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Effect of Metformin on tau pathology and proteostasis in cellular and a *Drosophila* model of Tauopathy

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Abstract

Alzheimer's disease is increasingly linked to metabolic dysfunction, with insulin resistance emerging as a key contributor to tau pathology. The antidiabetic drug Metformin has shown conflicting effects in neurodegeneration, underscoring the need to resolve its mechanistic impact on tau regulation. This study combines insulin-resistant neuroblastoma cells with *Drosophila* models of tauopathy to define how Metformin modulates tau phosphorylation via underlying signalling pathways.

Metformin treatment increased total tau levels while consistently reducing hyperphosphorylated tau at disease-relevant epitopes in endogenous neuroblastoma cells. This dissociation was associated with enhanced activation of AKT and AMPK signalling and maintenance of basal phosphatase activity. We further observed that Metformin at low concentrations promoted autophagic activity, whereas higher concentrations impaired autophagic clearance.

In the Tauopathy models, Metformin selectively ameliorated tau-induced neurodegeneration and tau-hyperphosphorylation in tau-0N4R but not in more pathogenic tau variants (tau-2N4R and tau-E14). The alteration in pathological tau levels did not correlate directly with the status of autophagy. On the contrary, autophagic status was influenced by diet and sex of these organisms.

Together, our findings demonstrate that Metformin modulates tau phosphorylation independently of total tau levels and reveals a critical dissociation between upstream metabolic signalling and downstream proteostatic outcomes.

Keywords: Alzheimer's Disease; Insulin-resistance; Metformin; Tau; Kinases; Autophagy

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Introduction

Alzheimer's Disease (AD) is the most prevalent form of dementia currently affecting over 50 million people [1,2]. Two of the neuropathological features of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles of hyperphosphorylated and aggregated tau protein [2,3,4].

Due to insulin resistance in the brain, AD has been referred to as Type 3 Diabetes [5,6]. Epidemiological studies have shown that people with Type 2 Diabetes have 1.5 to 2-fold increased risk of dementia including AD [7,8]. An overwhelming body of evidence suggests that the tau pathology correlates more strongly with the severity of the disease rendering pathological tau as an important target for therapeutic studies [9,10]. Interestingly, in people with Diabetes, instead of A β , an enhanced levels of total and phospho-tau (p-tau) has been observed in their CSF which suggests that the risk of AD in individuals with Diabetes might be linked to increased tau pathology [11,12].

Metformin is one of the potent anti-diabetic drugs that also has a beneficial effect on neurodegenerative diseases [13,14]. While clinical studies indicate that Metformin might confer a protective effect on AD in diabetic and non-diabetic patients [15,16], recent studies have shown that Metformin worsened the cognitive functions in preclinical AD rodent models and increased dementia risk in patients with Parkinson's Disease [17,18].

In diabetic mouse models Metformin has been reported to alleviate A β plaque formation and decrease levels of p-tau with improved spatial memory and reduction of neuronal loss [19,20]. The neuroprotective effects of Metformin have been also observed in a tau-seeded rodent model of tauopathy [21]. In contrast, in another study involving P301S mouse model of tauopathy, Metformin was found to increase the formation of insoluble tau oligomers, neurodegeneration and severe behavioural impairments [22]. Hence it is important to decipher the action of Metformin on total and hyperphosphorylated tau.

Drosophila melanogaster has been used for several decades in the study of neurodegenerative diseases and metabolic dysfunctions [23,24,25,26]. We and others have previously shown that the overexpression of the full-length human tau in the *Drosophila* eye generates a characteristic "rough-eye" phenotype with hyperphosphorylated and aggregated tau which is indicative of a neurodegenerative phenotype [25]. This tauopathy model has been extensively used for genetic screening wherein we have shown that the induction of insulin resistance by genetic manipulation exacerbates tau hyperphosphorylation and neuronal loss indicating an underlying connection between insulin response and tau pathology [26].

Hyperphosphorylation of tau is caused by the imbalance of kinases and phosphatases [27]. GSK3 β , one of the prominent tau kinases is downstream of the insulin signalling pathway. Dysregulation of GSK3 β causes tau hyperphosphorylation which eventually leads to the formation of NFTs within the neurons [28,29]. Hence, Metformin as an insulin-sensitizer could play a significant role in maintaining the optimal balance of tau phosphorylation and dephosphorylation.

Autophagic dysfunctions leading to the accumulation of toxic misfolded proteins have been one of the key features of neurodegenerative diseases including AD [30,31]. Impairment of autophagy in neurons exacerbates the deposition of A β plaques and the formation of NFTS [32,33,34]. Recent reports indicate that insulin-resistance which is a prominent feature of Type 2 Diabetes downregulates autophagy which might be one of the reasons why Diabetes is an important risk factor of AD [35,36]. AMPK, one of the Metformin-regulated kinases has been known to upregulate autophagy which might contribute towards neuroprotection [37,38]. However, the impact of Metformin on the interplay of kinases and phosphatases and its role in autophagic regulation needs to be investigated.

Using a combination of neuroblastoma (SHSY5Y) cells and *Drosophila* tauopathy models we have investigated the impact of Metformin on endogenous and overexpressed human tau. Our data in SHSY5Y cells shows that Metformin increases total tau while decreasing hyperphosphorylated tau by activating autophagy at low concentrations. Interestingly, we have been able to replicate these observations *in vivo* in the tauopathy models particularly in the context of hyperphosphorylated tau and autophagic clearance. We have observed a gender-specific response to the neuroprotective actions of Metformin providing further insight into the mode of action of Metformin *in vitro* and *in vivo*.

Results

Metformin increases the levels of endogenous tau under insulin resistant conditions

To induce insulin resistance SHSY5Y cells were cultured in high-glucose media (4.5g/l) for a period of 24 hours and incubated with the fluorescent glucose analogue 2-NBDG. Compared to cells grown in normal (low) glucose media (1g/l), the cells in high glucose media demonstrated significantly lower glucose uptake confirming insulin resistant conditions (Fig. S1 A). Upon treating these cells with progressively increased concentrations of Metformin we observed that the cells retained 80% viability in both low and high glucose conditions till 20 mM which was used as the highest concentration (Fig. S1 Bi and ii).

To explore whether Metformin impacts tau levels under insulin-resistant conditions, these cells were treated with progressively increased concentrations of Metformin (0mM to 20 mM) for 24 hours and the total tau levels were measured by western blotting (A0024, T46). We found that the total tau levels increased significantly at 5mM, 10 mM and 20 mM Metformin treatment compared to untreated controls (Fig. 1A). Our data was also confirmed by immunostaining with total tau antibody (Fig. 1B). We also observed both nuclear and cytoplasmic distribution of tau in the cells with the formation of mesh-like networks in the nucleus and cytoplasm at high Metformin concentrations (10 mM and 20 mM).

To investigate whether Metformin specifically increases both 3R and 4R isoforms of tau, the cells were treated with progressively increased concentrations of the drug and tau levels were assessed by immunocytochemistry. Our data showed a significant increase of both tau isoforms on treatment with 10 mM and 20 mM Metformin with the formation of punctate structures in the cytoplasm (Fig. S2 A).

116

117 **Metformin decreases the levels of hyperphosphorylated tau**

118 Neurofibrillary tangles composed of hyperphosphorylated and aggregated tau protein is one of
119 the neuropathological markers of AD [39,40]. In the next step, we investigated whether
120 Metformin was able to affect the levels of hyperphosphorylated tau protein. Interestingly, we
121 found that despite the increase of total tau, there was a significant decrease of phospho-tau at
122 Ser199/202 and Ser202/Thr205 (AT8) residues upon Metformin treatment at high
123 concentrations of 10mM and 20mM (Fig. 2A). This was also confirmed by immunostaining
124 with the phospho-tau antibodies where a progressive decrease of cytoplasmic punctate tau was
125 observed (Fig. 2B).

126 Since we observed this downregulation of endogenous phospho-tau at AD-specific disease
127 epitopes, we investigated whether this was also the case for overexpressed tau-0N4R and tau-
128 E14 (pseudo-hyperphosphorylated) transfected SHSY5Y cells [41,42]. Although we did not
129 observe any decrease of total tau upon Metformin treatment (Fig. S2 B i and ii), there was a
130 significant decrease of hyperphosphorylated tau in 0N4R transfected cells (Fig. S2 B i).

131 Taken together our data shows that Metformin decreases the levels of endogenous and
132 overexpressed hyperphosphorylated tau.

133

134 **Impact of Metformin on the GSK-3 β /AMPK/PP2A axis**

135 As our data consistently displayed a significant decrease in the levels of hyperphosphorylated
136 tau, in the next step we investigated the levels of kinases and phosphatases that are regulated
137 by Metformin. We observed that Metformin increased the ratio of phospho AKT/Total AKT
138 which indicated insulin sensitivity in the treated cells (Fig. 3A). It is well known that AKT
139 phosphorylates the Serine9 residue of GSK3 β which renders this kinase inactive [43]. On the
140 contrary, we found a significant decrease in the levels of inactive GSK3 β compared to untreated
141 controls especially at 10mM and 20 mM Metformin (Fig. 3B). To explore whether tau is
142 dephosphorylated by PP2A which is one of the key phosphatases regulated by Metformin, we
143 investigated the levels of PP2A [44]. In its active conformation, the C-terminal tail of the
144 PP2Ac subunit is methylated by the enzyme leucine carboxyl methyltransferase1 [45]. Despite
145 a significant decrease of PP2A at 20 mM, we found that the basal levels of active PP2A remains
146 unchanged, indicating the maintenance of endogenous phosphatase activity in the cells (Fig.
147 3C).

148 Next, we investigated the levels of another tau-associated kinase AMPK which is directly
149 regulated by Metformin. AMPK acts as the energy sensing component within cells by
150 inhibiting mTOR and activating autophagy [46]. As expected, we found a significant increase
151 in the ratio of phospho-AMPK/Total AMPK levels with progressively increasing Metformin
152 concentrations (Fig.4). Interestingly, while phospho-AMPK displayed enhanced punctate
153 staining, localized primarily in the cytoplasm; total AMPK was distributed in the nucleus and
154 the cytoplasm.

Taken together our data suggests that Metformin decreases tau hyperphosphorylation by maintaining a basal level of phosphatase activity and promoting robust AMPK activation. The differential subcellular distribution of phospho vs total AMPK suggests potential involvement in mediating tau phosphorylation/dephosphorylation effects under conditions of metabolic stress.

Low dose Metformin activates autophagy in neuroblastoma cells

The soluble and insoluble forms of hyperphosphorylated tau are primarily cleared by the autophagy-lysosomal and UPS pathways [47,48].

Given the robust activation of AMPK and its established role as a central regulator of autophagy, we next examined whether Metformin alters the status of early and late autophagy under insulin-resistant conditions. Recent studies have shown that Metformin at low concentrations inhibits mTORC1 and promotes autophagy in an AMPK-dependent manner [49]. We found that progressive Metformin treatment reduces mTOR-Thr172 phosphorylation indicating an upregulation of autophagy (Fig. S3A).

To explore whether Metformin upregulates late-stage autophagy, we investigated the status of autophagy by monitoring autophagic flux and measuring LC3-II and p62 levels [50,51]. Lipidated LC3-II protein is the standard marker of for autophagic induction while p62 is an adapter protein that links autophagy to the ubiquitin-proteasomal pathway.

Upon treatment of neuroblastoma cells with autophagy-blocker Bafilomycin we observed a significant increase of LC3 punctae due to the blockage of autophagic flux while treatment with Rapamycin cleared the punctate structures by activating autophagy. Interestingly, we observed low levels of LC3 punctate structures in untreated and low-dose Metformin treated cells (0 mM to 5 mM) which matched with that of the Rapamycin treated cells suggesting an autophagic activation. However, under high dose Metformin (10 mM and 20 mM) there was a gradual accumulation of LC3 punctate structures suggesting an impaired clearance of autophagosomes (Fig. 5A). This was also confirmed by a modest increase of LC3-II/LC3-I ratio by western blotting (Fig. 5B, upper panel, i). Given that an increase of LC3-II levels can be either due to autophagic induction or autophagic blockage, next we assessed the levels of p62 which accumulates when there is an autophagic impairment. Notably, we observed a concomitant increase of p62 protein levels implying a downregulation of autophagy at high Metformin concentration (Fig. 5B lower panel, ii). To further assess the state of autophagic flux, we pre-treated the cells with Bafilomycin to inhibit autophagy, prior to Metformin treatment. Interestingly, under conditions of forced autophagic blockage both low and high dosage of Metformin activated autophagy which was apparent in the increase of LC3-II/LC3-I ratio and the concomitant clearance of p62 protein indicating that Metformin activated autophagy under stressed conditions (Fig. S3 B).

Finally, the cells were subjected to lysotracker staining which specifically detects the acidic compartments of lysosomes that facilitate the degradation of misfolded proteins [48]. While we observed an increase in the number of lysotracker punctae at low Metformin concentration

(2.5 mM and 5 mM), we detected a significantly smaller number of larger sized punctate structures at high dosage of Metformin at 10mM and 20 mM (Fig.6).

Together, our data suggests while Metformin activates autophagy at low doses (2.5 and 5mM) there is a pronounced blockage of autophagy at higher dosage. However, artificially induced autophagic inhibition by Bafilomycin removes this blockage.

Metformin alleviates neurodegeneration and hyperphosphorylation in tau-0N4R transgenics

Our previous studies have shown that the misexpression of the longest human tau (2N4R) in the *Drosophila* eye by *GMR*-GAL4 produces a characteristic “rough eye” phenotype that is accompanied by increased tau hyperphosphorylation and aggregation [25]. To investigate whether Metformin is able to ameliorate human-tau induced neurodegeneration we overexpressed tau-0N4R and the pseudo-hyperphosphorylated phospho-mimetic tau-E14 selectively in the *Drosophila* eye by the eye-specific *GMR*-GAL4 driver and bred the flies in normal diet and high sugar diet respectively supplemented with and without 10 mM Metformin.

Overexpression of human tau-0N4R (Fig. 7C and Fig. 7K) generated a moderately “rough eye” phenotype compared to the severe eye-phenotypes in tau-2N4R and tau-E14 transgenics (Fig. 7 B, D and Fig. 7 J, L) in both normal and high-sugar diet. The treatment of tau-0N4R transgenics with 10 mM Metformin partially rescued the eye phenotype (Fig. 7G and Fig. 7O) but this effect was not observed in tau-E14 or tau-2N4R flies (Fig. 7 F, H vs Fig. 7 N, P) with advanced neurodegenerative retinal phenotypes.

Notably, while the number of ommatidia significantly (* $p < 0.01$) increased in control flies and tau-0N4R transgenics in normal food supplemented with Metformin (Fig.7 R i); in the high sugar diet we observed a decrease in ommatidial numbers for all the genotypes (Fig. 7R ii).

Since the “roughness” of human-tau induced *Drosophila* eye-phenotype is associated with tau hyperphosphorylation we investigated the impact of Metformin on total and hyperphosphorylated tau (Ser199/202) in these transgenics. Our western blot (Fig. 7Q) data showed that there were no alterations in total tau levels in the tau-0N4R transgenics (Fig.7R iii and iv). However, there was a significant decrease (* $p < 0.05$) of hyperphosphorylated tau (Ser199/202) in normal and high sugar diet supplemented with Metformin particularly in female tau-0N4R transgenics (Fig. 7R v and vi). This effect was also significant (* $p < 0.01$) in male tau-0N4R transgenics but only in normal food supplemented with Metformin (Fig. 7R v).

In case of tau-E14 transgenics we found a significant decrease of total tau in the female flies raised in normal food supplemented with Metformin, but this was not observed in the high-sugar diet. Total tau levels in male tau-E14 transgenics remained unaltered (Fig.7R iii and iv).

We also observed that while Metformin was able to induce insulin-sensitivity via increased phospho-AKT/total AKT ratio in normal food, this effect was reversed under high-glucose conditions especially in case of tau-0N4R and tau-E14 transgenics (Fig. S4).

Taken together, our data implies that Metformin has a diet and sex-specific effect on tau expression levels in both tau-0N4R and tau-E14 transgenics. Additionally, Metformin has a relatively weaker effect on insulin sensitivity in transgenics raised on high-sugar diet.

Tau clearance is mediated partially by autophagy

We investigated whether Metformin decreased total and phospho-tau levels in the transgenics due to activation of autophagy. Ref(2P) is the *Drosophila* orthologue of the mammalian p62 protein and accumulates in cells in a state of autophagic impairment [52]. Although not significant, we found a decreased trend of Ref2(P) in tau-0N4R transgenics in normal food supplemented with Metformin that correlated with the decrease of phospho-tau levels in male and female flies (Fig. 8 A).

In high-sugar diet we observed a significant ($***p<0.0001$) decrease of Ref(2P) in the female tau-0N4R flies that was concurrent with a significant decrease of tau hyperphosphorylation. Interestingly, in male flies we noted a significant increase ($**p<0.001$) of Ref(2P) implying a sex-specific divergence of autophagic levels (Fig.8 B).

In tau-E14 transgenics we found no significant change of Ref(2P) levels in normal food supplemented with Metformin in male or female flies (Fig 8 A). Interestingly, in high-sugar diet supplemented with Metformin a significant decrease ($*p<0.01$) of Ref (2P) was observed in tau-E14 females (Fig. 8 B). In control flies, Ref (2)P levels were significantly increased in males in normal food supplemented with Metformin, but this was not observed in high-sugar diet. The opposite effect was observed in control females (Fig. 8 A and Fig.8 B).

Altogether our findings suggest that while Metformin consistently modulates total tau levels across both dietary conditions in tau-0N4R and tau-E14, the associated changes in Ref (2)P levels vary depending on metabolic state, sex, and genotype, with minimal effects under normal conditions but more pronounced and divergent alterations under high-sugar conditions.

Discussion

Recent epidemiological studies suggest that long-term use of the antidiabetic drug Metformin can reduce the risk of dementia, including Alzheimer's disease (AD), supporting its use in individuals with insulin resistance or mild cognitive impairment prior to AD diagnosis [14,15].

Supraphysiological drug concentrations are widely used in neuronal cell models to overcome limited drug uptake and to elicit measurable intracellular responses, particularly in studies of metabolism and neurodegeneration [44,52]. To model this preclinical AD condition *in vitro*, we cultured SH-SY5Y cells under high-glucose conditions to induce insulin resistance and treated them with progressively increasing concentrations of Metformin ranging from 2.5 mM to 20 mM [53; 54]. In healthy brains, 3R and 4R tau isoforms are expressed at roughly equal levels, but pathological states disrupt this balance and drive isoform-specific tauopathies.

Metformin treatment markedly elevated total tau (3R + 4R), especially at 10–20 mM. This aligns with recent evidence that co-expression of both isoforms exacerbates tau pathology and behavioural deficits in bigenic mouse models [55]. In our study while high Metformin doses did not cause overt cell death, the presence of small cytoplasmic vacuoles suggests emerging microtubule network disruption.

Hyperphosphorylated tau is a hallmark of AD, forming paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) [39,40]. Phosphorylation at Ser199/202/Thr205 (AT8 epitope) is considered an early and critical marker of AD pathogenesis. Interestingly, we observed a consistent and significant decrease in AT8-tau following Metformin treatment, suggesting a potential neuroprotective effect. As Metformin acts as an insulin sensitizer, a progressive increase in the phospho-AKT/AKT ratio was expected and confirmed in our studies. AKT negatively regulates GSK3 β , a major tau kinase responsible for hyperphosphorylating tau at the AT8 site, by phosphorylating GSK3 β at its Ser9 residue [43]. However, despite increased AKT activation, we found a decrease in inactive GSK3 β , which was contradictory to the observed reduction in tau hyperphosphorylation. Given that overall phosphorylation reflects a dynamic balance between kinase and phosphatase activities, it is plausible that the effect of GSK3 β was mitigated by endogenous PP2A, a major tau phosphatase known to dephosphorylate tau rapidly and maintain microtubule stability [56].

Numerous studies show that Metformin through mitochondrial complex I inhibition increases the AMP/ATP ratio, promoting AMPK phosphorylation at Thr172 and subsequent suppression of mTORC1 signaling [57,58]. In this study, the observed reduction in phospho-mTOR at higher doses (10–20 mM) therefore reflects effective upstream activation of this canonical AMPK–mTOR axis.

However, the inhibition of mTOR signalling does not translate into efficient autophagic activation. Reduced LC3-II/LC3-I ratio together with p62 accumulation indicates impaired autophagic flux, reflecting a dissociation between signalling activation and autophagosome formation. Under normal conditions, mature autophagosomes fuse with lysosomes to form autolysosomes that degrade cellular debris. The observed autophagic gridlock at high Metformin doses (20 mM) was supported by lysotracker staining, which revealed fewer but enlarged lysosomes, reflecting impaired lysosomal function and incomplete autophagic progression. Interestingly under situations of cellular stress for instance preincubating cells with Bafilomycin, Metformin activates autophagy even at high concentrations.

The *Drosophila* model of tauopathy provides a robust system for investigating tau-induced neurodegeneration driven by hyperphosphorylation and aggregation [25,26]. Consistent with previous reports, we show that overexpression of tau-0N4R and phospho-mimetic tau-E14 similarly disrupts retinal architecture, leading to irregular ommatidial organisation and bristle loss [59,60]. Importantly, Metformin selectively ameliorated neurodegeneration in tau-0N4R transgenics but not in tau-2N4R or tau-E14 lines suggesting that Metformin might be more

effective for milder or earlier stages of tau pathology. *In vitro* and *in vivo* have shown that 4R Tau isoforms and tau-E14 are more prone to forming pathological tau aggregates [61,62].

Mechanistically, Metformin reduced tau hyperphosphorylation (Ser199/202) without altering total tau levels in tau-0N4R flies, reinforcing its role in modulating tau toxicity rather than expression. The reduction of pre-tangle phospho-tau markers further supports its neuroprotective potential, particularly under metabolic stress conditions such as high-sugar diet. Our data is in agreement with earlier studies showing that Metformin can reduce tau phosphorylation through activation of protein phosphatase pathways and metabolic signalling mechanisms [44].

In contrast, tau-E14 transgenics showed limited responsiveness to Metformin, with no clear correlation between total tau levels and phenotypic severity. This suggests that pathological tau variants may disrupt multiple signalling pathways, rendering them less sensitive to metabolic intervention. Intriguingly, although the levels of total tau in E14 females were 2-fold higher than 0N4R females in normal food this was not the case with the male flies irrespective of dietary conditions. At this stage we don't have a clear explanation of the mechanisms behind this gender specific difference of tau accumulation but it is worth acknowledging that sex differences are observed in Alzheimer's Disease with the females displaying more propensity towards tau tangle formation in the brain especially in the presence of high amyloid-b [63].

Given the established role of autophagy in tau clearance, we evaluated Ref(2P) which is the *Drosophila* homologue of the p62 protein as a marker of autophagic flux [64] Metformin-induced reductions in Ref(2P), particularly in tau-0N4R females, correlated with decreased tau hyperphosphorylation, supporting a role for autophagy in mediating its effects. However, the increase in Ref(2P) in tau-0N4R males under high-sugar conditions points to an autophagic impairment. These findings align with previous reports of sex-dependent differences in stress resistance and lifespan in *Drosophila*, such as the preferential lifespan extension observed in females following catalase overexpression [65].

It is worth noting that Ref(2P) levels do not always directly reflect efficient protein clearance. Studies have shown that Ref(2)P is not only a marker of degradation but can also participate in the formation of protein aggregates [66]. Evidence from mammalian models indicates that tau aggregates can bind to the autophagy receptor p62, but are not efficiently degraded, suggesting impairment in autophagic clearance [66,67]. More recent data show that tau fibrils may even block the recruitment of other autophagy proteins, further reducing degradation efficiency. Since tau-E14 has increased propensity to form fibrils and aggregate, these findings help explain why, in this study, reductions in tau phosphorylation were not always associated with lower Ref(2)P levels or improved proteostasis.

Overall, these findings suggest that under conditions of glucose dysregulation, Metformin induces a complex response characterized by altered tau kinase/phosphatase balance and autophagic clearance. In summary our study highlights a critical dissociation between upstream

metabolic signalling and downstream proteostatic outcomes, with important implications for understanding tau pathology and therapeutic targeting in neurodegenerative models.

Materials and Methods

Cell culture and induction of insulin resistance

Human SH-SY5Y neuroblastoma cells (ATCC, VA, USA; # CRL-2266TM) were maintained in DMEM (Sigma; #D6429) supplemented with 10% foetal bovine serum (Sigma; #F2442) and 1% penicillin-streptomycin (Sigma; #P4333) at 37°C in 5% CO₂. To model metabolic dysfunction relevant to Alzheimer's disease (AD), insulin resistance was induced by culturing cells in high-glucose DMEM (4.5 g/L glucose) for 24 h, while control cells were maintained in low-glucose DMEM (1.0 g/L glucose) (GibcoTM; #31885023) [68].

MTT Assay

Cell viability was evaluated using the MTT assay (Mosmann, 1983). The cells were seeded at a density of 5000 cells per well in 96-well plates in low-glucose and high-glucose media and incubated for 24 hours at 37°C in a humidified atmosphere containing 5%CO₂ to allow cell attachment. Following the initial incubation period, cells were treated with varying concentrations of Metformin (control, 0.2, 0.8, 1, 1.5, 2, 2.5, 5, 10, 20, 50 and 100 mM) and incubated for a further 24 hours under the same conditions. After drug treatment, 10µL of MTT (0.5mg/ml; #M2128, Merck) was added to each well, and the plates were incubated for approximately 2 hours to allow the formation of purple coloured formazan crystals. The culture medium was then carefully removed, and 100µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed crystals. The optical density (OD) of each well was then measured at 570 nm using a CLARIOSTAR microplate reader (BMG Labtech). Cell viability was expressed as a percentage cell viability relative to the untreated control group versus the concentrations, and graphs were generated using Graph Pad prism to represent cell viability as a function of drug concentration.

Glucose uptake assay

Cellular glucose uptake was quantified using the 2-NBDG Glucose Uptake Assay Kit (Abcam; #ab287845) according to the manufacturer's instructions. SH-SY5Y cells ($2-5 \times 10^4$ cells/well) were seeded in 24-well plates, incubated overnight, and treated under high-glucose conditions in reduced-serum medium (0.5% FBS). Cells were then incubated with a glucose uptake reaction mixture containing 2-NBDG and uptake enhancer at 37 °C. Following incubation, cells were washed with ice-cold buffer and analysed by flow cytometry (excitation: 488 nm), and mean fluorescence intensity was quantified. Intracellular 2-NBDG fluorescence, resulting

from transporter-mediated uptake and accumulation, was used as a measure of cellular glucose uptake.

Pharmacological treatments

Metformin (Merck; #PHR1084) was dissolved in sterile water and applied at concentrations of 0, 2.5, 5, 10 and 20 mM for 24 h. To modulate autophagic flux, cells were treated with Bafilomycin A1 (Sigma–Aldrich; #19-148; 100 nM, 24 h) to inhibit lysosomal acidification and autophagosome-lysosome fusion, or Rapamycin (Invitrogen™; #PHZ1235; 2 μM, 24 h) to induce autophagy.

Transient transfection of tau constructs

SH-SY5Y cells were seeded at a density of 5×10^5 cells per well in 6-well plates and allowed to adhere overnight. Cells were transfected 20–24 h post-seeding with EGFP-tagged human tau constructs pRK5-EGFP-Tau WT (Addgene; #46904), and pRK5-EGFP-Tau E14 (Addgene; #46907) using Magnetofectamine™ O2 (OZ Biosciences; #MTX2-1000) in accordance with the manufacturer's protocol. After a 24 h recovery period, cells were treated with Metformin for an additional 24 h. Subsequently, cells were harvested, and lysates were prepared for downstream protein analyses.

Protein extraction and western blotting in SHSY5Y cells

Cells were lysed in RIPA buffer supplemented with protease inhibitors, incubated on ice and centrifuged to obtain soluble protein extracts. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes. Membranes were blocked in 5% milk and incubated overnight with the following primary antibodies: Total Tau (#A0024, 1:10,000, DAKO; #136400, 1:1000, Thermo Fisher Scientific); phospho-Tau-Ser199/Ser202 (#44768G, 1:1,000, Thermo Fisher Scientific); AT8 (#MN1020, 1:1,000, Invitrogen); AKT (#58295S, 1:1,000, Cell Signaling Technology); phospho-AKT-Ser473 (#9271S, 1:1,000, Cell Signaling Technology); phospho-GSK-3β-Ser9 (#5558T, 1:1,000, Cell Signaling Technology); total GSK-3β (#124756T, 1:1,000, Cell Signaling Technology); demethylated-PP2A (#05-421, 1:1,000, Merck); total PP2A (#05-577, 1:1,000, Merck); LC3 (#4108S, 1:1,000, Cell Signaling Technology); p62/SQSTM1 (#5114, 1:1,000, Cell Signaling Technology).

Following primary antibody incubation, membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and subsequently incubated with HRP-conjugated secondary antibodies, including anti-rabbit (#A6154, 1:3,000, Sigma-Aldrich) and anti-mouse (#10301364, 1:3,000 Invitrogen), for 1 h at room temperature. After additional washes in TBS-

T, protein bands were detected using an enhanced chemiluminescence (ECL) reagent (Bio-Rad, #1705061) and visualised using an iBright™ CL1500 Imaging System.

Immunocytochemistry and confocal microscopy

SH-SY5Y cells were fixed in 4% (w/v) paraformaldehyde, followed by permeabilisation with Triton X-100 and blocking in 1% (w/v) bovine serum albumin to minimise non-specific binding. Primary antibodies against total Tau (#A0024, Dako); phospho-Tau-Ser199/Ser202 (#44768G, Thermo Fisher Scientific); phospho-Tau-Ser202/Thr205 (#MN1020, Invitrogen); AMPK (#5831T, Cell Signaling Technology); phospho-AMPK (#4188S, Cell Signaling Technology); and LC3 (LC3-I/II, 4108S, Cell Signaling Technology) were applied at a dilution of 1:200 overnight at 4 °C. The cells were incubated for an 1 hour with fluorophore-conjugated secondary antibodies, including Alexa Fluor anti-mouse (#62181, Invitrogen; 1:50) and Alexa Fluor anti-rabbit (#AB150077, Abcam; 1:50) washed in 1X PBST and mounted using antifade mounting medium (#AB104139, Abcam) following which the nuclei were counterstained by DAPI. Images were acquired using a Leica SP5 confocal microscope with a 63× oil-immersion objective. Quantitative analysis, including puncta counting and co-localisation studies, was performed using ImageJ software. Lysosomal compartments were labelled with LysoTracker™ Red DND-99 (50-75 nM) for 30-60 min at 37°C. Following washing, cells were immediately imaged using confocal microscopy. Z-stack imaging was used to assess lysosomal distribution and morphology.

***Drosophila* stocks and husbandry**

Drosophila stocks overexpressing human tau isoforms *UAS-tau2N4R* (51362) and *UAS-tau0N4R* (78847) and controls (2376) were obtained from the Bloomington *Drosophila* Stock Center (BDSC). The stock *UAS-tauE14* was a gift from Professor Mel Feany.

These transgenic lines were crossed to the retinal driver *GMR-GAL4-nina E* (a gift from Dr. Lovesha Sivanantharaja) to generate *GMR-GAL4/UAS-tau2N4R*, *GMR-GAL4/UAS-tau-0N4R* and *GMR-GAL4/UAS-tau-E14* respectively. *GMR-GAL4/UAS-OregonR* (2376) was used as a control. *Drosophila melanogaster* strains were maintained on Nutri-Fly Bloomington formulation medium (Genesee Scientific, #66-121) supplemented with 0.5% propionic acid (Sigma–Aldrich, #402907) under standard conditions (25 ± 1 °C, 60–70% humidity, 12 h light/dark cycle). Flies were transferred to fresh food every 2–3 days (25). Drug-supplemented diets were prepared by adding compounds to freshly prepared media prior to solidification. Briefly, Metformin (Sigma–Aldrich, #PHR1084) was added at 55–60 °C to a final concentration of 10 mM. For high-sugar conditions, D-(+)-glucose (Sigma–Aldrich, G7021) was added from a 1 M stock to achieve the desired concentration (24).

Scanning electron microscopy

Adult male flies were dehydrated through graded ethanol solutions, dried using hexamethyldisilazane, mounted on aluminium stubs and sputter-coated with gold. Samples were examined by scanning electron microscopy to assess eye morphology and ommatidial organisation following standard protocols (25).

Western Blotting

Adult age matched flies were snap-frozen in liquid nitrogen, and heads were subsequently isolated by mechanical separation. 10 fly heads were homogenized in lysis buffer at a ratio of 1 head per 5 μ L buffer (150 mM NaCl, 50 mM MES, 1% Triton X-100, protease inhibitor cocktail, 30 mM NaF, 40 mM β -glycerophosphate, 20 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, 1% SDS, and 10 μ M staurosporine). Homogenates were sonicated for 30 sec, centrifuged at $3,000 \times g$, and the resulting supernatants were collected while pellet debris was discarded. Samples were then mixed with $2\times$ SDS sample buffer and denatured by boiling at 95°C for 5 min. Protein samples were subsequently subjected to western blotting with the antibodies mentioned above following standard protocols (25).

Statistical analysis

Experiments were performed using at least three independent biological replicates. Statistical analysis was performed using GraphPad prism 10 software (GraphPad Software version 10; CA USA). Data normality was assessed by Shapiro-Wilk test and all data sets confirmed to a normal distribution. Data are presented as mean $\pm$ SEM, $n=3$. Pairwise comparisons were analysed using Student's t-tests, while multiple-group comparisons were analysed by one-way ANOVA with Tukey's post hoc tests. Statistical significance was defined as $P < 0.05$.

Data availability

All data supporting the findings of this study are provided within the manuscript and its supplementary Information. No additional data has been generated.

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CRedit Authorship Contributions

Srinithya Paruchari: Investigation, Methodology, Formal analysis, Data curation, Writing-Original Draft. **Lovesha Sivanantharajah:** Writing-review and editing. **Amritpal Mudher:** Conceptualization. **Shreyasi Chatterjee:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Conflicts of interest

There are no conflicts of interest disclosed by the authors.

Figure Legends

Fig.1: Tau expression is increased upon Metformin treatment.

A Representative immunoblots of total tau (T46 and A0024), and Actin in SH-SY5Y cells treated with increasing concentrations of Metformin (0, 2.5, 5, 10 and 20 mM). Quantification of T46 and A0024 band intensities normalised to Actin is shown (right). Data are mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ****p < 0.0001. *Full western blots are displayed in Supplementary Figure 5.* B Representative immunofluorescence images of total Tau (A0024; green) in SH-SY5Y cells treated with Metformin (0–20 mM). Nuclei are counterstained with DAPI (blue). Merged images are shown in the bottom panel. Quantification of A0024 fluorescence intensity is presented (right). Data are mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ****p < 0.0001.

Fig.2: Hyperphosphorylated tau is significantly increased upon Metformin treatment.

A Representative western blots of hyperphosphorylated tau (pSer199/202 and pSer202/Thr205) and Actin in SH-SY5Y cells treated with increasing concentrations of Metformin shows a significant decrease from 5mM to 20 mM. Quantification of band intensities normalised to Actin is shown (bottom). Data are presented as mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. *Full western blots are displayed in Supplementary Figure 6.* B Representative immunofluorescence images of phosphorylated tau at pSer199/202 (green) and pSer202/Thr205 (red) in SH-SY5Y cells treated with Metformin (0–20 mM) showing significant decrease. Nuclei are counterstained with DAPI (blue in merged images). Data are mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Fig.3 Metformin treatment modulates AKT/GSK3 β /PP2A axis impacting tau hyperphosphorylation.

A, B and C Representative western blots of phosphorylated AKT (pAKT), total AKT, phosphorylated GSK3 β (pGSK3 β -Ser9), total GSK3 β , demethylated PP2A catalytic subunit (DMPP2A-C), total PP2A-C and Actin in SH-SY5Y cells treated with increasing concentrations of Metformin (0, 2.5, 5, 10 and 20 mM). Quantification of pAKT normalised to total AKT, pGSK3 β normalised to total GSK3 β , active PP2A (DMPP2A-C normalised to total

PP2A-C (bottom). Data are presented as mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001. *Full western blots are displayed in Supplementary Figure 7.*

Fig.4 Metformin treatment activates AMPK.

A Phosphorylated AMPK (pAMPK; green) and total AMPK (green, separate panel) staining in SH-SY5Y cells treated with increasing concentrations of Metformin (0, 2.5, 5, 10 and 20 mM). AMPK staining is localized in the nuclei and cytoplasm and p-AMPK in the cytoplasm respectively. Nuclei are counterstained with DAPI (blue). B: Quantification of pAMPK fluorescence intensity normalised to total AMPK across Metformin treatments (0–20 mM). Data are presented as mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig.5 High-dose Metformin inhibits autophagic flux.

A LC3 (green) staining in SH-SY5Y cells cultured under high-glucose (HG) conditions and treated with Bafilomycin (negative control), Rapamycin (positive control) and increasing concentrations of Metformin (0, 2.5, 5, 10 and 20 mM). Progressive Metformin treatment displays a gradual accumulation of LC3 punctae. Nuclei are counterstained with DAPI (blue). Data are presented as mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; **p < 0.01; ***p < 0.001. B Representative immunoblots of LC3-I/LC3-II, p62 and Actin in SH-SY5Y cells treated with increased Metformin concentrations displays enhanced LC3-II and p62 levels. Quantification of LC3-II/LC3-I ratio normalised to Actin and p62 levels normalised to Actin respectively following treatment with Bafilomycin, Rapamycin or Metformin (0–20 mM). Data are presented as mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001. *Full western blots are displayed in Supplementary Figure 8.*

Fig.6 Metformin modulates the number and size of lysosomes.

A Representative fluorescence images of Lysotracker staining (red) in SH-SY5Y cells treated with increasing concentrations of Metformin (0, 2.5, 5, 10 and 20 mM) displays an enhancement in the size of lysosomes. Nuclei are counterstained with DAPI (blue). Merged images are shown. B Quantification of Lysotracker staining parameters, including puncta number, area, size and fluorescence intensity, across Metformin treatments (0–20 mM). Data are mean $\pm$ S.E.M, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Fig.7 Metformin modulates tau pathology in *Drosophila* under normal and high-sugar dietary conditions.

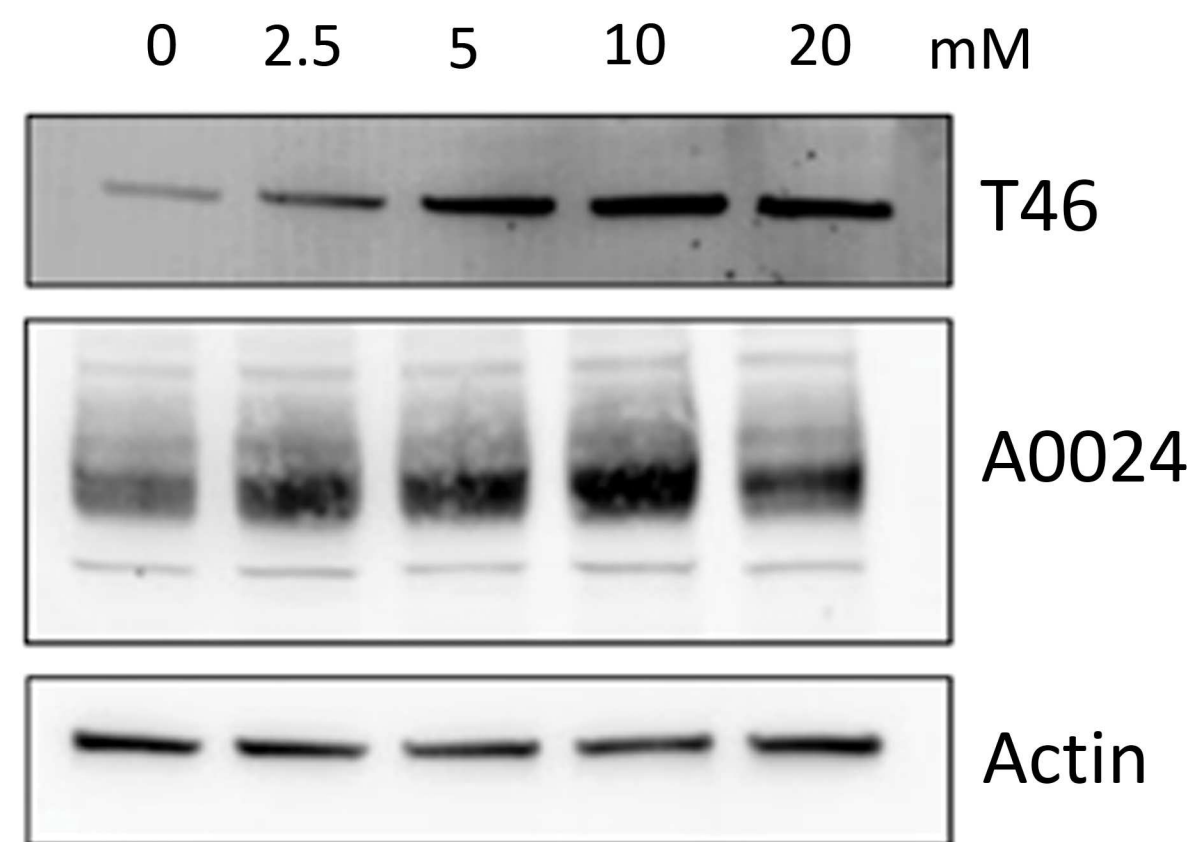
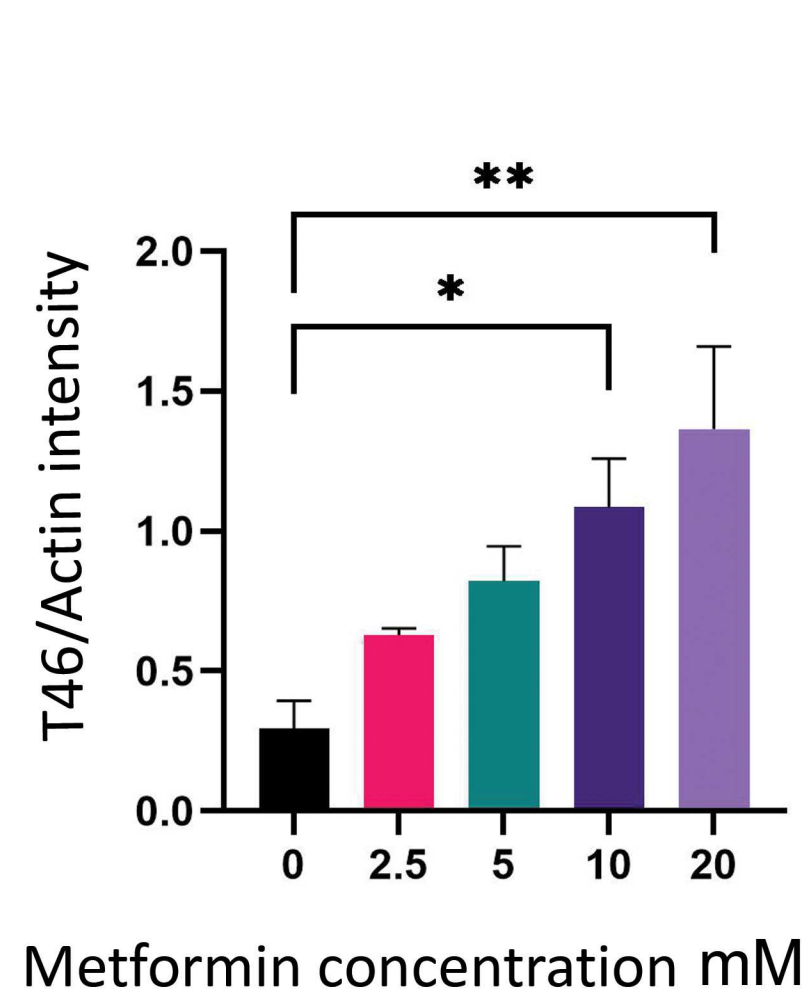
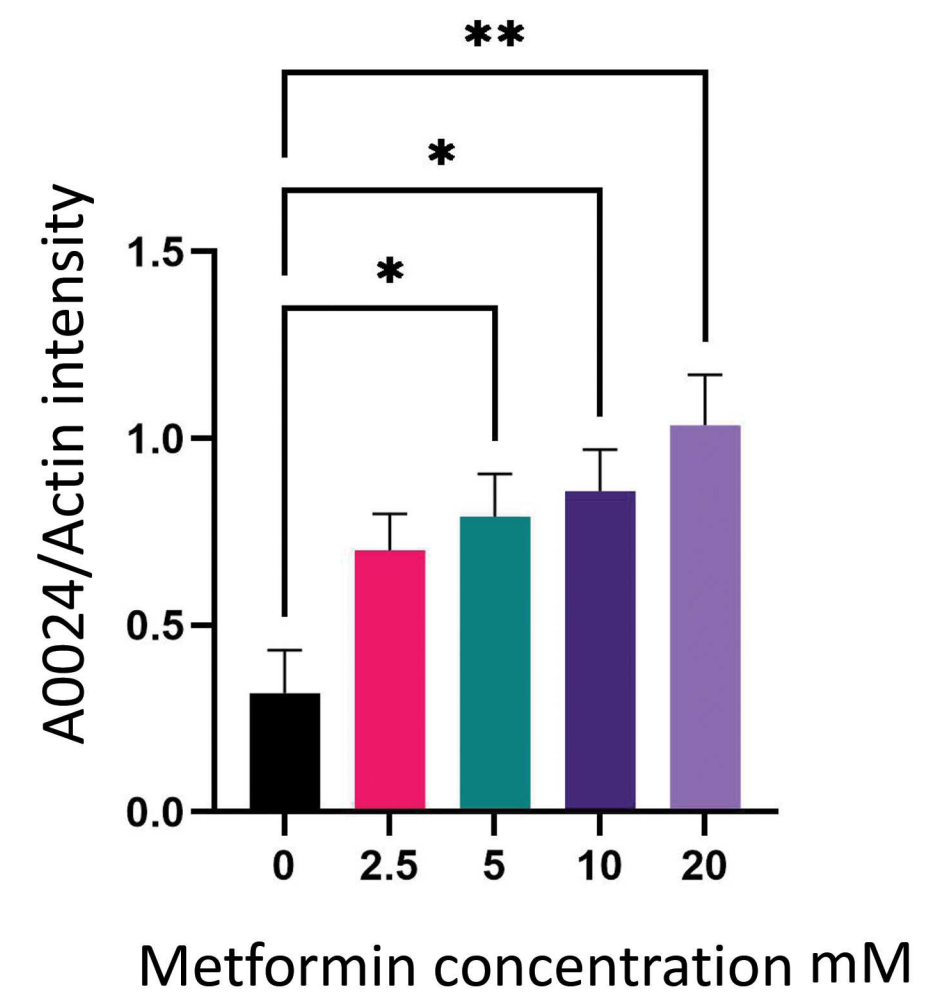
(A – P) Representative scanning electron microscopy images of *Drosophila* compound eyes expressing control, tau-2N4R, tau-0N4R and tau-E14 under normal food and high-sugar food conditions, with or without Metformin. Q Representative immunoblots of total tau and phosphorylated tau at pSer199/202 in *Drosophila* retina under normal food and high-sugar food

conditions, in the presence or absence of Metformin. Actin is the loading control. R (i) and (ii), Quantification of ommatidial numbers in male flies in normal and high sugar diet with and without Metformin. R (iii-vi), Quantification of total Tau and pSer199/202 levels in female (F) and male (M) flies under normal and high-sugar diet conditions with and without Metformin treatment. Data are mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. ns, not significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. *Full western blots are displayed in Supplementary Figure 9.*

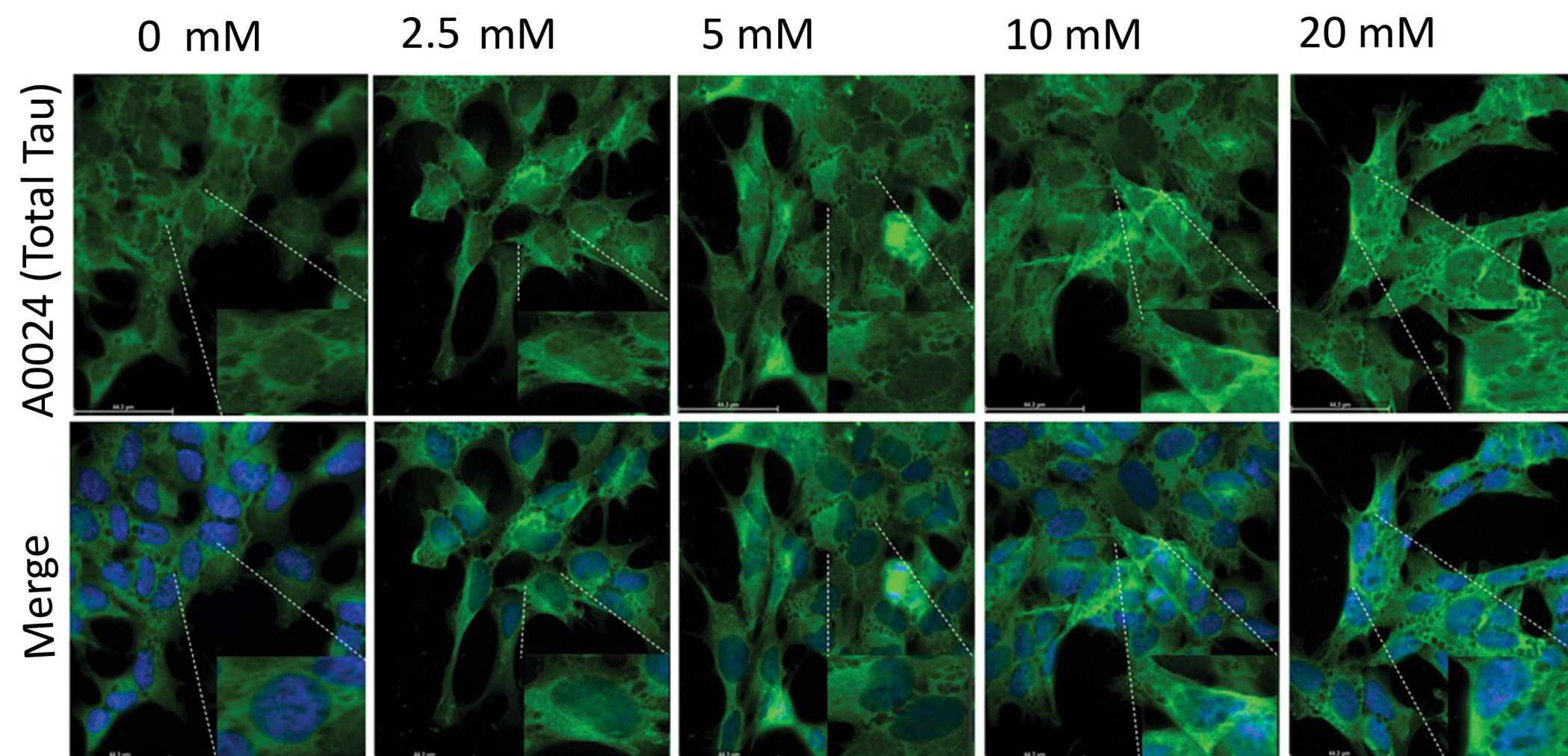
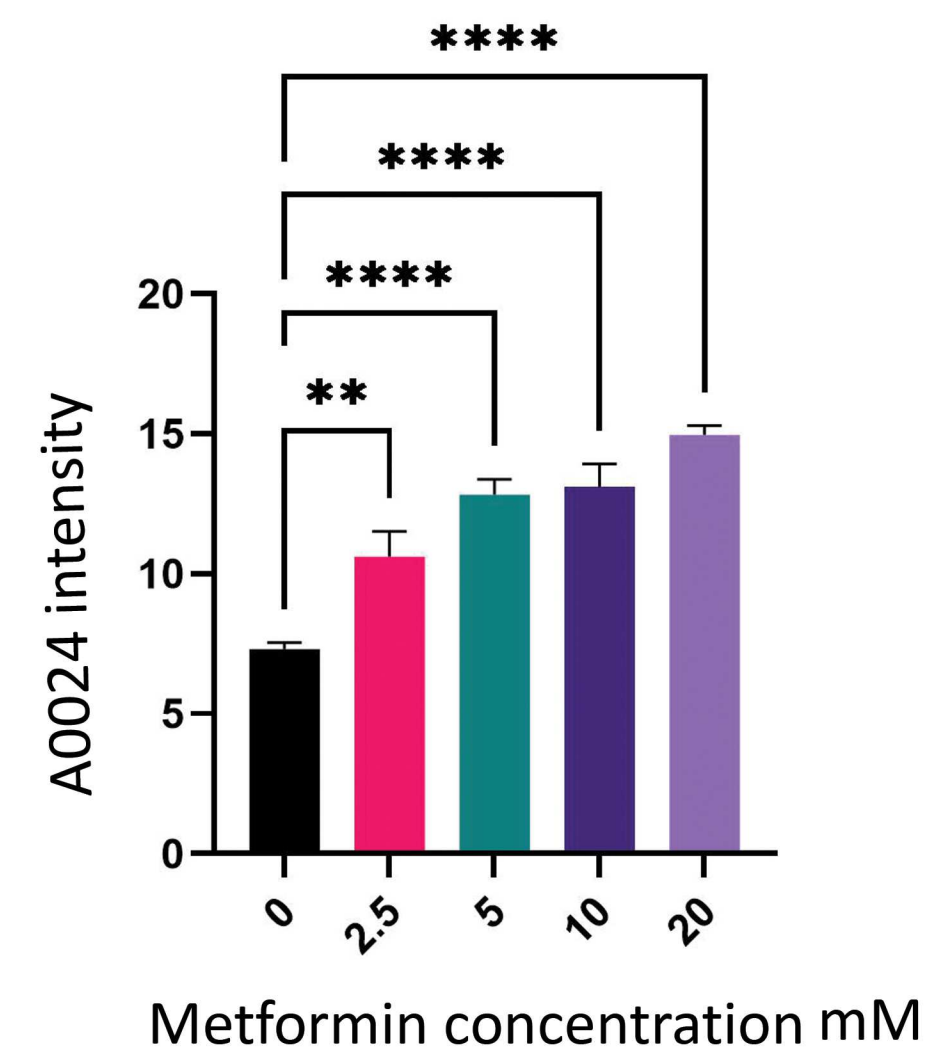
Fig. 8. Ref (2)P expression in tau-expressing flies under normal and high-sugar diets.

Representative western blots of Ref (2)P and Actin in controls, tau-0N4R, and tau-E14 flies reared on A normal food or B high-sugar diet (HSD) supplemented with and without Metformin. In female flies, both tau genotypes displayed higher Ref (2)P than controls on HSD that was significantly reduced by Metformin supplementation. In male flies, HSD reduced Ref (2P) levels in both tau transgenics and controls compared to normal food. Metformin in HSD significantly increased Ref (2P) levels but only in tau-0N4R males. Data are mean $\pm$ SEM (n = 3 biological replicates). ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. *Full western blots are displayed in Supplementary Figure 10.*

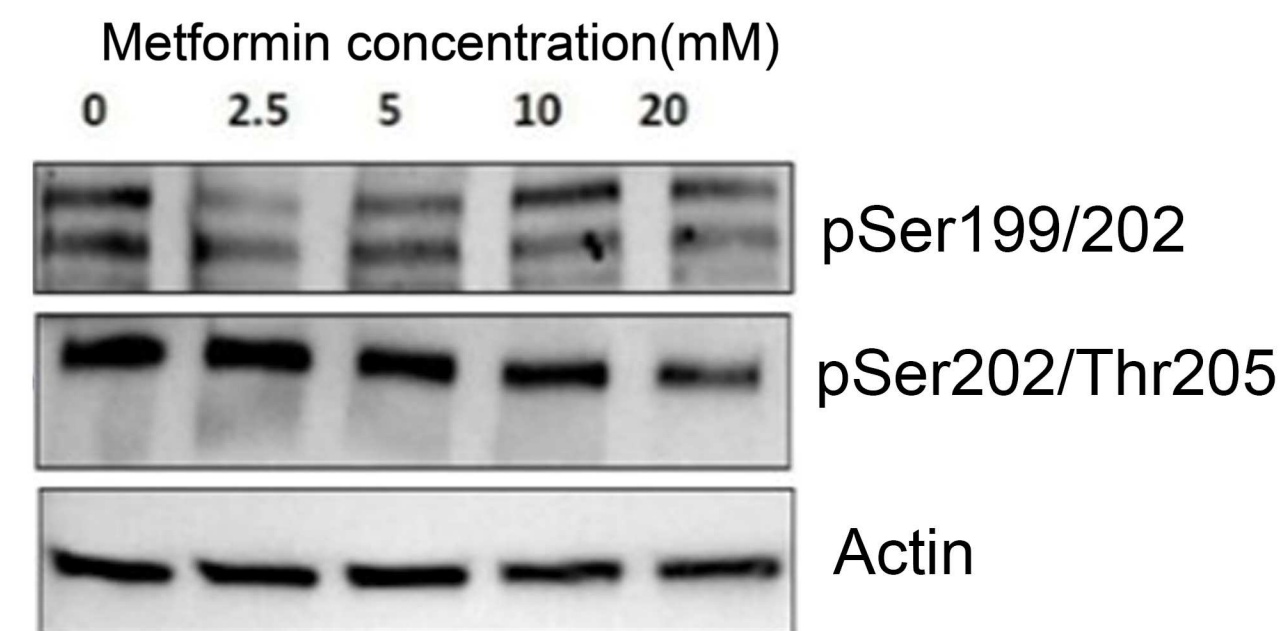
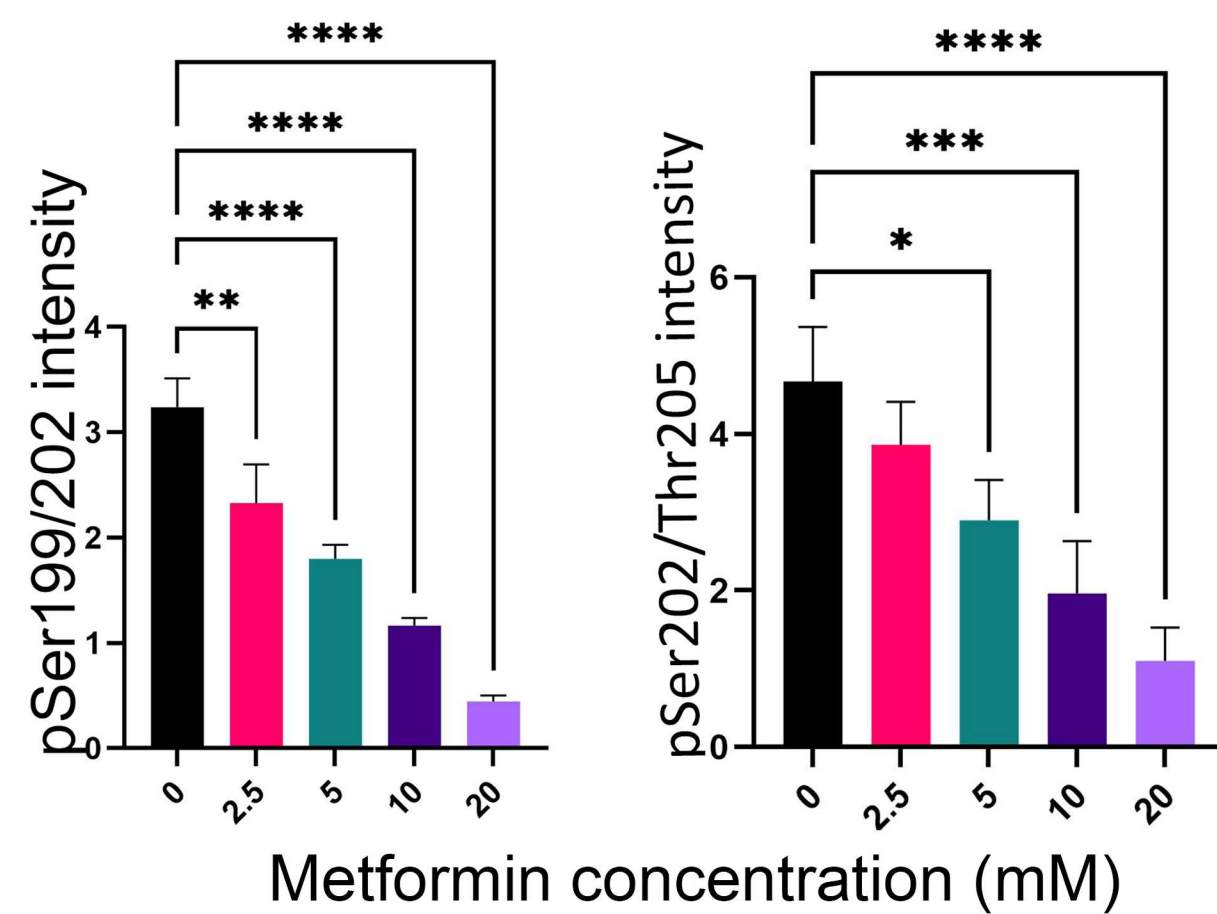
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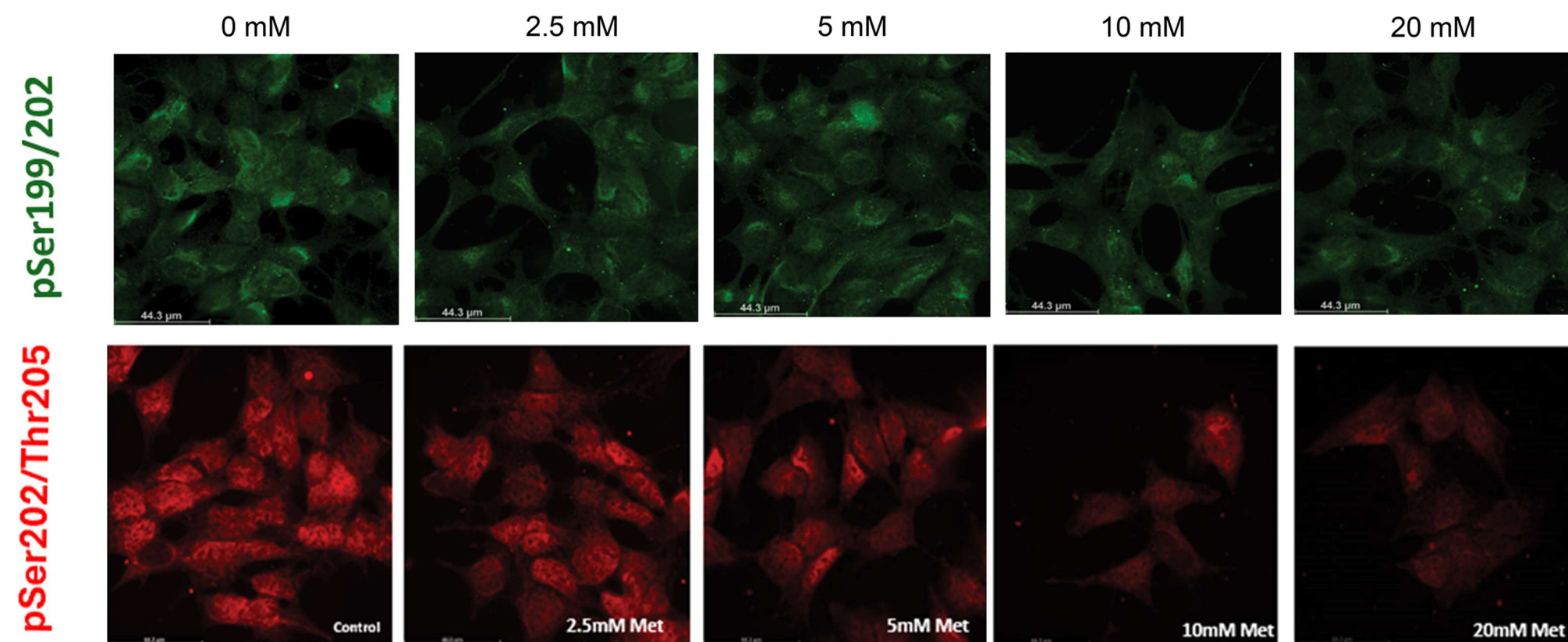
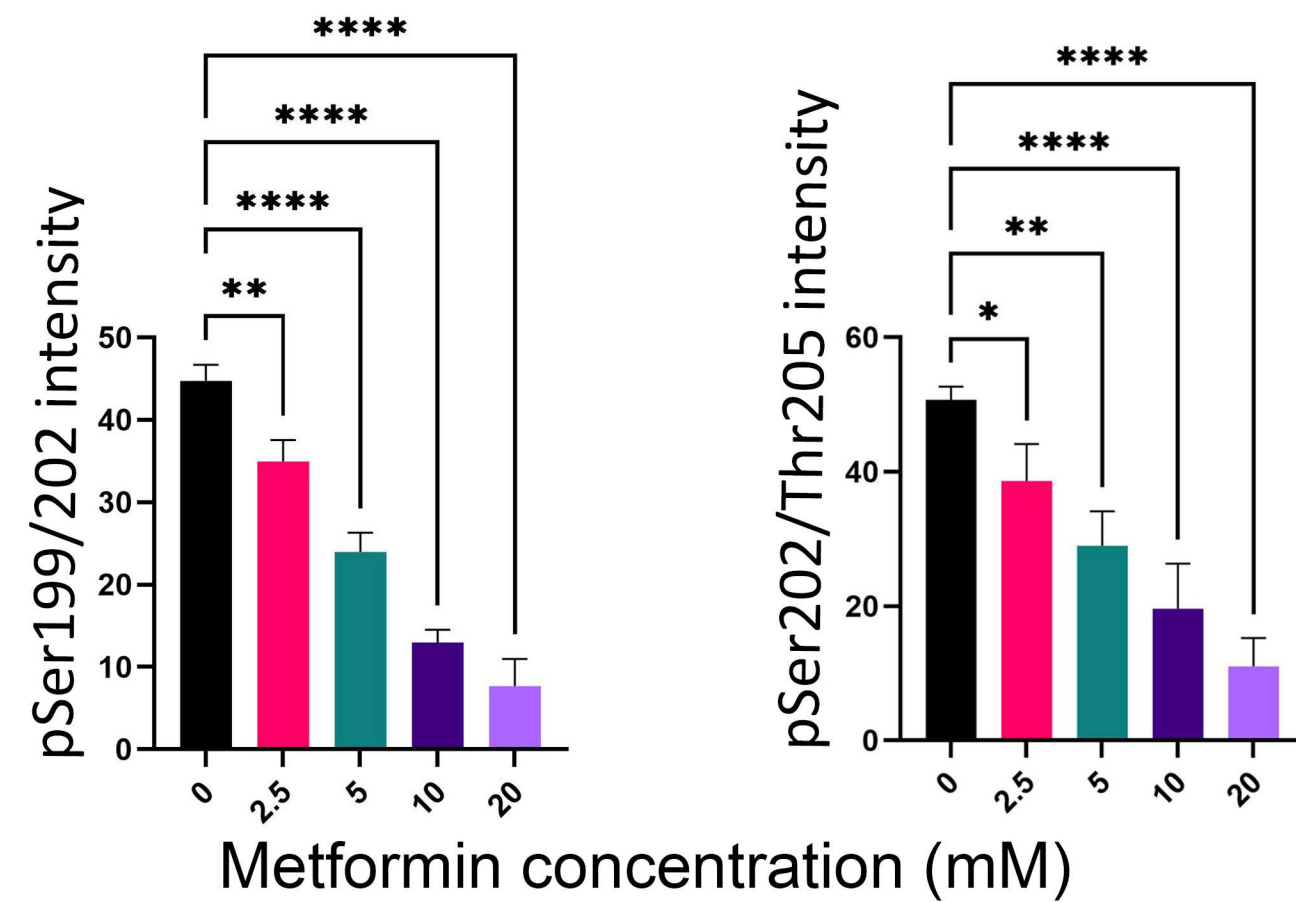
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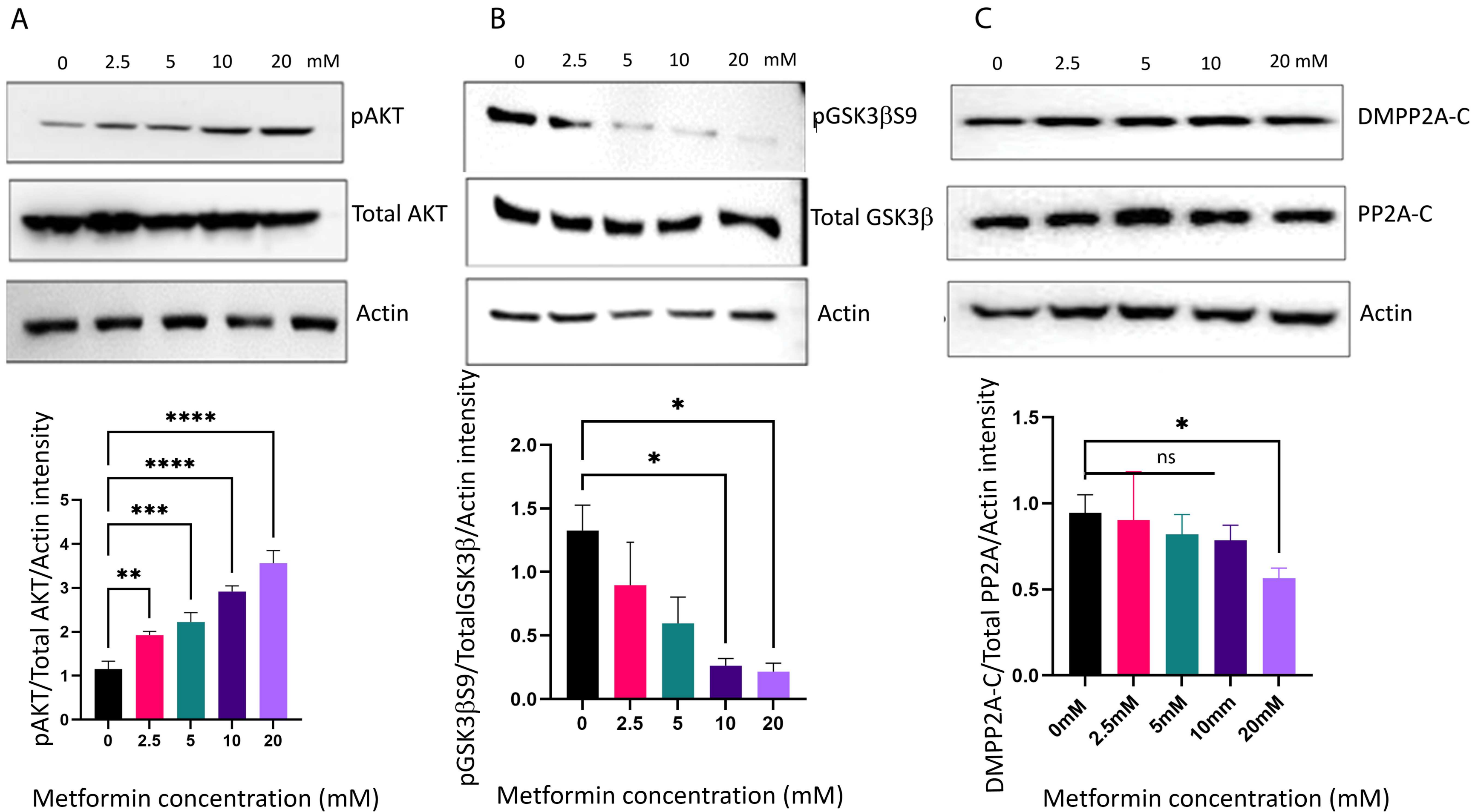


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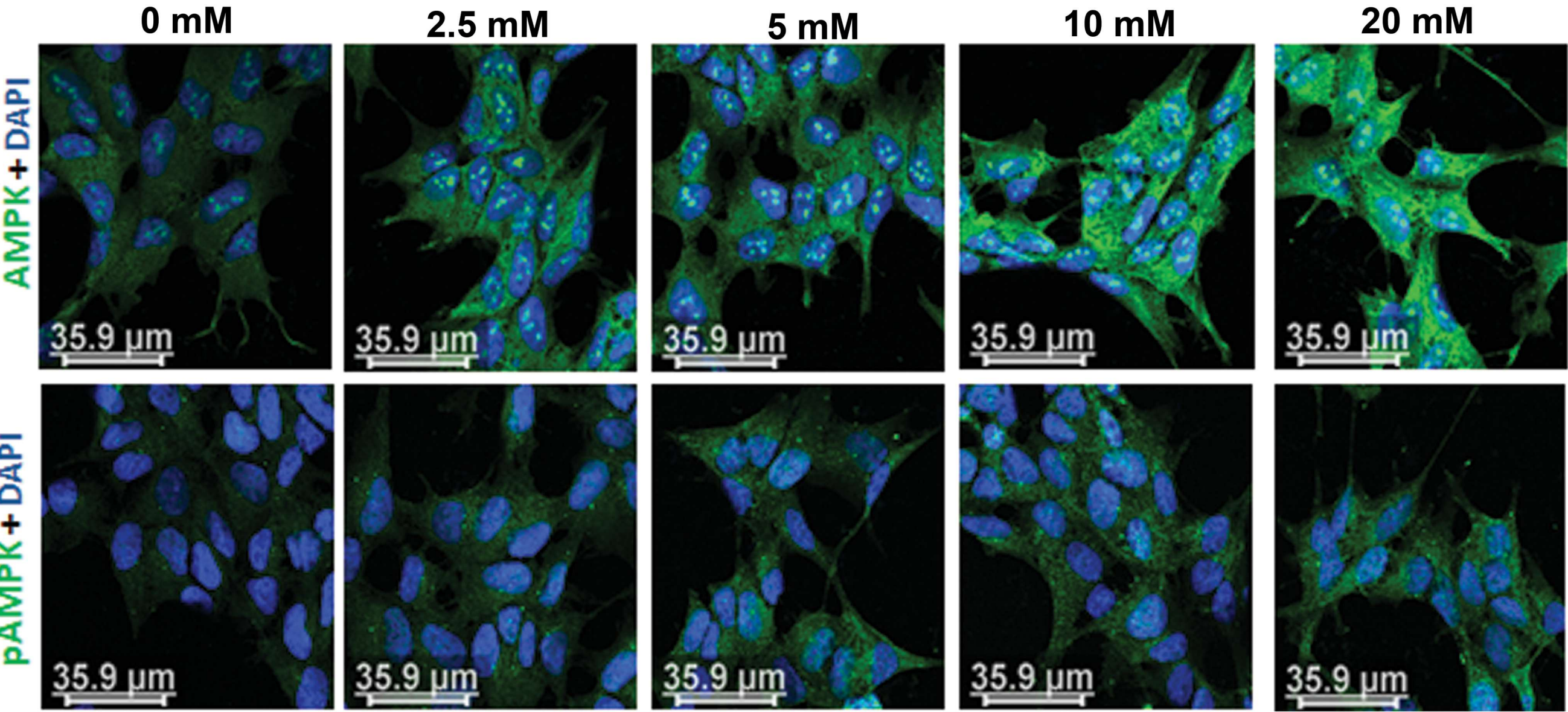
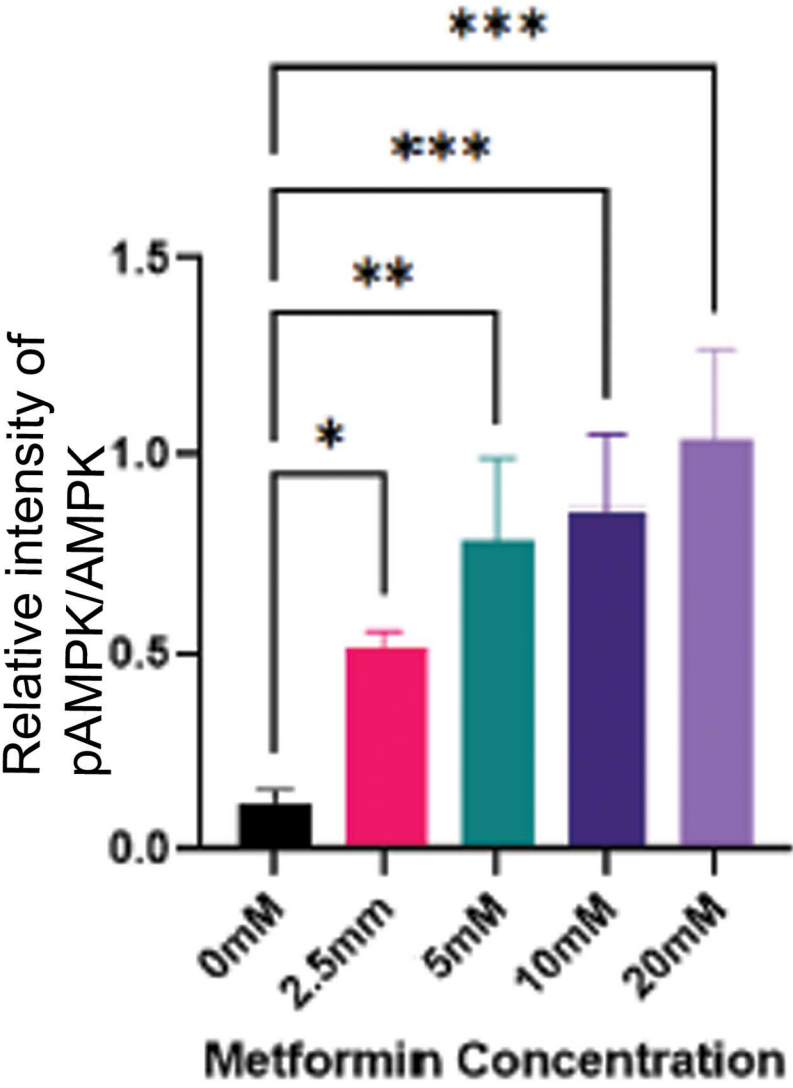


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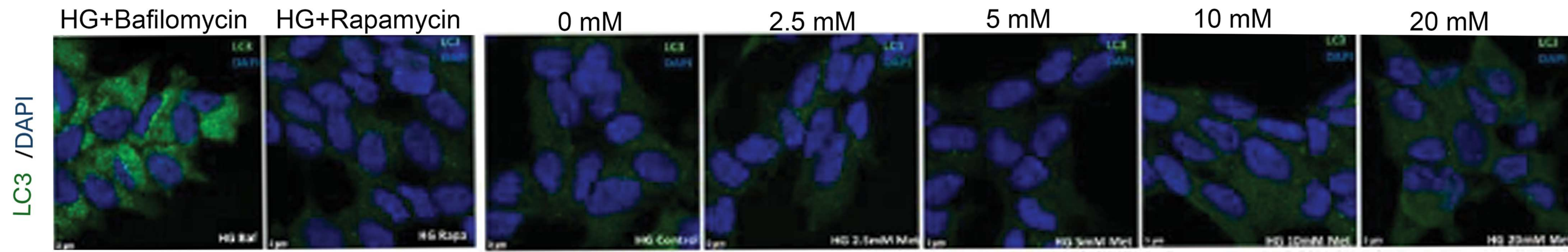
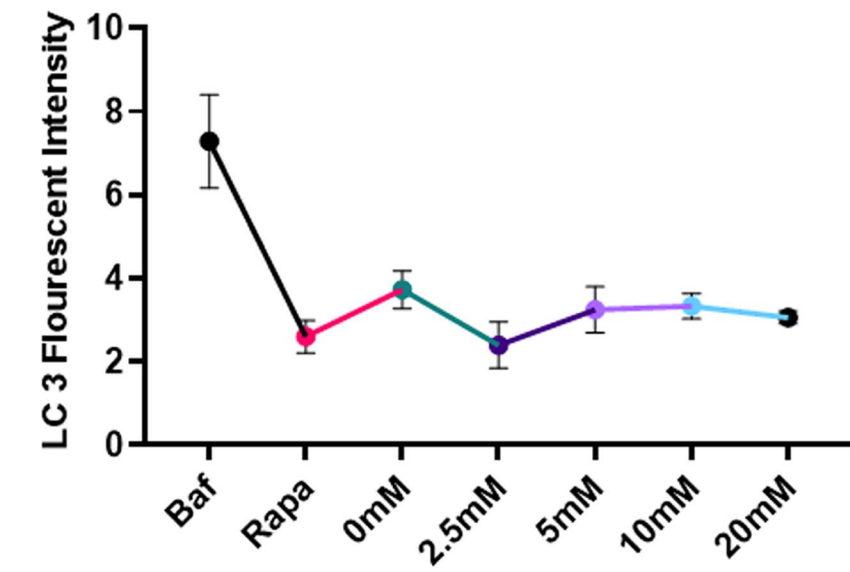




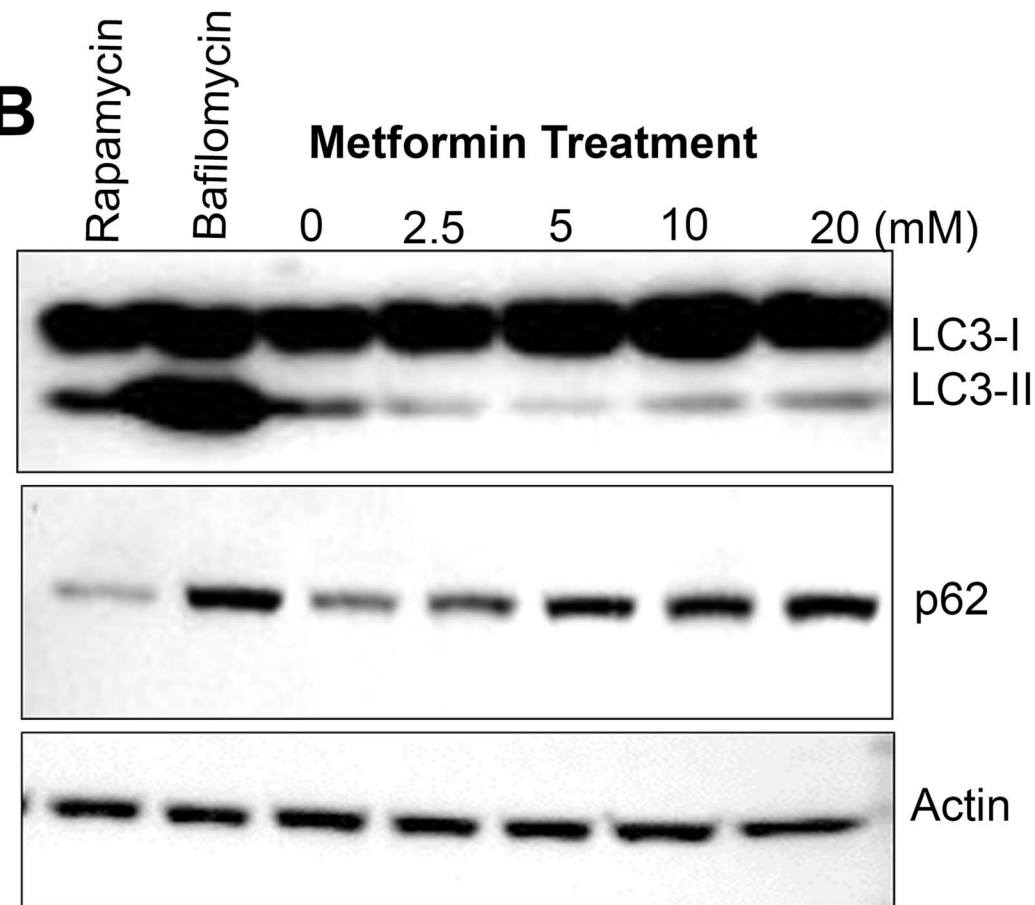
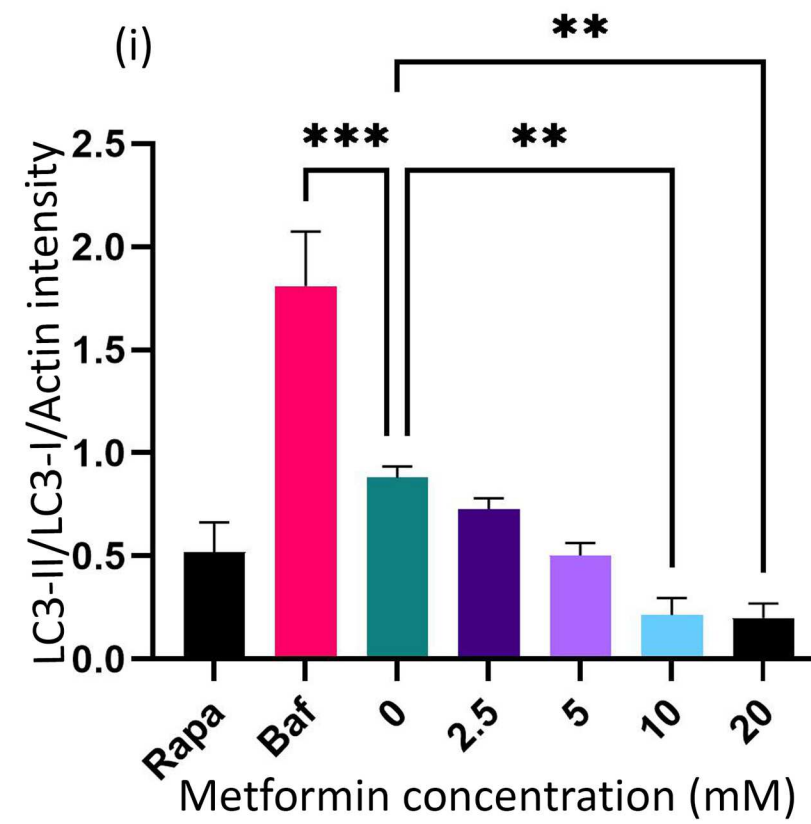
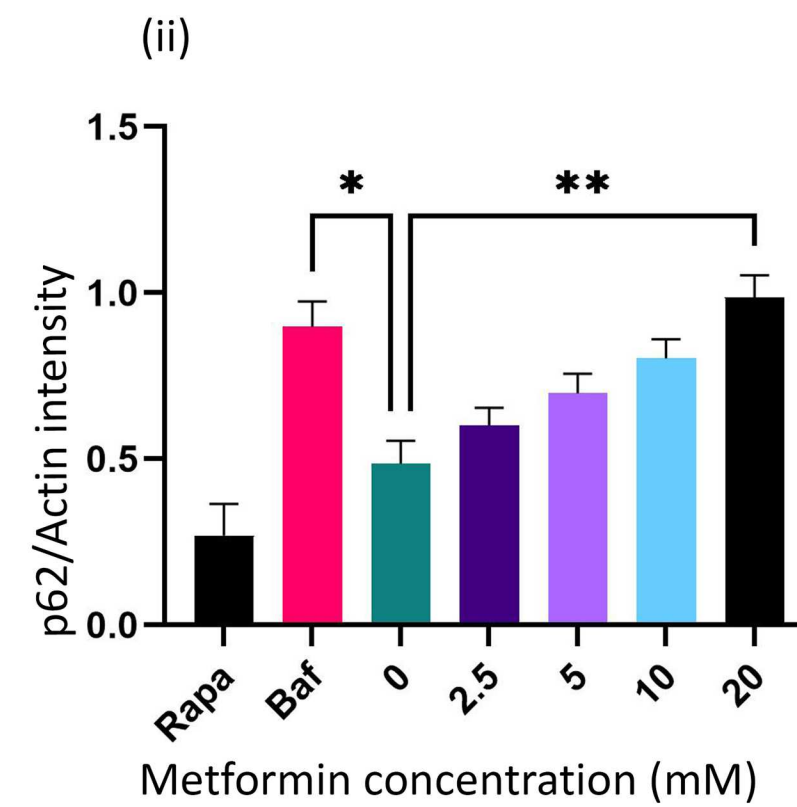
Metformin treatment



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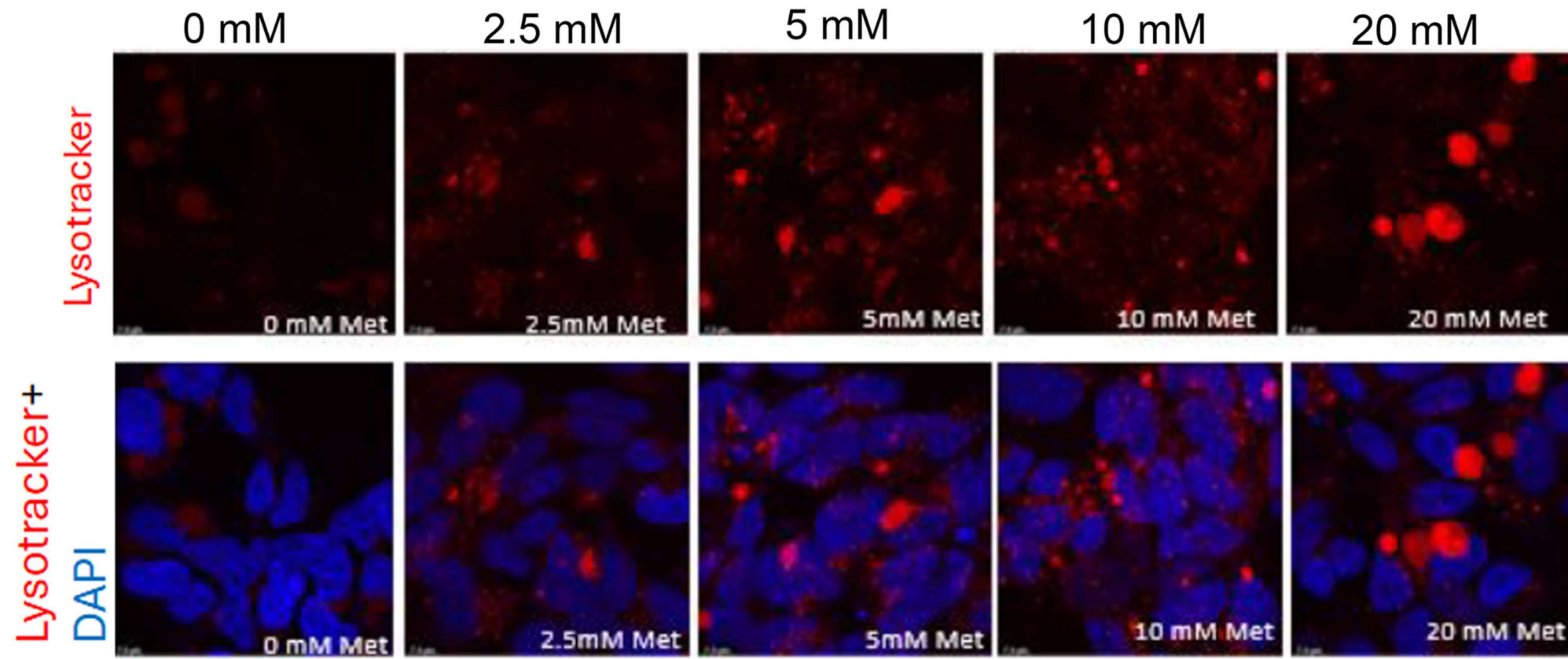


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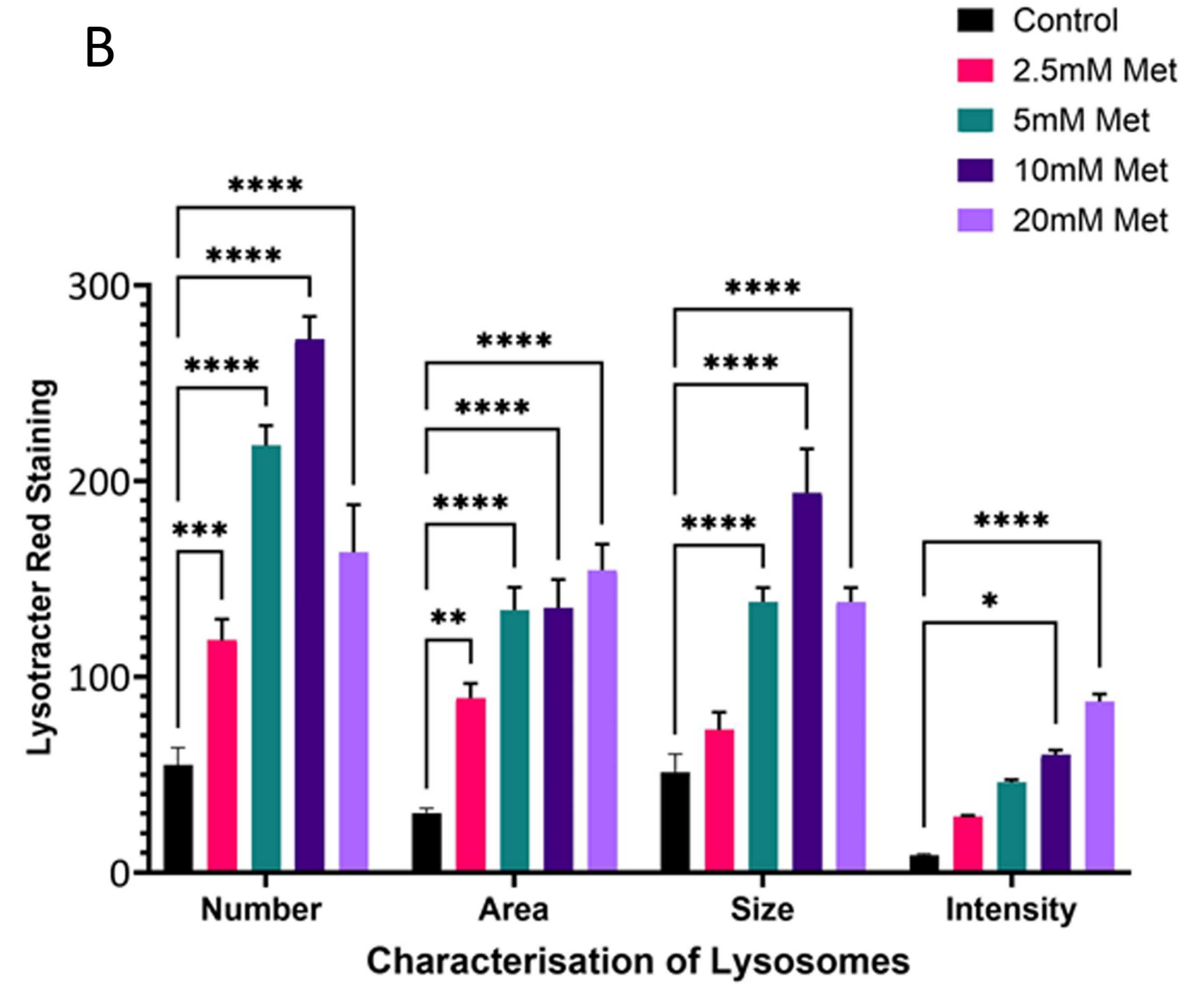


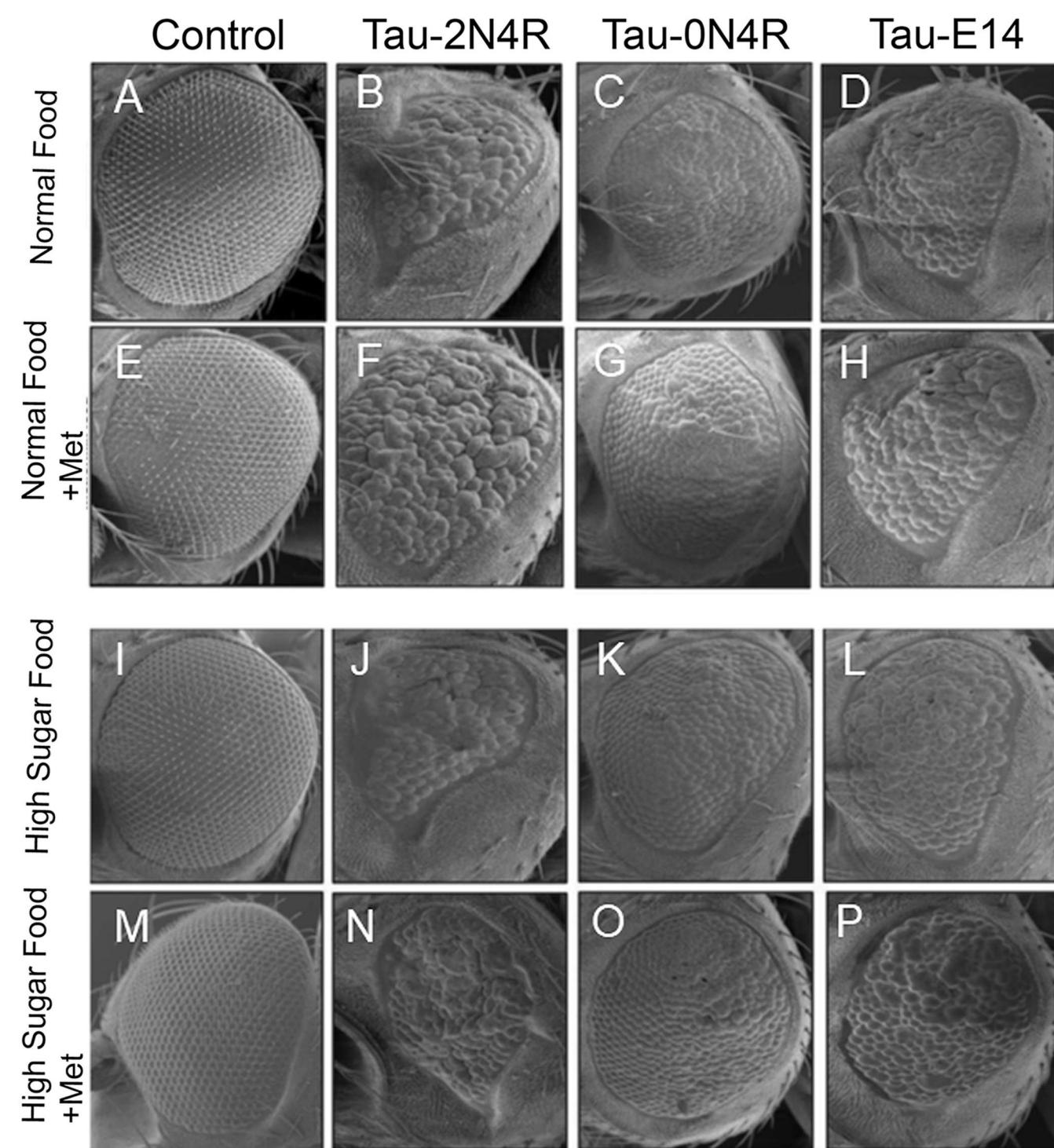
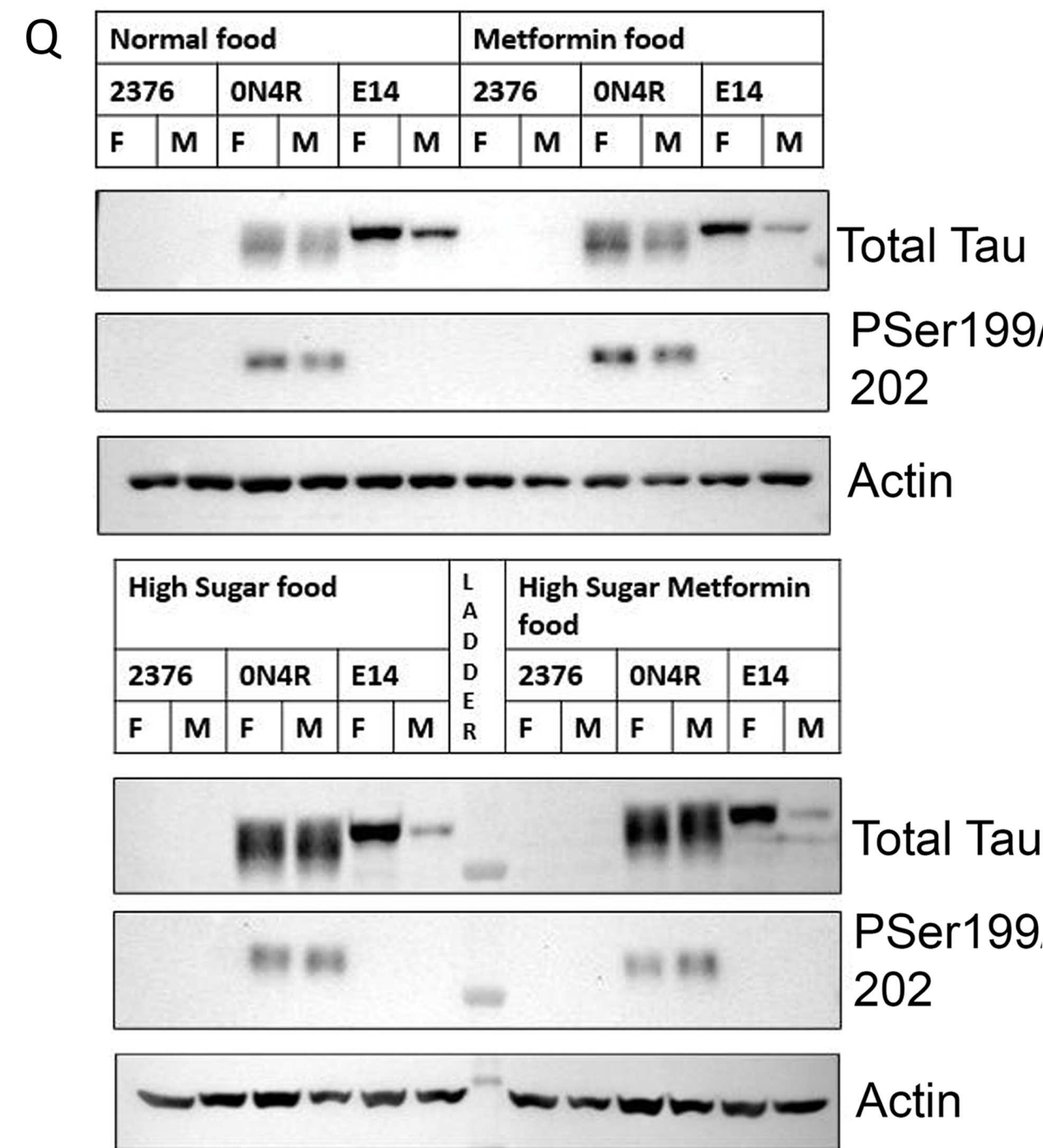
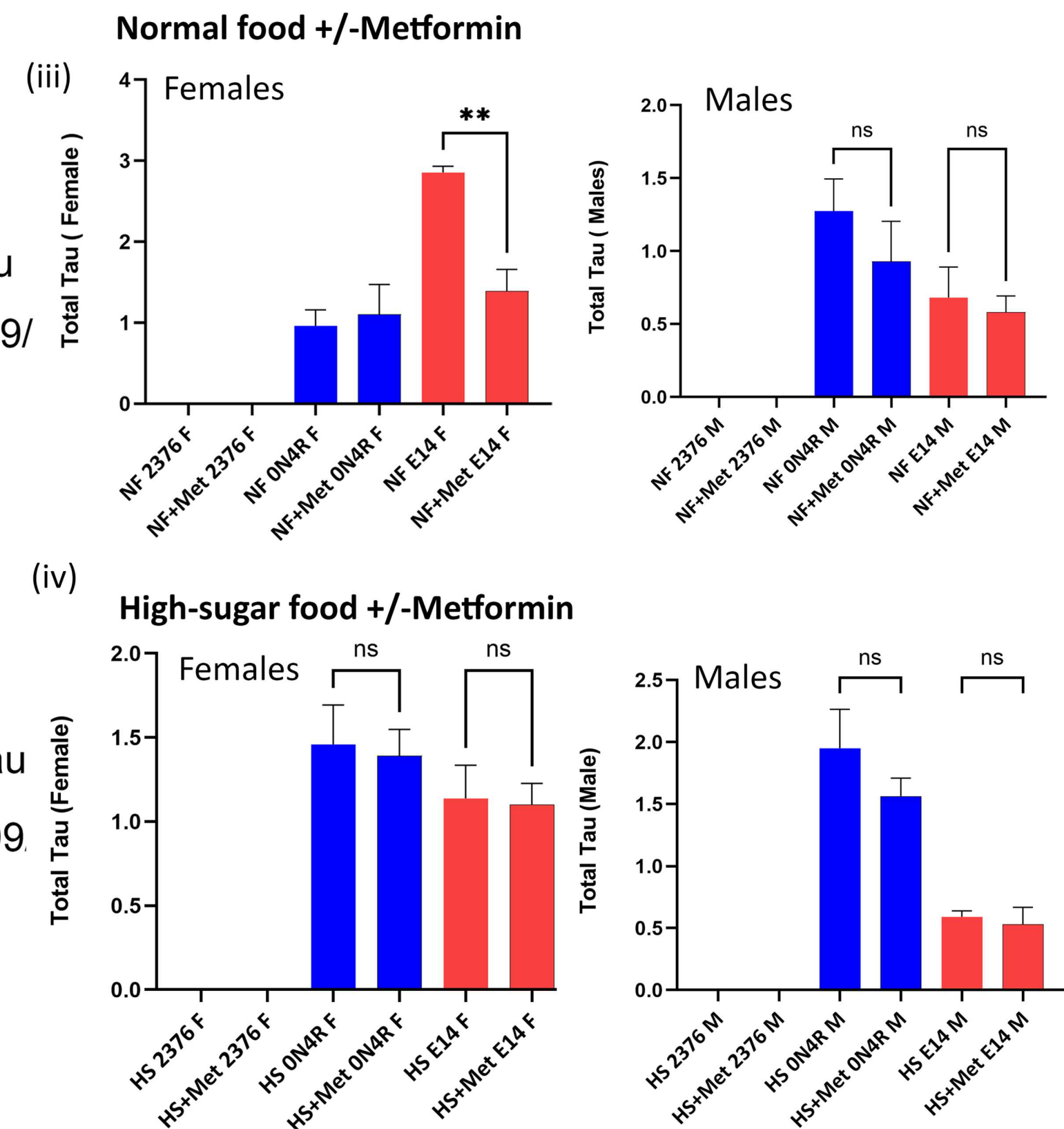
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Metformin concentration

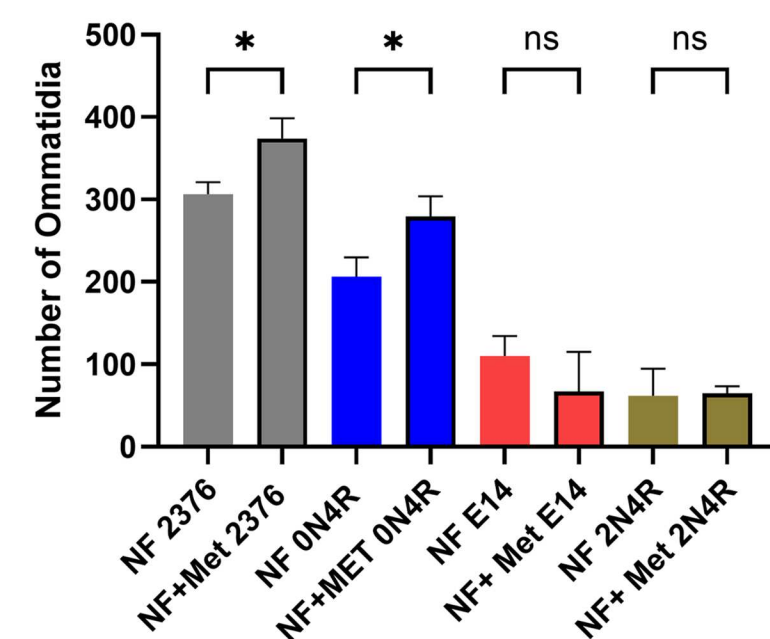


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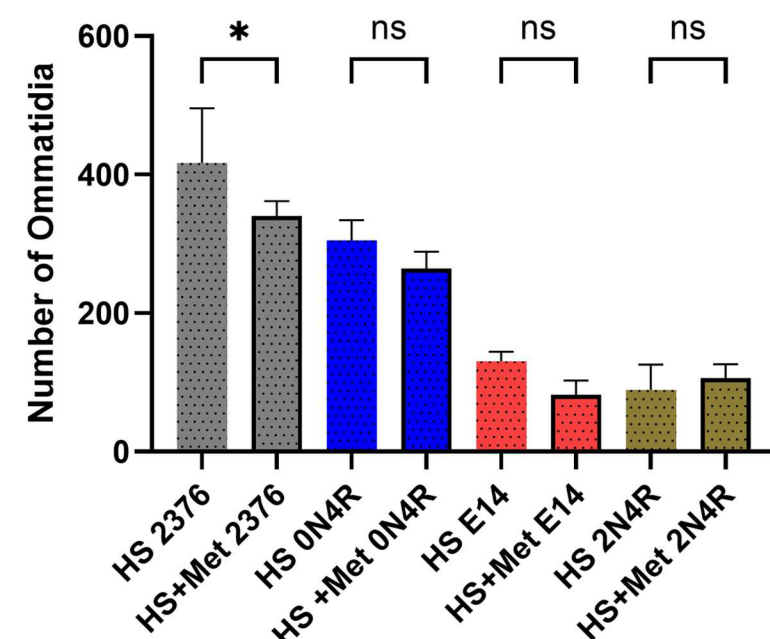




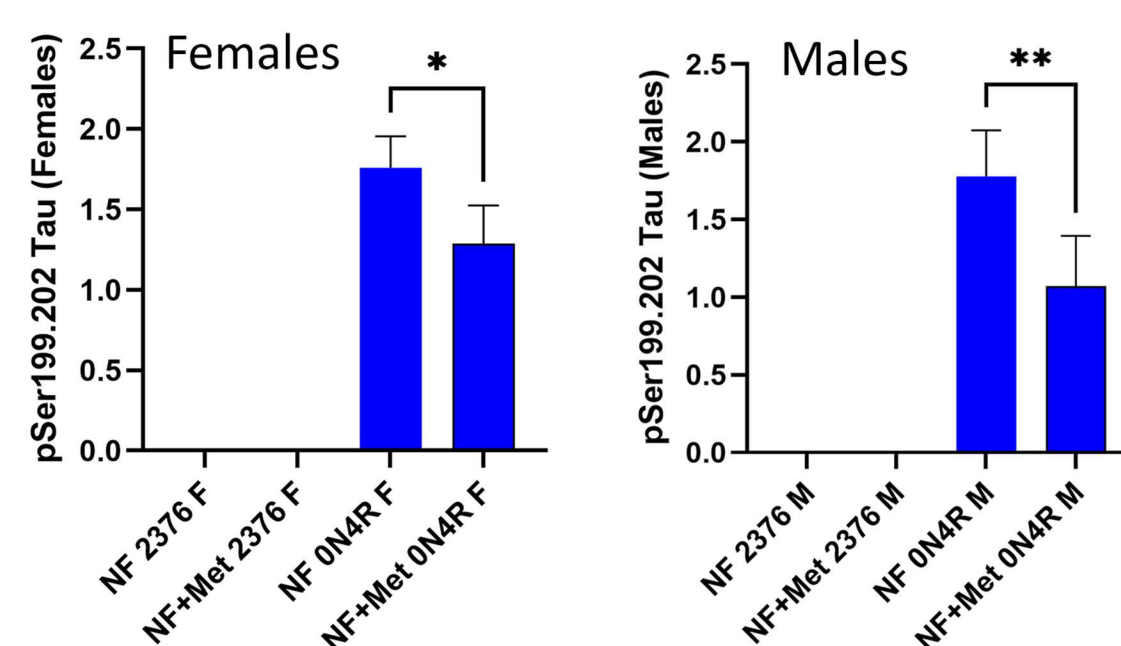
R (i) Normal food +/- Metformin



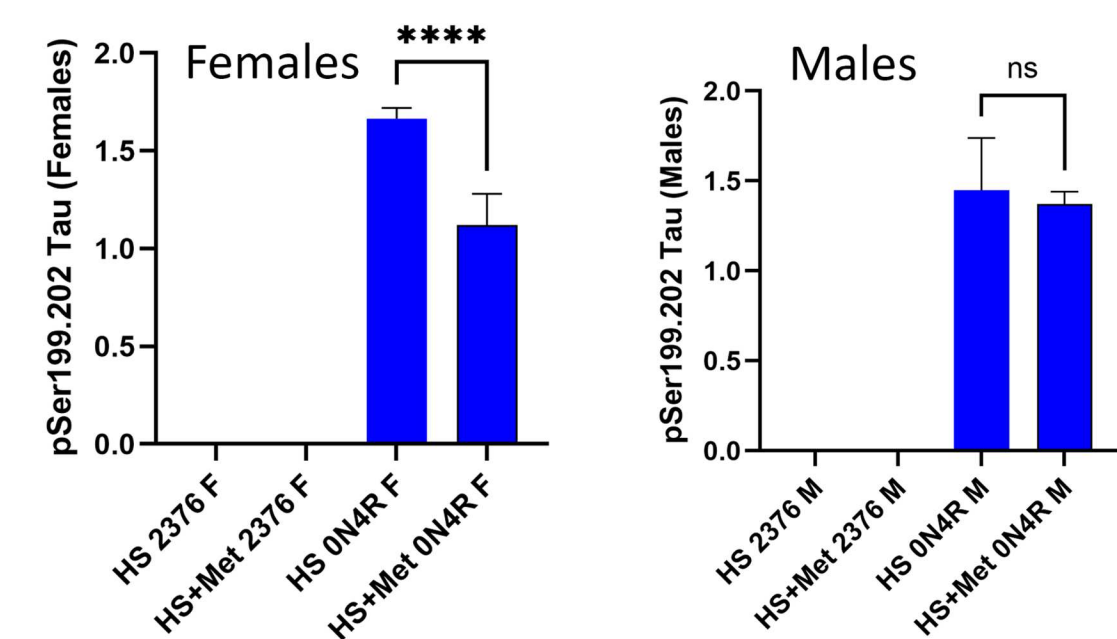
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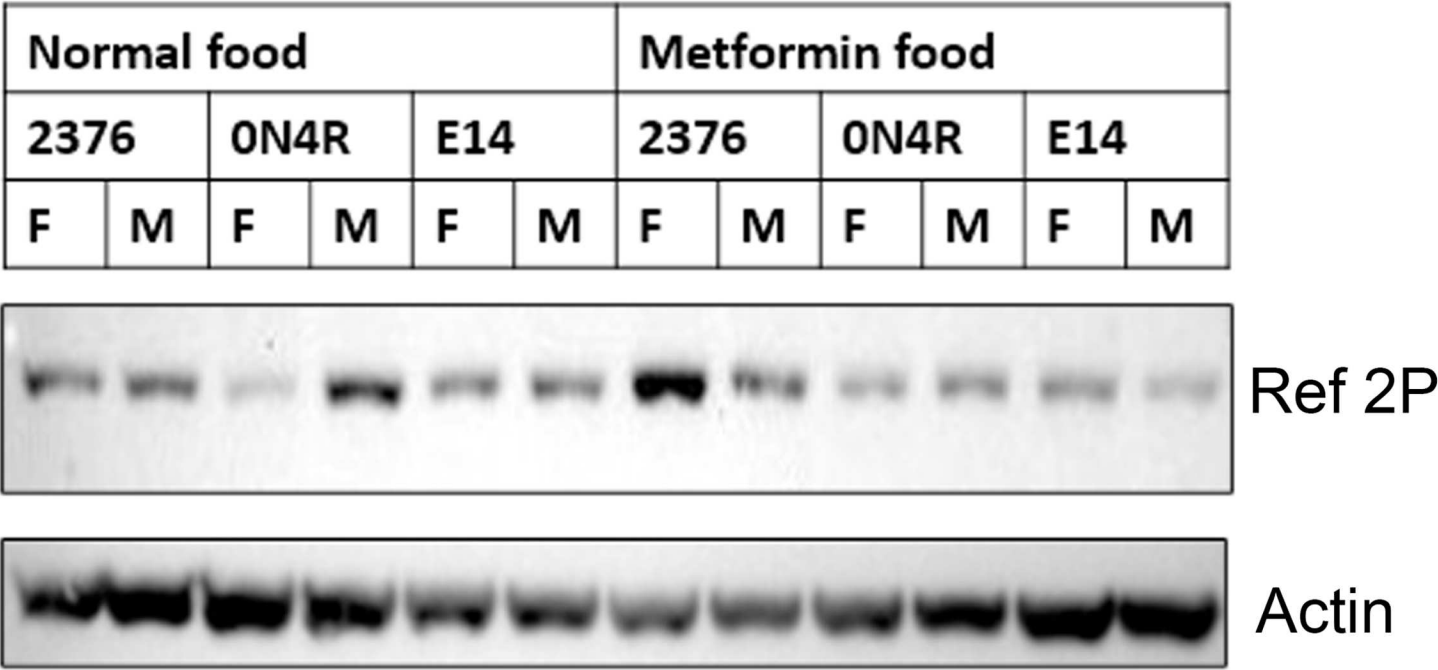
(v) Normal food +/- Metformin



(vi) High-sugar food +/-Metformin

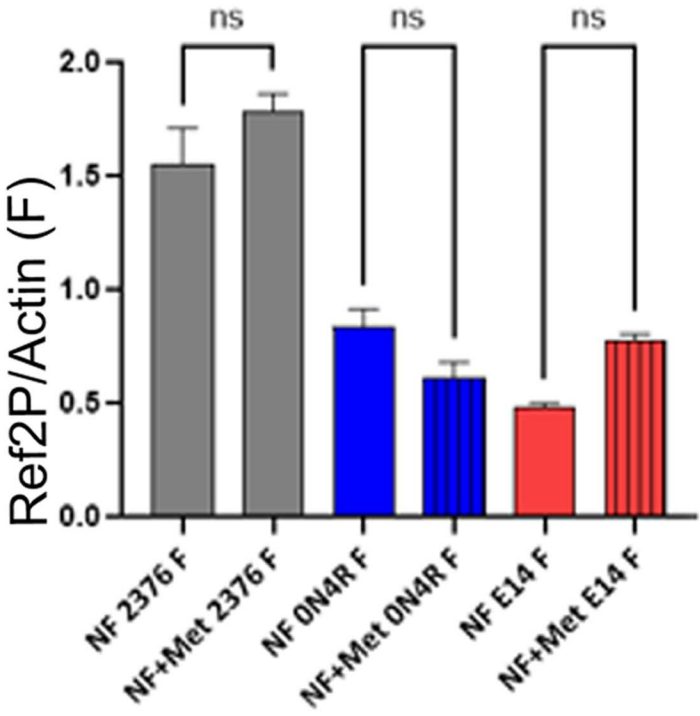


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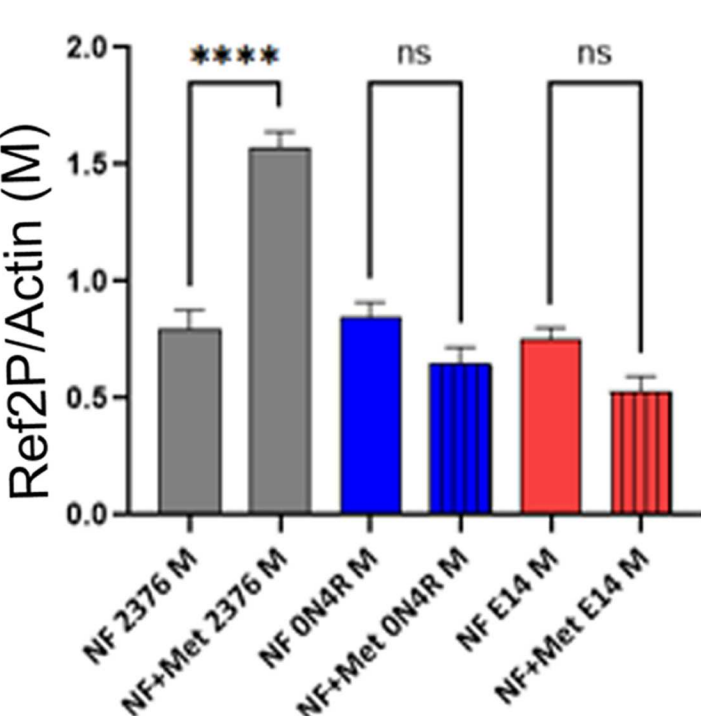


Normal Food +/- Metformin

Female



Male

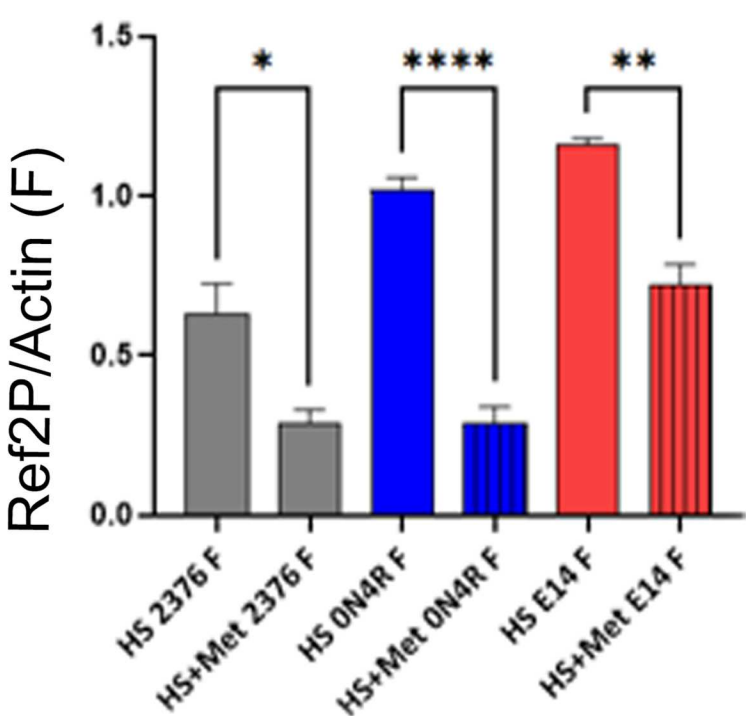


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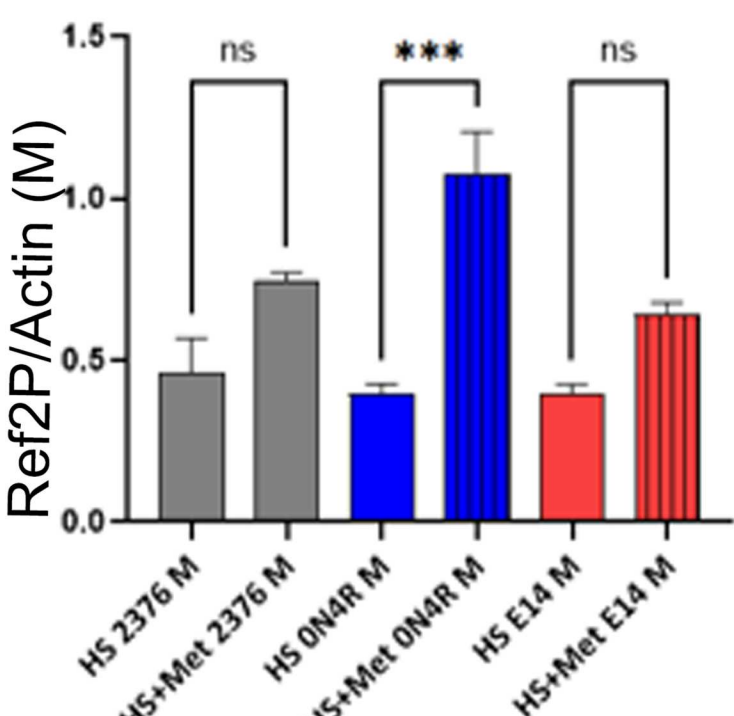


High-sugar Food +/- Metformin

Female



Male



Supplementary Files

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