Check for updates

RESEARCH ARTICLE

REVISED Effect of sex in the MRMT-1 model of cancer-induced bone pain [version 3; peer review: 2 approved]Sarah Falk¹, Tamara Al-Dihaissy¹, Laura Mezzanotte², Anne-Marie Heegaard¹¹Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, 2100, Denmark²Department of Radiology, Leiden University Medical Center, Leiden, 2333 ZA, The Netherlands

V3 First published: 31 Jul 2015, 4:445
<https://doi.org/10.12688/f1000research.6827.1>
 Second version: 18 Sep 2015, 4:445
<https://doi.org/10.12688/f1000research.6827.2>
 Latest published: 16 Nov 2015, 4:445
<https://doi.org/10.12688/f1000research.6827.3>

Abstract

An overwhelming amount of evidence demonstrates sex-induced variation in pain processing, and has thus increased the focus on sex as an essential parameter for optimization of in vivo models in pain research. Mammary cancer cells are often used to model metastatic bone pain in vivo, and are commonly used in both males and females. Here we demonstrate that compared to male rats, female rats have an increased capacity for recovery following inoculation of MRMT-1 mammary cells, thus potentially causing a sex-dependent bias in interpretation of the data.

Keywords

Cancer-induced bone pain, sex differences, in vivo model, x-ray, pain behavior, bioluminescence

Open Peer Review**Approval Status**

	1	2
version 3		
(revision)	view	view
16 Nov 2015		
version 2		
(revision)		view
18 Sep 2015		
version 1		
31 Jul 2015	view	

1. **Juan M Jiménez-Andrade**, Universidad Autónoma de Tamaulipas (UAT), Reynosa, Mexico
2. **Svetlana Komarova**, McGill University, Montreal, Canada

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Sarah Falk (saf@nexs.ku.dk)

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

Copyright: © 2015 Falk S *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Falk S, Al-Dihaissy T, Mezzanotte L and Heegaard AM. **Effect of sex in the MRMT-1 model of cancer-induced bone pain [version 3; peer review: 2 approved]** F1000Research 2015, 4:445 <https://doi.org/10.12688/f1000research.6827.3>

First published: 31 Jul 2015, 4:445 <https://doi.org/10.12688/f1000research.6827.1>

REVISED Amendments from Version 2

Further elaboration on the main results was added to the discussion. In addition, a section on estrogen-receptor status was added.

See referee reports

Introduction

A crucial step in translational research is development of animal models that can accurately mimic the human condition in relation to both symptoms and underlying mechanisms^{1,2}. To understand the molecular mechanism of complex human disease, and thereby create disease-specific treatment, the models need to be reproducible and with a high predictive value, requiring a detailed knowledge of the expected variation in the models used. Cancer-induced bone pain is a highly complex pain state involving cancer cells, bone cells, immune cells as well as neuronal and non-neuronal processing in the periphery and at spinal and supraspinal levels³. Numerous studies have reported on variation in the more than 45 animal models that have been used to model the pain state⁴. The most common variations are related to cell lines, species or injection site used⁴, and in addition we have previously demonstrated that sex does also affect the model⁵, thereby emphasizing the need to consider sex bias.

The majority of preclinical pain research is still performed using male animals, despite an overwhelming amount of both human and animal data demonstrating significant sex variations^{6–8}. Conversely, within the field of bone cancer pain, preclinical researchers have had more focus on the issue, likely due to a more intuitive choice of sex, based on the origin of the cancer cells used. A meta-analysis of studies on cancer-induced bone pain demonstrated that 49% of the studies used males, 22% used females and 29% did not report the sex of the animals used⁴. These numbers are indeed more balanced than the numbers previously reported, revealing that 79% of all animal studies published in PAIN from 1996 to 2005 were using male animals, whereas only 8% used females⁸. However, in this study we demonstrate that not only is sex an integral factor to be considered when choosing an animal model, intrinsic sex-dependent variation has to be carefully analyzed in order to avoid bias. The data presented in this study suggest, that in the MRMT-1-Luc2 carcinoma cell model of metastatic bone cancer pain, a sex-dependent bias related to recovery is skewing the behavioral responses observed in females to a greater extent compared to males, potentially masking effects in studies where female animals are used to model the pain state.

Methods

Experiments were performed on 10 male and 10 female Sprague-Dawley rats (Taconic M&B, Denmark) group-housed in cages under a 12-h alternating light/dark cycle with ad libitum access to food and water. In addition, unpublished data from four previous independent in-house experiments were analyzed; these included a total of 16 females and 42 males, and were performed as described in this method section (see [Figure 4C](#)). The only difference with respect to methods is that three experiments were performed prior to transfection of the MRMT-1 cells line, and this study and one of the prior experiments were conducted after transfection of the MRMT-1 cells line. For all animals bodyweight and general health was

monitored throughout the studies. All experiments were approved by the Danish Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (2014-15-0201-00031), and were carried out in accordance to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain⁹.

Generation of MRMT1-Luc2

Generation of MRMT1 cells expressing reporter proteins was achieved by genomic integration of the Luc2-copGFP reporter gene construct using lentiviral transduction. The lentiviral vector contains the EF1 α promoter sequence driving the equimolar expression of a codon-optimized firefly luciferase (Luc2) gene and a copepod green fluorescent protein (copGFP) thanks to the presence of a T2A sequence. Vector production and cell transduction were performed under appropriate biosafety level conditions (ML-II) in accordance with the National Biosafety Guidelines and Regulations for Research on Genetically Modified Organisms. Procedures and protocols were reviewed and approved by the LUMC Biosafety Committee (GMO permit 08-129). Vector production and transduction procedures have been described in detail elsewhere¹⁰. In brief, cells were seeded in 24-well plate at a cell density of 7.5×10^4 cells/well and maintained in a humidified incubator at 37°C and 5% CO₂. After attachment was accomplished, the cells were transduced using a MOI (multiplicity of infection) of 1. Subsequently cells were FACS sorted for high expression of copGFP and cell culture was expanded for experiments.

Cell culture

Syngeneic MRMT1-Luc2 rat mammary gland carcinoma cells (Leiden University medical Center, The Netherlands) were cultured in RPMI 1640 medium without phenol red (Invitrogen, Paisley, UK/Nærum, Denmark) supplemented with 10% heat-inactivated foetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin (Invitrogen, Paisley, UK/Nærum, Denmark). On the day of surgery, MRMT1-Luc2 carcinoma cells were released by brief exposure to 0.1% w/v trypsin-EDTA and centrifuged in medium for 3 min at 1200rpm. The pellet was washed twice with Hanks' balanced salt solution (HBSS) without calcium, magnesium or phenol red (Invitrogen, Paisley, UK/Nærum, Denmark) and centrifuged for 3 min at 1200rpm. Cells were re-suspended in HBSS and kept on ice until use.

Surgery

The inoculation of carcinoma cells was performed modified from [11](#) as previously described¹². Hence, following induction of isoflurane anaesthesia (induction 4%, maintenance 1.5–2%) the animal was placed with the abdominal side up. A small incision was made in a shaved and disinfected area on the anterior-medial surface of the tibia and the tibia carefully exposed. A hole was made in the tibia with a 0.7mm dental drill through which a thin polyethylene tube was fed 1cm into the proximal intramedullary cavity. Animals were injected with 5×10^3 MRMT1-Luc2 carcinoma cells in 10 μ l HBSS. Following removal of the tube, the hole was plugged with bone restorative material (IRM, Dentsply, Surrey, UK/Vallensbæk, Denmark). The wound was irrigated with saline and closed with two metal clips. The animals were placed under a heat source until fully awake. Postoperative analgesia was administrated by injection of Rimadyl (s.c. 5mg/kg, Pfizer, Denmark) and application of 2% Lidocaine gel (AstraZeneca, Denmark) to the wound.

Behavioral tests

Behavioral responses were assessed prior to surgery and on day 7, 10, 14, 17, 21 and 23. All animals were introduced to all behavioral tests 2–3 times prior to the start of the experiment. Animals reaching humane endpoint before day 23 (n=7) (predefined as limb use score 0) were euthanized by decapitation following brief exposure to isoflurane, and the behavioral scores from the last day carried forward for data analysis.

Von Frey test. Mechanical hypersensitivity, detected by von Frey filaments (North Coast Medicinal, Inc., Camino Arroyo, Gilroy, CA, USA), was used as an indicator of early pain behavior. The threshold was determined by the up and down method, as described previously¹³. Briefly, rats were placed in acrylic enclosures on a wire mesh floor and allowed to acclimatize for minimum 30 min. Starting at 6 g, filaments ranging from 0.4–26 g were applied to the plantar surface of the hind paw with a minimum of 3 min intervals between two stimulations. A stimulus was recorded as positive if paw withdrawal was observed within 3 s of stimulation with a given filament; if no response was observed it was recorded as negative. Following a positive response, the next stimulation was performed with a filament with a decreased bending strength, whereas a negative response was followed with stimulation with a filament with increased bending strength. According to the original protocol, optimal threshold calculation by this method requires 6 responses in the immediate vicinity of the 50% threshold, therefore recording of the 6 data points did not begin until the response threshold was first crossed (positive response changes to negative response or inverse). The 2 responses detecting the threshold were designated as the first 2 responses of the series of 6. In cases where continuous negative responses were observed to the maximum of the stimulus set, a value of 26 g was set as the cut-off and used as withdrawal threshold for data analysis. In all other cases the resulting pattern of positive and negative responses was calculated into a 50% response withdrawal threshold using the formula: $50\% \text{ g threshold} = (10Xf + k\delta) / (10,000)$, where Xf = value (log unit) of the final von Frey hair used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between filaments.

Weight-bearing test. Rats were placed in the incapacitance tester (MJS Technology Ltd., Buntingford, Herfordshire, UK) and allowed to acclimatize until calm. Measurements were performed over a period of 4 s and in triplicates. An average weight-bearing ratio was subsequently calculated as the amount of weight placed on the cancer-bearing leg divided by the total amount of weight placed on both legs and used for data analysis.

Limb use test. Rats were allowed to move freely in a transparent plastic cage without bedding (500 mm × 300 mm × 500 mm). Following 10 min of acclimation, each animal was observed for 3 min and assigned a limb use score from 3 to 0 as follows: 3: Normal use of leg, 2: mild or insignificant limping, 1: significant limping, 0: significant limping and part lack of use.

Bioluminescence imaging

Animals were anesthetized in an induction chamber with 2.5% isoflurane (Isobar Vet; 100%, Nomenco, Copenhagen, DK). D-Luciferin (PerkinElmer, Skovlunde, Denmark) dissolved in PBS was administered by intraperitoneal injection (40mg/kg). 10 min after

injection, animals were placed on their back in a nose cone in a Lumina XR instrument (Caliper Life Sciences, Terafene, Belgium) and anesthesia was maintained with a 2.5% isoflurane/oxygen mix. Image capture was performed with binning: M(4), F/stop: 1 and exposure time from 1 s to 60 s according to the power signal. The signal was adjusted according to the exposure time prior to data analysis. For each animal, an average of three images was used for analysis. Between each capture, the animal was repositioned to minimize bias caused by placement of the animals in the machine. Bioluminescence images were analyzed using IVIS Imaging Software (Living Image®, version 4.0.0.9801; Caliper Life Sciences, Terafene, Belgium). For each image, the threshold was adjusted to 35% of the signal, and the readout was measured in total flux, photos/s.

X-ray analysis

X-Ray images were captured subsequent to the bioluminescence images. The severity of bone degradation was analyzed using ImageJ (ImageJ 1.47v). Each X-Ray image was calibrated to a standard aluminum wedge. The mean grayscale value of a standard region of interest within the trabecular bone of the proximal tibia was measured and the average of two corresponding background regions in the soft tissue proximate to tibia was subtracted. The grayscale value was translated into millimeter aluminum equivalents (mmAl) according to the standard wedge and used as estimate of the relative bone density of the distal femur. Data analysis was blinded for sex of the animals.

Determination of active state

Animals with no bioluminescence signal or lack of osteolysis, hence no active cancer growth, were excluded from the active state group. All animals included in this study were assigned to an active or restored state based on presence of bioluminescence signal and osteolysis (active) or lack of bioluminescence signal and osteolysis (restored). Four previous in-house experiments were reanalyzed with regard to active and restored state based on presence bioluminescence signal and osteolysis or lack of bioluminescence signal and osteolysis, or based on osteolysis or lack of osteolysis alone. One study was similarly using bioluminescence as exclusion criteria, whereas the data from the three previous studies was analyzed with respect to lack of osteolysis. Lack of osteolysis was based on the last measure day, usually day 21–23, whereas one experiment was terminated at day 14, however on this day 10 out of 11 animals showed clear osteolysis.

Adverse events

No adverse events were observed during the experiment.

Statistical analysis

All analyses were blinded for the researchers. Analyses were performed using GraphPad Prism 6, (GraphPad Software, CA, USA), and for all data a 95% confidence interval was used as a measure of statistical significance. All data are expressed as mean ± standard error of mean (S.E.M.). *In vitro* analysis of bioluminescence signal was analyzed with linear regression. Behavioral, bone degradation and tumor progression data were analyzed using two-way repeated measure ANOVA followed by Bonferroni post-hoc test to compare baseline values to each additional time point, and in addition to compare females to males. Analysis of odds ratio of cancer clearance was

tested using Chi-square test. Level of significance for all tests was set at $*/\#p < 0.05$, $**/\#\#p < 0.01$, $***/\#\#\#p < 0.001$, $****/\#\#\#\#p < 0.0001$.

Results

Validation of bioluminescence emission in cancer cells

The bioluminescence signal was evaluated to validate successful transfection of Luc2 into the MRMT-1 carcinoma cells. *In vitro*, the signal increased linearly with the number of cells, $p < 0.0001$ (Figure 1A). *In vivo*, i.p. administration of D-Luciferin induced a robust plateau of the bioluminescence signal 5 min after injection and lasting approximately 20min (Figure 1B). Luc2 was hence successfully transfected into the carcinoma cells, linearly reflecting the number of living cells *in vitro*, and produced a robust signal *in vivo*.

Sex-dependent variation in disease progression

Pain behavior was quantified by detection of mechanical hypersensitivity, as well as limb use and weight-bearing deficits on days 7, 10, 14, 17, 21 and 23. Compared to baseline level, a significant decline in the 50% withdrawal threshold, reflecting mechanical hypersensitivity, was observed in both females and males from day 10 to 21 (Figure 2A, Dataset 1: rawdata_vonfrey). Limb use scoring was significantly reduced only on day 23 for the females, but on day 17–23 for the males (Figure 2B, Dataset 2: rawdata_limbuse). Decline in weight-bearing ratio was significantly reduced on day 17 and 23 for the females and on day 17–23 for the males (Figure 2C, Dataset 3: rawdata_weight-bearing). No significant difference was observed between the sexes on any of the test days, however females tended to present with a less pronounced limb use and weight-bearing deficit in the later phase of disease progression, days 17–23 (Figure 2B and C).

The relative bone density was significantly reduced compared to baseline measures in the males from day 14 and throughout the study (Figure 2D, Dataset 4: rawdata_xray). A similar, however insignificant, tendency was observed in the females (Figure 2D). No difference was found between males and females at any time point. Overall, the data suggest a less severe progression in females compared to males.

Dataset 1. Rawdata_vonfrey

<http://dx.doi.org/10.5256/f1000research.6827.d96687>

Animals were placed in acrylic enclosures on a wire mesh floor and allowed to acclimatize for minimum 30 min. Starting at 6 g, filaments ranging from 0.4–26 g were applied to the plantar surface of the hind paw with a minimum of 3 min intervals between two stimulations. A stimulus was recorded as positive if paw withdrawal was observed within 3 s of stimulation with a given filament; if no response was observed it was recorded as negative. Following a positive response, the next stimulation was performed with a filament with a decreased bending strength, whereas a negative response was followed with stimulation with a filament with increased bending strength. The resulting pattern of positive and negative responses was calculated into a 50% response withdrawal threshold using the formula: 50% g threshold = $(10Xf + k\delta)/(10,000)$, where Xf = value (log unit) of the final von Frey hair used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between filaments²⁹.

Dataset 2. Rawdata_limbuse

<http://dx.doi.org/10.5256/f1000research.6827.d96688>

Following 10 min of acclimation in a transparent plastic cage, each animal was observed for 3 min and assigned a limb use score from 3 to 0 as follows: 3: Normal use of leg, 2: mild or insignificant limping, 1: significant limping, 0: significant limping and part lack of use³⁰.

Dataset 3. Rawdata_weight-bearing

<http://dx.doi.org/10.5256/f1000research.6827.d96689>

Animals were placed in the incapacitance tester and allowed to acclimatize until calm. Measurements were performed over a period of 4 s and in triplicates. An average weight-bearing ratio was subsequently calculated as the amount of weight placed on the cancer-bearing leg divided by the total amount of weight placed on both legs and used for data analysis³¹.

Figure 1. (A) *In vitro* quantification of bioluminescence signal. Linear regression demonstrated that the signal increased linearly with the number of cells (dotted line), $p < 0.0001$. (B) *In vivo* quantification. The bioluminescence signal increased during the initial 5min following i.p. injection. After 5min the signal reached a stable plateau lasting for approximately 20min. Results from two experiments.

Figure 2. (A) A decrease in 50% withdrawal threshold was observed in both females and males as the disease progressed. (B and C) Males demonstrated a significant decrease in limb use and weight-bearing ratio on day 17–23. In contrast, females showed significant decrease limb use only on day 23, and decreased weight-bearing on day 17 and 23. (D) A significant decrease in relative bone density was observed in the males from day 14. A similar, yet insignificant, tendency was observed in the females. */**/****=males compared to baseline, #/##=females compared to baseline.

Dataset 4. Rawdata_xray

<http://dx.doi.org/10.5256/f1000research.6827.d96690>

The severity of bone degradation was analysed. The x-ray was calibrated to a standard aluminium wedge. The mean grayscale value of a standard region of interest within the trabecular bone of the proximal tibia was measured and the average of two corresponding background regions in the soft tissue proximate to tibia was subtracted. The grayscale value was translated into millimeter aluminium equivalents (mmAl) according to the standard wedge and used as estimate of the relative bone density of the distal femur³².

Females have increased capacity for recovery

All animals had a detectable bioluminescent signal on days 7 and 10, hence demonstrating successful inoculation of living cancer cells (Figure 3, Dataset 5: rawdata_BLI, Dataset 6: rawdata_bioluminescence). From day 14 an increasing number

of animals lost the bioluminescence signal, suggesting recovery in a subset of animals. Compared to females with consistent bioluminescence signal, a subset of females losing bioluminescence signal displayed a significant decrease in the signal from day 17 (Figure 3). A similar but later occurring tendency was observed in the males (Figure 3).

Loss of bioluminescence signal was observed in 40% of the females, but only in 20% of the males (Figure 4A and B). A significant trend for loss of signal was observed in both females and males, $p=0.0079$ and $p=0.0353$ respectively. A similar pattern in recovery was observed in previous independent in-house experiments. In these experiments, lack of osteolysis (determined by x-ray analysis) was used as an exclusion criterion. In one study, 5 out of 16 female animals displayed a lack of osteolysis (Figure 4C)¹⁴. Three additional experiments

Figure 3. Bioluminescence (BLI) signal reflecting progression of tumor growth. The signal increases in both males and females during the initial 14 days, and reaches a plateau for the remaining of the study. From day 14 an increasing numbers of animals lost the bioluminescence signal. #####=females without signal compared to females with signal.

Figure 4. (A and B) Cumulative numbers of females and males losing bioluminescence signal over time. **(C)** Numbers of animals with restored states in five independent in-house experiments. The recovery is based on either loss of bioluminescence signal or lack of osteolysis. **(D)** A significant difference is found between the odds ratio of recovering in females and males. *=females compared to males.

using male animals demonstrated lack of osteolysis at the end of the experiment in 3 out of 21, 1 out of 10 and 1 out of 11 animals, respectively¹² (Supplementary figure S1 and Supplementary figure S2). Pooling the data from the five experiments demonstrated a significant difference in the odds ratio between females and males, reflecting a significantly greater proportion of females clearing active cancer growth compared to males, as indicated by loss of bioluminescence signal and/or lack of cancer-induced osteolysis, $p=0.029$, (Figure 4D).

Dataset 5. Rawdata_BLI

<http://dx.doi.org/10.5256/f1000research.6827.d96691>

Images were obtained 10min after i.p. injection of 40mg/kg Luciferase. Image capture was performed with binning: M(4), F/stop: 1 and exposure time from 1 s to 60 s according to the power signal. For each animal, an average of three images was used for analysis. For each image, the threshold was adjusted to 35% of the signal, and the readout was measured in total flux, photos/s³³.

Dataset 6. Rawdata_bioluminescence

<http://dx.doi.org/10.5256/f1000research.6827.d96692>

Representative bio-luminescence images³⁴.

Loss of bioluminescence signal is associated with reversal of disease progression

Despite the presence of living cancer cells during the initial 10 days, neither pain behavior nor relative bone density was significantly changed in animals losing bioluminescence signal (Figure 5A–D). A transient but insignificant decrease in the 50% withdrawal threshold was observed in animals later losing bioluminescent signal (Figure 5A, rawdata_vonfrey). In addition, a slight decrease in limb use, weight-bearing and relative bone density was observed on day 14, however the decrease was insignificant and returned to baseline levels on the next measured day (Figure 5B–D, rawdata_limbuse, rawdata_weight-bearing, rawdata_xray). Overall this suggests initial

Figure 5. Pain-related behavior and relative bone density in animal losing bioluminescence signal during the experiment. (A) A transient, yet insignificant, decrease was observed in the 50% threshold in females. (B, C) Limb use and weight-bearing deficit was not observed in either females or males during the experiment. (D) The relative bone density remained the same throughout the experiment in both males and females.

normal disease progression, followed by subsequent recovery and hence normal relative bone density and lack of pain behavior. In addition, a strong correlation between bioluminescence signal and relative bone density was observed (Figure 6A) supporting the division of animals into two groups; animals with a clear bioluminescence signal and osteolysis, indicating active cancer growth (Figure 6A, B and C) and animals with no bioluminescence signal and no osteolysis, indicating recovery (Figure 6A, E and D).

Sex-difference screws progression of pain behavior

Exclusion of animals successfully recovering aligned the onset and severity of pain behavior in both sexes (Figure 7A–C). In addition, a similar effect was seen on the relative bone density, reflecting similar bone degradation in both females and males with active growing cancer (Figure 5D). No significant difference was observed between females and males at any time point.

Discussion

To increase the translational potential of preclinical research, it is essential to continually focus on refining and optimizing the animal models used. Increased focus has been given to sex as a variable, initially driven by the contradictory issue that preclinical research has been biased towards use of male animals despite the fact the chronic pain is highly overrepresented in women⁸. Although females are in most cases likely the most intuitive sex for models of metastatic breast cancer, such as the MRMT-1 model of cancer-induced bone pain, our

data suggest that care should be taken when interpreting the data. Our data demonstrates that the females have an increased capacity for recovering from the disease state, reflected by loss of bioluminescence signal accompanied by normal limb use and weight-bearing ratio as well as normal relative bone density throughout the study.

The increased odds ratio for recovery in the females introduces a potential bias in interpretation of the data. Importantly, although there is no significant difference between females and male, the increased recovery in the females pulls the mean of the females' readouts towards baseline to a much greater degree than the males, thereby increasing the risk of underestimating the severity of the disease in the females. In order to get a reliable model truly reflecting the human disease, it is critical to exclude the animals capable of recovery, as these animals are not reflecting the pain state observed in patients. This means that it is highly important to include a parameter for discrimination between an "active disease state" and a "recovered from disease state". Inclusion of animals with recovered states clearly mask the severity of the disease in the females, as limb use is only significantly decreased on the last day and the relative bone density is not significantly affected at any time point (Figure 2A and D). However, excluding females with recovered disease state reveals that females are in fact severely affected at an earlier time point, demonstrated by an significant decrease in limb use already on day 17 and in addition significant decreased relative bone density from day 21 (Figure 7B and D).

Figure 6. Correlation between relative bone density and bioluminescence signal. (A) Animals without bioluminescence signal all had high relative bone density (blue), whereas animals with high bioluminescence signal demonstrated low relative bone density (black). Examples of osteolytic (C) and non-osteolytic bone (D). Examples of high bioluminescence signal (B) and lack of signal (E).

In addition, our data demonstrates that bioluminescence signal is a reliable measure for the active disease state. All animals with continuous bioluminescence signal subsequently developed pain-related behavior and decreased relative bone density. In contrast, animals losing bioluminescence signal during the experiment did not develop pain-related behavior and demonstrated normal relative bone density. This is in agreement with other studies demonstrating that bioluminescence signal is predictable measure for quantification of cancer cells *in vivo*^{15,16}. To further validate the recovery state, histology could be made to confirm that loss of bioluminescence signal is actually caused by clearance of the cancer cells and not just loss of signal from the cancer cells. However, since the first model of cancer-induced bone pain was developed, bone degradation has been demonstrated to associate with tumor burden

numerous times¹⁷⁻²¹. In this study, the loss of bioluminescence signal is accompanied by lack of bone lesions and in addition lack of pain behaviors making it highly unlikely that there is still active cancer growth in the animals.

Importantly, this study emphasizes the need to consider sex-dependent variation beyond simple disease readouts. The data demonstrate that there are not significant difference between females and males with respect to pain behavior and bone degradation. However, there are sex-differences in the model which needs to be carefully considered when modeling cancer-induced bone pain in females, as the increased recovery rate in the females can potentially mask the progression. The specific mechanisms underlying the sex-dependent difference in recovery are currently not known, and requires an

Figure 7. Pain-related behavior and relative bone density in animals with a consistent bioluminescence signal throughout the experiment. (A, B and C) Both females and males demonstrate significant decreases in 50% withdrawal threshold, limb use score and weight-bearing ratio. (D) Both sexes show a similar extent of bone degradation. ****/****/****=males compared to baseline, ##/###/####=females compared to baseline.

extensive investigation of hormonal and cellular responses. One potential cause could be sex-dependent differences in the immune responses. It is now generally accepted that inherent properties and influence of sex hormones induce more potent immune and inflammatory reactions in females compared to males^{22,23}. An intuitive cause could therefore be, that the females' increased recovery rate is linked to an increased immune response to inoculation with the cancer cells possible modulated by sex hormones. It should be noted that the capacity for recovering was seen regardless of whether the cancer cells expressed luciferase or not, and is thus not due to an immune reaction to the luciferase enzyme. However, as the MRMT-1 are estrogen receptor-positive²⁰, the relationship might not be so simple. Estrogen generally cause increase growth in estrogen receptor-positive cells²⁴, suggesting that the female immune system would have to fight a more rapidly growing tumor, which would suggest a lower recovery rate. A full understand of the involvement of sex hormones on the progression of MRMT-1 requires extensive studies; however our data suggest a minor role in the overall progression of cancer-induced bone pain in this model. This is also supported by the similar progression in males and female rats despite the estrogen receptor-positives status of the cells.

In addition, recent work from Sorge *et al.* demonstrate that different cells mediate pain hypersensitivity in female and male mice²⁵. They found that although the males and females developed the same degree of mechanical allodynia following nerve injury, there was a fundamental difference in the cellular involvement. Whereas microglia activity was required for mechanical pain hypersensitivity in males, this was not the case in females, where the hypersensitivity was driven by adaptive immune cells, likely T lymphocytes. Our data suggest that a similar phenomenon could be involved in progression of MRMT-1 induced cancer pain. In agreement with Sorge *et al.*, we find a similar pain response in females and males, however since there is a significant difference in the recovery rate between the sexes, it might indicate the different systems are mediating the pain state in female and male rats.

Another factor potentially affecting the sex-dependent effect might be the local microenvironment in the bone. The microenvironment around the tumor might be different due to nonspecific sex-differences, hence potentially affecting the growth of the tumor cells following inoculation^{26–28}. Interestingly, the observed difference is likely species or cell line dependent. We have previously reported

that in a similar model of cancer-induced bone pain, based on 4T1-luc2 mammary cancer cell inoculation in femur of BALB/cJ mice, females have a significantly greater initial bioluminescence signal compared to males. The females had, in addition, an earlier onset of pain behavior, but a similar bone degradation rate⁵. This suggests that in the mouse model, intrinsic sex-dependent variation favors more aggressive progression in females compared to males, whereas the rat model displays better odds ratio for recovery in the females.

Data availability

F1000Research: Dataset 1. Rawdata_vonfrey, 10.5256/f1000research.6827.d96687²⁹

F1000Research: Dataset 2. Rawdata_limbose, 10.5256/f1000research.6827.d96688³⁰

F1000Research: Dataset 3. Rawdata_weight-bearing, 10.5256/f1000research.6827.d96689³¹

F1000Research: Dataset 4. Rawdata_xray, 10.5256/f1000research.6827.d96690³²

F1000Research: Dataset 5. Rawdata_BLI, 10.5256/f1000research.6827.d96691³³

F1000Research: Dataset 6. Rawdata_bioluminescence, 10.5256/f1000research.6827.d96692³⁴

Author contributions

S.F. conceived and designed the study and carried out all experimental procedures. T.A-D. assisted during experimental procedures. L.M. generated the MRMT-1-Luc2 cell line. A-M.H. provided advice and facilities. S.F. wrote the manuscript that was edited and approved by all coauthors.

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Supplementary Material

Supplementary figure S1. Representative images of animals demonstrating osteolysis (**A–F**) and lack of osteolysis (**G,H**).

Supplementary figure S2. Representatives of animals demonstrating bioluminescence signal (**A–C, E,F**) and lack of bioluminescence signal (**D**).

References

1. Barrett JE: **The pain of pain: challenges of animal behavior models.** *Eur J Pharmacol.* 2015; **753**: 183–90.
[PubMed Abstract](#) | [Publisher Full Text](#)
2. Ruggeri BA, Camp F, Miknyoczki S: **Animal models of disease: pre-clinical animal models of cancer and their applications and utility in drug discovery.** *Biochem Pharmacol.* 2014; **87**(1): 150–61.
[PubMed Abstract](#) | [Publisher Full Text](#)
3. Falk S, Dickenson AH: **Pain and nociception: mechanisms of cancer-induced bone pain.** *J Clin Oncol.* 2014; **32**(16): 1647–54.
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Currie GL, Delaney A, Bennett MI, *et al.*: **Animal models of bone cancer pain: systematic review and meta-analyses.** *Pain.* 2013; **154**(6): 917–26.
[PubMed Abstract](#) | [Publisher Full Text](#)
5. Falk S, Uldall M, Appel C, *et al.*: **Influence of sex differences on the progression of cancer-induced bone pain.** *Anticancer Res.* 2013; **33**(5): 1963–9.
[PubMed Abstract](#)
6. Mogil JS, Bailey AL: **Sex and gender differences in pain and analgesia.** *Prog Brain Res.* 2010; **186**: 141–57.
[PubMed Abstract](#) | [Publisher Full Text](#)
7. Greenspan JD, Craft RM, LeResche L, *et al.*: **Studying sex and gender differences in pain and analgesia: a consensus report.** *Pain.* 2007; **132**(Suppl 1): S26–45.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
8. Mogil JS, Chanda ML: **The case for the inclusion of female subjects in basic science studies of pain.** *Pain.* 2005; **117**(1–2): 1–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Zimmermann M: **Ethical guidelines for investigations of experimental pain in conscious animals.** *Pain.* 1983; **16**(2): 109–10.
[PubMed Abstract](#) | [Publisher Full Text](#)
10. Mezzanotte L, Aswendt M, Tennstaedt A, *et al.*: **Evaluating reporter genes of different luciferases for optimized *in vivo* bioluminescence imaging of transplanted neural stem cells in the brain.** *Contrast Media Mol Imaging.* 2013; **8**(6): 505–13.
[PubMed Abstract](#) | [Publisher Full Text](#)
11. Medhurst SJ, Walker K, Bowes M, *et al.*: **A rat model of bone cancer pain.** *Pain.* 2002; **96**(1–2): 129–40.
[PubMed Abstract](#) | [Publisher Full Text](#)
12. Falk S, Schwab SD, Frösig-Jørgensen M, *et al.*: **P2X7 receptor-mediated analgesia in cancer-induced bone pain.** *Neuroscience.* 2015; **291**: 93–105.
[PubMed Abstract](#) | [Publisher Full Text](#)
13. Dixon WJ: **Efficient analysis of experimental observations.** *Annu Rev Pharmacol Toxicol.* 1980; **20**: 441–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
14. Falk S, Ipsen DH, Appel CK, *et al.*: **Randall Selitto pressure algometry for assessment of bone-related pain in rats.** *Eur J Pain.* 2015; **19**(3): 305–12.
[PubMed Abstract](#) | [Publisher Full Text](#)
15. Kim JB, Urban K, Cochran E, *et al.*: **Non-invasive detection of a small number of bioluminescent cancer cells *in vivo*.** *PLoS One.* 2010; **5**(2): e9364.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
16. Jenkins DE, Oei Y, Hornig YS, *et al.*: **Bioluminescent imaging (BLI) to improve and refine traditional murine models of tumor growth and metastasis.** *Clin Exp Metastasis.* 2003; **20**(8): 733–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
17. Schwei MJ, Honore P, Rogers SD, *et al.*: **Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain.** *J Neurosci.* 1999; **19**(24): 10886–10897.
[PubMed Abstract](#)
18. Thorpe MP, Valentine RJ, Moulton CJ, *et al.*: **Breast tumors induced by N-methyl-N-nitrosourea are damaging to bone strength, structure, and mineralization in the absence of metastasis in rats.** *J Bone Miner Res.* 2011; **26**(4): 769–776.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Medhurst SJ, Walker K, Bowes M, *et al.*: **A rat model of bone cancer pain.** *Pain.* 2002; **96**(1–2): 129–140.
[PubMed Abstract](#) | [Publisher Full Text](#)
20. Doré-Savard L, Otis V, Belleville K, *et al.*: **Behavioral, medical imaging and histopathological features of a new rat model of bone cancer pain.** *PLoS One.* 2010; **5**(10): e13774.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Doré-Savard L, Beaudet N, Tremblay L, *et al.*: **A micro-imaging study linking bone cancer pain with tumor growth and bone resorption in a rat model.** *Clin Exp Metastasis.* 2013; **30**(2): 225–236.
[PubMed Abstract](#) | [Publisher Full Text](#)
22. Chrousos GP: **Stress and sex versus immunity and inflammation.** *Sci Signal.* 2010; **3**(143): pe36.
[PubMed Abstract](#) | [Publisher Full Text](#)
23. Furman D, Hejblum BP, Simon N, *et al.*: **Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination.** *Proc Natl Acad Sci U S A.* 2014; **111**(2): 869–74.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
24. Haldosén LA, Zhao C, Dahlman-Wright K: **Estrogen receptor beta in breast cancer.** *Mol Cell Endocrinol.* 2014; **382**(1): 665–672.
[PubMed Abstract](#) | [Publisher Full Text](#)
25. Sorge RE, Mapplebeck JC, Rosen S, *et al.*: **Different immune cells mediate mechanical pain hypersensitivity in male and female mice.** *Nat Neurosci.* 2015.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Fukusato T, Aoyama H, Mori W: **Age and sex differences in bone metastasis of hepatocellular carcinoma in Japanese autopsy cases.** *Gastroenterol Jpn.* 1989; **24**(2): 127–34.
[PubMed Abstract](#)
27. Sakaguchi S, Goto H, Hanibuchi M, *et al.*: **Gender difference in bone metastasis of human small cell lung cancer, SBC-5 cells in natural killer-cell depleted severe combined immunodeficient mice.** *Clin Exp Metastasis.* 2010; **27**(5): 351–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
28. Wright LE, Guise TA: **The microenvironment matters: estrogen deficiency fuels cancer bone metastases.** *Clin Cancer Res.* 2014; **20**(11): 2817–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
29. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 1 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)
30. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 2 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)
31. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 3 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)
32. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 4 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)
33. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 5 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)
34. Falk S, Al-Dihaissy T, Mezzanotte L, *et al.*: **Dataset 6 in: Sex-difference affects disease progression in the MRMT-1 model of cancer-induced bone pain.** *F1000Research.* 2015.
[Data Source](#)

Open Peer Review

Current Peer Review Status:

Version 3

Reviewer Report 22 December 2015

<https://doi.org/10.5256/f1000research.7974.r11695>

© 2015 Jiménez-Andrade J. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Juan M Jiménez-Andrade

Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas (UAT), Reynosa, Mexico

The authors have properly addressed all my previous concerns.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 30 November 2015

<https://doi.org/10.5256/f1000research.7974.r11231>

© 2015 Komarova S. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Svetlana Komarova

Faculty of Dentistry, McGill University, Montreal, QC, Canada

The revisions fully addressed my previous concerns.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 02 October 2015

<https://doi.org/10.5256/f1000research.7621.r10387>

© 2015 Komarova S. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Svetlana Komarova

Faculty of Dentistry, McGill University, Montreal, QC, Canada

I read the paper with interest and, although I do not always agree with the authors' interpretation of the data, I found the study thought-provoking and analysis thorough and complex.

1. In my view, the main result of the study is that even though initial analysis demonstrates some difference between male and female animals inoculated with breast carcinoma cells, after the animals that spontaneously recovered from the disease were excluded, this difference between sexes was no longer evident. To me, this says that the process of cancer establishment in the bone is very similar in animals of different sex. This is a very interesting conclusion, however, to strengthen it I suggest to run the statistical analysis to directly compare male and female animals for figure 2 and 7, since it is possible that even though male and female animals behave (somewhat) differently compared to their respective baselines, there could be no significant difference between the sexes on Fig 2, which would dilute the impact of the conclusion. If that is the case, then the main focus of the study would become the difference in the percentage of animals spontaneously recovered from cancer inoculation.
2. I think the critical information missing from the description is the status of MRMT-1 cells with regard to estrogen and progesterone receptors. I have never worked with this cell line and I couldn't find from the quick search if it is ER, PR positive or negative. I believe this information is critical both for interpretation of the difference in the cancer clearance rate between male and female animals, and the lack of difference in lesion progression between male and female animals.
3. I agree with Dr. Jiménez-Andrade that the sensitivity of the analysis of bone lesions based on grayness of X-rays is limited and that bone histomorphometry will significantly strengthen the conclusions.
4. I was confused with the last paragraph in Discussion on potential contributors to the difference in pain measures in male and female animals, when the conclusion of fig 7 is that there is no such difference.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 09 Nov 2015

Sarah Falk, University of Copenhagen, Copenhagen, Denmark

We thank Dr. Komarova for the helpful comments, and acknowledge that the study is somewhat untraditional in the sense that the main focus is on the increased variation observed in the females, which can potentially bias the model, rather than on a direct comparison on differences in readouts between males and females. Based on the reviewers useful criticism the following has been address and clarified in the discussion:

1. The reviewer is completely right; although the lack of difference between progression in female and male rats are on its own highly important for working with the model, the main result is not a direct comparison between sexes, but rather the differences in percentages of animals spontaneously recovering, and hence the indirect effect on variation in females and males. A statistical analysis comparing females to males was performed, and is mentioned in result section describing figure 2. The same analysis was performed for the data in figure 7, but was mistakenly not directly mentioned in the result section, this has been added. The main point is, as pointed out by the reviewer, the difference in ratio of spontaneous recovery between the sexes, which needs to be considered when working with the model in females, as the increased recovery rate can potentially mask the progression in females. Elaboration on this has been added to the discussion.
2. The MRMT1 cells line has been reported to be estrogen positive¹. This seems counterintuitive as a negative status for the MRMT1 cells could possible explain the lack of difference in progression between the sexes, but here other factors seems more important. In addition, as estrogen is known to increase the growth of ER-positive tumors, the ER-positive status does not explain the increased recovery in females. If anything the ER-positive status of MRMT-1 cells should in theory have an opposite effect, hence decrease the likelihood of recovery in females. A section on this has been added to the discussion.
3. It is true that X-ray analysis for quantification of bone lesions provides much less detailed information as compared to histomorphometry. However X-ray analysis for quantification of bone lesions is widely used in the literature, and has been extensively demonstrated to associate with degree of tumor burden quantified by histology since the first model was described by Schwei in 1999²⁻⁶. Also, in this study the conclusion that the rats recover from the cancer is not solely based on the x-ray data but also on bioluminescence and pain-related behavior. A further discussion of this has been added to the discussion.
4. The paragraph regarding the recent study by Sorge et al is included to make attention to the fact that although the outcome of a condition may be the same, the underlying mechanisms can be different. Sorge et al demonstrate that although male and females displays the same degree of mechanical allodynia following nerve injury, the underlying mechanisms are different. We suggest that a similar phenomenon

could be responsible for the data reported in this study. Despite a similar outcome in terms of pain behavior the underlying mechanisms driving the pain might be mediated by different systems, which are likely linked to the observed sex-differences in recovery rates. This has now been clarified in the discussion.

References

1. Doré-Savard L, Otis V, Belleville K, Lemire M, et al.: Behavioral, medical imaging and histopathological features of a new rat model of bone cancer pain. *PLoS One*. 2010; **5** (10). [PubMed Abstract](#) | [Free Full Text](#) | [Publisher Full Text](#)
2. Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, et al.: Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci*. 1999; **19** (24): 10886-10897 [PubMed Abstract](#)
3. Luger NM, Mach DB, Sevcik MA, Mantyh PW: Bone cancer pain: from model to mechanism to therapy. *J Pain Symptom Manage*. 2005; **29** (5 Suppl): S32-S46 [PubMed Abstract](#) | [Publisher Full Text](#)
4. Hu Z, Gerseny H, Zhang Z, Chen YJ, et al.: Oncolytic adenovirus expressing soluble TGF β receptor II-Fc-mediated inhibition of established bonemetastases: a safe and effective systemic therapeutic approach for breast cancer. *Mol Ther*. 2011; **19** (9): 1609-1618 [PubMed Abstract](#) | [Free Full Text](#) | [Publisher Full Text](#)
5. Feng S, Zhu G, McConnell M, Deng L, et al.: Silencing of atp6v1c1 prevents breast cancer growth and bone metastasis. *Int J Biol Sci*. 2013; **9** (8). [PubMed Abstract](#) | [Free Full Text](#) | [Publisher Full Text](#)
6. Juárez P, Mohammad KS, Yin JJ, Fournier PG, et al. *Cancer Res*. 2012; **72** (23): 6247-6256 [PubMed Abstract](#) | [Free Full Text](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

Reviewer Response 27 Nov 2015

Komarova Svetlana, McGill University, Montreal, Canada

Thank you for your response. I believe that new discussion provides a significant and novel focus for the study.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 20 August 2015

<https://doi.org/10.5256/f1000research.7341.r10035>

© 2015 Jiménez-Andrade J. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Juan M Jiménez-Andrade

Unidad Académica Multidisciplinaria Reynosa-Aztlán, Universidad Autónoma de Tamaulipas (UAT), Reynosa, Mexico

This is very interesting and well presented study; however the data does not support the title and conclusions at all of the present study. For this reviewer it is unclear what is the functional implication of females having an increased capacity for recovering from cancer state, given that there are non-significant differences in terms of pain behaviors and bone degradation between females and male rats. I think the authors are overstating the results and the results are not properly interpreted.

Additionally, the authors have not considered that the loss of bioluminescence signal over time may due to gender-related differences in terms of bioavailability and excretion of D-Luciferin. In fact, the authors are strongly recommended to present the *in vivo* quantification of bioluminescence signal after i.p. injection (as presented in figure 1B) in both females and males.

Finally, a histopathological analysis of the bone is strongly needed in order to suggest that females rats are recovering faster than male rats.

EDIT 21/08/2015: This review was mistakenly published with a 'Not Approved' status, this has now been amended to an 'Approved with Reservations'. The text has not been changed in any way.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Sep 2015

Sarah Falk, University of Copenhagen, Copenhagen, Denmark

Dear Professor Jiménez-Andrade

Thank you for your very constructive review. We have taken your comments into carefully consideration, and made the following changes:

We have changed the title to "Effect of sex in the MRMT-1 model of cancer-induced bone pain", as we realize that the original title could be misleading.

Regarding the functional implication of the study, we have extended the discussion to clarify that the main result of the study is the increased capacity for recovery from the

tumor-induced disease in the female rats compared to the male rats. This can potentially affect the interpretation of data produced with the model.

The last line in the abstract is in addition changed to “interpretation of data”, to emphasize that the increased capacity for recovery do not affect the overall progression in cancer-bearing animals, but that the recovery in some animals can mask the actual effect of the cancer, as the data from the recovered animals will shift the mean towards baseline.

Sex-related differences in terms of bioavailability and excretion of D-Luciferin should not affect the result in the study. The bioluminescence emission is based on acute injection of D-Luciferin at a level that saturates the system. Bioluminescence signal is measure 10min past injection on each measure day, meaning that if there were sex-differences in bioavailability and excretion is should be seen on all measuring day, and not only in a subset of animals from day 17 and beyond. Also as can be seen in supplement figure S2 the loss of signal is seen as an all-or-non response; either there is a signal or no signal at all. If the loss of bioluminescence signal were caused by changes in bioavailability and excretion in a subset of animals a more gradient decrease in signal would be expected and not a complete loss of signal as seen in these animals.

Additional histopathological analysis could be performed, however since loss of bioluminescence signal is accompanied by both lack of pain behavior and osteolysis it is unlikely that the animals should have living cancer cells in tibia. To emphasis this point, we have added a section in the discussion.

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research