Title: Rapamycin induced hyperglycemia is associated with exacerbated age-related osteoarthritis

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Running Title: Dietary rapamycin accelerates age-related OA

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Abstract

Objective: Determine if mechanistic target of rapamycin (mTOR) inhibition and/or AMP-acitivated protein kinase (AMPK) activation can modify primary osteoarthritis (OA).

Design: Dunkin-Hartley guinea pigs develop mild primary OA pathology by 5-months of age that progresses to moderate OA by 8-months of age. At 5-months, guinea pigs were fed either a control diet (n=8) or a diet enriched with the mTOR-inhibitor rapamycin (Rap, 14ppm, n=8), the AMPK-activator metformin (Met, 1000ppm, n=8), or the combination of rapamycin plus metformin (Rap+Met, 14+1000ppm, n=7) for 12 weeks. Knee joints were evaluated by OARSI scoring, micro-computed tomography, and immunohistochemistry. Glenohumeral articular cartilage was collected for western blotting.

Results: Dietary treatment with Rap and Rap+Met decreased body weight compared to control. Rap and Rap+Met inhibited articular cartilage mTORC1 but not mTORC2 signaling. Rap+Met, but not Rap or Met alone, stimulated AMPK. Despite lower body weight and articular cartilage mTORC1 inhibition, Rap and Rap+Met treated guinea pigs had greater OA severity in the medial tibial plateau due to articular cartilage structural damage and/or proteoglycan loss. Rap and Rap+Met increased plasma glucose compared to control. Plasma glucose concentration was positively correlated with OA pathology.

Conclusions: This is the first study to show that Rap induced increase in plasma glucose was associated with greater OA severity. Further, articular cartilage mTORC1 inhibition and bodyweight reduction by dietary Rap were insufficient to protect against primary OA during the prevailing hyperglycemia.

Key Words: Aging, mTOR, AMPK, Dunkin Hartley Guinea Pig, Degenerative Joint Disease

Introduction

Primary, age-related OA is estimated to account for as many as 90% of all knee OA cases in humans[1]. However, preclinical research commonly relies on experimental models of secondary OA. Although primary and secondary OA share similar pathological outcomes, there is a growing body of evidence to suggest they are driven by distinct mechanisms. Only 10% of differentially upregulated and 35% of differentially downregulated genes in OA vs non-OA samples are conserved between primary and secondary OA, meaning 65-90% of differentially expressed genes are unique to primary versus secondary OA[2]. Additionally, transgenic animal models have revealed that several genes are differentially involved in the progression of primary and secondary OA[3–8]. For example, deletion of *Panx3* protects against secondary OA yet dramatically worsens primary OA[3], and deletion of *JNK1/2* accelerates the development of primary OA while having no effect on secondary OA progression[8]. Together, these studies reinforce that unique mechanisms underpin these two forms of OA.

Age is one of the greatest risk factors for nearly every chronic disease, including primary OA. Two evolutionarily conserved kinases, mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK), are energy sensing pathways similarly dysregulated during aging and OA[9–12]. The mTOR inhibitor rapamycin (Rap) can extend lifespan in mice and delay the onset of several age-related morbidities[11,13]. The anti-diabetic drug metformin (Met) can activate AMPK, extend lifespan in mice[14], is retrospectively associated with decreased incidence of chronic diseases[15], and is the first drug being tested to slow age-related multi-morbidity in humans[16]. While the prospect of lifespan extension is tantalizing, extending lifespan without delaying the onset or slowing the progression of the most debilitating age-associated conditions could be viewed as detrimental. Therefore, it is imperative to understand if purported lifespan-extending therapies that target the fundamental biology of aging are also capable of delaying the onset of chronic diseases, such as primary OA.

84 mTOR exists as complex I (mTORC1) and complex II (mTORC2). mTORC1 regulates cellular
85 proliferation, protein synthesis, senescence, and survival while mTORC2 functions downstream
86 of insulin signaling on substrates such as Akt[11]. In articular cartilage, mTORC1 activity increases
87 with age and is sufficient to induce OA in young male mice[9]. Acute or intermittent Rap selectively
88 inhibits mTORC1 while chronic Rap administration for durations greater than 14 days also inhibits
89 mTORC2 activity[17], although this has not yet been tested in articular tissues. Cartilage-specific
90 deletion of mTOR and systemic or intra-articular injections of Rap and the mTORC1/2 inhibitor
91 Torin 1 lower secondary OA in young-male mice and rabbits[18–21]. While these findings support
92 mTOR-based therapeutics for OA, the completed studies were exclusively in injury-induced
93 models of OA and have not been investigated in primary, age-related OA.

94 Decreased AMPK activity disrupts chondrocyte homeostasis and contributes to more severe
95 OA pathology in male mice[10,22]. Met and other AMPK-activators have chondroprotective effects
96 against inflammatory-induced protease expression *in vitro*[22,23] and protect against injury-induced
97 OA in young male mice and rhesus monkeys[24]. Retrospectively, Met treatment in obese patients
98 is associated with a lower rate of medial tibiofemoral cartilage volume loss and risk of total knee
99 replacement[25]. However, metformin and AMPK activation have not been prospectively
100 investigated in primary OA.

101 The Dunkin-Hartley guinea pig is a well-characterized outbred model of primary OA. The
102 progression of OA in guinea pigs is related to bodyweight[26] and shares a similar age-related and
103 spatial progression to humans[27]. Mild OA pathology develops by 5 months in guinea pigs that
104 progresses to moderate OA by 8-9 months of age[27–29]. Therefore, at 5 months of age we treated
105 guinea pigs with lifespan-extending doses of Rap, Met, or a combination of Rap+Met for 12 weeks
106 to slow the progression from mild to moderate OA. This study is the first to evaluate if lifespan
107 extending treatments that slow the biological rate of aging can modify primary OA, the most
108 prevalent form of OA observed in older adults.

Methods

Animal Use

All tissues were collected at the University of Illinois Urbana-Champaign and approved by the Institutional Animal Care and Use Committee. Data collection and analysis were completed at University of Wisconsin-Madison. Male Dunkin-Hartley guinea pigs (Charles River) were singly housed in clear plastic, flat bottomed cages (Thoren, Model #6) with bedding. 12-hour light/dark cycles were used beginning at 0600. Guinea pigs acclimated for 2-3 weeks and were provided standard chow diet (Evigo 2040) fortified with vitamin C (1050 ppm) and Vitamin D (1.5 IU/kg) and water ad libitum until 5 months of age. Guinea pigs were then randomized to continue the standard diet (n=8), or receive diets enriched with encapsulated rapamycin (14 ppm, n=8), metformin (1000 ppm, n=8), or the combination of encapsulated rapamycin+metformin (14 ppm, 1000 ppm, n=8) for 12 weeks. Guinea pigs were randomized to match bodyweight between groups prior to beginning treatment. Diets were enriched with microencapsulated rapamycin (Rapamycin holdings) and/or metformin (AK Scientific, I506) at concentrations previously shown to extend lifespan in mice[13,14,30]. Food consumption was recorded on Monday, Wednesday, and Friday between 8 and 9 AM, and body weight was recorded before feeding on Monday. Guinea pigs treated with Rap, Met or Rap+Met diet had ad libitum access to food. Dietary Rap treatment has been shown to significantly reduce bodyweight in mice[31,32]. Therefore, we matched food consumption in the control group to the Rap diets to minimize the influence of food intake on dependent variables. One guinea pig in the Rap+Met group was euthanized early due to a wound on the gums which led to suppressed appetite and infection. Tissues from this animal were not collected for analysis. It could not be determined if this was due to a laceration or an oral ulcer, which is a known side effect of mTOR inhibitors[33].

133 Tissue Collection

134 Two animals were sacrificed daily between 7 and 10 AM for 4 weeks. Food and water
135 were removed from the cages 2-4 hours before euthanasia. Animals were anesthetized in a
136 chamber containing 5% isoflurane gas in oxygen and maintained using a face mask with 1.5-3%
137 isoflurane. Blood was collected by cardiac venipuncture followed by excision of the heart. The
138 right hind limb was removed at the coxofemoral joint, fixed in 10% neutral buffered formalin (NBF)
139 for 48 hours, and transferred to 70% ethanol until processed for histology. The soleus muscle was
140 collected from the left hind limb, snap frozen in liquid nitrogen, and stored at -80C for further
141 analysis. Because testicular atrophy has been observed following Rap treatment[34], the left testicle
142 was preserved in 10% NBF and weighed.

143

144 Analysis of Experimental Diets and Blood

145 Samples of diets enriched with Rap, Met or the combination of Rap+Met, and aliquots of
146 whole blood (n=4 per group) were sent to the Bioanalytical Pharmacology Core at the San Antonio
147 Nathan Shock Center to confirm drug concentrations in the diet and in circulation. Analysis was
148 performed using tandem HPLC-MS as described previously[13,35,36]. Frozen aliquots of plasma were
149 thawed to measure glucose and lactate concentrations using the YSI Biochemistry Analyzer (YSI
150 2900).

151

152 Micro Computed Tomography (µCT)

153 Right hind limbs from half of each treatment group (n=4 per group) were scanned using a
154 Rigaku CT Lab GX130 at 120 uA and 110 kV for 14 minutes, achieving a pixel size of 49um.
155 Scans were first processed in Amira 6.7 (ThermoFisher) where epicondylar width was measured

156 and a series of dilation, erosion, filling, and image subtraction functions were used to isolate
157 trabecular and cortical bone as described previously[37]. Scans were then resliced 4 times along
158 axes perpendicular to medial and lateral tibial and femoral articular surfaces and binarized using
159 identical thresholds. NIH ImageJ software and BoneJ plugin were used to quantify thickness,
160 spacing, and volume fraction measurements. Cortical thickness was measured by placing
161 polygonal ROIs in resliced scans to encompass the articular surfaces in each joint compartment.
162 Trabecular thickness, spacing, and bone volume fraction were measured by placing transverse
163 ROIs (2.4x2.4x1mm) in the trabecular bone of each joint compartment.

164

165 Histology

166 Knee joints were decalcified in a 5% EDTA, changed every 2-3 days for 6 weeks. Joints
167 were then cut in a coronal plane along the medial collateral ligament, paraffin embedded and
168 sectioned at 5um increments for Toluidine Blue staining and immunohistochemistry (IHC). Slides
169 were scanned using the Hamamatsu NanoZoomer Digital Pathology System, providing 460nm
170 resolution. Scan focus points were set manually along the articular cartilage. Imaged slides were
171 then scored by two blinded reviewers for OA severity following OARSI Modified Mankin
172 guidelines[29]. One guinea pig each from the Rap and Met groups were unable to be analyzed due
173 to off-axis transection before embedding, and one control animal was a statistical outlier as
174 detected by Grubb's test. Therefore, N=7 per group were used for histopathological analysis.

175

176 Immunohistochemistry (IHC) and Western Blotting

177 Phosphorylation of ribosomal protein S6 (RPS6), a downstream target of mTORC1, is
178 commonly used as a readout for mTORC1 activity. Therefore, IHC was performed using a primary
179 antibody against P-RPS6 Ser235/236 (4858, Cell Signaling) and visualized by 3,3'-

180 diaminobenzadine (DAB) to assess mTORC1 signaling. Staining in the medial tibia was quantified
181 as signal intensity and percentage of cells staining positive.

182 Western blot analysis was performed on 10ug of protein from glenohumeral cartilage
183 lysate for P-RPS6 Ser235/236, RPS6, P-Akt Ser473, Akt, P-AMPK Thr172, AMPK, LC3B,
184 ADAMTS5, and MMP-13. More detailed methods on IHC, western blotting, and antibodies are
185 available in supplementary materials.

186

187 Statistical Analysis

188 A two-way repeated measures ANOVA (time x treatment) was performed to determine
189 differences in food consumption and body weight. Upon a significant interaction, a Holm-Sidak
190 multiple comparisons test was used. A two-tailed unpaired t-test was used to confirm age-related
191 differences between 5- and 8-month-old guinea pigs. Because we were interested determining if
192 treatments impacted the trajectory of OA pathogenesis compared to aged controls, differences in
193 all other variables besides plasma glucose were made using one-way ANOVA comparing
194 treatment groups to 8-month controls with a Holm-Sidak multiple comparison test. Because there
195 is evidence that Met can rescue the hyperglycemic effects of Rap[32], comparisons were made
196 between all groups for plasma glucose. Pearson's R was used to determine correlation between
197 variables. P-values <0.05 were considered statistically significant. Data are presented as scatter
198 plots with mean or mean ± 95% confidence interval.

199

200

201 **Results**

202 *Influence of rapamycin and/or metformin on guinea pig physical and metabolic characteristics*

203 Figure 1A shows the average daily food consumption per week of standard diet or diet
204 enriched with Rap, Met, or Rap+Met. The average daily intakes of Rap and Met based on dietary
205 concentration are reported in Table 1. Compared to control, there was decreased food
206 consumption in guinea pigs receiving Rap+Met during week 2 (p=0.04). There were no significant
207 differences between treatments.

208 Despite largely matching food intake, there was a significant effect for time (p=0.049),
209 treatment (p=0.004), and an interaction between time and treatment (P<0.0001) on bodyweight.
210 Rap (p=0.03) and Rap+Met-treated guinea pigs (p=0.02) were smaller than control starting at
211 week 6 and week 3, respectively, until the end of the study (Figure 1B). At sacrifice, Rap and
212 Rap+Met-treated guinea pigs were 15% and 22% smaller than control. There were no differences
213 in body weight between DH guinea pigs treated with Met versus control.

214 Treatment with Rap (p<0.0001) and Rap+Met (p=0.008) increased plasma glucose by
215 64% and 39%, respectively, and the addition of Met to Rap non-significantly decreased plasma
216 glucose compared to Rap alone (p=0.07; Figure 1C). Lactate concentration was elevated by 66%
217 in Rap+Met-treated guinea pigs, only (P=0.05; Figure 1D). Testicle weight in guinea pigs receiving
218 Rap (p=0.01) and Rap+Met (p<0.0002) were 27% and 44% lower than control, respectively,
219 suggesting gonadal atrophy (Figure 1E). Treatment with Met alone exerted no apparent effects
220 on blood glucose (P=0.66), lactate (P=0.38), or testicular weight (P=0.38) compared to control.
221 We analyzed blood for the circulating Rap and Met concentrations ~3-hours after food had been
222 removed from the cage (Table 2). This timing aligns with a measurement of peak circulating Rap
223 and Met. We saw that experimental diets were sufficient to increase Rap and Met concentrations

224 in the blood, and that Rap and Met values were not different when providing diets individually or
225 in combination. There was no Rap or Met detected in circulation in control animals.

226

227 *Rapamycin and rapamycin+metformin treatment exacerbated the age-related progression of OA*

228 There was an increase in OA score from 5 to 8 months (P=0.03; Figure S1A) which is
229 consistent with the age-related progression of mild to moderate OA in guinea pigs. Surprisingly,
230 Rap and Rap+Met treatment resulted in a ~2-fold increase in total OA score in the medial tibial
231 plateau compared to age-matched control (P=0.048 for both Rap and Rap+Met; Figure 2B). This
232 was driven by increased scores for articular cartilage structure (p=0.03 for Rap only; Figure 2C)
233 and a trend for proteoglycan loss (P=0.07 for Rap, P=0.05 for Rap+Met; Figure 2D). Met alone
234 did not significantly affect OA score (p=0.21).

235

236 *OA pathology was correlated to plasma glucose*

237 Since Rap and Rap+Met treated guinea pigs displayed increased plasma glucose,
238 testicular atrophy, and worsened OA pathology, we evaluated the relationship between plasma
239 glucose, testicle weight, and OARSI score across all guinea pigs. Plasma glucose was
240 significantly correlated to both total OARSI score (R=0.38; p=0.048; Figure 3A) and proteoglycan
241 loss (R=0.47; p=0.01; Figure 3B). There was no significant correlation between testicle weight
242 and OA score (R=0.12; p=0.31; data not shown).

243

244 *Effects of rapamycin and/or metformin on mTOR, AMPK, and protease expression*

245 To evaluate mTORC1 signaling in articular cartilage, we measured the phosphorylation of
246 ribosomal protein S6 (P-RPS6) at Ser235/236 using IHC and western blotting. P-RPS6 was

247 decreased by 95% and 91% in the medial tibial articular cartilage of Rap and Rap+Met treated
248 guinea pigs as assessed by percentage of P-RPS6-positive cells (p=0.01 for both; Figure 4B).
249 mTORC1 inhibition was further supported by an 81% lower ratio of phosphorylated to total RPS6
250 in glenohumeral cartilage from Rap (p=0.003; Figure 4E). Rap+Met decreased RPS6
251 phosphorylation by 48% but did not reach significance (p=0.08). There were no differences
252 between groups in phosphorylation of the mTORC2 substrate Akt at Ser473 (Figure 4F; p=0.41).
253 AMPK activity was measured using western blot to assess phosphorylation of AMPK at Thr172
254 (P-AMPK). P-AMPK was not significantly changed by Rap alone (p=0.83; Figure 4G) but was
255 elevated 77% by Rap+Met (p=0.01). Met alone elevated AMPK phosphorylation by 30% but was
256 not significant (p=0.37). Rap, Met, or Rap+Met did not significantly change the conversion of
257 LC3B I to II (p=0.84 for all groups; Figure 4H) nor a disintegrin and metalloproteinase with
258 thrombospondin motifs 5 (ADAMTS5; Figure 4I; p=0.9 for Rap and Met, p=0.12 for Rap+Met).
259 Matrix metalloproteinase 13 (MMP13) was increased by Rap+Met only (p=0.02; Figure 4J).

260

261 *Rapamycin and rapamycin+metformin decreased subchondral and diaphyseal bone thickness*

262 Representative microCT images shown in Figure 5A were used to quantify the effect of
263 experimental diets on subchondral bone parameters. Mean subchondral cortical thickness was
264 significantly decreased by Rap and Rap+Met in the medial (29%, p=0.002 for Rap; 23%, P=0.007
265 for Rap+Met) and lateral (21%, p=0.03 for Rap; 20%, p=0.03 for Rap+Met) tibia (Figure 5B). Met
266 treatment alone exerted no apparent effects on any subchondral bone parameter measured. Rap
267 and Rap+Met decreased (p=0.02) trabecular spacing by 15% and 16%, respectively, in the lateral
268 tibia only (Figure S2B). Trabecular thickness, trabecular spacing in other compartments, and bone
269 volume fraction were not affected by any experimental diet (Figures S2A-C). Further investigation
270 revealed that cortical thickness at the femoral diaphysis was decreased proportionately to medial
271 tibial subchondral bone (Figure 5C) and was correlated to bodyweight (R=0.74, p=0.001; Figure

272 5D), suggesting the smaller body mass of Rap and Rap+Met treated guinea pigs was related to
273 decreased cortical thickness. Femoral epicondylar width (Figure 5E) was not statistically different
274 between groups (Rap, P=0.75; Met and Rap+Met, P=0.82), suggesting that our treatments did
275 not affect skeletal development.

276

277 **Discussion**

278 The purpose of this study to test if dietary Rap, Met, or Rap+Met at concentrations
279 previously shown to extend lifespan in mice can delay the onset of age-related OA in the outbred
280 Dunkin-Hartley guinea pig. Treatment with Rap and Rap+Met inhibited mTORC1 but not
281 mTORC2 signaling in articular cartilage. Rap+Met, but not Rap or Met alone, activated AMPK.
282 Surprisingly, Rap with or without Met accelerated the age-related progression of OA from 5- to 8-
283 months. Guinea pigs receiving Rap and Rap+Met also displayed increased plasma glucose, and
284 total OA score and proteoglycan loss were correlated with plasma glucose. These findings
285 suggest the off-target effects of dietary Rap treatment may exacerbate OA pathology. Further,
286 these data suggest that mTORC1 inhibition may not be sufficient to slow the progression of age-
287 related OA during high-glucose conditions.

288 Despite inhibiting mTORC1 in articular cartilage, our findings indicate that Rap, with or
289 without Met, worsens age-related OA in the medial tibial plateau. Further, Rap and Rap+Met
290 treated guinea pigs had greater OA scores even though they weighed less, which is contrary to
291 previous work where lower body weight was accompanied by lower OA scores in guinea pigs[26].
292 Although there is precedent that mTORC1 inhibition by intra-articular injection of Rap is
293 associated with exacerbated TMJ OA[38], our findings were opposite of our original hypothesis and
294 previous results using Rap in secondary models of knee OA[18,19]. The guinea pigs in the current
295 study received a dose of Rap that achieved similar circulating Rap concentrations shown to
296 extend lifespan[13,30]. Additionally, the dose of Rap in guinea pigs was similar to the dose shown to

297 protect against secondary OA in mice (0.7 in guinea pigs vs 1 mg/kg/day in mice)[18]. These findings
298 suggest that dose of Rap was not a likely factor contributing to differences between studies. In
299 our study, Rap treatment inhibited mTORC1 but not mTORC2 in articular cartilage. Previous work
300 has shown that deleting articular cartilage mTOR[21] or treating with Rap[18,19] or Torin-1[20] can
301 attenuate secondary OA in mice and rabbits. These non-selective genetic and pharmacological
302 methods likely disrupt the entire mTOR kinase and therefore could inhibit both mTORC1 and
303 mTORC2 signaling. However, this remains speculative since mTORC2 signaling was not
304 evaluated in these previous studies. Therefore, it continues to be unknown if mTORC2 inhibition
305 is necessary for protection against either primary or secondary OA. In support of the notion that
306 targeting mTORC2 modifies OA, inhibition of the mTORC2 substrate Akt protects against PTEN-
307 deletion-induced OA by decreasing cellular senescence and oxidative stress[39]. Further
308 investigation is needed to resolve the role of each mTOR complex in the initiation, progression,
309 and treatment of both primary and secondary OA.

310 While mTORC2 inhibition may be needed to preserve articular cartilage health, many
311 adverse side effects of Rap are mediated by off-target inhibition of mTORC2 in non-articular
312 tissues[17]. We showed that 12-weeks of dietary Rap and Rap+Met was accompanied by increased
313 plasma glucose and testicular degeneration. Met non-significantly lowered Rap-induced
314 hyperglycemia, however, the glucose values in Rap+Met treated guinea pigs were still greater
315 than control. These findings are in line with previous results that showed metformin alleviated
316 rapamycin induced glucose intolerance only in female mice[32]. The off-target effects of Rap in our
317 study are consistent with previous studies in mice and guinea pigs that show glucose intolerance,
318 insulin resistance, hypertriglyceridemia, immunosuppression, testicular atrophy and
319 cataracts[17,34,40]. In our study, OA severity in guinea pigs was correlated with plasma glucose, and
320 we propose that Rap-induced hyperglycemia may have contributed to worsened OA following
321 dietary Rap treatment. In support of this hypothesis, diabetic mice show accelerated OA after

322 injury and chondrocytes cultured in high glucose media display decreased expression of Collagen
323 II and increased MMP13 and inflammatory mediators IL-6 and NFkB[41,42]. However, intermittent
324 intraperitoneal injections of Rap lowered glucose and mitigated diabetes accelerated secondary
325 OA[43]. Previous work suggests Rap can have divergent effects where it is beneficial in some
326 diabetic models but causes adverse side effects in metabolically healthy models[17,44]. Collectively,
327 these data indicate that the adverse metabolic side-effects of dietary Rap treatment are
328 associated with a deleterious impact on primary OA pathology and could limit the utility of systemic
329 Rap as a healthspan extending treatment in humans.

330 Rap has been implicated in attenuating secondary OA by increasing autophagy and
331 decreasing protease expression[18,19]. While autophagy is a highly dynamic process, the static
332 marker of autophagy, LC3B, is commonly used as a surrogate for autophagic flux. In our study,
333 we saw no effect by any treatment on LC3B or ADAMTS5, while Rap+Met increased MMP13 in
334 glenohumeral cartilage. Therefore, the inability to increase markers of autophagy and decrease
335 proteases may be one contributing factor to why our lifespan-extending treatments did not protect
336 and even worsened OA during aging and hyperglycemia. More work is needed to determine the
337 molecular mechanisms responsible for the deleterious effects of Rap and Rap+Met.

338 Treatment with Rap and Rap+Met also decreased subchondral cortical bone thickness in
339 multiple joint compartments and the femoral diaphysis. As bone growth in guinea pigs ceases by
340 4 months[45], and epicondylar width was not different between groups, the differences in bone
341 thickness were likely not the result of disrupted development. Intra-articular injection of Rap into
342 the TMJ caused subchondral bone loss by inhibiting pre-osteoblast proliferation[38], and Rap
343 treatment also decreased osteoblast differentiation and bone matrix synthesis[46], which supports
344 the idea that Rap acted directly on the bone to decrease thickness. However, we also found that
345 subchondral thickness was highly correlated to bodyweight. This is in line with Wolff's law and
346 agrees with previous findings where bodyweight restriction decreased cortical bone thickness in

347 the femoral diaphysis while largely maintaining trabecular bone parameters[47]. Therefore, both
348 local and systemic effects of Rap likely contributed to reduced cortical bone thickness.

349 Met treatment resulted in a modest increase in AMPK activity in articular cartilage, which
350 only reached significance when combined with Rap. Met alone had no effect on OA score, and
351 the addition of Met was unable to completely rescue the detrimental effects of Rap-induced
352 hyperglycemia. This is in contrast to previous studies that showed Met protected against
353 secondary OA in mice and rhesus monkeys[24] and attenuated hyperglycemia-induced OA in
354 mice[48]. One major difference between studies, besides species, is metformin dose. After
355 correcting for species differences in fractional bioavailability following oral consumption of Met[49,50],
356 the doses used by Li et al. (2020; 100, 200 mg/kg/day), which elicited protection against
357 secondary OA, are more comparable to a clinically effective dose of Met in humans. Therefore,
358 the 40±2 and 45±6 mg/kg/day of Met consumed by our guinea pigs receiving Met and Rap+Met
359 is ~5-fold lower than a clinically relevant dose in rodents and humans. In agreement, Met whole-
360 blood concentration (277 ng/mL) 2-3-hours after removing food from the cage was 5-7-fold lower
361 than observed in healthy and diabetic humans following a normal therapeutic dose[50]. More work
362 is needed to determine if there are dose-dependent effects of Met on OA.

363 Although we provide new insight into the role of mTOR during primary OA progression,
364 we recognize some limitations of our study. While the guinea pig is an excellent model of primary
365 OA, it is not a widespread model for biomedical research and molecular probes are seldom
366 designed for reactivity with guinea pig tissue. Due to reactivity issues with IHC in guinea pig
367 cartilage (Figure S3), several of our analyses were restricted to western blot on cartilage from the
368 glenohumeral joint. Although guinea pigs also develop mild glenohumeral OA[27], this is not the site
369 at which we measured OA pathology. Our study could not conclusively determine if the
370 deleterious effects of Rap stemmed from its direct effects on the joint or off-target effects on other
371 tissues. However, our data suggest hyperglycemia induced by off-target actions of Rap was

372 associated with worsened age-related OA. Future work is needed to determine the role of articular
373 cartilage mTORC1 and/or mTORC2 in primary and secondary OA. Finally, while the Dunkin-
374 Hartley guinea pig more closely recapitulates both OA pathology and the genetic diversity in
375 humans, the outbred background lends itself to a high degree of variability that may have limited
376 our ability to detect more subtle differences in variables measured.

377

378 **Conclusion**

379 In summary, we have shown that a lifespan-extending dose of Rap and Rap+Met inhibited
380 mTORC1 but not mTORC2 in articular cartilage and accelerated the progression of age-related
381 OA. Rap- and Rap+Met-induced hyperglycemia was accompanied with worsened OA. Met alone
382 exerted no significant effect on OA pathology, which may be due to insufficient dose. These
383 findings raise concerns regarding the efficacy of dietary Rap as a life- and healthspan-extending
384 agent in humans and highlight need for further research regarding the individual and combined
385 role of mTORC1 and mTORC2 signaling in both primary and secondary OA.

386

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Author Contributions

Study design: AK. Data collection: DM, CE, AK, KS, MJ. Data analysis and interpretation: DM, AK. Manuscript preparation: DM, AK. All authors approved of the final manuscript.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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560 **Figure Legends**

561 **Figure 1: Characterization of animals on experimental diets.** Food consumption (A) and
562 bodyweight (B) of guinea pigs were recorded for the duration of the study (data presented as
563 mean +/- 95% CI). Plasma glucose (C) and lactate (D) and testicle weight (E) are shown. **P<0.01
564 vs Con, ***P<0.001 vs Con, ****P<0.0001 vs Con.

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566 **Figure 2: Rapamycin and rapamycin plus metformin worsened primary OA.** Representative
567 images of histology from the medial tibia are shown for each group (A; scale bars are 0.5mm and
568 0.25mm in 5x and 10x images, respectively). Histological images were graded for total OARSI
569 score (B; n=7 per group). The individual scores for articular cartilage structure (C), proteoglycan
570 loss (D) and cellularity (E) are also shown. *P<0.05 vs Con.

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572 **Figure 3: OA severity correlated with hyperglycemia.** Correlations of total OARSI score (A)
573 and proteoglycan loss (B) with plasma glucose are shown. Dotted lines represent 95% CI.

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575 **Figure 4: Rapamycin and rapamycin plus metformin inhibited mTORC1 but had no effect**
576 **on mTORC2 or autophagy**. IHC was performed on the medial tibia for P-RPS6 (A; n=7 per
577 group) and quantified as percent positive cells (B) and mean integrated intensity (C). Red
578 arrowheads indicate cells staining positive for P-RPS6. Western blot was performed on
579 glenohumeral cartilage (D) for P-RPS6 (E), P-Akt (F), P-AMPK (G), LC3B (H), ADAMTS5 (I), and
580 MMP-13 (J). n=8 per group for Rap and Met, and n=7 per group for Con and Rap+Met. *P<0.05
581 vs Con, **P<0.01 vs Con.

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583 **Figure 5: Decreased subchondral bone thickness in rapamycin and rapamycin plus**
584 **metformin treated guinea pigs.** Representative microCT sagittal cross sections from the medial
585 aspect of the joint are shown (A). Subchondral cortical thickness was measured in the medial and
586 lateral tibial plateaus and femoral condyles (B). Cortical thickness at the medial tibia presented
587 relative to cortical thickness at the femoral diaphysis (C), which indicates a proportionate
588 decrease at both sites. Medial tibial cortical thickness was highly correlated to bodyweight (D).
589 Femoral epicondylar width was found to be similar between groups (E). N=4 per group. *P<0.05
590 vs Con, **P<0.01 vs Con.

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604 **Individual Tables and Figures**

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606 **Table 1: Average consumption of rapamycin and metformin.** Using the concentration of
607 rapamycin and metformin from the diet analysis, the average doses were calculated for each
608 group. N=7-8 per group. Data are presented as mean with 95% CI (lower limit, upper limit).

	Experimental Diet		
	Rapamycin	Metformin	Rapamycin+Metformin
Rapamycin consumed (mg/kg/day)	0.72 (0.68, 0.76)	-	0.68 (0.59, 0.77)
Metformin consumed (mg/kg/day)	-	39.9 (38.7, 41.1)	44.5 (38.9, 50.1)

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628 **Table 2: Concentrations of rapamycin and metformin in circulation.** Whole blood was
629 collected ~3 hours after food had been removed from the cages of guinea pigs and was analyzed
630 for rapamycin and metformin concentration by tandem HPLC/MS. N=4 per group. Data are
631 presented as mean with 95% CI (lower limit, upper limit).

	Experimental Diet		
	Rapamycin	Metformin	Rapamycin+Metformin
Circulating rapamycin (ng/mL)	72 (65, 79)	-	78 (68, 88)
Circulating metformin (ng/mL)	-	274 (248, 299)	282 (229, 334)

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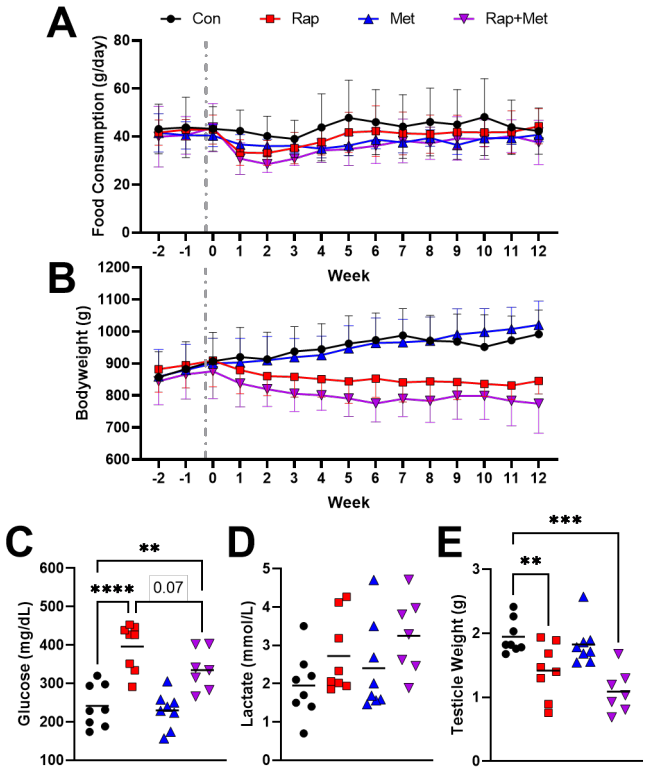
654 **Figure 1**



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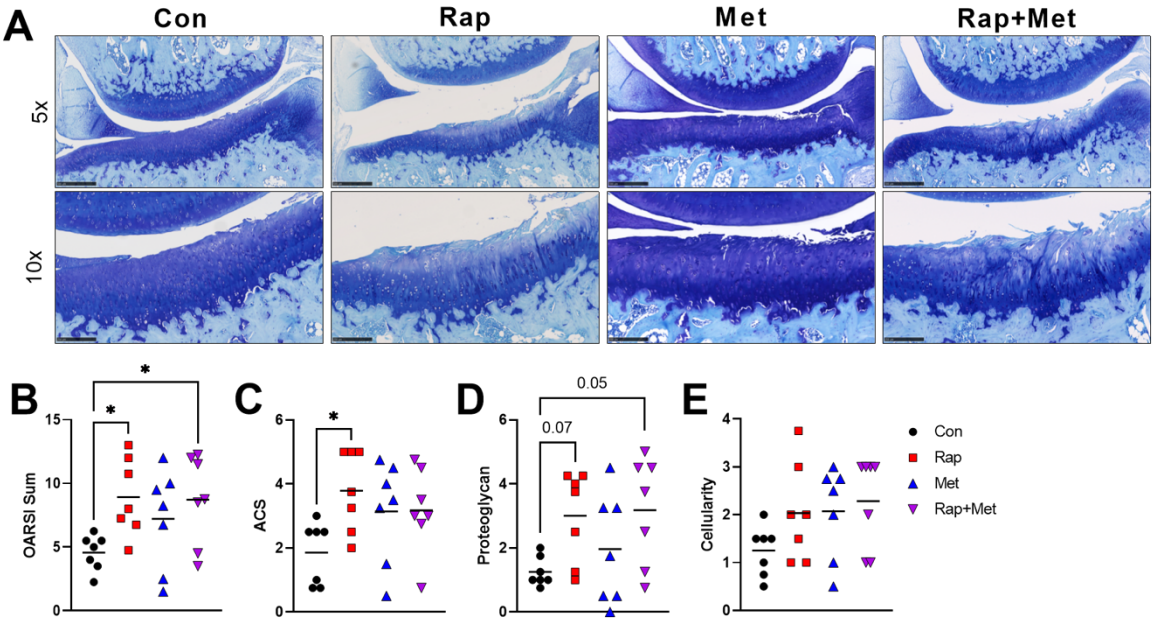
665 **Figure 2**



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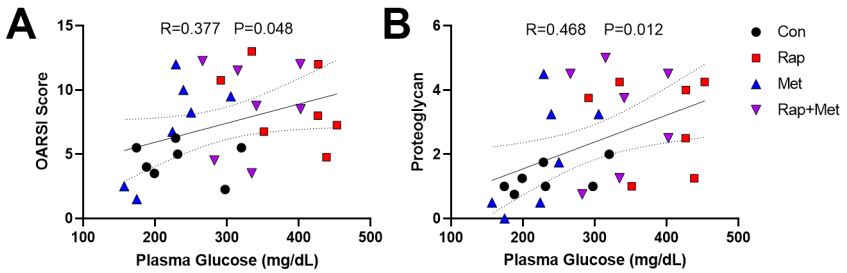
675 **Figure 3**



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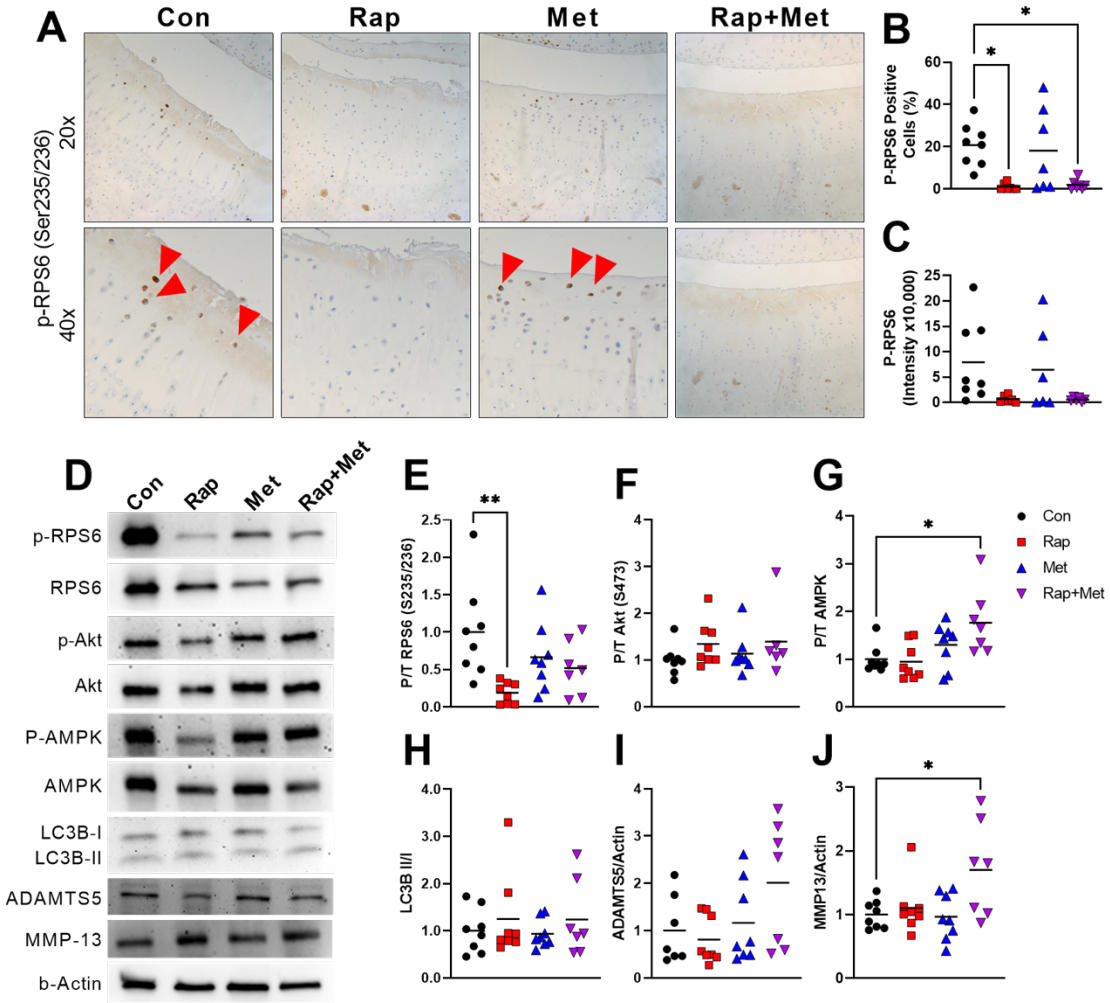
701 **Figure 4**



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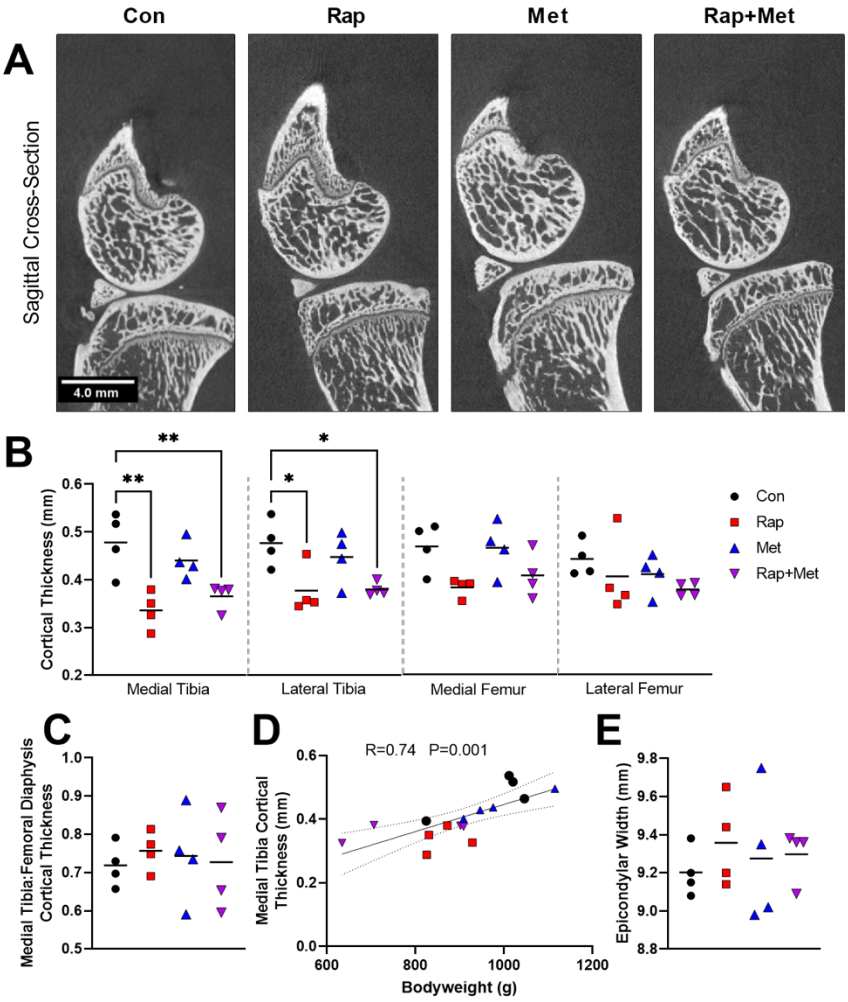
714 **Figure 5**



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