Progress Report for Diabetic Complications Consortium: In Vivo Removal of Advanced Glycation End-products Rescues Type-II Diabetes Skeletal Fragility

Rationale: In our first progress report, we discussed the results for the rat model which was used for the studies proposed in the aim 1 & 2 (please see the appendix for the report #1). We determined that although for this model, PTC was effective in removing AGEs and rescuing skeletal fragility, the animals did not develop type II diabetes. Therefore, we switched our studies toward the development of a mouse model with capabilities of developing type II diabetes (T2D) (SA1 – as originally proposed) and to investigate the efficacy of PTC in removing AGEs and rescuing skeletal fragility in a T2D model (SA2-as originally proposed).

Specific Aim 1 (SA1): Determine if extended high-fat diet and sugar water treatment led to sustained diabetes using a new mouse model

Specific Aim 2 (SA2): Determine if PTC administration reduced accumulation of advanced glycation end-products (AGEs) in rodent long bone

Significant results including major findings, developments and conclusions (referenced Figures are included in the attached appendix)

(a) Mice provided HFD developed diabetes when compared to mice on LFD. Twelve male C57BL/6J mice (8 weeks of age) were obtained from Taconic Biosciences (Rensselaer, NY). After 2 weeks of acclimation, the animals were randomly divided into two groups of 6 animals. One group was fed a low-fat diet, and the other fed a high-fat diet. Energy (kcal/g) 2 from fat, protein and carbohydrates was altered between LFD (10% fat, 72% carbs, 18% proteins) and HFD (46% fat, 36% carbs, 18% proteins. The dietary intervention started at 10 weeks of age for 22 weeks until 32 weeks of age. All animals were fed ad-libitum and given unrestricted access to filtered water while kept on a regular 12-hour light/dark cycle. As compared to the mouse model #1, the feeding and dietary intervention was introduced at different mouse age. Also, there was no sugar added to the animals' water. Body mass and glucose measurements were taken every 7 weeks to measure weight changes associated with the diet intervention. These time points also coincided with oral glucose tolerance tests (OGTTs) for the mice as a measure of impact from the dietary intervention on glycemic control. Fasting period prior to the start of each OGTT was 6 hours. Mice were given an oral glucose dose (2 grams/kg body mass) by feeding needle. Blood glucose measurements were collected at five intervals corresponding to 0, 15, 30, 60, and 120-minute time points [1]. Baseline measurements at time point 0 represents the fasting blood glucose (also after 6 hours of fasting), which was used to categorize the animal's glycemic control as normal (< 199 mg/dL), pre-diabetic (200-249 mg/dL), or diabetic (> 250 mg/dL) [2].

C57BL/6J male mice given a high-fat diet (HFD) had a significantly higher body mass ($p = 0.00013$, **Figure 1 A**) when compared to mice fed a low-fat diet (LFD). HFD-fed mice also showed an increase in fasting blood glucose (**Figure 1 B**) compared to LFD-fed mice. At 16-weeks of age, both groups of mice showed similar levels of blood glucose ($p = 0.6349$, **Figure 1 B**). However, by 23-weeks of age, HFD fed mice showed a significant increase in blood glucose ($p = 0.0181$, Figure 1B) that reached and surpassed the diabetic threshold for mice (> 250 mg/dL) [35]. Fasting blood glucose measurements taken at the end of the study demonstrated a stark contrast between LFD and HFD-fed mice with all mice fed a HFD becoming diabetic ($p = 0.0011$, **Figure 1 C**).

The area under the OGTT curve (AUC) was calculated and used as an index of glucose tolerance. In agreement with the fasting blood glucose, AUC results showed that HFD fed mice experienced significantly greater difficulty in restoring glucose levels back to baseline ($p = 0.00189$, **Figure 1 D**).

The increase seen in body mass correlated with the increase in blood glucose ($R = 0.6429$, $p = 0.0242$, **Figure 1 E**). Thus, it is likely that obesity and diabetic status were interconnected.

In summary, we developed dietary conditions which successfully induced T2D in this mouse model. ***The preliminary data on the characterization of the T2D mouse model are presented below (b and c)***.

(b) Analysis of skeletal characteristics of the T2D mouse model revealed changes which were similar to the ones observed for diabetic human bone. We established those diabetic mice showed altered microarchitecture and increased BMD (**Figure 2A-2E**), changed geometry and a significant reduction in marrow expansion (**Table 1**), and an increase in crosslinked and non-crosslinked AGEs (**Figure 3**). ***In summary, the developed T2D mouse model #2 mirrored skeletal condition observed in humans with T2D***.

(c) Removal of glycation end-products served as an effective in vitro treatment for rescuing bone toughness. Mechanical testing of T2D femurs demonstrated that AGE accumulation in T2D was associated with the loss of toughness as maximum bone toughness negatively correlated with an increase in fAGEs ($R = -0.55$, $p < 0.066$). In vitro PTC-treatment of femurs from T2D mice resulted in partial restoration of bone toughness where the mean initiation toughness non-significantly increased by 19.3% ($p = 0.3359$, **Figure 4 A**), and the maximum toughness significantly increased by 35% ($p = 0.04277$, **Figure 4 B**). In vitro PTC treatment was, therefore, effective in rescuing skeletal fragility.

(d) Extended provision of high-fat diet to mice did not maintain diabetic status (SA1).
Following our results demonstrating PTC's efficacy in ameliorating bone quality *in vitro*, we aimed to determine PTC's in vivo efficacy in rescuing skeletal fragility in a long-term diabetic mouse model, as long-term diabetes closely mirrors what is observed clinically. To this end, eight-week-old male C57Bl/6 (B6) mice were provided either a low-fat (n = 32) or high-fat diet (n = 32) ad libitum until 38 weeks of age. Mice on high-fat diet were also given 25% fructose water to increase the likelihood of animals to develop diabetes. Seven fasting blood glucose tests were performed during the study duration to monitor the progression of diabetes in all groups. At 13 weeks of age, half of the mice on low-fat diet (n = 16) and high-fat diet plus sugar water supplementation (n = 16) were given a single dosage of STZ to further ensure diabetic status, following recent work by Eckhardt and colleagues [3]. At 15 weeks of age, mice provided HFD, sugar water, and a single dose of STZ exhibited elevated fasting blood glucose relative to LFD controls, and hence were considered diabetic (> 250 mg/dL fasting blood glucose, **Figure 5A**). At 22 weeks of age, mice were given daily injections of PTC or vehicle solution (0.9% saline) as a control. This created four groups: low-fat diet + vehicle injections (n = 16), high-fat diet + sugar water + vehicle injections (n = 16), low-fat diet + STZ-injection + PTC treatment (n = 16), and high-fat diet + sugar water + STZ-injection + PTC treatment (n = 16). We found that fasting blood glucose and HbA1c at 38 weeks of age, following administration of PTC and vehicle solutions to mice, led to loss to diabetic status and improved glycemic control **(Figure 5A-5B).** Consequently, at 38 weeks of age, the amount of fAGEs was similar between PTC and control groups (**Figure 6**).

In summary, we established that in PTC is effective in vitro in removing impact of T2D on bone (a potential application for improving bone quality of bone grafts) but were unable to evaluate its impact in an in vivo T2D model due to complex effect of PTC and saline injection on glycemic control/AGE removal. Combined with results presented in Progress report 1, this project demonstrates that T2D as well aging causes accumulation of AGEs in bone and AGEs can be removed in vivo in aging bone and in vitro in T2D bone using PTC treatment. Further work is required to establish efficacy of PTC in vivo for rescuing T2D bone fragility. Based on part of these results we submitted an R01 application to NIH earlier this year (Feb 2021 cycle) and currently in process of revising/resubmitting the application (Nov. cycle). A manuscript based on this work is currently under revision (see Publications below)

Publications

Llabre, J.E., Sroga, G.E., Tice, M. JL., Vashishth, D. (2021). Induction and Rescue of Skeletal Fragility in a High-Fat Diet Mouse Model of Type 2 2 Diabetes: An In Vivo and In Vitro Approach. *Bone (BONE-D-21-00908, revision pending)*.

Bailey S, Magliochetti T, McNay E, Vashishth D. (2019). Removal of Advanced Glycation End-products In Vivo Rescues Bone Fragility. JBMR. (Abstract Accepted)

NIH Grant Application:

1 R01 AG075654-01 Effects of Advanced Glycation Endproducts on Type II Diabetic and Fragility Fractures (Submitted Feb 5, 2021; Score: Not Discussed; Status: Resubmission – November 5, 2021).

References:

[1] Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. Am J Physiol Endocrinol Metab. 2008; 295: E1323–E1332. doi:10.1152/ajpendo.90617.2008

[2] Fajardo RJ, Karim L, Calley VI, Bouxsein ML. A Review of Rodent Models of Type 2 Diabetic Skeletal Fragility. Journal of Bone and Mineral Research. 2014; 29(5):1025–1040. doi: 10.1002/jbmr.2210

[3] Eckhardt, B. A., Rowsey, J. L., Thicke, B. S., Fraser, D. G., O'Grady, K. L., Bondar, O. P., Hines, J. M., Singh, R. J., Thoreson, A. R., Rakshit, K., Lagnado, A. B., Passos, J. F., Vella, A., Matveyenko, A. V., Khosla, S., Monroe, D. G., & Farr, J. N. (2020). Accelerated osteocyte senescence and skeletal fragility in mice with type 2 diabetes. *JCI insight*, *5*(9), e135236. https://doi.org/10.1172/jci.insight.135236

Appendix

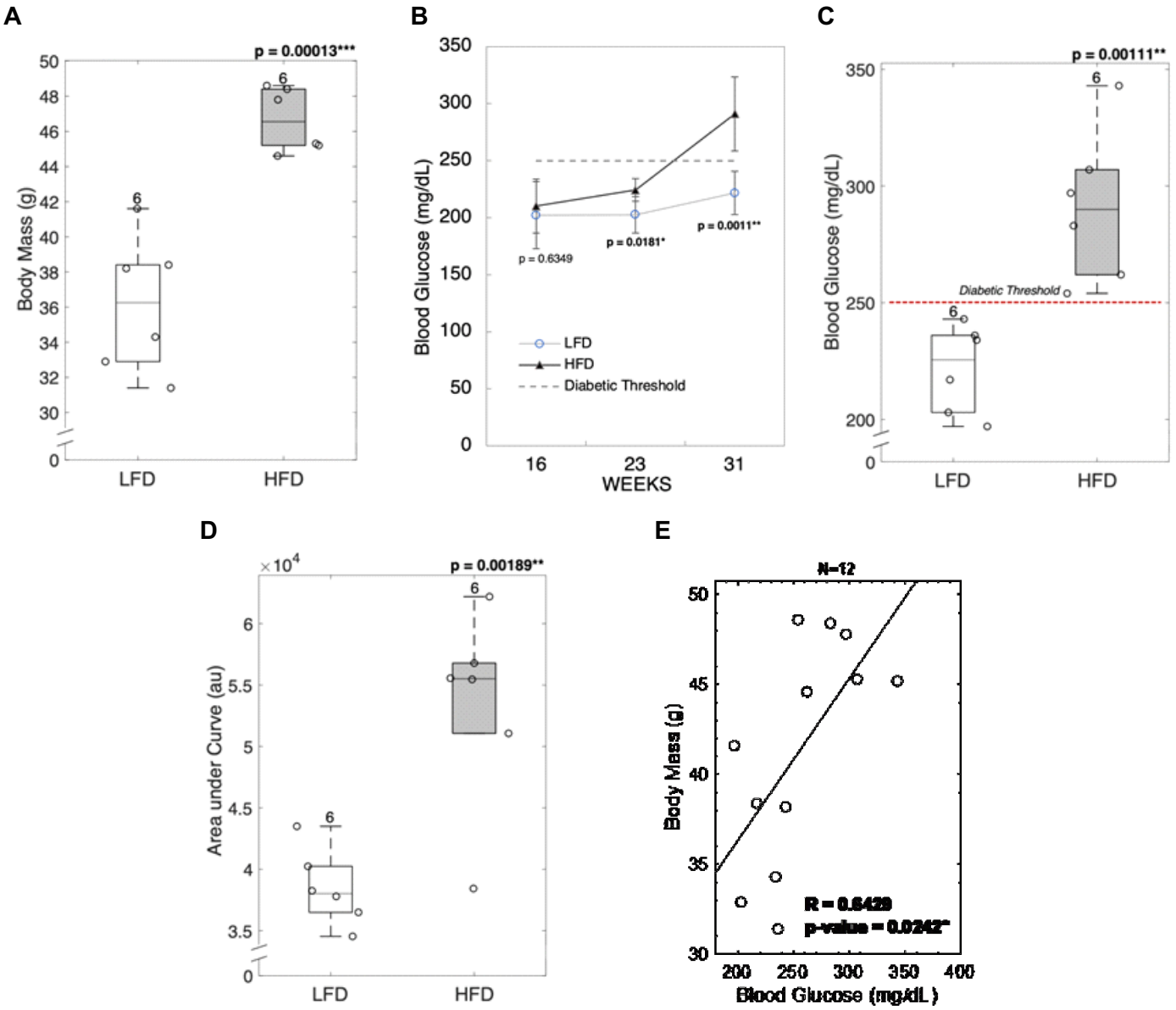


Figure 1: Characterization of the diabetic condition for the second mouse model. (**A**) C57BL/6J mice given a high-fat diet during development developed obesity and type II diabetes. HFD-fed mice showed greater body mass gain compared to LFD-fed mice. (**B**) HFD-fed mice had higher levels of fasting blood glucose throughout the study. (**C**) After diet treatment for 22 weeks, HFD-fed mice had fasting blood glucose in the diabetic range and (**D**) demonstrated impaired insulin production assessed by the AUC of final glucose tolerance test. (**E**) The increase in body mass correlated with the increase in blood glucose showing interconnection between obesity and diabetic status (E). LFD = low-fat diet; HFD = high-fat diet; AUC = area under curve. Results are shown as boxplots (with median and interquartile range) showing all data points. Statistically significant differences were determined by One Factor ANOVA with Replication (α=0.05). Number on top of each boxplot indicates sample size. Coefficient of correlation determined by Pearson's R test (α=0.05). Significant codes: $p < 0.05$ '*', $p < 0.01$ '**', $p < 0.001$ '***'.

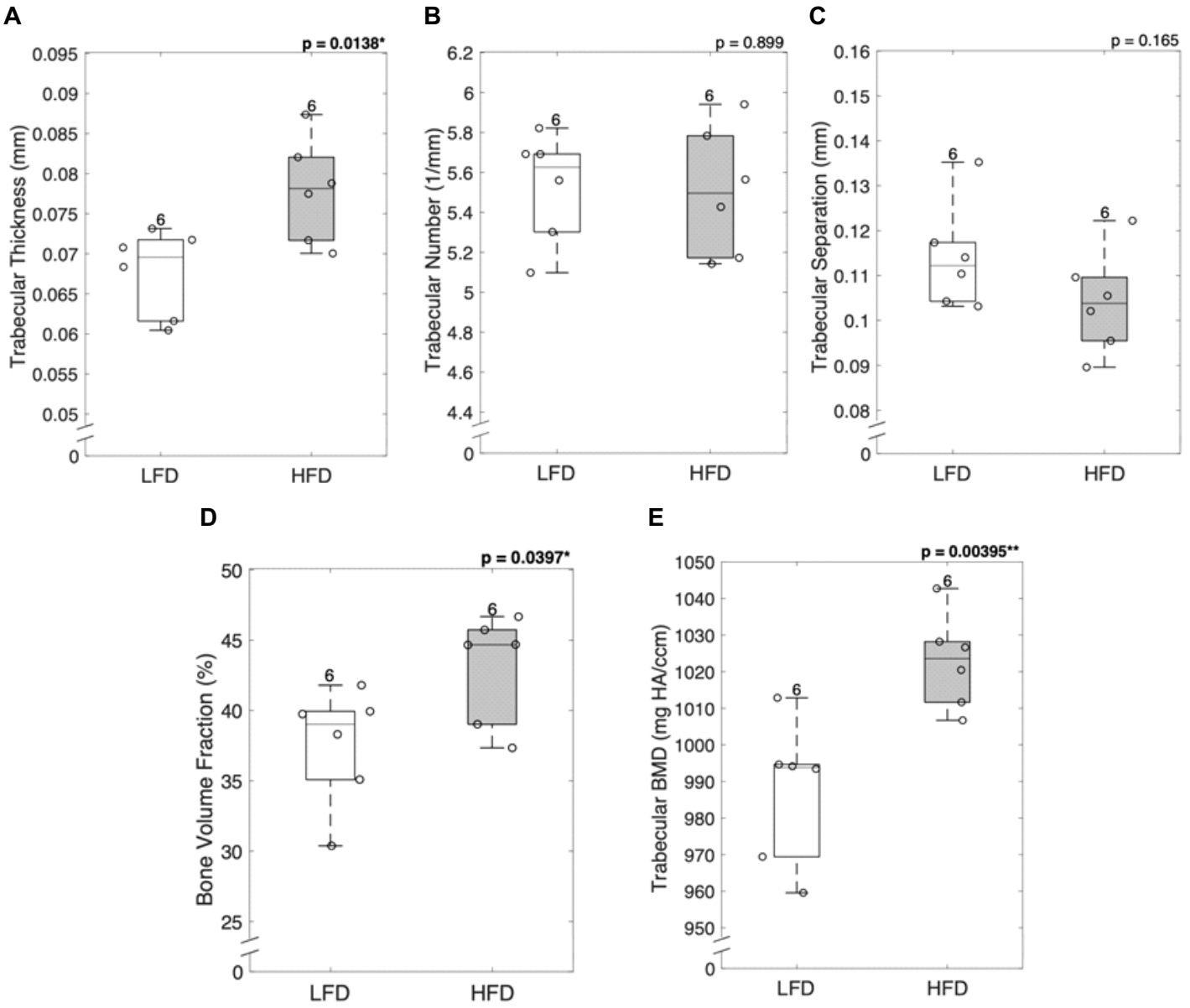


Figure 2: Diabetic mice showed altered microarchitecture and increased mineralization. (**A**) Trabecular structure analyzed at the distal femur showed that diabetic mice had increased trabecular thickness, but (**B**) no changes in trabecular number or (**C**) trabecular separation. (**D**) Diabetic mice demonstrated typical increase in bone volume fraction and (**E**) trabecular BMD. LFD = low-fat diet; HFD = high-fat diet; BMD = bone mineral density. Results are shown as boxplots (with median and interquartile range) showing all data points. Statistically significant differences were determined by One Factor ANOVA (α=0.05). Number on top of each boxplot indicates sample size. Significant codes: $p < 0.05$ '*', $p < 0.01$ '**'.

	LFD (n = 6)	HFD (n = 6)	
Endosteal Dia. (mm)	1.0472 ± 0.0520	1.0765 ± 0.0569	p = 0.3734
Periosteal Dia. (mm)	1.4188 ± 0.0623	1.4659 ± 0.0562	p = 0.1995
Ct. Th (mm)	0.1858 ± 0.0139	0.1947 ± 0.0072	p = 0.194
Ct.Ar/Tt.Ar (%)	41.28 ± 02.41	42.43 ± 01.06	p = 0.3119
I_{xx} (mm^4)	**0.2663 ± 0.0568**	**0.1943 ± 0.0538**	**p = 0.048***
cBMD (mg HA/ccm)	1258.77 ± 14.37	1249.39 ± 10.72	p = 0.2288

Table 1. Femoral cortical morphology assessed by microCT imaging. Femoral cortical structure analyzed at the mid-diaphysis showed non-significant increases in inner and outer surface expansion, cortical thickness, and cortical area fraction. However, there was a significant reduction in the marrow expansion of HFD-fed mice, as determined by the decreased in moment of inertia (I_{xx}). Cortical bone mineral density did not show significant changes with HFD intervention.

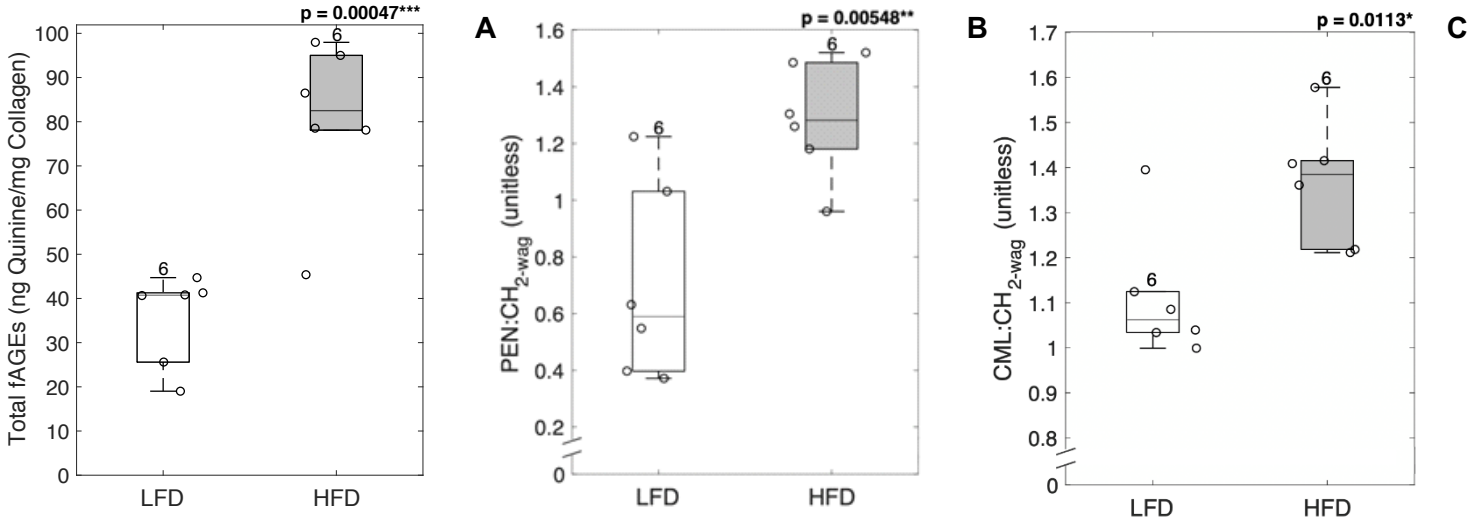


Figure 3: Diabetic mice showed an increase in AGEs. AGEs assay and confocal Raman spectroscopy were used to evaluate organic matrix properties from femoral cortical sections. (**A**) Diabetic mice showed a significant increase in total fAGEs, (**B**) pentosidine and (**C**) carboxymethyl-lysine. LFD = low-fat diet; HFD = high-fat diet, fAGEs = fluorescent advanced glycation end-products. Results are shown as boxplots (with median and interquartile range) showing all data points. Statistically significant differences were determined by One Factor ANOVA (α=0.05). Number on top of each boxplot indicates sample size. Significant codes: $p < 0.05$ '*', $p < 0.01$ '**', $p < 0.001$ '***'

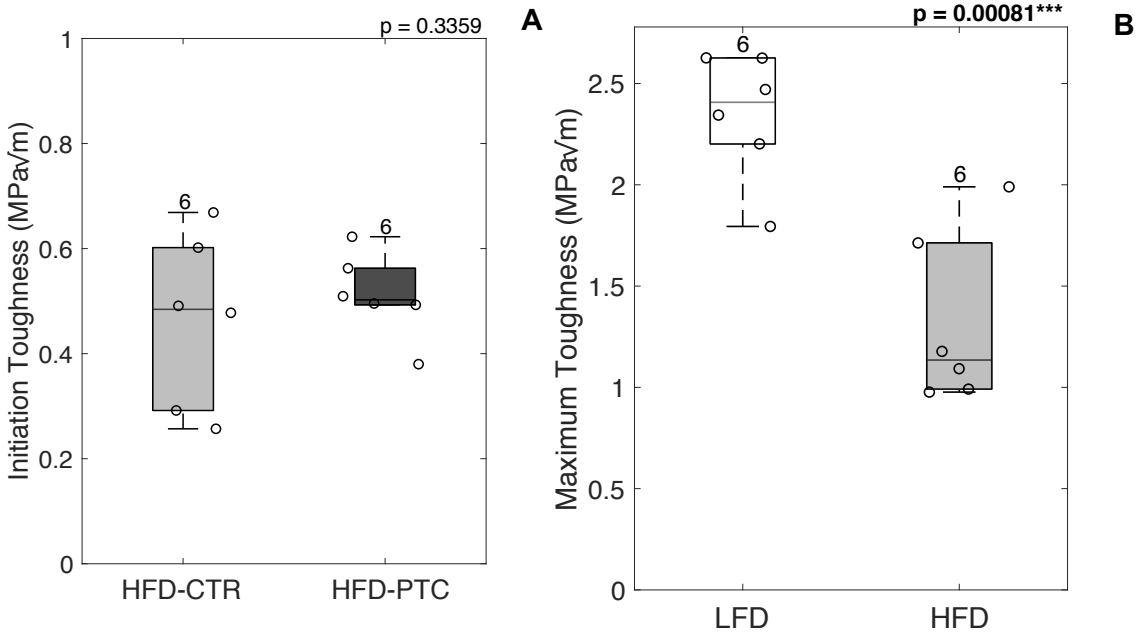


Figure 4: Removal of glycation end-products served as an effective in vitro treatment for rescuing bone toughness. (**A**) PTC-treated femoral samples showed a non-significant increase in initiation toughness and (**B**) significant increase in maximum toughness. HFD = high-fat diet; PTC = phenacyl thiazolium chloride; CTR = control. Results are shown as boxplots (with median and interquartile range) showing all data points. Statistically significant differences were determined by Paired T-test (two tailed, α=0.05). Number on top of each boxplot indicates sample size. Significant codes: $p < 0.05$ '*', $p < 0.001$ '***'.

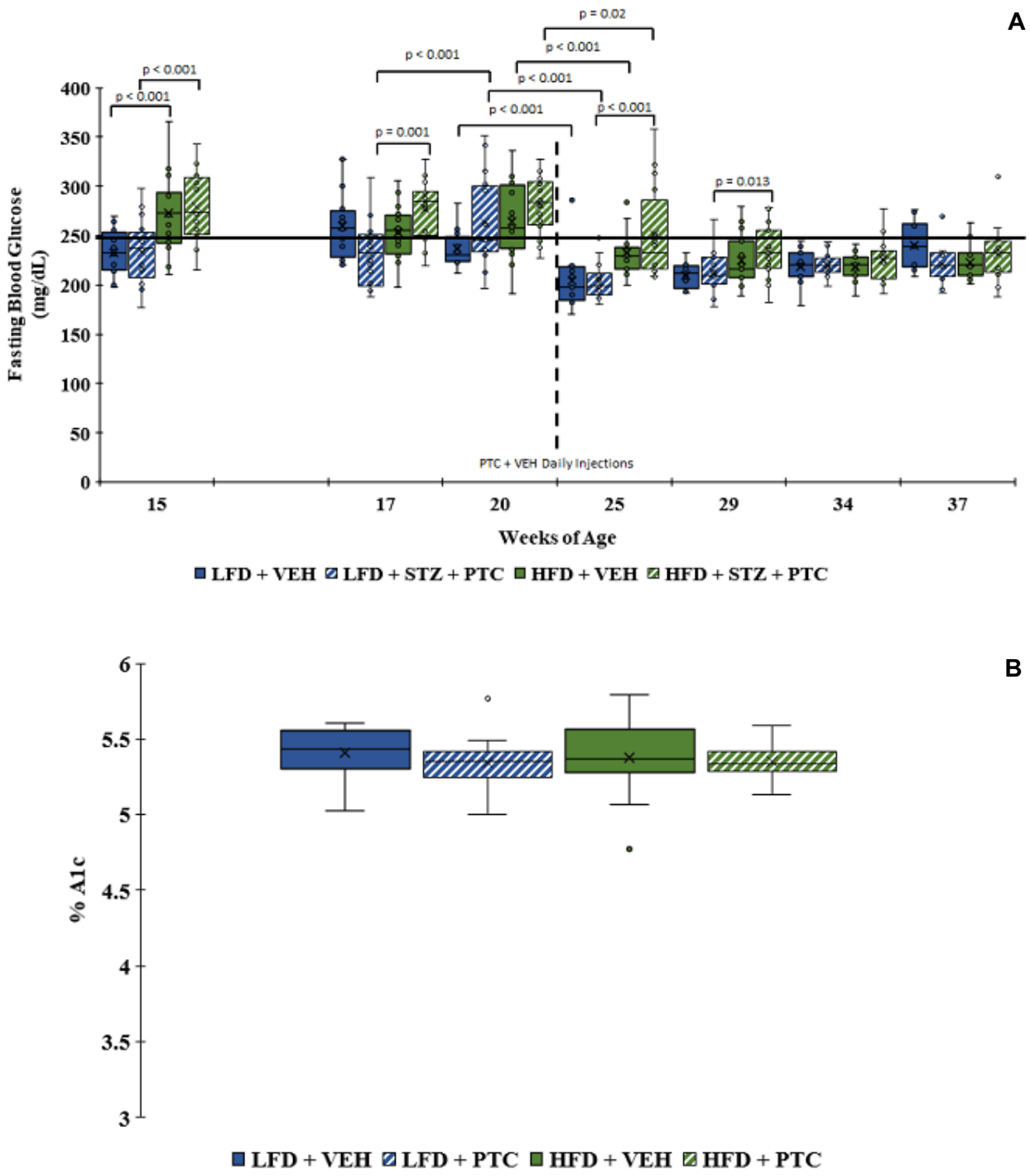


Figure 5: Administration of PTC and vehicle solutions did not maintain long-term diabetic status in mice. Fasting blood glucose demonstrated that mice on HFD showed elevated blood glucose as compared to mice on LFD. Glucose levels significantly decreased in all groups following PTC and vehicle solution administration (A). Analysis of glycated hemoglobin (HbA1c) showed that all groups reached "under diabetic threshold" of 6.5% glycated hemoglobin and were statistically similar to each other (B). Data are expressed as boxplots with interquartile range as whiskers, with significant p-value (< 0.05) as shown for the groups of interest.

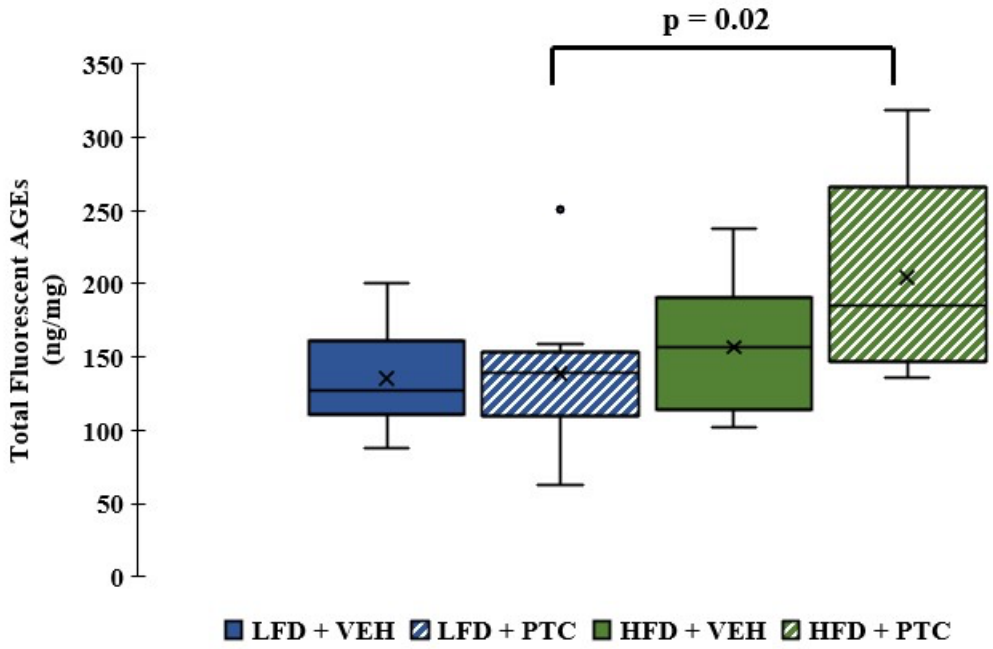


Figure 6: Quantitation of fluorescent advanced glycation end-products (fAGEs). High-fat diet fed mice injected with PTC exhibited elevated fAGEs compared to PTC-injected mice on a low-fat diet. Data is expressed as boxplots with interquartile range as whiskers, with significant p-value (< 0.05) explicitly written between groups of interest.

Diabetic Complications Consortium

Application Title: In Vivo Removal of Advanced Glycation End-products Rescues Type-II Diabetes Skeletal Fragility

Principal Investigator: Deepak Vashishth, PhD

1. Project Accomplishments:

The overall goal of the pilot and feasibility study was to determine the contribution of advanced glycation end-products (AGEs) to bone fracture in a diet-induced obesity model of type 2 diabetes (T2D), and to therapeutically rescue T2D bone fragility by removal of AGEs. To this end, six weeks-old male Sprague Dawley rats were used in the study. Animals were placed on a high fat diet or standard chow for 20 weeks. At 26 weeks, a subset of animals was treated with a daily injection of phenacylthiazolium chloride (PTC, 10µg/g of body weight) or saline for an additional 20 weeks to create four test groups (HFD+PTC, HFD+saline, standard+PTC, standard+saline, n=12/group). We found that administration of a high-fat diet (HFD) induced pre-diabetes. HFD animals were obese (increased body weight and gonadal fat pad) and glucose intolerant compared to standard chow-fed animals. *In vivo* treatment with PTC reduced the accumulation of total fluorescent AGEs in bone from both high-fat diet and standard chow-fed animals. While initiation toughness was significantly lower in PTC-treated groups, maximum toughness was significantly improved in the standard+PTC group only. We observed no differences in bone geometry or bone mineral density between the groups. While these results show the efficacy of PTC in rescuing bone fragility in vivo in controls due to natural aging, they do not help address rescue of T2D bone fragility as our chosen diet failed to induce T2D bone fragility. Using the remaining funds, under no-cost extension mechanism, we propose to repeat T2D studies on modified diet (addition of sugar to drinking water) and a precise control of light dark cycle. These experiments will help address the original goal of the grant and help submission of an R01 grant application to NIH.

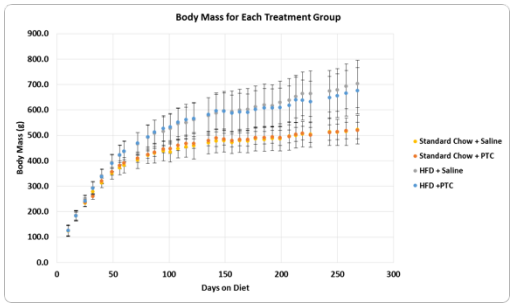
2. Specific Aims:

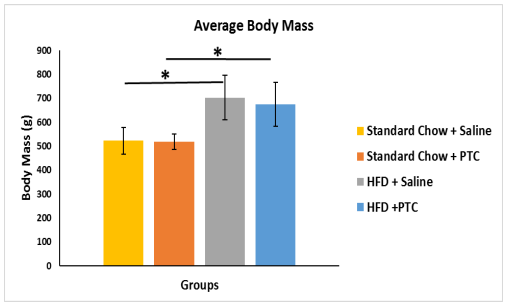
Specific Aim (1a): Establish the role of AGEs in causing increased bone fragility in a diet-induced obesity rat model of T2D.

Results:

Diet-induced Obesity Rat Model Characterization:

HFD animals were hyperglycemic, hyperinsulinemic, and significantly larger than standard chow-fed animals (Figures 1-4). We observed no differences in glycated hemoglobin between the groups (Figure 3c). HFD alone was insufficient to cause increased T2D bone fragility as we observed no differences in total fluorescent AGEs (fAGEs, Figure 5a) and bone toughness (HFD+saline vs standard+saline, Figure 5b). In the no-cost extension request submitted, we propose to use mice on controlled light/dark cycles and supplement the water with sugar (fructose) to achieve T2D bone fragility.





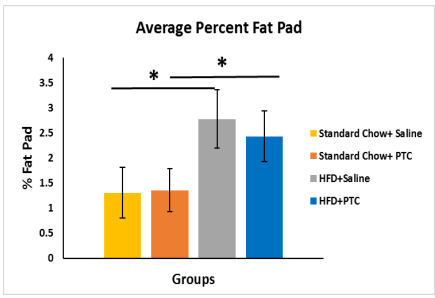
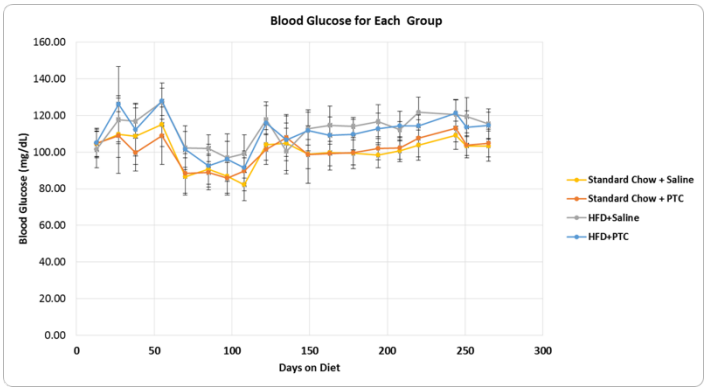


Figure 1: Body mass measured weekly for the duration of the study (a), and average body mass (b) and percent gonadal fat (c) at the end of the study. HFD animals were significantly obese and larger than standard chow animals (* indicates p<0.05). PTC treated groups were not different from their respective saline-treated groups (i.e. HFD+PTC vs HFD+saline) indicating a significant main effect of diet (i.e. HFD+saline vs Standard+saline) but no effect of drug.



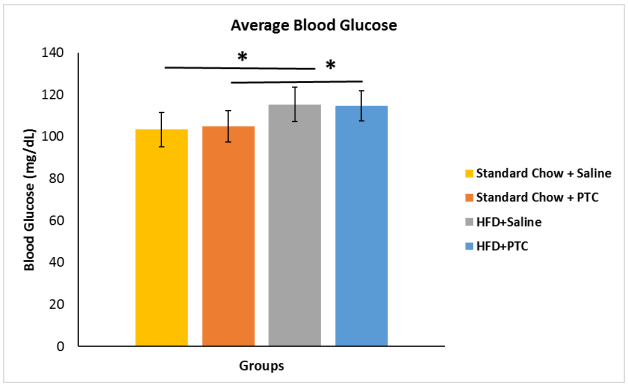
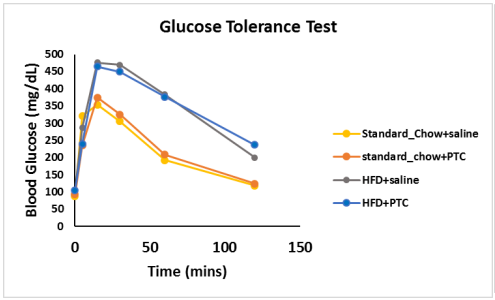
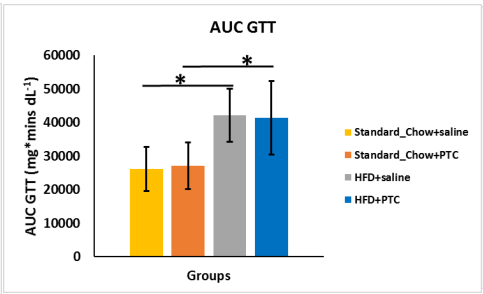


Figure 2: Fasting blood glucose measured bi-weekly for the duration of the study (a) and average fasting blood glucose at the end of the study (b). HFD animals were hyperglycemic compared to standard chow-fed animals. PTC treated groups were not different from their respective saline-treated groups (i.e HFD+PTC vs HFD+saline) indicating a significant main effect of diet (i.e. HFD+saline vs Standard+Saline) but no effect of drug (* indicates p<0.05).





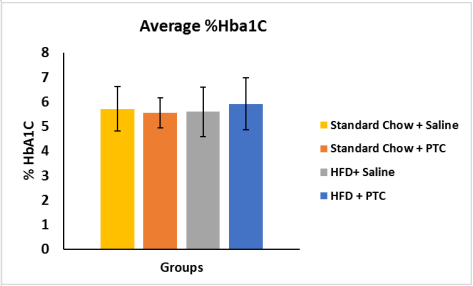


Figure 3: Glucose tolerance of the rats was assessed by the intraperitoneal glucose tolerance test over a period of two hours at the end of the study (a). HFD animals were significantly glucose intolerant compared to standard chow-fed animals shown by the area under the curve (b). PTC-treated groups were not different from their respective saline-treated groups (i.e HFD+PTC vs HFD+saline) indicating a significant main effect of diet (i.e. HFD+saline vs Standard+Saline) but no effect of drug. Average percent glycated hemoglobin (c) measured at the end of the study was also not different between the groups.

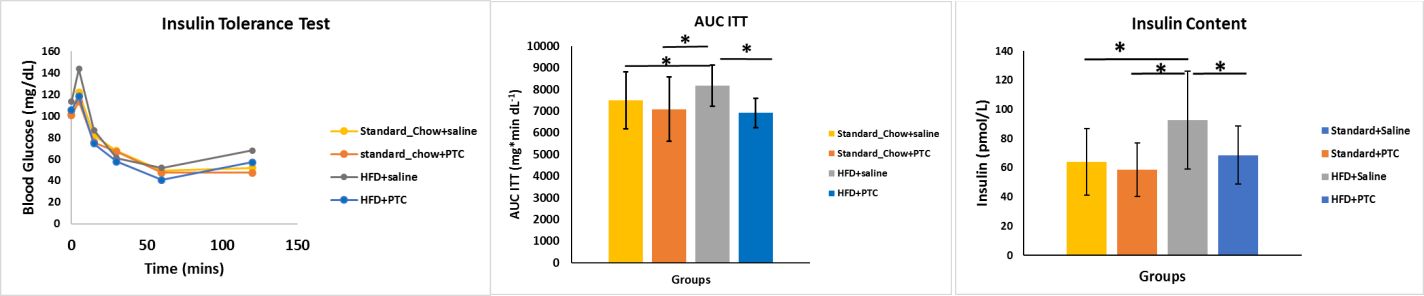


Figure 4: Insulin tolerance of the rats was assessed before euthanasia by bolus intraperitoneal administration of insulin and monitoring glucose concentration over a period of two hours (a). HFD animals were significantly hyperinsulinemic compared to standard chow-fed animals shown by the area under the curve (b). Interestingly, HFD+PTC group was significantly different from HFD+saline indicating a significant main effect of drug. These findings were confirmed by ELISA-based plasma insulin content (c).

Assessment of Bone Structure and Quality:

Groups	Inner Diameter (mm)	Outer Diameter (mm)	Ixx[mm^4]	Cortical Area[mm^2]	Total Area [mm^2]	Cortical Thickness (mm)	Tissue Mineral Density (mgHA/ccm)
Standard + Saline	2.59 (0.23)	4.23 (0.23)	13.86 (3.88)	9.01 (0.72)	14.03 (1.28)	0.79 (0.05)	1300.43 (20.22)
Standard + PTC	2.49 (0.21)	4.14 (0.21)	13.81 (2.51)	8.99 (0.63)	13.94 (1.04)	0.80 (0.044)	1291.56 (13.26)
HFD+ Saline	2.55 (0.20)	4.23 (0.22)	13.34 (4.84)	9.22 (0.87)	14.89 (2.73)	0.79 (0.08)	1303.46 (18.12)
HFD + PTC	2.58 (0.26)	4.39 (0.32)	16.06 (4.23)	9.92 (1.28)	15.15 (1.86)	0.82(0.10)	1299.33 (20.96)

Table: Average morphometric parameters and bone mineral density of femoral cortical bone from each group. There were no statistically significant differences on any parameters measured.

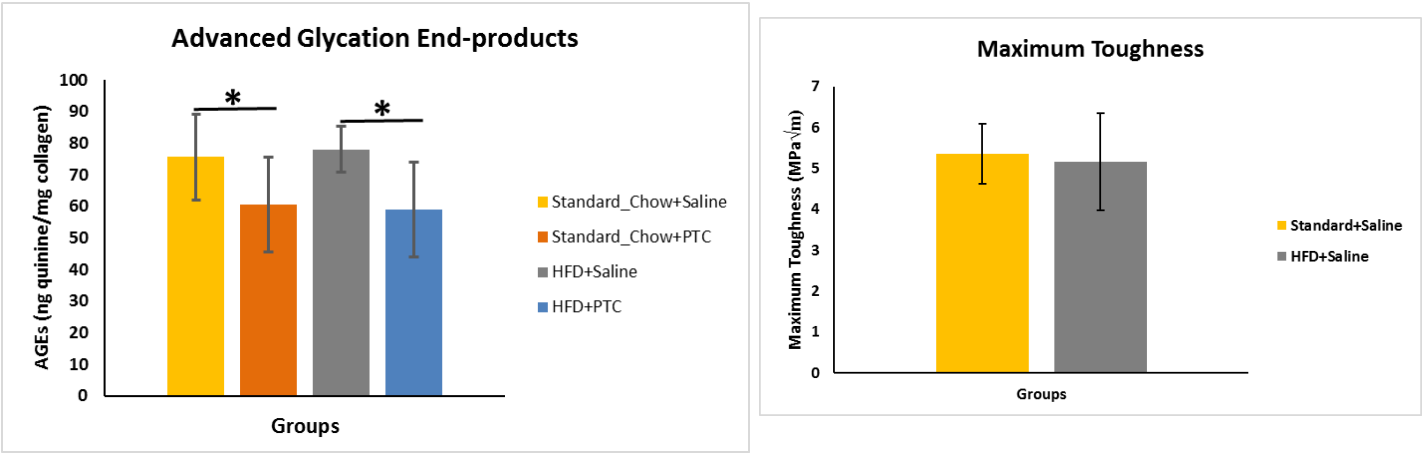


Figure 5: Total fluorescent AGEs (a) and maximum toughness (b) of femoral cortical bone. There were no significant differences between the groups.

Specific Aim (1b): Test whether PTC treatment can remove AGEs and rescue T2D bone fragility in-vivo.

Results

PTC was effective in removing AGEs *in vivo* measured from femoral cortical bone irrespective of diet (HFD+PTC vs HFD+saline, standard+PTC vs standard+saline $p<0.05$, Figure 6). Maximum toughness of femoral cortical bone was significantly higher in standard+PTC compared to all other groups ($p<0.05$, Figure 7).

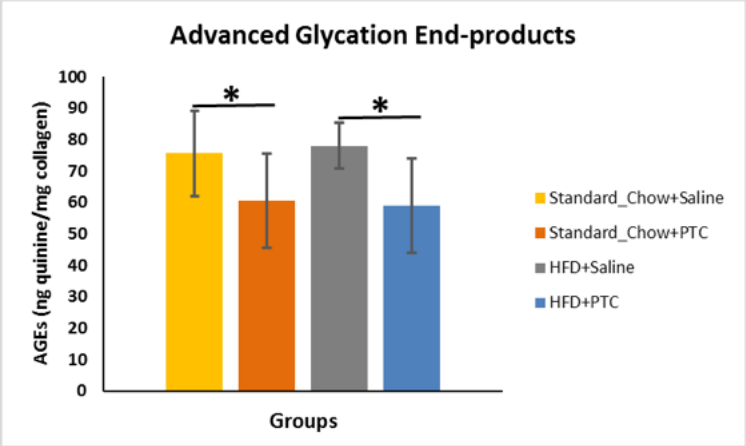


Figure 6: Daily injections of PTC significantly reduced the accumulation of AGEs compared to saline-treated controls (*indicates $p<0.05$).

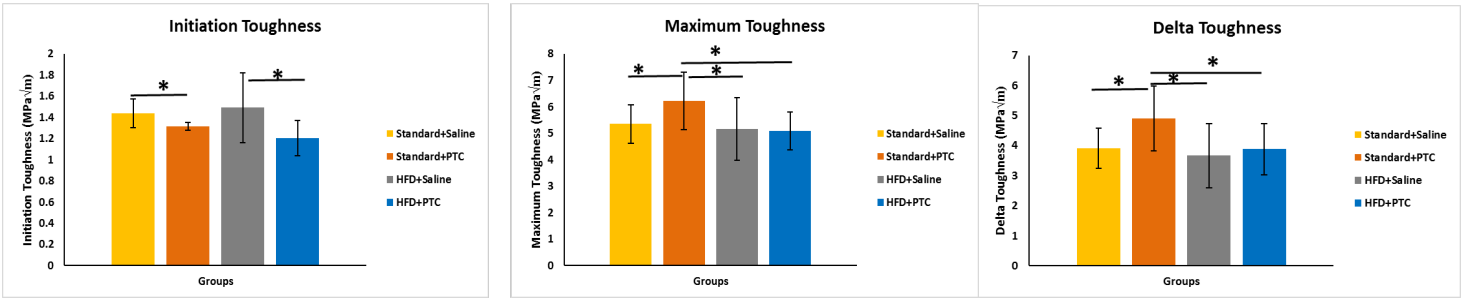


Figure 7: Daily injections of PTC significantly reduced initiation toughness compared to saline-treated controls (a). However, maximum toughness and delta toughness (maximum minus initiation, a measure of crack extension) was significantly higher in Standard+PTC group compared to all other groups.

3. Publications:

Bailey S, Magliochetti T, McNay E, Vashishth D. Removal of Advanced Glycation End-products *In Vivo* Rescues Bone Fragility. J Bone Mineral Res 2019 (Abstract Accepted)