AWARD NUMBER: W81XWH-18-1-0243

TITLE: Role of Osteopontin in Hepatocellular Carcinoma

PRINCIPAL INVESTIGATOR: Natalia Nieto

CONTRACTING ORGANIZATION: The Board of Trustees of The University of Illinois
The University of Illinois at Chicago

REPORT DATE: August 2020

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
August 2020	Annual	**07/15/2019 - 07/14/2020**

4. TITLE AND SUBTITLE
Role of Osteopontin in Hepatocellular Carcinoma

5a. CONTRACT NUMBER
W81XWH-18-1-0243

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Natalia Nieto

E-Mail: nnieto@uic.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

The Board of Trustees of The University of Illinois
The University of Illinois at Chicago

8. PERFORMING ORGANIZATION REPORT

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project aims to dissect the molecular mechanisms whereby Osteopontin (OPN) drives hepatocellular carcinogenesis and progression, to fill the gap in our knowledge on the pathogenesis of hepatocellular carcinoma (HCC). Our main hypothesis was that OPN signals via CD44 to inhibit DNA repair, apoptosis and the cell cycle by reducing p53 signaling. Our results we have shown that both overexpression and ablation of OPN in hepatocyte drive the onset of HCC. In mice lacking OPN, we observed DNA hyper-methylation and reduction of the response to the DEN chemical carcinogen, followed by increased cancer stem cells (CSCs) at 5 months. In mice overexpressing OPN in hepatocytes, we did not find an increase in the number of CSCs at 5 months, suggesting that high levels of OPN would rather stimulate the progression of CSCs to HCC. The last reporting period will address this hypothesis. Finally, we showed that the impact of OPN on HCC was independent from CD44.

15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU		19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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

Our **overall objective** is to dissect the molecular mechanisms whereby Osteopontin (OPN) drives hepatocellular carcinogenesis and progression, to fill the gap in our knowledge on the pathogenesis of hepatocellular carcinoma (HCC), a disease affecting the general population, which has a particularly profound impact on the health and well-being of military service members and US veterans.

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

Hepatocellular carcinoma, Osteopontin, cancer stem cells, diethylnitrosamine, CD44, p53

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 was to dissect if OPN binding to CD44 in hepatocytes inhibits DNA repair, apoptosis and the cell cycle by blocking p53, using the diethylnitrosamine (DEN) model at early time-points (24 and 48 h). We planned to address Specific aim 1 during the first 6 months of the project. In addition, we analyzed the impact of *Opn* overexpression or ablation on DNA methylation.

Specific aim 2 was to establish if hepatocyte-derived OPN stimulates the emergence of cancer stem cells (CSCs) and increased their maintenance and proliferation. We planned to address Specific Aim 2 during months 5 to 20 of the project.

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 was completed. We also analyzed the impact of *Opn* overexpression or ablation on DNA methylation. Specific Aim 2.1) was completed by analyzing the number of CSCs after 5 months in all groups of mice. The experiments for Specific Aim 2.2) are ongoing. We successfully transplanted the CSCs into our mice and started to monitor them for the development of HCC. In addition, we analyzed the tumor burden after 12 months of DEN injection in mice with *Opn* conditional ablation ($Opn^{\Delta Hep}$) or overexpression in hepatocytes (Opn^{Hep} Tg), with or without global ablation of *Cd44* ($Cd44^{-/-}Opn^{Hep}$ Tg). Below are our main findings:

1) Analysis of the tumor burden after 12 month of DEN injection in the different groups of mice shows that: **a)** both ablation and overexpression of *Opn* in hepatocyte increase the number of tumors; and **b)** this is independent from CD44, as *Cd44* ablation does not protect from the effect of OPN.

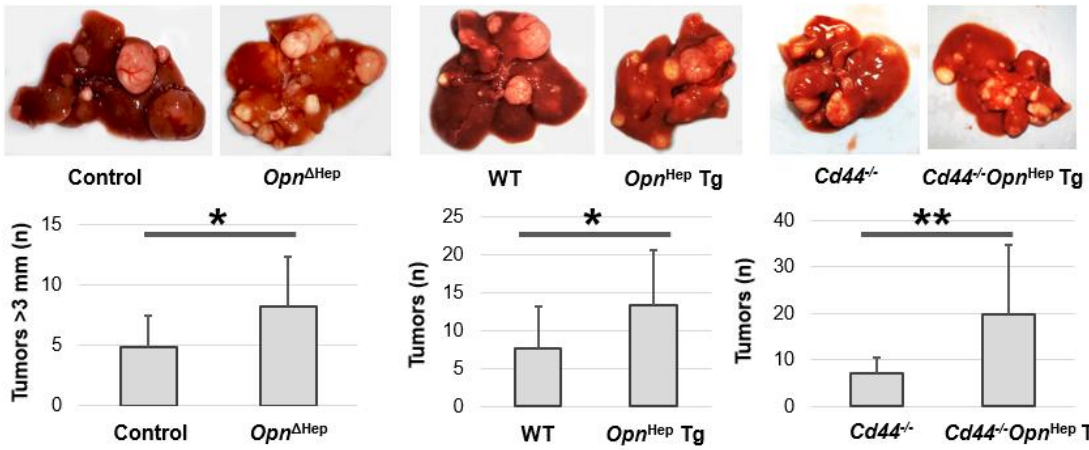

Figure 1. Representative gross appearance of livers from control (n=13), *Opn*$^{\Delta Hep}$ (n=17), WT (n=15), *Opn*^{Hep} Tg (n=22), *Cd44*$^{-/-}$ (n=14) and *Cd44*$^{-/-}$*Opn*^{Hep} Tg (n=14) mice injected DEN and sacrificed after 12 months and corresponding number of tumors per group. *p<0.05; **p<0.01.

2) *Opn*$^{\Delta Hep}$ mice showed an increase in global DNA methylation, independent of DEN injection (**Figure 2A**) and show reduction in the response to DEN at 48 h (**Figure 2B**) and higher number of CSCs at 5 months (**Figure 2C**), suggesting early onset of HCC.

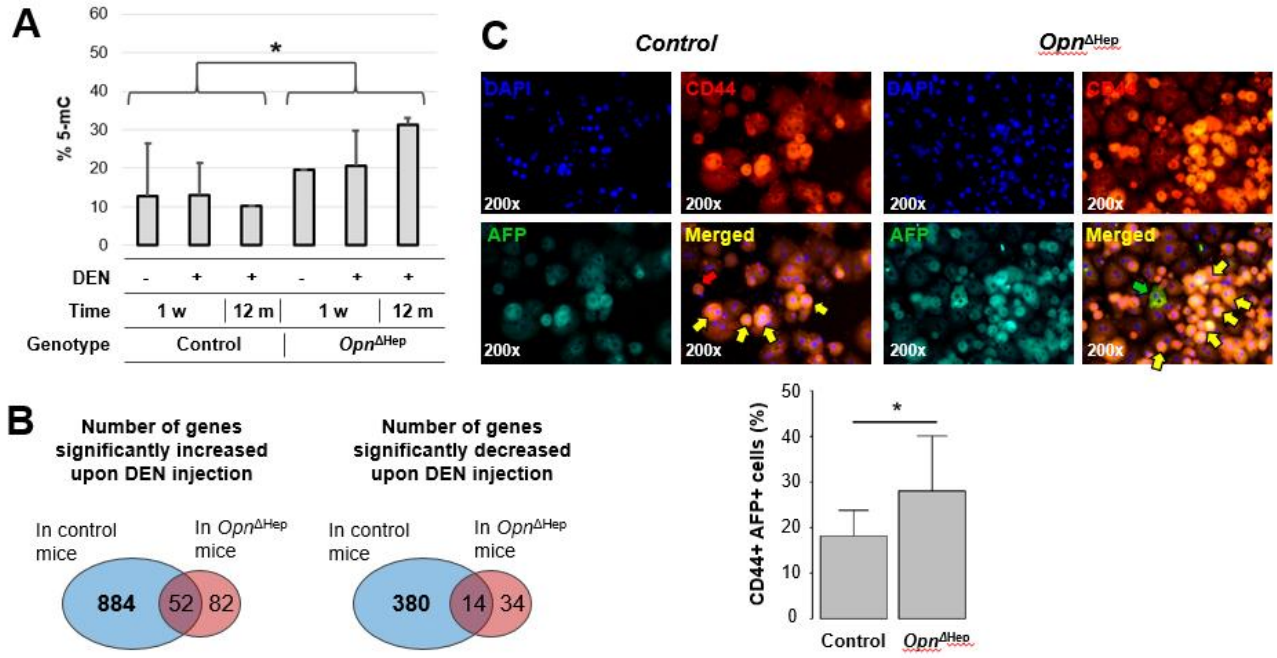

Figure 2. Global DNA methylation in control and *Opn*$^{\Delta Hep}$ mice 1 week or 12 months after DEN or PBS injection, assessed by LINE-1 (**A**). Venn diagram showing the number of differentially expressed genes in control and *Opn*$^{\Delta Hep}$ mice 48 h after DEN injection (**B**). Quantification of the number of CSCs in control and *Opn*$^{\Delta Hep}$ mice 5 months after DEN injection, assessed by immunohistochemistry for CD44 (red) and AFP (green). Yellow arrows, CSCs. *p<0.05

3) *Opn*^{Hep} Tg mice did not show changes in DNA methylation or an increase in the number of CSCs at 5 months, suggesting that the effect of high hepatocyte-derived OPN on HCC occurs at ;ater stages and may increase the progression of CSCs to HCC. To prove this hypothesis, we have started an experiment where we injected CSCs to WT and mice with global ablation of *Opn* (*Opn*$^{-/-}$) to monitor the effect of OPN on the progression of CSCs to HCC. This experiment was included in the original **Specific Aim 2**. We also planned to perform *in vitro* experiments with CSCs to study the effect of recombinant OPN on these cells. This was also included in **Specific Aim 2**. These ongoing experiments will be completed during the last reporting period.

Discussion: the overall objective of the project was to dissect the molecular mechanisms whereby OPN drives hepatocellular carcinogenesis and progression. During the two first reporting periods, we studied the potential contribution of CD44 and observed ablation and overexpression of *Opn* in hepatocytes had a similar effect although through different mechanisms. While *Opn* ablation affects the HCC onset, potentially through DNA methylation; *Opn* overexpression increases the number of CSCs transformation towards HCC. This divergence may be explained by different roles of intracellular compared to extracellular OPN. Indeed high expression of OPN, as it occurs in *Opn*^{Hep} Tg mice, enhances secretion of the protein and activation of extracellular signaling such as integrins. On the other hand, low expression of intracellular OPN, as it occurs in *Opn*$^{\Delta Hep}$ mice, appears to have a strong impact on hepatocyte behavior, based on the RNAseq data and the DNA methylation assay. This could be due to a physiological role of OPN in hepatocytes, which remains poorly understood.

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.

Dr. Desert worked as a postdoctoral fellow on this project and did most of the experiments during the reported period. He worked closely with Dr. Nieto that led the project. Dr. Desert was trained and assisted by Daniel Lantvit in the breeding and genotyping of mice as well as in the development of the mouse models and the writing of the IACUC protocols. This project has provided Dr. Desert skills in basic biology, biochemistry, animal models, data analysis and increased knowledge in the field of HCC biology. He also presented his data at the AASLD Meeting and at our Work in Progress seminars.

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

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.

1) Finish **Specific Aim 2** by monitoring for the development of HCC in mice transplanted with CSCs. This experiment will be completed within the next 6 months.
2) Perform the *in vitro* experiments as originally planned in the application.
These two experiments will address the hypothesis that *Opn*^{Hep} Tg mice induce the progression of CSCs to HCC. It will investigate the role of extracellular OPN on liver carcinogenesis.
3) Consolidate the results from *Opn*$^{\Delta Hep}$ mice by validating the changes in DNA methylation with alternative approaches (5hmC immunohistochemistry, MBD1, DNMTs and TET qPCR, etc.).
4) Submit the manuscript.

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).

Our results highlighted hepatocyte-derived OPN as a regulator of DNA methylation, response to a chemical carcinogen and early carcinogenesis. This improves our understanding of liver carcinogenesis and could improve the therapies and/or the monitoring of patients with HCC in the future.

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.

Our research suggest a major role for OPN in hepatocyte under physiological conditions, which was widely underestimated. This improves our comprehension of hepatocyte biology and could lead to multiple research projects in the future, potentially improving the therapies and/or the monitoring of patients with various liver diseases.

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:*

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.

Due to the COVID19 pandemic, we closed the lab four months. At the time of the submission of this progress report, lab operations have resumed at 50% capacity and a no-cost extension has been requested and approved to conclude the pending experiments.

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.

Nothing to report

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

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).*

A manuscript is currently in progress. A review on the role of OPN in chronic liver disease is under revision.

  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.

None

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

None

- **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.

None

- **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.*

None

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: Romain Desert
Project Role: Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12 months
Contribution to Project: Ms. Desert has participated to inject and sacrifice the mice. He also actively maintained and bred the mouse colonies. He has analyzed the samples to address Specific Aim 1 and Specific Aim 2. He has presented some of the data at conferences and seminars. He is currently preparing a manuscript.

Name: Daniel Lantvit
Project Role: Technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 4.2 months
Contribution to Project: Ms. Lantvit has assisted in maintaining and breeding the mouse colonies and has provided technical assistance for the sacrifice of the mice needed for specific aim 1 and specific aim 2.

Name: Natalia Nieto
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.8 months
Contribution to Project: D. Nieto directed the project, analyzed data and contributed to writing and editing the manuscript.

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*
- *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://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.*