ESMO Research Fellowship  
(November 2019 – October 2020)  
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FINAL REPORT  

Host Institute: EXTRO-Lab, Department of Therapeutic Radiology and Oncology, Medical University of Innsbruck  
Mentor: Prof. Ira-Ida Skvortsova, MD, PhD  
Project title: Breast cancer metastasis and lipid metabolism implication  

Home Institute: Alexandrovska University Hospital Sofia, Bulgaria

Introduction

Obesity, defined as a BMI ≥30kg/m², is currently diagnosed in more than 13% of adult population(1). It has strong impact on health since people with BMI between 30-35 kg/m² have reduced median overall survival (OS) with 2-4 years (2). In breast cancer (BC) patients obesity is negative prognostic factors for survival(3) and the strongest negative impact is observed in women with hormone receptor positive cancer (4) compared to almost no effect in the other subtypes (5). Obesity has been related to worse outcomes in all treatment modalities – surgery, radiotherapy and systemic therapy and the cosmetics.(6) Higher BC recurrence rate after radiotherapy has been found in women with increased BMI.(7) Since cancer progression is a main cause of death in patients with solid tumors up to 15 years after diagnosis (8, 9), investigating the obesity related pro-metastatic and pro-survival pathways diminishing radiation response of breast carcinoma cells is essential.

Obesity is associated with alteration in adipose tissue and lipid metabolism.(10) Changes in lipid metabolism and, consequently, lipid composition could modulate therapy response of cancer cells.(11) Many lipids have been found to be increased in cancer, as for example sphingolipid 1-phosphate in BC. Studies also suggested that the choline-containing lipids and phospholipids in cancer cells could increase during the metastatic process.(12)

Adipocytes in BC microenvironment can communicate with cancer cells and this crosstalk leads to phenotypical and functional changes of both cell types (13). Adipokines, secreted by adipocytes, promote breast cancer initiation, proliferation, invasion, can have anti-tumorigenic effect (14) or can modulate response to treatment.(15) Additionally, the co-culture of mature adipocytes and breast cancer cells results in heterogeneous and probably breast cancer cell type specific communications(16).

Dipeptidyl peptidase DPPIV/CD26 is an adipokine - type II transmembrane multifunctional glycoprotein involved in various biological and pathologic processes. DPPIV degrades incretins such as glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide,(17) leading to reduced insulin secretion and it is a target for treatment of diabetes mellitus. Its expression is positively correlated with BMI (18). In cultured cells, DPPIV knockdown induced epithelial to mesenchymal transition (EMT) and cell migration and inhibition of DPPIV increases the metastatic potential of breast cancer (19). Currently, there are several DPPIV inhibitors available, which have different binding modes in the DPPIV active center. Among them Teneligliptin has the highest inhibitory function toward DPPIV (18). It also has antioxidant properties and ameliorates oxidative stress (20).

Rationale and Aim

We aimed to elucidate whether affected lipid metabolism can contribute to therapy response in breast cancer patients.
### Experimental design

We investigated the lipid metabolism and radio sensitivity of three types of breast cancer cell lines: MDA – MB-231 (triple-negative, TN), T47D (hormone receptor positive, HR+) and AU565 (Her2-positive, HER2+) in correlation with their invasive capacities.

BC cells (MDA – MB-231; T47D and AU565 cell lines) were exposed to repetitive migration through an uncoated 8 µm-pore membrane in order to obtain invasive (INV) BC cells. Both parental cells and derived INV cells are accessed for their:

- INV potential - CytoSelect Invasion Assay (Collagen I or Laminin);
- Proteomics and lipidomics – nano-LC-mass spectrometry (Omics Technologies Inc., USA)
- Metabolic activities – Agilent Seahorse Metabolic Analyzer XFp
- Metastatic abilities - in vivo experiments using nude mice;
- Radiation response - exposure to photon-based ionizing radiation and assessment of their clonogenic survival (Figure 1);

![Diagram](image)

**Figure 1. Schedule for assessment of the parental and the invasive cells**

The response to radiotherapy was evaluated as we exposed parental and invasive BC cells to photon-based ionizing radiation and investigated how their clonogenic survival is affected. The parental and invasive MDA-MB-231 cells did not show differences in clonogenic survival; T47D-INV became markedly more resistant to ionizing radiation; Au565-INV cells became also more resistant, as the effect was less significant then the observed in T47D. We further applied this model of response to radiotherapy in the investigation of lipid profile of BC cells and their expression of adipokines.

1. Lipidomics - Lipid profile of the parental and invasive cell lines was analysed as each lipid class, identified from the samples analysis, was related to a category, class and, wherever possible, to a subclass according to the nomenclature of LIPID Metabolites and Pathways Strategy (LIPID MAPS). When a lipid was identified multiple times in the analysis of a certain cell line, the average value of the peak areas was calculated and used for further analysis.
2. Adipokines - The secretion of adipokines by the different BC cell lines was investigated with Proteomics Profiling Array (R&D Systems, Inc.).
3. DPPIV - The role of DPPIV /CD26 was tested by investigating its effect on BC cells viability, clonogenic survival and migratory abilities.
Results, Conclusions and Future Perspectives

1. Lipidomics

We first investigated how the lipid profile of the cell lines is changed in the process of acquirement of increased invasive abilities. We analysed the common lipids found in the parental cell lines and their invasive counterparts. The highest percentage of lipids in common was found in MDA-MB-231 cells (74%), followed by Au 565 (68.3%) and T47D (55.9%). We also found some lipids, which were unique for a specific subtype as PIP3 species, which were found only in Au 565 parental and were not detected in Au 565 invasive.

We further investigated the common lipids between parental and invasive cell lines in order to find differences in their expression. Lipids, which were expressed more than 1.5 times in invasive cells compared to parental were considered for upregulated; lipids, which levels of expression in invasive cells was 2/3 of the observed in parental were considered as downregulated. We found 7 lipids species, which were downregulated in all invasive cell lines and 8 lipid species which were all upregulated.

![Figure 2. Lipid species in common between parental and invasive cells and (a) downregulated in invasive; (b) upregulated in invasive.](image)

Taking into account the model of response to radiotherapy observed in the three cell lines we searched for a lipids class with the similar pattern of expression as the observed for the radiotherapy response. We investigated the ratio of lipid content from each main class in invasive to parental cells of each cell line. The results are shown on Table 1.

<table>
<thead>
<tr>
<th>Category</th>
<th>Main class</th>
<th>MDA INV to PAR</th>
<th>T47D INV to PAR</th>
<th>Au 565 INV to PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acyls (FA)</td>
<td>Fatty Acids and Conjugates</td>
<td></td>
<td>0.48165744</td>
<td>0.883675955</td>
</tr>
<tr>
<td></td>
<td>Fatty esters</td>
<td>2.837753047</td>
<td>4.512442898</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diradylglycerols</td>
<td>1.158191</td>
<td>0.450112281</td>
<td>2.661971867</td>
</tr>
<tr>
<td></td>
<td>Triradylglycerols</td>
<td>0.744085</td>
<td>1.115601537</td>
<td>0.509453434</td>
</tr>
<tr>
<td>Glycerolipids (GL)</td>
<td>Glycosyldiradylglycerols</td>
<td>0.563908</td>
<td>6.292520966</td>
<td>3.555041726</td>
</tr>
<tr>
<td></td>
<td>Other Glycerophospholipids</td>
<td>0.210936</td>
<td>0.352815478</td>
<td>0.630401557</td>
</tr>
<tr>
<td></td>
<td>Glycerophosphocholines</td>
<td>0.958423</td>
<td>1.439405398</td>
<td>1.231485791</td>
</tr>
<tr>
<td></td>
<td>Glycerophosphoethanolamines</td>
<td>0.750787</td>
<td>0.771262522</td>
<td>0.957254397</td>
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<tr>
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<td>Glycerophosphoserines</td>
<td>0.622584</td>
<td>0.29316928</td>
<td>2.139268982</td>
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<tr>
<td></td>
<td>Glycerophosphoglycerols</td>
<td>0.445577</td>
<td>2.312552594</td>
<td>1.299299303</td>
</tr>
</tbody>
</table>
2. Adipokines

The secretion of adipokines by the different BC cell lines was investigated with Proteomics Profiling Array (R&D Systems, Inc.). We compared the levels of expression of adipokines in invasive cells to the expression in their parental counterparts and again looked for the pattern of expression similar to the pattern of radiotherapy response. The adipokines, shown in Table 2, are the ones most corresponding to the pattern of radioresistance of interest.

Table 2. Adipokines, expressed in the same manner with the pattern of the observed response to radiotherapy and the ratio of expression INV/parental cells

<table>
<thead>
<tr>
<th>Adipokines</th>
<th>MDA-MB-231-IN V</th>
<th>T47D-IN V</th>
<th>Au565-IN V</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPIV/CD26</td>
<td>1.52</td>
<td>5.71</td>
<td>0.43</td>
</tr>
<tr>
<td>Nidogen-1/Entactin</td>
<td>1.68</td>
<td>4.55</td>
<td>0.77</td>
</tr>
<tr>
<td>Proprotein Convertase 9/PCSK9</td>
<td>0.90</td>
<td>2.27</td>
<td>0.73</td>
</tr>
<tr>
<td>LIF</td>
<td>1.17</td>
<td>2.11</td>
<td>0.79</td>
</tr>
</tbody>
</table>

3. DPPIV

We have decided to further evaluate the role of DPPIV/CD26 as possible key determinant of radiotherapy response in breast cancer cell lines.

First, we have detected that DPPIV/CD26 expression in the INV cells correlates with glucose uptake and total volume of intracellular lipids. Thus, invasive hormone receptor-positive breast carcinoma T47D-IN V cells reveal an upregulation of DPPIV/CD26 and enhanced glucose uptake and lipid content, whereas Her2-positive Au565-IN V cells with down-regulation of DPPIV/CD26 and significant decrease of glucose uptake and intracellular lipid content. MDA-MB-231-IN V changed neither DPPIV/CD26, nor glucose uptake nor total lipid volume.
Secondly, we investigated the effect of the DPPIV inhibitors Teneligliptin and p32/98 on both parental and invasive cells viability, clonogenic survival in combination treatment with radiotherapy or alone and migratory abilities.

For the purposes of those experiments all cell lines were grown in RPMI1640 medium supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum (FCS) (Thermo Fisher Scientific, Vienna, Austria). Cell cultures were maintained in a 5% CO₂ humidified atmosphere.

1) Parental and invasive cells viability
Parental MDA-MB-231 and Au565 cells and their invasive pairs were seeded (1 x 10⁵) in 3.0 mL in 6-well plates; parental T47D and T47D-INV cells were seeded 2 x 10⁵ and 2.5 x 10⁵, respectively. All cells were incubated for 24 hours at 37°C and then treated with different concentrations of Teneligliptin (0.1μM, 1.0μM, 3.0μM and 10μM). Cells were trypsinized and counted using Beckman Coulter Vi-CELL AS cell viability analyzer (Beckman Coulter, Fullerton, CA, USA) 48h and 72 hours after treatment with the inhibitors. Based on these experiments we couldn’t find significant effect of Teneligliptin on cells viability.

2) Clonogenic survival after treatment with DPPIV inhibitors alone and after combination treatment with radiotherapy
First, we investigated the effect of Teneligliptin and p32/92 alone on the clonogenic ability of the parental and invasive cells. Parental MDA-MB-231, T47D and Au565 and their invasive pairs were seeded 500 cells in 3.0-mL in 6-well plates. All cells were incubated for 24 hours at 37°C and then treated with different concentrations of Teneligliptin (0.1μM, 1.0μM, 3.0μM and 10μM) and p32/92 (1.0μM, 10μM, 50μM and 100μM) in duplicates. Each pair of parental and invasive cells were incubated for the same time and stained with crystal violet in the same day. The incubation time for the different cell lines was different, since they have different doubling time (as previously reported) and clonogenic abilities. We observed close, but different response to treatment with Teneligliptin and p32/98 among the MDA-MB-231 and Au 565 parental and invasive cells as Teneligliptin was suppressing or not affecting and p32/98 was stimulating the clonogenic abilities of the cells. In T47D invasive cells we observed 10 times increase in the surviving fraction (SF), when cells were treated with 50μM p32/98, compared to control and more than 6 times increase of SF, when treated with 0.1 μM Teneligliptin. (Figure 3).

These results are in line with our previous findings that T47D-INV cells reveal an upregulation of DPPIV, whereas Au565-INV cells reveal down-regulation and MDA-MB-231-INV revealed no change in DPPIV.

Secondly, we investigated the effect of combined treatment of Teneligliptin and radiotherapy (RT) on MDA-MB-231 parental and invasive cells in two treatment schedules— if the treatment is applied consequently or simultaneously. MDA-MB-231 parental and invasive breast cancer cells were seeded (1 x 10⁵) in 3.0-mL medium in 6-well plates. After 24 hours incubation cells were treated with different concentrations of Teneligliptin (0.1μM, 1.0μM, 3.0μM and 10μM) and incubate for another 24 hours. After that 500 cells from each treatment concentration were seeded in 6 well plates in duplicates and incubated for 24 hours, when radiotherapy in dose of 2Gy and 6Gy was applied. In the second approach 500 cells from the MDA-MB-231 parental and invasive cells were seeded in 6 well plates. In 24 hours the cells were treated with different concentration of Teneligliptin and 4 hours later were irradiated with dose of 2Gy and 6Gy.

Cells, treated without irradiation and with 2Gy, from both experiments were incubated for 9 days. At this point there were
no clones developed by cells treated with 6Gy. MDA-MB-231 invasive cells were able to form more clones compared to the parental. Cells, treated with 6Gy, were incubated for 16 days in total and then stained. Only big single clones were observed. We observed dose dependent response to treatment in both parental and invasive cells, most significant, when cells are treated with 3.0μM Teneligliptin. Further experiments are needed to confirm these results in this and the others cell lines.

3) Migratory abilities – scratch assay

Migratory capacities of the investigated BC cells were assessed with automated live cell imager Lionheart FX (BioTek, Vermont, USA). When a full cell monolayer was obtained a scratch was made in a straight line with a p200 pipet tip. The medium was then changed with medium containing different concentrations of Teneligliptin and cells were incubated in the live cell imager for 72 hours. Images were obtained every 2 hours. We observed the fastest closure in both control and treated cells in Au parental cells – for 22 hours and 18 hours, respectively. T47D invasive cells were not affected by Teneligliptin and the “scratch” in those cells monolayer was never closed by the end of the incubation period. In T47D parental cells the “scratch” was not closed at the 72hour in the control and was almost closed in the cells treated with 10μM Teneligliptin. Therefore we could conclude that Teneligliptin is affecting the migratory abilities of MDA-MB-231 INV cells, Au 565 parental and invasive cells and most significantly – the T47D parental cells.

Further experiments are needed in order to confirm the reported results.

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**List of Publications and Presentations Resulting from the Translational Research Project “Breast cancer metastasis and lipid metabolism implication”**


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**List of Publications and Presentations resulting from other projects during the fellowship period**

**Poster presentations:**


Presentations:


Publications:


Selection of Courses and Workshops Attended During the Fellowship

October 2020 – ESSO 2020 Virtual Congress
September/October 2020 – ESMO 2020 Virtual Congress
October 2020 - Breast Cancer in Young Women: Fifth ESO-ESMO International Symposium
August 2020 - ESMO Academy
March 2020 – Think Pink Europe Innovation award – second prize
December 2019 - International Conference „Cancer Metastasis“, Seefeld-in-Tirol, Austria
December 2019 - ESMO Advanced Course on Biomarkers for Precision Medicine, Zurich, Switzerland

Personal Statement

The ESMO translational research fellowship gave me once in a lifetime opportunity to learn and gain new experience in a field that I wouldn’t otherwise have the chance to explore in such details. In the year that has past I have learnt so much, every day. I have enriched my view and changed my perspective for research, not only translational, but in the whole field of oncology. Beside scientific work, the last year have taught me to think more for others and strengthen my belief that we, working together, can actually have an impact on our life and life of the others.

Before coming to the ESMO TR fellowship I wanted to see what would be to be involved in a cutting-edge research that can really make the change for people. Working in a lab, learning so much and seeing the gaps in research and treatment led to the development of the project ROSE: Rising the Oncology patients’ Survival among the European countries- a project for breast cancer digital diary, which is connecting translational and clinical research.

The year that has past was also very hard for me as I believe for many others. It was a year in which we all had to rethink our priorities, sacrifice our comfort and realize that we could be running out of time. This year was my 33th year and above all it was truly special one. I want to thank to ESMO, for giving me this chance, for changing my life and for supporting me all the way.
References


4. Pan Hongchao GRG, and on behalf of the Early Breast Cancer Trialists' Collaborative Group. Effect of obesity in premenopausal ER+ early breast cancer: EBCTCG data on 80,000 patients in 70 trials. Journal of Clinical Oncology 2014 32 503-.


SIGNATURES
Award Recipient full name
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Signature and Date
23 Oct 2020
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