AWARD NUMBER: W81XWH-13-2-0097

TITLE: Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation

PRINCIPAL INVESTIGATOR: Jeffrey M. Gimble MD PhD

CONTRACTING ORGANIZATION: Tulane University
New Orleans, LA 70118

REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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**Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation**

6. **AUTHOR(S)**
   Jeffrey M. Gimble MD PhD

   Email: jgimble@tulate.edu

7. **PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
   Tulane University
   New Orleans, LA 70118

9. **SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
   U.S. Army Medical Research and Materiel Command
   Fort Detrick, Maryland 21702-5012

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

14. **ABSTRACT**
    attached

15. **SUBJECT TERMS**
    Nothing listed

16. **SECURITY CLASSIFICATION OF:**
   a. **REPORT**  U
   b. **ABSTRACT** U
   c. **THIS PAGE** U

   17. **LIMITATION OF ABSTRACT**
   UU

   18. **NUMBER OF PAGES**
   15

   19a. **NAME OF RESPONSIBLE PERSON**
       USAMRMC

   19b. **TELEPHONE NUMBER** (include area code)
abstract

Over the past decade, improved personal protective equipment and medical support has reduced combat fatalities substantially among wounded war fighters. As a result, survivors are more likely to present with severe trauma to their arms and legs that will need multiple reconstructive surgeries or amputation during their recovery. The orthopaedic doctors caring for these wounded service personnel have been concerned by the fact that over 60% of these patients go on to form abnormal bone within the soft tissue of their injured limbs. This condition, known as Heterotopic Ossification (HO), causes pain, loss of mobility, and often requires additional surgeries to remove the rock hard tissue that has replaced their fat and muscle. While there are theories to explain why HO might occur, doctors still do not fully understand the mechanism(s) causing this disorder. Without knowing the mechanism, doctors find it difficult to predict which patients might be at risk for developing HO or to decide which drugs or treatments to use that would prevent HO from happening in these patients. The currently available treatments for HO have many undesirable side effects which can complicate the overall recovery process.

The Specific Aims of this Idea Development proposal address these important questions by using blood samples collected from wounded warriors and civilians with bone injuries. The study will compare the blood samples between patients who either have or have not developed HO during the first year after their injury. The first experiments will ask, does the blood or wound fluid contain any proteins that can stimulate fat or muscle cells to form bone in the laboratory? This will test whether patients with HO have factors circulating in their blood or around the wound that specifically stimulate bone formation as compared to patients without HO. If this proves true, it will be an important step forward in understanding how HO occurs. The second experiments will ask, what is the identity of the protein(s) in the HO blood that might cause bone to form? The study will use a state of the art technique that can analyze all of the proteins in the blood and find out which ones are present. Using computer technology, researchers can then learn the name and function of these proteins of interest. This type of information will be of particular value to the orthopaedic surgeons caring for HO patients. The presence or absence of these proteins in the blood can be used to predict which patients might develop HO or to monitor HO treatment. Also, by knowing the names of the proteins involved in HO, doctors and pharmacists might be able to tell which drugs can be used to prevent HO formation at the time of injury. Wounded warriors and civilians would benefit directly from these advances since doctors would be able to prevent HO with a pill or drug or, at the very least, reduce the number of surgeries required to treat the condition when it happens. There would be minimal risk to wounded war fighters and civilian patients enrolled in this study. Patients would only be required to provide several extra tablespoons of blood to doctors during the weeks to months following their injury. This might cause a bruise but no other complications and would not interfere with their recovery in any way. It is predicted that this information could be used to improve patient care within 5 years or less after the study is completed. As a result, war fighters recovering from blast injuries in the future will have a better outlook than today’s combat casualties. They will no longer have the same high risk of developing HO and can avoid the emotional, psychological
and physical damage sustained as a result of multiple orthopaedic surgical procedures. As a result, the effort, time, and cost of wounded warrior’s recovery from life threatening orthopaedic trauma could be substantially reduced and as such, accelerate their return to active duty.
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Heterotopic Ossification (HO), the ectopic formation of bone in soft tissues, has been found to complicate >60% of extremity war injuries in casualties from Afghanistan and Iraq. Elevated levels of circulating and local cytokines released in response to high energy blast injuries have been found to correlate with the onset of HO, indicating that the disease process will require early intervention; however, preventive therapies such as tissue radiation, bone morphogenetic protein antagonists, and cyclooxygenase inhibitors, carry substantial risks for patients recovering from orthopaedic trauma. Consequently, there is an as yet unmet medical need to develop assays of serum and wound fluid biomarkers to identify those patients at greatest risk of HO progression during their recovery.

The current project is using liquid chromatography/mass spectroscopy, in combination with other cell and protein biological assays, to evaluate the serum and wound fluid from civilian and military orthopaedic trauma patients for the presence of cytokines or factors capable of inducing HO. The studies are focusing on a select set of candidate biochemical pathways (bone morphogenetic, cyclic AMP, Wnt) that have been implicated in genetic models of HO. The research team includes expertise in civilian and military orthopaedic surgery, adipose and bone marrow stromal/stem cell biology, proteomics and mass spectroscopy, and regenerative medicine. During the first year of the project, the scope of the study was expanded to include the analysis of serum samples from established murine (burn) and rat (blast injury) models of HO and these analyses will be used to complement and support the initially proposed studies of human serum.

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

   Adenylate Cycle (AC)
   Adipose-derived Stromal/stem Cells (ASC)
   Bone Marrow-derived Stromal/stem Cells (BMSC)
   Bone Morphogenetic Protein (BMP)
   Fibrodysplasia Ossificans Progressiva (FOP)
   Heterotopic Ossification (HO)
   Liquid Chromatography Mass Spectroscopy (LC/MS)
   Progressive Osseous Heteroplasia (POH)
   Skeletal Muscle Stromal/stem Cells (SMSC)
   Wnt Pathway

**OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended...
manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer’s Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Training Specific Task 1: Mentoring of Dr. O’Brien

Current Objective: This Nested IDEA award included a component relating to the mentoring of Fred O’Brien MD. The intent is to advance Dr. O’Brien’s training as a clinician scientist.

Results, Progress and Accomplishments with Discussion: For several months during 2016, Dr. O’Brien was on active duty at Madigan Military Medical Center in Tacoma WA to cover for a fellow orthopedic surgeon who required medical leave. Despite this anticipated deployment, he made substantial progress during the past year. Dr. O’Brien has completed classwork during the Fall 2015, Spring 2016, and Summer 2016 semesters. On March 15-16, 2016, Dr. O’Brien visited the Tulane University program and presented grand rounds to the Department of Orthopaedic Surgery at LSUHSC-NO and met with his faculty in the Tulane University Masters of Science in Clinical Research program. Dr. O’Brien has taken an independent study class in conjunction with Dr. Gimble and Dr. Mark Hamrick, Director of the Bone Histology Core at the University of Augusta, Augusta GA. At this time, Dr. O’Brien is on track to complete the Master of Science in Clinical Research program in 2017. He is in the process of completing a review article on the topic of heterotopic ossification. Additionally, he has contributed as a participant on a grant application in collaboration with Dr. Hamrick and the Endocrinology Division at University of Augusta.

Research Tasks 2 & 3 (Military and Civilian Serum Samples Collection and Inventory): Current Objective: The intent of these Tasks is to use an existing biorepository of serum samples from orthopaedic trauma patients with and without HO from the Walter Reed National Military Medical Center-Bethesda and to begin developing a comparable biorepository from a similar aged civilian population at the Louisiana State University Health Sciences Center-New Orleans.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. IRB and HRPO approval of all aspects of the study at all of the involved sites has been renewed.
2. Samples from the inventory at Walter Reed National Military Medical Center-Bethesda have been evaluated at the Tulane University site. These human serum samples have been immunodepleted and analyzed by mass spectroscopy laboratory at Ohio State University.
3. Ms. Elaine Boos, study coordinator in the Dept. of Orthopaedic Surgery, LSUHSC-NO, continued to implement protocols and procedures for civilian HO serum collection until leaving the Dept. in June 2016. To date, twenty two
subjects have been recruited and seventeen remain actively enrolled. Eight of the seventeen subjects remaining in the study have developed radiographic evidence of HO in less than one year following the initial trauma event. This corresponds to an incidence of HO in civilian orthopaedic trauma patients of 47% based on those subjects remaining enrolled or 36% based on all subjects originally enrolled in the study. These values are lower than, but comparable to, the 65% incidence of HO observed in military orthopaedic trauma subjects. Ms. Cara Rowe was hired in September 2016 to replace Ms. Boos and will continue to recruit subjects moving forward.

4. An IRB protocol has been approved at LSUHSC-NO to perform a retrospective clinical study to assess frequency of heterotopic ossification in civilian orthopaedic trauma and elective surgical patients. Dr. Harry Molligan (PGY2, LSUHSC-NO) and Matt Fury (Medical Student LSUHSC-NO) are actively participating in this project and are nearing completion of their review of the patient records.

5. Serum collected from the civilian and military patients have been evaluated by mass spectroscopy. Follow up studies are being performed by western blot and ELISA to confirm the mass spectroscopy proteomic findings. Additional ELISA studies will be performed to determine the expression of those cytokines identified in military orthopaedic trauma patient serum samples as predictive of HO development. The ELISA kits required have been received at Tulane University and the assays will be performed within the next 6 to 12 months. Upon completion, this data will be compiled with relevant clinical and radiographic data into a manuscript describing the incidence of HO in a prospective study of civilian orthopaedic trauma.

6. Serum samples collected from murine (burn) and rat (blast injury) models of HO under approved IACUC protocols have been incorporated into the study design. The inclusion of these animal models will provide opportunities to better control the degree of injury and the timing of serum collection and, more important, will provide the team with access to serum samples that can be replaced (unlike the more limited and therefore valuable human specimens). Dr. Benjamin Levi (University of Michigan) has provided access to his murine burn model of HO while the rat blast injury model has been developed in the laboratories of Drs. Davis and Forsberg at Naval Medical Research Center. Mass spectroscopy of the rat serum samples has been completed and preliminary analyses demonstrate similarities to the findings obtained in the human serum. Additionally, tissue samples from the rat blast injury model, collected under the approved IACUC protocol, are being processed at Ohio State University for mass spectroscopy analyses. It is anticipated that these samples will provide complementary information to the serum analyses already underway.

Research Tasks 4-6 (Osteoconductive and Osteoinductive Biochemical and Cell Based Assay Evaluation with Serum Stimulation):
Current Objective: The intent of these Tasks is to develop biochemical and cell based assays for detection of factors activating signal transduction pathways associated with osteogenesis.
Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. Dr. Martin in collaboration with Ms. Claire Llamas (Tulane) and Dr. Ammar Qureshi (NMRC) completed the PCR and western blot studies with human ASC following induction with BMP, forskolin, PMA and IL-6 exposure as well as pooled human serum from military and civilian subjects with or without HO development. Studies have identified the AP1 transcription factors and the MAPK/ERK signal transduction pathway as early responsive biomarkers of osteogenic differentiation in the ASC. The final version of a manuscript describing these findings has been reviewed and approved by all co-authors. The manuscript is now being submitted for review and clearance approval at the Naval Medical Research Center. Pending approval, this manuscript will be submitted for publication to the Journal of Bone and Joint Surgery (Am). Parallel studies are nearly complete using the same inductive factors to evaluate the response of human bone marrow mesenchymal stem cells (BMSC) and human muscle derived stromal cells. Additionally, Dr. Martin and Dr. Qureshi have completed studies evaluating the expression of microRNA regulatory proteins in human ASC and human BSMC, respectively. A manuscript describing these findings is in the final stages of preparation. Once completed, the manuscript will be reviewed and approved by all co-authors prior to submission for review and clearance approval by the Naval Medical Research Center.

Research Task 7 (Manuscript and Oral Presentation of Biochemical and Cell Studies): Current Objective: The intent of this Task is to disseminate information gained through the biochemical and cell based assays relating to osteogenesis to the general scientific community.

Results, Progress and Accomplishments with Discussion:

Dr. Martin and Dr. Gimble (Tulane) along with Dr. Ammar Qureshi and Dr. Tom Davis at NMRC, Dr. Vinod Dasa at LSUHSC-NO, and Dr. Mike Freitas at OSU published a review article on miRNA in the context of adipogenesis, myogenesis, and osteogenesis. The manuscript was published in a special issue of Biochimie focusing on aging, metabolism and obesity that appeared in May, 2016. The paper has been cited twice based on the ISI Web of Science.


A manuscript that reports that AP1 transcription factors and the MAPK/ERK signal transduction pathway may be early responsive biomarkers for early bone development is now under review and for clearance approval by the Naval Medical Research Center and will be submitted for peer review to the Journal of Bone and Joint Surgery (Am).

A manuscript reporting the human BMSC and skeletal muscle HO and signaling studies will be prepared and submitted for peer review likely to the Journal of Bone
and Joint Surgery (Am), pending review and clearance approval by the Naval Medical Research Center.

One or two manuscripts reporting the expression of micro RNA processing apparatus in human ASC and human BMSC will be prepared for submission to the journal Adipocyte and/or Bone. A parallel manuscript reporting on the micro RNA processing in the rat blast injury model will be prepared pending further data collection.

Research Task 8 (Preparation and Proteomic Analysis of Serum Samples):
Current Objective: The intent of this Task is to develop LC/MS assays for detection of factors activating signal transduction pathways associated with osteogenesis.
Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:
1. Human serum samples obtained from military (NMRC) and civilian (LSUHSC-NO) subjects with and without HO were immunoselected using a commercial kit by Ms. Claire Llamas (Tulane University) and analyzed by mass spectroscopy by Dr. Michael Freitas and his graduate student (Michael Hoover) using bioinformatics approaches.
2. A panel of rat serum samples from blast injured and amputated animals were prepared by Dr. Qureshi and Dr. Davis at NMRC, shipped to Tulane, immunoselected and then transferred to OSU for mass spectroscopy. The mass spectroscopy data from the rat HO model identified and quantified time dependent changes in the profiles of 280 individual proteins. These specimens were further evaluated using bioinformatic approaches to correlate changes in protein levels to the time course of HO development.
3. Serum samples obtained by Dr. Levi (University of Michigan) from mice subjected to burns over 30% of their body surface area and Achilles tendonectomy to initiate HO in 100% of the treated animals have been immunoselected (by Ms. Llamas, Tulane) and shipped to Ohio State University. These samples, along with their appropriate controls, remain to be evaluated by LC/MS using new instrumentation following completion of the human and rat sample analyses.

Research Task 9 (Manuscript and Oral Presentation of Proteomic Studies):

Abstract Presentation for 64th Meeting of the American Society for Mass Spectrometry (San Antonio TX, June 4, 2016)
Lead Author: Michael Hoover
Title: Identification of serum protein biomarkers associated with early heterotopic ossification formation following traumatic injury

Two manuscripts are being prepared to report the human and rat serum proteome, respectively. These studies will describe the mass spectroscopy proteome as well
as confirmatory secondary protein analyses (ELISA, western blot). The investigators have not yet decided on the appropriate journal(s) for submission. A comparable study of the murine burn model serum will be prepared when that data is complete.

Two manuscripts will be prepared to report the incidence of heterotopic ossification in a civilian population retrospectively (review of the LSUHSC records) and prospectively (analysis of the current civilian orthopaedic trauma patient cohort). The investigators have not yet decided on the journal(s) for submission.

3. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.
   a. Mentoring – Dr. Fred O'Brien continues to progress in the Master’s Program.
   b. Research –
      i. Publication of review article on the role of micro RNA in ASC and BMSC differentiation in a special issue of the journal *Biochimie*.
      ii. Department of Orthopaedic Surgery Grand Rounds presentation at LSUHSC-NO by Dr. O'Brien (New Orleans LA, March 2016)
      iii. Presentation by Mr. Hoover and Dr. Freitas at the American Society for Mass Spectroscopy (San Antonio TX June 2016)
      iv. Poster presentation accepted for Dr. Martin at the Military Health System Research Symposium (Orlando FL, August 2016); however, presentation was cancelled due to severe flooding in Baton Rouge LA.

4. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and/or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The third year of this research project has continued to integrate productive and complementary activities across four distinct campuses. Critical established and validated assays necessary to complete the study goals were shared between campuses and used effectively to generate statistically significant and conclusive data. The team continues to advance its experimental findings to peer reviewed publication. The outcomes of the experiments now underway and/or nearing completion have begun to define biomarkers and signal transduction pathways involved in the onset of HO. These are being investigated and validated across species (human, mouse, rat) and will advance our understanding of the signal transduction pathways regulating osteogenesis in bone and soft tissue derived
stromal/stem cells. In the coming year, under an approved no-cost extension, the investigative team will seek to achieve the following goals:

1. Publication of primary research manuscripts regarding the response of human ASC, BMSC and skeletal muscle cells to osteogenic inductive factors and human serum from HO and non-HO civilian and military patients in a time dependent manner.

2. Publication of a detailed LC/MS study of serum biomarkers in the rat and human with accompanying manuscript submissions. Similar studies on the murine HO serum proteome will be completed thereafter and prepared for publication.

3. Publication of HO incidence based on prospective and retrospective analyses of civilian orthopaedic trauma patients.

4. Continue to present abstracts for poster and oral presentation at regional, national and international meetings from the investigative team.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:
  None

(2) Invited Articles:
  None

(3) Peer Reviewed Articles:

(4) Abstracts:

  13th Annual Meeting of the International Federation for Adipose Therapeutics and Science (New Orleans LA, November 8, 2015)
  Lead Author: Elizabeth Martin
  Title: Pro-inflammatory and osteo-inductive cytokines induce bone-related early response genes in adult human adipose stromal cells.

  Lead Author: Ammar T. Qureshi
Co-Authors: Martin EC, Gimble JM, Forsberg JA, Hayes DJ, Davis TA. 
Title: MicroRNA mediated inhibition of osteoblast mediated bone formation.

64th Meeting of the American Society for Mass Spectrometry (San Antonio TX, June 4, 2016) 
Lead Author: Michael Hoover 
Title: Identification of serum protein biomarkers associated with early heterotopic ossification formation following traumatic injury

Military Health System Research Symposium (August 18, Kissimmee FL) 
Lead Author: Elizabeth Martin 
Title: Serum from individuals with trauma induced-heterotopic ossification alters activator protein 1 complex in adipose derived stromal/stem cells (Poster not presented due to severe flooding in Baton Rouge LA immediately prior to the meeting)

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

13th Annual Meeting of the International Federation for Adipose Therapeutics and Science (New Orleans LA, November 8, 2015) 
Lead Author: Elizabeth Martin 
Title: Pro-inflammatory and osteo-inductive cytokines induce bone-related early response genes in adult human adipose stromal cells.

Lead Author: Ammar T. Qureshi 
Co-Authors: Martin EC, Gimble JM, Forsberg JA, Hayes DJ, Davis TA. 
Title: MicroRNA mediated inhibition of osteoblast mediated bone formation.

64th Meeting of the American Society for Mass Spectrometry (San Antonio TX, June 4, 2016) 
Lead Author: Michael Hoover 
Title: Identification of serum protein biomarkers associated with early heterotopic ossification formation following traumatic injury

6. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.
Nothing to report.

7. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. 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. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to report.

8. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Completion of Active Duty Deployment at Madigan Military Medical Center by Dr. Fred O’Brien.

For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”

9. REFERENCES: No references cited.

APPENDICES: No appendices attached.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

Dr. O’Brien has completed three semesters of classwork in the Masters of Clinical Science Research Program at Tulane University School of Medicine and has re-enrolled online.

COLLABORATIVE AWARDS: Not applicable.
QUAD CHARTS: Quad Chart submitted with annual report
MARKING OF PROPRIETARY INFORMATION: Not applicable.
Proteomic Analysis of Trauma-Inducted Heterotopic Ossification Formation

ERMS/Log Number OR120163 Task Title
Award Number W81XWH-13-2-0097

PI: Jeffrey Gimble MD PhD Org: Tulane University Award Amount: $962,052

**Study/Product Aim(s)**
- Mentor Dr. O’Brien as Clinical Research Scientist
- Recruit civilian & military orthopaedic trauma study population
- Harvest serial serum & wound fluid samples from subjects at risk for HO development
- Examine clinical fluids for osteogenic activity using in vitro assays
- Identify potential HO biomarkers using proteomic assays

**Approach**
Orthopaedic trauma patients from civilian and military hospitals will provide serial serum and wound fluid samples. These will be shared between collaborating laboratories in assays examining (a) activation of osteogenic biochemical pathways in human primary adipose, bone marrow and skeletal muscle cells and (b) proteome identification using mass spectroscopy, ELISA, and western immunoblotting technologies.

**Timeline and Cost**

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**Goals/Milestones**

**CY13 Goal** – HRPO Evaluation, Assay Development, Mentoring
☑ HRPO documents submitted, Assay development & enrollment in Masters in Clinical Research Program initiated

**CY14 Goals**
- In Vitro Assay Development, Mentoring
  - Complete clinical fellowship & complete initial Master’s classes
  - Develop primary cell culture assays & mass spec controls
  - Review article submissions

**CY15 Goal** – Civilian Patient Accrual & Assay Data Collection
- Meet enrollment goals for civilian patient accrual
- Identify target biomarkers in serum & wound fluid

**CY16 Goal** – Manuscript Submission, Mentoring, Final Data Analyses
- Complete 1st research article submissions to peer review journals - Ongoing
- Completion of Master’s Program – On track for 2017 completion
- Final analyses of data to determine HO biomarkers in serum - Ongoing

**Budget Expenditure to Date**
Projected Expenditure: $962K
Actual Expenditure: Approximately 85%; 4th Year No Cost Extension Approved

*Updated: 05- Oct-2016*