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TITLE: Novel In Vivo Genetic and Single-Cell Genomic Analyses for Understanding Congenital Heart Disease

PRINCIPAL INVESTIGATOR: Dr. Lisa Maves

CONTRACTING ORGANIZATION: SEATTLE CHILDREN'S RESEARCH INSTITUTE

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Development Command
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This research project addresses the FY19 PRMRP Topic Area Congenital Heart Disease. Congenital heart disease (CHD) is a birth defect that is characterized by improper cardiac development, resulting in the abnormal structure and function of the heart. It is the most common birth defect, affecting approximately 1 percent of the population at varying severities. Understanding the causes of CHD is critical for military personnel and their families because undiagnosed congenital heart defects can impact health and combat effectiveness. It is estimated that previous studies have thus far accounted for only about 10-20 percent of the genetic contribution to CHDs. Thus, many more human CHD genes, likely about 400 genes, await discovery. A major hurdle that remains for understanding the causes of CHD is the identification and validation of the many human CHD genes that are as yet unknown. If a better understanding of the genetic and molecular bases of CHD could be obtained, then advances in genomic technologies now make it feasible to use genomic screening of military personnel and recruits to identify at-risk individuals. The goal of this research project is to provide increased understanding of the genetic and molecular causes of CHD, leading to more accurate diagnoses of CHD. The hypothesis of this proposal is that we can demonstrate functions for a novel set of CHD-candidate genes in heart development, using in vivo functional and genomic assays in the zebrafish animal model.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

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

Congenital heart disease (CHD) is a birth defect that is characterized by improper cardiac development, resulting in the abnormal structure and function of the heart. It is the most common birth defect, affecting approximately 1 percent of the population at varying severities. It is estimated that previous studies have thus far accounted for only about 10-20 percent of the genetic contribution to CHDs. A major hurdle that remains for understanding the causes of CHD is the identification and validation of the many human CHD genes that are as yet unknown. If a better understanding of the genetic and molecular bases of CHD could be obtained, then advances in genomic technologies now make it feasible to use genomic screening of military personnel and recruits to identify at-risk individuals. The goal of this research project is to provide increased understanding of the genetic and molecular causes of CHD, leading to more accurate diagnoses of CHD. The hypothesis of this project is that we can demonstrate functions for a novel set of CHD-candidate genes in heart development, using in vivo functional and genomic assays in the zebrafish animal model. In Aim 1, we validate functions in heart development for a novel set of 128 human CHD-candidate genes, using in vivo functional screening and characterization in the zebrafish animal model. In Aim 2, we determine whether new CHD genes regulate shared or distinct gene regulatory programs in myocardial cells and other cell types, using single-cell RNA-seq analyses of whole mutant zebrafish embryos. The work proposed takes an innovative approach to discovering and classifying candidate CHD genes.

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

Congenital heart defects, genetics, zebrafish, CRISPR, single-cell RNA-seq

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.

Major Task 1: Screen functions of 128 genes in early zebrafish heart development, using F0 CRISPR screening. Target dates Months 1-3; 40% completion.

Major Task 2: Generate stable mutant strains for genes that show heart phenotypes in Major Task 1 F0 screen. Targets dates Months 3-12; 90% completion.

Major Task 3: Generate single-cell nuclei preps from control and mutant embryos from different classes of heart defect mutant strains. Targets dates Months 13-15; 30% completion.

Major Task 4: Analyze data from sc-RNA-seq experiments. Target dates Months 16-24; 0% completion.

Major Task 5: Preparation and submission of 2 manuscripts for publication. Target date Year 2; 30% completion.

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.

For Major Task 1, the major activities and specific objectives have been to obtain CRISPR guides for 61 of our 128 genes (Subtask 1) and to inject and screen 40 of our 128 genes for heart defects in F0 zebrafish embryos (Subtask 2). For these activities, we synthesized CRISPR guide RNAs in vitro and then injected them into 1-cell-stage zebrafish embryos. Heart development was assessed over the first 5 days of development by imaging myl7-gfp expression in live embryos. The key outcomes are that 13/40 of the genes screened thus far showed defects in heart development. These defects fall into 3 phenotypic classes. These activities thus far are exciting findings for two reasons. First, these results show that about 33% of our candidate genes for heart defects have requirements in zebrafish heart development. Second, these results point to important roles for proteasome genes in heart development and heart defects, since our efforts identified pomp (proteasome maturation factor), psmd6 (proteasome subunit), and psma6 (proteasome subunit) as required for heart development in zebrafish.

For Major Task 2, the major activities and specific objectives have been to generate stable mutant strains for the zebrafish pomp and psmd6 genes (Subtask 1). We used CRISPR to generate an allelic series of pomp and psmd6 strains (Table 1). To generate a spectrum of alleles, we used different CRISPR guide RNAs to target mutations at different sites within pomp and psmd6. We also used a CRISPR approach to delete the 5' end of each gene (Table 1). With the 5' end deletions, we increase the chance of generating null alleles. We used PCR-based approaches and DNA sequencing to define the mutations in our new strains and generated genotyping protocols (Table 1; Subtask 2).

Table 1 New zebrafish mutant strains for proteasome genes

***pomp* mutants generated**		
Strain	Mutation position	Mutation type
pomp scm35	Exon 2	Mis-sense with early stop codon at aa 38 (of 142 amino acids)
pomp scm37	Exon 4	Mis-sense with early stop codon at aa 83
pomp scm41	TSS (Transcription start site) deletion	2.4kb deletion removing 5'UTR and exon 1
***psmd6* mutants generated**		
Strain	Mutation position	Mutation type
psmd6 scm42	Exon 1	Mis-sense with early stop codon at aa 22 (of 386 aa)
psmd6 scm44	Exon 5	Mis-sense with early stop codon at aa 279
psmd6 scm39	TSS deletion	1.8kb deletion removing 5'UTR, exon 1, and exon 2

Also for Major Task 2, we have validated and characterized the heart defect phenotypes for pomp and psmd6 (Subtasks 2 and 3). We find that breeding pomp scm35+/- fish generates clutches in which the pomp scm35-/- embryos show cardiac edema, brain hemorrhage, craniofacial, and heart looping phenotypes (Fig. 1A-D). We also find that psmd6 scm44+/- fish generate clutches in which the psmd6 scm44-/- embryos share very similar phenotypes as the pomp scm35-/- embryos, including cardiac edema and heart looping defects (Fig. 1E-H). These results show that we have successfully generated mutant strains for pomp and psmd6 and that these proteasome genes share critical roles in heart development.

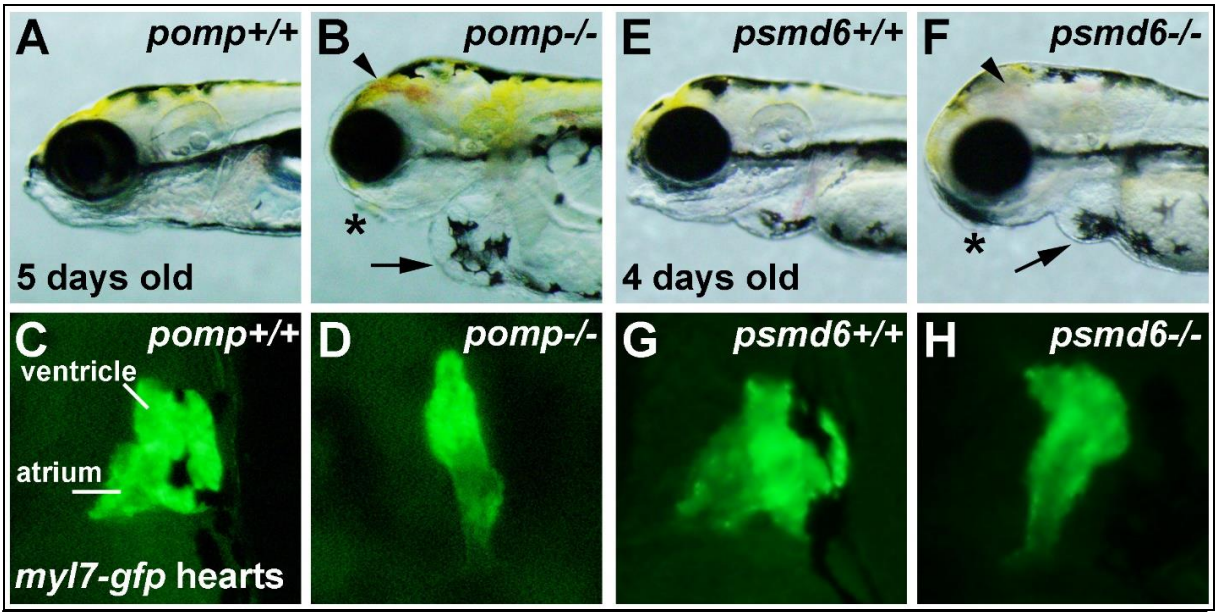

Fig. 1. (**A-D**) *pomp scm35* embryos. (A) 5 day-old wild type, (B) *pomp* mutant. (C) Right-side view of hearts labeled with *myl7-gfp* in 5 day-old control or (D) *pomp* mutant embryo, with un-looped heart. (**E-H**) *psmd6 scm42* embryos (E) 4 day-old wild type, (F) *psmd6* mutant. (G) Hearts in 4 day-old control or (H) *psmd6* mutant embryo, with un-looped heart. Arrows, pericardial edema. Arrowheads, blood pooling in brain. *, reduced craniofacial structures.

For Major Task 3, the major activities and specific objectives have been to continue to optimize our preparation of single-cell nuclei from whole zebrafish embryos (Subtask 1). This is a critical step in our efforts to do sc-RNA-seq on our mutant zebrafish embryos. Although we have not yet progressed as far as we had anticipated with Major Task 3, our protocols now put us in a great position to make progress on Major Tasks 3 and 4.

For Major Task 5, the major activities and specific objectives have been to continue work on a manuscript for publication. This manuscript will describe our results thus far from Major Tasks 1-3, as well as our PPI network analysis from Specific Aim 2. The title of the manuscript is “Systems genetics analysis identifies novel roles for proteosome factors in heart development and congenital heart defects”.

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.

Nothing to Report.

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.

For Specific Aim 1, we plan to complete our CRISPR screen and identify major phenotypic classes of heart development phenotypes.

For Specific Aim 2, we plan to perform single-cell RNA-seq and data analysis on mutant embryos from 2-3 different phenotypic classes from our screen, including from pomp mutant embryos (proteasome gene class).

For Major Task 5, we plan to submit the manuscript currently in preparation and prepare a second manuscript on the completion of our CRISPR screen.

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).

Thus far, the significant impact of this project is the advancement of our understanding of the causes of congenital heart defects (CHDs). Our zebrafish CRISPR screening shows that we are identifying several new genes with likely roles in CHDs in humans. We have also identified a new family of genes, proteasome factors, that are required for proper heart development. Our network analysis suggests that these proteasome factors may interact with genes like Notch1 that are already known to cause congenital heart defects. We expect that our studies will advance our understanding of the causes and diagnoses of CHDs.

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.

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.*

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:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

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.

Because of general delays and disruptions during the COVID pandemic, we were not able to make as much progress as originally expected on our single-cell RNA-seq studies in Specific Aim 2. We expect to be able to continue progress on this in the coming months.

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.

Our Animal Per Diem expenditures continued to be about half of what we had projected (about $15k vs $30k). This is in spite of generating several new strains (Table 1).

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

Not Applicable.

Significant changes in use or care of vertebrate animals

We had no significant deviations, outcomes, or changes in the care of, or procedures used on, our zebrafish vertebrate animals.

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).*

Nothing to Report.

  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".

<u>Example:</u>

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: Lisa Maves
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0002-9798-790X
Nearest person month worked: 1
Contribution to Project: Dr. Maves has performed work in the area of Specific Aim 1 in analyzing CRISPR screening data and generating and maintaining new mutant zebrafish strains and in the area of Specific Aim 2 in obtaining single cell nuclei protocols and analyzing PPI network analyses. Dr. Maves has also worked on preparing a manuscript for publication.
Funding Support: NIH, Additional Ventures, Seattle Children's Research Institute

Name: Gist Hank Farr III
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Mr. Farr has performed work in the area of Specific Aim 1 in preparing CRISPR guides, performing CRISPR screening, analyzing CRISPR screening data, generating genotyping protocols, and generating and maintaining new mutant zebrafish strains. Mr. Farr has performed work in the area of Specific Aim 2 in testing and optimizing single cell nuclei protocols.
Funding Support: NIH, Additional Ventures, Seattle Children's Research Institute

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.

The following awards providing other support have been activated in the past year.

Title: Defining Novel Roles for Proteasome Factors in Heart Development
Time Commitment: 1.2 calendar months (Maves PI)
Funding Agency: Additional Ventures
Performance Period: 01/15/2022 – 01/14/2025
Level of Funding:
Goal: The goal of this proposal is to use novel zebrafish and mouse mutant strains to characterize the roles of five proteasome factors in embryonic heart development
Specific Aims: Aim 1: determine the novel functions of a set of five proteasome factors in zebrafish and mouse heart development. Aim 2: determine how proteasome factors interact with Notch and Hedgehog pathways in heart development, using genetic, pharmacologic, and scRNA-seq approaches.

Title: Proteomics Analyses of Proteasome Factors in Zebrafish Heart Development
Time Commitment: 1.2 calendar months (Maves PI)
Funding Agency: Additional Ventures
Performance Period: 07/01/2022 – 06/30/2023
Level of Funding:
Goal: The goal of this project is to perform global proteomics analyses in zebrafish proteasome mutant embryos that exhibit defects in heart development.

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*
- *Other.*

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://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.*

Not Applicable.

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.*

No appendices attached.