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Uma D. Alappan March 30, 2022

Effect of Dietary Phosphorus on Incidence of Lung Cancer Metastasis to Bone

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Lung cancer is one of the most commonly diagnosed cancers in the US. In lung cancer patients, incidence of bone metastasis is ~40%. Malignant tumor cells can successfully colonize, grow, and flourish in secondary sites like bone by hijacking bone remodeling processes to create a feedforward loop (“vicious cycle”), promoting tumor growth. Phosphorus (Pi) – a vital nutrient for homeostatic processes and cell growth – can alter energy metabolism gene expression and create low-grade inflammation in bone microenvironments to support cancer proliferation/outgrowth.

Our study aims to further define the role of Pi in lung cancer progression & bone metastasis and identify robust gene marker(s) which can be used to identify occurrence of lung tumor bone metastasis. We hypothesize that 1) a high Pi-diet will increase lung cancer metastasis to bone, and 2) cells that metastasize to bone can be detected in bone marrow by lung “signature” genes using qRT-PCR to measure gene expression. Using a novel *KLL*$_{\text{Lenti}}$ model, 12–15-week-old mice were enrolled in a high or low Pi diet (HPD, LPD) 2-weeks post infection with tumor-inducing lentiviral-Cre (1x10ˆ6 i.u). Mice were fed diets similar in protein/Kcals/fat. At 30-weeks, mice were sacrificed, and RNA & qRT-PCR, lung histology (H&E stains), bone metastasis analysis (bone X-rays) were performed.

The results demonstrated that HPD significantly increased bone metastasis. HPD significantly increased tumor growth in females. Additionally, novel gene markers of

lung cells were identified in bone marrow with high specificity to detect bone metastasis. TTF1 and PTHrP expression in lung tissue correlated with both Pi-diet and tumor growth. Our identified genes could be used as powerful lung cell markers to perform further analyses (ex – single-cell analyses) to determine additional genes/signaling associated with lung cancer metastasis to bone. Other labs may benefit from using our novel KLL_{lenti} transgenic model to perform additional studies assessing direct relationships between lung adenocarcinoma and metastasis.

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Introduction:

Cancer is one of the leading causes of death with a mortality rate of 158.3 per 100,000 men and women per year **[1]**. Individuals diagnosed with metastasis, a secondary stage of cancer where malignant cells leave the primary tumor site and spread to other locations and vital organs, have few treatment options, which are rarely curative. Bone metastasis—a common site for metastasis in advanced cancer patients—remains one of the most challenging diagnoses with a particularly poor prognosis. Once bone metastasis has occurred, current treatment options are limited to repurposed osteoporosis drugs, such as bisphosphonates and Denosumab, an anti-RANKL antibody that inhibits bone reabsorption. These drugs reduce osteolysis (bone degradation) stimulated by tumor cells and therefore can reduce fracture risk and improve patient quality of life. However, this treatment is non-curative with median survival after diagnosis of bone metastases ranging from ~1 year for lung cancer patients to 3-5 years for those with breast cancer, prostate cancer, or multiple myeloma **[2,3]**. Surprisingly, metastasis is an inefficient process with most disseminated tumor cells failing to establish metastases **[3,4]**. Yet, given its impact on survival, it is imperative to understand how malignant tumor cells successfully colonize, grow, and flourish in secondary sites. A growing body of evidence is highlighting the important role of the bone microenvironment in promoting tumor growth. By understanding the changes in bone remodeling during tumor progression, we can develop new therapeutic strategies to specifically diminish the fertility of this environment and prevent bone metastasis and improve patient outcomes.

Bone constantly undergoes remodeling whereby osteoclasts (bone resorbing cells) remove old bone which is subsequently replaced by osteoblasts (bone forming cells). Growth factors and minerals released during this process have been shown to contribute to the fertile “soil” needed for colonization and growth of cancer cells **[5]**. Further, tumor cells hijack this process to create a feedforward loop, known as the “vicious cycle”, promoting growth factor and mineral release and resulting in tumor growth. Thus, the state of the bone microenvironment can modulate bone metastases and represents an important therapeutic target.

Phosphorus (Pi) is needed to maintain homeostatic, cellular, and metabolic processes in the body – including creation of ATP, phospholipids, nucleic acids; and regulation of bone metabolism & kidney absorption/reabsorption processes (**Fig. 1**). Thus, Pi is necessary for rapid cell growth, as seen in tumors. Further, *in vivo* studies have shown that tumors have increased Pi uptake, Pi metabolism, and Pi retention compared to normal tissue controls– including lung cancer (~2-fold increase) **[6,7,8].** Data also demonstrates that elevated Pi fosters rapid cell growth, transformation, and cancer progression in mouse models (reviewed in **[9]**). Transformation occurs when a healthy cell adopts traits of a malignant tumor cell – i.e., unrestricted growth, altered energy metabolism, etc. High Pi availability can alter energy metabolism by increasing oxidative

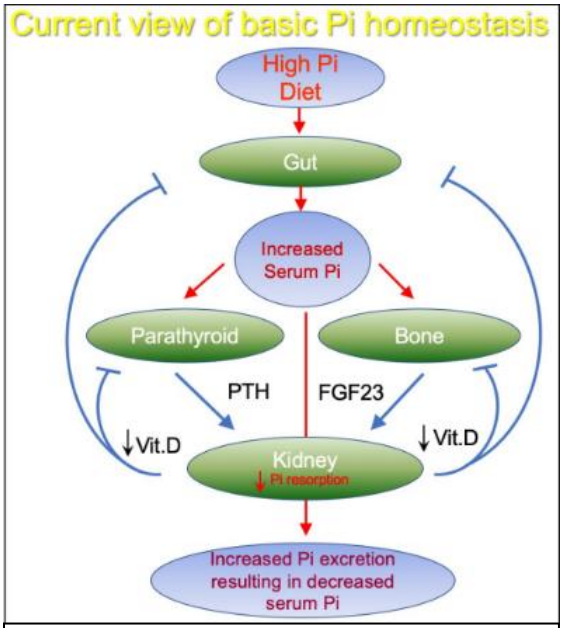

Fig. 1: Pi Homeostasis

phosphorylation, glycolysis, ATP production **[10,11,12,13]** and alter gene expression (ex. – osteopontin (OPN) **[14]**. Bone is the main site of Pi storage in the body leading to the idea that increased Pi availability in areas like bone, can alter cellular metabolism and cell growth which in turn promotes tumor growth.

Additionally, a high phosphate diet has been shown to increase bone turnover/remodeling thereby increasing the release of bioactive peptides, mineral salts, and growth factors **[15]**. These factors contribute to the “vicious cycle” observed in bone metastasis. Thus, increased bone remodeling by increased Pi uptake could create a favorable microenvironment for tumors, in effect “priming” bones to be more susceptible to tumor colonization and outgrowth. Pi has also been shown to increase the expression of genes linked to metastasis, such as osteopontin (OPN). Altogether, this indicates that Pi regulation has the potential to both alter tumor cell behavior and impact the tumor microenvironment at secondary sites, such as bone **[16]**.

Lifestyle factors like diet (which includes Pi consumption) have shown to have about 10-70% of an effect on cancer prevalence; thus, nutritional intervention does hold importance regarding cancer prevention, post-disease mitigation, and/or recovery **[17]**. It is therefore logical to consider treating cancer and bone metastasis by lowering Pi.

In alignment with increasing public transparency about Pi-related pathologies, the Beck lab aimed to explore further the role of Pi in mediating cancer metastasis to bone, specifically lung cancer. Lung cancer consistently ranks among the top – if not the first – most commonly diagnosed cancer in the US **[20,21].** In lung cancer patients, incidence of bone metastasis is ~40% **[18,19]**. According to the 2015 World Health Organization (WHO) classification of lung cancer, non-small cell lung cancer (NSCLC) includes

adenocarcinoma – the most common NSCLC comprising one-half of all lung cancer diagnoses – squamous cell carcinoma, and large cell carcinoma **[22]**. Although other epithelial-based cancers have specific genetic markers (ex. – BRCA-1, BRCA-2 for breast cancer), no current literature defines such a signature gene for lung cancer metastasis in bone **[23]**.

Aims/Goals of Project*:* Thus, ***our study goals were to not only further define the effect of Pi on lung cancer progression and metastasis to bone, but also define a robust gene marker(s) which can be used to identify the occurrence of lung tumor metastasis to bone (a genetic marker for the presence of lung cancer cells in the bone marrow).***

Hypotheses Tested: The overall hypothesis is that a diet high in Pi will increase bone metabolism and the subsequent release of factors in the bone marrow microenvironment and in the context of metastasizing cancer will increase the likelihood of establishment in the skeleton-bone metastasis. Specifically, we hypothesize that 1) a high phosphate diet will increase metastasis to bone relative to a low phosphate diet and 2) the cells that metastasize to bone can be detected in the bone marrow by lung "signature" genes using a sensitive technique to measure gene expression, qRT-PCR. We further predict that mice with bone metastases will have altered gene expression in the bone marrow including inflammatory, chemotactic, angiogenesis-related, etc. factors [ex. – RANK-L, OPN, TNFa, VegFa, IL-6] which would indicate occurrence of the "vicious cycle" of bone degradation & tumor growth. Whether the high phosphate diet will alter primary tumor growth is unknown but will be measured. If these results are

shown, it would establish a novel approach for disease prevention and possibly identify a novel therapeutic strategy in modulating phosphate consumption.

One of the obstacles to better understanding both the underlying mechanisms of bone metastasis as well as the influence of lifestyle factors such as nutrition on the process is that no mouse model that generates spontaneous metastasis to bone exists currently. Through a collaboration between the Beck lab and the labs of Adam Marcus and Melissa Gilbert-Ross, (Emory Winship Hematology & Oncology), a transgenic mouse model was identified that might generate spontaneous lung metastasis to bone. The genetically engineered mouse model (GEMM) is created by administering lentiviral-Cre which inactivates the *LKB1 (STK11)* tumor suppressor gene and activates the *KRAS* oncogene, promoting spontaneous generation of lung cancer – specifically adenocarcinoma. In adenocarcinomas, *LKB1* remains the second most frequently mutated tumor suppressor gene and is inactivated in ~30% of KRAS NSCLCs **[24,25,26,27,28].** KRAS (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) oncogene belongs to the Ras-family of GTPase proteins and regulates cell division. Mutation of Gly to Asp at amino acid 12 results in KRAS remaining “on” – causing cell transformation and increased resistance to EGFR-targeted chemotherapy and biological therapies **[29].** Assessment of the bones (tibias) of these mice by x-ray, approximately

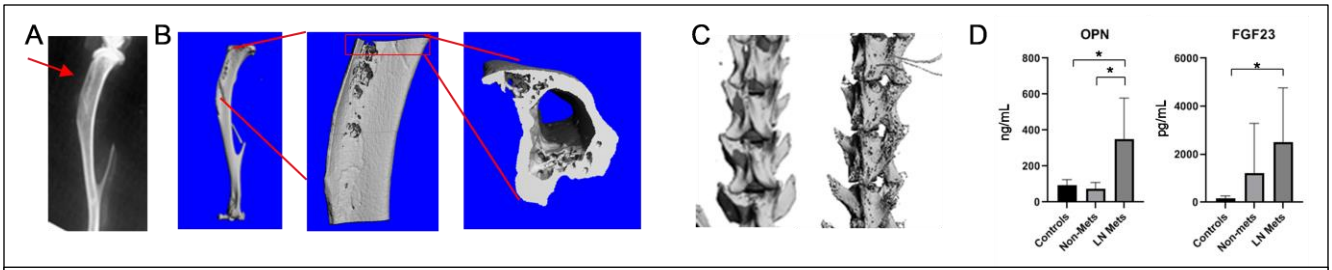

Fig. 2: Bone metastases in *Kras*^{G12D}*Lkb1*$^{fl/fl}$ mice after ~20 wks after Cre administration were sacrificed and bone assessed by (**A**) x-ray and (**B**) MicroComputed Tomography (µCT). (**C**) Spines (control and Met) were also assessed by µCT. (**D**) OPN and FGF23 plasma levels were measured by ELISA from control mice (n=6) and *Kras*^{G12D}*Lkb1*$^{-/-}$ without Mets (Non-Mets) (n=7) and with Lymph Node Mets (LN Mets) (n=6) at sacrifice. Mean ± StDev. *P<0.05 by one way ANOVA.

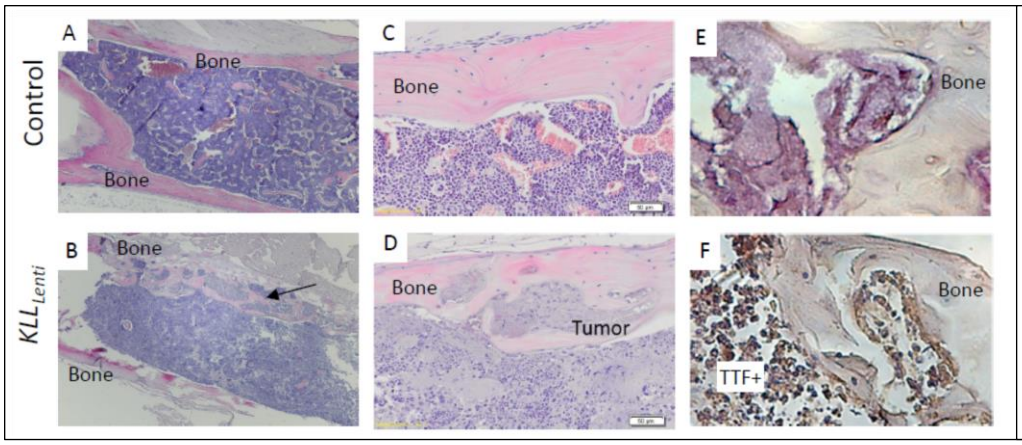

Fig.3: Histological sections of tibia (cortical) with H&E stain. (**A,B**) 4x, (**C,D**) 40x. Bone (pink), Bone erosion (arrow), and Tumor cells are indicted. (**E,F**) IHC using TTF-1 antibody, a marker of lung cells. Bar=50µm

20wks after lung cancer initiation, suggested the presence of bone metastases (**Fig.2A**). Detailed imaging by micro-computed tomography identified bone erosions consistent with bone metastasis (**Fig.2B**) including spine (**Fig.2C**). Phosphate responsive circulating factors including osteopontin (OPN) and Fibroblast growth factor 23 (FGF23) were measured by ELISA and found to be increased in mice that had distant metastases (lymph nodes) relative to mice without metastases **(Fig.2D**). The presence of lung tumor cells in bone was confirmed by histological analysis (**Fig.3A-D**), including immunohistochemical identification of the lung specific transcription factor TTF (**Fig.3E,F**). Because this data further suggests a potential role of Pi in lung cancer metastasis, our lab created a new study which subjected *KLL$_{lenti}$* mice to either a High Pi diet (HPD) or Low Pi diet (LPD). This model allows the Beck lab to assess differences between gene expression and correlate those differences with lung tumor burden/growth and the presence bone metastases. RT-PCR assessment of chemotactic, inflammatory, Pi-related, angiogenesis-related, etc. factors, ELISA assays, and lung & bone histology will allow us to determine ***1) if Pi plays a role in mediating these processes and 2) if the "vicious cycle" of bone damage and tumor growth is occurring.***

Experimental Approaches*:*

Transgenic Mouse Model: *KLL*$_{\text{Lenti}}$ mice were obtained from the Cancer Animal Models core at Emory University. All mice were housed and treated according to Institutional Animal Care and Use Committee protocol #202000126 by the Emory University Division of Animal Resources. Lung tumors were induced by intratracheal administration of lentiviral-Cre (1×10ˆ6 i.u.) to 12-15 weeks old mice. Mice were enrolled into either the high phosphate diet (HPD) or low phosphate diet (LPD) 2 weeks post infection. Mice were imaged starting at 6 weeks post infection at least every other week on an IVIS Spectrum (PerkinElmer). LivingImage software was used to compare longitudinally acquired calibrated signals. All mice infected with lentiviral Cre were monitored to a 30-week post infection endpoint until tumorigenic symptoms (i.e., weight loss greater than 10%, respiratory distress, inactivity) presented at which point the mice were euthanized according to IACUC guidelines **[30].**

Diets: Both male and female mice were randomly assigned to varying phosphate diets (fed ad libitum) consisting of low-phosphorus diet (LPD; 0.2% phosphorus [TD.110360]), or high-phosphorus diet (HPD; 1.8% phosphorus [TD.110362]) for the duration of the study starting 2 weeks post infection. Study diets were designed and manufactured by Envigo (Indianapolis, IN) to be isocaloric (3.7-3.9 kcal/g) and keep calcium (0.6%), vitamin D (2.2 IU), and other vitamin and minerals constant.

RNA Isolation (For Lung Tissue and Bone Marrow) & qRT-PCR: The left lobe of the lung was placed in Trizol (Austin TX) and homogenized. Bone marrow was harvested from hips and left femur and tibia by flushing with centrifugation and placed in Trizol. After homogenization, RNA was isolated using the manufacturers protocol (Trizol) and

the resulting pellet was diluted with RNA-se free H_2O. The concentration and A260/A280 protein ratio were estimated using NanoDrop instrument. cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City CA) as follows. A master mix was prepared with 10X RT Buffer, 25X dNTP Mix (100 mM), 10X RT Random Primers, MultiScribe™ Reverse Transcriptase, and Nuclease-free H_2O. qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems-StepOnePlus. Primers were designed using qPrimerDepot software as described previously **[51, 53]** (**Table 1).**

Table 1: Primer sequences for genes analyzed with qt-PCR

Gene	Forward	Reverse
SCGB1A1	5'- ATGAAGATCGCCATCACAATCAC-3'	5'- GGATGCCACATAACCAGACTCT-3'
SFTPC	5'- ATGGAGAGTCCACCGGATTAC-3'	5'- ACCACGATGAGAAGGCGTTTG-3'
SFTPB	5'-TGCTTCCTACCCTCTGCTG-3'	5'- CCAGGCTTTGGCACCAGAA-3'
TTF1	5'- ATGAAGCGCCAGGCTAAGG-3'	5'- GGTTTGCCGTCTTTGACTAGG-3'
PTHrP	5'- CATCAGCTACTGCATGACAAGG-3'	5'- GGTGGTTTTTGGTGTTGGGAG-3'
HPRT1	5'- GAGGAGTCCTGTTGATGTTGCCAG-3'	5'- GGCTGGCCTATAGGCTCATAGTGC-3'
SLC34A2	CCTTGGCCCGAGTTGGAAAAT	AGGTATGCTCGATTGTGGTCC
RANKL	GACTCCATGAAAACGCAGGT	GAAAGGCTTGTTTCATCCTCC
TNFa	GCCTCTTCTCATTCCTGCTT	CACTTGGTGGTTTGCTAGGA
Firefly	GTGGTGTGCAGCGAGAATAG	CGCTCGTTGTAGATGTCGTTAG
LUC.3	GCGCGGAGGAGTTGTGTT	TCTGATTTTTCTTGCGTCGAGTT
luciferase	CAACTGCATAAGGCTATGAAGAGA	ATTTGTATTCAGCCCATATCGTTT
DNA.Luc.	TGGGCTCACTGAGACTACATCA	CGCGCCCGGTTTATCATC
CXCL12	TGCATCAGTGACGGTAAACCA	TTCTTCAGCCGTGCAACAATC
CXCR4	GACTGGCATAGTCGGCAATG	AGAAGGGGAGTGTGATGACAAA
CXCR7	AGCCTGGCAACTACTCTGACA	GAAGCACGTTCTTGTTAGGCA
OPN.b	ATTTGCTTTTGCCTGTTTGG	TGGCTATAGGATCTGGGTGC
VegFα	GCTTCCTACAGCACAGCAGA	AATGCTTTCTCCGCTCTGAA
IL-6	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC

Following PCR, melt curves were observed for peak abnormalities, consistency between sample replicates was observed, and CT values were checked to make sure they were within range. Three technical replicates were made for each cDNA sample representing individual mice. Fold change was calculated using the $2^{(-\Delta\Delta Ct)}$ method **[51]**. Lung tissue histology was used to distinguish which samples supported tumor growth; samples without tumor growth were used as a control against tissues which did support

tumor growth for all genes except SCGB1A1, SFTPC, and TTF1. Non-tumor tissue from untreated mice were used as controls for SCGB1A1, SFTPC, and TTF1. HPRT1 was used as a normalization control gene.

Histology of lungs: The right lobe of the lung, was placed in formalin for 24h before being placed in 70% ethanol. Lungs were paraffin embedded, sectioned, and stained by Hematoxylin and Eosin (H&E) by the Emory Cancer Tissue and Pathology Core. Slides were scanned on an Olympus VS130 slide scanner at 40x, and tumor and lung volume calculated using VS-ASW application software by Olympus Soft Imaging Solutions.

Bone metastasis analysis: Right and left tibia and femur were excised and x-rayed on a Kubtec preclinical animal imaging scanner (Stratford, CT). Bone lesions appeared as shadows and were scored as “yes”, “maybe” or “no” based on two scorers. Although both tibia and femur were scored from both left and right limbs, since we only flushed marrow from right limbs, we decided to use right limbs for scoring. Scorers were blinded as to the diet and sex of the mice.

Statistical Analyses: 2-Sample T-Test for Means and Chi-Square for Independence/Association analyses were used to calculate significance at $p<0.05$ level.

Results:

*1) Study Design (***Fig. 4***)*:

15 male and 16 female KLL mice randomly selected at approximately 10wks of age were administered intratracheal Lenti-Cre to initiate tumorigenesis in the lungs. Two weeks later the mice were randomly divided and fed low (0.2% Pi and 0.6% Ca) or high Pi diets (1.8% Pi and 0.6% Ca) (8F-LPD, 8F-HPD and 8M-LPD, 7M-HPD). Mice were tracked every two weeks for tumor progression by Bioluminescent imaging. When the tumors progressed to interfere with normal functioning, mice were sacrificed and tissues including bone, bone marrow, and lungs were collected for further analysis. 28 of 31 mice (90.32%) generated lung tumors. Kaplan Meier's survival curves showed a LPD to increase probability of survival in females – however, this data was not significant (**Fig. 5**).

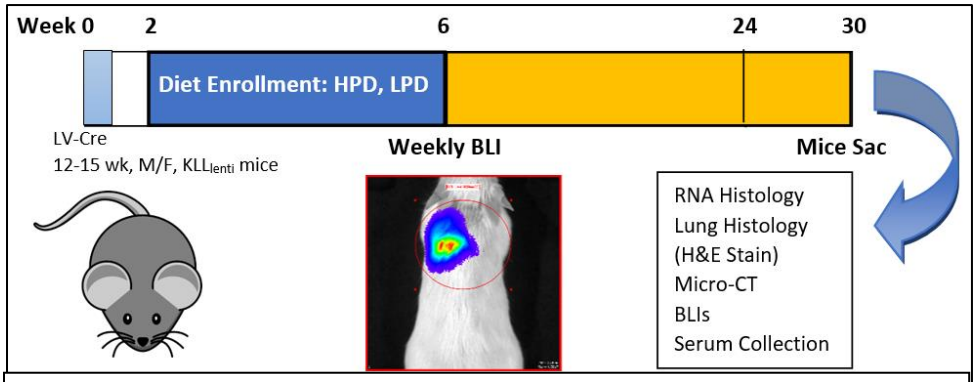

Fig. 4: Study Design. ~50% of mice were sacked at 24 weeks.

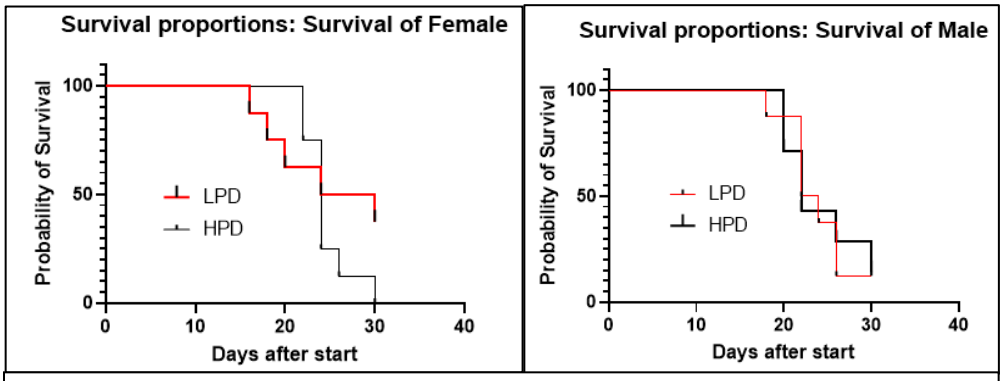

Fig 5: Kaplan Meier's Survival Curves for Female and Male Mice

2) *Dietary Pi consumption does not alter tumor burden but does influence tumor growth in female mice.*

Lungs were collected from sacrificed mice and fixed for histology and stained with hematoxylin and Eosin (H&E). Tumor burden was calculated by quantifying the tumor volume relative to lung tissue volume of H&E-stained histological sections using VS-ASW application software (**Fig 6A & 6B**). Tumor growth (average tumor volume) was calculated by dividing tumor burden by number of tumor initiation points in each sample. There were no significant differences in tumor burden between LPD vs. HPD overall (35.93 +/- 24.42 v 39.03 +/- 22.86, p=0.65), LPD-F vs. HPD-F (26.20 +/- 23.65 v 42.83 +/- 23.12, p=0.18), or LPD-M vs. HPD-M (45.66 +/- 22.42 v 34.69 +/- 23.55, p=0.37). Though there were no significant differences in tumor growth overall (6.13 +/- 9.46 v 7.73 +/- 6.98, p=0.82) or between LPD-M vs. HPD-M (9.56 +/- 12.53 v 3.95 +/- 2.07, p=0.26), there was a significant difference in tumor growth % between LPD-F vs. HPD-F (2.70 +/- 2.80 v 11.05 +/- 8.19, p=0.016) (**Fig 6C**). Thus, within females, a HPD may increase tumor growth.

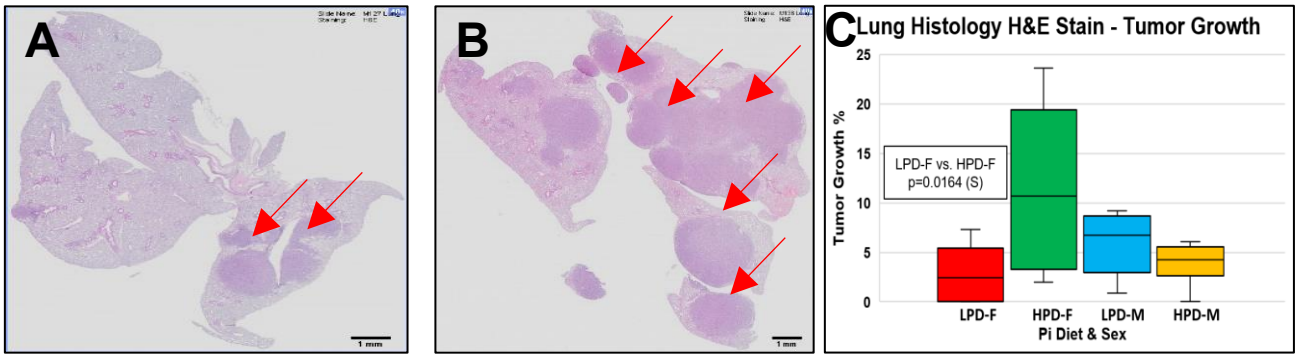

Fig. 6: Scoring of lung tumor volume and growth. (A) Histology of lung tissue harvested at the completion of the study. Slides stained with H&E. example of lung histology presenting fewer tumors (red arrows) and (B) higher tumor volume. (C) Box Plot of Tumor Growth % vs. Pi Diet & Sex, Lines & scalebars designate Q1, Q2, Q3 (Data Quartiles).

3) Genes/Pathways Potentially Associated with High-Pi Increase of Tumor Growth

Because **results in section 2** (**Fig. 6C**) showed evidence of Pi leading to increased tumor growth in females, we quantified gene expression in lung samples that could be related to this process.

Table 2

Genes	Diet-Sex			Bone Mets	Tumor Growth
Lung Tissue	LPD vs. HPD	LPD-F vs. HPD-F	LPD-M vs. HPD-M	Expression Level vs. Y/N	Expression vs. Low, Medium, High
PTHrP	NS	p=0.041 (S)	NS	NS	L v H: p=0.0095 (S) M v H: p=0.0023 (S)
Slc34a2	NS	NS	0.048 (S)	NS	NS
TTF1	NS	0.017 (S)	NS	NS	L v H: p=0.043 (S)
SFTPC	NS	NS	0.042 (S)	NS	L v M: p=0.033 (S) L v H: p=0.031 (S)

Table 2: Comparisons between Lung Tissue Gene Expression and Pi Diet, Bone Metastasis Incidence, and Tumor Growth Level; 2-Sample T-Test for Means were used ($p<0.05$).

PTHrP expression in lung tissue significantly differed between LPD v HPD females (8.63 +/- 11.25 v 16.42 +/- 9.41, p=0.0413) and between low and high tumor growth levels (Low v High: 3.71 +/- 5.00 v 19.31 +/- 12.71, p=0.0095) (**Table 2, Fig 7).**

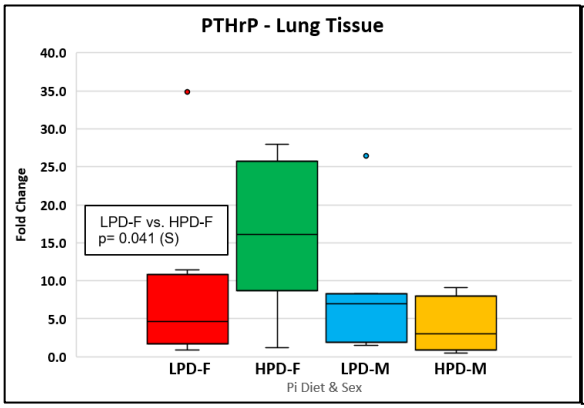

Fig 7. PTHrP expression in lung tissue. RNA was isolated from the lungs of mice at the completion of the study and qRT-PCR was used to quantify expression of genes as indicated. Fold change was calculated relative to three mice samples with 0% tumor burden using a HPRT1 loading control and the ΔΔCT method. 2-Sample T-Test for Means was used for analyses for significance ($p<0.05$). Points represent outliers.

TTF1 and SFTPC significantly differed between LPD v HPD females (p=0.017, 0.042) and between low and high tumor growth levels (p=0.043, p=0.031) (**Table 2)**. Slc34a2 correlated with Pi diet (p=0.048) but not metastasis or tumor growth (**Table 2**). In lung, CXCL12, CXCR4, CXCR7, OPN, TNFa, Vegfα, SCGB1A1 did not show significant associations with Pi diet, bone metastasis, or tumor growth. Though HPD did not correlate with tumor burden, we ran analyses to see if gene expression in lung tissue correlated with tumor burden in a non-Pi-mediated manner. Mean expression level for CXCR4, PTHrP, TNFa, and Slc34a2 were significantly different between low and medium tumor burden groups. CXCL12, CXCR7, OPN, VegFα, TTF1, SCGB1A1, SFTPC showed non-significant associations with tumor burden. Collectively the results identify PTHrP as strongly upregulated in lung tumor growth in female mice fed a high Pi diet and future studies will be needed to determine any functional significance of this response.

4) Dietary Pi consumption alters metastasis to bone.

Bone metastases were scored on x-ray images of both right and left tibia and femur by two independent and blinded reviewers. The presence of a bone metastasis (shadow on the bone) was scored as either 1) yes (both scorers agreed with a positive), 2) maybe (scorers did not agree) and no (both scorers did not detect). The results were then analyzed as if the Maybe was a Yes or the Maybe was a No (**Fig 8a**). Results suggested if “Maybe” was scored as “Yes,” HPD increased incidence of metastasis overall (LPD v HPD: 8 v 13, p=0.029) and within females (LPD v HPD: 2 v 7, p=0.012). There was no difference between LPD- and HPD-fed male mice, with almost all developing bone metastases. If genders were combined, 50% of LPD fed mice

developed bone metastases whereas 87% of HPD fed mice developed bone metastases (**Fig 8b**). If “Maybe” was factored as a “No,” no significant associations were found between metastases and sex or diet (**Fig 8c**). Additional confirmation of yes or no is underway in the lab using histology and micro-computed tomography (micro-CT).

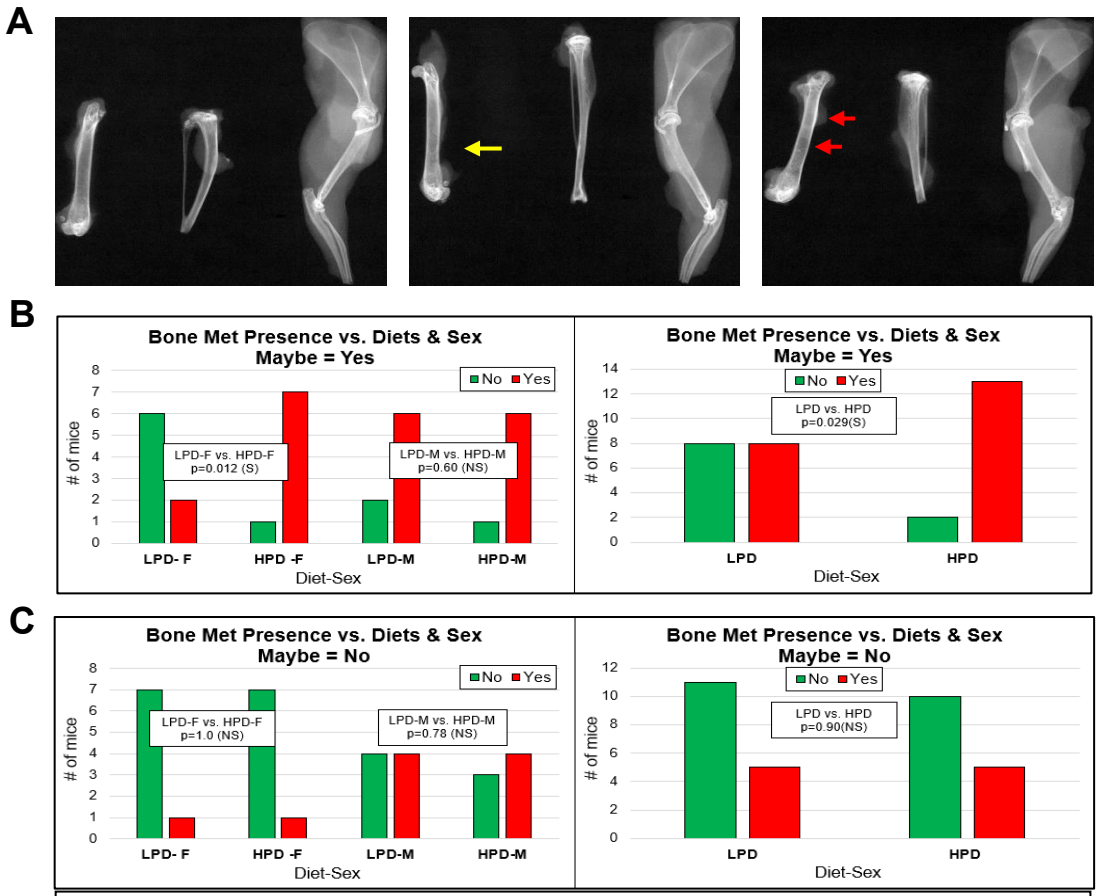

Fig 8: Incidence of Bone Mets by Diet & Sex. (A) Bone X-Ray Tibia and Femur. Arrows: No Arrow = No Met Present, Yellow = Bone Met Maybe Present, Red = Bone Met Present. (B) Quantification of bone metastases with “Maybe” scored as Yes. Separated by Sex (left panel) or total (right panel) (C). Quantification of bone metastases with “Maybe” scored as No. Separated by Sex (left panel) or total (right panel).

5) *Identification of Novel Lung-Specific Markers for Detection of Lung Cells in Bone Marrow*

To find a marker for lung cancer, we analyzed lung-specific genes, including TTF1 (thyroid transcription factor 1, NKX2-1), previously used but not well documented.

Because our KLL_{lenti} model induced mutations which led to expression of Luciferase in tumor-infected cells and our BLI imaging showed robust and reliable results for Luciferase expression (**Fig 4**), we surmised Luciferase should be robust in identifying metastases. We designed and analyzed several Luciferase genes, including Firefly, LUC.3, luciferase, DNA.Luc (**Table 1**); however, surprisingly, all primers failed to show consistent results. Although TTF1 is a lung-specific gene and Luciferase should be highly expressed in our model's tumor cells, qt-PCR analysis did not show high expression of TTF1 or any Luciferase genes in lung tissue or bone marrow (not shown). Thus, we decided to change strategy and test other lung-specific genes — i.e. not transcription factors — for example, genes coding for cell-surface protein receptors present in type II alveolar cells – which constitute 60% of alveolar epithelial cells, a common site for development of cancer **[31,32,48]**. We used previously published large scale "omic" studies aimed at identifying lung specific marker genes **[33,34]** to generate a list of target "lung specific" marker genes. The choice of genes was based on commonality between the datasets. The genes included SFTPB, SFTPC, SCGB1A1.

With the goal of using these to identify lung cells in the bone marrow we tested the specificity of these genes on RNA samples obtained from control lung and bone marrow samples (non-tumor generating mice). These non-tumor controls were obtained from 15-week-old female C57BL/6 mice. Samples were analyzed by qRT-PCR and abundance assessed CT (cycle threshold). Samples with relative high abundance are detected at 20-30 CTs and low abundance (<30 CTs). Samples less than 35 CTs are generally considered background and undetected considered 40 CTs (the max number of cycles). In our control samples, both SCGB1A1 and SFTPC were highly expressed in

100% of lung tissue samples (<19.61 and <23.83 raw CT, respectively), and either not detected or at essentially background CT levels expressed in all bone marrow samples (>35.46 and undetermined, respectively) (**Fig.9**). To determine background expression levels for SCGB1A1, we set a stringent cut off value of 35.7343 – equal to the mean raw

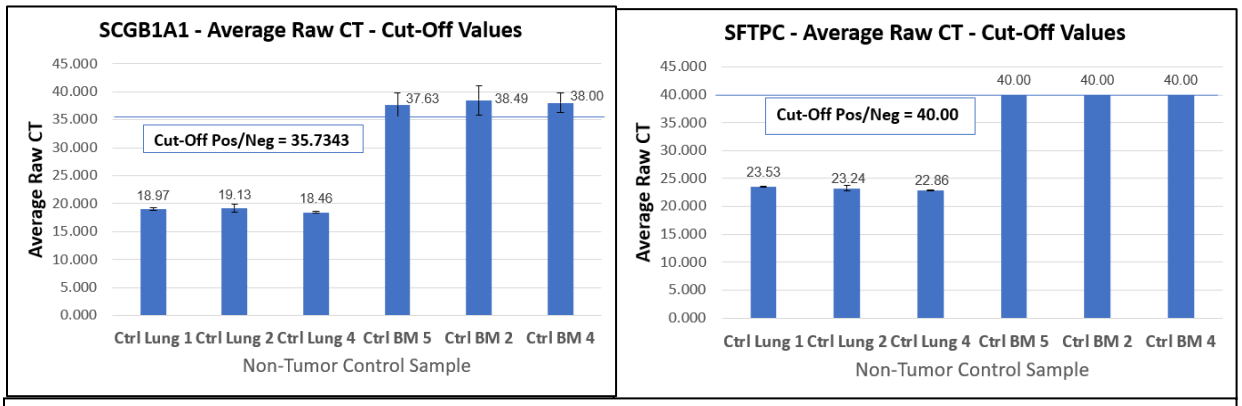

Fig 9. SCGB1A1 and SFTPC cut-off values for determination of whether gene is expressed (positive) or not (negative). qRT-PCR was used to quantify gene expression in control samples of lung tissue and bone marrow. Data are expressed as CT (cycle Threshold values for each sample. The higher the CT value the lower abundance of the gene. Values >35.7343 and >40.00 were considered negative for SCGB1A1 and SFTPC, respectively.

CT of all not-undetermined control samples minus their standard deviation values. Any raw CT value >35.7343 was considered undetermined/not truly expressed. Based on this analyses, SCGB1A1 and SFTPC genes will only be detected in bone marrow if lung cells are present, i.e. lung cancer has metastasized.

6) In vivo assessment of lung cell markers in bone metastasis mice:

Using our new lung marker genes, we analyzed lung and bone marrow RNA isolated from KLL mice at the completion of the analyses described above. All SCGB1A1 and SFTPC lung CT values were <24.34 CT and <23.12, respectively as expected from a specific lung expressed gene. Analysis of bone marrow samples identified a range of expression levels from undetected to reasonably well detected. We then compared this gene expression to the +/- bone metastases results obtained from x-ray (Fig.8). Out of

20 samples identifiable as “yes” or “no” for presence of bone metastases (“maybe” removed), expression of SCGB1A1 and SFTPC in bone marrow correctly predicted presence of bone metastasis in 16 of 20 samples (80%) and 15 of 20 (75%) overall; 9 of 9 samples (100%) and 8 of 9 samples (89%) in females; and 7 of 11 samples (64%) and 7 of 11 samples (64%) in males; respectively. SCGB1A1 and SFTPC only disagreed in prediction three (3) times out of 31 samples (9.68%). SCGB1A1 level in bone marrow was significantly associated with presence of bone metastasis (p=0.023) with higher expression indicating bone metastasis incidence (“yes”) (**Table 3**). Average SFTPC fold change in bone marrow was significantly higher in samples with bone metastasis than without (4.21 +/- 5.78 vs. 0.46 +/- 1.10, p=0.024) (**Table 3**).

Table 3:

Genes	**Diet-Sex**			**Bone Mets**
Bone Marrow	LPD vs. HPD	LPD-F vs. HPD-F	LPD-M vs. HPD-M	Expression Level vs. Y/N
IL-6	NS	NS	0.032 (S)	NS
TTF1	NS	NS	NS	p=0.031 (S)
SCGB1A1	NS	NS	NS	p=0.023 (S)
SFTPC	p=0.020 (S)	p=0.011 (S)	NS	p=0.024 (S)

Table 3: Comparisons between Bone Marrow Gene Expression and Pi Diet, Bone Metastasis Incidence; 2-Sample T-Test for Means were used ($p<0.05$).

The significant difference in SFTPC expression in bone marrow between mice with and without bone metastasis, LPD vs. HPD overall (0.53 +/- 1.44 v 4.39 +/- 5.87, p=0.020), and LPD vs. HPD within females (0.23 +/- 0.24 v 4.55 +/- 3.81, p=0.011), (**Table 3, Fig 10**), provides further support that SFTPC can be used as a novel robust lung marker in the bone marrow. The result is emphasized in the lack of association of

other genes tested: TNFa, RANK-L, IL-6, Vegfα, OPN, SFTPB, PTHrP, were tested in bone marrow but did not associate with both Pi and bone metastasis presence.

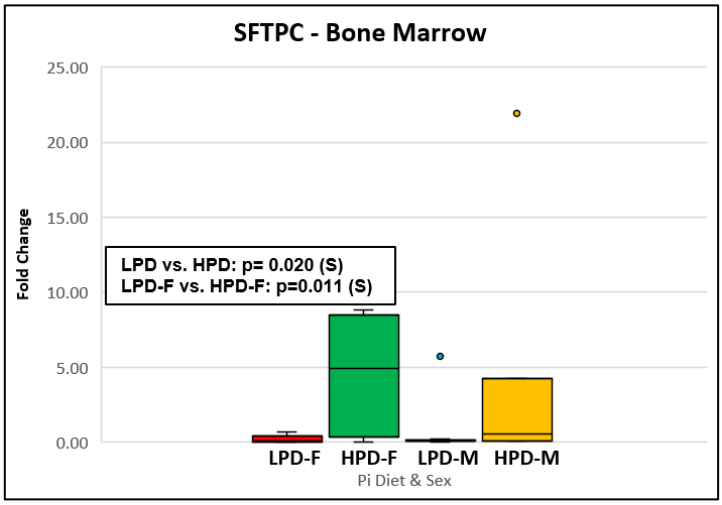

Fig 10. SFTPC expression in bone marrow. RNA isolated from femur and tibia at the completion of the study analyzed by qRT-PCR. Fold change was calculated relative to non-tumor control samples using the ΔΔCT methods. Error bars represent st. dev. 2-Sample T-Test for Means were used for statistical analyses ($p<0.05$). Points represent outliers.

7) Reassessment of bone metastases using novel Markers SCGB1A1 and SFTPC.

Given these results supporting SCGB1A1 and SFTPC as robust markers for lung tumor, we used bone marrow CT values for SCGB1A1 and SFTPC to decide whether "maybes" should be labelled yes (high expression) or no (low expression) from our initial analysis (Fig.8B,C). SCGB1A1 (35.7 CTs) and SFTPC (undetermined at 40CTs) remained the cut-off values for this analysis. Results showed that a HPD was associated with increased bone metastasis incidence overall (p=0.019) and within

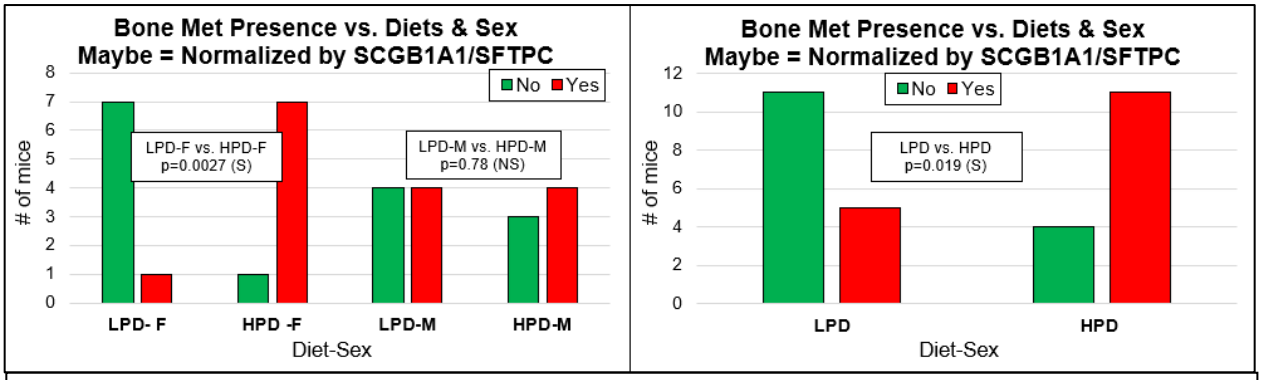

Fig 11. Analysis of bone metastasis incidence after implementing SCGB1A1 and SFTPC as lung cell markers to determine whether "maybe" should be classified as "yes" or "no."

females (p=0.0027) (**Fig. 11**). This data aligns with our findings from **Fig 8b** when "maybes" were classified as "yes." Given our data showing the high accuracy of both SCGB1A1 and SFTPC, we believe these findings will be further supported by studies underway to confirm these results using histology and microCT bone scanning. Thus, high Pi diet may increase incidence of lung cancer metastasis to bone– at least in females— if not in both males and females. This data also highlights the potential robust expression of SCGB1A1 and SFTPC as novel lung cell markers.

Discussion:

Our data demonstrated that a high Pi diet does not significantly affect overall survival or degree of tumor burden in a genetically engineered mouse model of lung cancer with associated metastases to bone. A high Pi diet did significantly increase incidence of tumor growth and bone metastasis, at least in females relative to a low Pi diet (**Fig 6c, 8b, 11**). This finding becomes important as it relates to the adverse health consequences of hyperphosphatemia and Pi overconsumption in US populations.

This study also identifies SFTPC and SCGB1A1 as novel markers of lung cells in bone marrow with high specificity in detection of bone metastasis via qt-PCR analysis. Additional analyses using micro-CT and histology can confirm our bone X-ray data and help further establish SFTPC and SCGB1A1 as lung tumor markers in the bone marrow. Since our **Fig. 11** data using SCGB1A1 and SFTPC to define "maybe" presence of bone metastasis showed that Pi diet significantly increased metastasis in females, if not males and females – this analysis not only highlights the novelty of these genes as lung cell markers in bone marrow but also the harmful impact of Pi in promoting bone metastasis. Elevated SFTPC in lung correlated with increased tumor

growth in males (**Table 2**), and elevated SFTPC in bone marrow correlated with both a HPD and increased bone metastasis in females, if not males and females (**Table 3, Fig. 10**). This data emphasizes how robust SFTPC is as a lung cell marker and shows that perhaps SFTPC expression and related Pi-mediated pathways could be key targets in the development of therapeutics to prevent lung tumor growth and bone metastasis.

Despite BLI imaging indicating robust and reliable expression of Luciferase correlated with tumor growth (**Fig 4**), none of our designed Luciferase primers consistently worked to identify tumor via qRT-PCR. Further analyses and refinement of techniques should be performed to troubleshoot Luciferase as identification of tumor cell via qRT-PCR of Luciferase would greatly expedite future experiments such as single-cell analyses.

An LPD did not seem to significantly affect survival of mice (**Fig. 5**). Increased tumor burden and growth do not seem to play a role in increasing bone metastasis incidence. It is important to note that since Pi did not alter tumor growth in males – only females – perhaps a non-Pi-related factor is responsible for this trend. There is less evidence supporting the role of Pi in increasing tumor growth compared to Pi increasing incidence of bone metastasis (**Fig 6c, Fig 8b, 11)**. Gender seemed to play a role in creating similar trends in **Fig 6c, 8b, 11** which could be due to differences in metabolism or hormonal signaling (ex. – estrogen-related pathways) between males and females **[50,52]**.

Numerous translational studies have been performed linking excess Pi consumption with reduced longevity in mammals **[35]**. Because commonly consumed sodas and processed foods often contain high Pi content—namely, "hidden preservatives" like sodium hexametaphosphate or inorganic Pi—Americans have historically consumed phosphorus well in excess of official Recommended Dietary Allowances (RDAs) which may lead to hyperphosphatemia **[36,37]**. Thus, it becomes important to find novel therapeutic approaches or prevention strategies to prevent hyperphosphatemia.

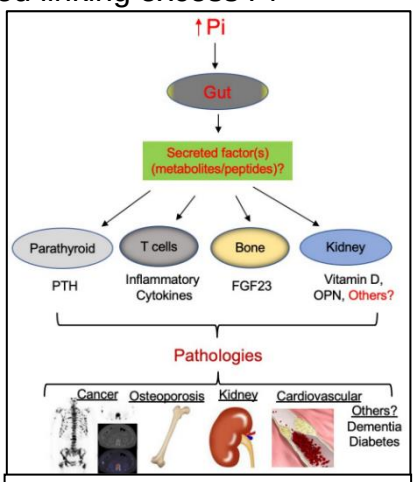

Fig 12: Pi-Related Pathologies

Our results suggest that reducing Pi consumption may be beneficial to cancer progression. In addition to reducing dietary intake, therapeutic strategies exist to reduce Pi absorption in the gut. The predominant clinical therapy to reduce serum Pi and related health complications of hyperphosphatemia is orally administered Pi-binders with meals, including the commonly used Lanthanum carbonate (LaC). While several *in-vivo* studies have claimed phosphate-binders work to reduce serum phosphorus, no study has provided quantitative evidence to support these claims **[38,39,40,41,42,43]**. Furthermore, many studies – including one of my own – show evidence that although LaC and commonly used binders do work to reduce serum Pi and some related health outcomes, they do not efficiently reduce all potentially life-threatening outcomes **[44]**. Although a new Pi-binder "Tenapanor" has been devised by Ardelyx company, more work must be done to test its efficacy in preventing potential long-term health complications associated with hyperphosphatemia **[49]**. Considering our lab's new

findings concerning the correlation between a HPD and increased incidence of tumor growth and bone metastases, we strongly urge researchers to focus attention on designing and testing novel therapeutics to prevent hyperphosphatemia and related negative health outcomes.

Furthermore, attention should be shifted to increasing public awareness of Pi and its potential health implications – particularly in disadvantaged and immunocompromised populations – due to its implications in progression of various pathologies (**Fig 12)**. In 2014, the American Society of Nephrology and National Kidney Foundation urged the FDA to mandate phosphorus (Pi) inclusion on their nutritional label; however, the 2016 final FDA regulation disregarded their pleas, stating “the label is not meant to be used...to treat chronic diseases”—i.e. limiting Pi intake in kidney function compromised, high-risk patients—but for consumers to “make more informed [dietary] choices” [**45**]. Though the FDA is aware Pi is a risk factor for immunocompromised patients, they fail to mandate that manufacturing companies include Pi content on their FDA nutrition label. In addition, by inhibiting full transparency on nutrition labels, the FDA could be perpetuating a systemic issue which amplifies racial disparity, jeopardizes public health, and disproportionately affects undereducated youth.

Because African Americans are three to four times more likely to develop Chronic Kidney Disease (CKD) and excess Pi is linked to CKD progression, they should avoid Pi [**46**]. Socially and economically disadvantaged youth often find themselves in “food deserts” with limited access to healthy options and therefore many may overconsume high Pi processed foods and beverages. In my 2018 FDA study, I found a

correlation between education level and beverage type in adolescent and children participants of the National Health and Nutrition Examination Survey (NHANES) [**47**]. In 2016, I estimated Pi and acidity content of commonly consumed sodas and conducted a soda consumption questionnaire in my hometown to assess consumption trends and current public knowledge of Pi. Further studies should be done to increase public transparency about Pi – particularly for disadvantaged and immunocompromised populations like cancer patients.

Future Directions: In the future, single-cell analyses can be expedited by "sorting" only for cells expressing SFTPC and SCGB1A1 at high levels. In these highly expressing cells, one could perform more experiments to determine additional genes and signaling pathways involved in lung cancer metastasis to bone – perhaps via Pi-mediated or non-Pi mediated regulation. These analyses could aid in creating novel drug therapies targeting specific pathways to prevent tumor growth and/or metastasis to bone. Furthermore, in clinical settings, SCGB1A1 and SFTPC could serve as powerful lung markers to perform ELISA assays on patients' extracted bone marrow to confirm presence of bone metastasis. This mouse model represents a novel platform to begin the developed therapeutic strategies block tumor metastasis to bone and/or eliminating bone established tumor colonies. Finally, other labs may benefit from the use of our novel KLL_{lenti} transgenic mouse model to perform additional studies assessing direct relationships between lung adenocarcinoma and metastasis.

Sources:

1. "Cancer Statistics." *National Cancer Institute*, https://www.cancer.gov/about-cancer/understanding/statistics.

2. Coleman, R. E. Clinical features of metastatic bone disease and risk of skeletal morbidity. Clin. Cancer Res. 12, 6243s–6249s (2006).
3. Coleman, R.E., Croucher, P.I., Padhani, A.R. *et al.* Bone metastases. *Nat Rev Dis Primers* **6,** 83 (2020). https://doi.org/10.1038/s41572-020-00216-3
4. Weilbaecher, K. N., Guise, T. A. & McCauley, L. K. Cancer to bone: a fatal attraction. Nat. Rev. Cancer 11, 411–425 (2011).
5. Boyce, Brendan F et al. “Osteoclasts have multiple roles in bone in addition to bone resorption.” *Critical reviews in eukaryotic gene expression* vol. 19,3 (2009): 171-80. doi:10.1615/critreveukargeneexpr.v19.i3.10
6. Elser, J. J.; Kyle, M. M.; Smith, M. S.; Nagy, J. D., Biological stoichiometry in human cancer. *PLoS One* **2007,** *2* (10), e1028.
7. Jones, H. B.; Chaikoff, I. L.; Lawrence, J. H., Phosphorus metabolism of neoplastic tissues (mammary carcinoma, lymphoma, lymphosarcoma) as indicated by radioactive phosphorus. *Cancer Research* **1940,** *40*, 243-250.
8. Thomas, C. I.; Harrington, H.; Bovington, M. S., Uptake of radioactive phosphorus in experimental tumors. III. The biochemical fate of P32 in normal and neoplastic ocular tissue. *Cancer Res* **1958,** *18* (9), 1008-11.
9. Arnst, J. L.; Beck, G. R., Jr., Modulating phosphate consumption, a novel therapeutic approach for the control of cancer cell proliferation and tumorigenesis. *Biochem Pharmacol* **2021,** *183*, 114305.
10. C.E. Camalier, M.R. Young, G. Bobe, C.M. Perella, N.H. Colburn, G.R. Beck Jr., Elevated phosphate activates N-ras and promotes cell transformation and skin tumorigenesis, Cancer Prev Res (Phila) 3 (3) (2010) 359–370.
11. D.F. Wilson, C.S. Owen, A. Holian, Control of mitochondrial respiration: a quantitative evaluation of the roles of cytochrome c and oxygen, Arch. Biochem. Biophys. 182 (2) (1977) 749–762.
12. P.C. Brazy, S.R. Gullans, L.J. Mandel, V.W. Dennis, Interactions between phosphate transport and oxidative metabolism in the rabbit proximal tubule, Adv. Exp. Med. Biol. 151 (1982) 65–69.
13. P.C. Brazy, S.R. Gullans, L.J. Mandel, V.W. Dennis, Metabolic requirement for inorganic phosphate by the rabbit proximal tubule, J. Clin. Invest. 70 (1) (1982) 53–62.
14. Camalier, Corinne E et al. “An integrated understanding of the physiological response to elevated extracellular phosphate.” *Journal of cellular physiology* vol. 228,7 (2013): 1536-50. doi:10.1002/jcp.24312
15. Vorland, Colby J et al. “Effects of Excessive Dietary Phosphorus Intake on Bone Health.” *Current osteoporosis reports* vol. 15,5 (2017): 473-482. doi:10.1007/s11914-017-0398-4

16. H. Colburn, T.P. Conrads, G.R. Beck Jr., An integrated understanding of the physiological response to elevated extracellular phosphate, J. Cell. Physiol. 228 (7) (2013) 1536–1550.
17. Doll, R.; Peto, R., The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* **1981,** *66* (6), 1191-308.
18. Coleman, R. E., Metastatic bone disease: clinical features, pathophysiology and treatment strategies. *Cancer Treat Rev* **2001,** *27* (3), 165-76.
19. Selvaggi, G.; Scagliotti, G. V., Management of bone metastases in cancer: a review. *Crit Rev Oncol Hematol* **2005,** *56* (3), 365-78.
20. Ferlay, Jacques et al. “Cancer statistics for the year 2020: An overview.” *International journal of cancer*, 10.1002/ijc.33588. 5 Apr. 2021, doi:10.1002/ijc.33588
21. Mattiuzzi, Camilla, and Giuseppe Lippi. “Current Cancer Epidemiology.” *Journal of epidemiology and global health* vol. 9,4 (2019): 217-222. doi:10.2991/jegh.k.191008.001
22. Clark, Shane B. and Saud Alsubait. “Non Small Cell Lung Cancer.” *StatPearls*, StatPearls Publishing, 9 September 2021.
23. Musolino, Antonino et al. “BRCA mutations, molecular markers, and clinical variables in early-onset breast cancer: a population-based study.” *Breast (Edinburgh, Scotland)* vol. 16,3 (2007): 280-92. doi:10.1016/j.breast.2006.12.003
24. Sanchez-Cespedes M, et al. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. Cancer Res. 2002;62(13):3659–3662.
25. Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M. Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. Oncogene. 2004;23(22):4037–4040.
26. Ding L, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455(7216):1069–1075.
27. Imielinski M, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell. 2012;150(6):1107–1120.
28. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature. 2014;511(7511):543–550.
29. Jancík, Sylwia et al. “Clinical relevance of KRAS in human cancers.” *Journal of biomedicine & biotechnology* vol. 2010 (2010): 150960. doi:10.1155/2010/150960
30. Gilbert-Ross, Melissa et al. “Targeting adhesion signaling in *KRAS, LKB1* mutant lung adenocarcinoma.” *JCI insight* vol. 2,5 e90487. 9 Mar. 2017, doi:10.1172/jci.insight.90487
31. Castranova, V et al. “The alveolar type II epithelial cell: a multifunctional pneumocyte.” *Toxicology and applied pharmacology* vol. 93,3 (1988): 472-83. doi:10.1016/0041-008x(88)90051-8

32. Hinck, Lindsay, and Inke Näthke. “Changes in cell and tissue organization in cancer of the breast and colon.” *Current opinion in cell biology* vol. 26 (2014): 87-95. doi:10.1016/j.ceb.2013.11.003
33. Song Y, Ahn J, Suh Y, Davis ME, Lee K (2013) Identification of Novel Tissue-Specific Genes by Analysis of Microarray Databases: A Human and Mouse Model. PLoS ONE 8(5): e64483. https://doi.org/10.1371/journal.pone.0064483
34. Xiong, Min et al. “Identification of lung-specific genes by meta-analysis of multiple tissue RNA-seq data.” *FEBS open bio* vol. 6,7 774-81. 16 Jun. 2016, doi:10.1002/2211-5463.12089
35. Kuro-o, Makoto. “A potential link between phosphate and aging--lessons from Klotho-deficient mice.” *Mechanisms of ageing and development* vol. 131,4 (2010): 270-5. doi:10.1016/j.mad.2010.02.008
36. Calvo MS, Park YK. Changing phosphorus content of the U.S. diet: Potential for adverse effects on bone. In: Journal of Nutrition. Vol 126. American Society for Nutrition; 1996. doi:10.1093/jn/126.suppl_4.1168s
37. Changing phosphorus content of the U.S. Diet: Potential for adverse effects. Mona S. Calvo and Youngmee K. Park. *J. Nutr.* 1996 *Apr*;126(*4 Suppl):1168S-80S*.
38. Brunner-Ziegler S, Fröschl B, Hiebinger C, Zsifkovits J. Effectiveness and cost-efficacy of phosphate binders in hemodialysis. *Ann Nutr Metab*. 2011;58(4):315-319. doi:10.1159/000331988
39. Negri AL, Torres PAU. Iron-based phosphate binders: Do they offer advantages over currently available phosphate binders? *Clin Kidney J*. 2015;8(2):161-167. doi:10.1093/ckj/sfu139
40. Ruospo M, Palmer SC, Natale P, et al. Phosphate binders for preventing and treating chronic kidney disease-mineral and bone disorder (CKD-MBD). *Cochrane Database Syst Rev*. 2018;2018(8). doi:10.1002/14651858.CD006023.pub3
41. Sekar A, Kaur T, Nally J V., Rincon-Choles H, Jolly S, Nakhoul GN. Phosphorus binders: The new and the old, and how to choose. *Cleve Clin J Med*. 2018;85(8):629-638. doi:10.3949/ccjm.85a.17054
42. Chan S, Au K, Francis RS, Mudge DW, Johnson DW, Pillans PI. Phosphate binders in patients with chronic kidney disease. *Aust Prescr*. 2017;40(1):10-14. doi:10.18773/austprescr.2017.002
43. Drüeke TB, Massy ZA. Phosphate binders in CKD: bad news or good news?. *J Am Soc Nephrol*. 2012;23(8):1277-1280. doi:10.1681/ASN.2012060569
44. Alappan, Uma Devi, et al. “Efficacy of Pi-Binder Lanthanum Carbonate in Reversing Systemic Effects of a High Phosphate Diet in Mice.” *Journal of the American Society of Nephrology*, vol. 32, 2021, pp. 624.

45. Borgi L. Inclusion of phosphorus in the nutrition facts label. Clin J Am Soc Nephrol. 2019;14(1):139- 140. doi:10.2215/CJN.07230618
46. Assari S. Racial Disparities in Chronic Kidney Diseases in the United States; a Pressing Public Health Challenge with Social, Behavioral and Medical Causes. Vol 5.; 2016. http://www.jnephropharmacology.com
47. Alappan U. D., Ferguson M., Wolpert B. J. Beverage Intake by U.S. Adolescents: Analysis of National Health and Nutrition Examination Survey (NHANES) Data. Journal of the American Society of Nephrology Abstract Supplement, 29: 1133; 2018.
48. Barkauskas, Christina E et al. “Type 2 alveolar cells are stem cells in adult lung.” *The Journal of clinical investigation* vol. 123,7 (2013): 3025-36. doi:10.1172/JCI68782
49. Ardelyx, Inc. 2022. *Ardelyx Announces FDA Acceptance for Filing of its New Drug Application of Tenapanor for the Control of Serum Phosphorus in Adult Patients with CKD on Dialysis* | *Ardelyx, Inc.*. [online] Available at: https://ir.ardelyx.com/news-releases/news-release-details/ardelyx-announces-fda-acceptance-filing-its-new-drug-0.
50. Orlando M. Gutiérrez, Anna K. Porter, Manjula Viggeswarapu, Joseph L. Roberts, George R. Beck, *Effects of phosphorus and calcium to phosphorus consumption ratio on mineral metabolism and cardiometabolic health*. *The Journal of Nutritional Biochemistry*, Volume 80, 2020,108374.
51. Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001,** *25* (4), 402-8.
52. Ha, S. W.; Jang, H. L.; Nam, K. T.; Beck, G. R., Jr., Nano-hydroxyapatite modulates osteoblast lineage commitment by stimulation of DNA methylation and regulation of gene expression. *Biomaterials* **2015,** *65*, 32-42.
53. Wang, X.; Spandidos, A.; Wang, H.; Seed, B., PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res* **2012,** *40* (Database issue), D1144-9.