# Title: Parathyroid Hormone-Related Peptide (PTHrP) as a New Target for Metastatic Breast Cancer: Evaluation in Preclinical Models

**Authors:** Dr Richard KREMER, Dr Anne CAMIRAND  
e-mails: richard.kremer@mcgill.ca  anne.camirand@mcgill.ca

**Organization:** Research Institute of McGill University  
2155 rue Guy, bureau 500, Montréal, QC Canada H3H 2R9

The purpose of this study is to investigate the role of parathyroid hormone-related protein (PTHrP) in cancer stem cell-driven triple-negative breast cancer. Our goal is to confirm PTHrP as a targetable molecular driver in order to develop better therapies against this notoriously hard-to-treat family of cancers. We have now constructed animal models with mammary epithelium fluorescent labelled cells to allow tracing of cancer stem cells from the primary mammary tumor to the metastatic site. We will use these models to compare cancer stem cell behavior in PTHrP-ablated and non-ablated animals. Using CRISPr technology, we are developing pre-clinical PTHrP-ablated human triple-negative breast cancer cell lines to test the cancer-blocking efficacy of our anti-PTHrP monoclonal antibodies and identify cell markers associated with cancer progression events. We are using a large tissue bank from triple-negative breast cancer patient tumors to investigate the relationship between triple-negative breast cancer tumoral PTHrP expression levels and cancer recurrence. The goal is also to develop a method to identify patients who would benefit from a novel anti-PTHrP therapy. We have already determined that PTHrP is overexpressed in the tumor tissue of the majority of triple-negative breast cancer cases.

**Subject Terms:** Breast cancer, metastasis, triple-negative breast cancer (TNBC), parathyroid hormone-related peptide (PTHrP), cancer stem cells (CSCs), tumor-initiating cells, targeted drugs, blocking monoclonal antibody, skeletal invasion, PyMT-MMTC, CXCR4, ALDH1.

**DISTRIBUTION / AVAILABILITY STATEMENT**  
Approved for Public Release; Distribution Unlimited
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>2- KEYWORDS</td>
<td>5</td>
</tr>
<tr>
<td>3- ACCOMPLISHMENTS</td>
<td>6</td>
</tr>
<tr>
<td>4- IMPACT</td>
<td>15</td>
</tr>
<tr>
<td>5- CHANGES – PROBLEMS</td>
<td>16</td>
</tr>
<tr>
<td>6- PRODUCTS</td>
<td>17</td>
</tr>
<tr>
<td>7- PARTICIPANTS and OTHER COLLABORATING INSTITUTIONS</td>
<td>18</td>
</tr>
<tr>
<td>8- SPECIAL REPORTING REQUIREMENTS</td>
<td>20</td>
</tr>
<tr>
<td>9- APPENDICES</td>
<td>21</td>
</tr>
</tbody>
</table>
INTRODUCTION

SUBJECT: Triple-negative breast cancer (TNBC) accounts for up to 24% of all diagnosed breast cancer cases. This family of cancers generally strikes younger patients and displays poor prognosis and outcome. TNBCs are notoriously difficult to treat since they lack the usual receptors that are targeted by modern specific treatments. The alternative therapeutic approach, traditional chemotherapy, is followed in TNBCs by more frequent and more aggressive relapse than is observed in receptor-positive cancers. The identification of targetable TNBC molecular drivers is therefore crucial to the development of efficient therapies. With this goal in mind, we have observed that nearly all surveyed TNBC cell lines overexpress the parathyroid hormone-related protein (PTHrP) while most receptor-positive breast cancer lines do not (Fig. 1). We also demonstrated that anti-PTHrP approaches inhibit breast cancer initiation, progression and metastasis (Li et al 2011, J Clin Invest., 121:4655). TNBCs are known to be driven by enrichment in cancer stem cells (CSGs, also known as tumor-initiating cells) (Bhola et al 2013, J Clin Invest. 123:1348), and we suspect a role of PTHrP in events triggering increase of breast CSCs involved in oncologic development. PURPOSE: We hypothesize that endogenous PTHrP is involved in expansion of a sub-population of CSCs responsible for tumorigenesis and metastasis and that a targeted anti-PTHrP approach will decrease or prevent metastases. To achieve practical anti-PTHrP therapy, we have developed anti-PTHrP blocking monoclonal antibodies (MAbs). SCOPE: In aim 1 of this application, we propose to validate the role of PTHrP in breast cancer progression through tracing in time and location of tumor cells from the primary tumor. In aim 2 we propose to use pre-clinical models of TNBC to test the efficacy of our PTHrP blocking MAb on the cancer stem cell subpopulation spreading to the skeleton. In aim 3 we will investigate the relationship between PTHrP expression and cancer recurrence in a large tissue bank from TNBC patients in order to explore the mechanism linking PTHrP to metastasis and develop a selective approach to optimise patient selection for future PTHrP-targeted therapy. If successful, our proposed studies could be tested shortly in early phase trials and translated rapidly into clinical practice.
2. **KEYWORDS**

Breast cancer

Metastasis

Triple-negative breast cancer (TNBC)

Parathyroid Hormone-Related Peptide (PTHrP)

Cancer Stem Cells (CSCs)

Tumor-Initiating Cells

Targeted Drugs

Blocking Monoclonal Antibody

Skeletal invasion

PyMT-MMTV

ALDH1

CXCR4
3- ACCOMPLISHMENTS

3.1- MAJOR GOALS AND OBJECTIVES OF THE PROJECT:

This project is based on the hypothesis that in triple-negative breast cancer, endogenous PTHrP is involved in the expansion of a sub-population of cancer stem cells destined for tumorigenesis and metastasis, and that a targeted anti-PTHrP approach will decrease or prevent metastases. The major goals are:

In aim 1: to validate the role of PTHrP in breast cancer progression through tracing in time and location of tumor cells from the primary tumor.

In aim 2: to use pre-clinical models of triple-negative breast cancer to test the efficacy of our PTHrP blocking monoclonal antibody on the cancer stem cell subpopulation spreading to the skeleton.

In aim 3: to investigate the relationship between PTHrP expression and cancer recurrence in a large bank of triple-negative breast cancer patients in order to better select patients for future PTHrP-targeted therapy.

A detailed timeline Gantt chart was prepared that follows the approved SOW. See SOW in Appendix 1. The timeline is shown in fig.2 on page 7 with the various tasks highlighted in colour according to each specific aim.

Figure 2: (next page) Gantt diagram illustrating the tasks and milestones for the DoD contract W81XWH-15-1-0723. The baseline ranges from September 2015 to September 2018. Red arrows on the baseline indicate the dates where the annual reports are due. Yellow and blue arrows indicate meetings with various other PIs. Specific aim 1 tasks are in green, specific aim 2 tasks are in orange and specific aim 3 tasks are in purple. Approximate dates for beginning and end of each task are on the left of the bars and descriptions are on the right.
3.2. ACOMPLISHMENTS IN THE FIRST YEAR OF THE CONTRACT:

3.2.1 aim 1: (green task lines)

- 3.2.1.1 ACURO approval: obtained (approval document in appendix 2).
  % COMPLETED: 100% OF TASK. (ON TIME).

- 3.2.1.2 Preparation of anti-PTHrP monoclonal antibodies:
  % COMPLETED: 100% OF TASK. (ON TIME).

Monoclonal antibodies were prepared from two of our clones (#158 and #6) according to standard protocols. The IgG purification (Fig 3) from the supernatant was conducted by Médilabs (Montréal, QC, Canada) and the analysis report confirms good yield for both antibodies (52 and 40 mg for #158 and #6 respectively).

Figure 3: Coomassie staining of SDS-PAGE protein gel illustrating purification of anti-PTHrP monoclonal antibodies #158 (lane 3) and #6 (lane 4). Lane 1: IgG standard, lane 2: molecular weight markers.

- 3.2.1.3 TdT (tomato-red fluorescent stain)-transformed mammary tumor cells: wild-type (WT) and PTHrP-knock-out (KO):
  % COMPLETED: 80% OF TASK. (ON TIME).

In order to conduct cell tracing experiments for comparison of wild-type and PTHrP-deleted tumors in cancer progression studies, we need to obtain mice in which a fluorescent indicator is expressed specifically in mammary epithelium. We have used the Jackson Laboratories ROSA26mTmG mouse model (stock 026862) which possesses two fluorescent indicators (tandem dimer Tomato and Green fluorescent protein, respectively red and green) on an FVB background. Production of ROSA26 mTmG PTHrP flox/flox PyMT MMTV-Cre mice with mammary epithelium-expressed TdT and GFP fluorescent indicators has now been accomplished by crossings described in Fig.4. We
are now genotyping the resulting animals and we will conduct genomic sequencing to confirm PTHrP deletion.

**Figure 4:** Protocol diagram for generation of ROSA26\(^{mTmG}\) PTHrP\(^{flox/flox}\) PyMT MMTV-Cre mice with mammary epithelium-expressed tandem dimer Tomato and Green fluorescent protein fluorescent indicators.

- 3.2.1.4 RNA sequencing of non-TdT WT and KO cells at various stages of tumorigenesis (hyperplasia, early carcinoma, late carcinoma):
  \% COMPLETED: 60\% OF TASK. (ON TIME).

RNA sequencing was conducted on tumor cells obtained from wild-type and PTHrP-deleted mice to initiate the search for crucial pathways in cancer progression and the changes associated with PTHrP ablation. Three different stages were used for this purpose: hyperplasia, early carcinoma, and late carcinoma. Profiles are being obtained and analysis is proceeding.

3.2.2 aim 2: (orange task lines)
- 3.2.2.1 KO of human TNBC cells by Clustered Regularly Interspaced Short Palindromic Repeats (Crispr):
  \% COMPLETED: 90\% OF TASK. (ON TIME).

A unique CRISPR sequence for PTHrP was chosen from pre-designed sites in the human genome using online tools (Sigma-Aldrich). The sites are designed to minimize off-targeting events. The DNA target sequence for the PTHrP gene (PTHLH exon 4) was: CGTCGCCGTAAATCTTGGATGG. The all-in-one Cas9 reporter vector U6gRNA-Cas9-2A-RFP was used with Red fluorescent protein (RFP) fused to the C-terminus of Cas9 with a 2A peptide to enable tracking of transfection efficiency and enrichment of genome editing activity through fluorescence-activated sorting (figure 5).
**Figure 5** Crispr summary: target ID and references sequence (Note, here, PTHLH is equivalent to PTHrP). Plasmid diagram: in blue: promoters, gRNA: guide RNA, Cas9: caspase 9, 2A: 2A peptide link, RFP: red fluorescent protein, Kanr: kanamycin resistance, pUC ori: plasmid origin of replication. The PTHrP exon 4-specific target sequence was inserted in the vector on the left.

**CRISPR Designs Summary Table**

<table>
<thead>
<tr>
<th>Target ID</th>
<th>Gene Symbol</th>
<th>Gene_ID</th>
<th>RefSeq</th>
<th>Target Ex Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS000266300</td>
<td>PTHLH</td>
<td>5744</td>
<td>NM_0002820, NM_198854, NM_198855, NM_198866</td>
<td>4 Human</td>
</tr>
</tbody>
</table>

Human triple negative breast cancer cells (MDA-MB-231, MDA-MD-549, MDA,MB-435 and MDA-MB-468) were transfected with the PTHrP Crispr plasmids using Lipofectamine 2000 reagent (Thermo Fisher Scientific). An example of transfected cells is shown in **figure 6**. The RFP positive cells will be separated by FACS, and DNA sequencing will be used to confirm PTHrP ablation.

**Figure 6**: Fluorescence microscopy of MDA-MB-549 cells transfected with CRISPR plasmid to ablate PTHrP. The white arrow indicates a cell with no PTHrP deletion while the yellow arrow indicates an RFP positive cell (red) where PTHrP is ablated.
3.2.2.2 Flow cytometry for marker analysis, mammosphere culture, growth and invasion characteristics for WT and PTHrP KO human TNBC cells.

% COMPLETED: 50% OF TASK. (ON TIME).

We have previously demonstrated that mammary tumor growth over time in PTHrP $^{\text{flox/flox}}$: Cre $^+$ (PTHrP ablated) mice is considerably delayed as compared to tumor growth in PTHrP $^{\text{flox/flox}}$: Cre $^+$ (control) animals (figure 7 A). In order to associate PTHrP expression with cancer stem cell expansion, we used the in vitro suspension technique known as mammosphere culture (figure 7 B), a method that enriches CSC populations and facilitates their characterization. An outline of the procedure is described in fig. 7 C.

**Figure 7: Mammosphere enrichment of CSCs.** A: PTHrP-ablated tumor cells develop significantly slower than tumor cells from control mice. B: mouse mammary tumor-derived mammosphere growing in vitro in suspension. C: Experimental design for enriching cancer stem-like cells from the PyMT MMTV spontaneous mammary cancer model. Cells are flow-analyzed for CSC markers such as CD24, CD49f, and EpCam. The lipophilic fluorescent PKH26 dye is used to monitor cell division (Akrap et al 2016 Stem Cell Reports 6:121-136). Secondary mammospheres are derived from cells taken from primary mammospheres.

A comparison was first established between CSC-like cells in tumors of control or PTHrP-ablated animals. Flow cytometric analysis showed that the majority of cells are
CD49f+ EpCam+, i.e. luminal progenitors. The mesenchymal-like CD49f+ EpCam- cells in contrast, are fewer in proportion, and interestingly, their proportion is significantly decreases in cells from PTHrP-ablated tumors vs cells from control tumors (2.17% vs 10.4-13.9% in controls) (Figure 8 A). This indicates that PTHrP removal favors the epithelial subpopulation, and that PTHrP presence favors the mesenchymal subpopulation, confirming the role of PTHrP in epithelial to mesenchymal transition (EMT).

Flow analysis was conducted of mammosphere-derived cells stained with the lipophilic fluorescent dye PKH26 as a cell division dilution marker (Akrap et al 2016 Stem Cell Reports 6:121-136). Fast-dividing cells are low-PKH26, whereas non-dividing cells are high-PKH26. In primary mammospheres, fast-dividing PHK26 low (epithelial-like CSC cells) represented 9.27% of CD49f+ EpCam-, but showed total absence in secondary mammospheres (0%). This indicates that the CD49f+ EpCam- subpopulation enriches the majority of the non-dividing mesenchymal-like CSCs, whereas fast-dividing (epithelial) cells remain in the CD49+EpCam+ (not shown).

**Figure 8 Example of flow analysis of markers in tumor cells.** A: flow analysis for cells derived from primary mammary tumors from control and PTHrP-ablated animals. B: flow analysis for mammospheres derived cells from tumors of non-ablated mice.
3.2.3 aim 3: (purple task lines)

- 3.2.3.1 IRB amendment for TNBC use. The 3 documents are included in Appendix 3.
  % COMPLETED: 100% OF TASK. (ON TIME).

- 3.2.3.2 HRPO approval: PENDING

- 3.2.3.2 PTHrP and other markers level assessments according to TNBC subtype
  % COMPLETED: DELAYED PENDING HRPO APPROVAL.

- 3.2.3.4 PTHrP expression and clinical outcomes.
  % COMPLETED: DELAYED PENDING HRPO APPROVAL

3.3- WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT DID THE PROJECT PROVIDE?

Several scientists are benefiting from this project. First, two senior employees, Anne Camirand, Ph.D. and Jiarong Li, Ph.D. are acquiring further professional development, i.e. increased knowledge and skills through conferences, seminars, as well as acquisition of new scientific techniques in view of developing new therapeutic approaches in breast cancer. Two post-doctoral fellows, Gloria Assaker, Ph.D. and Yoshihiko Nasai, Ph.D. will be receiving similar professional development, as well as training in new professional skills including biostatistics, with the goal of doing research in translational medicine. A technician, Karine Sellin, and a Ph.D. student, Rui Zhang, are being mentored and instructed in all techniques involved in our particular project, including the use of novel technologies such as CRISPr. Several summer students are also participating in the project and receive a first contact with cutting-edge cancer research in this project.

3.4- HOW WERE THE RESULTS DISSEMINATED TO THE COMMUNITIES OF INTEREST?

During the first year of this contract, we did not report results as they were still very preliminary. However, from now on, we plan to reach the communities of interest at the international level by our participation in meetings such as those of AACR (American Association of Cancer Research) and ASBMR (American Society of Bone and Mineral Research). Locally, we will present results at the annual meeting of the McGill University Health Centre Experimental Therapeutic Group, The McGill Experimental Medicine Meeting, and the annual McGill Endocrine Meeting. Furthermore, we will participate in the popular “Café Scientifique” organised by the Canadian Institute of
Health Research, and we plan a permanent web site for our laboratory that will allow dissemination in lay terms of salient results from our lab.

3.5- WHAT IS PLANNED FOR THE NEXT REPORTING PERIOD TO ACCOMPLISH GOALS AND OBJECTIVES?

For the second year of this contract, we will closely follow the SOW and the timeline in figure 2 to investigate mammary tumor cell tracing from early to late stages in our PyMT MMTV model, including skeletal and lung metastasis, and we will compare tumors from wild-type and PTHrP-ablated animals. We will also move to human systems and document the cellular pathways involved in TNBC progression through RNA sequencing and pattern analysis. We will proceed to intratibial injections of human TNBC cells (wild-type and PTHrP-ablated) in immunodeficient mice and begin RNA sequencing of TNBC cells colonising the bone marrow. Finally, we will continue our analysis of the TNBC patients tissue bank and investigate correlations between PTHrP expression levels and other significant markers.
4- IMPACT

4.1- IMPACT ON PRINCIPAL DISCIPLINE.

The main patient population targeted by our study is women suffering or at risk of developing bone metastases from triple-negative breast cancer. Skeletal metastases appear as complications in up to 70% of breast cancer patients and are usually incurable, causing considerable suffering and morbidity. Current therapies against skeletal invasion in breast cancer use bisphosphonates, however, these compounds are not curative and do not prolong survival. We are in the early stages of our project, but we can already state that the impact of achieving remission or even cure of the disease in chemoresistant patients through the identification of a novel target such as PTHrP would be very high. The fact that we already have developed anti-PTHrP blocking monoclonal antibodies indicates that the step to trials could be rapid if the present study achieves its goals. The development of methods to identify TNBC patients most likely to benefit from anti-PTHrP therapy would be a major advance in the fight against this hard-to-treat cancer.

4.2- IMPACT ON OTHER DISCIPLINES.

PTHrP is known to play a driving role in several other types of pathologies such as lung, melanoma, gastric and prostate cancer. Any observation of anti-cancer activity from our anti-PTHrP monoclonal antibodies against breast cancer is likely to be transferable to other pathologies where PTHrP is part of the driving mechanisms. The confirmation of PTHrP as a novel target will open the door to testing the anti-PTHrP treatment in many PTHrP-overexpressing oncological conditions that frequently spread to the skeleton.

4.3- IMPACT ON TECHNOLOGY TRANSFER.

From our present work, we envision the use of several anti-PTHrP blocking antibodies against breast (and other) cancers. We also aim to provide a new method to identify patients for targeted therapy.

4.4- IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY.

Derivatives from our work would be a step toward “personalized medicine” where therapeutic drugs will be administered to specific cancer types to allow optimized dosing and avoid destructive effects from current approaches such as chemo- and radiation therapy.
5- CHANGES – PROBLEMS

5.1- CHANGES IN APPROACH:

A minor change has been made to the reported protocols, we now use the lipophilic fluorescent yellow-orange PKH26 dye (Sigma-Aldrich) for monitoring mammosphere-derived cells during flow analysis. The reason for this choice is that PKH26 is an excellent cell division dilution marker that allows us to track fast-dividing cells (low-PKH26), and contrast with non-dividing cells (high-PKH26) (Akrap et al 2016 Stem Cell Reports 6:121-136). It can be used in vitro and in vivo and is compatible with many other fluorescent dyes emitting in the spectral regions of violet, green, red and far-red.

5.2- POTENTIAL PROBLEMS OR DELAYS:

We are waiting for HRPO approval to start implementing all steps of third specific aim.

5.3- CHANGES WITH IMPACT ON EXPENDITURES:

No changes have been made that have a significant impact of expenditures.

5.4- CHANGES IN VERTEBRATE ANIMALS:

A small change in the experimental mouse obtained from Jackson Laboratories has been effected: we purchased the newly-available mouse (ROSA26mTmG) which possesses two fluorescent markers (GFP and TdT, respectively green and red) instead of only the TdT (Jackson Laboratory stock 026862). The double-fluorescence model allows direct live visualization of both recombined and non-recombined cells at single-cell resolution, offering an internal control for phenotypic analysis of Cre-induced mosaic mutants, and providing a second marker for lineage tracing applications. The model has the considerable advantage of also possessing the FVB background which is appropriate for us and will therefore save us a lot of time in avoiding the need for backcrossing animals to uniformize the background.
6- PRODUCTS

6.1- PUBLICATIONS, CONFERENCE PAPERS, PRESENTATIONS.

During the first year of this contract, we have not presented conference papers of publications as our results are still preliminary. However, in the coming year, we will prepare presentations for several international (AACR, ASBMR) and local meetings.

6.2- WEBSITE OR OTHER INTERNET SITES

We will also start a website for our laboratory with appropriate links.

6.3- TECHNOLOGIES OR TECHNIQUES.

Nothing to report.

6.4- INVENTIONS, PATENT APPLICATIONS, LICENSES.

There are right now no patent applications, although our anti-PTHrP monoclonal antibody has already been patented.

**PTHrP, its isoforms and antagonist thereto in the diagnosis and treatment of disease**

**US 8501929 B2**

<table>
<thead>
<tr>
<th>Publication number</th>
<th>US8501929 B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication type</td>
<td>Grant</td>
</tr>
<tr>
<td>Application number</td>
<td>US 12/673,877</td>
</tr>
<tr>
<td>PCT number</td>
<td>PCT/CA2008/001478</td>
</tr>
<tr>
<td>Publication date</td>
<td>Aug 6, 2013</td>
</tr>
<tr>
<td>Filing date</td>
<td>Aug 18, 2008</td>
</tr>
<tr>
<td>Priority date</td>
<td>Aug 17, 2007</td>
</tr>
</tbody>
</table>

6.5- OTHER PRODUCTS.

In the first of this contract, we have created several human TNBC cell lines with ablation of the PTHrP gene. Furthermore, we have created a novel PyMT MMTV-based mouse model with mammary epithelium-expressed tandem dimer Tomato and Green fluorescent protein fluorescent indicators. We have the PTHrP-nonablated and the PTHrP-ablated versions of this model.
7- PARTICIPANTS and OTHER COLLABORATING INSTITUTIONS

7.1- INDIVIDUALS WHO WORKED ON THE PROJECT:

<table>
<thead>
<tr>
<th>Name</th>
<th>Jiarong Li</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Research associate</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Construction of TdT animals, CRISPr preparation of human TNBC KO lines, student mentoring.</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Anne CAMIRAND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Research associate</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>4</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Administration, planning, writing, mentoring, software instruction.</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Gloria ASSAKER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Research associate</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>4</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Testing anti-PTHrP Mab methods.</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Yoshihiko NASAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Post-doctoral fellow</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>3</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Rui ZHANG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Graduate student</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Analysis of mammospheres from human TNBC KO and WT cell lines.</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Karine SELLIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Research assistant</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>Antibody preparation, cell culture, general technical support</td>
</tr>
<tr>
<td>Funding support</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
7.2- CHANGE IN OTHER ACTIVE SUPPORT OF PI OR OTHER KEY PERSONNEL
No change to report.

7.3- OTHER ORGANISATIONS THAT HAVE BEEN INVOLVED AS PARTNERS.
No other organisations have been involved as partners.
8- SPECIAL REPORTING REQUIREMENTS

Not applicable.
9- APPENDICES

APPENDIX 1 : SOW (approved)

<table>
<thead>
<tr>
<th>Specific Aim 1: Cancer cell tracing for testing</th>
<th>Timeline</th>
<th>Responsible Party</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTHrP role progression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obtain ACURO approval</td>
<td>1-3</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td>Task 1.1: Generation of PyMT MMTV (PTHrP WT or KO) mouse model of BC animals with a mammary epithelium-expressed tandem dimer Tomato fluorescent indicator: determine the fate of cancer cells and their subpopulations time- and space-wise</td>
<td>1-12</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td>Task 1.2: Primary tumor tracing: time-course study of tdT PTHrP WT and KO LME cells progression from normal cells to hyperplasia, adenoma and early and late carcinoma. Examine tumor progression in relation to TIC phenotype at hyperplasia, early and late carcinoma, collect breast tissue, hematoxylin/eosin (H&amp;E) staining, IHC, IF for PTHrP, ALDH1, CXCR4, vimentin, e-cadherin.</td>
<td>13-24</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td>Task 1.3: Metastatic tracing: time-course study of PTHrP WT and KO tdT-labelled LME cells invasion of bone marrow.</td>
<td>13-24</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td>Task 1.4: RNA sequencing will be conducted on tdT labelled cells collected by sorting, TIC-enriched ALDH1 and CXCR4 and non-enriched populations; establish molecular profiles, compare real-time analysis of gene expression specific for PTHrP WT and KO populations in primary tumors, CTCs, bone marrow and lungs (72 samples);</td>
<td>1-12</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td>Biomathematics analysis, Biostatistics and</td>
<td>1 - 24</td>
<td>Dr. Kremer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dr. Ragoussis</td>
</tr>
<tr>
<td>Validation studies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Milestone Achieved:**
- Identify PTHrP as the driving factor in TIC dissemination to distal sites
- Identify specific pathways as targets for our PTHrP blocking mAbs.

**Specific Aim 2: Pre-clinical: mechanisms implying PTHrP in TNBC TIC expansion and therapeutic targeting of skeletal metastases:**

**Task 2.1** Studies *in vitro* of the TNBC lines from BT-549 and their TIC-like subpopulation (with or without Pthrp ablation):

<table>
<thead>
<tr>
<th>Task 2.1</th>
<th>Dr. Kremer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-24</td>
<td></td>
</tr>
</tbody>
</table>

**Subtask 2.1.1:** Construction of PTHrP-negative TNBC control cell lines stable KOs from BT-549 stable lines expected to have low PTHrP (WB, IF).

<table>
<thead>
<tr>
<th>Task 2.1</th>
<th>Dr. Kremer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-12</td>
<td></td>
</tr>
</tbody>
</table>

**Subtask 2.1.2:** Determine the effect of Pthrp ablation and *in vitro* mAb treatment on TIC phenotype: expected WT cells with enriched TIC phenotype to be more invasive compared to ablated or mAb-treated cells

<table>
<thead>
<tr>
<th>Task 2.1</th>
<th>Dr. Kremer</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-24</td>
<td></td>
</tr>
</tbody>
</table>

**Task 2.2.** Pthrp ablation and mAb treatment after intra-tibial injection:

<table>
<thead>
<tr>
<th>Task 2.2</th>
<th>Dr. Kremer</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-36</td>
<td></td>
</tr>
</tbody>
</table>

**Milestone(s) Achieved:**
- Reveal patterns for drug resistance/sensitivity
- Establish Pthrp wt giving rise rise to higher bone tumor burden than Pthrp KO
- mAb treatment mimics effect of ablation.

<table>
<thead>
<tr>
<th>Task 2.2</th>
<th>Dr. Kremer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12-18</td>
</tr>
</tbody>
</table>

**Specific Aims 3: Analysis of PTHrP expression and validation its relation to tumor recurrence in TNBC as a preliminary step to selection of patients for PTHrP-targeted therapy:**

**Task 3.1:** Obtain IRB, amendment for use of TNBC

<table>
<thead>
<tr>
<th>Task 3.1</th>
<th>Dr. Sabri</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td></td>
</tr>
<tr>
<td>Task 3.2: IHC of tissue samples: correlation with patients' data, treatment characteristics, survival outcome with patterns of or distant recurrence (liver, lung, brain, bone). Samples of each TNBC subtype analysed by IHC for PTHrP, CXCR4 expression (cytoplasmic and membranous) within each TNBC subtype.</td>
<td>3-24</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Task 3.3: Biostatistics</td>
<td>25-36</td>
</tr>
<tr>
<td><strong>Milestone(s) Achieved:</strong></td>
<td></td>
</tr>
<tr>
<td>- establish correlation of PTHrP expression to clinical outcomes</td>
<td>12-18.24</td>
</tr>
<tr>
<td>- identification of a patient sub-population eligible to anti-PTHrP mAb therapy</td>
<td></td>
</tr>
</tbody>
</table>

Note: The Government reserves the right to request a revised SOW format and/or additional information.
APPENDIX 2: ACURO approval

DEPARTMENT OF THE ARMY
HEADQUARTERS, US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

June 16, 2016

Director, Office of Research Protections
Animal Care and Use Review Office


Principal Investigator Richard Kremer
McGill University Health Centre Research Institute
Montreal,

Dear Dr. Kremer:

Reference: (a) DOD Instruction 3216.01, “Use of Animals in DOD Programs”
(b) US Army Regulation 40-33, “The Care and Use of Laboratory Animals in DOD Programs”
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol BC142405 entitled, “Parathyroid Hormone-related Protein (PTHrP) as a New Target for Metastatic Breast Cancer: Evaluation in Preclinical Models,” IACUC protocol number 2015-7713, Protocol Principal Investigator Richard Kremer, is approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 07-JUN-2016 for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the McGill University Health Centre Research Institute (RI MUHC) IACUC on 01-FEB-2016.

Required Actions: When updates or changes occur, documentation of the following action or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)

- IACUC actions involving this protocol regarding
  a. any noncompliance;
  b. any deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC
• USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

• AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

• Species used (must be approved by this office)
• Number of each species used
• USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usammc.other.auro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Original Signed

Bryan K. Ketzenberger, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:
Ms. Danielle L. Reckley, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Nicole M. Williams, Congressionally Directed Medical Research Program (CDMRP)
Dr. Lucie Cote, McGill University Health Centre Research Institute (RI MUHC)
Dr. Susan James, McGill University Health Centre Research Institute (RI MUHC)
APPENDIX 3: IRB approval (3 documents)

HREBA
Health Research Ethics Board of Alberta
Cancer Committee

01 September 2016

Dr. Bassam Abdulkarim
Radiation Oncology
McGill University

Dear Dr. Abdulkarim:

RE: 24868: Triple Negative Breast Cancer and Patterns of Local Recurrence for Breast Patients with Conservative Surgery Versus Mastectomy

Thank you for the submission of an Addition of Co-Investigator form in reference to the above named study, received 29 August 2016.

On behalf of the Health Research Ethics Board of Alberta (HREBA) - Cancer Committee, I acknowledge receipt of this document. It is noted that as of 29 August 2016 Dr. Siham Sabri has been added to the study as the co-investigator. Our records have been updated accordingly.

If there are any other changes to the protocol or consent form during the year, or if any adverse reactions to the treatment are found, the Cancer Committee requests that you forward a letter describing changes/reactions, together with an updated protocol and/or consent form to the HREBA-Cancer Committee Office.

Sincerely,

Raul Urtasun, M.D.
Associate Chair, Health Research Ethics Board of Alberta (HREBA) – Cancer Committee

/bm
01 September 2016

Dr. Bassam Abdulkarim
Radiation Oncology
McGill University

Dear Dr. Abdulkarim:

RE: 24868: Triple Negative Breast Cancer and Patterns of Local Recurrence for Breast Patients with Conservative Surgery Versus Mastectomy

Thank you for the submission of an Addition of Co-Investigator form in reference to the above named study, received 29 August 2016.

On behalf of the Health Research Ethics Board of Alberta (HREBA) - Cancer Committee, I acknowledge receipt of this document. It is noted that as of 29 August 2016 Dr. Siham Sabri has been added to the study as the co-investigator. Our records have been updated accordingly.

If there are any other changes to the protocol or consent form during the year, or if any adverse reactions to the treatment are found, the Cancer Committee requests that you forward a letter describing changes/reactions, together with an updated protocol and/or consent form to the HREBA-Cancer Committee Office.

Sincerely,

[Signature]

Raul Urtasun, M.D.
Associate Chair, Health Research Ethics Board of Alberta (HREBA) – Cancer Committee

/bm
Addition of Co-Investigator Form

This form must be completed if Co-Investigators are added throughout the duration of the study. This applies to both industry sponsored and in-house research.

Please attach current CV’s for the addition of new Co-Investigators.

ETHICS #:24868

Study Title: Triple Negative Breast Cancer and Patterns of Local Recurrence for Breast Patients with Conservative Surgery Versus Mastectomy

Principal Investigator (Lead Site): Bassam Abdulkarim, MD, PhD

Principal Investigator (Participating Site): Siham Sabri, PhD

Sponsor: NA

The undersigned Co-Investigator(s) acknowledge that he/she is aware of being listed as a Co-Investigator to the above named study, he/she has read the protocol and he/she agrees to participate in the above named study as outlined in the protocol. (Add extra lines as needed)

1. Name and Site (print): Dr Siham Sabri
   Assistant Professor, Department of Oncology, McGill University
   Principle Investigator, Cancer Research Program (CRP)
   The Research Institute of the McGill University Health Centre
   Glen Site, Bloc E; Office: EM2.3218; Lab: E01-4126
   1001 Decarie Blvd,
   Montreal, Quebec, H4A 3J1, Canada
   Phone: 514-934-1934 Ext.: 44686
   E-mail: siham.sabri@mcgill.ca

2. Signature:

3. Date: August 29, 2016

Submitted by: Dr Bassam Abdulkarim

Version: March 2014

Email address: bassam.abdulkarim@mcgill.ca