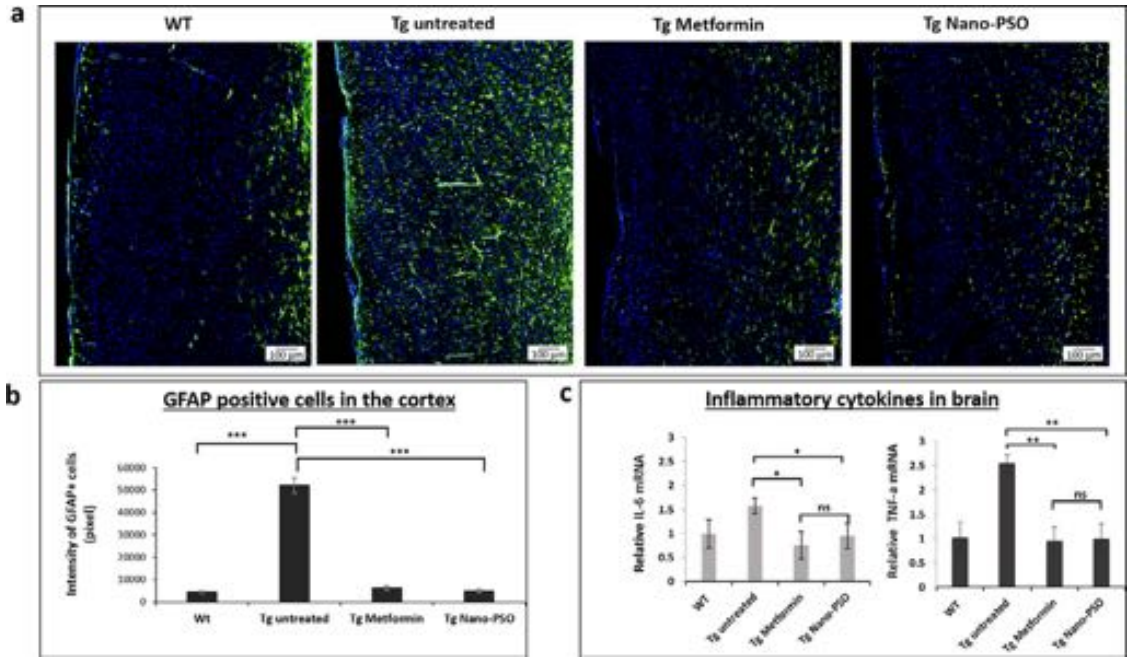

Fig. 5. Metformin and nano-PSO reduced inflammation factors in the brain. (A) GFAP Immunofluorescence of brain slices (cortex) from WT, untreated Tgs, and Tgs treated with Nano-PSO or Metformin (magnification x20). (B) Quantification of GFAP positive cells in the cortex. F = 158.436; $^{***}p < 0.0001$ (C) Relative IL-6 and TNF-a mRNA in brains from WT (n = 3), Tg untreated (n = 5), Tg metformin (n = 4) and Tg nano-PSO (n = 6) groups (long treatment), as measured by Real-Time PCR. Relative expression levels were normalized in reference to UBC and GAPDH. $^{*}p < 0.05$, $^{**}p = 0.01$.

establish if this is indeed the case in untreated TgMHu2ME199K mice and whether administration of either Metformin or Nano-PSO can restore normal levels of these markers, we immunostained relevant brain sections for GFAP (Glial fibrillary acidic protein), as well as tested the brain expression levels of IL-6 and TNFα by Real Time PCR. Fig. 5 A & B) show that, as expected, GFAP staining in brains sections of TgMHu2ME199K mice was considerably elevated (Friedman-Levi et al., 2011), but then reduced significantly following treatment of these mice with both Metformin or Nano-PSO. Concomitantly, we tested the mRNA expression levels of 2 pro-inflammatory cytokines TNF alpha and IL-6 in the brains of treated and untreated mice (Fig. 5c). We found that the levels of both cytokines were significantly elevated in TgMHu2ME199K brains as compared to wt mice, and restored to wt levels following administration of any of these reagents to the mice. These results indicate that both Metformin and Nano-PSO present anti–inflammatory properties.

3.7. Metformin does not delay disease advance in TgMHu2ME199K mice

We have demonstrated above that both Metformin and Nano-PSO fulfill the biochemical and pathologic criteria of candidate anti–aging compounds. Next, we compared the effect of both

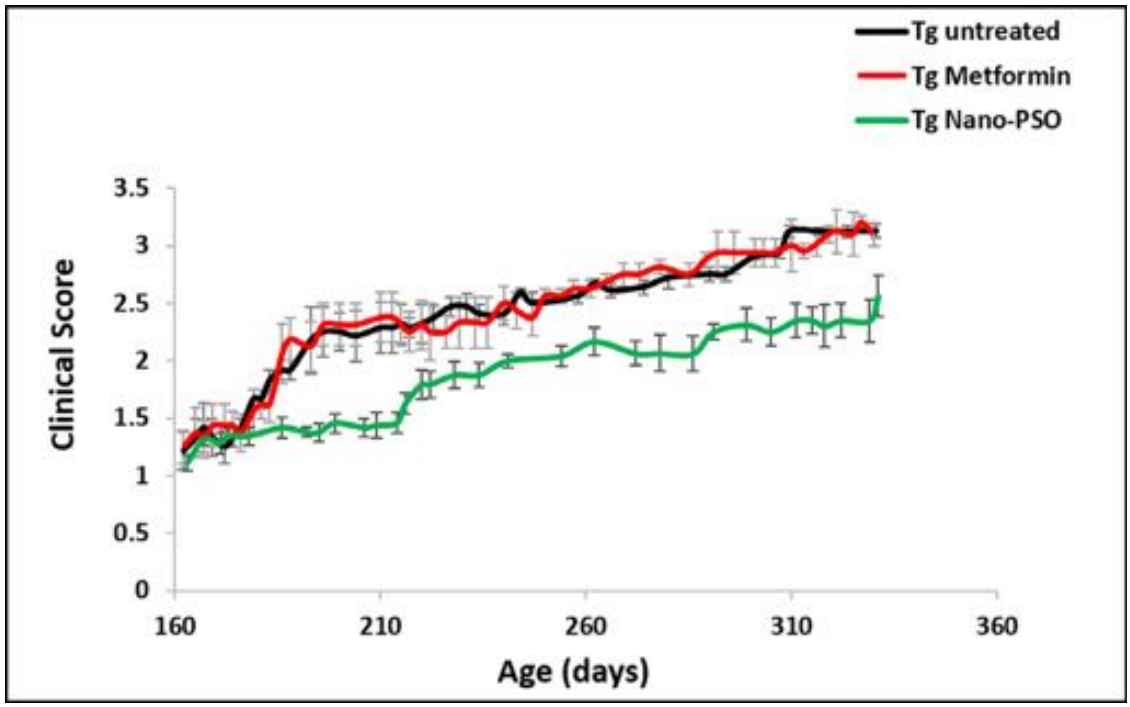

Fig. 6. Metformin treatment have no clinical effect on the disease advance in TgMHu2ME199K mice. Metformin (n = 8, 200mg/kg/day) and nano-PSO (n = 7, ~64 µL oil diluted in water per day) were administrated to TgMHu2ME199K mice from an early symptomatic stage until the age of 12 months. Treated and untreated mice (n = 8) were evaluated for disease score twice a week as described in the methods. The graph presents the average group score as related to age of mice. T-test (tg nano-PSO vs. untreated Tg and Tg metformin): days 180–220 $p < 0.001$; days 220–240 $p < 0.05$; days 240–280 $p < 0.01$; day 300–330 $p < 0.05$.

reagents on markers of prion disease. First and most important we tested the effect of Metformin as compared to Nano-PSO on the advance of clinical disease in TgMHu2ME199K mice. Fig. 6 confirms that, as we was shown before for initiation of Nano-PSO treatment at several ages, Nano-PSO administration significantly delayed disease advance in these mice (Binyamin et al., 2017; Frid et al., 2020; Mizrahi et al., 2014). On the contrary, administration of Metformin had no beneficial or deleterious clinical effect, and advance of clinical scores over time were the same as in untreated TgMHu2ME199K mice. This is a surprising and intriguing result in view of the similar effects of Nano-PSO and Metformin as regard to all tested aging hallmarks, and suggests for the presence of an independent factor that correlates with disease advance in genetic CJD.

3.8. Nano-PSO and metformin did not reduce disease related PrP accumulation

Accumulation of aggregated and protease resistant (PK) PrP (PrP^{Sc}) is the most important feature of prion diseases (Prusiner et al., 1987). In the TgMHu2ME199K mice, mutant PrP even at early age forms aggregated structures (Keller et al., 2019), which conformation may be dictated by the pathogenic E200K mutation, and subsequently converts into PK resistant PrP concomitant with appearance of disease signs (Canello et al., 2010; Friedman-Levi et al., 2011; Wang et al., 2016) (Friedman-Levi et al., 2013). We have shown previously that Nano-PSO administration did not reduce the levels of aggregated or PK resistant PrP, but rather allows for normal cell metabolism to proceed in the presence of these disease related PrP forms (Keller et al., 2019) (Binyamin et al., 2017). In Fig. 7A we show that this is also the case for treatment of TgMHu2ME199K mice with Metformin. The differences between the levels of aggregated PrP between untreated brain cortex samples and Metformin or Nano-PSO treated samples were not significant, as seen by immunofluorescence with an α PrP antibody. This can also be seen for immunoblots of total brain PrP in the presence or absence of PK digestion (Fig. 7B).

3.9. Metformin has no effect on GAGs accumulation and degradation

Glycosaminoglycans (GAGs) are highly polymerized sulfated polysaccharides (Khan et al., 2020). They are frequently associated with proteinaceous deposits in brains of patients affected by amyloid related neurodegenerative diseases, as is the case for CJD, and AD (Mah et al., 2021) (Diaz-Nido et al., 2002; Snow et al., 1989; Yin et al., 2007). In prion disease, the role of GAGs has been studied extensible (Papy-Garcia et al., 2011) (Hijazi et al., 2005; Vieira et al., 2014). In addition, it was shown that low molecular weight sulfated sugars may compete with GAGs for the binding to misfolded proteins, and also serve as anti–prion agents (Schonberger et al., 2003; Shaked et al., 2001).

To this effect, we examined brain samples from wt, untreated TgMHu2ME199K mice as well as those treated with either Nano-PSO or Metformin, for the accumulation of GAGs in brains, and urine samples. While both PrP and GAGs were shown to aggregate in these mice in correlation with disease advance in untreated mice, only GAG levels were reduced following continues administration of Nano-PSO (Binyamin et al., 2017). Fig. 8A shows that while no alcian blue and/or PAS-stained GAGs could be observed in normal brain sections, a significant staining was observed in the TgMHu2ME199K brains. As shown before, administration of Nano-PSO decreases such staining significantly (Binyamin et al., 2017), however no reduction in the levels of brain GAGs was detected in the brains of Metformin treated TgMHu2ME199K mice (see quantification panel next to picture). This is the second parameter, in parallel to disease advance which differentiates between the effects of Nano-PSO, and Metformin on TgMHu2ME199K mice.

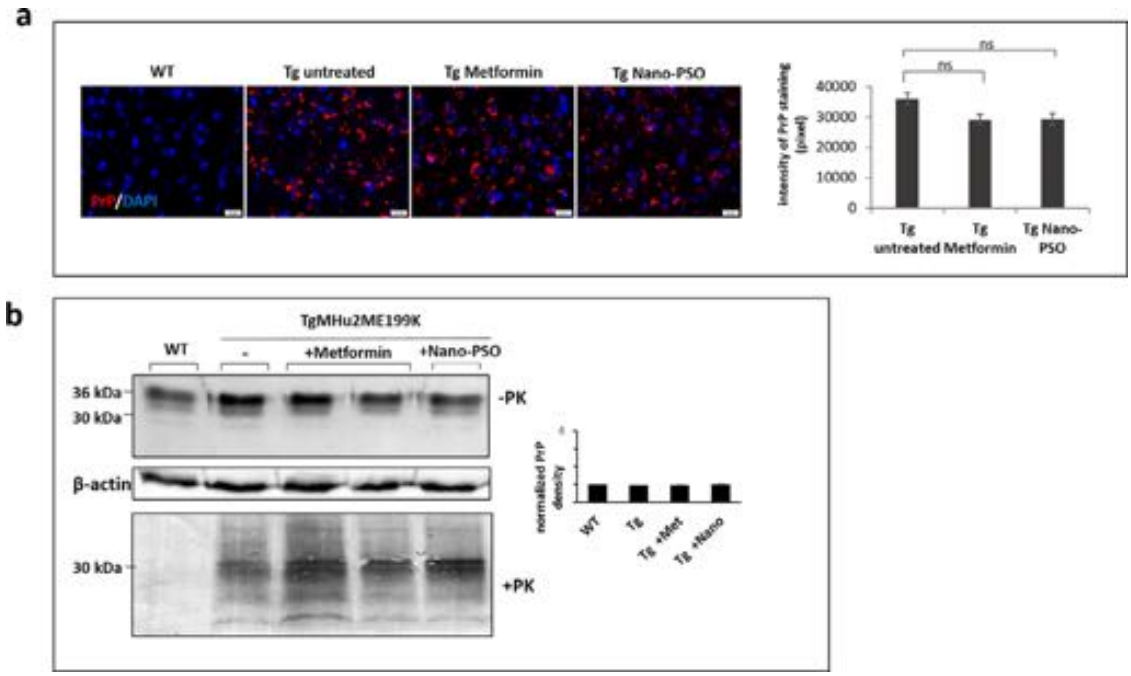

Fig. 7. Both Metformin and nano-PSO do not affect aggregated and PK resistant PrP accumulation. Brain samples of WT, Tg untreated, Tg metformin and Tg nano-PSO mice were analyzed for disease related PrP (A) Immunofluorescence evaluation of aggregated PrP accumulation in 12 months old WT, Tg untreated, Tg metformin and Tg nano-PSO mice. Paraffin embedded brain slices were treated for epitope revealing as described in the methods and subsequently immunostained with α PrP pAb RTC (red), and counter-stained with dapi (blue). Magnification of cortex area by $\times$ 40 lens. Pixels of red stain from Tg groups measured and quantified, presenting no statistical difference between the groups. $F = 3.950$ (B) Western blot analysis of mice brain extracts. Each sample was digested in the presence or absence of PK, as described in the methods, and immunoblotted with α PrP pAb RTC. Total PrP levels (-PK) were normalized to beta actin. No statistical difference between the groups (Color version of the figure is available online.)

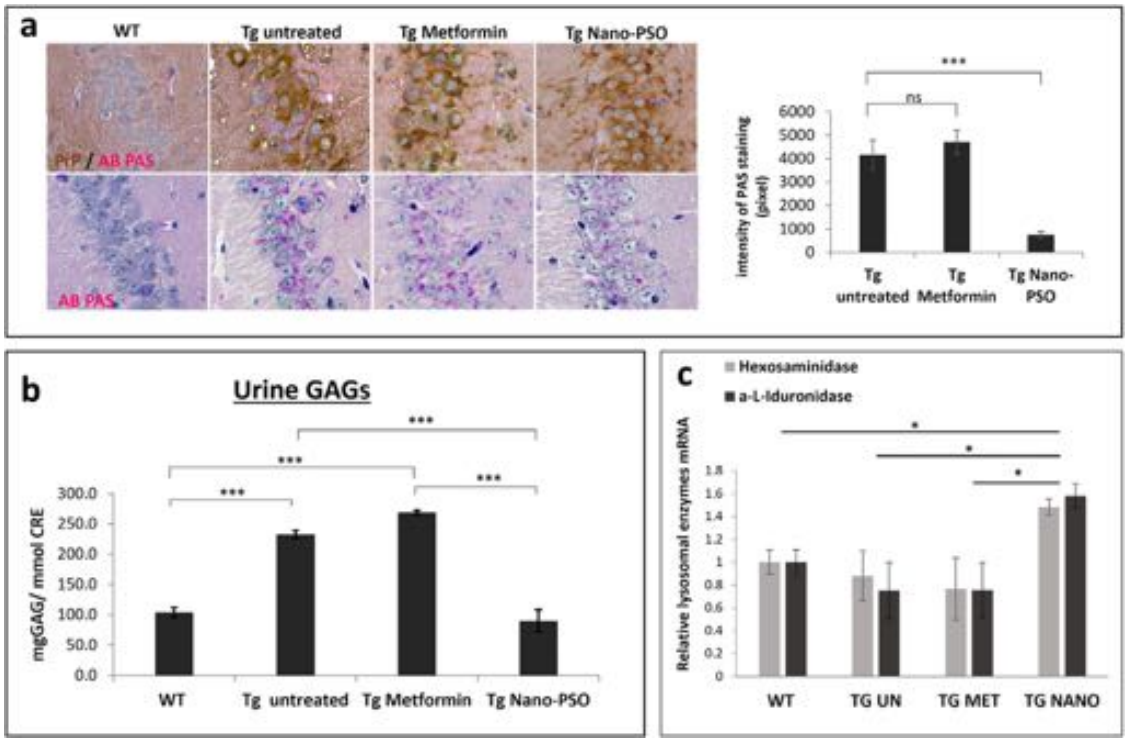

Fig. 8. Metformin does not affect GAGs metabolism. (A) Upper panel: brain slices of mice from the 4 groups were double-stained for alcian blue/PAS (red stain) and PrP (brown stain). Magnification of hippocampus area by $\times$ 40 lens. Lower panel present only alcian blue/PAS stain of serial sections from the same brains. AB/PAS intensity of Tg groups measured and normalized to wt stain. $F = 17.763$; $^{***}p = 0.0001$ (B) quantitative analysis of GAGs in mice's urine. Urine were processes as described in the methods. Total GAGs concentration is expressed as mg GAGs/nmol creatinine. $F = 48.969$ $^{***}p \leq 0.0001$ **c.** Relative Hexosaminidase and a-L-iduronidase mRNA levels in brains from WT($n = 4$), Tg untreated ($n = 3$), Tg metformin ($n = 4$) and Tg nano-PSO ($n = 4$) groups,as measured by Real-Time PCR analysis. Relative expression levels were normalized in reference to UBC and GAPDH. statistics presented related for both Hexosaminidase and a-L-iduronidase $^{*}p \leq 0.01$ (Color version of the figure is available online.)

In addition of brain GAGs, we have previously demonstrated that urinary GAGs levels were significantly elevated in CJD patients (genetic and sporadic) as well as in prion infected mice, hamsters, and macaques (Mayer-Sonnenfeld et al., 2005). To this end, we next tested the levels of GAGs in urine of untreated TgMHu2ME199K mice as compared to those in urine of mice treated with Nano-PSO or Metformin. Fig. 8B shows that GAGs were also elevated in the urine of TgMHu2ME199K mice, mimicking for genetic CJD, but reduced to levels of normal mice following administration of Nano-PSO to these Tgs. Contrarily, no such reduction was observed in the urine of TgMHu2ME199K mice treated with Metformin, although Metformin was shown to reduce GAGs levels in urine of diabetic patients suffering from nephritis (Jura-Poltorak et al., 2019), suggesting this drug may be less active in brains than in kidney when concerned to lysosomal protective activity.

High levels of GAGs in both brains and urine can be detected in the mucopolissacharidoses, genetic diseases in which the activity of lysosomal enzymes degrading GAGS is impaired (Tomatsu et al., 2014) (Kakkis and Marsden, 2020). Therefore, we tested by RT-PCR in wt, as well as in treated and untreated TgMHu2ME199K brains the expression levels of alpha-L-iduronidase, the most common GAG degrading lysosomal enzyme (Dorfman and Matalon, 1976). As a general marker of lysosomal activity, we tested the expression levels of hexosaminidase α, a lysosomal enzyme involved in ganglioside degradation. We found (Fig. 8C) that while there was no significant difference in the expression levels of these enzymes between the wt, untreated and Metformin treated Tgs, mRNA levels of these enzymes were significantly elevated in the brains of TgMHu2ME199K mice treated with Nano-PSO. While the mechanism by which Nano-PSO as opposed to Metformin increases the expression of lysosomal enzymes is unknown at this point, this may explain why Metformin as opposed to Nano-PSO did not reduce GAGs accumulation in both brains, and urine.

4. Discussion

TgMHu2ME199K mice represent a model for spontaneous genetic prion disease linked to mutations in the PRNP gene (Friedman-Levi et al., 2011). These mice are born healthy but at 5–6 months of age develop initial signs of neurodegenerative diseases which aggravate in parallel to age advance, until they have to be sacrificed at 12–15 months due to severe neurologic malfunction. In this work, we tested whether Metformin, an anti–diabetic drug with recognized anti–aging properties (Barzilai et al., 2016; Kulkarni et al., 2020), can activate anti–aging hallmarks, and delay disease progression in TgMHu2ME199K mice. Experiments had to be terminated at 1 year of age, which constitutes adult but not old age in mice, since the untreated mice cannot survive much longer after that mark. In parallel, we tested whether Nano-PSO, a nanoformulation of pomegranate seed oil shown to delay disease onset and/or progression in several neurodegenerative mice models (Binyamin et al., 2015; Binyamin et al., 2019; Mizrahi et al., 2014), can activate aging hallmarks similar to Metformin. Both compounds were administrated to these mice for either 2 weeks or 7 months and followed for signs of disease advance and aging hallmarks until they were about 1 year of age, as compared to untreated Tgs.

Our results presented in Figs. 1–5 show that both Nano-PSO and Metformin delayed hallmarks of aging in a similar way. This includes activation of AMPK, increased expression of Nrf2, elevation of mitochondrial markers such as COX IV-1 and HIF-1α, as well as reduced expression of IGF-1, GFAP and inflammation markers (Lopez-Otin et al., 2013). Interestingly, both treatments had no effect whatsoever on the accumulation of disease related PrP forms, which constitute the main prion marker. Nevertheless, while Nano-PSO administration significantly reduced the rate of disease advance in these mice, Metformin treatment did not show any significant clinical effect. Whether administration of Nano-PSO to wt mice or other appropriate models can elongate life span, as was shown for Metformin, or have an effect on diabetes will need to be tested separately (Martin-Montalvo et al., 2013).

Aging is the obvious risk factor for the presentation and progression of late onset diseases. Therefore, it was extensively hypothesized that delaying the activation of aging hallmarks, could delay the advance of neurodegenerative conditions (Markowicz-Piasecka et al., 2017). Here, we present evidence that this is not always the case, at least for genetic prion disease in the TgMHu2ME199K mice model. Interestingly, Metformin did not exert a beneficial clinical effect also in a transmissible model of prion disease (Abdelaziz et al., 2020). Indeed, the question of whether Metformin can delay neurodegeneration is still under debate (Kim et al., 2020). While in some animal models Metformin treatment reduced dementia and APP accumulation (Ou et al., 2018), in others it was considered detrimental (Picone et al., 2015). Still, it should be mentioned that Metformin as well as other anti–diabetic drugs have an intrinsic beneficial effect on dementia in human patients, probably due to the correction of metabolic abnormalities caused by both diabetes, and aging (Kim et al., 2020) (Akimoto et al., 2020).

An additional interesting observation that can be drawn from this work is the different pattern of AMPK activation in brains as opposed to other organs. While both lungs and livers present low levels of pAMPK, subsequently activated by Nano-PSO or Metformin administration, we hereby demonstrate that in wt brains AMPK is activated constantly. Contrarily, this constitutive activation of AMPK is absent in brains from TgMHu2ME199K mice, but can be achieved by administration of both Nano-PSO, and Metformin. The different mechanism of AMPK activation as well as its role in neurodegeneration is being studied extensively (Muraleedharan and Dasgupta, 2021).

Yet another interesting point, is the different effect of Nano-PSO and Metformin administration toward the accumulation of GAGs in brains and urine form the TgMHu2ME199K mice. It may well be that a general elevation of lysosomal enzyme expression by Nano-PSO, which probably contributes to the reduction of GAGs accumulation, is a key factor in delaying disease advance in TgMHu2ME199K mice. Whether this is also the case in human patients is under investigation.

Credit authorship contribution statement

OB: conceptualization of experiments; data curation, writing of manuscript, analysis of data
KF: data curation, analysis of data
GK: data curation
AS: analysis of data
RG: conceptualization of experiments, writing of manuscript, analysis of data

Funding

This work was funded by the Abramsky-Prusiner foundation and by a grant from Granalix Biotechnologies.

Disclosure statement

RG is the founder and Scientific Officer of Granalix Biotechnologies.

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