AWARD NUMBER: W81XWH-21-1-0577

TITLE: Antibiotic-Induced Dysbiosis Promotes Lung Metastasis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Recent studies by various groups have revealed that antibiotics (ABX)-induced dysbiosis has systemic consequences including changes in vasculature beds at distal sites, and ultimately acceleration of lung carcinogenesis. These observations and their clinical implications motivated us to investigate whether dysbiosis also has an influence on lung metastasis. The **long-term goal** of our work is to define the systemic effects of ABX-induced dysbiosis, to develop strategies to quell metastasis. The **overall objective** of this proposal is to determine the influence of ABX on lung metastasis progression and dissemination. Attaining this objective will be a major step in understanding the processes of lung metastasis and off-target effects of ABX. In this concept award, we test our **central hypothesis** is that ABX-induced dysbiosis increases fibronectin in the perivasculature stroma, creating a favorable pre-metastatic niche.
None of the tasks of the original approved Statement of Work (SOW) were changed.

15. SUBJECT TERMS

Lung metastasis, antibiotics, dysbiosis, cooperative human tissue network (CHTN), lung cancer biospecimen resource network (LCBRN), stroma.

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1. Introduction

Antibiotic-induced microbial imbalance, or dysbiosis, has systemic and long-lasting deleterious effects on the host. Although they fight infections, antibiotics (ABX) severely alter microbiomes by disrupting commensal bacteria crucial for maintaining various host homeostatic mechanisms. The use of ABX has significantly increased by 30% in recent years, yet the influence of ABX-induced dysbiosis on metastasis is largely unknown. Metastasis, the successful spreading of primary tumor cells to distant organs is the primary cause of cancer morbidity and mortality, and is estimated to account for 90% of the cancer death. Importantly, military personnel are at a higher risk of developing lung cancer and metastasis than the general population due to increased rates of smoking as well as an increased likelihood of being exposed to environmental carcinogens during their service. Thus, there is a pressing need to reach a deeper understanding of the biological consequences of ABX-induced dysbiosis on lung metastasis to uncover strategies to intervene and reverse by restoring and promoting beneficial microbiota.

2. Keywords

Lung metastasis, antibiotics, dysbiosis, cooperative human tissue network (CHTN), lung cancer biospecimen resource network (LCBRN), stroma.

3. Accomplishments

Please note: we were granted a 12-months no-cost extension (NCE) due to unforeseen challenges. There were no significant changes in the project or its direction. Please see more detail under **Section 5**. Accomplishments and approximate percentage of completion, as outlined in the approved SOW:

- **What were the major goals of the project and what was accomplished under these goals?**

Major Goal 1: Identify ABX primarily responsible for stromal modulation and metastasis dissemination.
In order to identify which ABX was primary responsible for the stromal modulation and metastasis dissemination we used broad-spectrum ampicillin and neomycin, Gram-positive targeting vancomycin and anaerobic targeting metronidazole, in the context of ciprofloxacin and bactrim that are prescribed in high frequency in the clinic. This also has the potential of identifying the beneficial bacteria, or concostium, regarding stromal homeostasis. In the experimental (by iv injection) lung metastasis models of Lewis Lung Carcinoma (LLC) and B16-F10 with ABX-induced dysbiosis, we determined the metastatic spread and pulmonary (peri-) vascular stroma remodeling.

Please see **Table 1** for our subtasks and milestones and our interim achievements and key outcomes as updates below it.

Table 1. Interim achievements and key outcomes of major goal 1.

Major Goal 1: Identify ABX primarily responsible for stromal modulation and metastasis dissemination.	**Timeline (months)**	**Completion (%)**
Major Task 1	Months	
Subtask 1.1 – Isolate lung and GI tract bacteria and perform 16S rRNA analysis by Argonne National Library.	1-3	100%
Subtask 1.2 – Define the ABX-induced stromal changes as determined by IHC and IF.	1-6	80%
Subtask 1.3 – Quantify experimental (iv injected) and spontaneous (from sc implanted) LLC and B16-F10 lung and kidney metastases (nodules and overall burden), by microscopic, macroscopic and whole animal imaging (IVIS). Mice obtained from JAX and cell lines from ATCC.	5-11	80%
Subtask 1.4 – Correlate bacteria taxa changes with stromal changes and subsequent lung metastasis progression and dissemination.	10-12	60%

Milestones:

1. identify which antibiotic causes which changes in bacteria taxa.
2. identify which antibiotic(s) is/are responsible for stromal changes.
3. determine which stromal changes occur due to antibiotic-induced dysbiosis.
4. identify which bacteria are important for stromal homeostasis.
5. determine the increase in the number of micrometastases, dissemination and accelerated mortality rate due to ABX-induced dysbiosis per tumor model.

Update Subtask 1.1 and Milestone 1 and in part Milestone 2 and 4: In order to identify microbial taxa that are lost per antibiotic, we induced dysbiosis by 6 specific antibiotics in C57BL/6J mice and quantified and analyzed the changes by 16S rRNA gene exact sequence variants (ESVs) to compare these to untreated control or orthobiotic conditions. Namely, using broad-spectrum ampicillin and neomycin, Gram-positive targeting vancomycin and anaerobic targeting metronidazole would identify which bacterial taxa are beneficial, in the context of ciprofloxacin and Bactrim that are prescribed in high frequency in the clinic. We found that the cocktail of antibiotics (ABX), ampicillin, and vancomycin affected the microbiota the most (**Fig. 1** – please see appendix at end of the document). Namely, as measured by cecum weights, only ampicillin and vancomycin mimicked the approximate 4-fold cecum enlargement seen when treated with the ABX cocktail: from an average cecum weight of 0.5 grams to approximately 2 grams (**Fig. 1A**). Although we are finalizing the bioinformatics data analysis, the initial taxonomy bar plots revealed clear differential consequences in abundance, composition and diversity per ABX. This was also noted in both the *unweighted* (qualitative) and weighted (quantitative) *UniFrac* analysis (**Fig. 1B** and **C**). The *unweighted UniFrac* only considers their presence or absence, whereas the weighted UniFrac reflects the abundance of observed organisms. While every antibiotics induced distinct chances in the frequency and disappearance of certain microbial taxa, the cocktail, broad-spectrum ampicillin, and gram-positive targeting vancomycin displayed the greatest effects (**Fig. 1D**). This is also reflected in the changes seen in the gross pathology of the ceca. We are now further analyzing the ESVs by the Quantitative Insights Into Microbial Ecology (QIIME 2) pipeline.

Update Subtask 1.2 and Milestone 2, 3 and 4: Identified that some stromal adhesion molecules change during dysbiosis (e.g. CD54, CD106, MECA79, whereas others do not e.g. CD146). We are currently finalizing the analysis on potential changes in extra cellular matrix proteins, i.e. collagen and fibronectin. We are finalizing these staining and quantifications.

Update Subtask 1.3 and Milestone 5. In order to determine whether dysbiosis changes lung metastasis progression and dissemination, we induced experimental metastasis by injected luciferase+ B16-F10 i.v. (**Fig. 2 –** appendix). Dysbiosis accelerated the morality rate due to succumbing of lung metastasis (**Fig. 2A**). Namely, as shown in the Kaplan-Meier curve, all the mice under dysbiotic conditions succumbed by 25 days, whereas the orthobiotic controls lived until day 29. At least in part, this was due to an overall increase in total amount of lung metastasis and change in dissemination as measured by tumor nodule weights at the conclusion (**Fig. 2C-E**), as well as by bioluminescence (luciferin detected by IVIS) longitudional (**Fig. 2F**).

Update Subtask 1.4 and Milestone 1-5. This subtask is the accumulation and integration of the previous 3 subtasks. Thus, we preliminary conclude that Gram-positive clostridial cluster IV (*Ruminococcaceae*) and XIVa (*Lachnospiraceae*), are important for stromal homeostasis, as dysbiosis induced by ampicillin and vancomycin induced the greater changes in microbiota, and consequently accelerated mortality by increasing and altering the micro-metastasis dissemination.

Major Goal 2: Determine differences in bacteria taxa in Bronchoalveolar lavage (BAL) fluids and stromal changes in matched NSCLC specimen.

BAL fluids and matched NSCLC specimens – a mix of adenocarcinoma and squamous cell carcinoma along with their respective adjacent normal tissue were obtained from the lung cancer biospecimen resource network (LCBRN), now integrated into the cooperative human tissue network (CHTN). Cryo-tissue sections were generated (5 μm thickness) and mounted on glass slides for histopathology and immunohistochemistry (IHC). Stromal and extracellular matrix changes (i.e. alpha smooth muscle actin, desmin, NG2, PDGFRs and fibronection, collagens, laminins), lipids (Oil Red O, BODIPY) and mucins/glycogen (Periodic Acid Schiff) were identified. Bacterial metabolites were identified in matched serum samples by Liquid Chromatography - Mass Spectrometry (LC-MS), to correlate the serum bacterial metabolites with the changes in stromal tissue signatures.

Please see **Table 2** for our subtasks and milestones and our interim achievements and key outcomes as updates below it.

Table 2. Interim achievements and key outcomes of major goal 2.

Major Goal 2: Determine differences in bacteria taxa in BAL fluids and stromal changes in matched NSCLC specimen.	**Timeline (months)**	**Completion (%)**
Major Task 2		
Subtask 2.1 – Acquisition of 30 BAL fluids and their matched NSCLC specimen from LCBRN/CHTN.	1-3	66%
Subtask 2.2 – Isolate bacteria in bronchoalveolar lavage fluids and perform 16S rRNA analysis by Argonne National Library.	2-10	30%
Subtask 2.3 – Stain for proteins, lipids and mucins in human specimen.	2-10	30%
Subtask 2.4 – Correlate changes in bacteria taxa, stroma, medical history and clinical outcomes.	9-12	30%
Milestones achieved: 6. defined bacteria in BAL fluids. 7. defined the stromal differences in NSCLC specimen as compared to normal tissues. 8. correlated bacteria in BAL, ABX use, medical history and clinical outcomes with stromal signatures in NSCLC specimen.		

Update Subtask 2.1 and 2.2, and Milestone 6: We obtained 20 matched NSCLC specimen from LCBRN/CHTN. However, due the lack of availability of BAL specimen, esp. as these are collected prospectively and the peak of SARS-CoV-2 pandemic policies in place, we obtained serum samples in addition. We are still pursuing BAL fluids, but decided to broaden this subtask to include bacterial metabolites in the serum. This is a relative less invasive strategy and clinically more pragmatic for patients with advanced lung cancer. Effectively it is the bacterial metabolites. While the epithelial barrier ensures that bacteria are largely confined to the lung alveolar space, microbial metabolites cross-over the epithelial barrier, allowing the metabolites to enter the host circulatory system maintaining various processes of cell homeostasis. Thus, we established and optimized a new targeted and untargeted Liquid Chromatography - Mass Spectrometry (LC-MS) acquisition work flow to analyze bacterial derived short-chain fatty acids (**SCFA**; **Fig. 3 –** please see appendix).

Update Subtask 2.3 and Milestone 7: We obtained 20 matched NSCLC specimen from LCBRN/CHTN. These are currently being processed and analyzed, as we now have our LC-MS up and running an optimized for bacterial SCFA analysis.

Update Subtask 2.4 and Milestone 8: This subtask integrates the previous 3 subtasks. As we finalize those subtasks in our NCE, we preliminary conclude that ABX-induced dysbiosis causes significant changes in bacterial metabolites in the serum, i.e. short chain fatty acids. Particularly, butyrate (C4) showed the inverse profile of dysbiosis induction by ampicillin and vancomycin (**Fig. 1**).

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

  Although not a major objective of this project, it did provide training for a High School student (Kinsey Garofalo), two Undergraduate Research Students (Savannah Huyvaert and Hailey Campbell), a Research Assistant (Amir Mortazavi) and Junior Faculty fellow (Samir Jenkins, Ph.D.).

- **How were the results disseminated to communities of interest?**

  We have one manuscript under final review (federal support acknowledged) and the results were presented at local and international symposia and scientific meetings.

  Conferences (poster presentation):
  American Association of Cancer Research
  Location: New Orleans, LA; Date(s): 04/8-13, 2022
  Title: T-cell trafficking and extravasation is suppressed in distal tumors during gastrointestinal tract dysbiosis

  Arkansas Undergraduate Research Symposium
  Location: Little Rock, AR; Date(s): 07/27/2022
  Title: The paracrine effects of lung cancer on dysbiotic stromal compartment

  Metabolomics Association of North America
  Location: Alberta, Canada; Date(s): 09/16-19, 2022
  Title: Delineating dysbiosis-induced metabolomics signatures to optimize precision medicine
  Authors: Renny Lan, Hailemariam Abrha Assress, Samir V. Jenkins, Ruud P.M. Dings.
  *won 1st place award for highly meritorious research in metabolomics by the society

  Invited talks:
  - Harnessing the power of the tumor microenvironment. Department of Physiology and Cell Biology, UAMS (02/17/2022)
  - T-cell trafficking and extravasation is suppressed in distal tumors during gastrointestinal tract dysbiosis. Rhode Island Microbiome Symposium (01/14/2022).
  - 1, 2, 3, Go – Bacterial Scan. COM Research Council (12/1/2021).
  - Biomarkers of treatment resistance. Metabolism in health and disease - University of Arkansas Fayetteville (11/16/17 2021).
  - Trainee S. Huyveart presented some of the results at the annual UAMS Summer Undergraduate Research Fellowship Symposium on 06/01/2022.
  - Trainee K. Garofalo presented some of the results at the annual UAMS Division for Diversity, Equity, and Inclusion (DDEI) Summer Research Internship (SRI) on 07/29/2022.

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

  During our NCE we will continue and finalized our subtasks as described in our originally approved SOW and address the comments we received on our submitted manuscript.

4. Impact

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

  At least in mice, we preliminary conclude that certain antibiotics have the potential to accelerated mortality by increasing and altering lung micro-metastasis dissemination. Particularly, broad-based ampicillin and Gram-positive targeting vancomycin are potential harmful as Gram-positive clostridial cluster IV (*Ruminococcaceae*) and XIVa (*Lachnospiraceae*) produce short-chain fatty acid butyrate important for stromal homeostasis.

- **What was the impact on other disciplines?**

  This study generated a foundation for future large-scale studies aimed at intervening or even preventing metastatic spread, and clinical decision-making regarding the use of certain antibiotics in individuals at high risk for developing lung cancer, i.e. veterans. Additionally, although further studies are warranted, we envision that bacterial metabolites in specimens can be used as a supplemental screen for lung cancer prognosis. Of note, the society of Metabolomics Association of North America recognized our work with an award and deemed the work described in this report highly meritorious research in metabolomics.

- **What was the impact on technology transfer?**

  Nothing to report at this interim.

- **What was the impact on society beyond science and technology?**

  We now have a better understanding of the potential negative consequences of antibiotic use. Many of the antibiotics, although effective and necessary, have undisclosed 'off target' effects.

5. Changes/Problems

- **Changes in approach and reasons for change**

  Nothing to report.

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

  There were no significant changes in the project or its direction. We did encounter some challenges, which were all practical in nature. We were granted a 12-months no-cost extension (NCE) as progress under this award was delayed by the SARS-CoV-2 pandemic, causing challenges on multiple levels, including
  - Timely delivery of laboratory supplies due to persistent supply chain issues
  - Hiring-freeze: the animal-use certified technician ended up not available anymore and there was a freeze on hiring someone else
  - Suppressed clinical trial recruitment: the prospective collection of human specimens has taken more time than anticipated
  - as was much needed, our whole Institute (clinical and academic) transitioned to a cloud-based enterprise management platform, Workday. This included all of our financial and human resources management systems. In order to train everyone and switch over as efficient as possible there was purchasing and hiring hiatus for June and part of July of 2022.

  Update and resolve: We have overcome most of these challenges: supply chain issues are persistent but less frequent for now; hiring freeze was lifted as of Q2 2022; hired and trained a new technician; clinical trial recruitment is increasing. We are successfully transitioning over to Workday since July 2022. Although not everything is functional yet for all people in the institute, the issues are being addressed expeditiously and should be resolved in the near future. All the tasks in the original approved SOW are unchanged and moving forward. The extra time was requested for the completion of the repeats of the animal studies (SOW Aim 1, subtasks 3 and 4), acquire additional human specimen from the federal Cooperative Human Tissue Network (CHTN) and analyze the acquired data further (SOW Aim 2, subtasks 1-4).

- **Changes that had a significant impact on expenditures**

  Please see previous sub-heading.

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

  Nothing to report.

- **Significant changes in use or care of human subjects**

  Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

  Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

  Nothing to report.

6. Products

- **Publications, conference papers, and presentations**

  - **Journal publications.**

    Authors: Jenkins SV, Shah S, Jamshidi-Parsian A, Mortazavi A, Boysen G, Vang KB, Griffin RJ, Rajaram N, Dings RPM.
    Title: Acquired radiation resistance induces thiol-dependent cisplatin cross-resistance'.
    Under final review at the IJROBP. Acknowledgement of federal support (yes)

  - **Books or other non-periodical, one-time publications.**

    Nothing to report.

  - **Other publications, conference papers, and presentations.**

    Nothing to report.

- **Website(s) or other Internet site(s)**

  Nothing to report.

- **Technologies or techniques**

  Nothing to report.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**

  Nothing to report.

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name	Ruud P.M. Dings
Project Role:	PI
Researcher Identifier:	0000 0001 7686 1331
Nearest person month worked:	2 – no change
Contribution to Project	Dr. Dings is the PI of the project and has overseen all elements.
Funding Support	Nothing to Report

Name	Amir Mortazavi
Project Role:	Research Assistant
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project	Mr. Mortazavi has performed animal work – to collect and analyze samples for 16S rRNA.
Funding Support	Nothing to Report

Name	Hailey Campbell
Project Role:	Research Assistant
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project	Ms. Campbell has performed work in the area of flow cytometry and immunohisto chemistry and fluorescence to analyze the stromal changes.
Funding Support	Nothing to Report

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

  Nothing to report.

- **What other organizations were involved as partners?**

  Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

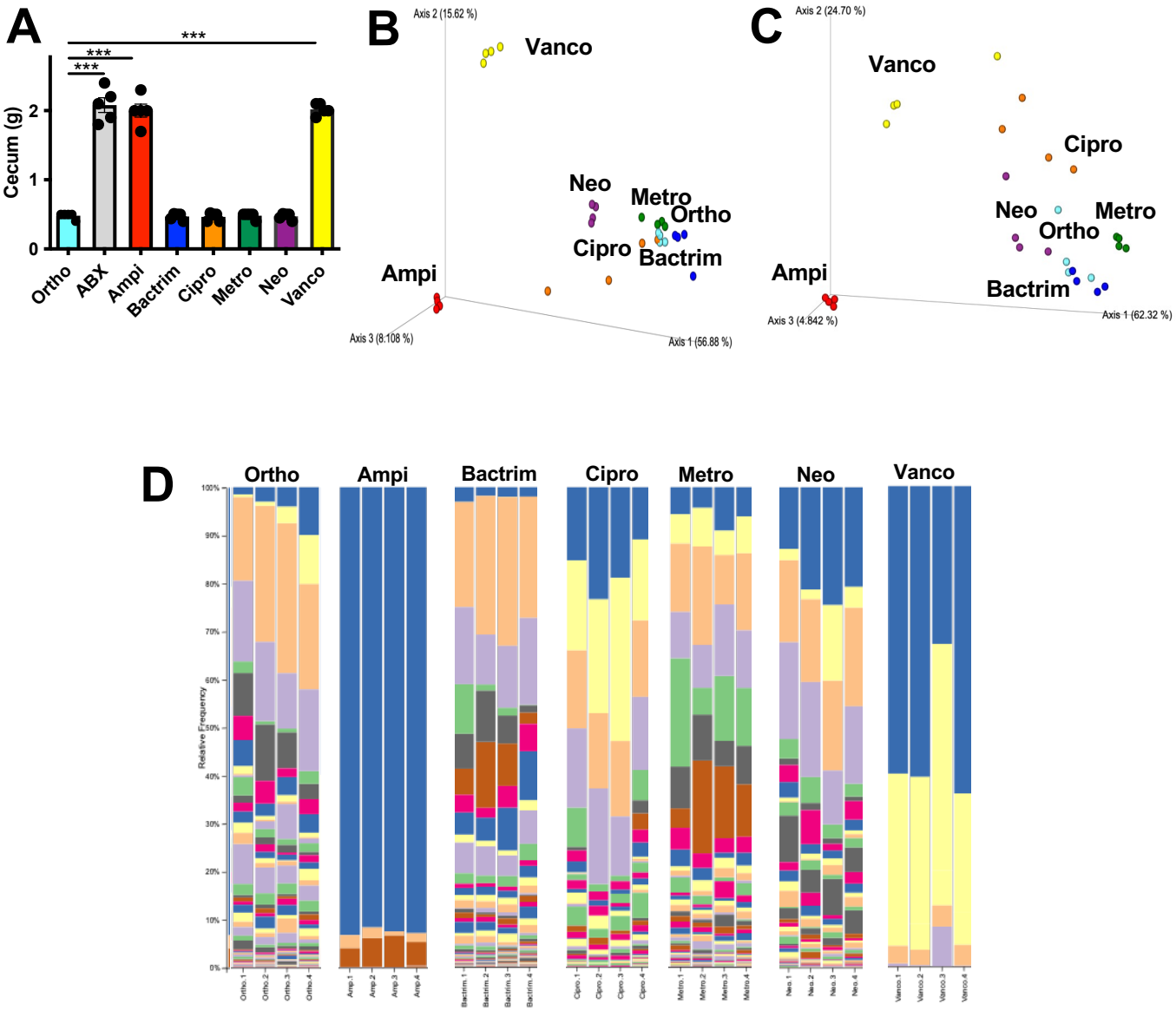

Figure 1, Dysbiotic microbial community composition after individual antibiotics. (**A**) The cocktail of ABX (ABX), ampicillin and vancomycin affect the host the most, as measured by cecum enlargement. (**B**) Beta diversity comparisons, unweighted (qualitative) and (**C**) weighted (quantitative) UniFrac. (**D**) Taxonomy bar plots of microbial fecal matter composition obtained from 16S rRNA sequence analysis in healthy controls (Ortho) and antibiotic exposed mice. Biological independent samples per group (n = 4 each). Assignment of the individual colors in the taxonomy bar plots have been omitted for clarity reasons but available upon request. Ortho = healthy control (water); Dysbiosis was induced by adding antibiotics to their drinking water for 14 days: Ampi = ampicillin [250 mg/L]; Bactrim [125 mg/L]; Cipro = ciproflaxin [125 mg/L]; Metro = metronidazole [250 mg/L]; Neo = Neomycin [250 mg/L]; Vanco = vancomycin [125 mg/L]. ABX = cocktail of Ampi, Metro, Neo and Vanco at the listed doses. Data presented as mean ± SEM (n=4/group) from female C57/BL6 mice. ***$P < 0.001$ two-sided *t*-test.

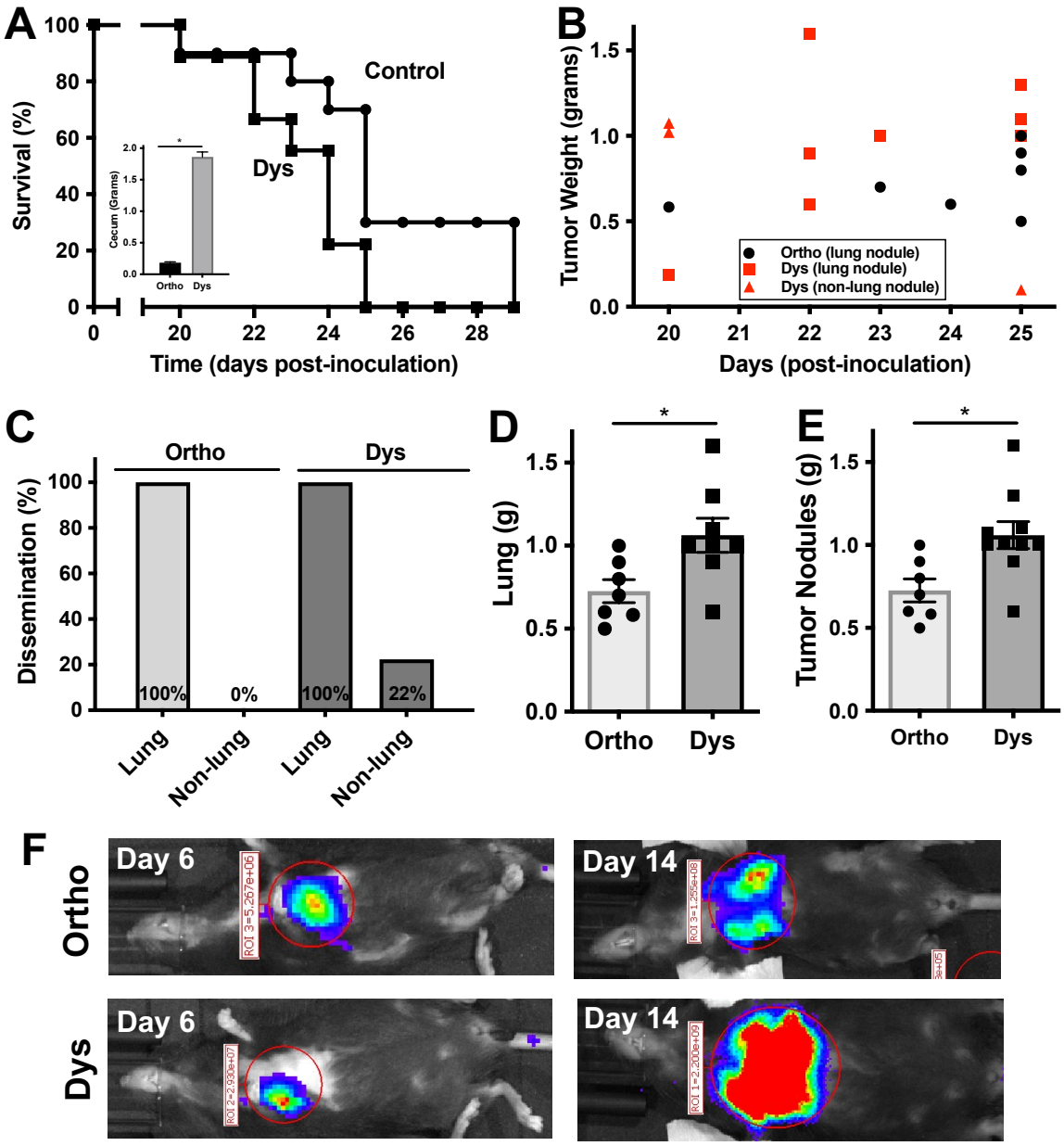

Figure 2, ABX-induced dysbiosis (Dys) increases lung metastasis and alters dissemination. (**A**) Dysbiosis accelerates the mortality rate in lung metastasis-bearing mice. Dysbiosis was confirmed by cecum enlargement (insert). (**B**) Dysbiosis increases the total amount of lung metastasis and (**C**) dissemination. (**D**) Dysbiosis increases overall lung metastasis as measured by overall lung weight and (**E**) in weights of all disseminated metastatic nodules. (**F**) Exemplary IVIS images of luciferase+ B16-F10 lung metastasis.

Ortho = healthy control (water); Dysbiosis was induced by adding antibiotics to their drinking water for 14 days: Ampi = ampicillin [250 mg/L]; Bactrim [125 mg/L]; Cipro = ciproflaxin [125 mg/L]; Metro = metronidazole [250 mg/L]; Neo = Neomycin [250 mg/L]; Vanco = vancomycin [125 mg/L]. ABX = cocktail of Ampi, Metro, Neo and Vanco at the listed doses. Data presented as mean ± SEM (n=7-8 / group) from female C57/BL6 mice i.v. injected with experimental metastasis of B16-F10 tumor cells. *$P < 0.05$ two-sided *t*-test.

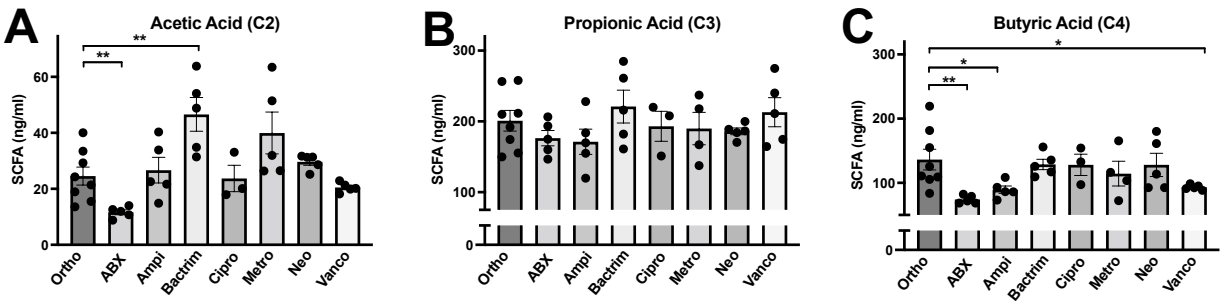

Figure 3. Butyric acid serum levels are reduced after ampicillin, vancomycin and a cocktail of ABX. (**A-C**) Changes in SCFA serum levels as induced by the different antibiotics. Ortho = healthy control (water); Dysbiosis was induced by adding antibiotics to their drinking water for 14 days: Ampi = ampicillin [250 mg/L]; Bactrim [125 mg/L]; Cipro = ciproflaxin [125 mg/L]; Metro = metronidazole [250 mg/L]; Neo = Neomycin [250 mg/L]; Vanco = vancomycin [125 mg/L]. ABX = cocktail of Ampi, Metro, Neo and Vanco at the listed doses. Data presented as mean ± SEM (n=4-8 / group) from female C57/BL6 mice. *$P < 0.05$, **$P < 0.01$ two-sided *t*-test.