AWARD NUMBER: W81XWH-19-1-0105

TITLE: Somatic Mutation Rate as Determinant of Breast Cancer Penetrance in BRCA1/2 Familial Cases

PRINCIPAL INVESTIGATOR: Dr. Jan Vijg, PhD

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine REPORT

DATE: MAY 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAY 2023	Annual	1MAY2022 - 30APR2023

4. TITLE AND SUBTITLE

Somatic Mutation Rate as Determinant of Breast Cancer Penetrance in BRCA1/2 Familial Cases

5a. CONTRACT NUMBER
W81XWH-19-1-0105

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Jan Vijg, PhD

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albert Einstein College of Medicine
1300 Morris Park Ave
Bronx, NY. 10461

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

Analysis of how mutations accumulate in pretumor tissue, although widely presumed to occur, has been extremely difficult to study. This is principally because, with most such mutations being unique to individual cells within a tissue, their detection is technically challenging. In this study we propose to apply "Single Cell Multiple Displacement Amplification (SCMDA)" that we recently developed for high accuracy detection of a spectrum of mutations from single nucleotide substitutions to indels and aneuploidy in individual cells within pre-tumor tissues of women who inherited mutations in the *BRCA1* or *BRCA2* genes. We hypothesize that mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy accumulating as consequence of defects in homology dependent DNA repair in mammary epithelial cells are the underlying cause of increased cancer risk in these women. We further hypothesize that estrogen, which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of *BRCA1/2* cancer penetrance by working in concert with the *BRCA1/2* repair defects to increase the somatic mutation rate in the cells of *BRCA1/2* carriers. In **Aim 1**, we will utilize SCMDA to test if mutation frequencies are elevated in individual *BRCA1/2* heterozygous mammary epithelial cells. In **Aim 2**, we will directly test the hypothesis that estrogen increases mutation frequencies in *BRCA1/2* mutant cells.

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			USAMRDC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   Cancer is a genetic disease caused by mutations that accumulate in somatic cells during aging, environmental exposure or other endogenous factors. How these somatic mutations acquired by cells transform them into tumors remains largely unknown. This is due, in part, to the technical difficulties of studying non-clonal casual mutations accumulated in tissues before transformation. In this application we will test the hypothesis that mutations accrued as consequence of endogenous DNA damage caused by high estrogen levels cause increased breast cancer risk. By leveraging *in house* generated approaches including "Single Cell Multiple Displacement Amplification (SCMDA)" we aim to map all forms of mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy using as a model woman who inherited germline mutations in *BRCA1* or *BRCA2* because of their intrinsic defect in the homologous repair (HR) pathway which greatly increase their susceptibility to develop tumors. We hypothesized that estrogen, from endogenous production, contraceptives, pregnancy, hormone replacement therapy, which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of *BRCA1/2* cancer penetrance by working in concert with the HR repair defects to increase the somatic mutation rate in the cells of *BRCA1/2* mutation carriers. Our hypothesis is being tested along two specific aims: in Aim 1, we apply SCMDA to test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* mutation carriers relative to age-matched control women undergoing reduction mammoplasty purely for cosmetic reasons. In Aim 2 we define how the hormonal microenvironment of the mammary epithelium influence genomic instability to promote transformation by acquisition of a cascade of genetic event increasing the risk for tumor transformation. To do so we established a unique collection of primary organoids obtained from *BRCA1/2* mutation carriers or age matched controls which we exposed to estrogen levels mimicking those found in the mammary gland.

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

   Breast cancer, BRCA1, BRCA2, germline mutations, mutations, genomic instability, transformation, estrogen, 17b estradiol, inherited cancer, DNA damage

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.

   Specific Aim 1: Test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* carriers. While it has been proposed that *BRCA1/2* mutation carriers exhibit increased genomic instability due to haploinsufficiency, this has not actually been measured in humans yet. This is because accurate detection of the full complement of mutations in normal somatic cells requires a single cell approach, which has proven to be a major technical challenge. Herein we will take advantage of our recently developed, validated and highly accurate procedures (SCMDA and SVS) to comprehensively determine the frequency and spectrum of small mutations and structural

variations in mammary epithelial cells isolated from *BRCA1/2* carriers before tumor development, relative to age-matched controls undergoing cosmetic reduction mammoplasty.

Specific Aim 2: Determine the effects of estrogen exposure on mutation rate in *BRCA1/2* mutant cells. Epidemiological studies have proposed estrogen exposure as a major modifier of penetrance in *BRCA1/2* carriers. The mechanism remains unknown. We will test if estrogen mechanistically acts as genotoxic agent to alter mutation frequency and/or spectrum in *BRCA1/2* defective cells. In **Aim 2A,** primary 3D mammary organotypic cultures established from the breast tissue of controls and *BRCA1/2* mutation carriers before tumor development will be maintained in the presence or absence of physiological levels of 17b estradiol (E2). Modulation of mutation frequency will be tested in this system using SCMDA and SVS. In **Aim 2B,** we will analyze the sequence of events (i.e. mutation in gatekeeper/caretaker genes) versus loss of heterozygosity (LOH) of the *BRCA1/2* wt allele in conjunction with estrogen exposure in non-transformed cells. Locus-specific LOH as observed in full blown tumors will be introduced using genome-editing technologies in *BRCA1/2* mutant primary mammary epithelial cells, and genomic instability will be quantified using SCMDA and SVS in the presence and absence of estrogen.

What was accomplished under these goals?

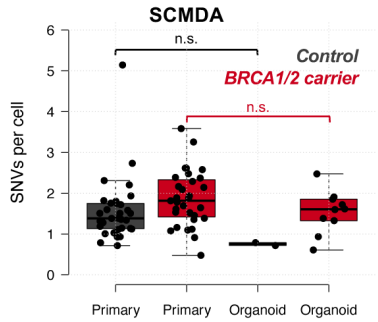
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include 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.

Specific Aim 1: Test if mutation frequency in normal mammary epithelial cells is increased in *BRCA1/2* carriers

Specific Aim 1 accomplishments: Specific Aim 1 is largely completed and a manuscript describing the findings has been published "*Single-cell analysis on somatic mutation burden in mammary cells of pathogenic BRCA1/2 mutation carriers*" in the Journal of Clinical Investigation (PMID: 35025760).

A major outcome of aim 1 was the establishment a unique biobank of primary human organoids from *BRCA1/2* mutation carriers before tumor development and form age matched controls with no history of personal or familial cancer. The breast organoids retain viability over many passages and can be cultured for over 1 month, which allows for exposure to 17b estradiol (E2). To further validate the model, and to ensure that our culture conditions did not introduce significant bias that would prevent the use of 3D organoids for genomic studies (Aim 2), we performed preliminary analyses by Single-Cell Multiple Displacement Amplification (SCMDA) of two single cells from the organoids of a *BRCA1*-wt donor and ten cells from a *BRCA1*-mut carrier and compared the results with those obtained from uncultured cells (**Figure 1**). As in

Figure 1: Comparison of mutation frequency measured in single primary cells and 3D organoids. SNV levels measured by SCMDA in *BRCA1/2* wt controls (dark gray) and *BRCA1/2* mutant (red) cells (each dot corresponds to one single cell). **Left:** mutation frequency in primary cells obtained from the tissue (n=32 *BRCA1/2*-wt cells, n=31 *BRCA1/2*-mut cells). **Right:** single cells obtained from 3D organoids after 4 weeks of culture (n=2 *BRCA1*-wt cells, n=10 *BRCA1*-mut cells).

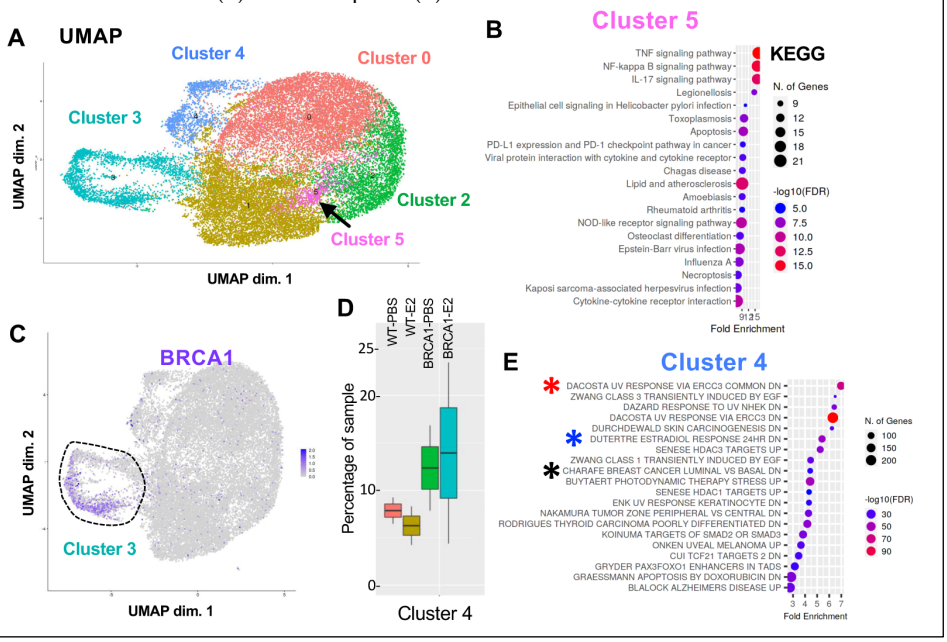


uncultured cells, significantly more mutations were detected in 3D organoids from the *BRCA1*-mut carriers, but we observed no statistical differences between primary cells and those obtained from the organoids. These results validate our protocol of 3D mammary organoids.

Specific Aim 2: Determine the effects of estrogen (E2) exposure on mutation rate in *BRCA1/2* mutant cells.

Specific Aim 2 accomplishments: To mimic the exposure of mammary epithelial cells to estrogen levels present in the breast tissue we exposed organoids derived from *BRCA1*-wt or *BRCA1/2*-mut carriers to estradiol for 4 weeks. E2 in the breast tissue has been reported to have higher concentration than the circulating levels. To remain within physiological levels, we established a regiment of hormonal treatment of 20nM E2 for 4 weeks (the circulating levels during the second trimester of pregnancy). Our pipeline for establishing the 3D organoid model of E2 exposure is now fully established; efforts are continuing to expand the cohort.

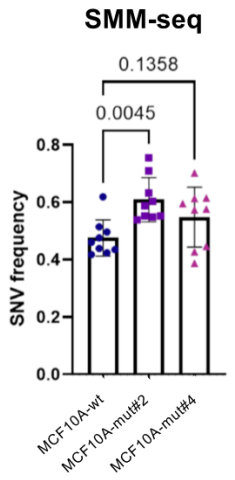
Figure 2: sc-RNA-seq analysis of human 3D organoids. Preliminary analysis of *BRCA1-wt* and *BRCA1*-mut organoids grown in the presence or absence of 20mM E2. **A)** Uniform Manifold Approximation and Projection (UMAP) depicting five epithelial clusters identified based on aggregate gene expression analysis. **B)** Genes markers expressed in Cluster 5 are enriched for KEGG pathways associated with TNF and NF-κB signaling. **C)** t-SNE projection of cells that express *BRCA1* point to cluster 3. **D)** Percentage distribution of cells comprising cluster 4 across samples. **E)** Cluster 4 is enriched for the expression of basal markers (*) and is associated with DDR (*) and E2 response (*).



We performed sc-RNA-seq using single cells dissociated from 3D organoids established from two *BRCA1*-wt and two *BRCA1*-mutant donors, grown in the presence or absence of 20nM E2 (**Figure 2**). While we continue the analysis of the data set, some observations underscore the strengths of 3D organoids to study loss of *BRCA1/2* function. Five clusters, all of epithelial origin, could be identified (**Figure 2A**). Cluster 5 points to cells enriched in gene ontology terms significantly associated with TNF and NF-κB signaling (**Figure 2B**). Cluster 3 defines cells in which *BRCA1* is expressed (**Figure 2C**). Most importantly cluster 4 is associated with GO terms enriched in basal cells (**Figure 2D-E**). This cluster is expanded in *BRCA1* mutant cells as previously reported. Most notably, Gene Set Enrichment Analysis (GSA) of the core markers expressed in cluster 4 points to curated GSA signatures M11171 and to M2156, core sets of genes upregulated as consequence of loss of DNA repair function and response to estrogen respectively. The relative size of cluster 4 measured as percentage of cells in the cluster normalized to sample size varies across genotypes (*BCRA1*-wt vs *BRCA1*-mut) but most notably across treatment (PBC vs 20nM E2)(**Figure 2D**).

SMM-SEQ TO DETECT SNVS AND LARGE-SCALE STRUCTURAL VARIANTS ACROSS CELL SUBTYPES: While SCMDA is highly sensitive, it is based on single cell whole genome analysis and requires the sequencing of each individual patient reference genome as well as each single cell at ~30X coverage. We normally sequence 3-5 cells per individual, thus the assay is not applicable to the analysis of multiple cell types from the same individual. We have therefore explored the possibility to adapt a new methodology recently developed by partnering PI Dr. Jan Vijg (Albert Einstein college of medicine) named single-molecule mutation sequencing SMM-seq (PMID: 35394831). SMM-seq is a novel two-step library preparation protocol validated using MCF10A isogenic *BRCA1-wt* and *BCRA1-mut* cell lines (**Figure 3**). Rolling circle-based linear amplification (RCA) is utilized to produce single-stranded DNA (ssDNA) molecules composed of multiple concatemerized copies of equally represented DNA strands of each DNA fragment followed by multiple cycles of denaturation-annealing-extension in a reaction we termed pulse-RCA. Since all these copies are independent replicas of the original DNA fragment, potential errors of amplification remain unique for each copy and do not propagate further. Copies of opposite strands are in an end-to-end orientation and separated by common spacers used as PCR priming sites during the second step of the process when concatemerized copies are individually amplified and converted into a sequencing library. Thus, the resulting sequencing library is composed of PCR-duplicates of multiple independent copies of an original DNA fragment assembled in RC-amplicons. Sequencing reads originating from the same fragment are recognized based on unique molecular identifiers (UMIs) introduced as part of hairpin-like adapters during library preparation. UMI families composed of reads originating from both strands of the original fragments are then used to identify the consensus sequence of each fragment. Consensus calls different from the corresponding positions on the reference genome are compared with a list of single nucleotide polymorphisms (SNPs) of this DNA sample as well as with dbSNP. This allows to filter out germline variants and identify potential *de novo* somatic mutations. A list of germline SNPs is obtained by analysis of conventional sequencing data of the same DNA sample performed in parallel with SMM-Seq. The resulting list of potential somatic SNVs is further filtered to exclude low confidence candidates and then saved for further analysis.

Figure 3: Validation of SMM-seq. Frequency of somatic SNVs in MCF10A *BRCA1*-wt cells (left), and two independent MCF10A clones (#2) and (#4) heterozygous *BRCA1*-mutant (*BRCA1*-185 Del AG). Each dot represents an independent SMM-seq assay. Median and standard error are plotted.



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.

To our knowledge, no single cell whole genome sequencing technologies have been applied to the analysis of 3D organoids established from human primary tissues. The analysis of these samples provided a unique dataset to apply and develop analytical tools for students and postdoctoral trainees in the Dr. Vijg laboratory. Collection of primary mammary tissues provided postdoctoral trainees in Dr. Montagna's laboratory opportunities to validate the 3D in culture model using single cell RNA sequencing methodologies.

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.

Publication of *Single-cell analysis on somatic mutation burden in mammary cells of pathogenic BRCA1/2 mutation carriers*" was published in the Journal of Clinical Investigation (PMID: 35025760).
Poster entitled: "Estrogen Induced Somatic Genomic Instability as a Modulator of Breast Cancer Penetrance in BRCA1/2 Mutation Carriers" by Yi Zhang, Shixiang Sun, Moonsook Lee, Ben H Park, Jan Vijg and Cristina Montagna was presented at the 2022 -Mammary Gland Biology
Gordon Research Conference entitled "Normal Breast Biology and Its Relationship to Breast Cancer Initiation and Progression".

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.

Due to Dr. Montagna relocation to the Rutgers Cancer Institute of New Jersey work on this project was suspended between May1st 2021 and April 30th 2022. This was due to the relocation of Dr. Montagna's laboratory and to allow for the award to be transferred to Rutgers. Work has fully resumed and in the next period of time we plan on completing the goals of the project as originally proposed under a no cost extension agreement.

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).

Nothing to report

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.

Nothing to report

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

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.*

Nothing to report

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:*

There were no major changes to the experimental approach.

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.

We do not anticipate problems or delays as the execution of the experiments is now ongoing.

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.

In addition to delays caused by the PI relocation to the Rutgers Cancer Institute of New Jersey, work on this project was substantially affected by the pandemic caused by SARS-CoV-2. While most laboratory activities resumed in June-July 2021, most institutions, including Rutgers, still operated with limited and staggered shift personnel to maintain safe physical spacing. As a result, we experienced delays and we were unable to perform experiments with the timelines initially proposed.

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

Nothing to report

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).*

Sun S, Brazhnik K, Lee M, Maslov AY, Zhang Y, Huang Z, Klugman S, Park BH, Vijg J, Montagna C. Single-cell analysis of somatic mutation burden in mammary epithelial cells of pathogenic BRCA1/2 mutation carriers. J Clin Invest. 2022 Mar 1;132(5):e148113. doi: 10.1172/JCI148113. PMID: 35025760; PMCID: PMC8884908.

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".

Name: Jan Vijg, no change, NCE

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:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (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*
- *Other.*

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://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) 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.*