Project proposal submitted to the OeGHO
to apply for the ASHO Clinical Research Grant

Dissecting the interplay of the long non-coding RNA NORAD and PARP inhibitors in triple negative breast cancer allowing optimization of treatment concepts

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Abbreviations: **BC:** breast cancer; **BER:** base-pair excision repair; **BRCA:** breast cancer susceptibility gene; **CIN:** chromosomal instability; **DMSO:** dimethyl sulfoxide; **DSB:** double strand breaks; **ER:** estrogen receptor; **Her2**: human epidermal growth factor receptor 2; **HR:** homologous recombination; **lncRNA:** long non-coding RNA; **NORAD:** non-coding RNA activated by DNA damage; **PARP:** Poly-ADP-ribose Polymerase; **PARPi**: PARP inhibitor; **PR:** progesterone receptor; **SSB:** single strand breaks; **siRNA:** short interfering RNA; **TNBC:** triple negative BC.

Keywords: long non-coding RNAs, PARP inhibitors, triple negative breast cancer, therapy optimization

1. BACKGROUND AND SCIENTIFIC SIGNIFICANCE

1.1. Molecular subtypes of breast cancer and their consequences on therapeutic options

Breast cancer (BC) is the leading cause of cancer death in women aged 20 to 60 years. About 63.960 cases of female BC carcinoma are expected to be diagnosed in 2018 only in the US and BC alone accounts for 30% of all new cancer diagnoses in women[1]. BC is a heterogeneous disease in terms of underlying biology, clinical course, treatment strategy and prognosis[2]. In general, BC can be classified into several molecular subtypes[3,4] depending on gene expression patterns of breast carcinomas. Triple-negative BC constitutes the most aggressive BC subtype, is characterized by a lack of expression of estrogen receptor (ER^-), progesterone receptor (PR^-) and human epidermal growth factor receptor 2 ($HER2^-$)[5] and is often associated with mutations in the breast cancer susceptibility protein (*BRCA)* genes, which are known tumor-suppressor genes involved in DNA damage repair. Patients with $BRCA1^{Mut}$ are more likely to develop TNBC, with high nuclear grade and a larger tumor burden[6]. Patients with TNBC have a poor prognosis for disease-free as well as for overall survival[5] and an increased risk for early relapse or distant recurrence within the first five years after initial diagnosis. Currently, due to a lack of molecularly characterized targets, chemotherapy is still the mainstay of treatment for TNBC[7] in clinical routine. Therefore, new treatment options are urgently needed to fight this deadly disease.

1.2. Chromosomal instability, PARP Inhibitors and BRCA1 mutations in TNBC

Chromosomal instability (CIN) has been added to the hallmarks of cancer and is characterized by a frequent gain or loss of chromosomes during mitosis[8]. These chromosomal aberrations are one of the defining features of cancer and hallmark of gene deregulation and genome instability[9]. Markers for chromosomal aberrations facilitate cancer detection, prediction of clinical outcomes and response to therapy. Furthermore, identification of genes involved in regions of recurrent aberrations may be an attractive target for the development of new therapies[9], as many therapies are based on increased CIN which causes aberrant cells to undergo apoptosis[10]. BRCA1 and BRCA2 breast tumors develop by specific and distinct evolutionary paths, as their gene profiles[11] and genome aberration spectra differ from each other and from those in sporadic BC[12,13]. Poly-ADP-ribose Polymerase (PARP) inhibitors (PARPi) are gaining medical attention for therapy of TNBC patients harboring a $BRCA1/2^{Mut}$ which can be found in 20-25% of TNBC cases[14]. The proteins PARP1 and PARP2 are activated by DNA damage and facilitate repair pathways involving single-

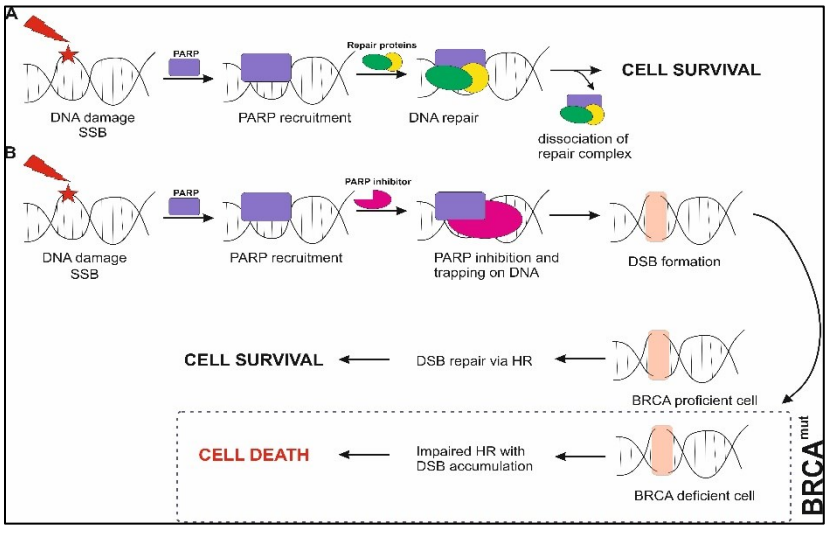

Figure 1 I Schematic representation of PARPi. (A) PARP-mediated DNA repair mechanism after DNA damage results in cell survival. **(B)** PARPi blocks recruitment of repair complex and results in DSB formation. $BRCA^{WT}$ cells are able to repair DBS via HR. $BRCA^{Mut}$ cells, have impaired HR which leads to DSB accumulation and ultimately to cell death.

strand breaks (SSB) and base pair excision repair (BER). Subsequently, these two proteins bind to areas with single strand damage via their zinc-finger DNA-binding domain. This binding is increasing the catalytic activity of PARP1 and PARP2 allowing them to use NAD^+ to create polymers of poly(ADP-ribose) and transfer them to acceptor proteins. As a result, various other proteins are recruited to the damage site and a repair complex is initiated. As depicted in **Figure 1**, BRCA-deficient cells are unable to repair accumulating double-strand breaks (DSB) which inevitably result in cell death. DSB repair by homologous recombination (HR) is required for the repair of DNA damage arising from many currently used DNA damaging chemotherapy agents, as well as DNA single strand breaks that are generated by PARPis[15]. Since BRCA deficient cells are incapable of HR, tumors arising in carriers of germline mutations in BRACA1/2 are highly sensitive to DNA damaging chemotherapy and PARPi. The PARPi olaparib is already in phase III clinical trials and has recently been granted accelerated approval based on clinical data showing its effectiveness[16].

1.3. The role of (long) non-coding RNAs in breast carcinogenesis

Non-coding RNAs are a class of RNA transcripts that are usually not translated into proteins but exert important functions through different types of molecular mechanisms[17]. Generally spoken, they can be divided into small (<200 nucleotides in length[18]) and long non-coding RNAs (lncRNAs, >200 nts in length)[19], whereas both families have been demonstrated to be involved in physiology and pathophysiology[20-22]. The lncRNA Non-Coding RNA Activated by DNA Damage (NORAD) has been demonstrated to contribute to the progression of several cancer entities[23-28]. It shows strong evolutionary conservation and is ubiquitously and abundantly expressed in human tissues and cell lines. NORAD is maintaining genomic stability by sequestering a significant fraction (50-100%) of PUMILIO proteins PUM1 and PUM2, which normally repress the stability and translation of their target mRNAs. Among these targets are factors critical for mitosis, DNA repair and replication whose excessive repression in the absence of NORAD perturbs accurate chromosomal segregation and can induce tetraploidization or in other words, NORAD inactivation produces a CIN phenotype in previously karyotypically stable cell lines[29]. Thus, targeting NORAD could potentially represent a new strategy for the treatment of cancer.

As NORAD plays a role in preserving genomic stability and in DNA repair, treatment of TNBC cells with PARPi (e.g. olaparib) and simultaneous silencing of NORAD should lead to synthetic lethality and therefore to cell death. This approach could be trailblazing for future treatment options of TNBC patients.

2. PRELIMINARY DATA that is relevant to the current proposal

The involvement of lncRNAs and chromosomal instability have gained tremendous importance in cancer research, therefore, we have studied current literature on this topic. Two recent publications by Lee et al.[29] and Munschauer et al.[30] caught our attention where authors showed the contribution of the lncRNA NORAD to genomic stability. Together with the findings described above that NORAD influences progression of several cancer entities, we decided to further characterize this lncRNA. In order to clarify the potential clinical significance of NORAD in human TNBC, we explored the association of NORAD mRNA levels and clinical endpoints. Using a publicly available microarray-based large dataset[31], high levels of NORAD were identified to be a negative prognostic factor for disease-free survival (n=161, p=0.0075, **Figure 2A**) in TNBC patients. Furthermore, analysis of several BC studies by using the database cBioPortal[32] revealed that in up to 3% of BC cases an amplification of the NORAD gene is present (**Figure 2B**). Supportingly, data derived from the GEPIA server[33] show that NORAD expression levels are increased in tumor tissue vs. non-tumor tissue in BC patients (**Figure 2C**). In order to further investigate the effects of NORAD manipulation in BC under laboratory conditions, we verified overexpression of this lncRNA in several BC cell lines by qRT-PCR compared to normal breast cells MCF-12A (**Figure 2D**). This screening showed an at least 4-fold increased expression of NORAD in BC cell lines. Next, we established an siRNA-mediated silencing approach in two triple-negative cell lines (SUM159 and MDA-MB-231) by using two independent siRNAs against NORAD, reaching a knock-down efficiency of at least 60% in both cell lines (data not shown). Silencing of NORAD resulted in a slightly reduced proliferation when compared to control conditions (data not shown). Since MDA-MB-231 cells have been reported to be insensitive to olaparib treatment, we determined the IC_{50} of olaparib in SUM159 cells (35 µM; data not shown) and used this concentration for further experiments.

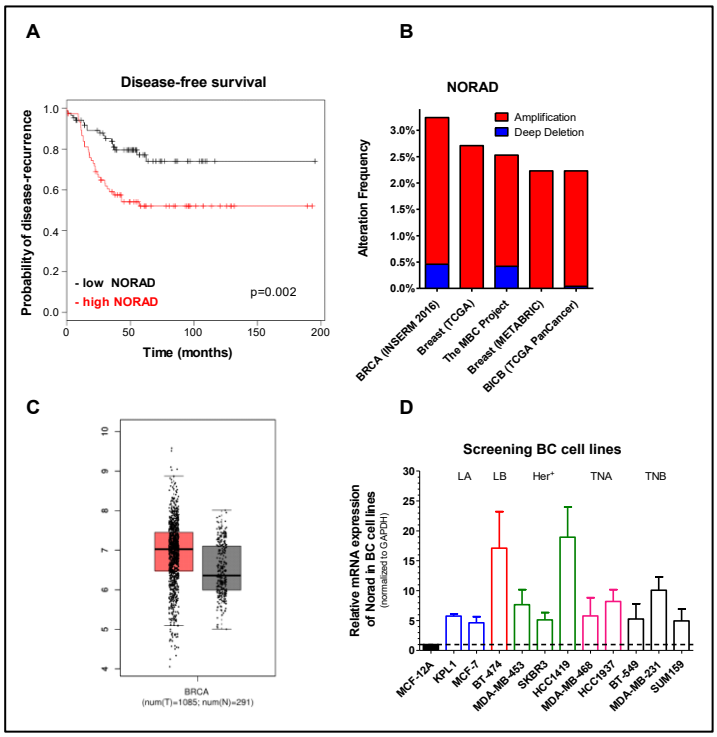

Figure 2 | (A) Patient data validating high NORAD expression as negative prognostic factor for disease-free survival of TNBC patients. HR with 95% confidence interval: 2.4 (1.35-4.25), n=161, p=0.002 with log-rank test. **(B)** Alteration profile of NORAD in BC studies based on the cBioPortal data. **(C)** NORAD expression in tumor vs matched non-tumor controls. **(D)** qRT-PCR data of mRNA expression levels of NORAD in BC cell lines compared to normal breast cells MCF-12A. For normalization the housekeeper GAPDH was used. LA…luminal A, LB…luminal B, Her+…Her2-enriched, TNA/B…triple negative A/B. mean±SD, n=3.

3. RATIONALE, HYPOTHESIS AND SPECIFIC AIMS OF THIS PROPOSAL

The concept of the involvement of lncRNAs in carcinogenesis is an evolving field[34] and the identification of the link between lncRNAs and genomic instability seems to be promising for the discovery of novel mechanisms and new treatment strategies. The broad goal of this research proposal is to investigate the role of the lncRNA NORAD in TNBC and to deepen the current knowledge of its contribution to genomic instability. Results emerging from this project could be trailblazing for further investigations towards optimized therapy options of TNBC.

We hypothesize that a combination of NORAD knock-down and PARPi treatment increases inhibition of DNA repair mechanisms and leads to more pronounced "anti-tumorigenic" phenotypes. This approach could lead to the optimization of existing or to the development of novel treatment concepts.

Hypothesis 1: *Combining NORAD knock-down and PARPi treatment amplifies respective effects on TNBC cells in vitro resulting in augmented anti-carcinogenic phenotypes.*

AIM 1: Based on the established siRNA-mediated NORAD knock-down approach, we aim to identify if combining it with PARP inhibition results in reduction of (I) cancer cell specific features such as proliferation, colony and mammosphere formation. Furthermore, the expected augmented (II) CIN phenotype should lead to increased apoptosis which will be investigated with several independent apoptosis assays.

Hypothesis 2: *Combining NORAD knock-down and PARPi treatment amplifies respective effects on TNB carcinogenesis in vitro by inducing genomic instability.*

AIM 2: In order to completely unravel the cellular consequences of our approach, we will investigate if the combination of NORAD silencing and PARPi treatment is impacting carcinogenesis due to induction of genomic instability. As described above, genomic instability is a hallmark of cancer and associated genes are under intensive investigation as therapeutic targets. Therefore, it is essential, to clarify if our combinatorial approach of PARP inhibition and NORAD silencing results in genomic instability. We hypothesize that the parallel application of NORAD knock-down and olaparib will result in DNA damage accumulation, which would serve as indicator for a defective DNA repair mechanism, ultimately resulting in genomic instability.

Hypothesis 3: *Combining NORAD knock-down and PARPi treatment amplifies respective effects on carcinogenesis in vivo.*

AIM 3: Based on the preceding *in vitro* experiments, we aim to identify if combining NORAD silencing with PARP inhibition results in reduced cancer cell growth *in vivo*.

4. WORKING PLAN ACCORDING TO THE SPECIFIC AIMS

SPECIFIC AIM 1: Combination of NORAD knock-down and PARPi treatment amplifies respective effects on TNB carcinogenesis *in vitro* (months 1–6).

Rationale: *In order to investigate if the combination of the PARPi olaparib and NORAD silencing could be a promising anti-cancer therapy, TNBC cells will be treated with a combination of siRNA-mediated knock-down of NORAD and treatment with olaparib. Respective controls will be included i.e. control siRNA, DMSO control and as positive control cisplatin treatment (as this compound is also inducing DNA damage[35]).*

AIMs and corresponding experimental steps

(I) Influence of combinatorial approach on cancer cell phenotype

- **Proliferation**: Before measuring the functional effects, we will investigate if the combination of the PARPi olaparib and NORAD silencing results in more pronounced proliferation defects. To assess if a combination of olaparib treatment and NORAD knock-down results in a more pronounced anti-proliferative effect, then the single treatments, we will perform a standard proliferation assay. Therefore, TNBC cells will be seeded (3500 cells/well) and transfected in 96-well plates with siRNAs against NORAD or negative control siRNA. On the next day, cells will be treated with 35 µM olaparib, 3.3 µM cisplatin or the respective controls (DMSO or NaCl). From this point on, WST-1 proliferation agent (Roche Applied Science, Vienna, Austria) will be applied every 24 hours according to manufacturer's recommendation. The colorimetric changes will be measured ever day using a SpectraMax Plus (Molecular Devices, San José, CA, USA) at a wavelength of 450 nm with a reference wavelength at 620 nm.

- **Colony formation**: As the ability to form colonies represents an essential characteristic of cancer cells, we will conduct colony formation assays. 48 hours after transfecting/treating cells, they will be trypsinized, counted and seeded for colony formation assay in 6-well plates at a density of 100-400 cells/well (depending on the cell line). After 10-14 days, cells will be fixed and stained with crystal violet and numbers of colonies will be counted and compared between experimental conditions.

Mammosphere formation: We will perform a spheroid growth model as previously described[36] with slight modifications. In detail, the adherent growing BC cell lines will be dissociated 48 hours after transfection into single cells using trypsin/EDTA and 2,000 single cells per well will be seeded in ultra-low attachment 6-well plates using serum-free medium (SFM). SFM is supplemented with 1xB27 supplement, 20 ng/ml human epidermal growth factor EGF, 10 ng/ml human basic fibroblast growth factor FGF 20 IU/ml Heparin and 1% antibiotic/antimycotic solution. After 10-21 days mammospheres will be counted.

(II) Influence on combinatorial approach on TNBC apoptosis

- **Apoptosis assays**: To elucidate if combining olaparib treatment with NORAD knock-down results in apoptosis induction, we will perform three independent apoptosis assays. *a)* Activity of caspases 3 and 7

will be determined by Caspase-Glo 3/7 assay (Promega) 48 hours after transfection/treatment according to the manufacturer's instructions. Cells will be seeded in 96-well plates and after adding the substrate, luminescence will be recorded using a luminometer (LumiStar, BMG). *b)* To assess the ratio between full-length PARP1 and cleaved PARP1 (the more cleaved PARP, the more apoptosis), Western Blots will be performed with TNBC cell lysates 48 hours after transfection/treatment according to standard Western Blot procedures. Antibodies will be used in the following concentrations: anti-PARP1 1:1000 (Cell Signaling), anti-β-Actin (housekeeper): 1:5000 (Sigma Aldrich) and HRP-conjugated anti-rabbit or anti-mouse secondary antibodies, respectively (Dako, Glostrup, Denmark). (*c)* Upon apoptosis induction, mitochondria are changing their shape from reticulo-tubular to spherical. Mitochondrial shape will be measured 48 hours after transfection of TNBC cells in 6-well plates on 30 mm coverslips. Mitochondria will be stained with 0.5 µM MitoTracker® Red FM and visualized on a confocal spinning disk microscope (Axio Observer.Z1 from Zeiss) equipped with a 100x objective lens and a Nipkow-based confocal scanning unit (CSU-X1). Z-stacks of mitochondria with 0.2 µm increments will be imaged and mitochondrial morphology including volume, surface, form factor and aspect ratio will be analyzed using the 3D-Suite[37] (3D Geometrical Measure and 3D Ellipsoid Fitting) plugin in ImageJ.

Expected Results (ER) and potential pitfalls. At the end of AIM 1, we will be able to reliably assess the potential of a combinatorial approach of NORAD silencing and PARPi treatment of TNBC cells *in vitro* and its potential significance as therapeutic approach. These data allow the estimation of the potential of this combinatorial approach in *in vivo* experiments. Since I am experienced in the above mentioned techniques and the knock-down approach as well as cell treatment with chemotherapeutic agents are established, I do not foresee any problems and experiments can be started straightforward.

SPECIFIC AIM 2. Combining NORAD knock-down and PARPi treatment amplifies respective effects on TNB carcinogenesis *in vitro* by inducing genomic instability.

Rationale: *To realize this aim, we will visualize if the combined treatment induces DNA damage, a key feature of genomic instability, by applying immunofluorescence against proteins specifically detecting DNA double-strand breaks. Furthermore, as genomic instability manifests as aneuploidy (loss or gain of chromosomes) or as rearrangement of chromosomal structures, we will perform karyotyping, chromosomal banding and multi-color FISH in cooperation with Dr. Emberger and his team of the Department of Human Genetics (Head: Dr. Michael Speicher), in order to decipher if the treatment is inducing genomic instability and if so, which chromosomes and chromosomal regions are involved.*

AIMs and corresponding experimental steps

(I) Influence of combinatorial approach on DNA damage

Immunofluorescence: In order to visualize if combining NORAD silencing and PARPi treatment increases DNA DSB in TNBC, we will perform immunofluorescence experiments. Therefore, we will use antibodies against Rad51 and γH2AX-pS139 (abcam, Cambridge, UK), both indicators for DSB and HR efficiency[15,38].

10.000 SUM159 cells/well will be seeded on 8-well chamber slides and transfected with siRNA against NORAD or negative control siRNA. One day after transfection, cells will be treated with olaparib, cisplatin or the respective controls for 48-96 h and subsequent immunofluorescence procedure will be conducted according to manufacturer's protocol. Rad51 and γH2AX-pS139 will be used in dilutions of 1:500 and 1:1000, respectively. As secondary antibody Alexa Fluor 488 antibody (1:500; ThermoFisher) will be used. Nuclei will be visualized using Dapi-containing ProLong® Gold Antifade Mountant (ThermoFisher). Fluorescent images and Z-stacks will be taken using on a Nikon A1 confocal microscope. Foci quantification (total amount of foci per DAPI-positive cell) will be performed manually by displaying the maximal intensity projection of the individual Z-stacks with the software NIS-Elements Viewer 4.20 (Nikon, Tokyo, Japan) allowing the quantification of all foci present in the respective cell.

(II) Influence of combinatorial approach on chromosomal arrangements

These experiments will be conducted by our collaboration partner Dr. Emberger and members of his specialized laboratory on mFISH and chromosomal arrangement studies.

Karyotyping: As a first step, we will perform chromosomal G-banding, a technique allowing the visualization of gross chromosomal abnormalities, including gains or losses of whole or partial chromosomes as well as large structural abnormalities such as additions, deletions, inversions and, translocations.

Metaphase chromosomal spreads will be prepared as described previously[39] with custom adaptions to the respective cell lines. Briefly, 2.5 hours before harvesting, cells will be treated with colcemid solution (0.03 µg/ml; Sigma Aldrich) to arrest cell cycle at metaphase. Cells will then be fixed with hypotonic solution (0.0075 M KCl), fixed three times with fixative (3:1 methanol to acetic acid) and spread on glass. Slides will be baked at 55-60°C overnight. Slides will be incubated with 0.025% trypsin solution for 10 sec, followed by trypsin inactivation with 1% FBS. Finally, slides will be stained with Giemsa staining solution for 10 minutes and chromosomal banding will be visualized on a standard light microscope[40,41].

multi-color FISH: If G-banding results in chromosomal alterations, multicolor-FISH (mFISH) will be applied for more detailed evaluation of the occurring aberratins. mFISH is a method to facilitate analysis of each single chromosome or chromosome part of a metaphase. Thus, marker chromosomes, complex chromosomal rearrangements, and all numerical aberrations can be visualized simultaneously in a single hybridization experiment.

mFISH of TNBC cells treated with a combination of NORAD silencing and PARPi treatment, will be conducted as described previously[41-44]. Briefly, a probe cocktail containing 24 differentially labeled chromosome-specific painting probes (24xCyte kit MetaSystems, Altlussheim, Germany) will be denatured and hybridized to denatured metaphase chromosome spreads according to the manufacturer's protocol. The slides will be incubated at 70°C in saline solution (2xSSC), denatured in NaOH, dehydrated in ethanol

series, air-dried, covered with 10 µl of probe cocktail (denatured) and hybridized for two days at 37°C. The slides will then be washed with post-hybridization buffers, dehydrated in ethanol series and counter-stained with 10 µl of DAPI/antifade. The signal detection and analysis of subsequent metaphases used the Metafer system and Metasytems' ISIS software (software for spectral karyotypes).

Expected Results (ER) and potential pitfalls. At the end of AIM 2, we will be able to reliably assess if a combinatorial approach of NORAD silencing and PARPi treatment is leading to induction of DNA damage accumulation and subsequent chromosomal instability. The expected arising chromosomal instability will be further characterized by detailed analysis of chromosomal alterations. As the applicant is experienced with immunofluorescence and the laboratory is well equipped for such experiments, we do not foresee any difficulties with AIM 2 (I). As chromosomal aberration analyses will be performed by our collaboration partner, a leading expert in the application of the described techniques, experiments can be started straightforward and we do not expect any complications. The results of AIM 2 will allow us to conclude if the treatment is leading to chromosomal instability, indicating if it is a suitable candidate as therapeutic strategy.

SPECIFIC AIM 3. Combining NORAD knock-down and PARPi treatment amplifies respective effects on carcinogenesis *in vivo* (months 5-8).

Rationale*: Our preliminary data point towards a clinical significance of NORAD in TNBC. Furthermore, its association with chromosomal instability suggests a role in DNA-repair mechanisms. Therefore, we aim to investigate if a combination of NORAD silencing with PARPi treatment is increasing the therapeutic effect of the PARPi. To investigate this, we will perform in vivo experiments in a xenograft mouse model to visualize potential anti-tumor effects of a combinatorial treatment.*

Technical details:

To substantiate the *in vitro* findings of AIM 1 and AIM 2, *in vivo* experiments will be performed to elucidate a potential therapeutic significance of combining NORAD silencing and PARPi treatment. Therefore, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes loaded with siRNAs against NORAD or control siRNAs will be administered into the mice as described previously[45]. Female athymic nude mice between 6–8 weeks of age will be inoculated with $1x10^6$ SUM159 cells in the subcutaneously mammary fad pad (local tumor growth). After tumors have reached a size of 20 mm^3, we will start to treat the mice with siRNAs in nanoliposomal-encapsulated particles (siRNA against NORAD or control siRNA; 200 mg/kg body weight twice weekly) as well as with the treatment with either olaparib (50 mg/kg i.p.), cisplatin (6 mg/kg, i.v.) or corresponding vehicle control . All groups will receive treatment for 4 weeks. The read out of this experiment will be the reduction in tumor burden (local growth) after 4 weeks. Tumors will be harvested after scarifying the mice to further analyze cellular parameters (Ki67, CD31, cleaved Caspase3/7) and the RNA target by *in situ* hybridization. In case we will find significant differences in efficacy, we will perform *in vivo* toxicity studies to rule out toxicity and inflammatory events. For this, after 72 hours of

treatment with siRNA-loaded nanoliposomes of 200 mg/kg body weight, mice will be euthanized by exsanguination following IACUC approved protocols. Blood samples and tissues (fixed and embedded in paraffin) will be collected at necropsy for further analyses. Blood samples will be processed for blood chemistry and hematology testing (liver enzymes, kidney function and blood cell counts). Paraffin-embedded tissue sections will be stained with hematoxylin and eosin for routine histopathology. To assess serum cytokine levels and exclude pro-inflammatory effects of siRNA delivery, mice will be treated with single i.v. injections of either anti-NC-DOPC (n=10) or anti-lncRNA-DOPC (n=10).

Expected Results (ER) and potential pitfalls. At the end of AIM 3, we will be able to draw a conclusion if a combinatorial approach of NORAD silencing and PARPi treatment of TNBC cells has therapeutic potential. As our laboratory is experienced with the nanoliposomal-mediated *in vivo* knock-down we do not foresee any difficulties. Furthermore, professional personal of the Core and Animal Facilities will handle and take care of the animals according to institutional rules as well as analyzing the routine blood parameters and cytotoxicity tests.

5. WORK AND TIMELINE FOR THIS PROJECT

Time in Months												
Work plan	01	02	03	04	05	06	07	08	09	10	11	12
AIM 1												
AIM 2												
AIM 3												

The above described methods are well established in our laboratory, the applicant is experienced in performing such experiments and therefore, the proposed work plan can realistically be conducted in the given period.

6. PERSONAL provided by Medical University of Graz (not funded by the OeGHO)

Principal Investigator

The applicant of this grant application (**Christiane Klec, BSc, MSc, PhD**) has recently completed her PhD studies in the international program DK-MCD (Metabolic and Cardiovascular Disease) at the Medical University of Graz. During her PhD studies she was involved in several projects ranging from designing, establishing and applying genetically encoded Ca^{2+} sensors, over unravelling crucial aspects and components important for the regulation of mitochondrial Ca^{2+} homeostasis to the metabolic characteristics and settings of cancer cells. The contribution to this broad spectrum of projects on the one hand resulted in co-authorships of several publications in prestigious journals and on the other hand allowed the applicant to acquire a comprehensive range of methods, techniques and knowledge. Since April 2018 Ms. Klec is

working in Prof. Dr. Martin Pichler's Research Unit for Non-coding RNAs and Genome Editing in Cancer combining her profound molecular biological and microscopy experience with already established techniques of the research group with a main focus on the role of non-coding RNAs in solid tumors. Starting with April 2019, the applicant will continue as PostDoctoral Fellow in Prof. Pichler's Research Unit, where she will further pursue her aims in cancer research.

Co-Investigators

Assoc. Prof. PD Mag. Dr. Martin Pichler (Division of Clinical Oncology, Medical University of Graz and MD Anderson Cancer Center) extensive experience in the field of oncology and non-coding RNAs. Besides his clinical training as an oncologist, he acquired several skills for a successful research career. Since 01.01.2016, his scientific achievements led to his promotion and acceptance to tenure track as Associate Professor at the Medical University of Graz. To further substantiate his knowledge in the field of non-coding RNAs, he joined in August 2013 the lab of Prof. George Calin, at The University of Texas MD Anderson Cancer Center (Houston, USA), where he is still affiliated as an Adjunct Ass. Professor at the Division of Cancer Medicine. In the Calin lab, Dr. Martin Pichler obtained a lot of expertise in many novel and innovative RNA techniques necessary for successfully performing this proposal. He is currently the head of the Research Unit of Non-Coding RNAs and Genome Editing in Cancer. He published more than 220 peer-reviewed publications (cumulative IF: 800), most of them in the cancer-associated non-coding RNA field.

Sen.-Scientist Dr.med.univ. Werner Emberger (Diagnostic and Research Institute of Human Genetics, Medical University of Graz – Head: Univ.-Prof. Dr.med.univ. Michael Speicher) is the head of the FISH-laboratory of the Department of Human Genetics, which is a leading laboratory in the performance of routine and research chromosomal alteration analysis. The team around Dr. Emberger will conduct the experiments concerning detection and specification of chromosomal alterations. The gold standard method of mFISH used worldwide to study and decipher chromosomal aberrations, has been developed at the Institute of Human Genetics[43].

7. INFORMATION ON THE RESEARCH INSTITUTION

The achievement of the outlined goals requires an excellent local research environment with basic laboratory hardware, technical expertise, and intellectual inspiration, as well as the access to high end analytical equipment. The academic context of three scientific Universities ("BioTechMed Cluster" including the Karl-Franzens-University of Graz, University of Technology and Medical University of Graz) and a large University Clinic in Graz offer all these assets. This grant will be embedded in a highly competitive research environment with a strong international standing in cancer research. The laboratory is part of the Division of Oncology and is located in a newly built campus part called the "Center for Medical Research" (http://www.meduni-graz.at/zmf). This relatively new building complex (opened in 2004) encompasses 4000 m^2 of modern laboratory and office space, including 6 Core facilities (Molecular biology, FACS and

Cell biology, Microscopy, Ultrastructure analysis and Proteomics) with state of the art technical equipment necessary for successfully performing this project. Notably, since 2009 Martin Pichler is the Head of the Research Unit of Non-coding RNAs and the Research Managing Director of the whole Division of Oncology. In addition, a recently opened and associated "Centre for knowledge and technology transfer in medicine" (http://www.zwt-graz.at/en/zwt) adds additional 11.000 m^2 research area to this campus complex, which is one of the largest space expansions of research buildings in Austria within the last five years. The Division of Clinical Oncology, the institution where this project will be affiliated, is one of three core areas in the recently (2014) approved COMET1 program, an ambitious research project for novel biomarker identification in cancer patients (funded with more than 30 Mio Euros over seven years). Finally, the Biobank Graz at the Medical University of Graz offers one of the world's largest collections of diseased and control tissues, which has been developed under the 7th EU Framework programme with the aim of the establishment of a pan-european network of biobanks and biomolecular resource centres, their innovative further development and sustainable financing. The overall European coordination of this EU-infrastructure project with the name, „Biobanking and Biomolecular Resources Research Infrastructure (BBMRI)" has been carried out by the Medical University of Graz. Consistent with this infrastructure, both the Medical University of Graz (MUG) designated cancer research as a top priority research area.

8. FINANCIAL ASPECTS

The costs for the consumables are based on previous experiences from the last years.

Material costs	**Subtotal**
siRNAs (Qiagen)	2.000.-
RNA isolation, Reverse Transcriptase, Primer, SYBR Green Kit from Standard supplier for RT-PCR (Qiagen, Roche)	8000.-
Consumables and plastic ware, Cell culture media, disposables	4000.-
Chemotherapeutic agents (olaparib, cisplatin)	2000.-
WST-1 proliferation Assay (Roche)	3000.-
Matrigel for mammosphere Assay (Corning)	2500.-
HiPerFect Transfection Reagent (Qiagen)	3500.-
Antibodies (PARP, β-Actin, secondary antibodies; Cell Signaling, Sigma Aldrich, DAKO) and Western Blot components	3000.-
Confocal microscopy (MitoTracker® Red, cover slips, EHL-buffer ingredients; ThermoFisher, Sigma Aldrich, Roche)	3000.-
Caspase 3/7 Apoptose Kit (Promega)	3000.-
Chromosomal aberration analysis: karyotyping, mFISH	12000.-

Mouse experiments (mice, handling, care, routine blood parameter and cytotoxicity measurements, anaesthetics)	4000.-
Sum (total)	**50.000.-**

9. DISSEMINATION STRATEGY

The results of our data arising from this project will be prepared for publication in prestigious international journals during and after the research project. The data will be presented in the form of poster presentations as well as oral communications at national and international congresses and scientific meetings such as the Annual Meeting of the American Association for Cancer Research (AACR), ASCO, ESMO or Keystone Symposia, besides smaller national meetings. The OeGHO grant will be acknowledged in all of the arising presentations and publications.

10. ETHICAL CONSIDERATIONS

As this proposal does not include human samples (or just publicly available international datasets) I do not foresee any major ethical hurdles. For animal studies we will follow strict institutional rules according to local as well as international clear recommendations. The animals will be handled and taken care by professional personal of the Core and Animal Facilities according to institutional rules.

11. REFERENCES

1 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2018. *CA Cancer J Clin* **68**, 7-30 (2018).

2 Pasculli, B., Barbano, R. & Parrella, P. Epigenetics of breast cancer: Biology and clinical implication in the era of precision medicine. *Semin Cancer Biol* **51**, 22-35 (2018).

3 Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**, 10869-10874 (2001).

4 Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747-752 (2000).

5 Schlacher, K. PARPi focus the spotlight on replication fork protection in cancer. *Nat Cell Biol* **19**, 1309-1310 (2017).

6 Chen, H., Wu, J., Zhang, Z., Tang, Y., Li, X., Liu, S., Cao, S. & Li, X. Association Between BRCA Status and Triple-Negative Breast Cancer: A Meta-Analysis. *Front Pharmacol* **9**, 909 (2018).

7 Brenton, J. D., Carey, L. A., Ahmed, A. A. & Caldas, C. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J Clin Oncol* **23**, 7350-7360 (2005).

8 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

9 Albertson, D. G., Collins, C., McCormick, F. & Gray, J. W. Chromosome aberrations in solid tumors. *Nat Genet* **34**, 369-376 (2003).

10 Vargas-Rondon, N., Villegas, V. E. & Rondon-Lagos, M. The Role of Chromosomal Instability in Cancer and Therapeutic Responses. *Cancers (Basel)* **10** (2017).

11 Hedenfalk, I., Duggan, D., Chen, Y., Radmacher, M., Bittner, M., Simon, R., Meltzer, P., Gusterson, B., Esteller, M. *et al.* Gene-expression profiles in hereditary breast cancer. *N Engl J Med* **344**, 539-548 (2001).

12 Tirkkonen, M., Johannsson, O., Agnarsson, B. A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R. B. *et al.* Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res* **57**, 1222-1227 (1997).

13 Wessels, L. F., van Welsem, T., Hart, A. A., van't Veer, L. J., Reinders, M. J. & Nederlof, P. M. Molecular classification of breast carcinomas by comparative genomic hybridization: a specific somatic genetic profile for BRCA1 tumors. *Cancer Res* **62**, 7110-7117 (2002).

14 Peshkin, B. N., Alabek, M. L. & Isaacs, C. BRCA1/2 mutations and triple negative breast cancers. *Breast Dis* **32**, 25-33 (2010).

15 Graeser, M., McCarthy, A., Lord, C. J., Savage, K., Hills, M., Salter, J., Orr, N., Parton, M., Smith, I. E. *et al.* A marker of homologous recombination predicts pathologic complete response to neoadjuvant chemotherapy in primary breast cancer. *Clin Cancer Res* **16**, 6159-6168 (2010).

16 Ito, S., Murphy, C. G., Doubrovina, E., Jasin, M. & Moynahan, M. E. PARP Inhibitors in Clinical Use Induce Genomic Instability in Normal Human Cells. *PLoS One* **11**, e0159341 (2016).

17 Riefolo, M., Porcellini, E., Dika, E., Broseghini, E. & Ferracin, M. Interplay between small and long non-coding RNAs in cutaneous melanoma: a complex jigsaw puzzle with missing pieces. *Mol Oncol* **13**, 74-98 (2019).
18 Slaby, O. Non-coding RNAs as Biomarkers for Colorectal Cancer Screening and Early Detection. *Adv Exp Med Biol* **937**, 153-170 (2016).
19 Anastasiadou, E., Jacob, L. S. & Slack, F. J. Non-coding RNA networks in cancer. *Nat Rev Cancer* **18**, 5-18 (2018).
20 Klec, C., Gutschner, T., Panzitt, K. & Pichler, M. Involvement of long non-coding RNA HULC (highly up-regulated in liver cancer) in pathogenesis and implications for therapeutic intervention. *Expert Opin Ther Targets*, 1-10 (2019).
21 Klec, C., Prinz, F. & Pichler, M. Involvement of the long noncoding RNA NEAT1 in carcinogenesis. *Mol Oncol* **13**, 46-60 (2019).
22 Schanza, L. M., Seles, M., Stotz, M., Fosselteder, J., Hutterer, G. C., Pichler, M. & Stiegelbauer, V. MicroRNAs Associated with Von Hippel-Lindau Pathway in Renal Cell Carcinoma: A Comprehensive Review. *Int J Mol Sci* **18** (2017).
23 Zhang, J., Li, X. Y., Hu, P. & Ding, Y. S. LncRNA NORAD contributes to colorectal cancer progression by inhibition of miR-202-5p. *Oncol Res* (2018).
24 Miao, Z., Guo, X. & Tian, L. The long noncoding RNA NORAD promotes the growth of gastric cancer cells by sponging miR-608. *Gene* **687**, 116-124 (2019).
25 Liu, H., Li, J., Koirala, P., Ding, X., Chen, B., Wang, Y., Wang, Z., Wang, C., Zhang, X. *et al.* Long non-coding RNAs as prognostic markers in human breast cancer. *Oncotarget* **7**, 20584-20596 (2016).
26 Li, H., Wang, X., Wen, C., Huo, Z., Wang, W., Zhan, Q., Cheng, D., Chen, H., Deng, X. *et al.* Long noncoding RNA NORAD, a novel competing endogenous RNA, enhances the hypoxia-induced epithelial-mesenchymal transition to promote metastasis in pancreatic cancer. *Mol Cancer* **16**, 169 (2017).
27 Li, Q., Li, C., Chen, J., Liu, P., Cui, Y., Zhou, X., Li, H. & Zu, X. High expression of long noncoding RNA NORAD indicates a poor prognosis and promotes clinical progression and metastasis in bladder cancer. *Urol Oncol* **36**, 310 e315-310 e322 (2018).
28 Huo, H., Tian, J., Wang, R., Li, Y., Qu, C. & Wang, N. Long non-coding RNA NORAD upregulate SIP1 expression to promote cell proliferation and invasion in cervical cancer. *Biomed Pharmacother* **106**, 1454-1460 (2018).
29 Lee, S., Kopp, F., Chang, T. C., Sataluri, A., Chen, B., Sivakumar, S., Yu, H., Xie, Y. & Mendell, J. T. Noncoding RNA NORAD Regulates Genomic Stability by Sequestering PUMILIO Proteins. *Cell* **164**, 69-80 (2016).
30 Munschauer, M., Nguyen, C. T., Sirokman, K., Hartigan, C. R., Hogstrom, L., Engreitz, J. M., Ulirsch, J. C., Fulco, C. P., Subramanian, V. *et al.* The NORAD lncRNA assembles a topoisomerase complex critical for genome stability. *Nature* **561**, 132-136 (2018).
31 Gyorffy, B., Lanczky, A., Eklund, A. C., Denkert, C., Budczies, J., Li, Q. & Szallasi, Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* **123**, 725-731 (2010).
32 Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L. *et al.* The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**, 401-404 (2012).
33 Tang, Z., Li, C., Kang, B., Gao, G., Li, C. & Zhang, Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* **45**, W98-W102 (2017).

34 Del Vecchio, F., Lee, G. H., Hawezi, J., Bhome, R., Pugh, S., Sayan, E., Thomas, G., Packham, G., Primrose, J. *et al.* Long non-coding RNAs within the tumour microenvironment and their role in tumour-stroma cross-talk. *Cancer Lett* **421**, 94-102 (2018).
35 Basu, A. & Krishnamurthy, S. Cellular responses to Cisplatin-induced DNA damage. *J Nucleic Acids* **2010** (2010).
36 Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J. & Wicha, M. S. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**, 1253-1270 (2003).
37 Ollion, J., Cochennec, J., Loll, F., Escude, C. & Boudier, T. TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. *Bioinformatics* **29**, 1840-1841 (2013).
38 Kinner, A., Wu, W., Staudt, C. & Iliakis, G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res* **36**, 5678-5694 (2008).
39 Bayani, J. & Squire, J. A. Preparation of cytogenetic specimens from tissue samples. *Curr Protoc Cell Biol* **Chapter 22**, Unit 22 22 (2004).
40 Bayani, J. & Squire, J. A. Traditional banding of chromosomes for cytogenetic analysis. *Curr Protoc Cell Biol* **Chapter 22**, Unit 22 23 (2004).
41 Rondon-Lagos, M., Verdun Di Cantogno, L., Marchio, C., Rangel, N., Payan-Gomez, C., Gugliotta, P., Botta, C., Bussolati, G., Ramirez-Clavijo, S. R. *et al.* Differences and homologies of chromosomal alterations within and between breast cancer cell lines: a clustering analysis. *Mol Cytogenet* **7**, 8 (2014).
42 Speicher, M. R., Petersen, S., Uhrig, S., Jentsch, I., Fauth, C., Eils, R. & Petersen, I. Analysis of chromosomal alterations in non-small cell lung cancer by multiplex-FISH, comparative genomic hybridization, and multicolor bar coding. *Lab Invest* **80**, 1031-1041 (2000).
43 Speicher, M. R., Gwyn Ballard, S. & Ward, D. C. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat Genet* **12**, 368-375 (1996).
44 Geigl, J. B., Uhrig, S. & Speicher, M. R. Multiplex-fluorescence in situ hybridization for chromosome karyotyping. *Nat Protoc* **1**, 1172-1184 (2006).
45 Nishimura, M., Jung, E. J., Shah, M. Y., Lu, C., Spizzo, R., Shimizu, M., Han, H. D., Ivan, C., Rossi, S. *et al.* Therapeutic synergy between microRNA and siRNA in ovarian cancer treatment. *Cancer Discov* **3**, 1302-1315 (2013).