**Innovative mRNA Vaccine against NSCLC: designing a platform of targeted polymeric nanoparticles for efficient personalized therapies.**

**Abstract:**

Although non-small cell lung cancer (NSCLC) treatment benefits from an unprecedented improvement with immune checkpoint inhibitors (ICIs), only a minority of NSCLC patients (25%) demonstrate long-term benefice. We **hypothesize** that therapeutic mRNA vaccination, coupled with co-targeting of antigen presenting cell (APC) immunosuppressive functions will strongly enlarge the spectrum of patients that could benefit from ICIs. While, the Covid-19 pandemic leaded to a paradigm shift in vaccine development, making key the role of mRNA vaccines, we predict that these novel cost-effective end flexible technologies will revolutionize personalized cancer care. Based on the complementary expertise of the consortium we **aim** to obtain proof of concept (PoC) for a novel NSCLC **mRNA vaccine**, through the evaluation of an innovative **mRNA + siRNA** combination therapy to **modulate APC** toward a restructuration of tumor microenvironment (TME). For that, we will use **our proprietary polymeric nanoparticles (NPs)** to selectively deliver therapeutic nucleic acids in myeloid cells.

The program will be structured around 6 work-packages (WP). **WP1** will establish mRNA and siRNA cargo NPs allowing model tumor associated antigens (TAA) expression in APC together with inhibition of ATP degradation and adenosine production by siRNA-mediated knockdown of ENTPD1/CD39 in APC. Alternatively, siRNA-mediated DICER knockdown to avoid immunosuppressive polarization of APC will be evaluated. In addition, we will design and synthesize novel poly(beta aminoester) polymers, selectively targeting monocytes of the TME and formulate NPs able to be administered through intravenous and nasal routes (low invasiveness). In **WP2,** we will characterize the particles for each monotherapy alone or, combinations of therapeutic siRNA plus mRNA. **WP3** will test the safety and efficacy of nanoparticles using tumor naïve mice and *in vitro* models simulating the TME. **WP4** will evaluate the most effective nanomedicine in mice including autochthonous NSCLC models and determine if it can sensitize these refractory models to anti-PD1, anti-CTLA4 therapies. **WP5** is defined to reach nanotherapeutics personalization. We will establish a method allowing TAA prediction from genome sequencing of circulating tumor cells (CTC) and biopsies, for stratifying patients and developing personalized TAA/mRNA cocktails. This will be a key step towards the definitions of minimal criteria required for appropriated clinical evaluation of the new vaccine candidate. **WP6** will initiate technology transfer, including analysis of patients CTC genomic alterations, toward a presentation of clinical trial protocols to the regulatory authorities for initiating clinical evaluation.

By the end of the project, we **expect to** have designed a novel targeted nano-immunomodulatory therapy that will become a turning point in lung cancer treatments and thus **impact** notably patient’s quality of life and costs to healthcare systems.

**Project Description**

**Non-small cell lung cancer** (NSCLC) remains the leading cause of tumor-related deaths1,2, however, **immune checkpoint inhibitors (ICIs)** have revolutionized its management and anti-PD-1 antibody became a standard of care (KEYNOTE-042). Indeed anti-PD1 treatment yield highly promising results that could be explained by the fact that NSCLC tumors are characterized by an elevated tumor mutational burden3 leading to the appearance of neo-antigens and non-mutated tumor-associated antigens (TAA). Direct cytolysis of cancer cells by tumor specific CD8 T cells is the major mechanism of ICI activity4,5. However, a large percentage of the NSCLC patients (75%) do not show response to ICIs and many develop immunoresistance6.

In this context, combining ICIs with **cancer vaccines** that will mobilize adaptive immune response against TAA and **target resistance mechanisms** of the TME appears as a promising strategy.

Recent studies discovered some common TAA across NSCLC tumor specimens. But previous clinical investigations failed to demonstrate a significant improvement of patient survival using well-known TAA (e.g. MUC-1). Resistance against TAA vaccines was associated with reduced MHC-I expression and default in transporter associated with antigen processing (TAP1/2) in the cancer cell. However, alternative processing and MHC-I presentation of non-mutated TAA, shown to be independent of the proteasome and TAP, can drive an efficient anti-tumor T cell response7. **Hence, patients stratification and selection of the right TAA**8 **cocktail represents one of the key issues in cancer vaccination**9.

Vaccination based on the use of **antigenic mRNA** could be advantageous given its immunogenic character, which does not require adjuvants. In addition, mRNA vaccines can easily be personalized depending on the specific patient mutations and cancer TAA processing by only modifying the sequence of the TAA-coding mRNA.

Besides the selection of the right TAA, **controlling tumor immunosuppressive microenvironment is another challenge.** Recent work from our consortium and data from the literature highlighted important immunosuppressive mechanisms linked with neutrophil infiltration, M2 tumor associated macrophages (TAM) and regulatory T cells (Treg)1 in NSCLC. Each of these cell types being associated with adverse outcome. In contrast, patients showing a strong proportion of M1-macrophages, showed a longer overall survival10. Hence, enhancing the priming of TAA specific Th1 and cytotoxic T cell response together with the **eradication of myeloid cells immune subversion** seems to be necessary to increase ICI effectiveness.

Among the immunosuppressive mechanisms, our consortium and others demonstrated the importance of the entonucleotidase-I ENTPD1/CD39 that degrades extracellular ATP and ADP into AMP, that is then converted into adenosine by CD73. Thus, CD39 and CD73 convert an important danger signal (ATP) into an immunosuppressive metabolite (adenosine)11. Alternatively, we hypothesize that avoiding macrophage polarization toward M2 might be key to enhance the anti-tumor immunity by favoring the emergence of anti-tumor T cells, reducing Treg expansion and avoiding tumor neo-angiogenesis. Importantly, an emerging research field demonstrated that M2 and immunosuppressive DC polarization could be reverted by inhibiting microRNA interference in these cells (abolishing DICER expression)12,13.

Thus, we are proposing that **silencing CD39 and/or DICER1 genes by using small-interfering siRNA** directly formulated together with mRNA coding for TAA using **polymeric nanoparticles (NPs)**, will allow eradicating the suppressive activity of the APC toward a stronger initiation of the adaptive immunity. This should lead to a deep reprograming of the TME architecture and reduce ICI resistance in mouse models and patients.

Our proprietary NPs were designed to protect therapeutic mRNA and specifically target myeloid cells, including APCs that will present mRNA encoded TAA to prime and/or reactivate the adaptive anti-tumor immunity. Our consortium demonstrates an extensive experience in the design of **poly (beta aminoesters) (pBAE)** NPs to encapsulate nucleic acids forming polyplexes. We have designed pBAEs with end-oligopeptides (OM-pBAEs) that efficiently transfect a variety of cell types, given their ability to escape from endosomes. OM-pBAEs are biocompatible and biodegradable polymers that can be produced using an easy to scale-up cost-efficient method. Tailoring these polymers with cationic terminal peptides is simple and enables them to self-assemble with nucleic acids. Therefore, OM-pBAEs hold great promise as nucleic acid carriers for *in vivo* applications18–20. Indeed, we already demonstrated their capacity to transfect tumor APCs18 and they are advantageous as i) they enable the encapsulation of an mRNA cocktail in combination with other nucleic acids (e.g. siRNA); ii) polymers can be modified, including mucus penetration moieties, for a local administration. Iii) NPs can be lyophilized without losing their integrity and functionality, that prolongs their stability without ultra-cold temperatures, thereby decreasing storage and distribution costs19. iv), they enable the personalization required in therapeutic anticancer vaccines due to the high patient-specific antigen profile of NSCLC21.

**The aim of this project is to establish the PoC of a novel personalized combined nano-immunotherapy for NSCLC patients, based on polymeric NPs (OM-pBAE) loaded with antigenic mRNAs and immunomodulator siRNAs**.

We already showed:

1. The NPs capacity to selectively target APC after intravenous administration (Fornaguera et al., Adv Healthcare Mat, 2018)18.
2. That macrophages show M2-like phenotype and express CD39 in subcutaneous lung tumors (Figure 1)
3. The selective binding of NPs to macrophages and neutrophils inside subcutaneous tumors after intratumoral injection (Figure 1).
4. The capacity to lyophilize NPs without losing their integrity and functionality (Fornaguera et al., Int J Pharm, 2019)22.
5. MALDI-MSI of NSCLC specimen analysis (Figure 2).

The project has been organized around 6 work packages (WP).

**WP1**: **Selection and validation of nanotools**

In order to adapt formulations for an administration through non-invasive routes, we will advance on formulations by designing and synthesizing: a) polymers to penetrate mucosa and enable nasal administration; and b) negatively charged polymers to cross the muscular extracellular matrix and enable the intramuscular delivery. The transfection efficacy of the novel formulations will be tested by assessing GFP expression by flow cytometry using of eGFP mRNA in different cell types and the safety by measuring the metabolic activity (e.g. MTT assay). Next, we will analyze NPs biodistribution in tumor bearing mice following nasal and intramuscular routes by measuring the *in vivo* and *ex vivo* bioluminescence of NPs encapsulating Fluc mRNA. We will also adapt matrix-assisted laser deposition/ionization mass spectrometry imaging (MALDI-MSI) to detect NPs and to characterize the metabolome of the TME (in mice). This technique is well-established in the consortium for glioma23 and was recently adapted for NSCLC24. It has been widely used to determine metabolic alterations determination in tumor and peritumoral tissues23,25,26.

**WP2: Full characterization of selected therapeutic immunotherapy NPs**

Formulation and characterization of the following therapeutic NPs will be performed: a) mRNA vaccine encapsulating OVA as model antigen; b) siRNAs targeting immunosupressive mechanisms, and c) mRNA + siRNA cocktail. Routinelly, DLS, NTA will be used. CryoTEM will be applied, as well as DSC, for the fine characterization of candidates, based on the long expertise of our consortium members27–29. Next, *in vitro* tests of novel formulations’ interaction with physiological components to discard protein corona modification of particles integrity will be studied using the same techniques. Finally, storage and *in use* stability studies will be performed by repeated measurement of the quality control attributes.

*In vitro* studies of safety of a) candidate vaccine; b) gene silencing; and c) combination therapy will be performed in an array of cultured cells. *In vitro* studies of safety of a) candidate vaccine; b) gene silencing; and c) combination therapy will be performed in an array of cultured cells.

**WP3: Validation of mono and combined therapy using *in vitro* and *in vivo* models**

First, anti-OVA (or any other model antigen) specific T cell induction in vitro will be determined for the vaccine, by flow cytometry and ELISA tests (e.g. IFN as imunostimulatory cytokine). Second, repolarization capacity of the novel nano-therapy to increase the capacity of myeloid cells to prime anti-tumor T cells response will also be tested. Third, combined therapy (coming from a single NP with a cocktail of the oligonucleotides or a predefined administration schedule of individual therapies) immune modulation in models of TME will be tested by flow cytometry and ELISA tests. Finally, selected candidates will be validated in complex *ex vivo* cell cultures and in healthy mice to determine the immunogenic potential of the combined therapy and tested in tumor naïve mice to monitor ovalbumin specific adaptive immunity induction (clonal T cell expansion and polarization, endogenous anti-ova antibodies).

**WP4: Efficacy of the selected candidate combined nano-therapy in mice models**

*In vivo* impact on the anti-tumor activity of mRNA vaccine monotherapy, siRNA particles monotherapy and siRNA + mRNA particles combination will be tested on transplanted mice tumor models using M8 and Clem2 cell lines engineered to constitutively express the OVA gene as TAA. Usage of these two lung cancer models are of particular interest as Clem2 cells form immunogenic tumors sensitive to anti-PD1 while the M8 model is highly infiltrated by M2-macrophages and tumor promoting neutrophils and is resistant to anti-PD1 treatment30. Hence this first part of *in vivo* investigations will allow to identify the best vaccine candidate (NPs containing ovalbumin mRNA +/- CD39 and/or DICER1 siRNA) to enhance anti-tumor immunity and efficacy of anti-PD1/anti-CTLA4 Ab co-treatment. We will analyze mice survival and tumor growth together with a deep characterization of the tumor immune landscape and anti-OVA systemic and intratumor T cell response using dextramer staining.

Next, similar experiments will be performed using autochthonous lung cancer model KrasLSL-G12D/WT; p53fl/fl engineered to specifically express the OVA gene in the cancer cells (using a lentiviral vector driving Cre recombinase expression to initiate tumor formation together with the coding sequence for the OVA), as already published by members of our consortium1. This autochthonous lung cancer model will be treated with the best vaccine candidate identified. In addition to tumor growth monitoring using computed tomography, mouse survival and flow cytometry immune-monitoring as proposed before, the impact of the novel nanomedicine on the TME will be determined by image mass cytometry (Hyperion) using an already validated panel of 28 antibodies against mouse tumor stroma. This will allow an in-depth characterization of the TME modulation following NPs administration to better understand, how our novel vaccine might change the response to ICI treatment.

**WP5: Developing nano-immunotherapy personalization in clinic**

We will setup TAA prediction from next gene sequencing (NGS) approach on our genomic data set of NSCLC patients10, and compare formalin-fixed paraffin-embedded biopsies of NSCLC patients with healthy tissues, to design and synthetize mRNA TAA-cocktail, as we previously described21. To go further toward the possibility to develop personalized TAA cocktails and stratify patients according to tumor cell ability in presenting TAA, we will setup a method based on purified circulating cancer cells (CTCs) from patients’ blood. These CTCs will be subjected to NGS for identifying the best possible TAA cocktail and evaluate TAP complexes and MHCI expression on cancer cells (patients’ stratification). During this development phase, results obtained from CTCs will be compared with similar analyses performed on biopsies from the same patient.

Then, we will validate the TAA presentation by APCs in co-culture experiments with human cells (PBMC, Cell lines). In parallel, we will select, design and synthesize siRNA to inhibit the immunosuppressive pathways (e.g. CD39/CD73 and/or DICER) and evaluate them alone or in combination in the same *in vitro* models.

**WP6: Technology transfer work package**

At the end we will initiate technology transfer. Since we aim to obtain a preclinical PoC, we want to leave the pBAE platform prepared for the clinical study. To achieve that, we will perform the scaling up of the synthesis methodology for production of big batches using automated microfluidics methods (PoC done; see Figure 3, Annexes). We will also prepare the production under GMP, for clinical trials. In addition, we will set up the preclinical regulatory strategy to follow.

**NOVELTY AND ORIGINALITY OF THE PROJECT**

mRNA vaccines have revolutionized the society after their rapid success in the Covid-19 pandemic, thanks to the easy tailoring of nanosystems, which only required a change on the antigens encoded by the mRNA. Our approach differs from the two marketed mRNA vaccines (Pfizer and Moderna lipid NPs) in the material used as carrier. Indeed, we will use pBAE proprietary polymers, which are advantageous over lipid competitors as they: 1) show long term stability, thus not requiring ultra-cold temperatures; 2) no PEG moieties, which have produced some adverse reaction and 3) demonstrate a referential transduction potential on myeloid and APCs in vivo. Furthermore, we will also take advantage of pBAE as a novel platform not only to deliver TAA coding mRNA but also to modulate the functionality of targeted APC. This will be the first time that such combination approach into the same NP will be evaluated. This specific development relays on the observation that most of the APC found in tumor are characterized by their immunosuppressive function leading to inhibition of TAA specific T cells. Our NPs are highly tunable. To address essential challenges toward clinical development, our project includes a development phase designed to enable stratification of the patients and personalized TAA-cocktail determination based on CTC analyses. Our objective here being to overcome limitations linked to biopsies access in patients that do not receive surgery. To Summarize, the technological and industrial development of effective vaccination system based on RNA-loaded NPs as we are proposing here, paves the way toward new immunotherapies for cancer and infectious diseases treatment.

**FEASIBILITY OF THE PROJECT, MANAGEMENT STRUCTURE AND IMPLEMENTATION PLAN**

To successfully deliver the project, we have assembled an **interdisciplinary and transnational consortium that** combines complementary knowledge areas as bioengineering, experimental medicine and biomedical product development, matching completely the proposal’s objectives. The **IQS-URL partner** is expert on the design of poly(beta aminoesters) (patented technology) and has demonstrated their use form RNA vaccination. Together with the **Technion partner,** expert in advanced techniques for characterization of nanovehicles, they will design the nanoformulations. The performance of the nanoformulations will be analyzed using highly advanced imaging techniques, namely MALDI-MSI, where **BielefeldUni partner** are experts. In parallel, the **UniErlangen partner** has a long trajectory on the use of mRNA for tumor immunotherapeutics, and they will contribute to the selection and encapsulation of the antigenic mRNAs and their test *in vitro*. The **INSERM partner** has a strong experience on tumor immune escape mechanisms (CD39/CD73 as example) leading to refractoriness to ICI treatment in various tumor models. They have a strong expertise in manipulating transplantable and autochthonous mouse models of NSCLC that are accessible in their institute. Furthermore, the team is leading the Hyperion (image mass cytometry) and flow cytometry platforms warranting full access to latest imagery technics allowing deep characterization of the TME in mouse models. Additionally, the consortium includes the **IGTP partner**, an international recognized medical oncology group, specialized in lung cancer immunotherapies, who will assist in designing the tools allowing personalized TAA design for further development toward clinical evaluation of our novel vaccine. All the partner institutions have the appropriate resources, facilities and personnel to develop the activities defined in the proposal.

The project will be coordinated by C. Fornaguera. A Gantt chart was designed, as well as a contingency plan, for the risk management (detailed in Annexes as Figures 4 and 5).

**IMPACT ON CANCER IMMUNOTHERAPY**

From a **scientific perspective**, this application has a profound translational character and aims at shifting the current tumor therapeutic paradigm by establishing feasibility of new strategies. We will develop and evaluate a highly innovative tool to reinvent cancer vaccine and increase ICI effectiveness in NSCLC patients. Currently, targeted nanodelivery of drugs and nano‐therapeutics are included in drug companies’ portfolios; these areas being among the most innovative nanomedicine. But, complications associated with advanced stages of the disease, along with the poor performance due to an inadequate carrier design are limiting the clinical benefice of current TAA-vaccine strategies. In our project, we will address these two limitations by i) using a novel type of NPs allowing specific myeloid cell targeting and simultaneous down regulation of APC immunosuppressive functions, ii) developing methods allowing patients stratification and determination of a personalized TAA-cocktail from CTC and biopsies analyses. We plan to carry out the dissemination of our results through presentation at national and international conferences and through the publication of results in international journals of high impact.In this project, we aim to introduce innovation by designing and testing new types of NP‐based therapy platforms. In this regard, the development of the technology, tested as a PoC for NSCLC, will be adapted to the treatment of other tumor types relatively readily with few research studies to determine the antigens to be encoded for each tumor type, thus decreasing R&D costs. In this way, the paradigm shift that is already becoming visible over the world by mRNA vaccines will enable to move from traditional treatment with existing drugs/tools, towards a personalized medicine.

Through the generation of new agents, polymeric NPs encapsulating gene material for their administration, this project constitutes a significant improvement in therapeutic technologies with a relevant **industrial impact** and should have a substantial impact on competitiveness of European pharma/biotech companies. We assume the PoC that will be produced during this project (WP1 to 4) together with the setting of an innovative pipeline allowing rapid and efficient transfer of our technology toward a clinical evaluation as personalized medicine (WP5 and WP6) will be attractive for multiple European industrial partners.

For the **non-scientific community**, this project aims to improve patient’s quality of life and increase their overall survival. The COVID-19 pandemic crisis showed that the general audience is sensitive to the emergence of innovative technologies such as mRNA vaccine, but lack of information and communication on these new technologies is a source of anxiety in a nonscientific public. We will work on the creation of a dedicated website to provide updated information on mRNA nanomedicine and on our specific research program. We think that a better communication on this very important technological development, emanating from non-profit organizations, is required for a better consideration and acceptation by the European population, toward a full deployment of its clinical potential in the coming years.

**Ley Summary**

Lung cancer, and specifically non-small cell lung cancer (**NSCLC**), is among the top six leading causes of death worldwide, according to WHO. Current treatments enable only 8-10-month survival upon diagnosis. Hence, more efficient therapies are needed. Traditional chemotherapies based on drugs that are toxic to fast-growing cells have very limited efficiency and cause dramatic side effects. In addition, they do not target other cell types included in the tumor microenvironment (TME), whose role in cancer malignancy has been remarked recently. A change in paradigm cancer treatment has shifted to more efficient methods based on immunotherapies, which can double patient 5-year survival, giving signs of hope in other cancer types. Immunotherapy is based on the re-activation of the immune system against cancer cells, thus involving the whole TME. However, the effect of this novel therapy is still limited to some subsets of patients and more studies stratifying patients are required to determine which are the specific altered mechanisms in each.

In this context, we **aim** to design a personalized enhanced and combined nano-immunotherapeutic approach, to treat lung cancer patients, based on the use of proprietary polymeric nanoparticles encapsulating nucleic acids as active principles. These nucleic acids will: a) act as antigens to immunize patients against tumor antigens and achieve the self-killing of tumor cells (mRNA cancer vaccination); and b) immunomodulate the TME by silencing key immunesupressor genes (targeted therapy).

To achieve such an ambitious goal, we built a consortium composed of six experienced and recognized European groups in the field, each of which is a key pillar for the project success. Specifically, we have clinical oncologists, experts on lung tumors, that will determine the key antigens to vaccinate against (WP1). We have molecular biologists and polymer chemistry experts on the synthesis of antigen mRNAs, which will be encapsulated in polymeric nanoparticles (WP2), taking into account the requirements of low invasive administration routes. In addition, we have experts on the fine characterization at the nanoscale, who will characterize mRNA vaccine (+/- combined silencing therapy) and their interaction with biological fluids components (WP3). To further advance the mRNA vaccine , we also have experts on the *in vitro* characterization of safety, efficacy and immunization capacity (WP4) who will work to determine which formulation/s (or combinations) will be selected for the final *in vivo* therapeutic efficacy test, performed by expert partner on orthotropic lung cancer models (WP5). Last but not least, we will finish the project by transferring the technology to facilitate its arrival to the market (WP6). Thus, at the end of the project, we will have a validated combined personalized nano-immmunotherapy against NSCLC patients, prepared to start regulatory preclinical and clinical trials.