AWARD NUMBER: W81XWH-19-1-0213

TITLE: Investigation into the Mechanisms of Acute Myeloid Leukemia (AML) Tumorigenesis and Chemoresistance via Systems Analysis of Mitochondrial Form and Function.

PRINCIPAL INVESTIGATOR: Kelsey Fisher-Wellman

CONTRACTING ORGANIZATION: East Carolina University
Greenville, NC, 27858-2502

REPORT DATE: JUNE 2020

TYPE OF REPORT: Annual Technical Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2020	Annual	1JUN2019 - 31MAY2020

4. TITLE AND SUBTITLE Investigation into the Mechanisms of Acute Myeloid Leukemia (AML) Tumorigenesis and Chemoresistance via Systems Analysis of Mitochondrial Form and Function

5a. CONTRACT NUMBER W81XWH-19-1-0213

5b. GRANT NUMBER CA180334

5c. PROGRAM ELEMENT NUMBER PRCRP

6. AUTHOR(S)
Dr. Kelsey Fisher-Wellman

E-Mail: fisherwellmank17@ecu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
East Carolina University / 1000 E 5th ST, Greenville, NC, 27858-2502

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Mitochondrial content and basal respiration have been repeatedly demonstrated to be higher in leukemia. Although these findings are suggested to reflect higher 'reliance' on oxidative ATP production, bioenergetic efficiency in leukemic mitochondria has not been empirically tested. That is, it remains unclear if higher basal respiration in leukemia reflects accelerated demand for ATP regeneration and/or intrinsic limitations in bioenergetic efficiency. Distinguishing between these potential outcomes is critical, as the molecular 'cause' and thus potential treatment strategies are distinct. Herein, we sought to comprehensively evaluate bioenergetic efficiency in blood cancer mitochondria using a discovery-based biochemical platform. This platform encompasses two key technological advancements; 1) comprehensive unbiased analysis similar in scope to traditional 'omics', and 2) utilization of the creatine kinase clamp that allows assays to be performed across physiological ATP free energies (ΔG_{ATP}), thus permitting quantitative evaluation of bioenergetic efficiency. Experiments were performed in intact cells, permeabilized cells, and isolated mitochondria derived from human leukemia (HL-60, KG-1, and MV-4-11), as well as peripheral blood mononuclear cells (PMBCs). Results revealed three key insights related to substrate preference, electron transport regulation, and bioenergetic efficiency. 1) Contrary to PBMCs, leukemic mitochondria were completely refractory to glycerol-3-phosphate oxidation. 2) When respiration experiments were performed in the presence of ΔG_{ATP}, the addition of FCCP, following oligomycin, failed to stimulate respiratory flux in leukemia. Parallel ADP titration experiments revealed that this effect was dependent on ATP free energy, suggesting that electron transport flux is allosterically and/or post-translationally blunted by extra-mitochondrial ΔG_{ATP} (e.g., glycolytic ATP) in leukemia. 3) Compared to PBMCs, the fraction of electron transport flux coupled to ATP synthesis was consistently reduced in leukemic mitochondria, indicating that reductions in bioenergetic efficiency are an intrinsic bioenergetic signature in leukemia. DOD-W81XWH1910213

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	25	19b. TELEPHONE NUMBER *(include area*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

Page

1. **Introduction - Page 4**
2. **Keywords - Page 4**
3. **Accomplishments - Pages 4-9**
4. **Impact - Page 9**
5. **Changes/Problems - Pages 10-12**
6. **Products - Pages 12-14**
7. **Participants & Other Collaborating Organizations - Page 15-18**
8. **Special Reporting Requirements - Page 18**
9. **Appendices - Pages 18-25**

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The identification of ***discrete*** mitochondrial phenotypes across cancers is a prerequisite to successful implementation of mitochondrial precision medicine. At present, while several reports exist documenting differences in AML mitochondrial quantity and quality, the molecular details underpinning these observations remain largely unknown. Identifying the precise molecular cause of a given mitochondrial shift demarcates the difference between effective targeted drug delivery and catastrophic secondary toxicity. The natural sequitur to this is the central goal of this application; identify the unique bioenergetic signature(s) of AML tumorigenesis and chemoresistance. To accomplish this task, the current application proposes to employ a state-of-the-art assay platform which unites discovery-based molecular omics with comprehensive bioenergetic characterization. Given the unprecedented diagnostic utility of this methodological platform, we anticipate the discovery of distinct bioenergetic parameters and proteomic signatures which can subsequently be used to guide future AML-targeted therapies. Collectively, the data generated from the current proposal will represent the necessary first steps in the design and development of efficacious mitochondria-targeted chemotherapeutics.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Mitochondrial bioenergetics, respirometry, proteomics, acute myeloid leukemia, OXPHOS efficiency

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1: Determine the unique bioenergetic phenotypes that differentiate AML cells from non-cancerous hematopoietic controls, as well as those linked to AML chemoresistance, the major cause of treatment failure.

Major Task 1 – Seek IRB and HRPO approval for the use of PBMCs. Perform mitochondrial diagnostics workflow on specified AML cell lines, as well as human PBMCs. All experiments will be performed using isolated mitochondria.

Subtask 1 - Experiments will be carried out on 6 distinct AML cell lines (KG-1a, MV4-11, HL-60 control, HL-60/Ara-C, HL-60/DNR, HL-60/VCR), as well as PBMCs. To reach the target sample size per group of N=10 for AML cell lines and N=30 for human subjects will require ~90 full experimental work days (1 day is required to run a given mitochondrial preparation through the entire diagnostics workflow). Assuming mitochondrial preparations are performed 2-3 days/week, we anticipate all functional data being generated in the first 9 months of the project.

Expected Milestone(s) for Major Task 1 – At the conclusion of these experiments we will have generated a comprehensive bioenergetic profile of AML, as well as AML chemoresistance. We anticipate that these data will unveil several AML-specific bioenergetic signatures which can subsequently be utilized by the entire scientific community to determine the potential druggability of these novel mitochondrial targets.

Major Task 2 – Validate the diagnostic potential of a permeabilized cell model for bioenergetic characterization.

Subtask 1 - Experiments will be carried out on a subset of AML cell lines (MV4-11, HL-60 control) and PBMCs using a truncated version of our mitochondrial diagnostics workflow adapted for the utilization of a permeabilized cell model. For these experiments a sample size of N=10 will be achieved for all groups.

Expected Milestone(s) for Major Task 2 – The goal of these experiments is to determine which, if any, of the AML-specific bioenergetic phenotypes observed in isolated mitochondria can also be observed using a permeabilized cell model. While the use of permeabilized cells limits the diagnostic capabilities of the platform, such a system is more amendable to the translation of this work to the clinic where it may be difficult to obtain the necessary volume of blood needed for a full mitochondrial preparation. Validation of a permeabilized cell approach to blood cancer mitochondrial bioenergetic characterization could open the door for mitochondrial precision medicine in which unique bioenergetic profiles can be screened for on a patient to patient basis to determine whether a given “mito-therapeutic” should be prescribed.

Aim 2: Determine the phospho-proteomic landscape of mitochondria derived from hematopoietic controls and AML cells (sensitive and refractory to several chemotherapeutics) using bottom-up mass spectrometry.

Major Task 3 – Characterize the mitochondrial proteome and phosphoproteome of AML tumorigenesis and chemoresistance.

Subtask 1 – Using the same mitochondrial preparations used for the diagnostics workflow, phosphoproteomics analysis will be performed using nLC-MS/MS. Initial experiments will be performed using aliquots from each mitochondrial prep which were either not subjected to the diagnostics workflow (i.e., flash-frozen immediately upon isolation) or collected at the completion of the respiration assays in which ATP free energy was maximal.

Subtask 2 – Additional experiments will be carried out in isolated mitochondria from AML cell lines in which mitochondrial aliquots are incubated with increasing ATP free energies. Phosphoproteomics analysis will then be performed on these samples to characterize phosphorylation dynamics as a function of ATP free energy.

Subtask 3 – Using phosphoproteomics data, prediction of kinase-substrate relationships will be performed using KinomeXplorer.

Subtask 4 – Integrate bioenergetic flux data with phosphoproteomics data into on-line resource which can be used by the entire scientific community to mine for potential mitochondrial-specific AML drug targets.

Expected Milestone(s) for Major Task 3 – We anticipate the discovery of novel phospho-signatures associated with AML tumorigenesis and chemoresistance. These findings, while preliminary, are anticipated to serve as a powerful resource for future studies tasked with validating the druggability of implicated kinase families identified herein.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

As outlined in the SOW, the first year of the project was focused on completing the Major Tasks and Subtasks associated with Aim 1. Related to Major Tasks 1 and 2, we have already completed bioenergetic phenotyping using our mitochondrial diagnostics workflow using the stated 3 AML cell lines (KG-1, MV-4-11, HL60) compared to PBMCs isolated from healthy human subjects. Sample size for the AML cell lines was taken to N=12, while PBMCs isolations were performed from over 30 human subjects. These sample sizes are in agreement with that proposed in our SOW. Related to Major Task 2, while performing our experiments in isolated mitochondria, we simultaneously developed and optimized protocols in permeabilized cells and thus all preparations included data generated using intact cells, permeabilized cells, and isolated mitochondria. Such protocols are critically important, as it allows for bioenergetic experiments in the AML cell lines to be compared to future studies involving primary acute leukemias. There were 2 primary milestones set out for Major Tasks 1 and 2:

1) Generate a comprehensive bioenergetic profile of AML.
2) Determine which, if any, of the AML-specific bioenergetic phenotypes observed in isolated mitochondria can also be observed using a permeabilized cell model.

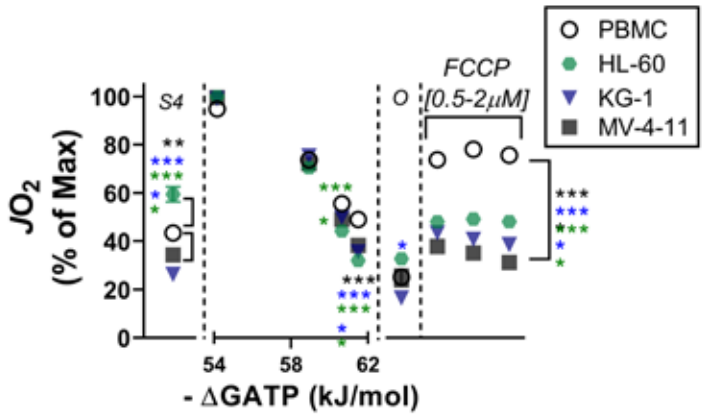
Accomplishments of stated milestones: Both Milestones were achieved in year one of the project and we are currently writing up the results from this work for publication in Cancer and Metabolism. **Appendix 1** contains an abridged description of the primary bioenergetic phenotypes observed in AML, relative to PBMC, complete with data depiction and methodology description. In brief, we discovered that AML mitochondria are characterized by intrinsic limitations in bioenergetic efficiency, linked to impairments in ADP phosphorylation potential. In addition, leukemic mitochondria were found to be deficient in the metabolic enzyme, glycerol-3-phosphate dehydrogenase, thus representing a novel bioenergetic vulnerability of AML. In addition to the primary manuscript, the methodology describing the permeabilized cell technique in PBMCs is currently in press in Methods in Molecular Biology and will be published later this year.

Related to chemoresistance, we have performed a full bioenergetic analysis of HL-60 naïve cells, relative to vincristine resistant counterparts (HL60/VCR). This data is currently being written up as a separate manuscript in collaboration with Co-I Myles Cabot. The primary bioenergetic phenotype identified for VCR resistance was bioenergetic inefficiency, due to specific impairments in respiratory complex I.

Other Accomplishments – As part of our initial bioenergetic phenotyping of AML, we performed proteomics screens using the isolated mitochondria samples prepared for all Aim 1 experiments. These experiments were critical in identifying glycerol-3-phosphate loss-of-function in AML. Moreover, the methodology by which we can integrate bioenergetic phenotyping data with the proteome was developed for this study and then leveraged for a separate project in the lab and was recently published in Scientific Reports (PMID: 32107436).

Stated Goals not met – The only milestones for year 1 of the project that were not met relates the cytarabine (Ara-C) and daunorubicin (DNR) resistant HL60 cells. Bioenergetic phenotyping of these cell lines, relative HL60 control cells is ongoing in the lab and is expected to be completed in year 2 of the project. Similar to HL60/VCR cells, we plan to publish each resistant cell lines phenotype separately.

Stated Goals for Aim 2 – The Major Task associated with Aim 2 was to characterize the mitochondrial proteome and phosphoproteome of AML tumorigenesis and chemoresistance. While we have already performed whole proteome screens comparing AML to PBMC, the focus of year 2 will be on the phosphoproteomics experiments. In our preliminary data in the grant we presented data that AML mitochondria are completely refractory to mitochondrial uncoupling in the presence of physiological ATP free energy, implying that ATP-mediated phosphorylation may be a novel mechanism of respiratory control in leukemia. In our full dataset, we validated this phenotype in the 3 main AML cell lines, as well as all chemoresistant cell lines (See figure below - note that respiration is blunted in AML mitochondria in the presence of the uncoupler FCCP in the presence of ATP free energy).



The lack of response to FCCP in the presence of adenylates suggests a potential phosphorylation mechanism. This is the focus of Aim 2 and we plan to test this mechanism in year 2 of the project.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Preliminary results from this project were presented by PI Fisher-Wellman and Dr. Margaret Nelson (the postdoc assigned to this project) at the 2019 NHLBI Mitochondrial Biology Symposium on Mitochondrial Networks and Energetics. Appendix 2 is a PDF of the abstract and poster presented at this conference.
Results from our work were also to be presented at the 2020 Keystone Symposia on Tumor Metabolism; however, this meeting was canceled due to COVID-19. Appendix 3 is a PDF of the abstract and poster that would have been presented at this conference.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The focus of year 2 will be on the phosphoproteomics experiments outlined in Aim 2, as well as on publishing our work from year 1. Related to this, we generated some preliminary findings that suggest that the respiratory inhibition seen with ATP free energy in leukemia may be mediated by the adenine nucleotide translocase (ANT) and may require TRAP1 activity. We plan to test these potential mechanisms in our Aim 2 experiments using gain and loss of function molecular techniques.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The work in year 1 represents the most comprehensive characterization performed to date, related to the intrinsic bioenergetic phenotypes that underly leukemia. We discovered that leukemic mitochondria are highly inefficient with respect to mitochondrial oxidative phosphorylation and thus are likely under chronic energetic stress. Such findings are likely to inform the development of more targeted therapeutics designed to impinge on the specific bioenergetics of leukemia. Moreover, the findings that leukemia mitochondria cannot oxidize glycerol-3-phosphate indicates that pharmacological targeting of the malate-aspartate shuttle in mitochondria may have therapeutic potential in leukemia.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Aside from the implications of our work to leukemia, our results demonstrate that using intact respirometry alone to diagnose mitochondrial 'heath' in cancer masks potential unique bioenergetic phenotypes of cancer that only become apparent when comprehensive approaches are leveraged to diagnose the mitochondrial network. The methodologies developed for this work thus have the potential to shift the paradigm with respect to mitochondrial 'function' in cancer and open up an entirely new field of research focused on understanding and leveraging the unique, intrinsic bioenergetic phenotypes of human malignancy.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

As detailed above, the methodologies developed for this work thus have the potential to shift the paradigm with respect to mitochondrial 'function' in cancer and open up an entirely new field of research focused on understanding and leveraging the unique, intrinsic bioenergetic phenotypes of human malignancy. While it will likely take some time for our work to translate to new therapies, our work in year 1 represents a critical step towards new mitochondrial-localized therapy relative to leukemia and potentially other cancers.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Like most labs in the US, we are currently in a period of reduced experimentation due to the COVID-19 pandemic. This had an impact on all expenditures beginning in March 2019 and will likely continue into year 2 of the project.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Like most labs in the US, we are currently in a period of reduced experimentation due to the COVID-19 pandemic. This had an impact on all expenditures beginning in March 2019 and will likely continue into year 2 of the project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Continuing review by the institutional review board of East Carolina University was granted on 04.07.2020. For the continuing review, we removed the age stipulation of 18-35 and extended our age of recruitment to 70. The intent of this change was to better reflect the age range of the natural history or acute myeloid leukemia. Continuing review was approved on 04.07.2020. We will refrain from subject recruitment pertaining to this study until we receive approval from the U.S. Army Medical Research and Development Command`s (USAMRDC) Office of Research Protections, Human Research Protection Office (HRPO).

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

· **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

McLaughlin, K. L., Kew, K. A., McClung, J. M. & Fisher-Wellman, K. H. **Subcellular proteomics combined with bioenergetic phenotyping reveals protein biomarkers of respiratory insufficiency in the setting of proofreading-deficient mitochondrial polymerase.** *Sci. Rep.* (2020). doi:10.1038/s41598-020-60536-y. Federal Funding was acknowledged.

Margaret A.M. Nelson[1,3], Kelsey L. McLaughlin[1, 3], James T. Hagen[1,3], Hannah S. Coalson[1,3], Cameron Schmidt[1,3], Kimberly A. Kew[2], P. Darrell Neufer[3], Myles C. Cabot[2,3], and Kelsey H. Fisher-Wellman[1, 3]*. **Intrinsic limitations in bioenergetic efficiency characterize the mitochondrial network in leukemia.** Cancer and Metabolism. In preparation. Federal Funding will be acknowledged.

Margaret A. M. Nelson[1,2] and Kelsey H. Fisher-Wellman[1,2,*.] **Mitochondrial Diagnostics: A discovery-based biochemical platform for phenotyping human peripheral blood cell mitochondria**. Methods Mol Biol. In Review. Federal Funding was acknowledged.

Li-Pin Kao[1], Miki Kassai[1], Todd E. Fox[2], Su-Fern Tan[3], David J. Feith[3,4], Mark Kester[4], Thomas P. Loughran Jr[3,4], Kelsey H. Fisher-Wellman[5+] and Myles C. Cabot. **Vincristine resistance reshapes sphingolipid composition and mitochondrial bioenergetics in acute myelogenous leukemia.** In preparation. Federal Funding will be acknowledged.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

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

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

· **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Kelsey Fisher-Wellman*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*ORCID ID: 0000-0002-0300-829X*
Nearest person month worked:	*4.2*
Contribution to Project:	*Dr. Fisher-Wellman is involved with all aspects of the project.*
Funding Support:	*Co-I on the following NIH R01 grants: DK110656, DK125812*

Name:	*Margret Nelson*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Nelson is involved with all aspects of the project.*
Funding Support:	

Name: *Kelsey McLaughlin*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*

Contribution to Project: *Ms. McLaughlin assisted with data collection*
Funding Support:

Name: *Tyler Hagen*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*

Contribution to Project: *Mr. Hagen assisted with data collection*
Funding Support:

Name: *Patricia Brophy*
Project Role: *Human Subjects Coordinator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.2*

Contribution to Project: *Ms. Brophy assisted with human subject recruitment*
Funding Support:

Name: *Myles Cabot*
Project Role: *Co-I*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.8*

Contribution to Project: *Dr. Cabot provided cell lines and participated in the chemoresistance arm of the project.*
Funding Support: *NIH P01 CA171983*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix 1:

Reduced bioenergetic efficiency is a common feature of human leukemia: We recently applied our mitochondrial diagnostics workflow to investigate how mitochondrial bioenergetics are modified in leukemia. Initial respiration experiments were performed using intact peripheral blood mononuclear cells (PBMC) from healthy controls compared to various leukemia cell lines (HL-60, KG-1, MV-4-11). Oligomycin similarly inhibited respiration across groups and spare reserve capacity was consistently lower in leukemia, compared to PBMC (**Fig. 1A**). Based on field standards, the interpretation of these data would be that mitochondrial efficiency is identical in leukemia, but that leukemic mitochondria simply respire at a greater percentage of their OXPHOS capacity. Indeed, this is the current understanding of mitochondrial bioenergetics in human acute myeloid leukemia (AML)[16].

However, our ability to intentionally interrogate the mitochondrial network again highlights the flawed diagnostic capabilities of the 'traditional' approach. For example, adjusting normalization from percent of basal to total protein, reveals that FCCP-stimulated flux is 2-fold lower in HL-60 cells relative to PBMC (**Fig. 1B**). This is particularly striking given that mitochondrial content (i.e., mitochondrial number assessed via MitoTracker (MTG-FM) relative to nuclear volume) was 2-fold higher in HL-60 cells (**Fig. 1C-D**). Similar discrepancies between mitochondrial content and maximal respiratory flux were observed in MV-4-11 cells (2-fold increase in content but only 30% increase in flux). Interestingly, across all leukemia cell lines, maximal respiration induced by FCCP titration occurred at much lower concentrations (i.e., lower Km in leukemia), with higher concentrations leading to overt bioenergetic collapse (**Fig. 1B;** compare JO_2 at FC [2.0µM] vs FC [5.0µM]). Considered together, these data indicate that human leukemia cells are bioenergetically inefficient resulting in chronic bioenergetic stress.

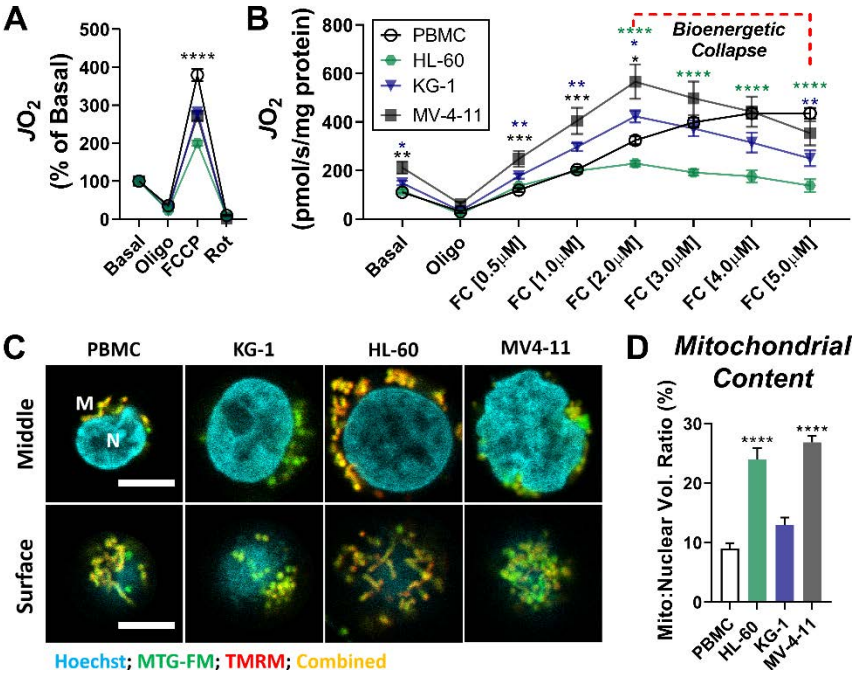


Fig 1. Bioenergetic profile of intact human leukemia cells: (**A**) Respiration in intact leukemia cell lines, compared to PBMC, depicted as a % of basal. (**B**) Respiration data normalized to total protein. (**C**) Representative confocal images of the mitochondrial network, assessed via MTG-FM and TMRM fluorescence. (**D**) Mitochondrial content derived from the ratio of mitochondrial volume (calculated for MTG-FM) to nuclear volume (Hoechst). N=15-30/group, *P<0.05.

To more directly test bioenergetic efficiency in leukemia, we developed 2 complementary assays, both using digitonin permeabilized cells. In the first assay, cells are suspended in potassium-based respiration buffer and then permeabilized with digitonin. Mitochondria are energized with saturating carbon substrates (pyruvate/malate/glutamate/octanoyl-carnitine/succinate – 'Multi') and respiration is stimulated at minimal ATP free energy (ΔG_{ATP}; i.e., an ATP/ADP ratio in vivo that would reflect 'maximal' OXPHOS flux). Cytochrome C is added to check the integrity of the mitochondrial inner-membrane, and ΔG_{ATP} is titrated via sequential additions of phosphocreatine (PCr). The key point is that the rate of respiration in the presence of the fully saturated 'fuel' node at the minimal ΔG_{ATP} represents the maximal conductance of the mitochondrial proton current through the OXPHOS system. This value is denoted as JH^+_{OXPHOS} and is represented by #4 in **Figure 2A**. To assess the maximal capacity of the electron transport system (ETS), independent of OXPHOS flux, in a parallel assay, cells were once again permeabilized with digitonin and energized with the same carbon substrates as in the ΔG_{ATP} titration assay. Instead of stimulating respiration with ΔG_{ATP}, FCCP was added until maximal respiration was observed. The key point is that the maximal FCCP-stimulated respiration rate reflects the absolute capacity of the ETS, denoted as JH^+_{Total} and is represented by point #7 in **Figure 2B**. Together, JH^+_{OXPHOS} and JH^+_{Total} provide a quantitative index of bioenergetic efficiency as they reflect the proportion of the entire respiratory system that can be harnessed by the OXPHOS system (**Fig. 2C**). A ratio of '1' reflects maximal bioenergetic efficiency, whereas a ratio of '0' indicates that the mitochondrial

proton current cannot be utilized for ATP synthesis. Strikingly, calculated bioenergetic efficiency in leukemic mitochondria was consistently decreased compared to PBMC, corresponding to a factor of ~ 0.5 (**Fig. 2D**), indicating that only half of the available ETS capacity can be dedicated to OXPHOS. Consistent with our observations in permeabilized cells, additional experiments in isolated mitochondria revealed decreased OXPHOS efficiency in 2 of the 3 leukemia cell lines (**Fig. 2E**). Interestingly, this effect was only observed in the presence of NADH-linked substrates (Pyr/M), suggesting specific derangements in CI supported energy transduction.

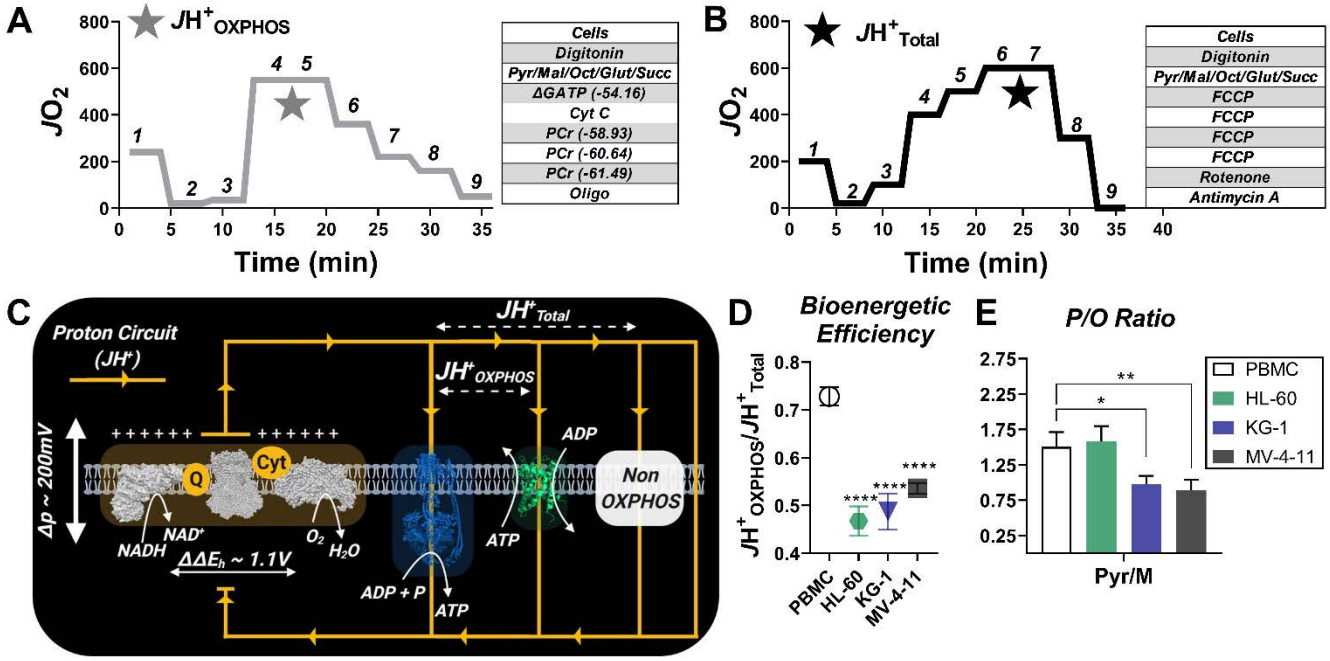
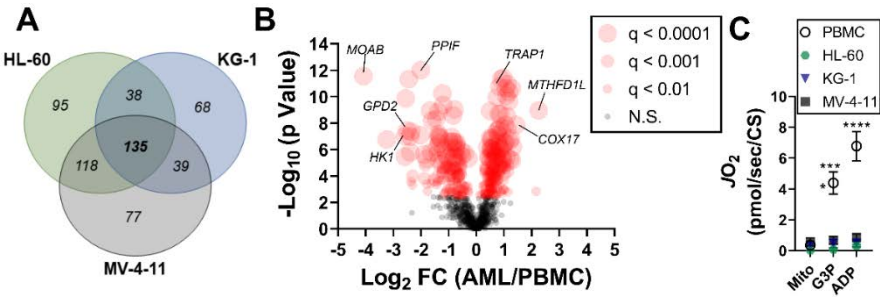


Fig 2. Decreased bioenergetic efficiency is a hallmark of leukemic mitochondria. (**A**) Computer generated trace depicting *J*O2 during a ΔGATP titration or (**B**) FCCP titration protocol in permeabilized cells. (**C**) Cartoon depicting the process of total mitochondrial energy transduction (JH^{+}_{Total}), as well as that dedicated to the phosphorylation of ADP (JH^{+}_{OXPHOS}). (**D**) Quantified bioenergetic efficiency. (**F**) Mitochondrial P/O ratio in mitochondria energized with either Pyr/M or Succ/R. N=15-30/group, *P<0.05.

We then further interrogated reduced bioenergetic efficiency in leukemia by conducting a proteomics screen using TMT-labeled peptides prepared from the same isolated mitochondria samples used for functional characterization. In total we identified 135 differentially expressed proteins (adjusted P value < 0.01) comparing PBMC to each of the 3 leukemia lines (**Fig. 3A-B**). Several of these proteins have previously been implicated in cancer biology, such as decreased MAOB[17] and HK1[18], and increased TRAP1[19] and MTHFD1L[20] (**Fig. 3B**). Interestingly, the most significant differentially expressed protein between PBMC and leukemia was peptidyl-prolyl cis-trans isomerase F (PPIF), also known as cyclophilin F. Like the more well-known cyclophilin D, PPIF binds cyclosporin A and catalyzes cis-trans isomerization of tetrapeptide substrates[21]. Cyclophilin D has previously been demonstrated to regulate ATP synthase activity and supramolecular assembly of synthesomes[22,23]. It is possible that loss of PPIF in leukemia disrupts bioenergetic efficiency by impinging on protein components of the phosphorylation system (e.g., ATP synthase, ANT). In addition to PPIF, we observed a striking leukemia-specific downregulation of the metabolic enzyme glycerol-3-phosphate (G3P) dehydrogenase (GPD2). We used this proteomics-based discovery to design a bioenergetic assay targeting GPD2 activity in isolated mitochondria. In PBMC mitochondria, robust respiration was observed with GDP2's substrate G3P; however, in agreement with reduced GPD2, all leukemic mitochondria were completely refractory to G3P oxidation (**Fig. 3C**). Taken together, our work in leukemia provides strong proof of principle that traditional assays of mitochondrial function (i.e., respiration in intact cells) are diagnostically limited at assessing intrinsic OXPHOS efficiency. Our application of mitochondrial diagnostics allowed us to demonstrate, for the first time, that human leukemia is characterized by overt OXPHOS inefficiency.

Fig 3. (**A**) Overlapping number of differentially expressed proteins between leukemia lines, relative to PBMC. (**B**) Volcano plot of mitochondrial proteome in leukemia vs PBMC. Larger circles reflect differentially expressed proteins (adjusted p value < 0.01, < 0.001, < 0.0001). (**C**) G3P oxidation in isolated mitochondria.



Appendix 2

Comprehensive bioenergetic profiling of human leukemia.

Margaret A.M. Nelson[1,2], Kelsey L. McLaughlin[1, 2], and **Kelsey H. Fisher-Wellman**[1, 2]*

[1]Physiology Department, Brody School of Medicine, [2]East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC
*fisherwellmank17@ecu.edu

Background: Research related to cancer mitochondrial function relies heavily on intact cellular respiration assays whereby bioenergetic conclusions are derived from sequential additions of OXPHOS inhibitors (e.g., oligomycin, FCCP, rotenone/antimycin). The inherent limitation of this approach is that identical rates of oxygen consumption can reflect completely opposite phenotypes. Such limitations become problematic when generalized inferences regarding 'mitochondrial health' are made from intact cell respirometry alone. For example, compared to non-cancer controls, mitochondrial content and basal respiration have been repeatedly demonstrated to be higher in leukemia. Although these findings are suggested to reflect higher 'reliance' on oxidative ATP production, bioenergetic efficiency in leukemic mitochondria has not been empirically tested. That is, it remains unclear whether higher basal respiration in leukemia reflects accelerated demand for ATP regeneration and/or lower bioenergetic efficiency. Distinguishing between these potential outcomes is critical, as the molecular 'cause' and thus potential drug targets for each are distinct. In the present study, we sought to comprehensively evaluate bioenergetic efficiency in blood cancer mitochondria using a recently described discovery-based biochemical platform. This platform encompasses two key technological advancements; 1) comprehensive unbiased analysis similar in scope to traditional 'omics', and 2) utilization of the creatine kinase clamp that allows bioenergetic assays to be performed across physiological ATP f

Comprehensive bioenergetic profiling of human leukemia. #166

Margaret A.M. Nelson[1,2], Kelsey L. McLaughlin[1, 2], and Kelsey H. Fisher-Wellman[1, 2*]

[1]Physiology Department, Brody School of Medicine. [2]East Carolina Diabetes and Obesity Institute. East Carolina University, Greenville, NC. *fisherwellmank17@ecu.edu

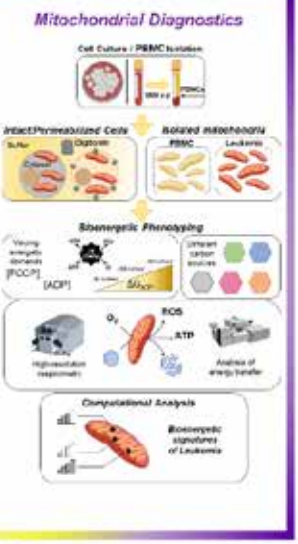
Background: Research related to cancer mitochondrial function relies heavily on mixed cellular respiration assays whereby bioenergetic conclusions are derived from sequential additions of OXPHOS inhibitors (e.g. oligomycin, FCCP, rotenone/antimycin). The inherent limitation of the approach is that identical rates of oxygen consumption can reflect completely opposite phenotypes. Such conditions become problematic when generalized inferences regarding mitochondrial health are made from single cell respirometry alone. For example, compared to non-cancer controls, mitochondrial content and basal respiration have been repeatedly demonstrated to be higher in leukemia. Although these findings are suggested to reflect higher reliance on oxidative ATP production, bioenergetic efficiency in leukemic mitochondria has not been empirically tested. That is, it remains unclear whether higher basal respiration in leukemia reflects accelerated demand for ATP regeneration and/or lower bioenergetic efficiency. Distinguishing between these potential outcomes is critical as the molecular 'cause' and thus potential drug targets for each are distinct. In the present study, we sought to comprehensively evaluate bioenergetic efficiency in blood cancer mitochondria using a recently described discovery-based biochemical platform. This platform encompasses two key methodological advancements: 1) comprehensive untargeted analysis similar in scope to traditional 'omics', and 2) utilization of the unaltered tissue sample that allows bioenergetic assays to be performed across physiological ATP free energies (ΔG_{ATP}) that mimic the cell thermodynamic energy constraints.

Methods: Experiments were performed in intact cells, permeabilized cells, and isolated mitochondria derived from human leukemia (HL-60, KG-1, and MV4-11), as well as peripheral blood mononuclear cells (PBMCs). Using a combination of high-resolution respirometry and fluorescence spectroscopy, mitochondrial respiration, membrane potential, $NAD(P)H/NAD^+$, vH_2O_2 emission, cytochrome *c* flux, and ATP synthase were measured across a ΔG_{ATP} span. Additional measures included ADP and FCCP titration experiments, all performed with multiple carbon substrate combinations.

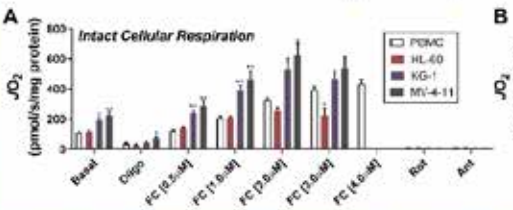
Results: Results revealed three key insights related to substrate preference, electron transport regulation, and ATP synthase in leukemia. 1) Contrary to PBMCs, respiration supported by glycerol-3-phosphate was completely absent in leukemia. 2) When isolated mitochondrial respiration experiments were performed in the presence of ΔG_{ATP}, the addition of FCCP following oligomycin, failed to stimulate respiratory rate. Parallel ADP titration experiments revealed that this effect was dependent on ATP free energy, suggesting that electron transport flux may be allosterically and/or post-translationally blunted by ΔG_{ATP} in leukemia. 3) Despite no change in oligomycin sensitive respiration using intact HL-60 and KG-1 cells, maximal respiratory mechanisms in isolated mitochondria was ~ 2.5-fold higher in KG-1. Remarkably, in HL-60 mitochondria exposed to ΔG_{ATP}, mitochondrial membrane potential assumed dormant activity by ATP synthase reversal. These data argue against the currently accepted experimental paradigm across scientific disciplines that oligomycin-sensitive respiration in intact cells reflects bioenergetic efficiency or coupling.

Conclusions: The collective findings support the necessity of comprehensive mitochondrial phenotyping using physiological ATP free energies in order to draw conclusions about mitochondrial health in cancer. DOD-W81XWH1810213

Mitochondrial Diagnostics

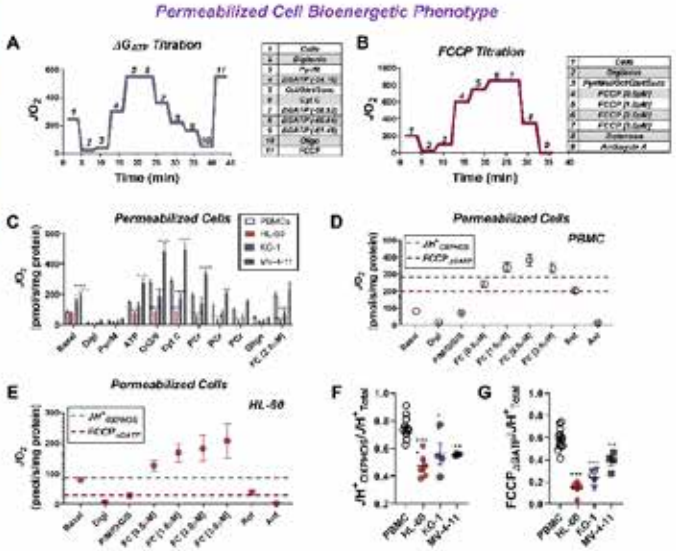


Intact Cell Bioenergetic Phenotype



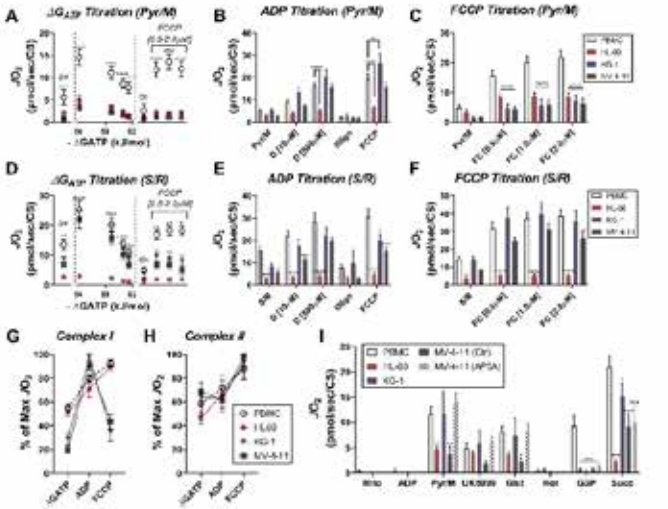
The spare reserve capacity of electron transport is universally blunted in leukemia cell lines. (A) Intact cell respiration was assessed in 3-5 x 10^6 cells in 1mM glucose, 1mM glutamine, 1mM pyruvate, 0.5mM carnitine, 0.2mM malate, 2.5uM oligomycin, 1uM FCCP, and 0.5uM rotenone/antimycin A. (B) Respiration data represented as a percentage of basal respiration. The FCCP value was selected from the maximum recorded value observed during the titration. Across all figures, data are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

Permeabilized Cell Bioenergetic Phenotype



A large portion of the H^+ circuit in leukemic mitochondria does not contribute to ATP synthesis. All assays are performed in sucrose-free mitochondrial respiration buffer (105mM potassium-MES (pH=7.1), 0.25% fatty-acid free BSA, 20mM KCl, 1mM EGTA, 10mM KH_2PO_4, 5mM $MgCl_2$, and 5mM creatine). (A) Computer generated trace depicting oxygen consumption (JO_2) during a ΔG_{ATP} titration protocol in permeabilized cells. Basal respiration (Cells) is recorded prior to digitonin (0.01mg/mL) followed by pyruvate/malate (PyrM, 5mM/2mM), creatine kinase clamp components to establish extramitochondrial ΔG_{ATP} (20U/mL CK, 1mM phosphocreatine-PCr, 5mM ATP), and additional carbon substrates (potassium-α-ketoglutarate/succinate: Oct/Glut/Succ, 0.2mM/1mM/5mM). Cytochrome C (Cyt C) is added to assess the integrity of the inner membrane and PCr is then further titrated to manipulate ΔG_{ATP} followed by oligomycin and FCCP. JO_2 observed with saturating carbon at ΔG_{ATP} of -64.16 kJ/mol is considered to reflect the maximum proton conductance by the OXPHOS system (JH^+_{OXPHOS}). (B) Computer generated trace depicting oxygen consumption (JO_2) during an FCCP titration protocol in permeabilized cells. Maximum JO_2 observed during the FCCP titration is considered to reflect the maximum proton conductance of the respiratory system (JH^+_{max}). Note, there are no adenylates present during this assay. (C) Permeabilized cell protocol involving ΔG_{ATP} titration. (D-E) FCCP titration in permeabilized (D) PBMC and (E) HL-60 cells. Dashed lines represent mean JH^+_{OXPHOS} and the maximum JO_2 elicited by FCCP at the end of the ΔG_{ATP} titration depicted in panel C. (F) Fraction of the total proton conductance attributable to ATP synthesis. (G) FCCP-supported JO_2 in the presence of an extra-mitochondrial ΔG_{ATP} equal to -61.40 kJ/mol relative to maximum uncoupled flux in the absence of adenylates.

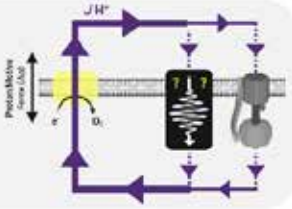
Isolated Organelle Bioenergetic Phenotype



Extramitochondrial ΔG_{ATP} blunts respiratory flux stimulated by an uncoupler in leukemic mitochondria. All assays are performed in sucrose-free mitochondrial respiration buffer (105mM potassium-MES (pH=7.1), 0.25% fatty-acid free BSA, 20mM KCl, 1mM EGTA, 10mM KH_2PO_4, 5mM $MgCl_2$, and 5mM creatine). (A) Relationship between JO_2 and ΔG_{ATP} in isolated mitochondria energized with PyrM, tetrahydrophosphorylating JO_2 (state 4; S4) is recorded prior to the addition of the CK clamp. At the conclusion of the S4 titration, oligomycin is added, followed by an FCCP titration. Note, the FCCP is omitted while the extramitochondrial ΔG_{ATP} is maintained at -61.40 kJ/mol. (B) JO_2 observed in the presence of PyrM, ADP (150μM), AOA (500μM), oligomycin, and FCCP. This assay is performed in the presence of the adenylate kinase inhibitor APSA (0.15mM). Note, FCCP is titrated during the assay but only the maximum respiration rate during the titration is plotted. (C) JO_2 observed in the presence of PyrM and increasing concentrations of FCCP. Note, there are no adenylates present during this assay. (D-F) Identical assay conditions to that depicted in A-C, but with succinate/rotenone (S/R, 10mM/0.5uM/mM) as the substrate combination. (G) Maximum JO_2 recorded during the 2 distinct respiratory protocols (ΔG_{ATP} titration, ADP titration, FCCP titration). Data are depicted as a percentage of the maximum rate from across the 2 assays. Data from 3 substrate combinations are pooled (PyrM, glutamate/malate: 10mM/2mM; succinate/rotenone: 5.0mM/0.5mM). Each of these substrate combinations supplies NADH to complex I. (H) Identical analysis as in panel G, but using only the substrate combination of S/R which supplies electrons directly to the quinone pool via succinate dehydrogenase (complex II). (I) Respiration measured in response to mitochondria alone (veh), ADP (clamped to 0.5mM with the hexokinase clamp), PyrM (1mM/2mM), the pyruvate carrier inhibitor UK5099 (1μM), glutamate (Glut, 10mM), Rot (0.005mM), glycerol-3-phosphate (G3P, 10mM), and succinate (Succ, 10mM). In MV4-11 mitochondria, the assay was repeated, but in the presence of APSA (0.15mM).

Common Bioenergetic Phenotypes of Leukemia:

1) In leukemic mitochondria, ATP synthase conducts only ½ of the available proton current. This may reflect inhibition of the phosphorylation system and/or the presence of additional non-ATP synthase resistors across the inner membrane.
2) Extramitochondrial ΔGATP restricts maximal uncoupled respiratory flux in blood cell mitochondria. This effect is more pronounced in leukemic mitochondria.
3) Leukemic mitochondria are completely refractory to G3P oxidation



Appendix 3

Intrinsic limitations in bioenergetic efficiency characterize the mitochondrial network in leukemia.

Margaret A.M. Nelson[1,2], Kelsey L. McLaughlin[1, 2], and Kelsey H. Fisher-Wellman[1, 2]*
[1]Physiology Department, [2]East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC

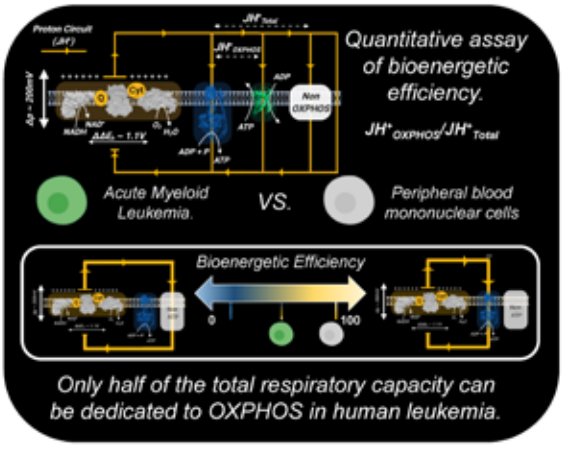
Mitochondrial content and basal respiration have been repeatedly demonstrated to be higher in leukemia. Although these findings are suggested to reflect higher 'reliance' on oxidative ATP production, bioenergetic efficiency in leukemic mitochondria has not been empirically tested. That is, it remains unclear if higher basal respiration in leukemia reflects accelerated demand for ATP regeneration and/or intrinsic limitations in bioenergetic efficiency. Distinguishing between these potential outcomes is critical, as the molecular 'cause' and thus potential treatment strategies are distinct. Herein, we sought to comprehensively evaluate bioenergetic efficiency in blood cancer mitochondria using a discovery-based biochemical platform. This platform encompasses two key technological advancements; 1) comprehensive unbiased analysis similar in scope to traditional 'omics', and 2) utilization of the creatine kinase clamp that allows assays to be performed across

Intrinsic limitations in bioenergetic efficiency characterize the mitochondrial network in leukemia.

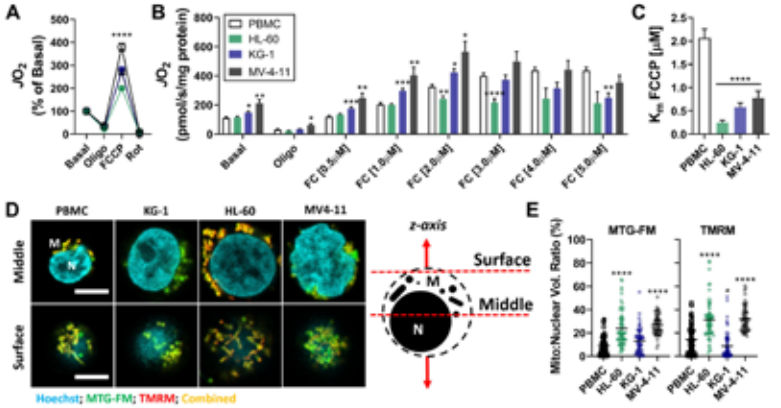
Margaret A.M. Nelson[1,2], Kelsey L. McLaughlin[1, 2], and **Kelsey H. Fisher-Wellman[1, 2*]**

[1]Physiology Department, Brody School of Medicine, [2]East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC, *fisherwellmank17@ecu.edu

Graphical Abstract



Bioenergetic profile of human leukemia cells indicates bioenergetic limitations.



Hoechst; MTG-FM; TMRM; Combined

Fig 1. (A) Respiration in intact leukemia cell lines, compared to PBMC, depicted as a % of basal. (B) Respiration data normalized to total protein. (C) Km for FCCP during the intact cell respiration assay. (D) Representative confocal images of the mitochondrial network in leukemia vs PBMC, assessed via MTG-FM and TMRM fluorescence. (E) Mitochondrial content derived from the ratio of mitochondrial volume (calculated for MTG-FM and TMRM) to nuclear volume (Hoechst). N=15-30/group. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

Decreased bioenergetic efficiency is a hallmark of leukemic mitochondria.

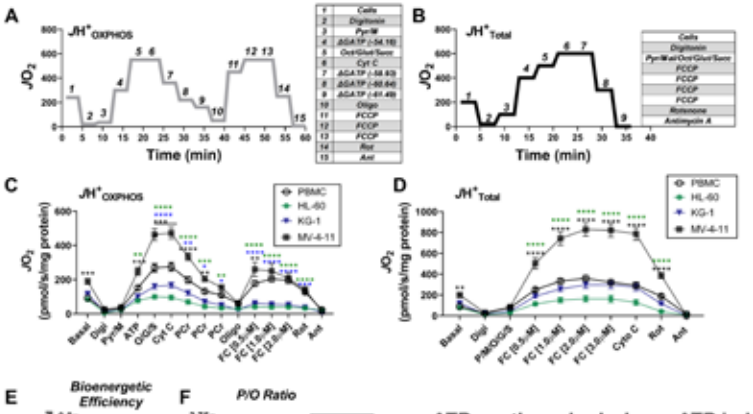


Fig 2. (A) Computer generated trace depicting JO2 during a ΔGATP titration for JH^+_{OXPHOS} quantification. (B) Computer generated trace depicting JO2 during an FCCP titration protocol for JH^+_{Total} quantification in permeabilized cells. (C-D) Respiration data from the PCR titration and FCCP titration protocols in permeabilized cells. (E) Quantified bioenergetic efficiency. (F) Mitochondrial P/O ratio in mitochondria energized with either PyrM or SuccR. N=15-30/group. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

Mitochondrial proteomics predicts G3PDH deficiency.

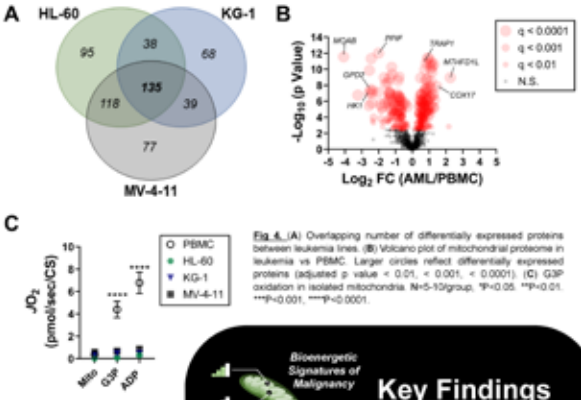


Fig 4. (A) Overlapping number of differentially expressed proteins between leukemia lines. (B) Volcano plot of mitochondrial proteome in leukemia vs PBMC. Larger circles reflect differentially expressed proteins (adjusted p value < 0.01, < 0.001, < 0.0001). (C) G3P oxidation in isolated mitochondria. N=5-10/group. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

ATP synthase hydrolyzes ATP in HL-60 mitochondria.

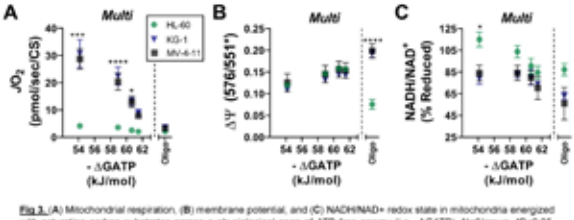


Fig 3. (A) Mitochondrial respiration, (B) membrane potential, and (C) NADH/NAD+ redox state in mitochondria energized with saturating carbon substrates across a physiological span of ATP free energy (i.e., ΔGATP). N=6/group. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

Key Findings

- ***Respiration assays using intact cells are diagnostically limited. Such assays reflect the 'capacity' of respiration and thus are not useful tools to assess OXPHOS efficiency.***
- ***Only 50% of respiratory capacity can be dedicated to ADP phosphorylation in leukemic mitochondria.***
- ***Leukemic mitochondria can NOT oxidize G3P. Potential vulnerability if malate-aspartate shuttle is inhibited.***

Funding: DOD-W81XWH1910213