

AWARD NUMBER: W81XWH-13-1-0307

TITLE: Why Do Only Some Women Develop Post-Menopausal Osteoporosis?

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14. ABSTRACT The proposed project addresses a novel and potentially important mechanism of osteoporosis which may determine which women suffer the disease. Confirmation and understanding of this mechanism will lead to new prediction methods and treatments for osteoporosis patients and greatly improve the lives of affected civilian, military and veteran populations. At the onset of menopause, lack of estrogen and other sex steroids results in increased bone turnover and net bone loss. Fortunately, only 25-30% of post-menopausal women will develop vertebral fractures - the hallmark of post-menopausal osteoporosis - because of differences in the amount of bone lost. This proposal presents a novel theory to explain why only some women get osteoporotic fractures and proposes a first experiment to test this theory. The theory suggests a critical link between the estrogen loss, the advanced glycation endproduct (AGE) content of the bone, and signaling via the receptor for advanced glycation endproducts (RAGE) and that the combination of these three components determines the extent of bone loss post menopause. The broadly accepted ovariectomized female rat will be used to model post menopausal bone loss. A special diet will induce advanced glycation endproduct formation.					
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1. INTRODUCTION:

The impact of osteoporosis on individual patients and their families, health care systems, and national economies is enormous. 10% of Western populations have osteoporosis (44 million Americans in 2002). One in four women over 50 years of age has osteoporosis and their quality of life is poor. Health care costs associated with osteoporosis are staggering, estimated at around \$17 billion a year in the US (2005). The proposed project addresses a novel and potentially important mechanism of osteoporosis which may determine which women suffer the disease. Confirmation and understanding of this mechanism will lead to new prediction methods and treatments for osteoporosis patients and greatly improve the lives of affected civilian, military and veteran populations. At the onset of menopause, lack of estrogen and other sex steroids results in increased bone turnover and net bone loss. Fortunately, only 25-30% of post-menopausal women will develop vertebral fractures – the hallmark of post-menopausal osteoporosis – because of differences in the amount of bone lost. This proposal presents a novel theory to explain why only some women get osteoporotic fractures and proposes a first experiment to test this theory. The theory suggests a critical link between the estrogen loss, the advanced glycation endproduct (AGE) content of the bone, and signaling via the receptor for advanced glycation endproducts (RAGE) and that the combination of these three components determines the extent of bone loss post menopause. The broadly accepted ovariectomized female rat will be used to model post menopausal bone loss. Some of these rats will be fed a special diet to induce advanced glycation endproduct formation. Their blood serum will be analyzed to confirm AGE formation, bone turnover and RAGE expression. Bone morphometry will be measured with microCT and histomorphometric techniques. Osteoclast expression of RAGE and co-localization with AGEs in the bone matrix will be tested with immunohistochemistry.

2. KEYWORDS:

Osteoporosis, Advanced glycation endproducts, estrogen, receptor for advanced glycation endproducts,

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Task 1. Apply for and receive Animal Use Protocol approval (months 1-4) COMPLETE

- 1a. Write and submit Animal Use Protocol to the Chief Veterinarian and Animal Ethics Committee, Division of Comparative Medicine (DCM), University of Toronto.
- 1b. Respond to questions and concerns of the Animal Ethics Committee in writing.
- 1c. Receive ACURO approval
- 1d. Receive Approval and submit to Grants Officer Ms. Angela Fong.

Task 2. Order and delivery of 60 three-month old female rats (month 5) COMPLETE

- 2a. Submit order for rats to Charles River Laboratories through the Division of Comparative Medicine, University of Toronto
- 2b. Receive rats at the Division of Comparative Medicine, University of Toronto
- 2c. Randomly assign rats to the six experimental groups (n = 10 each group)

Task 3. Experimental Period 1 – all six groups of 10 (months 6-8) COMPLETE

- 3a. Take time-zero baseline blood samples from all sixty rats (2 days; DCM staff)
- 3b. Start the controlled research diets - three Normal diet groups (n=30) and three High Fructose Diet groups (n=30) (DCM staff; months 4-6)

3c. At the end of Period 1, euthanize one Normal diet group (n=10) and one High Fructose Diet group (n=10) to characterize the effect of the High Fructose Diet. (DCM staff; end of month 6)

3c.1. Collect blood and bone specimens (DCM and student)

Task 4. Experimental Period 2 – remaining four groups of 10 (months 9-11)

COMPLETE

4a. Perform ovariectomy or sham operations on the four remaining groups (DCM staff; 2 days)

4b. All four groups continue on Maintenance diet, designed to promote longevity (months 7-9)

4c. 4 weeks after ovariectomy and sham operations, collect blood samples (DCM staff; month 8)

4d. Euthanize four remaining groups (Normal diet + Sham, Normal Diet + OVX, HFD + Sham, HFD + OVX)

4d.1. Collect blood and bone specimens (DCM staff and student)

Task 5. Data Acquisition and Analysis (months 11-15)

IN PROGRESS

5a. MicroCT scanning and analysis (student; months 10-11) **COMPLETE**

5b. Preparation of histomorphometry specimens (student; months 10-11)

5c. Histomorphometry data collection and analysis (student; months 12-13)

5d. Blood and Bone Biochemistry (student; months 12-13) **COMPLETE**

5e. Histology and Immunohistochemistry (student; months 12-15)

Task 6. Finalization and Dissemination (months 16-18)

IN PROGRESS

6a. Data analysis (student; months 16-17)

6b. Report and manuscript writing (student; months 17-18)

What was accomplished under these goals?

1) Major Activities: Tasks 1 thru 4 are COMPLETE. Tasks 5 and 6 are IN PROGRESS.

2) Specific Objectives: The animal model was successfully implemented. Data collection under way.

3) Significant Results or Key Outcomes:

- a. To date, our sole item to report is that the High Fructose diet had a very minimal effect in almost all measured parameters. When examining the amount of bone loss and deterioration of microarchitecture, it became obvious that any observed effects were predominately due to the OVX surgery with little effect due to High Fructose diet, as shown below. (Table 3, Fig 1, Fig 2 and 3). When examining the serum content of the AGEs, pentosidine and carboxymethyllysine, and soluble RAGE, we observed that all of these factors increased as the rats were fed their respective diets (between the first and second blood withdrawals), however when the diet was discontinued, these values remained stagnant or decreased (Figs 4, 5 and 6). Circulating CML was found to be increased due to the control diet at the third time point (one month after surgeries), and soluble RAGE was found to be increased at the third time point due to the OVX surgery (Figs 7&8).

Table 3. MicroCT results of femur scanned at mid-diaphysis

	HF+OVX	HF+SHAM	CD+OVX	CD+SHAM
vBMD	1.010 ± 0.022*	0.990 ± 0.041	1.013 ± 0.069*	0.944 ± 0.060
Cross Sectional Bone Area	7.52 ± 0.59	7.16 ± 0.47	7.35 ± 0.50	7.29 ± 0.48
Cortical Thickness	0.803 ± 0.046	0.804 ± 0.036	0.788 ± 0.036	0.832 ± 0.034
Mean Major Diameter	4.49 ± 0.20	4.38 ± 0.23	4.55 ± 0.24	4.48 ± 0.21
Mean Minor Diameter	3.23 ± 0.15	3.23 ± 0.14	3.23 ± 0.12	3.178 ± 0.17

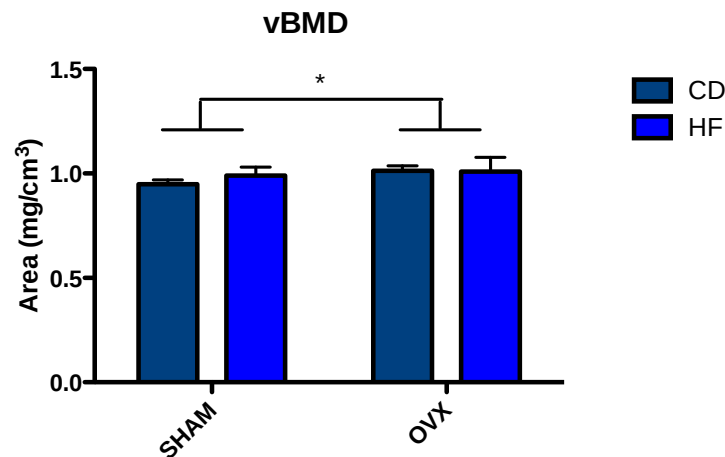


Figure 1. MicroCT results of femur at mid-diaphysis scanned at 11.6µm voxel size. Mean ± SD, n=9, two-way ANOVA, Surgery p=0.031, diet p=0.297, interaction p=0.222. Values reported as mean ± standard error. * Significant (p<0.05) compared to SHAM controls.

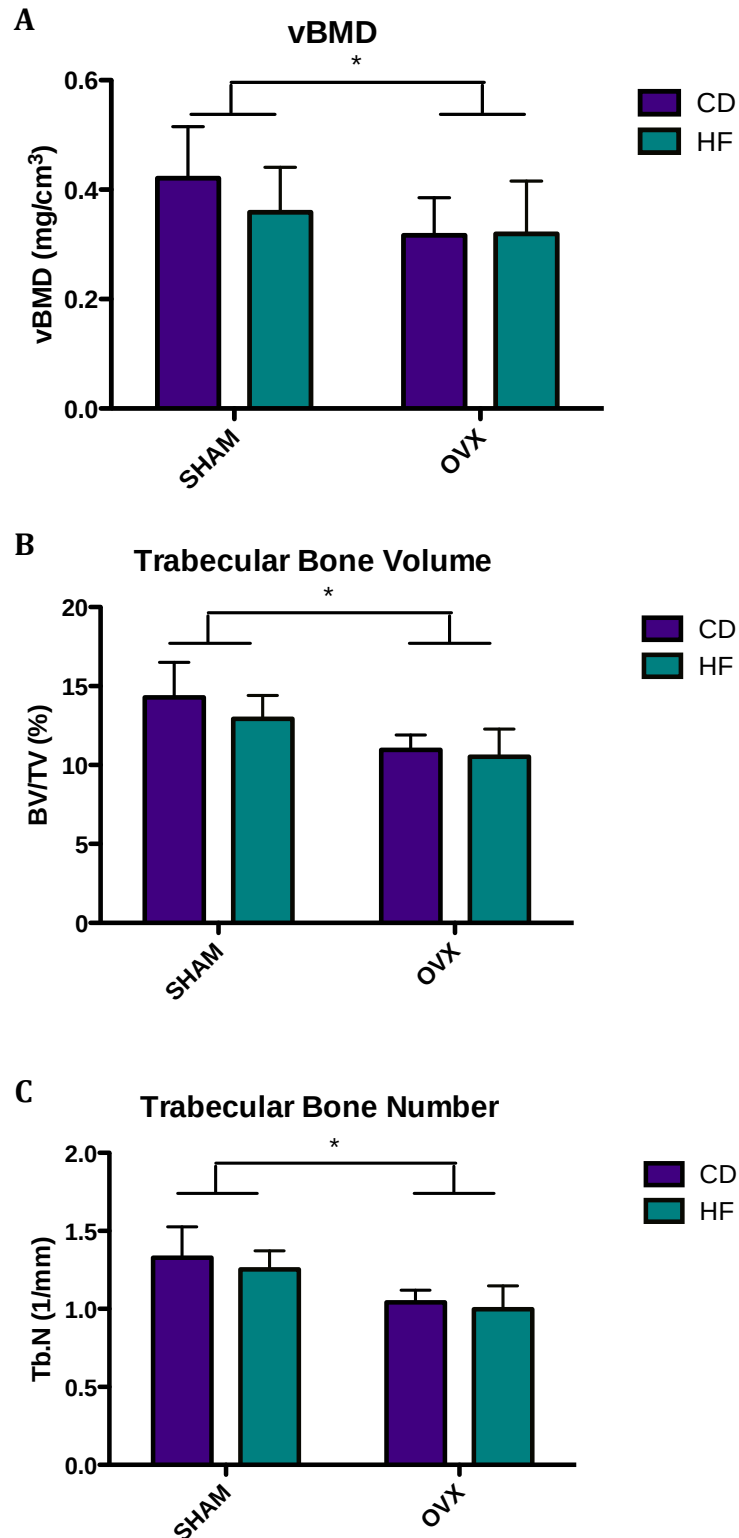


Figure 2. MicroCT results of secondary spongiosa in 6th lumbar vertebrae scanned at 11.6 μ m voxel size. Mean $\pm$ SD, two-way ANOVA, n=9. (A) surgery p=0.019, diet p=0.311, interaction p=0.271; (B) surgery p=0.000, diet p=0.128, interaction p=0.418; (C) surgery p=0.000, diet p=0.234, interaction p=0.766.

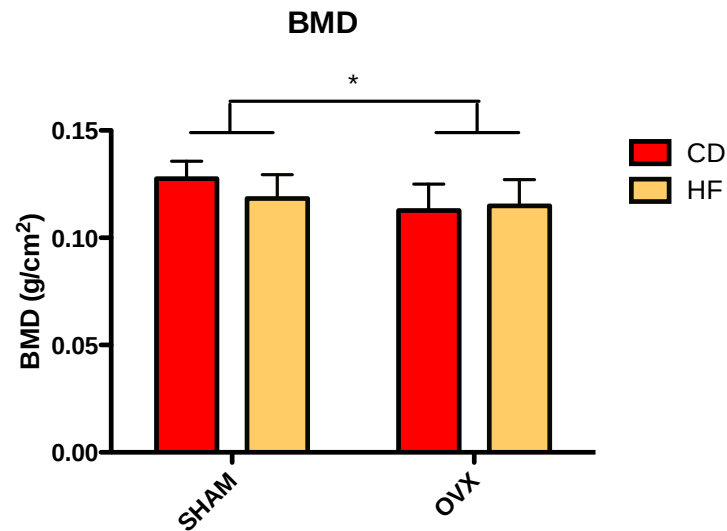


Fig 3ure. DEXA results of secondary spongiosa in 6th lumbar vertebrae. Mean $\pm$ SD, two-way ANOVA, n=9, surgery p=0.0155, diet p=0.3382, interaction p=0.1191. * indicates the detected effect of surgery.

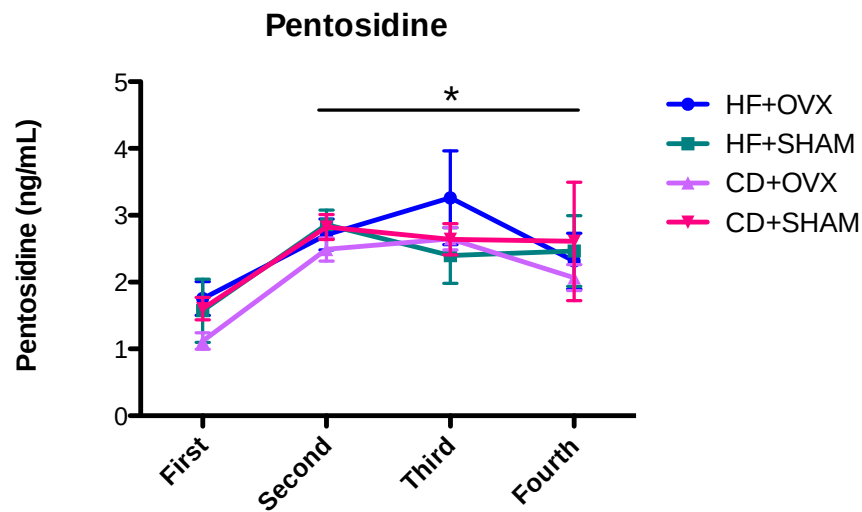


Fig 4. ELISA results for serum pentosidine measured at four time points. All groups showed a detectable increase in circulating pentosidine between the first and second time points (indicated by the bar with *). Mean, n=9, Repeated Measures Two-Way ANOVA, p<0.05 for all groups between time point one and two.

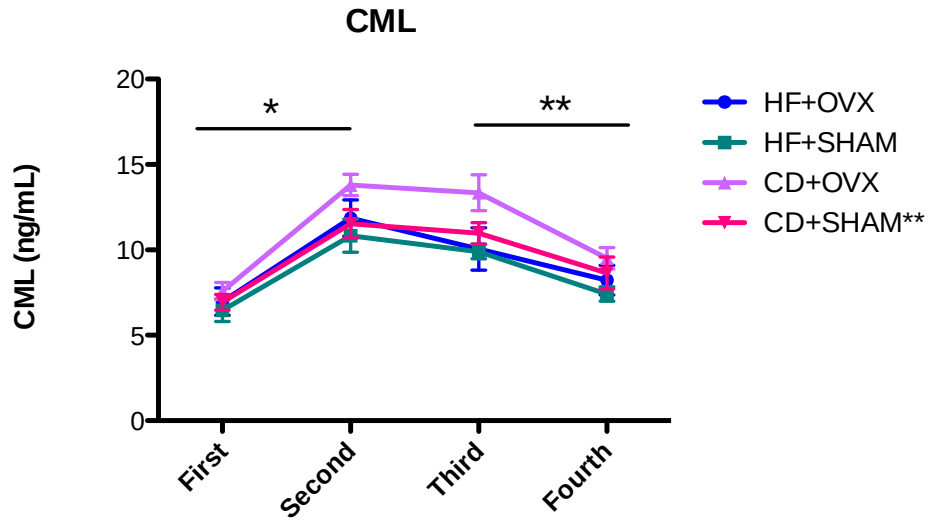


Fig 5. ELISA results for serum carboxymethyllysine measured at four time points. All groups showed a detectable increase in circulating CML between the first and second time points (bar with *). All groups, excluding HF+OVX, showed a detectable decrease between the third and fourth time points (bar with **). Mean, n=9, Repeated Measures Two-Way ANOVA, $p < 0.05$.

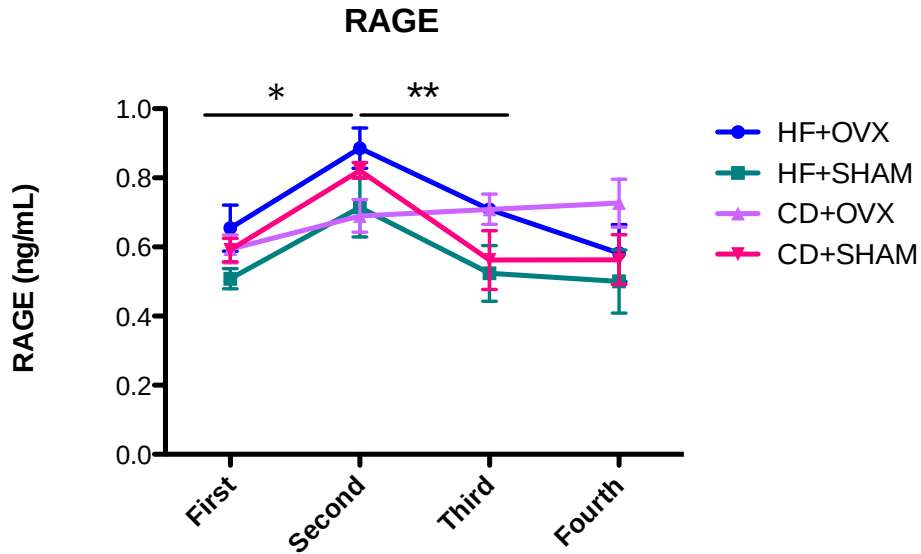


Fig 6. ELISA results for soluble RAGE measured at four time points. All groups showed a significant increase in circulating RAGE between the first and second time points (bar with *) and the HF+OVX and HF+SHAM groups showed a significant decrease between the second and third time points (bar with **). Mean, n=9, Repeated Measures Two-Way ANOVA, $p < 0.05$ for all groups between time point one and two.

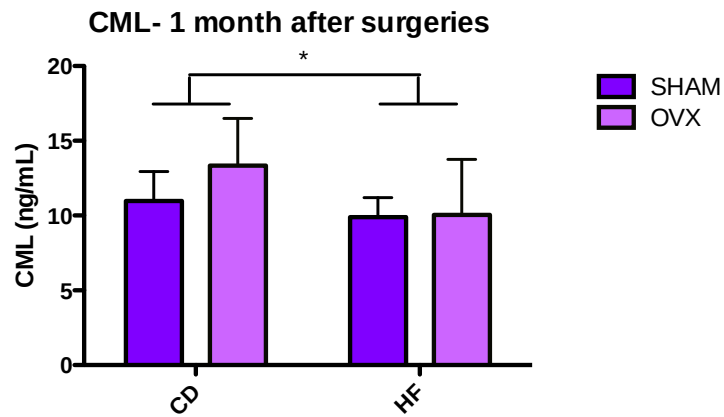


Fig 7. ELISA results for serum CML measured one month after surgeries. Mean $\pm$ SD, n=9, two-way ANOVA, surgery p=0.162, diet p=0.019, interaction p=0.216. * indicates that CML levels were detectably higher in the control diet groups at this time point.

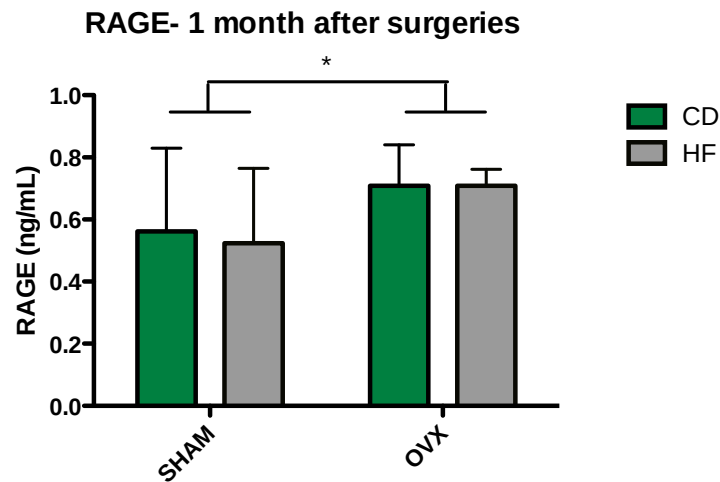


Fig 8. ELISA results for serum RAGE measured one month after surgeries. Mean $\pm$ SD, n=9, two-way ANOVA, surgery p=0.015, diet p=0.767, interaction p=0.767.

4) Other Achievements: None

What opportunities for training and professional development has the project provided?

Ms. Julia Pasquale has worked towards her MSc in Laboratory Medicine and Pathology and has had and will continue to have opportunities for excellent training in scientific process, methods and techniques.

How were the results disseminated to communities of interest?

Ms. Julia Pasquale presented her project and findings to date at the following venues:

1. 2nd Annual Toronto Musculoskeletal Centre Research Day, University of Toronto; February 11, 2014
2. 17th Annual Laboratory Medicine and Pathobiology Graduate Research Conference University of Toronto; March 11, 2014. First place poster prize for Masters students.
3. 20th Canadian Connective Tissue Society Conference, London, Ontario; June 8-10, 2014
4. 3rd Annual Toronto Musculoskeletal Centre Research Day, University of Toronto; February 12, 2015
5. 18th Annual Laboratory Medicine and Pathobiology Graduate Research Conference, University of Toronto; April 8, 2015

What do you plan to do during the next reporting period to accomplish the goals?

Work on Tasks 5 and 6 will be completed by early 2016.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No changes were required through Tasks 1 thru 4. However, as data collection and analysis commenced, it became obvious that any effects due to the High Fructose diet were very small and not detectable in many cases. This has lead to small changes in approach by simply concentrating on the tissue and cellular level measurements rather than whole bone level.

Actual or anticipated problems or delays and actions or plans to resolve them

- 1) Effect of High Fructose diet is small and often undetectable. This has lead to small changes in approach by simply concentrating on the tissue and cellular level measurements rather than whole bone level. Also, we are moving to more sensitive detection using confocal laser microscopy rather than conventional light microscopy.
- 2) Commissioning of immunohistochemical techniques has taken much longer than anticipated due to technical challenges. It has proven difficult to purchase effective primary antibodies and secondary detection systems to RAGE especially. The student and technician have invested a great deal of time in this. Negative and positive controls (Fig 9) have worked well but bone specimens have yielded poor results. High amounts of background staining in bone have made qualification of immunohistochemical staining difficult (Fig 10). Currently we are working to optimize a protocol using immunofluorescence to detect if RAGE is present within bone cells. Immunofluorescence has enhanced sensitivity compared to chromogenic techniques, and previous protocols have been optimized to work on bone tissues within our lab.

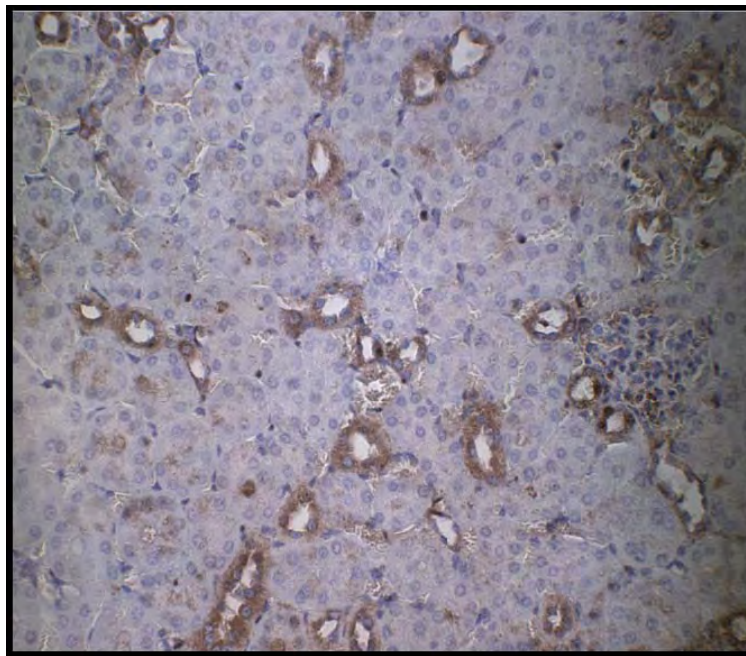


Fig 9. Immunohistochemical staining of positive control tissue (mouse kidney). Antibody (staining black/dark brown) is rabbit anti-RAGE from Abcam. Picture taken under 20x magnification.

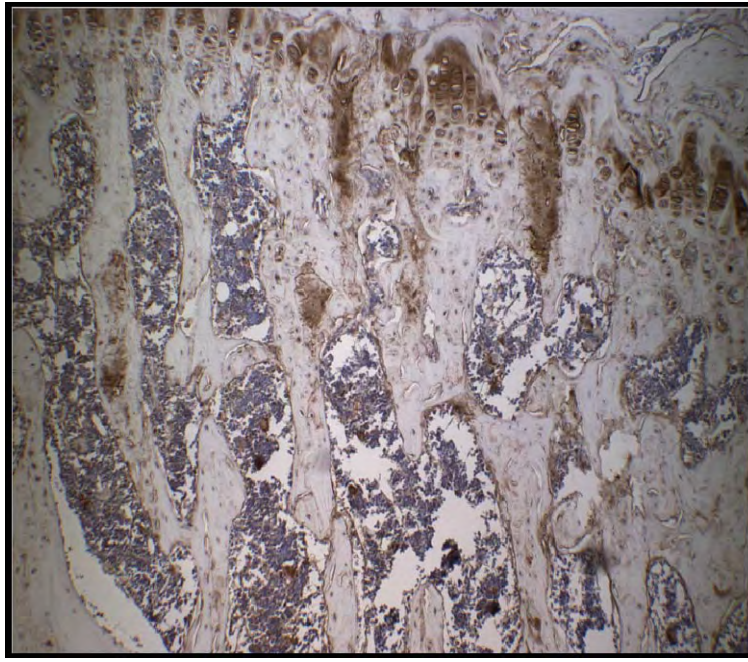


Fig 10. Immunohistochemical staining of tibia from an OVX control rat. Antibody (staining black/dark brown) is rabbit anti-RAGE from Abcam. Picture taken under 20x magnification.

Changes that had a significant impact on expenditures

Histology technician resigned, leading to significant delays in developing protocols. Immunohistochemical detection of RAGE and AGEs has proven difficult to achieve. Therefore greater expenditures on reagents, etc have been incurred.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

6. PRODUCTS

Other publications, conference papers, and presentations

1. 2nd Annual Toronto Musculoskeletal Centre Research Day, University of Toronto; February 11, 2014
2. 17th Annual Laboratory Medicine and Pathobiology Graduate Research Conference University of Toronto; March 11, 2014. First place poster prize for Masters students.
3. 20th Canadian Connective Tissue Society Conference, London, Ontario; June 8-10, 2014
4. 3rd Annual Toronto Musculoskeletal Centre Research Day, University of Toronto; February 12, 2015

5. 18th Annual Laboratory Medicine and Pathobiology Graduate Research Conference,
University of Toronto; April 8, 2015

7. PARTICIPANTS & OTHER COLLABORATIGN ORGANIZATIONS

What individuals have worked on the project?

Name:	Julia Pacquale
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	20
Contribution to the project:	All data collection and analysis
Funding support:	

Name:	Kate Banks
Project Role:	Veterinarian
Researcher Identifier:	
Nearest person month worked:	10
Contribution to the project:	Supervision of animal work
Funding support:	

Name:	Marc Grynepas
Project Role:	PI
Researcher Identifier:	
Nearest person month worked:	10
Contribution to the project:	Supervision and management
Funding support:	

Name:	Thomas Willett
Project Role:	PI
Researcher Identifier:	
Nearest person month worked:	10
Contribution to the project:	Supervision and management
Funding support:	

Name:	Tanya Raaphorst
Project Role:	Histology technician
Researcher Identifier:	
Nearest person month worked:	32
Contribution to the project:	Histological methods
Funding support:	

Name:	Iryna Storozhuk
Project Role:	Histology technician
Researcher Identifier:	
Nearest person month worked:	32
Contribution to the project:	Histological methods
Funding support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: University of Toronto Division of Comparative Medicine

Location of Organization: Toronto, Ontario, Canada

Partner's contribution to the project:

- Facilities: Animal housing, care and surgery
- Collaboration: Dr. Kate Banks