Infrastructure & Equipment for Research Activities

This item includes the creation, expansion and modernization of research infrastructures in particular the **Gene Therapy Center** – based on the already existing facilities at OPBG and Fondazione Tettamanti **-** and the development of the **RNA Production Platform** – devoted to RNA synthesis and formulation that will serve the research groups of the Center - in addition to the **Spoke Flagship**. As previously mentioned, the **Gene Therapy Center** is allocated on Spoke 10, the **RNA Production Platform** on the Hub and **Spoke Flagships** on the Spokes. Total Costs **87,1 M€**.

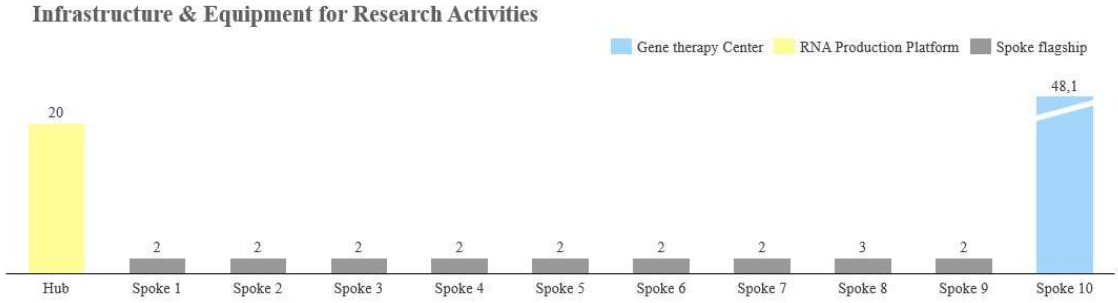


Figure B.13 – Infrastructure & Equipment for Research Activities Budget per Spoke

Open Calls & TRL Development Grants

139,8 M€ have been allocated for Open Calls & TRL Development Grants (32,4 M€ for Open Calls), corresponding to around 10% of the total budget.

Hub Services and Cross-Spokes Activities

As previously anticipated, the activities have the goal to address and coordinate research activities carried out by Spokes and speed up the technological development. Services and Cross-Spokes Activities include the following items for a total cost of **25,3 M€**:

- National Industrial PhD Programme – **3,2 M€** (*ca* 22 PhD at the total cost of *ca* 70 k€ each, including research costs, for the first Cycle; *ca* 22 PhD at the total cost of *ca* 70 k€ each, including research costs, for the second Cycle); **920 k€** to support PhD mobility and Network-wide training.
- Technology Transfer – **13 M€**
- Start-up Package for RTDA **from other EU and non-EU countries1,6 M€**
- Spin Off and Start-Up accelerators – **4 M€**
- Coordinating Structure and Contingencies **– 3,5 M€**

Lifelong-learning consisting in PharmaTech Academy –3 **M€** to be allocated to **Spoke 8** as it will be enrolled by UNINA. **Costs include**: **PharmaTech Academy infrastructure** (open-spaces functionalization, Labs set-up and functionalization): total cost of **2M€; Academy Activities**, Dissemination and communication, Recruitment, orientation and assessments, Trainers, Facilitators, Support to students- Allowance for out-of-towners, mini-grants, Traineeships: total cost of **0,4M€** for about 30 Master students enrolled (1st PharmaTech Academy) and total cost of **0,6M€** for about 50 Master students enrolled (2nd PharmaTech Academy).

Total Budget by Territory

Each Spoke has a percentage of investments in South Regions, that – overall –guaranties 41% of investments in South Regions

Figure B. 64 – Budget by Territory

Breakdown by Intervention Fields

The Budget considers the constraints imposed by the Call. Particularly, the Call requires to meet the following targets:
- **Constraint Climate: at least 36% of the Budget.** All the Investment (87,1M€) and the TRL development grants (107,4M€) totaling 194,5 M€ (60% of the Budget) fall within this intervention field, therefore the target is met;

- **Constraint Digital at least 16% of the Budget.** The Budget meets this target given that all the Investments (87,1M€) are pursue the progressive digitalization and automatization of processes, both in production and in quality control (27% of the Budget);
- **Constraint 006 - Investment in intangible assets in public research centers and education at least 50% of the Budget.** The TRL development grants (107,4M €), the Lifelong learning initiatives (3M€), the National PhD programme (3,2M€), the Technology Transfer and the feasibility studies (13M€), the Spin off and Start-up accelerators (4M€), the Gene Therapy (48,1M€) and the RNA Production Platform (20M€) totaling 198,7M€ (62% of the Budget) fall within this intervention field, therefore the target is met.

Budget Overview
In the figure B.12 the overview for the budget in M€

To pursue these goals, we confirm that the Total Cost of the Program financed by the Minister is 320,2 M€. In addition, the NC will be supported by private companies with other contributions amounting to 8,77 M€. Therefore, the Total Budget of the program financed both by the Minister and private companies is 328,97 M€. Finally, to ensure the possibility to continue its activities at the end of the program the NC receives additional resources from the Founders of the Foundation for 4.9 M€ per year with a total of 14.9 M€ for the entire duration of the PNRR for National Research Centers.

	GRANT	HUB	SPOKE 1	SPOKE 2	SPOKE 3	SPOKE 4	SPOKE 5	SPOKE 6	SPOKE 7	SPOKE 8	SPOKE 9	SPOKE 10
Permanent Staff	36,0	0,0	3,5	6,6	4,3	3,9	3,5	3,5	2,7	3,9	2,7	1,6
RTDa	22,7	0,0	2,2	4,3	2,7	2,4	2,4	1,9	1,6	2,4	1,9	0,8
PhD c/o spoke	7,9	0,0	0,7	1,5	0,9	0,8	0,8	0,6	0,5	0,8	0,6	0,5
Personnel	**66,6**	**0,0**	**6,4**	**12,4**	**7,9**	**7,1**	**6,7**	**6,0**	**4,9**	**7,1**	**5,2**	**2,9**
TRL development grants	**107,40**	**12,00**	**8,40**	**13,60**	**9,00**	**9,50**	**8,50**	**8,00**	**5,90**	**10,50**	**10,00**	**12,00**
Open Call	**32,4**	**2,0**	**2,0**	**2,4**	**2,0**	**2,0**	**2,0**	**5,7**	**1,8**	**7,2**	**2,3**	**3,0**
Gene Therapy Center	**48,1**	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	48,1
RNA Production Platform	**20,0**	20,0	0,0	0,0	0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Spoke Flagship	**19,0**	0,0	2,0	2,0	2,0	2,0	2,0	2,0	2,0	3,0	2,0	0,0
Infrastructure & Equipment for Research Activities	**87,1**	**20,0**	**2,0**	**2,0**	**2,0**	**2,0**	**2,0**	**2,0**	**2,0**	**3,0**	**2,0**	**48,1**
Life Long Learning	**3,0**	**3,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
National PhD	**3,2**	**0,0**								**3,2**		
Technology Transfer & Feasibility Studies	**13,0**	**13,0**										
Spin Off and Start-Up accelerators	**4,0**	**4,0**										
Management Expenses	**3,5**	**3,5**										
TOTAL	320,2	57,5	18,7	30,4	20,9	20,6	19,2	21,7	14,6	31,1	19,5	66,0

Figure B. 15 – Budget Overview M€

B.5 COHERENCE BETWEEN THE RESEARCH ACTIVITIES AND SKILLS OF THE EXECUTING SUBJECTS (SPOKE AND AFFILIATES)

The Spoke and affiliates were selected with care, using the criteria of excellence presented in section A. Here below we provide an overview of the Competence and Infrastructural Resources for each Spoke and affiliate demonstrating their coherence with the research workplan.

Spoke #1: Genetic Diseases

Partner	Competence	Infrastructural Resources
Spoke Leader UNIMORE	The participants of UNIMORE have consolidated skills and international leadership in the development of gene therapy for genetic diseases (Eye, Skin, Blood and Blood-Liver genetic diseases) with a consolidated background in developing protocols for gene editing, combined cell and gene therapy, viral and non-viral gene delivery.	Stefano Ferrari Center for Regenerative Medicine with commercial-grade GMP manufacturing capacity; Center for Genomic Medicine and Rare Diseases; Interdepartmental Center for Genomic Research CGR
Affiliate TELETHON-TIGEM	TIGEM is a multidisciplinary research institute devoted to studying the mechanisms underlying rare genetic diseases and developing new therapies with acquired fundamental skills to develop innovative DNA and RNA therapeutics for rare genetic diseases. TIGEM is currently performing a clinical trial based on AAV gene therapy for a rare genetic condition, known as Mucopolysaccharidoses type VI.	Advanced Microscopy and Imaging, Generation of Animal Models, Retinal phenotyping, High-Content Screening, Bioinformatics and biocomputing core, Biosafety level 3 laboratory
Affiliate UNIBA	The participants of UNIBA have consolidated skills in mitochondrial biogenesis and function.	BLS-2; Platform for pharmacodynamics and biodistribution in small animals.

Affiliate UNIBO	UNIBO scientists have been internationally recognized for their discoveries on the primary mechanism of neuroinflammation at the cellular and molecular levels and depicting the pathogenesis of spastic paraplegia, autism, epileptic encephalopathy, mitochondrial encephalomyopathy, neuromuscular disorders and optic neuropathy.	Center for Applied Biomedical Research, Experimental animal facilities
Affiliate UNICA	UNICA scientists are internationally recognized for their studies on genetic characterization of rare anemias with special reference to genotype-phenotype correlation in beta thalassemia including identification of the most relevant modifier genes.	CeSAR: The University Center for Advanced Research
Affiliate UNIMI	Expertise in genetics and genetic disease research. Strong expertise in the generation of experimental models (cellular, including IPS cells, animal disease models genetically engineered) of genetic diseases and inflammation and a large collection of such models is available for the community. Strong connections with IRCCS for rare diseases specimens, access to several human bank tissues.	UNITECH: technological platforms with advanced technologies in 'omics, imaging and cell biology, bioinformatics; BL2/3 facilities
Affiliate UNINA	UNINA scientists have been internationally recognized for their discoveries focused on identifying genetic determinants and developing innovative therapies for hereditary defects of red blood cells, hereditary and sporadic pediatric cancers in the Nervous System, neurodevelopmental disorders and tubulinopathies.	Facilities of microscopy, sequencing, cytofluorimetry and animal house, Heart Failure and Pulmonary Hypertension Unit, Laboratory for Biosensing, Cardiovascular Rehabilitation Facility
Affiliate UNIPD	UNIPD scientists have recognized international leadership on RNA based technologies, gene therapy and Hematopoietic Stem Cells genetic engineering for treating different genetic diseases, such as mitochondrial diseases, neurometabolic diseases (MPS II, CLN1), leukodystrophies (MLD, ALD), and hemophilia A. Engaged industrial partners can bring these therapies to advanced clinical development.	BioImaging Facility; HiTS@UniPD
Affiliate UNISI	UNISI scientists have consolidated skills in identifying novel gene therapeutic strategies for patients affected by Amyotrophic lateral sclerosis (ALS), skeletal muscle tissue diseases, cardiomyopathies, Alport and Rett syndromes.	Sequencing facility; Opera Phenix high content screening system.

Spoke #2: Cancer

Partner	Competence	Infrastructural Resources
Spoke co-Leader-UNIMI	Research in oncology has always been a pillar of the research campus with research teams with strong and documented expertise in cancer pharmacology as well in studies on the molecular mechanisms controlling cell proliferation, metastasis formation and tumor angiogenesis. Strong interaction with clinicians at National Institute of Cancer, European Institute of Oncology and Niguarda Hospital.	Platforms for 'omics science, imaging, and cell biology; Clinical Technoshots-translational research center of genomic sciences
Spoke co-Leader UNIROMA1	Long-standing experience in the analysis of non-coding RNAs, with a particular focus on their role in cancer development.	Mass spectrometry Platform MALDI TOF & Spatial-omics
Affiliate CNR	CNR scientists made essential contributions in studying the relevance of lncRNAs such as MALAT1 and Golgi proteins in cancer and developing new personalized medicine approaches.	Euro Bioimaging
Affiliate UMG	UMG participants have a consolidated international leadership in the field of molecular and translational oncology and have gained expertise in the development of miRNA-based therapeutic reagents to help treat refractory cancer patients.	Genomic Facility, Cellular Facility and Pathology Facility
Affiliate UNIBA	The participants of UNIBA have consolidated skills in molecular oncology and medical genetics, developing a new RNA-based strategy for cancer treatment using Tunneling Nanotubes (TNTs) as a new delivery strategy for novel short interfering RNAs	Elixir, Euro Bioimaging

	(siRNAs)/small hairpin RNAs (shRNAs)/circular RNAs (circRNAs).	
Affiliate UNIBO	The expertise of participants @UNIBO ranges from molecular and cellular oncology to clinical trials, with worldwide recognized excellence in hematology, a broad portfolio of clinical studies. They are active in Orthopedic oncology, Cancer immunology and immunotherapy, Gastrointestinal cancer and rare tumors.	Center for Applied Biomedical Research, Big data management and analysis platform
Affiliate UNIBS	UNIBS participants have consolidated expertise in the identification of novel therapeutic targets using a multi-omic approach, identification of hot spots gene mutations in tumors and prediction of their impact on drug response.	Animal facility for rodent and zebrafish; Proteomic, genomic and big data analysis facility; Imaging facility.
Affiliate UNICT	UNICT participants have a well-proven experience in identifying and characterizing genetic and epigenetic alterations involved in the development and progression of tumors, which can be used for therapeutic strategies.	BRIT and CAPiR Facility; Research Center for Prevention, Diagnosis and Treatment of Cancer (PreDiCT)
Affiliate UNIFI	UNIFI participants have relevant expertise in identifying new cancer type-specific targetable genes/hubs, deregulation of energetics and cancer cachexia, development of RNA-based strategy for the targeting of the tumor-stroma metabolic crosstalk.	Metabolomic Facility; Confocal Imaging Facility; Histomorphology Facility
Affiliate UNIMORE	UNIMORE scientists incorporate experienced research groups and industrial partners in a venture aiming at multimodal biotherapeutics based on gene deliveries for adult solid tumors and leukemias.	Cell factory of 500 sqm for cell and gene therapy production in collaboration with Rigenerand srl.
Affiliate UNINA	UNINA participants have a well-proven experience in the identification and validation of new genomic, immunological and metabolic biomarkers predictive of response to immunotherapy, in the validation of new therapeutic targets for RNA drugs and gene therapy related to tumor cell metabolism for otherwise untreatable patients focusing on metabolic biomarkers related to thyroid hormones.	Next Generation Sequencing Platform; Facilities of microscopy, sequencing, cytofluorimetry and animal house
Affiliate UNIPD	The UNIPD scientists participating in this spoke are internationally recognized for their studies on signal transduction and molecular mechanisms involved in physiology and cancer providing seminal contributions in basic mechanisms of tumor progression and metastasis, in mitochondria biology and ion channels relevant for controlling apoptosis and metabolism.	Single cell RNA-seq; SPF and conventional animal house, BLS2/ BLS3 facilities; in vivo imaging of tumor injected cells, multiplexing molecular and histopathological data.
Affiliate UNIPI	UNIPI participants have robust expertise in identifying circulating mutations of KRAS or NRAS in liquid biopsies as a predictive tool in the evolution of liquid tumors and gene editing with CRISP/Cas9 technique.	Multimodal Molecular Imaging Italian Node of Eurobioimaging; Center for Instrument Sharing of the University of Pisa (CISUP).
Affiliate UNISI	UNISI scientists are internationally recognized for Gene editing and vaccine based on mRNA technologies for cancer treatment (novel therapeutic strategies of hematological tumors using CRISP/Cas9 system) and restoring cell cycle regulation by microbubbles encapsulated mRNA-coding to treat lung cancer.	DepArray Single cell sorting; Opera Phenix high content screening system; Sequencing facility
Affiliate UNITO	UNITO scientists are internationally recognised for their competencies in the development of RNA-based approaches to target tumor genes, and identification of micro-RNA targets	Animal house, histology and microscopy service, imaging facility.
Affiliate UNITS	UNITS is a reference institution for cancer research with recognized experience in the study of molecular pathways controlling tumor formation, progression, metastasis and resistance to therapy. Researchers from UNITS will provide their know-how concerning the identification and functional characterization of non-coding RNAs that modulate key biological processes and pathways in cancer.	Electron microscopy; Correlative Light Electron Microscopy; Multi-photon microscopy; High-throughput screening facility; clean animal facility with live imaging

Affiliate UNICAMPANIA	UNICAMPANIA incorporates a research group on Inherited Retinal Dystrophies (IRDs) focusing on Gene Therapy applications, participating in the first gene therapy clinical trial for the treatment of ocular diseases.	Center for Rare Ocular Disease (European Reference Network for Rare Eye Diseases) dedicated to clinical studies for Inherited Retinal Dystrophies

Spoke #3: Neurodegeneration

Partner	Competence	Infrastructural Resources
Spoke Leader IIT	IIT scientists have consolidated expertise in neuroscience and in the study of neurodegenerative diseases and neurodevelopmental disorders. Since 2020 IIT has launched The RNA Initiative, bringing together twenty research groups studying non-coding RNAs and developing new RNA-based drugs for brain diseases.	Genomic facility; Proteomics; SPF animal facility with automatic behavioral analysis; Structural biology (NMR, Crystallography).
Affiliate CNR	CNR scientists have provided significant contributions to the development of innovative therapeutic approaches for the treatment of age-related neovascular macular degeneration. One team member is also co-founder of a biotech focusing on the development of angiogenesis inhibitors.	IGB animal house including animal imaging IVIS and micron III apparatus for laser-induced choroidal neovascularization model
Affiliate UMG	UMG participants have consolidated expertise, and the technological skills for generating 3D brain organoids (3D-BO) from iPSCs reprogrammed from lymphocytes obtained from patients affected by progressive supranuclear palsy (PSP), a rare brain disorder or which no effective treatment is currently available.	Facilities for iPSC and organoids from human cells and tissues; Proteomics; Functional genomic Facility
Affiliate UNIBA	The participants of UNIBA have consolidated skills in the pathogenesis of neurodegenerative diseases, such as ALS and Alzheimer's, with competencies in super-resolution microscopy and functional water transport assays, migration / invasion assays, calcium and patch clamp measurements on tissue slices and two-photon microscopy.	Facility for super-resolution microscopy; animal facility with units for behavior and cognitive studies; Interdepartmental Center MIRROR
Affiliate UNIBO	UNIBO combines a complete set of basic, translational and clinical expertise in different fields of cellular, biochemical and molecular biology, biocomputing, genetics and neurology for tackling NDDs using nanostructured RNAzymes targeting specific exosomal miRNAs and Gene-editing with CRISPR-based technologies for previously characterized monogenic disorders.	Center for Applied Biomedical Research, Experimental animal facilities
Affiliate UNIFI	UNIFI participants have relevant expertise in the adoption and development of in vitro and in vivo models of neurodegeneration, focusing on stroke, progressive multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and neuropathic pain associated with these disorders.	CESAL (animal facility) CISPIM (pre-clinical imaging) LENS (Lab for Non-Linear Spectroscopy)
Affiliate UNIMI	Former Center of Excellence on Neurodegenerative diseases with strong basic and clinical research in Neuroscience. UNIMI has developed cellular and animal models for the study of Huntington, ALS, Alzheimer, motor neuron disorder and developed NA-based therapeutic tools and innovative delivery methods.	Core labs with advanced technologies in 'omics, imaging and cell biology, drug discovery labs.
Affiliate UNINA	UNINA participants have a well-proven experience in the identification and validation of miRNAs as theranostic agents in stroke and other neurodegenerative diseases, delivery of miRNA/small drugs combination through nanocarriers and gene therapy products for cerebral and retinal sphingolipidoses (Tay–Sachs e Sandhoff diseases).	Dedicated rooms for the use of Adeno-associated virus (AAV) vectors; Animal housing; Drug Discovery Laboratories; Instrumental Analysis Laboratory (IAL)
Affiliate UNIPD	The Center for Neurodegenerative diseases (CESNE) has multidisciplinary competences and is world leader in the genetic, neurophysiological and phenotypic characterization of Parkinson and Alzheimer disease as well as of rare genetic conditions including triplet diseases. They have preclinical animal models,	The three facilities are located at the Vallisneri: Small animal facility, in vivo Microscopy, iPS and oganoid cell lab

	laboratory facilities for iPS and organoids and for the implementation of RNA-based therapeutics and gene editing. They are Italian leaders in Phase II and III clinical trials in neurodegenerative conditions.	
Affiliate UNIPI	UNIPI participants have a well-proven experience in identifying early molecular biomarkers of neural diseases and developing new therapeutic strategies to restore RNA homeostasis by using murine models of ALS and AD and human brain organoids.	Center for Instrument Sharing (CISUP); European Reference Network for Rare Diseases - ERN-EuroNMD
Affiliate UNIROMA 1	UNIROMA1 participants have a well-proven experience in therapeutic applications to treat muscular or neurodegenerative diseases.	3T MRI station fully implemented for clinical research in neuroimaging
Affiliate UNITO	UNITO participants have a well-proven experience in investigating target RNA-protein interaction in neurodevelopmental and neurodegenerative disorders.	Genomic platform for NGS sequencing; Imaging

Spoke #4: Metabolic and cardiovascular diseases

Partner	Competence	Infrastructural Resources
Spoke leader UNIPD	UNIPD has a unique cardio-metabolic research group spanning basic, translational and clinical science. The group has world-class experience in cellular metabolism, mitochondrial biology, cancer and muscle metabolism, metabolic regulation of cardio-vascular homeostasis and disease, and metabolic cross-talks among tissues, organs and systems. The clinical group has international leadership in obesity and diabetes research, designing, promoting and leading studies fertilized by pre-clinical findings.	SPF animal house; mouse metabolic phenomapping platform), 2-photon confocal microscopy with STED, FACS facility with imaging flow-cytometry; genomic facility with NGS; Vevo echocardiography, BLS2 room; Operetta screening platform
Affiliate HUNIMED	The team has developed protein-based tolerising cardiac oligopeptides to modRNA and use them in heart failure tolerising vaccination experiments. Also, they use RNA antisense approach for inhibiting disease allele in LMNA mutation and microRNAs involved in atherosclerosis.	STED microscope; Hyperion; FACS (Fortessa, ARIA III), Vevo 2100 echocardiography, 10X NGS
Affiliate RI.MED	RI.MED has a well-proven experience in single-cell RNA sequencing of cardiac progenitor cells from human diabetic patients and mouse hearts with diabetic cardiomyopathy.	Research Infrastructure for Precision Medicine
Affiliate UMG	UMG participants have consolidated international leadership in stem cell biology and molecular cardiology to improve regeneration and function in diabetes mellitus and related mice models and diabetic human cardiac organoids.	Genomic Facility: Cellular Facility: Pathology Facility
Affiliate UNIBA	The participants of UNIBA have consolidated skills in the molecular mechanisms of non-transmissible high-prevalence diseases with an inflammatory basis, including obesity, type 2 diabetes and their chronic complications.	Elixir platform, animal enclosure
Affiliate UNIMI	UNIMI has a long history of research in atherosclerosis, immunometabolic disorders and other cardiometabolic related syndromes. UNIMI participants are already active in the study of innovative approaches to control the over-activation of the immune-inflammatory response and the metabolic reprogramming with siRNA, ASO and other innovative techniques to determine the pharmacogenomics signature influencing siRNA therapeutics response.	Technological platforms with advanced technologies in 'omics, imaging and cell biology. Clinical research centers and Niguarda and Monzino Hospitals
Affiliate UNINA	Participants have consolidated expertise in deciphering the role of snoRNA as diagnostic and therapeutic tools in experimental models of cardiovascular pathophysiology and disease, in designing combination miRNA/Peroxisome Proliferator-Activated Receptor (PPAR) agonists as new therapeutic strategy for the treatment of metabolic disorders and cardiovascular diseases, and identifying specific epigenetic profiles involved in the development of obesity and in adipose tissue dysfunctions.	Animal housing; Drug Discovery Laboratories; Instrumental Analysis Laboratory (IAL)

Affiliate UNIPV	The participants have consolidated expertise in proposing therapeutical solutions based on RNA and novel gene therapies for cardiovascular diseases and skills to prepare therapeutic constructs and viral vectors.	BLS2 space, confocal microscopy (x2), light-sheet microscopy, Cryo-EM, NMR,
Affiliate UNISA	UNISA participants have internationally consolidated expertise on the identification of molecular regulators for the treatment of cardiovascular diseases and their experience in multi-omics analyses to elucidate the molecular changes in biofluids and tissues, as well to elucidate the efficacy and safety of these novel therapies.	Animal house LAMeTa
Affiliate UNITS	SUNITS will provide its well-established expertise in gene therapy, cardiomyocyte biology, and cellular/animal models of human cardiac diseases, to identify and manufacture novel RNA therapeutics for cardiovascular disorders, with the particular goal of inducing cardioprotection, neo-angiogenesis, and cardiac regeneration.	Facilities for AAV vector production; LNP formulation and testing; HTS for lead RNA identification; Bio-experimentation facility for in vivo RNA and AAV therapeutics

Spoke #5: Inflammatory and infectious diseases

Partner	Competence	Infrastructural Resources
Spoke Leader UNISI	UNISI participants have a long lasting and strong expertise in the analysis of cellular and circulating small RNAs using NGS sequencing technologies; expertise in the isolation and quality controls of cell culture derived and plasma extracellular vesicles. In addition, UNISI hosts the Tuscany Center for Precision medicine (CREMEP), a consortium involving Siena University Hospital and Toscana Life Science, fully devoted to translational research activities in the field of chronic as well as of rare diseases.	Sequencing facility (NovaSeq6000, NextSeq550, MiSeqDX sequencers; Proteomic core facility with several Mass spectrometry instrumentations
Affiliate HUNIMED	Participants have developed humoral innate immunity components (the long pentraxin PTX3 and the collectin MBL) as potential therapeutic drugs in infectious conditions of fungal, bacterial and viral origin.	STED microscope; Hyperion; FACS ARIA
Affiliate UNIFI	UNIFI participants have relevant expertise in design and use RNA/DNA oligonucleotides (ASOs) to modulate the expression of both mutated and non-mutated genes and characterisation of adaptive immune response evaluating activation markers by flow cytometric analysis multiparametric, activation of specific signaling pathways and transcription factors.	FloCEN: Florence Center for Electron Nanoscop; CERM; CISPIM (Center for the Preclinical Development of Molecular Imaging)
Affiliate UNIMI	Technical and scientific competence spread in numerous research teams interested in mechanistic studies of inflammation and immune response in respiratory diseases. Consolidated experience in the design and synthesis of novel formulations of nucleic acid-based drugs and generation of models for in vivo testing of their activity.	UNITECH: technological platforms @UNIMI. Selex platform clinical collaborations with Humanitas and San Paolo Hospital.
Affiliate UNINA	UNINA participants have consolidated expertise in harnessing siRNA therapeutics for the local therapy of lung inflammation up to preclinical validation and in developing RNA technologies to improve the control of parasitosis.	Molecular Biology Laboratory (MBL); Cell Culture Laboratory; Mice animal house
Affiliate UNIPD	The UNIPD scientists have consolidated skills on engineered biomaterials and signal transduction and molecular mechanisms involved in physiology and diseases.	3D printing infrastructure, material chemistry lab. for in vivo cell boil. biology, microfluidics, BL2 and BL3 facilities. Confocal microscopy.
Affiliate UNIVR	UNIVR participants have consolidated expertise in cell-based therapy to control graft-versus-host disease using GMP-certified encoding FLIP for in vitro transfect monocytes/dendritic cells with fully automated and scalable cell manufacturing procedures.	Centro Piattaforme Tecnologiche (University of Verona), cell factory and Clinical Research Center

		(Verona University Hospital)
Affiliate UNIROMA 2	The team has a long experience in the field of human genetics and developed an innovative gene targeting strategy based on oligonucleotide to restore a normal gene function by homologous recombination using small DNA fragment (SFHR) (Small Fragment Homologous Replacement). Participants are currently involved in Covid-19 host-genetics identifying life-threatening mutations in genes coding for interferons and characterized monoclonal antibodies against SARS-CoV-2.	Tor Vergata Oncoscience Research (TOR); Interdepartmental Research Center for Regenerative Medicine (CIMER); Center for Pharmaceutical Biotechnology

Spoke #6: RNA Drug Development

Partner	Competence	Infrastructural Resources
Spoke Leader CNR	The CNR scientists are internationally recognized for their studies 1) on the molecular biology of RNA and the impact of RNA metabolism on human health and ageing and 2) on therapeutic oligonucleotides and aptamers.	ESFRI LifeSciences Infrastructures including ELIXIR and EUROBIOIMAGING
Affiliate IIT	IIT scientists present expertise in imaging and high-resolution microscopy; RNA post-transcriptional modifications and genomics.	Nanopore Third-Generation Seq. platforms; Bioimaging Center; a large computational infrastructure
Affiliate RI.MED	Expertise in the identification of cmRNAs that revert senescent phenotype in human and mouse cardiac stem/progenitor cells in vitro.	CheMISt Computational Molecular Design and Screening
Affiliate - UNIBA	The participants of UNIBA have consolidated skills in RNA editing in degenerative disease and cancer.	ELIXIR infrastructure
Affiliate UNIBO	UNIBO Chemical Sciences represent excellence at National and International level in developing multiple technological platforms based on small molecule, stereopure oligonucleotides, PNA and hybrid conjugates molecules to modulate RNA function.	Center for Applied Biomedical Research, and analysis platform
Affiliate UNINA	UNINA participants are well-recognized for the development of more efficient processes for in vitro transcription of mRNA and construction of modified mRNA molecules with higher translation efficiency, design, synthesis and biophysical characterization of new therapeutic aptamers as therapeutics or targeting agents, validation of the safety and therapeutic efficacy by a multi-omic approach.	Drug Discovery Laboratories; Instrumental Analysis Laboratory, Mass Spectrometry Laboratory Molecular Biology Laboratory, Cell Culture Laboratory (LCC)
Affiliate - UNIPD	UNIPD participants are internationally recognized for research in RNA molecular biology, chemistry and bioinformatics and the role of non-coding RNAs, including microRNAs, lncRNAs, and circular RNAs, in diseases and hematological malignancies.	Slovenian NMR Center (ref to grant CERIC)
Affiliate UNIROMA 2	UNIROMA2 has a long-lasting experience in DNA/RNA chemistry and biosensors for diagnostic and therapeutic applications. The results achieved during these years cover diverse fields of DNA/RNA chemistry, analytical and physical chemistry and synthetic biology making the research extremely multidisciplinary.	Confocal microscopy; Fluorimeter; Fluorescence Plate reader
Affiliate UNISI	UNISI researchers have a strong experience in the preparation of library of photo-modulable RNA aptamer and their photophysical and photochemical characterization, including the development and validation of hybrid QM/MM computational models of RNA aptamers hosting a chromophore.	Laboratory for Computational Photochemistry and Photobiology (LCPP); High Performance Computer Cluster.

Spoke #7: Biocomputing

Partner	Competence	Infrastructural Resources
Spoke Leader UNIBA	UNIBA participants have a long-standing experience in the field of bioinformatics, computational biology and omics sciences.	ELIXIR-IT, The Italian node of the European Infrastructure for Life Science Data and Bioinformatics

Affiliate CNR	CNR scientists are internationally recognised for applying state-of-the-art computational techniques for the analysis of biological and biochemical systems, particularly pre-mRNA splicing, involved in the onset of human diseases and in the analysis of epitranscriptome.	ELIXIR CNR-BIOMICS
Affiliate IIT	Computational models of RNA structures and interactions.	HPC Franklin: petascale compute INFINIBAND cluster Energon computer cluster
Affiliate UNIMI	UNIMI participants have gained specific competencies in the biocomputing space over the years, more precisely focusing on AI digital technologies to support gene therapy and RNA drug analysis and discovery and bioinformatic identification and characterisation of RNA therapeutic targets.	UNITECH: technological platforms @UNIMI, Elixir-ITALY
Affiliate UNINA	UNINA participants have consolidated expertise in the dissection of the role of chromatin 3D architecture in congenital disorders and in the development of computational/experimental platforms for the prioritisation of anticancer drug targets integrating data from different sources.	Nano-Phor (Nano-Photonics for Life) and SCOPE datacenter and HPC infrastructure; Molecular Modeling Laboratory; Drug Discovery Laboratories; Instrumental Analysis Laboratory.
Affiliate UNIPD	UNIPD participants have consolidated expertise in developing and applying informatics/computational methodologies to support the design and industrial development of RNA drugs in different therapeutic areas such as cancer, neurodegenerative diseases, and viral infections.	Cloud-Veneto, computing and storage resources; Elixir-ITALY (https://elixir-italy.org/)
Affiliate UNIROMA 2	UNIROMA2 has a long experience in developing computational methods for RNA analysis as potential therapeutic target/agent in cancer or genetic diseases. Functional annotation of non-coding RNA, identification of motifs involved in RNA-protein interaction, RNA and DNA base-editing systems, experimental tests and validation of the computational studies through siRNA, CRISPR cas9, KO.	Elixir- ITALY (https://elixir-italy.org/)

Spoke #8: Platforms for RNA/DNA delivery

Partner	Competence	Infrastructural Resources
Spoke Leader UNINA	UNINA participants have a long-lasting expertise in developing advanced delivery strategies for different nucleic acids, covering the crucial aspects of their translation to the patient's bedside. Their current activities are focused on the development of nano-platforms for precision delivery, engineering of adenovirus and AAV, production technologies, paper-based sensors, and in vitro/in vivo models developed ad hoc to test delivery efficiency with the support of omics sciences.	Nanoparticle fabrication lab, drug and peptide synthesis and characterization, preGMP Drug Delivery Laboratory (DDL), Microfluidic lab, NMR and MS Facility, imaging Facility, Cell culture lab, animal house
Affiliate CNR	The participants have consolidated expertise in developing innovative nanomaterials for drug delivery, novel genetic methodologies for in vivo neuronal cell reprogramming based on AAV9 vectors with superior brain transduction, and application of extracellular vesicles of natural origin as delivery tools.	ESFRI LifeSciences Infrastructures, including ELIXIR and EUROBIOIMAGING
Affiliate IIT	The participants have consolidated expertise in engineering nanoplatforms for precision medicine, GLP production by microfluidics, additive manufacturing of microneedles for drug delivery.	Electron microscopy, Nanomanufacturing and characterisation
Affiliate UNIMI	The participants have strong experience with extracellular vesicles that are already close to the clinical application and developing delivery platforms (super selective nanoparticles) and testing efficacy	RNA/DNA carriers at pilot scale production; Large animal facilities. Hospital

	in a variety of animal models. Expertise in bioimaging of NA. Expertise in NA chemistry and in developing medicinal products in the EMA regulatory framework.	with large animal imaging technologies and surgical rooms
Affiliate UNIMIB	The participants have consolidated expertise in the design, implementation and study of delivery non-viral biocompatible nanosystems for nucleic acids in the treatment of oncological, inflammatory and neurodegenerative diseases taking advantage of cutting-edge automatised platforms for the synthesis and characterisation of nanocarriers for nucleic acid delivery.	NanoCosPha platform for nanoformulation, Nanpobiolab for the synthesis and characterisation of nanomaterials, microscopy facility
Affiliate UNIPA	The participants have consolidated skills in the development of tailor-made lipoplexes/polyplexes generating nanostructured RNA/DNA delivery systems.	ATeN Center – Advanced Technologies Network Center –
Affiliate UNIPD	The participants have consolidated expertise in the generation of innovative supramolecular platforms for cell-selective and controlled RNA/DNA delivery (polyplexes, lipoplexes and nanoparticles) and their biological characterization by already available preclinical tools, i.e. 2D/3D cells, Zebrafish and advanced ex-vivo/in-vivo organ perfusion.	Physicochemical and biological analytical infrastructure (Mass spectrometry, NMR, DLS, TEM, Confocal microscopy etc.); HTS Facility; gene delivery facilities
Affiliate UNIPI	The participants have consolidated expertise in extracellular vesicles (naturally obtained by specific cell culture) or camouflaged soft nanosystems (obtained by coating natural cationic polymers, chitosans, lipids or cyclodextrins, with specific cell membrane fragments.	CISUP, Center of Instrument Sharing, Pisa University; NIKON Imaging Center @IIT, Genoa, Italy
Affiliate UNISA	UNISA participants have consolidated skills in advanced technologies for powder production (supercritical fluids), microfluidics and development of drug delivery systems, and ADMET by MS-based spectroscopic and LC-MS methods.	AREA SCIENCE PARK, CERIC ERIC, ELETTRA SINCROTRONE, PREMIO
Affiliate UNITO	UNITO researchers have consolidated expertise in developing delivery systems based on the engineering of naturally occurring extracellular vescicles (EVs) and nanovectors (NVs).	Molecular imaging; Cell factory; animal house.
Affiliate Antares	Antares is active in the pharmaceutical market and guarantees protection of products, profit, people, and planet through inspection systems for quality control, Track & Trace solutions for anti-counterfeiting and supply chain transparency, smart data management tools for maximized efficiency and digitalization of the supply chain, from raw materials to the final consumer.	artificial intelligence services, automated inspection machines for the pharmaceutical industry, software for serialization and traceability, inspection systems for glass and metal containers, in software solutions for digitalization and supply chain transparency.

Spoke #9: From target to therapy: pharmacology, safety and regulatory competence center

Partner	Competence	Infrastructural Resources
Spoke Leader UNIMI	Internationally known technical and scientific competence in preclinical and clinical pharmacology, with particular reference to biotech drugs. Ability to generate innovative experimental models for in vitro to in vivo studies, competence in toxicology and immunology. Organization and participation in large interdisciplinary national and international networks involved in drug research and development, molecular biology and genetics. Strong connections with international and national biotech and pharma companies. Experienced TTO support for biomedical research translation. Unique expertise in clinical pharmacology and interactions with EU and Italian regulatory bodies.	Centralized facilities for cellular and animal studies (behaviour, toxicology, surgery advanced in vivo imaging). Organoid Center. Core labs for 'omics, imaging, bioinformatics, chemistry, spectroscopy and diffractometry, (UNITECH: technological platforms @UNIMI)

Affiliate CNR	The participants have expertise in the study of the molecular mechanisms underlying: i) synapse dysfunction and the impact of immune molecules on this process to identify new targets suitable for innovative treatments, ii) the contractility/ relaxation of cardiomyocytes and formulations of biomimetic nanoparticles for the administration of specific drugs for the heart.	Hyperion™, zetasizer, nanosight
Affiliate HUNIMED	Participants have recently developed a class of RNA aptamers, called AptaDiRs, selectively inhibiting DNA methyltransferase 1 (DNMT1) activity and exploited to target the methylation profile of disease genes to rescue their expression. This highly versatile approach can potentially target complex neurodegenerative diseases in which hypermethylation of specific genes (TREM2, BDNF, GAD1) is responsible for the pathogenetic mechanisms.	State-of-the –art animal facilities, functional genomics lab, access to clinical specimens and patient biobanks
Affiliate UNINA	The participants have consolidated expertise in developing 2D/3D models and organoids (EMT, methylomics, transcriptomics) and transgenic models to study pharmacodynamics, efficacy, organ/systemic toxicity. Animal models are available for cardiovascular and metabolic diseases, inflammation and cancer, dermatology, skeletal muscle diseases, neuropharmacology, respiratory and gastrointestinal pharmacology/toxicology. Expertise in pharmacoeconomic model creating frameworks for building regulatory reimbursement models with RWE is also relevant.	Molecular Biology Laboratory, Cell Culture Laboratory (LCC) Mice animal house (about 5000 rodents)
Affiliate UNIPD	The UNIPD group is internationally recognised for having generated SPLICS reporters for microscopy applications for mapping organelle contact sites in vitro and in vivo. This competence is crucial for applying splitGFP technology to nucleic acids-based therapy efficiency testing and is well complemented by consolidated skills in extracellular vesicles drug delivery and gene-therapy toxicity clinical assays.	MINIATURE with multispectral imaging, confocal microscopy (with FLIM), new high content microscopy, scanning TEM, X ray microscopy, Mass spectrometer and mass spec imaging; HTS facility; Facility for analytical chemistry, cell biology and animal house
Affiliate UNIPV	The participants have consolidated expertise in the application of natural polymers in drug delivery for the local treatment of infections and tissue repair and evaluation of safety profile.	Centro Grandi Strumenti - CGS (Pavia, Italy) - http://cgs.unipv.it
Affiliate UNIROMA 1	Participants have consolidated experience in the treatment of rare disorders of lipid metabolism with antisense RNA technology They have developed innovative approaches that use extracellular vescicles (EVs) for delivery targeted RNA therapy.	Confocal Microscopy; actuator/transducer and stimulator; reverse microscope with FLIM, facilities for cell culture.

Spoke #10: Preclinical development, GMP manufacturing and clinical trials of GTMP

Partner	Competence	Infrastructural Resources
Spoke Leader OPBG	The Institution has well-known expertise in developing, validating and manufacturing clinical grade Advanced Therapy Medicinal Products (ATMPs) based on gene-modified somatic cells (AGTMPs). The Institution has already activated and conducted 3 academic clinical trials on CAR T cells in B-cell malignancies and solid tumours.	Process Development Lab, Accredited GMP Facility, Vector production facility, Unit of Gene Therapy/Technology Transfer; Clinical Trial Center
Affiliate CNR	The participant has consolidated expertise in setting up disrupting strategies, including multiplex gene editing (NPs-MGE) based on CRISPR/Cas9 technology coupled with homologous recombination (HR), for a wide range of therapeutic scenarios in the field of gene therapy.	Biotech facilities, Technopole of Precision medicine (1200 m^2), Lab equipped for nano fabrication, cellular and molecular biology.

Affiliate TELETHON-TIGET	SR-TIGET has consolidated expertise in developing gene therapies with a focus on thalassemia, lysosomal storage disorders, *ex vivo* expansion of genetically-engineered hematopoietic stem and progenitor cells, *in vivo* intracerebral lentiviral gene therapy for metachromatic leukodystrophy.	GLP Lab for toxicology and safety studies, Vector Integration Core, Process Development Lab, Clinical Lab, Clinical Trial Center.
Affiliate Tettamanti	Consolidated expertise in translational/clinical research on leukaemias. The Institution has already activated and conducted an academic clinical trial on CAR-CIK cells in B-cell ALL.	GMP Facility for non-viral transduction of cells, Lab for pre-clinical proof-of-concept; Clinical Trial Center.
Affiliate UNICH	The participant has consolidated skills in cellular biology, animal models, manipulation and expansion of T- and NK-cells as well as on extracellular vesicles characterization.	Research labs, animal facility, clinical center.
Affiliate UNIPD	The Institution conducts bench-to-bedside research on pediatric diseases, including cancer and monogenic blood disorders, to develop innovative treatment modalities.	Research labs, animal facility, clinical center.
Affiliate UNISR	A leading healthcare university with remarkable competences in cutting edge research on rare genetic diseases, gene and cell therapy, with a focus on genetic diseases.	Academic Infrastructure for young researchers.
Affiliate UNICAMPANIA	The Unit is conducting a National Clinical Trials for ocular gene therapy in patients with Inherited Retinal Dystrophies (IRDs).	Research lab, Clinical center

Research activities the executing subjects (Spoke and Affiliates)

The research activities recognized as strategic to advance knowledge in Gene Therapy and Drugs based on RNA Technology are covered by 5 "Vertical" Spokes and 5 "Horizontal" Spokes. The "Vertical" Spokes aim to identify the most promising candidate targets for RNA-based drugs in five major areas of human diseases (genetic diseases, cancer, metabolic and cardiovascular, neurodegenerative, and inflammatory/infectious disorders). The "Horizontal" Spokes will build and disseminate the technological know-how necessary for designing, delivering and producing gene therapy products and RNA drugs. The expected outcomes are based on three pillars:

- Identification and validation of targets for both RNA-based drugs and gene-therapy/genome editing approaches;
- Technological advancement in the KET;
- Implementation of the core facilities for manufacturing RNA and gene therapy products.

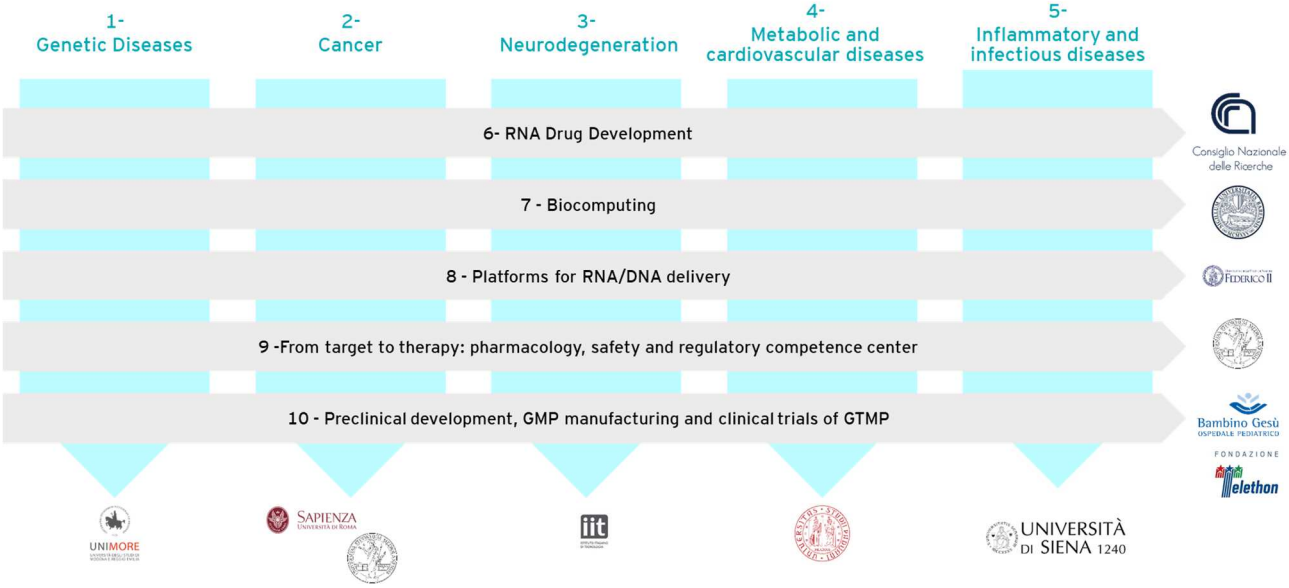
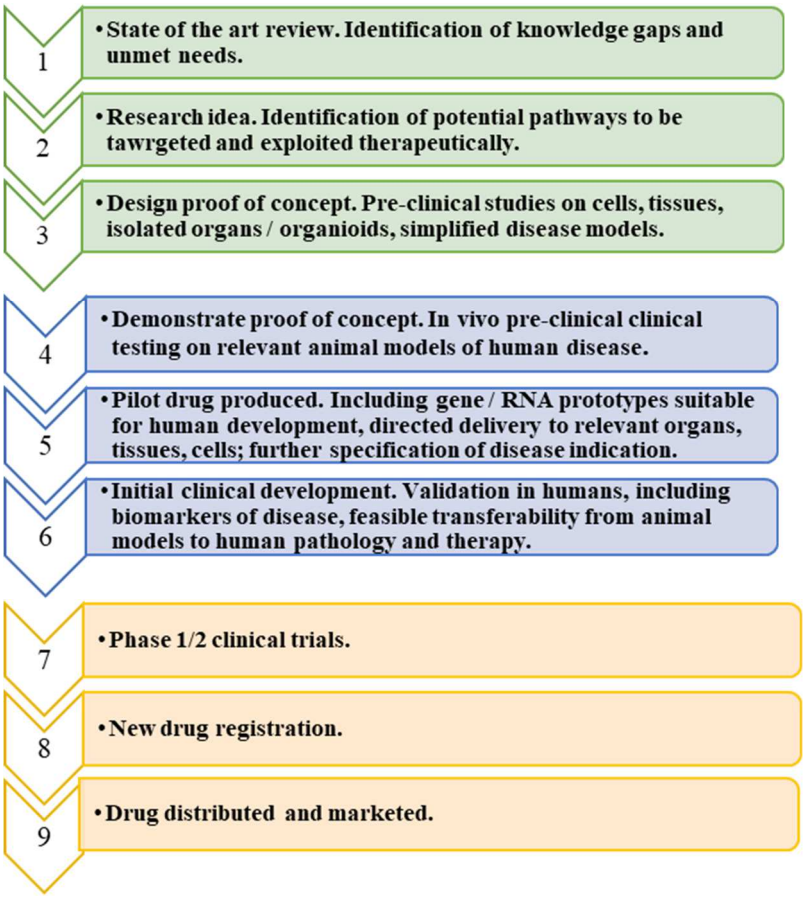


Figure B. 16 – Representation of the Spoke Model

The research activities carried out in each Spoke will strongly benefit from a synergic interaction between Academia and Private partners, with the mutual provision of expertise and competence. For these purposes, the research activities inside each Spoke are intimately connected, aligned in linking the technological developments of horizontal Spokes in providing tools and know-how for application in diseases (vertical Spokes) and take benefits of the strategic collaboration with

multinational (Pfizer, BioNtech, Novartis, AstraZeneca, Sanofi, Orgenisis) and Italian biomedical and Pharma companies (Stevanato, Chiesi, Dompè, Bracco, IRBM, Antares, Eurofins, PBL), of the largest Italian bank (Banca Intesa), and a number of biotech companies active in the rapidly expanding field of RNA therapeutics and gene. This tight interconnection will be instrumental in translating the results obtained in the proof-of-concept studies in academic laboratories and identifying opportunities to transfer knowledge from the Academia to Private players.
We describe in the following, for each thematic Spoke, the state of the field and unmet needs and how the Spoke will contribute to advance knowledge. The research activities are organized in work packages (WP) illustrating the general objectives and methodologies, the expected outcomes and progress in TRL of each specific task, and the main scientific deliverables and milestones that facilitate monitoring the progresses.
Herein the TRL scale applied to drug discovery process

TECHNOLOGY READINESS LEVELS- TRL



B.5.1 VERTICAL SPOKES
Spoke #1: Genetic Diseases. Spoke Leader: UNIMORE
State of the field and unmet needs. Most of the 6-8,000 known rare diseases have a genetic origin, affecting 6-8% of the worldwide population. Since the Orphan Drug Act (ODA) passed in 1983, more than 600 orphan drugs and biological products have been approved. However, the unmet medical need is still huge, as only 5% of rare diseases have an approved treatment. EURORDIS-Rare Diseases Europe has launched in 2021 a campaign to improve the lives of people living with a rare disease. Gene therapy may represent a definitive cure for many monogenic diseases, although its development provides little commercial incentive due to the limited size of the potential market. A main goal of the EURORDIS action is therefore “to bring 1,000 new medicinal products, including gene and cell therapies”.
How the Spoke will contribute to advance in this scenario. This Spoke assembles a consortium of academic research groups and industrial partners that are leaders in the field of genetic diseases and innovative medicine. By potentiating existing cutting-edge research groups and implementing targeted spin-off and industrial partnerships, we aim at fostering further innovation to accelerate “bench to bedside” translation and develop gene therapies and RNA therapeutics to treat a selected group of uncurable genetic diseases. Recent advances in the generation, purification and cellular delivery of RNA have enabled development of RNA-based therapeutics, a rapidly expanding category of drugs that are cost effective, relatively simple to manufacture, and can target previously undruggable pathways. RNA-based therapeutics include antisense oligonucleotides, aptamers, small interfering RNAs, microRNAs, and messenger RNA. The overall objective of

the research programme is to develop cutting-edge viral vector-based or genome and base editing technology for gene therapy of a selected group of paradigmatic, uncurable genetic diseases up to pre-clinical or clinical proof of concept, in collaboration with biotech/pharma companies interested in licensing, developing and marketing the therapies. The Spoke is articulated on six disease-oriented programmes addressing, at various phases, the development of safe and efficacious genetic therapies for severe and incurable monogenic diseases, based on the use of new generations of viral vectors, systemically or locally delivered microRNAs and interfering RNAs, nanomedicine-based delivery of drugs/gene materials (RNA/DNA, etc..) and gene- and base-editing technology. Avoiding operlapping in terms of aims and activities, specific projects and cross-actions will be implemented with **Spoke #3**, regarding neurodegenerative and neurodevelopment disorders, with **Spoke #7**, to optimize biocomputing, with **Spoke #8** in delivering strategies, and with **Spoke #10** to share protocols and medicinal products, respectively. In particular, a tight collaboration will be implemented with **Spoke #3** to target: a) mitochondria disorders, by sharing in relevant disease-models RNA activators/inducers of anti-oxidative stress response and mitochondria biogenesis, b) neuro-neuromuscular diseases due to hereditary haploinsufficiencies, by jointly developing and testing mRNA-specific enhancer of translation, such as SINEUPs lncRNAs, to increase the molecular tools to rescue physiological amounts of proteins and homestasis of diseased cells. These new approaches may represent ideal solutions in a contingency plan in the case of unexpected experimental pitfalls and drawbacks. In addition, **Chiesi**, a leading Italian pharmaceutical company affiliated to Spoke #1, whose mission is the identification of new biological entities and advanced therapies in the area of rare diseases, is interested in partnering and contributing in regard to specific projects within WP1.2 (Task 1.2.1), WP1.3 (Task 1.3.2-.4) WP1.4 and WP1.5.

WP 1.1 – Skin	
General objectives. Epithelial stem cell-gene therapy (GT) for genetic skin diseases **Methodology.** Gene & Cell therapy, Viral Vectors, Nanomedicine development, in vitro and in vivo testing, Omics analysis, Clinical grade development	
Task 1.1.2	**GT for Epidelmolysis bullosa (Task leader: De Luca-UNIMORE)**
TRL	**3→9**
	Completion of viral vectors development and Initiation of a phase 1/2 gene therapy (GT clinical trial (CT) for Epidermolysis bullosa (EB)
Task 1.1.3	**GT for Lamellar ichthyosis (Task leader: De Luca-UNIMORE)**
TRL	**3→7**
	Filing phase 1/2 GTCT for Lamellar ichthyosis (LI)
Deliverables WP 1.1	D1.1 Retroviral vector for RDEB; D1.2 CTA dossier for gene therapy of RDEB; D1.3 CTA dossier for gene therapy of LI
Milestones WP 1.1	M1.1 Completion of viral vectors development/RNA delivery by lipid nanoparticles (M12); M1.2 completion of safety and efficacy studies (M48); Initiation of a phase 1/2 GTCT for RB / filing a phase 1/2 GTCT for LI (M36)
WP 1.2 - Hemoglobinopathies, coagulation and genetic iron loading disorders	
General objectives. Gene and RNA therapy for blood-related genetic diseases **Methodology.** Viral Vector development, Gene therapy, Gene Editing, Omics analysis, Nanomedicine development, siRNA/ASO/small molecules development and validation, in vitro and in vivo testing	
Task 1.2.2	**Gene editing of Hematopoietic Stem Cells (HSCs) for the treatment of Sickle Cell Disease (SCD) Mavilio-UNIMORE**
TRL	**3→7**
	Pre-clinical proof of safety and efficacy of reversing the fetal-adult Hb switch by gene editing in HSCs from SCD patients
Task 1.2.3	**Targeting iron overload (IO) by miRNAs in thalassemia (Task leader: Origa-UNICA);**
TRL	**2→5**
	Validation of siRNAs & ASO in Thalassemia (Tal)
Task 1.2.4	**Small Molecule (SM) therapy in hemochromatosis and Ferroportin Disease (Task leader: Pietrangelo-UNIMORE)**
TRL	**2→5**
	GE and SM efficacy in Genetic Iron Overload Disorders
Task 1.2.5	**GE and RNA therapy (RT) in Hemophilia A (Task leader: Simioni-UNIPD)**
TRL	**2→5**
	Efficacy studies to correct Factor VIII defects
Task 1.2.6	**RT for red blood cell diseases (RBCD) (Task leader: Iolascon-UNINA)**
TRL	**2→5**
	siRNAs & ASO validation in RBCD
Deliverables WP1.2	D2.1 Phase-1/2 CTA dossier for gene-edited hematopoietic stem cells for treatment of SCD; D2.2 Identification of iron overload-specific-miRNAs in Tal; D2.3 GE & SM protocol in hemochromatosis and Ferroportin Disease; D2.4 GE/GT and RT in Hemophilia; D2.4 ASO protocols and models for hereditary RBCD
Milestones WP1.2	M2.1 Production of HbF by genome-edited SCD cells Enrollment of patients with iron overload -analysis of circulating miRNAs and identification of ASOs and siRNA; Protocol for NGS-based patient selection; Design of gene/base editing protocols; Catolog of target genes (M12). M2.2 Safety and efficacy studies; Generation of ASOs and siRNAs carrier prototypes; Pre-clinical validation; Testing of snRNAs for FVIII mutations; Identification of ASO (M48). M2.3 Completion of non-clinical studies;

	Development of multisensor-integrated organ-on-chip model prototypes; SM and GE protocols for hemochromatosis and ferroportin disease; Efficacy studies on patient-derived iPS cells; Pre-clinical validation in RBCD (M36)
WP 1.3 - Eye	
General objectives. Pre-clinical and early clinical Gene and RNA therapy for inherited retinal distrophies and degeneration **Methodology.** Gene Therapy, Gene Editing, Viral vectors, Nanomedicine development, Omics and biocomputing analysis, in vitro and in vivo testing	
Task 1.3.2	**Corneal stem cell-GT for corneal dystrophies (Task leader: Pellegrini-UNIMORE)**
TRL	**2→7**
	Completion of viral vectors development and Initiation of a phase 1/2 GT/CT for Meesman syndrome (MS)
Task 1.3.3	**Novel RNA-DNA treatments for inherited retinal degeneration (IRD) (Task leader: Marigo-UNIMORE)**
TRL	**2→5**
	Development of novel neuroprotective treatments for inherited Development and validation of non-viral delivery to the eye and nanomedicine-based eye delivery of gene-editing complexes
Task 1.3.4	**Nanomedicine and gene-editing (GE) complexes for IRD (Task leader: Recchia-UNIMORE)**
TRL	**2→5**
	Completion of micro-RNA development, testing and validation for Retinitis Pigmentosa (RP)
Task 1.3.5	**Micro-RNA development for optic neuropathies (Task leader: Bruno-TIGEM)**
TRL	**2→5**
	Validation of efficacy for miR-181a/b downregulation in Leber optic neuropathy
Deliverables WP1.3	D3.1 Lentiviral vector and CTA dossier for MS; Nanomedicine for delivery to the eye; D3.2 gRNAs for allele-specific ko of RP mutations; D3.3 microRNA-181a/b for neuroprotection
Milestones WP1.3	M3.1 Viral vector development; non-viral delivery systems development; Nanomedicine delivery of gene-editing complexes; OMIC analysis of miR-181a/b pathways (M12). M3.2 Completion of safety and efficacy studies; Nanomedicine delivery of gene-editing complexes; miR-181a/b "sponge" development (M48). M3.3 Phase 1/2 GT CT Initiation for Meesman syndrome; Pre-clinical efficacy studies completion; Pre-clinical efficacy studies completion (M36)
WP 1.4 - Storage Diseases	
General objectives. Gene and RNA therapy for Storage genetic diseases **Methodology.** Viral Vectors, Gene Editing, Gene Therapy, Omics analysis, in vitro and in vivo testing, Clinical grade development	
Task 1.4.2a	**HSC GT for Lipofuscinosis1 (CLN1) (Task leader: Biffi-UNIPD)**
TRL	**5→9**
	LV-based HSC GT Phase ½ clinical trial for CLN1
Task 1.4.2b	**Gene addition in HSCs for neuropathic SD (Task leader: Biffi-UNIPD)**
TRL	**3→7**
	Targeted Gene Addition in HSCs (TGAinHSCs) for neuropathic SD
Task 1.4.3	**GT of Mucopolisaccaridosis type VI (M-VI) (Task leader: Brunetti-TIGEM)**
TRL	**5→9**
	GTCT for Mucopolysaccharidosis type VI (M-VI)
Task 1.4.4	**GT of Pompe Disease models (PD) (Task leader: Parenti-TIGEM)**
TRL	**3→6**
	Validation of AAV-therapy in Pompe Disease models (PD)
Deliverables WP1.4	D4.1 CLN1 HSC GT dossier; D4.2 Validated TGAinHSCs protocol for SDs; D4.3 CTA dossier for MPSVI clinical trial; D4.4 mTORC /TRPML1 pathways modulator(s) in PD
Milestones WP1.4	M4.1 Completion of CTA-enabling safety studies; Optimization of TGA at the selected locus in HSCs; Completion of the ongoing phase-I/II clinical trial for an AAV-based gene therapy for MPSV; Pharmacological modulation of mTORC pathway (M12). M4.2 POC of efficacy in relevant SD models; CMC activity finalization and regulatory submission; Evaluation of safety and efficacy data of the trial; M4.3 Pharmacological modulation of TRPML1 pathway (M24). Initiation of a phase-I/II clinical trial of GT for CLN1; TGAinHSCs protocol scale up and validation; Design, regulatory interactions and filing AAV-based liver-directed gene therapy for MPSVI; Efficacy evaluation on AAV-GAA-mediated gene transfer in PD mouse model (M36)
WP 1.5 - Kidney	
General objectives. Gene and RNA therapy for kidney genetic diseases **thodology.** Viral vectors, Gene therapy, Gene editing, Omics analysis in vitro and in vivo testing	
Task 1.5.2	**RNA-based silencing of transcription factors (TF) (Task leader: Ballabio-TIGEM)**
TRL	**2→5**
	Validation of TFEB gene silencing by AAV-(sh)RNA and siRNAs
Task 1.5.3	**GE in Alport syndrome (Task leader: Renieri-UNISI)**
TRL	**2→5**
	GE validation for Alport syndrome
Deliverables WP1.5	D5.1 Delivery of (sh)RNA/siRNAs to kidney for TFEB silencing; D5.2 Kidney targeting-GE protocol
Milestones WP1.5	M5.1 Identification of the most effective delivery method for TFEB silencing in kidney cells; Completion of strategy design (M12). M5.2 PoC efficacy studies in-vitro on cells from animal models (M24); M5.3 Reduction of renal cysts and cancer cell growth after TFEB silencing in vivo; Completion of in-vivo biodistribution, toxicological, safety and pre-clinical efficacy studies (M36)
WP 1.6 - Mithocondria (MT)/Neurometabolic, Neuro/Neurodevelopmental-Neuromuscular diseases	
General objectives. Mithocondria (MT)-Neurometabolic, Neuro-Neurodevelopmental- Neuromuscular disorders. **thodology.** Gene editing, Gene Therapy, viral vectors, nanomedicine platform development, Omics analysis, in vitro and in vivo testing	
Task 1.6.2	**Nanocluster therapy for NMD (Task leader: Torrente-UNIMI)**

TRL	**4→7**
	CTA-enabling study for Au8-pX nanoclusters in Friedreich's ataxia
Task 1.6.3	**Nuclease and RT targeting mtDNA (Task leader: Garone-UNIBO)**
TRL	**2→5**
	Effective RNA/nuclease therapy for defective/mutant mtDNA
Task 1.6.4	**miRNA- and GT for MT-diseases (Task leader: Scorrano-UNIPD)**
TRL	**2→5**
	Effective ex-vivo and in-vivo GE
Task 1.6.5	**RNA and GT of MT disorders of nuclear origin (Task leader: Palmieri-UNIBA)**
TRL	**2→5**
	mRNA/AAV-GT of MT diseases of nuclear origin
Task 1.6.6	**Nanocarriers-mediated RT therapy for Duchenne MD (Task leader: Messina-UNIMI)**
TRL	**2→5**
	Validation of muscle-specific nanocarriers for shRNA delivery
Task 1.6.7	**GE for neuronal targeting (Task leader: Meloni-UNISI)**
TRL	**2→5**
	GE Validation in vitro and in vivo models
Task 1.6.8	Neurodevelopmental Disorders (NDD) (**Task leader:** Zollo-UNINA)
TRL	**2→5**
	Efficacy and pharmacokinetics in pre-clinical models
Task 1.6.9	**Micro-RNA therapy for Central core disease (CCD) (Task leader: Sorrentino-UNISI)**
TRL	**2→5**
	Efficacy of miR-486 in pre-clinical models of CCD
TRL	**2→5**
Deliverables WP1.6	Au8-px nanocluster manufacturing CT; mtDNA targeted nuclease /RNA drugs and small molecules; miRNA- and AAV-based gene replacement therapy for mitochondrial diseases; Novel polymeric/lipidic nanocarriers for RNA/AAV-GT; muscle-specific liposome-based nanocarriers (Lipo_shNfix) for DMD; protocols for neuronal targeting; PoC in NDD; AAV.miR-486 viral vectors
Milestones WP1.6	M6.1 Efficacy study of Au8-pX gold quantum nanoclusters in FA cells; Efficacy and safety of a nucleases library targeting mtDNA mutations in vivo models and GMP production for clinical trial development; Opa1 miR148/152 antagomirs, SINEUPs and AAV delivery and efficacy; In vitro validation of RNA/AAV-GT; design, development, *in vivo* testing liposome-based delivery of shNfix; Strategy design completion; Validation of mRNA therapeutics in carrying vectors (AAV2); completion of validation studies of miR-486 expression in CCD mouse models (M12). M6.2 Development of large-scale GMP manufacturing protocols for Au8-pX gold quantum nanoclusters; Efficacy and safety of RNA drugs for mtDNA replication defects in 2D and 3D models ; AAV9 CoQ genes vectors to correct CoQ-deficiency in vivo; Development and in vitro validation of nanocarriers for selective RNA/AAV delivery; efficacy of the Lipo_shNfix; Completion of in-vivo biodistribution studies in animal models; Validation of mRNA therapeutics in carrying vectors of nanoparticles (SNALPS) (M24). M6.3 CTA-enabling studies and CTA filing; Efficacy, safety and delivery of CRISPR-based gene therapy for mtDNA replication defect in vivo models; scAAV9-delivered genes in Ndufs4, Surf1 and Cox15 KO mice/AAV-mediated miRNA-1281 delivery and preclinical efficacy; In vivo validation of nanocarriers-delivered RNA/AAV-GT; development of GMP manufacturing protocols; Pre-clinical safety and efficacy studies and toxicological analysis completion; Pharmacokinetics for best therapeutic approach in NDDs; efficacy evaluation of AAV.miR-486 expression in pre-clinical models of CCD (M36)

Spoke #2: Cancer. Spoke co-Leaders: UNIROMA1, UNIMI.

State of the field and unmet needs. Almost 50 years ago, the "war on cancer" was declared with the premise that knowledge on alteration of cellular and molecular mechanisms of cancer would translate into effective treatments. On this wake, constant refinement of conventional chemotherapy and radiotherapy has been enriched by an increasing number of drugs targeting specific oncogenic proteins and by immune-oncology. Yet, in spite of few notable exceptions, the war on most cancers is far from being won. Why and how the RNA therapeutics can transform this scenario? To start, druggable genetic alterations are rare, diverse, present in restricted subsets of patients for each tumor type, with efficacy of targeted therapies often becoming limited by acquired resistance and by the tumor cellular heterogeneity. Second, the war on cancer has largely focused on mutant cancer cells; a wealth of evidence now indicates that tumors are complex ecosystems in which tumor cells are embedded in a microenvironment of normal, yet "corrupted" cells contributing to tumor phenotypic heterogeneity, or "plasticity". In this respect, metastasis, the ultimate cause of >90% of cancer deaths, is increasingly recognized as the product of normal regenerative programs hijacked by malignant cells, endowing immune evasion, resilience to stress and ability to co-option organ-specific niches for growth. Metastases display the same genetic alterations of primary tumors, indicating that their addictions and vulnerabilities must be searched in transcription factors, epigenetics and signaling factors currently considered undruggable. Finally, although immune checkpoint inhibitors (ICI) do provide proof that the immune system can be powerfully induced to eradicate tumors in patients, their benefit remains frustratingly restricted to a minority of cases.

How the Spoke will contribute to advance in this scenario. After the spectacular success of COVID vaccines, the remarkable efficacy, safety and flexibility of RNA therapeutics promise to forever change the landscape of cancer therapy. The Spoke will focus on the implementation of RNA therapeutics and biomarkers to tackle cancer trunk addictions with unprecedented specificity, notably signaling, transcriptional and epigenetic changes driving, and preserving, the tumor or metastatic cell states. Second, we will take advantage of the safety of RNA therapeutics to exploit synthetic combinatorial

approaches, co-targeting at the same time interdependent hallmarks of cancer leading to the collapse of the tumor/metastatic ecosystem. This is currently impossible with the current armamentarium of proteins-targeting drugs, given the excessive cumulative toxicities of drug combinations. Third, we plan to take full advantage of RNA to extend the applicability of ICI, by turning immunologically cold tumors into hot, and vastly expand the anti-cancer potentials of the immune system through vaccination strategies.

Our Spoke will converge on the three keywords: the undruggable, the unconventional and the unseen. We will consider research projects at different TRL levels with main emphasis on those that may be translated more readily into clinical applications. To start, we will identify novel targets using omics and artificial intelligence (AI) approaches and focus on new and already identified targets that are established determinants of tumorigenesis, or able to restore oncosuppressor circuits, but whose targeting has so far proven unfeasible. At the same time, we will continue research at a lower TRL aiming at the identification of novel targets and biomarkers that will fuel the Center in the near future with potentially groundbreaking tools. Targets will be protein-coding genes but also on elements of the vast and unexplored non-coding RNA fraction of the cancer genome, a potential treasure throve of therapeutic applications. We will focus on targets acting in malignant cells or in the tumor stroma, and particularly on strategies fueling anti-cancer innate immunity and unleashed anti-cancer adaptive immunity. We will discover novel diagnostic, prognostic and response to treatment biomarkers in cancer based on high-throughput sequencing technologies and AI approaches.

Activities of this Spoke will include interactions with **Spokes #6**, in particular on the encapsulation of small RNAs on human ferritin constructs thus developed, **Spokes #7**, in particular by expanding epitrascriptomic in silico screening with effective inhibition of RNA post transcriptional modifying enzymes, **Spoke #8** (Platforms for RNA/DNA Delivery), **Spoke #9** (From Target to therapy pharmacology, safety and regulatory competence/center) and **Spoke #10** for the translation of pre-clinical research into clinical trials (Preclinical development GMP manufacturing and clinical trials of GTMP). In addition, a collaboration is planned with **Spoke #**5 for target and biomarkers tuning protocols. The long standing cooperation with **IRBM** research group, capable of innovative research and manufacturing, will pave the way across all aspects of drug discovery and early development in order to accelerate the development of vaccines and medicine Clinical implementation will also be carried out in the framework of cooperation with existing facilities of the **SANOFI** group. **Antares**, also in collaboration with **Spoke #8**, will lead design and implementation a lab-scale prototype integrated modular system for continuous monitoring of formulations of nanosized EVs loaded with RNA under continuous or pulsed flow with steady-state fill volumes. The prototype will aim to characterize the formulations for EV size (based on dynamic light scattering), RNA content (based on UV-vis spectroscopy) and (selected) membrane properties (based on microarray technology). With respect to the EV physicochemical properties, the system will aim at performances analogous to state-of-the-art low-volume batch laboratory characterization, while it will aim at outperforming them in terms of processing time.

WP2.1 aims at identifying novel target genes that belong to the following categories: **A**) **Transcription factors**. Cancer is ultimately a disease of aberrant gene-expression mediated by Transcription factors (TFs) that are either mutated, or specifically dysregulated in cancer. TFs are typically chemically intractable with small molecules, but ideal targets of inhibitory RNA therapeutics. Here we will concentrate of main unmet needs, that is inhibition of TFs known to play paramount relevance in endowing tumor aggressiveness and metastatic traits, including YAP, HIF, Myc, NF-Y isoforms, SOX and FOXA TFs. **B**) We will also extend our analyses to inhibition of **epigenetic regulators**, DNA-repair/Nuclear envelope and heterochromatin packaging factors (such as ATR, Pin1 and SMC1A) for the dual purpose that loss of these factors should on the one hand increase the tumor mutational burden and, on the other, unleash innate immunity by spilling of nuclear DNA (e.g. through cGAS/IFI/AIM2 signaling), phenocopy of microsatellite instability, and/or reactivation of endogenous retroviruses, that are, in turn also superantigens; **C**) **metabolic pathways** critical in specific tumor types, for example iron metabolism in AML; and **D) mitochondrial pathways**, such as inhibition of the VDAC1 and potassium channels. The mitochondria can be induced to activate potent cell death cascades, that, through activation of mitochondrial RNA-sensors, can also immunologically re-heat the tumor microenvironment. A further layer is represented by altered expression of regulatory noncoding RNAs (lncRNA, miRNA, circular RNA) contributing to cancer development. We also plan to expand our investigation to other non-coding RNA species, such as long-non-coding RNAs, and circular RNAs, that are either specifically expressed in tumors or that interact with transcription factors relevant for cancer biology; as examples, lncAS-YC in RB-mutated tumors and lncRNAs specifically expressed by regulatory T cell and involved in the definition of their identity that can be targeted to enhance anti-tumor immune responses. Additionally, RNA-binding proteins (e.g., MEX3A, ELAVL1) and epitranscriptomic modifiers/editors (ADAR, METTL3 and FTO) represent an emerging theme in cancer research; the latter are also relevant to inform **Spoke #6** on what chemical modifications should to be introduced in RNA therapeutics to influence the fate of clinically relevant RNAs. Finally, and well connected to the WP3 below, another innovative layer of related intervention is represented by Alternative splicing (AS). More than 95% of multi-exonic genes are alternatively spliced, whose alterations alone generate more neo-epitopes than point mutations. Focus will be here devoted to RNA-mediated interference with Splicing Factors overexpressed in many types of cancers, such as MBNL1/2, CELF2 aiming at increase tumor immunogenicity, activation of DC cells and effector T-cell infiltration. Cancer patients

necessitate not only novel targets genes for RNA based innovative personalized treatments but also the tools for better patient management. Biomarkers can satisfy this urgent medical need providing clinicians a tool that can be used for diagnosis, prognosis, follow-up, patient stratification and management. To this end, we will identify novel tissue and circulating non coding RNA biomarkers using high-throughput technologies and AI.

WP2.2, we will prioritize our targets depending on two criteria: in vivo effectiveness and cancer specificity. Pending the results on optimization and delivery from **Spoke #8**, we plan to validate most of our work on the above listed targets by modulating target genes and RNA molecules through shutdown of key enzymes, RNA modifications, gene editing, siRNA, miRNA, Circular RNA, Antisense oligonucleotides (ASO), aptamers. Intratumoral delivery of RNA-therapeutics will be achieved through Lipid Nanoparticle mediated delivery (LNPs) that are currently used clinically. The latter may nevertheless immediately translate into a number clinical applications, such as treatment of unresectable tumors, or currently untreated and yet accessible metastatic lesions. This will concentrate the therapeutic effects on the tumor bed, but limit side- and confounding effects associated to systemic treatments. Even with this approach, it is plausible that only minority of cells will achieve effective target inhibition. Priorities for upgrading toward the clinic will be thus given to the most effective treatments in vivo, including use of Patient Derived Avatars, such as **PDX mice** and **syngeneic** mouse cancer **models**, when given either as monotherapy or in combination with standard therapies existing for each tumor type in human patients, and particularly ICI. Indeed, we will drive particular attention on treatments that, even if effective only on a fraction of tumor cells, may be able to ignite a "domino-like" effect with eradication of the whole tumor mass and signs of systemic disease attack (abscopal effects), thanks to the reactivation of the immune system, reiterated priming of cancer immunity through epitope spreading, all-in-all ultimately turning the tumor as its own vaccine. Previously identified tissue and circulating biomarkers will be validated and assessed for their diagnostic, prognostic and response to treatment power.

In parallel to the discovery of novel RNA therapeutics directed against tumor targets, we are developing novel immune-RNA therapeutics, drawing on a pipeline of newly identified neo-antigens and tumor associated antigens. To this end, in **WP2.3** we are analyzing systematically the tumor immune-peptidome by proteomics of tumor cells exposed to clinically available drugs (in particular epigenetic drugs stimulating expression of novel transcripts), and generation of novel "fusion proteins" with high antigenic potential. We are also screening the potentially vast antigenic space associated to fetal antigens, to unannotated reading frames (nuORFs), and potential products of alternative splicing. This information may generate a pool of tumor specific and potent immunogenic peptides which can be fast-tracked for the development of mRNA-based vaccines. We have already obtained some remarkable successes with the identification of effective antigens (e.g., RID proteins) for in preclinical models of breast cancer and melanoma. We will also enhance the efficacy of immunotherapy by developing combination therapies using RNA based therapies with ICI. RNA-based approaches will also be used for the transient manipulation of immune cells haplotypes. Clearly, the development of a robust pipeline to generate a large array of mRNA-based cancer vaccines represent a key deliverable for the **NC** and a nexus of the efforts planned in its several Spokes. **In WP2.4,** in the framework of a recently established cooperation with the group Intesa San Paolo, we have addressed financial aspects that are directly connected with Tech transfer and fund rising activities. We will ensure that the new technologies create concrete opportunities for the integration of all stakeholders involved in the Spoke value chain to join the specific results, in particular SMEs, Startups which may contribute to enhance the actions' outcome. We will collect and exchange, as well as connect what individual subjects, industrialists and research institutions, can offer, thus lowering the level of complexity in the context of communications. In parallel we will contribute to create the opportunity to represent, the different "capabilities" of the actors present in the HUB and of any other future participants that will be added, also in the sense of the prospective ability to fine-tune research and industrialization initiatives.

WP 2.1 – Identification of novel targets for RNA-based therapeutics and biomarkers	
General objectives. This WP aims at discovering novel therapeutic targets and biomarkers in cancer. Targets will be protein-coding genes but also elements of the vast and unexplored non-coding RNA fraction of the cancer genome. In this WP will be explored the possibility of introducing Artificial Intelligence techniques for the diagnosis of cancer starting from the acquired knowledge **Methodology.** The determinants of tumorigenesis will be addressed in patients derived malignant cells or in the tumor stroma, by means of multi-omic platforms. The effort will be integrated by high-throughput sequencing technologies and artificial intelligence (AI) approaches. The work will be also carried out in cooperation with the biocomputing Spoke #7. As a whole, this WP will serve as engine to fuel activities of horizontal Spokes.	
Task 2.1.1	**Identification of novel RNA targets: TFs, epitrascriptomic factors, ncRNAs, aberrant alternative splicing factors (Task leader: Mantovani-UNIMI)**
TRL	**1→4**
	• Novel RNAs from multi-omic platforms. • Novel RNA targets for RNA-based therapies in rare tumors, soft tissues sarcomas, cholangiocarcinoma, pediatric brain tumors, thyroid, melanoma, neuroendocrine, myeloid neoplasm, glioblastoma and prostate cancer. • RNA-protein interactions in prostate cancer.

Task 2.1.2	**Identification of novel RNA targets in cancer metabolism and metastasis mechanisms (Task leader: Caffarelli-CNR)**
TRL	**1→4**
	• Novel RNAs able to interfere with selected hubs sustaining cancer metabolic reprogramming. • Models for metabolic networks upon gene targeting through lncRNA gapmers. • RNA therapeutics to target metastases from solid tumours. • small ncRNAs able to target proteins involved in cancer metabolism and radio-adaptation of tumor cells.
Task 2.1.3	**Identification of novel RNA targets in the tumor micro environment (Task leader: DeiTos-UNITS)**
TRL	**1→4**
	• Secreted ncRNA that can modulate the TME impacting in breast, lung and liver cancer. • List of miRNA in osteosarcoma TME. • ncRNA in therapy response and TME modulation
Task 2.1.4	**Identification of Diagnostic, Prognostic and Response to therapy Biomarkers (Task leader: Ferretti-UNIROMA)**
TRL	**1→4**
	• List of RNA biomarkers in multiple myeloma, prostate cancer, pediatric brain tumors, melanoma, thyroid cancer and neuroendocrine tumors. • Use of AI technique for cancer diagnosis (e.g. kidney cancer). • Use of biomarkers for diagnostic, prognostic and response to therapy identified through AI approaches.
Deliverables WP 2.1	1.1 Libraries of novel identified RNA target sequences (M12); D2.1.2 List of novel identified miRNA biomarkers prostate cancer, thyroid cancer and neuroendocrine tumors (M12); D2.1.3 List of novel RNAs able to interfere with cancer metabolic reprogramming (M18). D2.1.4 Models for metabolic networks upon gene targeting through lncRNA gapmers (M18); D2.1.5 List of miRNA in osteosarcoma TME (M18); D2.1.6 List of novel RNA biomarkers in multiple myeloma, pediatric brain tumors and melanoma (M24); D2.1.7 Development of novel AI technique in cancer diagnostics (M24); D2.1.8 List of RNA-protein interactions in prostate cancer (M24); D2.1.9 List of Novel RNA therapeutics to target metastases from solid tumours (M32); D2.1.10 Identification and characterization small ncRNAs able to target proteins involved in cancer metabolism and radio-adaptation of tumor cells (M32); D2.1.11 Identification and characterization of secreted ncRNA that can modulate the TME impacting in breast, lung and liver cancer. (M32); D2.2.12 Identification and characterization of RNAs interfering with hedgehog pathway in medulloblastoma and glioblastoma.
Milestones WP 2.1	MS2.1.1 Identification of target RNA sequences (10-20) in selected tumor types. (M12); MS2.1.2 Identification of targets in cancer metabolism and metastases. (M18); MS2.1.3 Identification of RNA targets in tumor microenvironment (M18). MS2.1.4 Identification of protein RNA targets (M24); MS2.1.5 Identification and validation of diagnostic and prognostic biomarkers (M32). MS2.1.6 In vitro validated therapeutic RNAs (M36).
WP 2.2 - Prioritization and validation of targets toward clinical applications	
General objectives. We plan to modulate target genes and RNA molecules through shutdown of key enzymes, RNA modifications, gene editing, siRNA, miRNA, Circular RNA, Antisense oligonucleotides (ASO), aptamers. Validation of diagnostic, prognostic and response to therapy biomarkers. Preclinical, animal models and patient derived avatars will be used. The latter may immediately translate into clinical applications **Methodology.** We will prioritize our targets depending on vivo effectiveness and cancer specificity. Pending the results on optimization and delivery from other Spokes of the NC, we plan to validate most of our work on the above listed targets by systemic or intratumoral delivery of RNA-therapeutics assembled with LNPs currently used clinically or eventually developed by Spoke #8.	
Task 2.2.1	**Specific shutdown of key enzymes, RNA modification and gene editing (Task leader: Pizzi-UNIBS)**
TRL	**2→4**
	• Novel targets for hotspot mutations of tyrosine kinase in melanoma, prostate cancer and myeloma cancers. • Silencing RNA targeting ion channels in pancreatic ductal adenocarcinoma and triple negative breast cancer. • Gene editing on KRAS or NRAS mutants in multiple myeloma. • Post trascriptional RNA modifications as novel therapeutic targets. • Suicide genes approaches to hematological tumors.
Task 2.2.2	**In vitro and in vivo efficacy tests and delivery of RNA based therapies (siRNA, miRNA, Circular RNA, ASO, aptamers) (Task leader: Boffi-UNIROMA)**
TRL	**2→4**
	• ncRNAs targeting m6A methyltransferase in AML. • Novel targets for cytotoxic genes in glioblastoma. • anti-GOLPH3 siRNA in lung cancer. • novel short RNAs to inhibit tumorigenic circRNAs and microRNAs. • ASO to control splicing isoform in prostate cancer. • Generation of new conjugates aptamers to deliver miRs/antimiRs or siRNAs tumor and stroma cells in breast cancer and melanoma. • Development of small RNA molecules stimulating cytoplasmic sensors in glioblastoma and myeloid neoplasm. • Development of ASO with senolytic activity in prostate and pancreatic cancer. • Development of antitumor siRNA, miR mimics, antagomiR for EV loading. • Massive intracellular RNA and drug release in glioblastoma.
Task 2.2.3	**Validation of Diagnostic, Prognostic and Response to therapy biomarkers in preclinical models (Task leader: Viglietto-UNICZ)**
TRL	**2→4**
	• Validated RNA biomarkers in pediatric brain tumors, thyroid, melanoma, prostate, neuroendocrine, lung, colon and rectum. • Development of patients Avatars for Biomarker validation.

Deliverables WP2.2	2.1 List of novel targets for hotspot mutations of tyrosine kinase in selected cancers. (M12); D2.2.2 Silencing RNA targeting ion channels. (M12); D2.2.3 Identification of suicide gene sequences targeting hematological tumors (M12); D2.2.4 Gene editing protocols on KRAS or NRAS mutants in multiple myeloma (M18); D2.2.5 Identification of post trascriptional RNA modifications as novel therapeutic targets. (M18); D2.2.6 Preparation of ncRNAs targeting m6A methyltransferase in AML (M18); D2.2.7 Identification of novel targets for cytotoxic genes in glioblastoma and methods of targeted delivery (M24); D2.2.8 Novel silencing anti-GOLPH3 siRNA in lung cancer (M24); D2.2.9 Identification of novel short RNAs to inhibit tumorigenic circRNAs and microRNAs. (M32); D2.2.10 Preparation of ASO to control splicing isoform in prostate cancer. (M36);
Milestones WP2.2	MS2.2.1 Generation of new conjugates aptamers to deliver miRs/antimiRs or siRNAs tumor and stroma cells in cancers (M18); MS2.2.2 Development of antitumor siRNA, miR mimics, antagomiR for EV loading. (M18); MS2.2.3 Generation of self assemblying SiRNA targeting METTL3 (24); MS2.2.4 MS2.2.5 Development of patients Avatars for Biomarker validation (32);

WP 2.3 - Discovery of novel immune-RNA therapeutics

General objectives. Novel immune-RNA therapeutics, based on a pipeline of newly identified neo-antigens and tumor associated antigens, will be developed. The generation of novel "fusion proteins" with high antigenic potential will be achieved, including chimeric antigen receptors.
Methodology. The tumor immune-peptidome will be analyzed by proteomics of tumor cells exposed to clinically available drugs, and generation of novel "fusion proteins" with high antigenic potential. We are also screening the potentially vast antigenic space associated to fetal antigens, to unannotated reading frames and potential products of alternative splicing.

Task 2.3.1	**Identification of Novel immune-RNA targets in immune system (Task leader: Minucci-UNIMI)**
TRL	**2→4**
	• Identification of tissue-specific miRNA/mRNAs as prognostic factor for the activation of tumor-related inflammation. • Identification of tumor neoantigens, stimulatory molecules and/or PD-1/PD-L1 antagonists with antitumor effect. • Characterization of ncRNA landscape in tumor infiltrating lymphocytes in breast, ovarian and colorectal cancers.
Task 2.3.2	**Development of novel tumor RNA vaccines (Task leader: Piccolo-UNIPD)**
TRL	**2→4**
	• Development of mRNA- or adenoviral-vaccines towards immunogenic antigens including fetal antigens. • Novel candidates for anticancer therapy on colorectal cancer (CRC), using patient derived xenografts (PDXs) and matched organoids. • Development of a pipeline of novel RNA-based therapeutics in breast, melanoma and pancreatic cancer. • Development of neoantigens in lung and pancreatic cancers, glioblastoma and leukemias by novel ex-vivo strategies. • Targeting mitochondrial pathways controlling cancer survival
Task 2.3.3	**Development of Combination therapies to enhance efficacy of immunotherapy and CAR-T Therapies (Task leader: Mari-UNISI)**
TRL	**3→5**
	• DNA hypomethylating Agents (DHA) in combination with the administration of ICI. • LNP therapies targeting miRNAs involved in resistant to ICI melanoma and NSCLC. • Genetic manipulation of the haplotype in syngeneic models of breast cancer. • Engineer modified CAR-T cells able to target otherwise untreatable solid tumors.
Deliverables WP2.3	D2.3.1 Identification of tissue-specific miRNA/mRNAs as prognostic factor for the activation of tumor-related inflammation (M12); D2.3.2 Identification of tumor neoantigens, stimulatory molecules and/or PD-1/PD-L1 antagonists with antitumor effect (M12); D2.3.3 Characterization of ncRNA landscape in tumor infiltrating lymphocytes in breast, ovarian and colorectal cancers (M18); D2.3.4 Novel mRNAs or adenoviral-vaccines towards immunogenic antigens including fetal antigens. (M24); D2.3.5 Identification of novel candidates for anticancer therapy on colorectal cancer using patient derived xenografts (PDXs) and matched organoids. (M24); D2.3.6 Genetic manipulation methods of the haplotype in syngeneic models of breast cancer. (M32); D2.3.7 DNA hypomethylating Agents (DHA) in combination with the administration of ICI. (M32); D2.3.8 Methods for manipulation of the haplotype in syngeneic models of breast cancer. (M32); D2.3.9 Novel protocols for therapeutic use of DNA hypomethylating Agents (DHA) in combination with the administration of ICI. (M32);
Milestones WP2.3	MS2.3.1 Development of a pipeline of novel RNA-based therapeutics in breast, melanoma and pancreatic cancer. (M12); MS2.3.2 Development of neoantigens in lung and pancreatic cancers, glioblastoma and leukemias by novel ex-vivo strategies. (M18); MS2.3.3LNP therapies targeting miRNAs involved in resistant to ICI melanoma and NSCLC. (M24); MS2.3.4 Engineered modified CAR-T cells able to target solid tumors. (M32); MS2.3.5 Novel targets on mitochondria.

WP 2.4 – Maximizing impact

General objectives. The main objective is ensuring that the new technologies create concrete opportunities for the integration of all stakeholders involved in the Spoke value chain to join the specific results, in particular SMEs, Start-ups which may contribute to enhance the actions' outcome
Methodology. We will collect and exchange, as well as connect what individual subjects, industrialists, and research institutions, can offer, lowering the level of complexity in the context of communications, or creating the opportunity to represent, also in the sense of the prospective ability to fine-tune research and industrialization initiatives, the different "capabilities" of the actors present in the HUB and of any other future participants that will be added

Task 2.4.1	**Identification and evaluation of opportunities for market introduction (Task leader: Pacifici, Lunghi-Intesa San Paolo)**
TRL	**2→5**
	• A roadmap for the coordinated and integrated deployment of the relevant Spoke research and innovation results. • Technological market trend analysis and studies on the development and structural trends of specific industrial sectors. • Memos that cover the assessment of technological market needs and opportunities.
Task 2.4.2	**Business Model and Business acceleration (Task leader: Ascioni-Intesa San Paolo)**

TRL	**4→5**
	• Promotion of emerging business models. • Activation of open innovation methods between scientific partners and large private entities, as well as with startups and technological SMEs (not only in the strictly biotech / pharmaceutical sector, but also for example carriers of solutions artificial intelligence and machine learning for data processing and management, etc.). • Scouting and selection of the companies with high potential.
Task 2.4.3	**Networking and synergies with other programme (Task leader: Campolucci-Intesa San Paolo)**
TRL	**1→5**
	• Matchmaking with innovative companies and investors (belonging to the private or institutional sectors in Italy and Europe). • Implementation of networking activities and synergies with other National and EU initiatives (e.g. Mission Cancer, Partnership etc. in Horizon Europe). • Enabling multi-level funding synergies at EU and national level and private investments, as well as synergies between the EU financial instruments developed (from EIC Fund to INVESTEU), in order to accelerate the scale and speed necessary for Industry deployment.
Deliverables WP2.4	D2.4.1 Assessment of a roadmap for the deployment of the Spoke research and innovation results (M12); D2.4.2 Assessment of technological market trend analysis and studies on the development and structural trends of specific industrial sectors (M12); D2.4.3 Memos that covers the assessment of technological market needs and opportunities (M12); D2.4.4 Guidelines for promotion of emerging business models (M18); D2.4.5 Activation of open innovation methods between scientific partners and large private entities, as well as with startups and technological SMEs (M24); D2.4.6 Scouting and selection of the companies with high potential (M24); D2.4.7 Match-making with innovative companies and investors (M32); D2.4.8 Implementation of networking activities and synergies with other National and EU (M32); D2.4.9 Set-up of multi-level funding synergies at EU and national level and private investments. (M32);
Milestones WP2.4	2.4.1 Tech market reports and analysis (M12); MS2.4.2 Reporting on innovation trends (M12); MS2.4.3 Up to date layout of emerging technologies in data managment in pharma sector. (M24); MS2.4.4 Business meetings with investors. (M24); MS2.4.5 Reporting on networking matchmaking (M32); MS2.4.6 Reporting on identification of funding opportunities (M32);

Spoke #3: Neurodegeneration Spoke Leader: IIT

State of the field and unmet needs. 300 million people suffer of a form of brain disease in Europe. Among them, Alzheimer's disease (AD) and dementia are the most frequent. Given their slow progression, the amount of societal and economic costs as well the burden on families is staggering: the total cost of healthcare assistance in Europe is 800 billion per year. In Italy there are 600000 patients affected by AD and 240000 with Parkinson's disease (PD) with a healthcare cost for AD of 11 billion. All these diseases remain currently untreatable and no drugs can reverse or slow the neurodegenerative process. Concerning neurodevelopmental disorders, Autism spectrum disorders (ASDs) and Intellectual Disabilities (ID) involve more than 1 out of 100 children in Italy. Cerebral ischemia is the second cause of death and the major trigger of disabilities with tissue plasminogen activators the only available treatment but with limited success. Classic drug discovery approach has posed tremendous challenges to identify new compounds for neurodegenerative and neurodevelopmental diseases, resulting in staggering costs, poor specificity and difficulties in the synthesis of multitarget drugs. The list of failed clinical trials in neurodegenerative diseases is discouragingly long and the amount of money spent is so high that pharmaceutical companies are considering abandoning this field. We believe this state of the art is due to several largely unsolved issues in basic research departments, current traditional clinical settings and drug development strategies. The major issues are as follows: i. the majority of knowledge of the pathways involved in neurodegenerative diseases derives from the study of genes mutated in familial cases; ii. these diseases are highly heterogenous leading to failures of clinical trials for poor selection of patients; iii. many proteins remain difficult to be targeted so that all FDA- and EMA-approved drugs combined, target not more than 1000 human proteins leaving the large majority of the genome unchallenged; iv. most attention has been given to protein-coding genes; v. these approaches have not addressed the potential role of regulatory elements, lncRNAs, and repetitive sequences both as disease modifiers and pharmacological targets; vi. because drug development is based on single-gene-mutation models, combinatorial drug treatments have been largely ignored. In summary, we are suffering from the lack of understanding of the molecular basis of neurodegeneration and of the extreme heterogeneity of genomic, molecular and clinical features leading to a lack of implementation of precision medicine. These observations may also account for the state of the art in neurodevelopmental conditions where the extreme heterogeneity in genomic and clinical data as well the need to intervene during the first years of life represent insurmountable roadblocks to disease-modifying therapeutic interventions.

How the Spoke will contribute to advance in this scenario. We plan to take advantage of the specific properties of RNA therapeutics to treat brain diseases: i. RNA molecules can extend the portion of the druggable genome targeting in principle any molecules of interest including regulatory elements; ii. RNA molecules are scalable; iii. multitarget RNA molecules can be designed challenging different genes and pathways at the same time. In this context, we aim to promote down-regulation of gene expression, in conditions of dominant neurodegenerative diseases caused by the expression of a pathological, toxic target gene. We will also plan to design and validate the use of RNA molecules that can increase gene expression *in vivo*. Augmented levels and/or activity of modifiers of pathogenic pathways may contribute to reestablish a homeostatic cellular environment. For patients with haploinsufficiencies, as in a large portion of neurodevelopmental disorders, a therapeutic approach based on RNA drugs to rescue the physiological amount of the target protein would in principle be curative. We also aim to develop new classes of programmable RNAs resorting to the modular organization of

lncRNAs, combining RNA domains with specific structures and RNA-protein interactions patterns with antisense sequences tethering the biological activity of the interacting protein to a specific RNA or DNA sequence.
This Spoke will carry out a coordinated effort to establish Precision Medicine for brain diseases. This will be accomplished by: i. providing preclinical data to file for RNA-based Investigational New Drugs (INDs) within the time frame of 3 years for known RNA molecules with therapeutic potentials; ii. providing long-term sustainability of the pipeline with new programmable RNA drug platforms or with new single RNA drug candidates; iii. identifying new drug targets. Special attention will be devoted to i) tailoring RNA molecules to a specific group of patients according to their genomic repertory of diseases-causing mutations or risk-associated variants; ii. establishing multi-target drugs modifying the activity of more than one pathway at the same time. Known or new candidate RNAs with therapeutic potentials will be studied and these include siRNA, ASO, agoMir, antagomiRs, aptamers, coding and non-coding circular RNAs, lncRNAs including SINEUPs and mRNA vaccines. Projects will take advantage of established animal models for neurodegenerative and neurodevelopmental diseases. Therapeutic RNAs will be synthesized and delivered *in vivo* by stereotaxic injections, by *in utero* electroporation or by the use of AAVs. Mice will be characterized with behavioral tests, electrophysiological recordings and immunohystochemical analysis. Patient-derived cells will be differentiated into neurons *in vitro* or into brain organoids to assess phenotypic rescues. A large genomic facility for gene expression studies and the experience in the analysis of transcriptomics data from bulk tissues or single cells of coding and non-coding RNAs, long and short, and their epitranscriptomic modifications will be central to assess phenotypic rescues and identify new RNA targets.
We believe that a tight collaboration with the activities of other Spokes will be instrumental to the success of the scientific plan. In detail, we will collaborate with **Spoke #1** to share knowledge and tools to confront genetic diseases that involve the central nervous systems. Several collaborations are currently ongoing with project leaders in the two Spokes, especially for the optimization of SINEUP RNAs to treat haploinsufficiencies. We will resort to **Spoke #6** for the development of new technological platforms of programmable RNAs to be applied to the nervous system, for the identification of post-transcriptional modifications of therapeutic value with nanopore sequencing technology and for imaging techniques *in vivo* to study RNA drugs dynamics in cells and tissues. **Spoke #7** will provide knowledge and computational infrastructure to carry out predictions and simulations of RNA secondary structure and interactions with other nucleic acids and proteins. We will tightly collaborate with **Spoke #8** for the optimization of delivery technologies to the central nervous system: in particular, we will take advantage of expertise in the fields of nanoparticles optimization, microvesicles/exosome loading and delivery, technologies to pass the blood-brain barrier, molecules and peptides for homing nanoparticles to specific neuronal cell types. On the way to IND filing, a crucial step is represented by pharmacokinetics and pharmacodynamics analysis as carried out in **Spoke #9**. **Spoke #10** will be instrumental to streamline GMP production of RNA drug candidates.
To carry out this plan and reach our goals, we aim to take advantage of calls for the Italian scientific community to fill some gaps in the knowledge currently present in the Spoke. Among the topics, linear and circular RNAs in AD and poly(Qs) diseases, analysis of functional domains in lncRNAs and mice models for haploinsufficiences in neurodevelopmental disorders.
We also plan to promote the creation of infrastructures dedicated to the implementation of the project. Special attention, and the majority of the budget, will be dedicated to the affiliated Spokes located in the South of Italy. The long-term plan for the sustainability of the Spoke is based on i. taking advantage of licensing agreements with the Private sector to carry out clinical trials for the RNA drugs that reach IND approval during the time frame of the project and ii. promote the establishment of new RNA-based platforms that can attract investments aimed to tailor the use of new disruptive technologies to specific proof-of-principles applications. The optimization of these technologies may also provide preliminary data for competitive grant applications.
The Scientific plan comprises 5 WPs. In **WP3.1**, we will describe the transcriptional landscapes of small (miRNAs and piwiRNAs) and long non-coding RNAs, of circular RNAs (coding and non-coding) as well as of their interactions with proteins in AD, PD, ALS and PSP. Special attention will be dedicated to the analysis of their epitranscriptomic modifications as studied with different -omics approaches. We aim to establish new platforms for programmable RNAs technologies including those sponging miRNAs activities, circular RNAs to deliver neuroprotective factors, lncRNAs able to enhance translation of an mRNA of interest and aptamers modifying protein-RNA interaction networks. We will also setup brain organoids from patient-derived iPSCs as a tool to validate the biological activity of RNA-based drugs. In **WP3.2**, we will prioritize candidate therapeutic RNAs and identify promising RNA targets to streamline the acquisition of preclinical data from patient-derived differentiated neurons or brain organoids *in vitro* and in animal models of the neurodegenerative disease of interests. These include modifiers of RNA-protein interaction networks and tools to down-regulate gene expression or modify splicing patterns. We will also tackle neuroinflammation developing RNAs able to interfere with its role in pathogenesis. We will establish a pipeline to develop mRNA vaccines to proteins prone to aggregation starting with α-synuclein as Proof-of-concept. In **WP3.3**, we will explore the use of RNA molecules to treat neurodegenerative diseases caused by pathologically expanded nucleotide repeats. On one hand, we aim to interfere with pathological RNA translation. On the other, we will identify and assess the therapeutic values of modifiers of poly(Qs) toxicity. In **WP3.4**, we aim to exploit non-coding RNA biology for precision medicine of neurodevelopmental disorders. Given the large heterogeneity of

these diseases we will focus on a specific repertory of gene mutations while searching for common dysregulated drug targets and linear and circular RNA molecules with therapeutic potentials. In **WP3.5**, we will discover and assess the therapeutic use of RNAs involved in Stroke and other diseases of the brain. In summary, we aim to collect preclinical data on the most promising RNAs to reach IND filings and therefore lay down the premises for clinical trials.

WP 3.1 – RNA platforms for neurodegenerative diseases	
General objectives. Our goal is to identify candidate RNAs as therapeutics or drug targets by taking advantage of the biology of small, circular and long non-coding RNAs and of their expression patterns in neurodegenerative diseases. Protocols for brain organoids will be optimized. **Methodology.** We will resort to RNA-seq experiments for profiling small and long RNAs. Single-cell transcriptomics and epigenetic analysis of brain organoids. RNA-protein interaction predictions as well experimental validation with –omics approaches (CIRSseq, 2'-OmeSeq, m6Aseq, m1Aseq, m5Cseq, hm5Cseq, RAPiD-MaPseq, scRNAseq, RIPseq, PARCLIPseq) and bioinformatics tools for RNA data (MREdictor, RNA structure framework, DRACO). Protocols for brain organoids from patient-derived iPSCs.	
Task 3.1.1	**Identification and study of small (miRNAs, piRNAs) ncRNAs, lncRNAs and circular RNAs altered in neurodegenerative diseases (Task leader: Bozzoni UNIROMA1)**
TRL	**1→4**
	• Identification of circular and lncRNAs altered in neurodegenerative diseases: AD, PD, ALS. • Identification of piRNA in neuroinflammation. • Identification of miRNAs altered in the retina of AD and ALS mouse models.
Task 3.1.2	**RNA-protein interaction networks in neurodegenerative diseases (Task leader: Neri UNITO).**
TRL	**1→4**
	• Identification of lncRNAs-protein interactions in neurodegenerative diseases: of lncRNAs-protein interactions: secondary structure analysis and predictions, epitranscriptomic modifications etc. • Identify RNA-RNA and RNA-protein interactions involved in aggregate formation in ALS. • Analysis of RNA-protein interaction networks of FUS, HuD and FMRP in 2D co-cultures and in 3D neuromuscolar organoids.
Task 3.1.3	**Develop artificial RNA sequences to target proteins involved in neurodegenerative diseases (Task leader: Tartaglia IIT).**
TRL	**1→4**
	Design and validation of RNA aptamers targeting proteins involved in neuromuscular diseases including TDP-43 and FUS.
Task 3.1.4	**Identification of linear and circular natural lncRNAs with SINEUP-like activity (Task leader: Gustincich IIT).**
TRL	**1→4**
	Identification of linear and circular natural lncRNAs with SINEUP-like activity.
Task 3.1.5	**Brain organoids as models of neurodegenerative diseases (Task leader: Oliviero UNITO).**
TRL	**1→4**
	• Optimization and characterization of brain organoids as models of neurodegenerative diseases (single cell transcriptomics, epigenetic analysis, gene networks in cell populations etc including cortical and striatal organoids, midbrain organoids.
Deliverables WP 3.1	D3.1.1 Gene expression profiling of lncRNAs, circRNAs, piRNAs, miRNAs and validation of data (M12); D3.1.2 Experimental data on RNA-protein interaction network in models of AD and ALS (M12); D3.1.3 Experimental data on RNA-RNA interaction network in ALS (M12); D3.4 Computational prediction of aptamers binding TDP-43 and FUS and experimental validation (M12); D3.1.5 Computational analysis of lncRNAs with embedded repeats of the SINE type in the mouse and human transcriptomes and library preparation for screening (M12); D3.1.6 Optimization of protocols for brain organoids (M12); D3.1.7 Priority list of RNAs to be further studied (M24); D3.1.8 Analysis of epitranscriptomics modifications of RNAs (M24); D3.1.9 Computational predictions of RNA domains involved in protein interactions, secondary structure analysis (M24); D3.1.10 Analysis of RNA-protein interactions network for FUS, HuD and FMRP (M24). D3.1.11 Analysis of the biophysical properties of aptamer-protein interactions (M24). D3.1.12 Characterization of cell composition of brain organoids by single cell transcriptomics, cell-type specific markers and various -omics techniques (M 24).
Milestones WP 3.1	M3.1.1 List of differentially expressed miRNAs and piRNAs (M12); M3.1.2 List of differentially expressed lncRNAs (M12); M3.1.3 List of differentially expressed circular RNAs (M12); M3.1.4 List of lncRNAs that bind proteins (M24); M3.1.5 Set of aptamers with therapeutic potentials (M24); M3.1.6 List of natural linear and circular non-coding RNAs with translational enhancing activities (M24). M3.1.7 Priority list of RNAs with therapeutic potentials (M24). D3.1.8 Brain organoids as models of neurodegenerative diseases (M24).
WP 3.2 Neurodegenerative diseases	
General objectives. We aim to test new programmable linear and circular non-coding RNAs, small and long, as identified in WP1, for their ability to modify gene expression *in vitro* and *in vivo* as a therapeutic strategy for AD, PD, ALS and PSPs. **Methodology.** We will take advantage of *in vitro* and *in vivo* models of neurodegenerative diseases including brain organoids.	
Task 3.2.1	**Engineering synthetic miRNAs sponges, agomiRs or antagomiRs, aptamers, ASO, siRNAs, lncRNAs and SINEUP lncRNAs and test their activity in vitro (Task leader: Bozzoni UNIROMA1)**
TRL	**5→7**
	• Design and validation of synthetic multiSINEUPs as therapeutics of neurodegenerative diseases. • Design and validation ASOs against lncRNAs involved in neurodegenerative diseases. • Design and validation ASOs against lncRNAs involved in ALS. • Design and validation of synthetic circular RNAs to sponge miRNAs involved in AD. • Design and validate agoMir and antagoMir for retinal injections.

	• Design and validate ASOs, siRNAs and AAV vectors for inhibition of SARM expression and increase the expression of NMNAT2 to modulate NAD metabolome in neurodegenerative diseases.
Task 3.2.2	**RNA-protein interactions as drug targets (Task leader: Neri UNITO)**
TRL	**5→7**
	• Modify RNA-protein interaction networks in ALS. • Modify lncRNAs-protein interaction networks by the use of ASOs. • Modify RNA-protein interactions with RNA aptamers in neuromuscular diseases including TDP-43 and FUS.
Task 3.2.3	***In vivo* data on the use of therapeutic RNAs in AD, PD, ALS and PSPs (Task leader: Bozzoni UNIROMA1).**
TRL	**5→7**
	• Proof-of-concept of the therapeutic effects of multiSINEUPs in animal models of neurodegenerative diseases. • Proof-of-concept of the use of ASOs and lncRNAs activity in ALS models. • Proof-of-concept of the therapeutic effects of agoMir and antagoMir on the retina of mouse models of AD and ALS. • Proof-of-concept of the use of RNA aptamers in mouse models of neuromuscular diseases. • Proof-of-concept of the use of circular RNAs sponge for microRNAs in AD models. • Proof-of-concept of manipulating *AS AQP4* to interfere with vascular dysfunction and inflammation in AD and ALS.
Task 3.2.4	**Brain organoids as models of neurodegenerative diseases (Task leader: Oliviero UNITO).**
TRL	**5→7**
	• Analysis of brain organoids of lncRNA knockouts. • Gain of function therapy by RNA delivery to brain organoids. • AAV delivery of therapeutic molecules to cortical and striatal brain HD organoids. • Analysis of miRNAs expression and delivery of antagomiRs to midbrain organoids to treat PSP. • Manipulating RNA expression in brain organoids to tackle neuroinflammation.
Task 3.2.5	**Gene editing in mouse models of neurodegenerative diseases (Task leader: Siciliano UNIPI).**
TRL	**5→7**
	• Gene editing of lncRNAs in ALS. • Gene editing in the retina in AD/ALS models.
Task 3.2.6	**Targeting neuroinflammation (Task leader: Pizzo UNIPD).**
TRL	**5→7**
	• Characterization of the role played by the eATP/P2X7R signalling axis in AD neuroinflammation and phenotype and of the effects of its manipulation by siRNA for P2X7R and Panx-1 in IPSc-derived microglia and in AD mouse models. • Proof-of-concept of the use of selected miRNAs and siRNA to interfere with the microglia pro-inflammatory response. • Proof-of-concept of the use of selected piwiRNAs as antiinlfammatory drugs in AD and PD. • Proof-of-concept of the use of nanostructured RNAzymes to modulate neuroinflammation in AD and ALS. • Analysis and manipulation of the expression of AQP4 and of its antisense lncRNAs AS-AQP4 as a strategy to modulate inflammation and neurovascular dysfunction in AD and ALS. This study will be carried out by taking advantage of AQP4 KO mouse models including two in which AQP4 is unable to form the classic supramolecular structures in the plasma membrane.
Task 3.2.7	**mRNA vaccines (Task leader: Antonini UNIPD)**
TRL	**5→7**
	• Identification of alpha-synuclein (a-syn) epitopes recognized by a repertoire of autoantibodies in PD patients; evaluation of the aggregation properties and toxicity of the 3 lead epitope peptides *in vitro* and in cell models; synthesis of the mRNAs, development of the nanocarriers (NCs) for RNA delivery; validation of 1-2 leads *in vivo* in WT and Tg a-syn mouse models.
Task 3.2.8	**IND filings (Task leader: ALL)**
TRL	**5→7**
	• IND filing/approval of at least one therapeutic molecule
Deliverables WP 3.2	D3.2.1 Design and validate miRNA sponge targeting miRNAs involved in AD (M12); D3.2.2 Design and validate ASOs for lncRNAs identified in WP1 (M12); D3.2.3 Design and validate agoMirs and antagoMirs for retinal miRNAs (M12); D3.2.4 Design and validate ASOs, siRNAs and AAV vectors for inhibition of SARM expression and increase the expression of NMNAT2 (M12); D3.2.5 Design and validate miRNA sponge targeting miRNAs involved in AD (M12); D3.2.6 Characterize the role played by the eATP/P2X7R signalling axis in AD neuroinflammation and phenotype (M12); D3.2.7 Analysis of the effects of *AS AQP4* lncRNAs on AQP4 expression (M12); D3.2.8 Identification of α-syn epitopes recognized by a repertoire of autoantibodies in sporadic and genetic Parkinson's patients and in healthy controls (M12); D3.2.9 Validate SINEUP activity for endogenous single and multi-targets (M24); D3.2.10 Experimental data on interfering with RNA-protein interaction networks (M24); D3.2.11 Delivery of ASOs agoMir, antagomir, aptamers and lncRNAs in mouse models (M24); D3.2.12 Establishment of lncRNA knockouts in brain organoids (M24); D3.2.13 Delivery of therapeutic molecules in brain organoids (M24); D3.2.14 Delivery of immunomodulatory nanostructured RNAzymes (M24); D3.2.15 Gene editing in mouse models (M24); D3.2.16 Evaluation of the aggregation properties and toxicity of the 3 lead epitope peptides *in vitro* and in cell models and molecular characterization of the isolated autoantibodies (M24); D3.2.17 Phenotypic analysis of the effects of multiSINEUPs in animal models of neurodegenerative diseases (M36); D3.2.18 Effects of ASOs and lncRNAs on mouse models (M36); D3.2.19 Analysis of phenotypes of agoMir and antagoMir delivery to the retina of mouse models of AD and ALS (M36); D3.2.20 Analysis of phenotypes of ASOs, siRNAs and AAV vectors for inhibition of SARM expression and increase the expression of NMNAT2 (M36). D3.2.21 Analysis of phenotypes of brain organoids of lncRNA knockouts (M36); D3.2.22 Data on delivery to brain organoids (M36); D3.2.23 Phenotype analysis of the effects of gene editing in mice (M36); D3.2.24 Validation of selected nanostructured RNAzymes activities (M36); D3.2.25 Analysis of the effects of manipulating AQP4 on neuroinflammation and neurovascular dysfunction (M36). D3.2.26 Validation of 1-2 leads *in vivo* in WT and Tg α-syn mouse models (M36).

Milestones WP 3.2	M3.2.1 Engineered active circular RNAs with miRNAs sponge activities, agomiRs or antagomiRs, aptamers, ASO, siRNAs, lncRNAs, circular RNAs and SINEUPs (M12); M3.2.2 Role of the eATP/P2X7R signalling axis in AD neuroinflammation (M12); M3.2.3 Biological activity of *AS AQP4* lncRNAs (M12); M3.2.4 lncRNA knockouts in brain organoids (M24); M3.2.5 role of piRNAs in neuroinflammation (M24); M3.2.6 characterization of lead epitope peptides and characterization of the isolated autoantibodies for a-synuclein (M24); M3.2.7 *In vivo* data of biological effects of circular RNAs with miRNAs sponge activities, agomiRs or antagomiRs, aptamers, ASO, siRNAs, lncRNAs, circular RNAs and SINEUPs (M36); M3.2.8 Data on the therapeutic effects of modifying RNA-protein interaction networks (M36); M3.2.9 Phenotypes of lncRNAs KOs to prioritize candidate RNA targets or therapeutic RNAs (M36); M3.2.10 Proof-of-concept of the use of gene editing for neurodegenerative diseases (M36); M3.2.11 Proof-of-concept of interfering with neuroinflammation with ASOs, piRNAs. siRNAs, RNAzymes, agoMir and antagomir (M36); M3.2.12 Proof-of-concept of active immunization safety and target engagement in mouse models of an mRNA vaccine for a-synuclein (M36). M3.2.13 IND filing of at least one therapeutic RNA (M36).
WP 3.3 - Expanded nucleotide repeats	
General objectives. We aim to target diseases wth expanded nucleotide repeats by suppressing RAN translation of pathogenic nucleotide repeats, by inhibiting G4C2 toxicity and by expressing gene modifiers of diseases with expanded poly(Qs) tracts. **Methodology.** Reprogramming will be performed with non-integrating mRNA system to generate high quality, transgene-free iPSCs. NPCs will be obtained by differentiation of iPSCs. to generate cortical organoids, striatal organoids and "mini-brains". Motorneurons will be differentiated from patient-derived iPCSs. As mouse models, we will use a C9-500 BAC transgenic for ALS/FTD, a mouse overexpressing a mutant TDP43 for ALS, a KI AR polyQ mice for SBMA and two mouse HD models with a pathological number of Poly(Qs).	
Task 3.3.1	**Targeting RAN translation to treat C9ORF72-G4C2 repeats in ALS/FTD (Task leader: Poletti UNIMI).**
TRL	**5-7**
	• Optimized siRNA, ASO and Morpholino for their efficacy on PKA-Cbeta subunits. • Identification of positive impacts of the selected hits on RNA translation, the degradative pathways and stress granule formation in patient-derived C9orf72 models of ALS. • Identification of the effects of the best candidate hits on C9-500 BAC transgenic mouse.
Task 3.3.2	**Targeting C9ORF72-G4C2 repeats to mitigate C9orf72 and atxn2 RNA toxicity (Task leader: Corti UNIMI).**
TRL	**5→7**
	• Production and assessment of MO-CPP conjugates targeting C9ORF72 and ATXN2 expansion. • Therapeutic effect of C9orf72 conjugate in vitro in iPSC-Motorneurons and *in vivo* in C9-500 BAC transgenic mouse. • Therapeutic effect of ATXN2 conjugate in vitro in iPSC-Motorneurons and *in vivo* in TDP-43 mouse model.
Task 3.3.3	**Treating SBMA by targeting AUG usage (Task leader: Poletti UNIMI).**
TRL	**5→7**
	• Preparation and screening of libraries of ASOs, ASO-MO, siRNA, and LNA to be screened for their activity to block AUG of ARpolyQ facilitating translation from AUG of AR-A. • Evaluation of the protective activities of AR alternative translation on SBMA-NPC-MNs differentiation, survival and ARpolyQ induced neurotoxicity. • Evaluation of the protective activities of AR alternative translation on KI ARpolyQ mice.
Task 3.3.4	**Treating HD by delivering modifiers of polyQ toxicity (Task leader: Martello UNIPD).**
TRL	**5→7**
	• *in vitro* validations of 5 modifiers of poly(Qs) toxicity in a HD model and in zebrafish. • Test effect of therapeutic genes in human HD-NPCs and in HD-brain organoids including one modifier already vaidated *in vitro* and in 2 mouse models.
Task 3.3.5	**IND filings (Task leader: ALL)**
TRL	**5→7**
	IND filing/approval of at least one therapeutic molecule
Deliverables WP 3.3	D3.3.1 Optimization, preparation and analysis of siRNA, ASO and Morpholino that target the PKA-Cbeta subunits in SH-SY5Y neuronal cells to inhibit RAN translation of (G4C2)n of C9orf72 (C9) ALS patients (M12); D3.3.2 Production of MO-CPP conjugates targeting C9ORF72 and ATXN2 expansion (M12); D3.3.3 Preparation and screening of library of ASO (available from IONIS), ASO-MO, siRNA, and LNA) for their activity to block AUG of ARpolyQ facilitating translation from AUG of AR-A (M12); D.3.3.4 Validation of 4 candidates as suppressors of polyQ toxicity (M12); D3.3.5 Delivery of therapeutic genes by AAV o RNA-based strategies in wild-type NPCs (M12); D3.3.6 Identification of the capability of the selected active hits (siRNA, ASO or Morpholino) to block the formation of the five DPRs in C9-NPC-MNs (M24); D3.3.7 Identification of positive impacts of the selected hits on the degradative pathways and stress granule formation in C9 models of ALS (M24); D3.3.8 Analysis of the effects of the selected active hits on the differentiation potential of the C9-NPC-MNs and iso-NPC-MNs (M24); D3.3.9 Assessments of *in vitro* molecular efficacy of MO-CPP conjugates (M24); D3.3.10 Therapeutic effect of C9 conjugate *in vitro* in iPSC-MNs/CNs (M24); D3.3.11 Assessment of selected hits activity of AR-A expression and ARpolyQ downregulation in motor neurons differentiated from NPCs derived from iPSCs of SBMA patients (SBMA-NPC-MNs) and isogenic control (iso-SBMA- NPC-MNs) (M24); D3.3.12 Test effects of therapeutic genes for poly(Qs) toxicity in human HD-NPCs (M24); D3.3.13 Identification of the effects of the best candidate hits on the levels and activity of the PKA-Cbeta subunits in motor cortex and spinal cord of C9-500 BAC transgenic mouse, on the formation of polyGA and polyGP inclusions, on the neuroinflammatory response and on their behavioural alterations (M36); D3.3.14 Therapeutic effect of C9 conjugate *in vivo* in C9-Bac murine model (M36); D3.3.15 Therapeutic effect of ATXN2 conjugate *in vivo* in TDP43 murine model (M36); D3.3.16 Evaluation of the protective activities of AR alternative translation on SBMA-NPC-MNs differentiation, survival and ARpolyQ induced neurotoxicity (M36); D3.3.17 Evaluation of the protective activities of AR alternative translation on KI ARpolyQ mice (M36); D3.3.18 Test effect of therapeutic genes in human HD-brain organoids (M36).
Milestones WP 3.3	M3.3.1 A set of optimized siRNA, ASO and Morpholino that block RAN translation in cells stably expressing C9RAN GFP(polyGP) reporter vectors (M12); M3.3.2 MO-CPP conjugates targeting C9ORF72 and ATXN2 expansion (M12); M3.3.3 A set of optimized ASOs to modify translation start sites in ARpolyQ (M12); M3.3.4 A list of additional modifiers of Poly(Qs) toxicity (M12); M3.3.5 Blocking RNA translation *in vitro* in C9-NPC-MNs (M24); M3.3.6 Therapeutic effect of C9 conjugate *in vitro* in iPSC-MNs/CNs (M24); M3.3.7 Modifying translation start sites *in vitro* in SBMA-NPC-MNs (M24); M3.3.8 Therapeutic activity for poly(Qs) toxicity of genes in human HD-NPCs (M24); M3.3.9 Proof-of-concept of blocking RNA

	translation in C9-500 BAC transgenic mouse (M36); M3.3.10 Proof-of-concept of MO-CPP conjugates to modify C9orf72 and ATXN2 toxicity (M36); M3.3.11 Proof-of-concept of RNA ability to modify translation start site usage in KI ARpolyQ mice (M36); M3.3.12 Proof-of-concept of a set of modifiers of poly(Qs) toxicity in brain organoids (M36). M3.3.13 IND filing of at least one therapeutic RNA (M36).
WP 3.4 – Neurodevelopmental disorders	
General objectives. We aim to exploit non-coding RNA biology for precision medicine of neurodevelopmental disorders. Given the large heterogeneity of these diseases we will focus on a specific repertory of gene mutations while searching for common dysregulated drug targets and linear and circular RNAs with therapeutic potentials. **Methodology.** We will resort to RNA-seq experiments for profiling small and long RNAs. We will take advantage of *in vitro* and *in vivo* models of neurodevelopmental disorders.	
Task 3.4.1	**Identification of circular and lncRNAs as candidate therapeutic RNAs and drug targets (Task leader: Presutti UNIROMA1)**
TRL	**1→4**
	• Identification of small, long non-coding and circular RNAs in mouse models of ASD. • Preparation of iPSCs from monogenic forms of ASDs and their characterization with –omics approaches to identify drug targets.
Task 3.4.2	**Small, circular and lncRNAs as therapeutics for ASD (Task leader: Presutti UNIROMA1).**
TRL	**3→5**
	• Investigate the use of miRNAs, circRNAs and lncRNAs as theraputics of ASD. • Optimise production of small and long non-coding RNAs with modifications derived from plant RNAs.
Task 3.4.3	**Aptamer-based therapy for neurodevelopmental disorders (Task leader: Ruocco IIT).**
TRL	**5→7**
	Develop and validate an RNA-based drug aptamer to rescue the pathological clustering of the mutated form of MLL4 associated to Kabuki Syndrome.
Task 3.4.4	**lncRNAs as therapeutics of neurodevelopmental epilepsies (Task leader: Barberis IIT).**
TRL	**5→7**
	RNA-based treatment for neurodevelopmental epileptic encephalopathies caused by GABRA1, GPHN and SLC6A1 haploinsufficiency leading to deficient neuronal inhibition.
Task 3.4.5	**IND filings (Task leader: ALL)**
TRL	**5→7**
	IND filing/approval of at least one therapeutic molecule
Deliverables WP 3.4	D3.4.1 Analyze ncRNA de-regulation in mouse models of ASD, select those conserved in human and characterize the molecular pathways in which they are involved to elucidate the link with the pathology (M12); D3.4.2 Complete evaluation of altered miRNA profiles in patient's fibroblasts (M12); D3.4.3 Computational design and validation of an RNA aptamer for MLL4 (M12); D3.4.4 Design and experimental validation *in vitro* of the activities of SINEUP-GABRA1, SINEUP-GPHN and SINEUP-SLC6A1 (M12); D3.4.5 Understanding the role of specific ncRNAs (miRNAs and circ-RNA) in the regulation of gene expression in ASD with a particular attention to the control of the RNA Binding Protein CPEB1 (M24); D3.4.6 Definition of the miRNA-mediated response on the mRNAs coding for epigenetic factors in ASD (M24); D3.4.7 Completed characterization of cellular defective phenotypes of ASD patient's fibroblasts and gene-editing (M24); D3.4.8 IPSCs generation from individuals with monogenic forms of ASD/NDD (M24); D3.4.9 Establishing a novel microfluidic printhead capable of depositing and printing multi-biomaterials and cell types (e.g. induced pluripotent stem cells (iPSCs)-derived neurons and Mesenchymal stem cells (MSCs)) carrying the genetic mutations affecting KS to create unique vascularized nervous tissue substitutes (M24); D3.4.10 Assessment of aptamer ability to rescue the pathological clustering of the mutated form of MLL4 protein associated to KS *in vitro* (M24); D3.4.11 Assess the restoration of GABAergic inhibition with ex-vivo electrophysiology and IHC experiments upon AAV-mediated SINEUP delivery (M24); D3.4.12 Genome editing of iPSCs lines to create isogenic controls from patient-derived cells (M36); D3.4.13 Characterization of iPSCs by -omic approaches (M36); D3.4.14 Effects of miRNAs, circular and lncRNAs on ASD models (M36); D3.4.15 Optimize the production and targeting of these factors using miRNAs and non-coding RNAs as modified in plants (36); D3.4.16 Delivery of the aptamer to KS mouse model and assessment of its therapeutic effects (M36); D3.4.17 Assessment of the rescue of the epileptic phenotype with in-vivo EEG and EMG recordings (M36); D3.4.18 Validation of the human target engagement with electrophysiology and ICC experiments in patient-derived iNeurons (M36).
Milestones WP 3.4	D3.4.1 List of small, circular and non-coding RNAs in ASD (M12); D3.4.2 Aptamers binder of MLL4 (M12); D3.4.3 Validated SINEUP RNAs for GABRA1, GPHN and SLC6A1 (M12); D3.4.4 Set of iPSCs from ASD patients (M24); D3.4.5 *in vitro* system of vascularized nervous system from KS-derived iPSCs (M24); D3.4.6 Electrophysiological data on rescuing GABAergic inhibition with SINEUPs (M24); D3.4.7 Proof-of-concept of the use of small and long ncRNAs as therapeutics of ASD (M36); D3.4.8 Proof-of-concept of phenotype rescue of KS with RNA aptamers (M36); D3.4..9 Optimization of therapeutic RNAs with plant-derived chemical modifications (M36); D3.4.10 Proof-of-concept of phenotype rescue with SINEUP RNAs in neurodevelopmental epileptic encephalopathies (M36). M3.4.11 IND filing of at least one therapeutic RNA (M36).
WP 3.5 – Stroke and other neurological diseases	
General objectives. We will use a combination of miRNAs biology, ASOs, siRNAs, mRNAs, transcriptional repressors and gene therapy for precision medicine of a selection of currently unmet neurological diseases including stroke, idiopathic normal pressure hydrocephalus, hepato-encephalopaties, cerebral and retinal sphingolipidosis and age-related macular degeneration. **Methodology.** We will resort to RNA-seq experiments for profiling small and long RNAs. We will take advantage of in vitro and in vivo models of stroke. In collaboration with Spoke #8 we will use nanoparticles for delivery. We will carry out stereotaxic injections into brain parenchima or into the suretinal space. For preclinical data, we will also use non-human primates.	
Task 3.5.1	**Identification of stroke-related miRNAs (Task leader: Taglialatela UNINA; Toni UNIROMA1).**
TRL	**1→4**
	• Identification of plasma miRNAs, lncRNAs, mRNAs and exosomal RNAs altered in post-stroke (Tagliatela UNINA, Toni UNIROMA1). • Development of a spectrofluorometric and fluorescence-based microgel device for measuring micro-RNAs levels (Tagliatela UNINA).

Task 3.5.2	**RNA-based drugs for the treatment of stroke and stroke-related neurological diseases (Task leader: Taglialatela UNINA).**
TRL	**5→7**
	• Selection and validation of stroke-related miRNAs targeting ion channels and transporters. • Pharmacological strategies to modulate relevant stroke-related targets using miRNAs. • Optimization of the CNS delivery strategies and validation of nanovector-encapsulated miRNAs as therapeutics.
Task 3.5.3	**RNA-based drugs for enhancing endogenous post-stroke human neurogenesis (Task leader: Toni UNIROMA1).**
TRL	**5→7**
	• Validation of therapeutic value of post-stroke RNAs in a cellular model of hypoxia. • Validation of therapeutic value of post-stroke RNAs in animal models.
Task 3.5.4	**Anti-NKCC1 RNA therapy to rescue core symptoms of a mouse model of normal pressure hydrocephalus (Task leader: Cancedda IIT).**
TRL	**5→7**
	In vivo data on the use of an artificial amiR to NKCC1 on a mouse model of pressure hydrocephalus.
Task 3.5.5	**mRNA Therapeutics for the Treatment of Mitochondrial Hepato-Encephalopathies (Task leader: Comi UNIMI).**
TRL	**5→7**
	• To test the therapeutic efficacy of lipid nanoparticles (LNP-) mediated mRNA-delivery of SURF1 (LNP-SURF1) and MPV17 (LNP-MPV17) in patients-derived cellular models. • Optimization of systemic delivery in wild-type animals followed by rescue of MPV17 KO mice phenotype by LNP-MPV17. • Optimization of in utero fetal delivery of LNP-SURF1 in a large animal model.
Task 3.5.6	**Gene editing to treat Spastic Paraplegia 9 (Task leader: Bonora UNIBO).**
TRL	**1→4**
	• Development of a mouse model of Spastic Paraplegia 9. • Gene editing to treat Spastic Paraplegia 9.
Task 3.5.7	**Develop synthetic transcriptional repressors to treat cerebral and retinal sphingolipidosis (Task leader: Surace UNINA)**
TRL	**5→7**
	• Development of transcriptional repressors (TRs) targeting sphingolipids biosynthesis genes. • *In vivo* validation of TRs targeting sphingolipids biosynthesis genes in mouse models and NHPs. • Phenotypical and genotypical characterization of patients with Tay-Sachs and Sandhoff diseases.
Task 3.5.8	**Gene therapy to inhibit pathological neovascularization in age-related macular degeneration (Task leader: De Falco CNR)**
TRL	**5→7**
	• Validation of CRISPR-CAS9 technology to knocking out a PlGF variant, named PlGF-DE, through a single subretinal injection using lipid nanoparticles carrying mRNA for CRIPRS-CAS-9 system and donor DNA.
Task 3.5.9	**IND filings (Task leader: ALL)**
TRL	**5→7**
	IND filing/approval of at least one therapeutic molecule
Deliverables WP 3.5	D3.5.1 Analysis of plasmatic levels of miRNAs (miRNAs)/exosomal miRNAs i.e. miR17~92 family and biomarkers of neurogenesis/axonogenesis i.e. Netrin-1 and Semaphorine 3A (Sema3A) in human brain at different ages of development (preterm newborns, children and adults) (M12); D3.5.2 Identification of miRNAs modulated in adult animal models of stroke (permanent middle cerebral artery occlusion, pMCAO; transient middle cerebral artery occlusion, tMCAO; haemorragic stroke, HS) (M12); D3.5.3 Design and synthesis of CNS-permeable nanovectors (M12); D3.5.4 Design and *in vitro* validation of an artificial microRNAs (amiRs)) to downregulate endogenous levels of NKCC1 (M12); D3.5.5 Optimization of delivery lipid nanoparticles (LNPs) mediated mRNA-delivery of SURF1 (LNP-SURF1) and MPV17 (LNP-MPV17) in patients-derived cellular models (M12); D3.5.6 Development of TRs targeting the cis-regulatory sequences in the promoter regions of the sphingolipids biosynthesis genes (M12); D3.5.7 Design of the components for CRISPR CAS9 PlGF-DE editing with at least one L-NPs composition for subretinal delivery (M12); D3.5.8 Development of a spectrofluorometric and fluorescence-based microgel device for measuring micro-RNAs levels (M24); D3.5.9 Synthesis and validation of miRNA-based nanovectors *in vitro* and *in vivo* models (M24); D3.5.10 Analysis of the effects of miRs mimics or miRs locked nucleic acid (LNA) inhibitors and antagomirs in NPCs developed from human iPSC and in neuroblastoma cell lines (M24); D3.5.11 Optimization of systemic delivery in wild-type animals addressing liver targeting, pharmacokinetics, toxicity followed by rescue of MPV17 KO mice phenotype by LNP-MPV17 (M24); D3.5.12 Optimization of *in utero* fetal delivery of LNP-SURF1 in a large animal model: demonstration of therapeutic efficacy (M24); D3.5.13 SPG9 KI mouse behavioral/phenotype and molecular analysis (M24); D3.5.14 *In vivo* selection of TRs by AAV (AAV-TRs) retinal gene transfer in wild-type pigs (M24); D3.5.15 *In vivo* validation of TRs by CNS-directed AAV gene transfer in SD mouse model (M24); D3.5.16 Validation of L-NPs -PlGF-DE gene therapy approach *in vivo* (M24); D3.5.17 Evaluation of miRNA-based nanovectors in relevant *in vitro* and *in vivo* disease models (M36). D3.5.18 Data on the role of the selected miRNAs in post-stroke brain repairing *in vivo,* at different stages of development by using the middle cerebral artery occlusion mouse model of focal ischemia (M36); D3.5.19 Assessment of rescue of core behavioral symptoms in a mouse model of iNPH (M36); D3.5.20 SPG9 KI mouse gene editing test and mouse behavioural/phenotype analysis of treated SPG9 KI (M36); D3.5.21 *In vivo* AAV-TRs doses escalation assessments by CNS-directed AAV gene transfer in pigs (M36); D3.5.22 One-month pilot toxicity study of AAV-TRs in Monkeys (M36); D3.5.23 Clinic, laboratory, and instrumental evaluation of children affected by TSD and SD defining a genotype-phenotype correlation to establish clinical endpoints and design a novel therapeutic protocol to approach a clinical trial (M36); D3.5.24 Validation of L-NPs -PlGF-DE gene therapy approach in preclinical model of age-related macular degeneration (M36).
Milestones WP 3.5	M3.5.1 List of miRNAs modulated in human plasma and in animal models of stroke (M12); M3.5.2 Optimization of CNS-permeable nanovectors (M12); M3.5.3 An artificial microRNAs (amiRs)) to downregulate endogenous levels of NKCC1 (M12); M3.5.4 Lipid nanoparticles (LNPs) for mRNA-delivery of SURF1 and MPV17 (M12); M3.5.5 TRs targeting the promoter

	regions of the sphingolipids biosynthesis genes (M12); M3.5.6 A spectrofluorometric and fluorescence-based microgel device for measuring micro-RNAs levels (M24); M3.5.7 miRNA-based nanovectors for in *in vitro* and *in vivo* applications (M24); M3.5.8 Delivery *in utero* of LNP-SURF1 in a large animal model (M24); M3.5.9 SPG9 KI mice (M24); M3.5.10 CNS-directed AAV gene transfer of TRs in SD mouse model (M24); M3.5.11 L-NPs -PlGF-DE for gene therapy *in vivo* (M24); M3.5.12 Proof-of-concept of manipulating miRNAs in stroke in *in vivo* models (M36); M3.5.13 Proof-of-concept of manipulating miRNAs to enhance neurogenesis (M36); M3.5.14 Proof-of-concept of amiR effects on pressure hydrocephalus (M36); M3.5.15 Proof-of-concept of mRNA effects in mitochondrial hepato-encephalopaties (M36); M3.5.16 Proof-of-concept of gene editing to treat Spastic Paraplegia 9 (M36); M3.5.17 Proof-of-concept of gene therapy of sphingolipidoses (M36); M3.5.18 Proof-of-concept to treat age-related macular degeneration (M36). M3.5.19 IND filing of at least one therapeutic RNA (M36).

Spoke #4: Metabolic and cardiovascular diseases. Spoke Leader: UNIPD.

State of the field and unmet needs. Among chronic non-communicable diseases, muscular, metabolic and cardiovascular disorders pose major threats to global health and account for a large proportion of avoidable deaths. Worldwide, 1 in 2 individuals are overweight or obese, with consequent risk for diabetes, hypertension, dyslipidemia, and fatty liver disease, ultimately leading to atherosclerosis, ischemic heart disease, heart failure, and death. Muscle loss/atrophy and weakness occur in many diseases like cancer, diabetes, cardiac and renal failure, unhealthy aging, and infections, causing respiratory insufficiency, loss of independency and metabolic perturbations that ultimately, reduce the quality of life and increase morbidity and mortality. Aging is the primary risk factor for most chronic diseases driving both morbidity and mortality, e.g. diabetes, cancer, cardiovascular diseases, atherosclerosis, dementia and, as mentioned above, sarcopenia. These diseases, in their heterogeneity, share common pathogenic mechanisms, including adaptation to stress, loss of proteostasis, stem cell exhaustion, metabolism derangement, macromolecular damage, epigenetic modifications, and inflammation. Given the high prevalence, these diseases also account worldwide for a high proportion of the pharmaceutical expenditure. Cardio-metabolic and muscle disorders are responsible for a large quantity of avoidable deaths and continue to represent a primary focus of pharmaceutical research and development. Patients with cardio-metabolic diseases exhibit huge variability in clinical manifestations and their response to therapy and outcome. This is because most available treatments tackle the final clinical manifestations, while the multifactorial disease pathogenesis still lacks targeted drugs. The systemic cross talk between dysmetabolism and the final cardiovascular consequences are being elucidated. Scientists at partner Institutions within the Spoke have uncovered molecular targets to interrupt the accelerated aging cascades leading to cardio-metabolic disease and the underlying inflammation elicited by hemodynamic and metabolic stressors.

The Spoke addresses this need, by identifying novel targets that can become druggable with the RNA technology and developing a variety of new drugs allowing personalized treatments of muscular, cardiovascular and metabolic diseases.

How the Spoke will contribute to advance in this scenario. The game-changing opportunity provided by existing RNA technologies relies on the possibility to manipulate virtually any cellular process through the design of RNA antagonists (short hairpin, short interfering, antago-miRs) or agonists (mRNA or miRNA). As a result, identification of specific molecular mechanisms of disease inherently implies the opportunity to manipulate the respective coding or regulating RNAs. In the field of metabolic and cardiovascular disease, pharma companies have already developed dedicated pipelines exploiting RNA technologies. The team holds a portfolio of patents for leveraging the technological readiness level (TRL) of flagship proposals from conceptualization to first-in-human. Biotech companies are actively onboarding this roadmap to gene/RNA-based solutions for cardio-metabolic diseases. In the various thematic areas, top-tier projects currently at TRL 4-5 will lead validated targets to complete *in vitro* and in animal testing, reaching the stage of pilot production for in vivo use. In parallel, a pipeline of "TRL advancement projects" will be focused on targets that are well defined in pathogenesis but require validation as potential drug. Here, we present a pipeline to advance highly specific molecular therapies that target the pathogenic roots of cardio-metabolic and muscular disorders with gene/RNA-based technologies. The short-term aim is to advance the TRL of projects already focused on solid and validated molecular targets, with the highest probability of succeeding as a therapy. In parallel, the Spoke will nourish projects in an earlier development stage, selecting those that will show promising progress along the pipeline, for subsequent developments. On the long run, capitalizing on the success of flagship drug proposals, the Spoke will establish as a national and international reference for advanced therapies in the cardio-metabolic field, strengthen the relationships with industries, and maintain a wide R&D portfolio. The Spoke includes three main WPs wherein projects and tasks are grouped by therapeutic area.

WP4.1 will cope with the notion that a disproportionate number of people who reach old age (≈80% of people aged ≥80 years) suffer from cardiovascular and metabolic diseases, cellular and cognitive decline, cancer, sarcopenia and frailty. By using sh/siRNAs and miRNAs we have identified a subset of targets that, when inhibited in mouse muscles, result in recovery of mass, force and mitochondrial function/metabolism in different catabolic conditions such as cancer, immobilization, loss of innervation, diabetes/obesity, and aging. We reached this important goal by using local RNA-based therapy on single muscles. Therefore, we plan to extend our findings to whole body muscles to induce systemic improvement of muscle mass, exercise tolerance, and metabolism. Next, we will identify the best cocktail of RNAs and thus of targets that will preserve muscle function in each catabolic condition. We will target, either alone or in combination, a selection of genes involved in protein degradation (the muscle-specific E3 ligase MuRF1, the transcription factors FoxO1 and 3 and the autophagy factor Bnip3), in anabolism (muscle-specific mIGF-1), and in mitochondrial function and metabolism (players of mitochondrial energy production PDK1-4, MICU2 and miR-1, and the myokines FGF21 and

GDF15).We will target miR-206 to develop therapies against cardio-metabolic features of genetic myopathies. The longevity associated variant (LAV-) in BPI fold containing family B member 4 (BPIFB4) has proved strategic to cope with aging-related events, mainly cardiovascular ones and immune dysfunction achieving both an atheroprotective effect in ATS preclinical model and reducing the IS burden in aged mice. Thus, we will overexpress LAV-BPIFB4 mRNA using endothelial and mono-macrophage-targeted nanoliposomes (NLs-LAV) to restore healthy ageing and cardiovascular homeostasis. In aging and senescence-associated diseases, accumulation of senescent cells induces chronic inflammation and impairs tissue functions, and their clearance can delay features of aging and aging related diseases. FOXO4 is essential in senescent cell viability but is dispensable in non-senescent cells. Therapeutic targeting of senescent cells can restore tissue homeostasis in multiple conditions, such as muscular atrophy and sarcopenia in the elderly, degenerative diseases, pulmonary fibrosis, atherosclerosis, hepatic steatosis, type 2 diabetes, osteoarthritis and many others. The possibility to target FOXO4 via ASO/siRNA delivery opens a treatment option that has many advantages in comparison with the state of the art.

WP4.2 will address the outstanding issues for a modern molecular approach against obesity, type 2 diabetes, and their associated metabolic abnormalities and end-organ complications. Adipose tissue remodelling and inflammation is a major driver of obesity-associated insulin resistance and cardiovascular diseases. Tackling the dysfunctional adipose tissue will avoid the progression towards the complications of metabolic diseases. To pursue this task, we will target the immune cell receptor CD300, the mitochondrial calcium uniporter (MCU) and the ZMAT3/Zfp423 pathway. In addition to insulin resistance driven by adipose tissue inflammation, type 2 diabetes is due to beta-cell failure. As a strategy to prevent diabetes development and progression, we will silence p66Shc mRNA, encoding a redox protein mediating lipotoxicity-induced beta-cell dysfunction and death, specifically in beta cells. Hypertension, the most common among metabolic syndrome components, is characterized by hyperaldosteronism, with 6-20% of patients having primary aldosteronism. Since antagonizing the mineralocorticoid receptor has drawbacks, we propose to block the aldosterone synthase gene by siRNA. Worldwide, with eradication of viral hepatitis, dysmetabolic steatohepatitis is becoming the major cause of liver fibrosis and hepatocellular carcinoma. To address this unmet need, we will work on i) optimising hepatic lipidome remodelling to reduce lipotoxicity and preventing disease progression by over-expressing PPARGC1B in the liver; ii) delivering extra-cellular vesicles loaded with an anti-fibrotic cargo of miRs to the liver; iii) capitalizing on the known interplay between the main PNPLA3 I148M variant and oestrogen levels in promoting fatty liver in females and silencing PNPLA3 and HSD17B13. Furthermore, liver metabolism is recognized as a bridge to systemic complications of the metabolic syndrome. Individuals with loss-of-function mutations in ATP-citrate lyase (ACLY), which is involved in lipid synthesis and immune cell function, are protected against cardiometabolic disorders. Therefore, we will test ACLY siRNA in the development of obesity and atherosclerosis. In established diabetes, meta-inflammation contributes to complications and is sustained by Oncostatin M (OSM), that fuels pro-inflammatory cells and compromises endogenous stem cells. We will pursue OSM silencing as a strategy to restore a normal response to ischemia, which is altered by diabetes, and prevent atherosclerosis progression, which is accelerated by hyperglycemia. Diabetic nephropathy, which occurs in up to 40% of patients, remains a major cause of end-stage renal disease. To tackle the newly-discovered pathway of lysine 63 ubiquitination promoting diabetic nephropathy, we propose to silence UBE2v1 and to overexpress regulating miRs (27b-3p and 1228-3p). Finally, a series of miRs have been identified as mediators of perivascular fibrosis associated with metabolic diseases, as well as atherosclerosis development and progression. Thus, we aim to exploit miR-214 and miR-21 antagonism as a viable tool to control metabolic syndrome and its complications.

In WP4.3, the overall effort is to bring a number of targets and sophisticated methodologies identified or set up by the investigators as close as possible to clinical application. Myocardial diseases include a number of different pathologies unchained either by intrinsic, often inherited, defects of the cardiomyocyte, the contractile cellular unit of the myocardium, or by extrinsic noxae, as is the case of myocardial infarction due to coronary atherosclerosis or myocardial fibrosis following uncontrolled high blood pressure. In all cases, myocardial tissue undergoes a profound remodelling, during which the contractile tissue area is decreased and substituted by fibrotic, non-functional tissue, usually after tissue inflammation. During the last decade, it has been possible to study cardiac biology at an unprecedented level. The progress in DNA sequencing technology has allowed the identification of new RNA molecules which play a critical role in many aspects of cell biology or to study myocardial cellularity at a single-cell level. Similarly, advances in genomic reprogramming have allowed the generation of human disease models “in a dish”, through manipulation of patient-specific induced pluripotent stem cells. RNA has emerged as a vast field of study from which therapeutic opportunities could stem. WP2 aims, therefore, at exploiting RNA and gene therapy approaches for curing myocardial diseases as well as using cellular reprogramming for establishing models of myocardial disease or for cardiac cell replacement therapy. Cardiac organoids, thus 3D cultures, will be used for studying diabetic cardiomyopathy as well as a step-up, compared to 2D cultures, for studying cardiac inherited disorders. Starting from genetic diseases affecting cardiac rhythm and inotropism due to mutations of genes involved in excitation–contraction coupling or cell–cell tethering (LQT8, RYR2, LMNA, DSG2, PKP2, DSP), investigators of this WP have generated, or are in the process of, generating mouse or larger animal models of cardiac disorders, to be used for RNA allele-specific down-regulation of gene expression or for reconverting recombination animal models of cardiac diseases to

normal conditions through homologous recombination, before entering the clinical arena. Cardiac replacement therapy will be tested by stimulating cell cycle with microRNAs; microRNAs will be also tested for their capacity to favour homologous recombination in genetic models of cardiomyopathy and for silencing mutations causing arrhythmogenic cardiomyopathy. Modified RNAs will be used *in vitro* and *in vivo* for cardiogenesis, stimulating myocardial cell replacement therapy *ex vivo* and *in vivo*. The RNAi approach will be used for down-regulating the expression of genes critical during cardiac hypertrophy and failure, including urokinase-type plasminogen activator (uTPA), which is involved in transthyretin (TTR) cleavage and amyloidosis. An RNAi approach will be employed for stimulating angiogenesis *in vivo*. Similarly, the expression of non-coding RNAs significantly regulating cardiac function will be modulated by modRNA or an antisense approach. Modified RNAs will be used for modulating myocardial inflammation, a critical component of the remodelling process. New tools for targeting gene expression selectively in cardiac cells and for cardiomyocyte-selective homologous recombination will be generated, focusing in particular on lipid nanoparticles and new vectors for cardiac-specific gene expression.

While Spoke's projects at high baseline TRL are considered to be more ready for advancing in the clinical development of a RNA-based drug, more immature lower-TRL projects will take advantage from the collaboration with other vertical Spokes and from the cross-contamination of fundamental research within different areas of investigation and different therapeutic areas. For example, several biological pathways and cellular processes controlled by candidate RNAs are common to aging/metabolism (**Spoke #4**), cancer (**Spoke #2**) and inflammation (**Spoke #5**). Therefore, results of fundamental research within these lower-TRL projects will be shared among the various **vertical Spokes**. In fact, a given RNA target initially developed to tackle inflammation may turn out to be primarily involved in the regulation of metabolism or vice-versa. Similarly, targets initially included in **Spoke #4** may end up demonstrating promising results in the fight against cancer and its related conditions (*e.g.*cancer-induced cachexia). Thus, we envisage a strong exchange of information on the progresses of Spoke's #4 molecular targets, which may be of interest to **other Spokes**.

The strategic collaboration with **AstraZeneca** and **Stevanato** will enable the Spoke enable the Spoke to identify opportunities for the transfer of knowledge from the Academia to industries and develop applicable and scalable solutions. AstraZeneca maintains an entire R&D line dedicated to the CaReMe (cardio-renal-metabolic) philosophy, addressing unmet medical needs to create a seamless disease management pathway for millions of patients worldwide. AstraZeneca's pipeline in this area includes drugs for cardiovascular diseases and heart failure, chronic kidney disease, and metabolism. As such, AstraZeneca's commitment and vision are perfectly aligned and integrated with the aims of **Spoke #4**. Researchers within the Spoke will keep close contacts with AstraZeneca's R&D department and are actively partnering for developing RNA-based drugs within the CaReMe area. Stevanato is pioneering clinical-grade containment solutions for storing, delivering, and administering RNA-based drugs, by helping mitigate challenges posed by RNA technologies. Common challenges with RNA-based drugs that **Spoke #4** will face include issues due to RNA solutions being highly sensitive and requiring low-temperature storage. Stevanato's Analytical Services team investigates the suitability of containers at freezing conditions with regards to integrity, functionality, and performance. Further, Stevanato is developing prototype advanced injectors and wearables for customized delivery of parenteral drugs in the diabetes care area that would well suite need of **Spoke #4**.

WP 4.1 – Muscle atrophy and aging	
General objectives. WP1 will pursue the objective of tacking muscle atrophy, sarcopenia, and to develop therapies against aging-associated cardiovascular diseases and cardio-metabolic features of genetic myopathies **Methodology.** The spoke will take advantage of the Spokes' multi-Institution discovery platform relying on technological innovation and strong translational approach (cell-tissue-animal-human), to bring to light new targets of RNA-based drugs. We herein selected top-tier projects of drug development with a base of TRL4-5 and great advancement potential to TRL6-7. Availability of a set of diversified targets at different base TRL builds an ideal pipeline for the competitive advance to a new RNA-based therapy.	
Task 4.1.1	**AtroHit_RNA-based therapy to preserve muscle function and metabolism in disease and aging (Task leader: UNIPD-Sandri)**
TRL	**5→6**
	• Definition of atrophy-specific shRNA cocktails. In vivo skeletal-muscle specific delivery strategy. • Tailored RNA-based treatment for several myopathies.
Deliverables WP 4.1.1	D1 (Type Data) Generation and in vitro optimization of shRNA oligos-M3; D2 (Type Data) In vivo muscle delivery and validation of shRNA cocktails to treat atrophy caused by immobilization-M6; D3 (Type Data) In vivo muscle delivery and validation of shRNA cocktails to treat denervation atrophy-M9; D4 (Type Data) In vivo muscle delivery and validation of shRNA cocktails to treat cancer cachexia-induced atrophy-M12; D5 (Type Data) In vivo muscle delivery and validation of shRNA cocktails to treat diabetes-induced atrophy-M15; D6 (Type Data) In vivo muscle delivery and validation of shRNA cocktails to treat aging sarcopenia-M18; D7 (Type Data) Optimization of AAV-shRNA systemic delivery to specifically target skeletal muscle-M21; D8 (Type Data) Optimization of shRNA oligos systemic delivery via biological vesicles to specifically target skeletal muscle-M24; D9 (Type Data) delivery of cancer cachexia-targeted shRNA cocktail-M28; D1.0 (Type Data) Systemic delivery of diabetes-targeted shRNA cocktail-M32; D1.1 (Type Data) Systemic delivery of aging sarcopenia-targeted shRNA cocktail-M36
Milestones WP 4.1.1	MS1. shRNA oligos suitable for silencing of target genes-M3; MS2. A suitable shRNA cocktail to treat disuse atrophy-M9; MS3. A suitable shRNA cocktail to treat systemic atrophy-M15; MS4. A suitable shRNA cocktail to treat sarcopenia-M18; MS5. Skeletal muscle-specific shRNA cocktail delivery system-M21; MS6. A treatment for systemic atrophy for human use is available-M32; MS7. A treatment for aging sarcopenia for human use is available-M36
Task 4.1.2	**NLs-LAV- Endothelial and macrophage-targeted LAV-BPIFB4 mRNA overexpressing nanoliposomes (NLs) as a novel tool for cardiovascular therapy in elderly (Task leader: Puca, Vecchione-UNISA,)**
TRL	**3-7**

	• Validate a lab grade and biocompatible LAV-BPIFB4 mRNA loaded nanoliposomes system (formulation and design, fabrication parameters optimization and functional characterization of the cellular effects on endothelial and macrophage compartment.) • Confirm the delivery efficiency and monitor mRNA activity inside the cellular target. • Prove the efficacy of LAV-BPIFB4 mRNA loaded NLs system to prevent endothelial dysfunction and immuno-metabolic deleterious changes in: a) 26 months aged mice, b) ApoE knockout mice under HFD and in c) Göttingen Minipigs under 22-weeks of HFD for crosspecies validation. • Evaluate the in vivo modulated pathways by metabo/lipidomics analyses • Identify the most suitable therapeutic indication based on short and long-term efficacy/tolerability profile • Devise a phase I safety / efficacy clinical trial
Deliverables WP 4.1.2	D1 (Type R) Report functionality and biocompatibility of LAV-BPIFB4 mRNA loaded-NLs-M7; D2 (Type R) Report of functionalization of LAV-BPIFB4 mRNA loaded nanoliposomes for targeted delivery-M11; D3 (Type R) Report of efficacy in ApoE knockout mice under HFD-M17; D4 (Type R) Report of efficacy in Göttingen Minipigs-M18; D5 (Type R) Report of efficacy against immunesenescence and age-related changes in old-M19; D6 (Type Data) Metabo/lipidomics analyses of all above mentioned pre-clinical models-M20; D7. Analysis of undesired effects / toxicology-M30; D8. Design and prepare first in human clinical trial-M36
Milestones WP 4.1.2	MS1. A LAV-BPIFB4 mRNA suitable for human use is available-M8; MS2. A specific endothelial and mono-macrophage directed delivery is available-M12; MS3. Animal Ethics Committee authorization forAnimal Experimentation-M15; MS4. A multi-omic profile of in vivo models is provided-M20; MS5. Choose the most suitable indication for human age-related condition-M24; MS6. A safety profile is available in mice-M30; MS6. A clinical trial is ready-M36.
Task 4.1.3	**SUnFox Treatment of aging-associated diseases and senescence-associated diseases by inducing the apoptotic death of the senescent cells knocking down FOXO4 via systemic and local ASO/siRNA delivery: a novel senolytic approach (Task leader: Arancio-Ri.MED)**
TRL	**3→6**
	• Selection and validation of required ASO an/or siRNA from literature and de novo developed by specific in silico design • Design, synthesis and optimization of carriers and delivery protocol to study the effects of co-administration of canonical therapies in order to obtain synergic effects • Adapt the approach to treat respiratory and integumental systems • Preclinical validation in mouse models • Target organs and biodistribution can be evaluated thanks to non-invasive imaging techniques
Deliverables WP 4.1.3	D1 (Type:R. and Data); ASO and siRNA identification-M3; D2 (Type:R.) Carrier definition-M6; D3.(Type: R.) In vitro studies of co-administration of canonical drugs: identifying synergic molecules-M15; D4 (Type: R.) A report on the effect on other systems (e.g. respiratory and integumental systems)-M24; D5 (Type: R.) efficacy to slow muscular loss in mice-M36; D6 (Type: R.) analysis of undesired effects/toxicology-M36.
Milestones WP 4.1.3	MS1. In vitro study of the effects in knocking down FOXO4-M3; MS2. In vitro studies on cell vitality, toxicity, time and dose dependencies, and kinetic of the compound-M6; MS3. In vitro studies on synergic effect with commercial drugs and miRNA mimic/inhibitors-:M15; MS4. In vitro studies on other cellular models-:M24; MS5. Histological, transcriptomic and toxicology studies in mice. Analyses of desired and side effects relevant to human disease-:M26; MS6. Validation of safety studies are accomplished-M36
Task 4.1.4	**MiRHIT_Develop therapies against cardio-metabolic features of genetic myopathies (miR-206) (Task leader: Sandri, Zaglia-UNIPD-)**
TRL	**5→6**
	• MiR206 pattern of localization Cardiac and Sympatheitic system characterization after MiR206 delivery • Tailored antago-mir-206 treatment in diseases with high miR206 plasma level
Deliverables WP 4.1.4	D1 (Type Data) monitoring pattern of miR206 levels in plasma and tissues of autophagy deficient (ATG7 KO) and sarcopenic mice-M3; D2 (Type Data) monitoring pattern of miR206 levels in plasma and tissues of SOD1.G93A and mdx mice-M6; D3 (Type Data) monitoring pattern of miR206 levels in plasma of sarcopenic, dystrophin-deficient and ALS patients-M9; D4 (Type Data) monitoring pattern of miR206 levels in plasma and tissues of GAA KO mice-M12; D5 (Type Data) In vivo plasma delivery of miR206-:M18; D6 (Type Data) Generation and in vitro/in vivo optimization of AntagoMir206-:M24; D7 (Type Data) Treatment and characterization of sarcopenic mice-M28; D8 (Type Data) Treatment and characterization of SOD1.G93A mice-M32; D9 (Type Data) Treatment and characterization of GAA KO mice-M36
Milestones WP 4.1.4	MS1.MiR206 expression pattern in unhealthy ageing-M3; MS2.MiR206 expression patter in animal model of ALS and dystrophin deficient mice-M6; MS3.MiR206 plasma levels in patients-M9; MS4.MiR206 expression patter in animal model of Pompe disease-M12; MS5.Cardiac function and innervation. Skeletal muscle mass and innervation-M18; MS6.Blocking miR206-mediated effect on: 1) cardiac function and innervation and 2) Skeletal muscle mass and innervation-M24; MS7.Cardiac morphology/function and Skeletal muscle force in dystrophin deficient mice-M28; MS8.Cardiac morphology/function and Skeletal muscle force in SOD1.G93A mice-M32; MS9.Cardiac morphology/function and Skeletal muscle force in GAA KO mice-M36.
Milestones WP 4.2 Obesity, metabolic syndrome, type 2 diabetes.	
General objectives. WP2 will address the outstanding issues for a modern molecular approach against obesity, type 2 diabetes and the metabolic syndrome. **Methodology:** The spoke will take advantage of the Spokes' multi-Institution discovery platform relying on technological innovation and strong translational approach (cell-tissue-animal-human), to bring to light new targets of RNA-based drugs. We herein selected top-tier projects of drug development with a base of TRL4-5 and great advancement potential to TRL6-7. Availability of a set of diversified targets at different base TRL builds an ideal pipeline for the competitive advance to a new RNA-based therapy	
Task 4.2.1	**SiOSM:Silencing Oncostatin M in bone marrow macrophages as an anti-inflammatory pro-regenerative therapy for diabetic vascular disease (Task Leader: Fadini-UNIPD)**
TRL	**4→7**
	• Validate an OSM-RNA silencing therapy to block macrophage derived inflammation in the bone marrow.

	• Confirm efficacy against diabetic vascular disease, including acute (response to ischemia) and chronic manifestations (atherosclerosis progression). • Identify the most suitable therapeutic indication based on short and long-term efficacy/tolerability profile Devise a phase I safety / efficacy clinical trial
Deliverables WP 4.2.1	D1. Develop clinical-grade anti-OSM siRNA-M3; D2. Develop bisphosphonate loaded-liposomes with anti-OSM siRNA-M6; D3. Efficacy against meta-inflammation in mice-M12; D4. Efficacy to improve response to ischemia in mice-M15; D5. Efficacy to slow atherosclerosis in mice-M21; D6. Analysis of undesired effects / toxicology-M24; D7. Design and prepare first in human clinical trial-M36.
Milestones WP 4.2.1	MS1. An ASO suitable for human use is available-M3; MS2. A bone marrow directed delivery is available-M6; MS3. Choose the most suitable indication for human disease-M21; MS4. A safety profile is available in mice-M24; MS5. A clinical trial is ready-M36.
Task 4.2.2	**ASSET A sex-specific approach to NAFLD targeting (Task Leader: Valenti-UNIMI)**
TRL	**4→7**
	• Validate RNA silencing approach against PNPLA3/HSD1.7B13 to block lipid deposition and inflammation in the liver under a lipogenic diet • Confirm efficacy against fatty liver, NAFLD and NASH in mouse models (wt and Pnpla3-I148M KI and human liver organoids) • Identify the most suitable therapeutic indication based on short and long-term efficacy/tolerability profile • Devise a phase I safety / efficacy clinical trial
Deliverables WP 4.2.2	D1. Develop clinical-grade anti-PNPLA3/HSD1.7B13 siRNA-M4; D2. Develop galactose-conjugated liposome nano-particles (Gal-LipoNP) bearing siRNA -M8; D3. Efficacy against fatty liver development and progression in male and female mice (wt and Pnpla3 I148-Knock In) and in human liver organoids and assembloids. -M16; D4. Efficacy to limit liver inflammation-M20; D5. Efficacy to prevent primary liver cancer (hepatocellular carcinoma) under Western diet-M24; D6. Analysis of undesired effects / toxicology-M24; D7. Design and prepare ex vivo testing during perfusion in marginal organs discarded for transplantation due to excess fat accumulation and of the first in human clinical trial (cascading grant with hospital)-M36.
Milestones WP 4.2.2	MS1. An ASO suitable for human use is available-M8; MS2. A liver directed delivery is available-M12; MS3. Choose the most suitable indication for human disease-M24; MS4. A safety profile is available in mice-M24; MS5. A clinical trial is ready-M36.
Task 4.2.3	**HALIFIB: Hyaluronic acid-based delivery of extracellular vesicles for the treatment of liver fibrosis (Task Leader: Chinnici-RIMED)**
TRL	**4→7**
	• Isolation of MSC EVs and selection of anti-fibrotic miRNAs (either based on previous works or newly identified by in silico analysis); • Isolation of EV from engineered MSCs overexpressing one or more anti-fibrotic miRNAs; testing the in vitro anti-fibrotic effect of both EVs and EV-eng (LX-2 cell lines) • Computational analysis to select the best biomaterial (HA derivative) properly interacting with HA receptor CD44, which is overexpressed in activated HSCs during fibrosis; • Production of HA-coated EVs Clinical-grade production of "free" EVs and HA-coated EVs Safety and efficacy of EV-based therapy in a mouse model of hepatic fibrosis (NAFLD) To launch phase I clinical trials
Deliverables WP 4.2.3	D1. Production of EVs and EV-eng from MSCs of different sources; in vitro efficacy of EV-based treatment -M3; D2. Identification of the most suitable biomaterial for coating; Production of HA-coated EVs; comparing the efficacy of uncoated vs HA-coated EVs in vitro-M9; D3. Production of clinical-grade EVs; in vitro efficacy of clinical-grade EVs -M15; D4. Safety and efficacy of EV-based therapy in a mouse model of hepatic fibrosis -M25; D5. Designing phase I clinical trials-M36.
Milestones WP 4.2.3	MS1. Identification of the best EV product-M3; MS2. Releasing HA-coated EVs with an improved efficacy -M9; MS3. Releasing clinical-grade Evs-M15; MS4. Validated safety and efficacy of HA-based treatment -M25; MS5. Ready to launch clinical trials-M36.
Task 4.2.4	**MORDRED miRNA antagonists for the pRevention anD tReatment of mEtabolic Diseases. (Task Leader: Lavecchia-UNINA)**
TRL	**2→4**
	• Assess the efficacy of miR-214 and 21 antagonists in 2D and 3D in vitro models of metabolic syndrome and cardiovascular disease Validate miR-214 and 21 antagonism for the control of hypertension and hyperlipidaemia in murine models (including models of diet-induced atherosclerosis and AngII-dependent hypertension) • Evaluate changes at the mechanistic levels by using state-of-the-art technologies such as single-cells RNA sequencing, cytometry by tome of flight, and multiphoton microscopy real-time imaging • Identify the most suitable therapeutic indication based on short and long-term efficacy/tolerability profile
Deliverables WP 4.2.4	D1. report on the development of clinical-grade miR-214 and miR-21 antagonists -M6; D2 report on the formulation of a lipid-based delivery system for miR-214 and miR-21 antagonists-M12; D3.report on the efficacy of miR-214 and miR-21 antagonism in vivo-M24; D4.report on the safety/toxicity of developed miR antagonists-M36
Milestones WP 4.2.4	MS1 miR-214 and miR-21 antagonists suitable for human use are available-M6; MS2 a delivery platform for antimiRs is available-M12; MS3 Efficacy of miR-214 and miR-21 antagonists in mouse models-M24; MS4 A safety profile is available in mice-M36
Task 4.2.5	**CMCU Silencing CD300e and MCU in myeloid and adipose tissue as an anti-inflammatory and insulin-sensitivity restoring therapy for insulin-resistance patients (Task Leader: Vettor-UNIPD)**
TRL	**3→5**
	• Hijacking the insulin cascade and inflammation by CD300e and MCU: • definition of the pathway(s) in insulin-targeted cells Characterization of CD300e and MCU total KO mice in terms of insulin sensitivity and inflammation (in CFD and HFD) • Characterization of conditional CD300e and MCU KO mice in adipose and myeloid compartment in terms of insulin sensitivity and inflammation (in CFD and HFD) • Design of the most suitable therapeutic approach based on CD300e- and MCU-targeting RNA and validation of the efficacy of silencing (in macrophages and adipocytes) in vitro, in terms of restoring the insulin pathway

	• Validation of the efficacy of CD300e and MCU silencing (in adipose and myeloid compartment) in a murine model of insulin resistance
Deliverables WP 4.2.5	D1. Characterization of the impact of CD300e on insulin pathway-M3; D2. Characterization of the metabolic and inflammatory profile of adipocytes upon MCU silencing -M3; D3. Characterization of the metabolic and inflammatory profile of macrophages upon MCU silencing -M6; D4. Characterization of CD300e total KO mice-M12; D5. Characterization of the metabolic and inflammatory profile of MCU+/- mice.-M12; D6. Characterization of conditional CD300e KO mice in adipose and myeloid compartment-M24; D7. Characterization of conditional MCU KO mice in adipose and myeloid compartment-M24; D8. Design of the most suitable therapeutic approach based on CD300e-targeting RNA -M24; D9. Design of the most suitable therapeutic approach based on MCU-targeting RNA -M24; D1.0. Validation of the efficacy and safety of CD300e silencing in a murine model of insulin resistance-M36; D1.1. Validation of the efficacy and safety of MCU silencing in a murine model of insulin resistance-M36.
Milestones WP 4.2.5	MS1. Definition of the impact of CD300e on insulin pathway-M3; MS2. Definition of the impact of MCU on lipid metabolism and adipose-tissue inflammation-M6; MS3. Demonstration of the role of CD300e and MCU in modulating immune metabolism-M12; MS4. Definition of the contribution of two different CD300e- and MCU-expressing compartments in modulating immune metabolism-M24; MS5. Definition and validation of the most suitable silencing approach -M24; MS6. In vivo confirmation of the insulin sensitivity restoring following CD300e or MCU silencing-M36.
Task 4.2.6	**ACLYsi ATP-citrate lyase gene silencing to improve metabolism and immunometabolic responses in obesity and atherosclerosis (Task Leader: Norata-UNIMI)**
TRL	**4→7**
	• Validate a therapy with siRNA directed toward hepatic or haematopietic ACLY to improve liver lipid metabolism and immunometabolic responses in cardiometabolic diseases. • Confirm the efficacy of this strategy in metabolic disturbances related to obesity and atherosclerosis progression in experimental models Evaluate safety profile and off target effects. • Devise a phase I safety / efficacy clinical trial
Deliverables WP 4.2.6	D1. Development of clinical-grade anti-ACLY siRNA (collaboration with spoke 6); Report and Data-M6; D2 Development of GalNAc conjugated ACLY-siRNA (collaboration with spoke 7); Report and Data-M12; D3.Development of lipid–polymer nanoparticles ACLY-siRNA (collaboration with spoke 7); Report and Data-M12; D4.Efficacy of GalNAc conjugated ACLY-siRNA to improve metabolic syndrome development in obese mice (C57BL6J mice fed HFD diet); Data and Report-M24; D5.Efficacy of GalNAc conjugated ACLY-siRNA to improve lipid metabolism and atherogenesis in atherosclerotic prone animal models (LDL-R KO mice fed cholesterol rich diet); Data and Report-M24; D6.Evaluation of off-target effects and safety profile of GalNAc conjugated ACLY-siRNA in ASGR1 deficient animal models-M24; D7.Efficacy of lipid–polymer nanoparticles containing human ACLY-siRNA targeting haematopoietic niche to improve metabolic syndrome development in obese mice via immunometabolic modulation (Rag2/IL2/CD47; Triple KO mice humanized with human CD34+ cells and fed HFD diet); Data and Report-M32"; D8.Efficacy of lipid–polymer nanoparticles containing human ACLY-siRNA targeting haematopoietic niche to improve lipid metabolism and atherogenesis in humanized atherosclerotic prone animal models (Rag2/IL2/CD47/LDL-Rquadrupole KO mice humanized with human CD34+ cells and fed cholesterol rich diet); Data and Report-M32; D9. Design and prepare first in human clinical trial-M36.
Milestones WP 4.2.6	MS1. siRNA targeting mouse or human ACLY are available-M9; MS2. Liver directed delivery is available-M15; MS3. Bone marrow hematopoietic niche directed delivery achieved-M15; MS4. The efficacy and safety profile of GalNAc ACLY-siRNA is available in obese mice-M24; M5. The efficacy and safety profile of GalNAc ACLY-siRNA in atherosclerotic prone mice-M24; M6 GalNAC/liver independent side effects of ACLY silencing and immunosensitization response related to delayed siRNA catabolism -M24; M7 The efficacy and safety profile of human ACLY-siRNA directed toward bone marrow cell on obesity development is available in humanized experimental models-M32; M8. The efficacy and safety profile of human ACLY-siRNA directed toward haematopoietic cells on atherosclerosis is available in humanized experimental models-M32; M9. Clinical trial is ready to start-M36.
Task 4.2.7	**ASTRA Aldosterone Synthase Inhibition with siRNA to treat human Arterial Hypertension (Task Leader: Rossi-UNIPD)**
TRL	**4→7**
	• Synthesis of new Aldosterone Synthase (AS) ligands by organic chemistry followed by in vitro screening and characterization. In vivo delivery optimization of the selected molecules. • Selection of CYP11B2 siRNA by in vitro screening and in vivo delivery optimization • Demonstration of preclinical efficacy of AS ligands and CYP11B2 siRNA Translation to clinical use and to technology transfer activities
Deliverables WP 4.2.7	D1. Prototype preparation-M6; D2. Report on monitoring results-M12; D3.Report on monitoring results-M18; D4.patents filing -M24; D5. press & media actions-M28; D6.Ethics-M36.
Milestones WP 4.2.7	M1. Production of AS ligands with selective binding domains for the haeme group-M6; M2. Proof of the selectivity and efficacy of the AS ligands in animals-M12; M3. Proof of the selectivity and efficacy of the CYP11B2 siRNA in animals-M18; M4. Optimized formulations patented-M24; M5. Presentation to investors and incubators for fundraising -M28; M6 Design of a clinical trial-M36.
Task 4.2.8	**TAZZ Targeting ZMAT3 and ZFP423 to overcome adipose tissue dysfunction and prevent its negative consequences on metabolism and risk of type 2 diabetes (Task Leader: Beguinot-UNINA)**
TRL	**4→7**
	• Validate a ZMAT3/ZNF423 siRNA based therapy to improve adipocytes function in vitro • Confirm efficacy of ZMAT3/ZNF423 siRNA against impaired adipose tissue remodeling, glucose tolerance, insulin resistance and inflammation in vivo • Identify the most suitable therapeutic indication based on short and long-term efficacy/tolerability profile • Devise a phase I safety/ efficacy clinical trial
Deliverables WP 4.2.8	D1. Develop clinical-grade ZMAT3/ZNF423 siRNA-M6; D2 Develop lipid conjugated- ZMAT3/ZNF423 siRNA-M12; D3. Efficacy to favour proper adipose tissue remodelling in diet-induced obese mice-M18; D4.Efficacy against meta-inflammation in diet-induced obese mice-M20; D5.Efficacy to improve glucose homeostasis and insulin sensitivity in diet-induced obese mice-M22; D6.Analysis of undesired effects/toxicology in mice-M24; D7.Design and prepare first in human clinical trial-M36.
Milestones WP 4.2.8	M1. A siRNA suitable for human use is available-M6; M2. An adipose tissue directed delivery is available-M12; M3. Choose the most suitable indication for human disease-M24; M4. A safety profile is available in mice-M24; M5. A clinical trial is ready-M36.

Task 4.2.9	**PGC1B-NASH Overexpressing PPARGC1B in the liver as a strategy to improve hepatic outcomes in NASH (Task Leader: Moschetta-UNIBA)**
TRL	**4→6**
	• Optimise the therapeutic range for optimal balance overexpression/safety; • confirm effectiveness of the genetic tool in ameliorating NASH; • Carry a first translation in Humans using ex vivo Organ-On-Chip microtissues;
Deliverables WP 4.2.9	D1. Development of clinical-grade mRNA construct embedded in a hepatocyte-specific targeted delivery tool-M6; D2. Dose ranging, and tool safety/toxicity studies -M18; D3. Effectiveness of the genetic tool in ameliorating NASH-M36.
Milestones WP 4.2.9	MS1. Tool for hepatocyte-specific PPARGC1B delivery for human use is available-M6; MS2. Tool for hepatocyte-specific PPARGC1B delivery for human use is used to test safety and therapeutic range in vitro-M12; MS3. Tool for hepatocyte-specific PPARGC1B delivery for human use is used to test safety and therapeutic range in vivo-M18; MS4. Confirmation of effectiveness in vivo (DIO-NASH models)-M30; MS5. Confirmation of effectiveness in advanced FDA-approved in vitro human NASH models -M30; MS6. Data integration, report preparation; Drafting candidate Phase 1/2 study design -M36.
Task 4.2.10	**Sip66Shc-β-cells Silencing p66Shc in pancreatic beta-cells as a strategy to prevent the onset/progression of type 2 diabetes (Task Leader: Giorgino-UNIBA)**
TRL	**4→7**
	• Develop a beta-cell-targeted delivery systems for the down-regulation of the p66Shc mRNA and protein expression; • Validate a p66Shc-siRNA silencing therapy to prevent the lipotoxicity-induced loss of beta-cell functional mass; • Confirm the efficacy against the onset/progression of type 2 diabetes in mice; • Envision a "proof of concept" to establish the feasibility of using this pharmacological approach in the target patient population (Devise a phase I safety/efficacy clinical trial)
Deliverables WP 4.2.10	D1. Development of a clinical-grade p66Shc-siRNA construct embedded in a beta-cell-specific targeted delivery tool-M6; D2. Efficacy against lipotoxicity-induced beta-cell functional loss in human pancreatic islets-M12; D3. Confirmation of its effectiveness in vivo (High-fat diet-fed mice)-M20; D4. Analysis of undesired effects/toxicology-M24; D5. Design and prepare first in human clinical trial-M36.
Milestones WP 4.2.10	MS1. Tool for beta-cell-specific p66Shc-siRNA delivery for human use is available-M6; MS2. Efficacy against lipotoxicity-induced beta-cell functional loss ex vivo is confirmed-M12; MS3. Efficacy against lipotoxicity-induced beta-cell functional loss in mice is confirmed-M18; MS4. Efficacy against the onset and/or progression of type 2 diabetes in mice is confirmed-M20; MS5. A safety profile is available in mice-M24; MS6. A clinical trial is outlined-M36.
Task 4.2.11	**IN.DIA.NEPH INhibition of the UBE2v1 gene and overexpression of miR-27b-3p and miR-1228-3p to slow the progression of DIAbetic Nephropathy (Task Leader: Gesualdo-UNIBA)**
TRL	**4→7**
	• Optimise the genetic tools and their therapeutic range for optimal balance effect/safety; • Optimise the nanoparticles tissue-specific delivery approaches: • Confirm efficacy of the genetic tools in ameliorating diabetic nephropathy and the molecular effects; • Carry a first translation in Humans using human organoids.
Deliverables WP 4.2.11	D1. Development of clinical-grade anti-UBE2v1 siRNA and miRNA vectors in human kidney cells-M6; D2. Vectors dose ranging, and safety/toxicity studies -M18; D3. Effectiveness of the genetic tool in ameliorating diabetic nepharopathy-M36.
Milestones WP 4.2.11	MS1. Assessment of genetic vectors and nanoparticles delivery systems for human use-M6; MS2. Assessment of therapeutic range and safety in vitro-M12; MS3. Assessment of therapeutic range and safety in vivo (mice models)-M18; MS4. Confirmation of effectiveness in vivo (mice models)-M30; MS5. Confirmation of effectiveness in human organoids -M30; MS6. Phase I/II study design-M36.
WP 4.3 – Myocardial Diseases	
General objectives. The overall effort of WP3 is to bring a number of targets and sophisticated methodologies identified or set up by the investigators as close as possible to clinical application for the treatment of myocardial diseases. **Methodology:** The spoke will take advantage of the Spokes' multi-Institution discovery platform relying on technological innovation and strong translational approach (cell-tissue-animal-human), to bring to light new targets of RNA-based drugs. We herein selected top-tier projects of drug development with a base of TRL4-5 and great advancement potential to TRL6-7. Availability of a set of diversified targets at different base TRL builds an ideal pipeline for the competitive advance to a new RNA based therapy.	
Task 4.3.1	**Cardiac Tissue Regeneration Using RNA –RETURN (Task leader: Bellin-UNIPD)**
TRL	**3→6**
	• The overall aim of Task 4.3.1 is to drive human heart regeneration through partial reprogramming of terminally differentiated cardiomyocytes (CMs) in vivo and reacquisition of proliferation-competent state. Targeted mRNA carriers will be used for treating in vitro and ex vivo human cardiac tissue models (TRL3-4), a large-animal ischemic-model (TRL5), and for compassionate use in selected patients (TRL6);
Deliverables WP 4.3.1	D1. Demonstration of reacquisition of hiPSC-cardiomyocyte proliferation and coupling (M16); D2. Demonstration of reacquisition of cardiomyocyte proliferation in heart tissue slice (M24); D3. Demonstration of reacquisition of cardiomyocyte proliferation in treated pigs (M30); D4. Clinical and echocardiographic evidence of improved heart function (M36);
Milestones WP 4.3.1	MS1. generation of cardiomyopathic hCOs (M12); MS2. Design of optimal editing tools (M24); MS3. Assessment of on- and off-target evaluation and functional recovery (M36);
Task 4.3.2	**modRNA, RNA Therapeutics, and CRISPR/Cas9 for Heart Failure Therapeutics (Task leader: Condorelli-HUMANITAS)**
TRL	**3→5**
	• We plan to generate knowledge on the use of modified RNAs for inducing tolerance as a therapeutic tool in heart failure, define the use of a long non-coding RNA or siRNA for heart failure therapy and modify the genome for curing LMNA cardiomyopathy. • Use of modified RNA and siRNA technology. • Use of CRISP/Cas9 approach for generating models of disease and therapeutic DNA recombination in vivo

Deliverables WP 4.3.2	D1.1 Validation of the tolerogenic antigen payloads (M12); D1.2 Validation of the non-immunogenicity of the lipid (or alternative) nanoparticle carrier (M24); D1.3 Proof-of-principle HF vaccination experiments in in vivo models of disease using tolerogenic antigens in non-immunogenic nanoparticle carriers (M36); D2.1 Demonstration of the efficacy of siRNA TAZ in vitro in primary cultures of cardiomyocytes (M6); D2.2 Generation of a mouse model of TAZ cardiac-specific KO (M12); D2.3 Characterization of the mouse model of TAZ1 (M18); D2.4 Demonstration of the efficacy of TAZ1 siRNA nanoparticle delivery in vivo in mice (M24); D2.5 Demonstration of the efficacy of TAZ1 siRNA nanoparticle delivery in vivo in pigs (M36) (according to resource availability); D3.1 Full characterization of the CHHEAF-KO mouse model (M12); D3.2 Determination of the effects of modCHHEAF in the mouse KO model (M18); D3.3 Determination of the effects of modCHHEAF in the mouse WT model (M24); D3.4 Identification of the human homologue of CHHEAF and testing of human modRNA in the iPSC-derived cardiomyocyte model (M36); D4.1 Generation of a R190W or K219T knock-in mouse model (M12); D4.2 Characterization of the above model (M18); D4.3 Generation of a CRISPR-Cas9 approach for correction of LMNA mutation in vivo (M24) and testing in vitro (M24); D4.4 Correction of LMNA mutation in vivo (M36).
Milestones WP 4.3.2	M1. Knowledge and use of tolerogenic antigens for heart failure treatment (m 36); M2. Knowledge and use of siRNA TAZ antigens for heart failure treatment (m 36); M3. Knowledge and use of CHHEAF lncRNA as a therapeutic target for heart failure (m 36); M4. Generation of mouse models of laminopathies and DNA recombination in vitro and in vivo (m 36).
Task 4.3.3	**Small Nucleolar RNA SNORD3a in Ischemic Heart Disease and Heart Failure (Task leader: Esposito-UNINA)**
TRL	**1→5**
	• To test the role of small nucleolar RNA SNORD3a as diagnostic, prognostic and therapeutic tool in several different experimental systems of myocardial ischemia and heart failure. To reach our goal, we will employ in vitro models of hypoxia, in vivo models of myocardial infarction and post-ischemic heart failure and human samples from patients with ischemic heart disease and heart failure.
Deliverables WP 4.3.3	D1 Role of SNORD3a in cellular responses to hypoxia (M28); D2 Role of SNORD3a as a diagnostic, prognostic, or therapeutic tool (M28); D3 Role of SNORD3a as a biomarker in humans (M36).
Milestones WP 4.3.3	MS1. SNORD3a Antisense Oligonucleotides (ASO) and SNORD3a enriched vesicles (M18); MS2. SNORD3a in vivo delivery through enriched vesicles (M32); MS3. SNORD3A as diagnostic and prognostic biomarker in patients (M36);
Task 4.3.4	**RNA Therapies for Cardiovascular Repair, Regeneration, and Gene Editing (Task leader: Giacca-UNITS)**
TRL	**4→6**
	• The overall objective of this WP is to develop RNA therapies, based on both mRNAs and small non-coding RNAs, for cardiac and vascular regeneration and for cardiac gene editing. We will generate SNALP LNPs for the cardiac delivery of microRNAs that stimulate cardiac regeneration and neovascularization after myocardial infarction, and LNPs that deliver the CRISPR/Cas9 RNA suite and miRNAs inducing homology-directed repair in animal models of inherited cardiomyopathies
Deliverables WP 4.3.4	D1.0a LNP formulations for efficient cardiomyocyte RNA transfer (M12); D1.0b LNP-miR-199 and LNP-miR-1825 for cardiac regeneration and their proof of efficacy in rodents and pigs (M36) D2.0 microRNAs that enhance cardiac therapeutic angiogenesis and their cardiac delivery after myocardial infarction in mice (M24); D3.0a A gene editing RNA suite) and microRNAs enhancing gene editing (M24); D3.0b Efficacy of RNA-based cardiac gene editing in three animal models of inherited cardiomyopathy (M36).
Milestones WP 4.3.4	MS1.1. Identify LNPs for myocardial delivery (M12); MS1.2 Efficacy of selected miRNAs at inducing cardiac regeneration (M36); MS2.1. LNPs for myocardial delivery (M12); MS2.2 Efficacy of selective miRNAs at stimulating therapeutic angiogeneis in vivo (M36); MS3.1. LNPs for gene editing RNAs (M24); MS3.2 Efficacy of RNA-based gene editing in vivo (M36).
Task 4.3.5	**Capturing Harmful Intercellular Crosstalk via miRNA in Arrhythmogenic cardiomyopathy (CHICA) (Task Leader: Mongillo-UNIPD)**
TRL	**3→5**
	• Circulating and cell-type specific miR-landscapes will be quantified at different time-points, in DSP-KI AC vs. WT mice, and controlled with those of cultured iPSC derived from AC patients. Bioinformatics will identify the AC miRNA signature, infer the pathogenic signalling mechanisms most likely affected by miRNAs in different cardiac cell types. These analyses will determine the progression of myocardial remodelling, and the relative expression profile of circulating and cell specific miRNAs in DSP-dependent AC during disease development. Based on observational and in vitro data, miRNA mimics and miRNA inhibitors (GapmeRs) will aim to interfere with cell specific miRs found elevated/repressed in AC- CMs/non-CM cells. Our data strongly support the key pathogenic role of AC-mutant sympathetic neurons, qualifying them as cell culprits of both arrhythmias and myocardial remodelling. Based on a mechanism-driven hypothesis, we aim to target NPY signalling, with cardiac specific interference with NPY receptors, to ablate neurogenic effects on myocardial remodeling
Deliverables WP 4.3.5	D1.1 Identification of miRNA profiles biomarking AC progression (M18); D2.1 Reduction of AC myocardial remodelling through targeted NC RNA therapeutics (M24); D3.1 Interference with AC through NC-RNA-mediated modulation of NPY signalling (M36).
Milestones WP 4.3.5	MS1. A specific miRNA profile is identified in murine DSP dependent AC model (M9); MS2. miRNA profile is validated in patient-derived hIPSC (M12); MS3. Effect of miR modulation on DSP-specific AC remodeling (M24); MS4. Validation of cardiac specific NPY-R interference in AC remodeling (M36).
Task 4.3.6	**Development of RNA Gene Therapies for the Treatment of Inherited and Acquired Cardiomyopathies (Task Leader: Priori-UNIPV)**
TRL	**3→6**
	• The aim is to develop novel RNA therapies to treat cardiac disorders that increase the risk of sudden death. The project will address the unmet need of developing novel drugs for inherited conditions, which affect mostly young individuals, and cardiac amyloidosis, which is a significant acquired contributor to morbidity and mortality in the elderly. We will develop allele specific silencing in a knock-in pig model of LQT8 and a multi-target RNA-interference strategy for CPVT1 in hiPSC-CMs. We will test the modulation of plasminogen or uTPA expression to treat cardiac amyloidosis. We will also develop a computational model of the electro-mechanics of the heart for arrhythmic risk assessment.

Deliverables WP 4.3.6	D1. Patent submission (M30); D2 Agreement with an industrial partner for a first-in-human study (M36); D3. Patent submission and publication (M30); D4. Patent submission and publication (M30); D5. Identification of a partner for a first-in-human study (M36); D6. Complete data package for hiPSC characterization as required by hPSCreg and publication (M30); D7. Release the software open source and publishing of results (M36);
Milestones WP 4.3.6	MS1. Conclusion of in vitro siRNAs screening and identification of best siRNA candidate for LQT8 (M12). MS2. In vivo testing of AAV9-miRNA in LQT8 pigs (M24); MS3. In vivo testing of siRNA-loaded nanoparticles inhalation in LQT8 pigs (M36); MS4. Conclusion of in vitro siRNAs screening and identification of best pair of siRNA candidates for RYR2 SNPs (M18); MS5. siRNA testing on CPVT1 hiPSC-CMs (M30); MS6. Identification of the best siRNAs for plasminogen and dose optimization (M18); MS7. selection of the safest and most bioavailable formulation (M30); MS8. Identification of the best siRNAs for urokinase-type plasminogen activator and dose optimization (M24); MS9. Selection of safest and most bioavailable formulation (M36);
Task 4.3.8	**RNA-based Strategies to Treat Arrhythmogenic Cardiomyopathy (Task Leader: Rampazzo-UNIPD)**
TRL	**2→4**
	• Since available therapy for arrhythmogenic cardiomyopathy (ACM); has only a marginal impact on long-term prognosis, there is both room and need for the development of innovative therapies. We aim at testing antagomirs/miRNA mimics as a novel RNA-based therapeutic strategy for ACM. Our data indicate that miR-708, -217 and -499 are dysregulated in hearts of transgenic mice (tgQ); with cardiac-specific overexpression of mutant hDSG2. miRNA and mRNA sequencing will be carried out in iPS-CMs carrying the p.Q558* mutation in DSG2 and from their isogenic controls. In order to provide a human in-vitro proof-of-concept for the therapeutic efficacy of selected candidate antagomirs/miRNA mimics, we will explore the effects of patient-derived iPS-CM treatment by transcriptome analysis and luciferase assays.
Deliverables WP 4.3.8	D1.0 Identification of novel miRNA shared between ACM mouse model and patients (M8); D2.1 Design and synthesis of antagomirs/miRNA mimics (M4); D2.2 Pilot study to select the proper dose for in vivo treatment (M18); D2.3 In vivo antagomir treatment (M24); D3.0 In vitro antagomir/miRNA mimics treatment and molecular characterization (M36).
Milestones WP 4.3.8	MS.1 Identification of dysregulated miRNA and mRNA in ACM mouse model and iPS-CM carrying the same DSG2 variant (m 12); MS2.identification of most interesting dysregulated miRNA based on its targets (m 16); MS3. Design and synthesis of antagomirs/miRNA mimics in relation to miRNA expression (m 20); MS4 Pilot study to select the proper dose of antagomirs/miRNA mimics for iPS-CM treatment (m 28); MS5 In vitro antagomir/miRNA mimics treatment and molecular characterization (M36).
Task 4.3.9	**Reverting Cardiac Regenerative Cell Senescence to Improve Diabetic Cardiomyopathy (ReCARES) (Task leader: Torella-UMG)**
TRL	**1→7**
	• We aim at understanding at single cell level the molecular basis of heart regenerative deficit in diabetic cardiomyopathy while assessing its reversal by the combination of two chemically modified mRNAs (cmRNAs); for Bmi-1 and β-Catenin, which are dysregulated in the myocardium of diabetic subjects, scRNASeq and gain/loss of function assays to unveil the detrimental diabetic phenotype of cardiac regenerative cells; • cmRNAs loaded into functionalized nanoparticles and released by lyotropic liquid crystals tested in animal and pre-clinical human models of diabetic cardiomyopathy.
Deliverables WP 4.3.9	D1.0a mRNA and miRNA networks underlying diabetic cardiac regenerative defects (M18); D1.0b Efficacy of novel cmRNAs and miRNAs mimics/antagonists in vitro (M24); D1.0c Efficacy of novel cmRNAs and non-coding RNAs mimics/antagonists in vitro and in vivo (M36); D2.0a Develop glyceryl monooleate-based lyotropic liquid crystals (LLC); patches (M6); D2.0b Develop functionalized nanoliposomes (FNP); loaded with Bmi-1 and β-catenin cmRNAs (M18); D2.0c Efficacy of Bmi-1 and β-catenin cmRNAs-loaded FNPs in mouse and human cardiac progenitors in vitro (M24); D2.0d Efficacy of Bmi-1 and β-catenin cmRNAs-loaded FNPs through LLC patches release in mice (M36); D3.0a Establishment of human diabetic cardiac organoids (M12); D3.0b Efficacy of Bmi-1 and β-catenin cmRNAs-loaded FNPs in human diabetic cardiac organoids (M36).
Milestones WP 4.3.9	M1. A preclinical human model of diabetic cardiomyopathy is available (M12); M2. cmRNAs-loaded FNPs are available (M18); M3. Efficacy of Bmi-1 and B-Catenin cmRNAs-loaded FNPs is available in mice and human preclinical model (M24); M4. A safety profile of Bmi-1 and B-Catenin cmRNAs-loaded FNPs is available in mice (M30); M5. Efficacy of Novel cardio-regenerating RNAs is available in mice and human preclinical mode (M36).

Spoke #5: Inflammatory and Infectious diseases. Spoke Leader: UNISI.

State of the field and unmet needs. RNA-based technologies are profoundly impacting the way to treat and to prevent infectious diseases and immune-based disorders. As an example, mRNA-based vaccines are dominating the SARS-CoV-2 pandemic scenario, being demonstrated to be of rapid development and highly effective in eliciting antigen-specific immune response. Of more, mRNA vaccine-based approach is now harnessed also to suppress, rather than prime, antigen-specific immune responses, thus being useful to treat other immune disorders (i.e. autoimmune diseases). In addition, RNA represents a strong candidate to be specifically targeted by ad hoc designed drugs (CRISPR, ASOs, siRNAs). Moreover, there is an increasing interest in non-coding RNAs as sensitive non-invasive biomarkers for disease diagnosis, prediction and patient stratification as well as novel therapeutic targets. In line with this, circulating extracellular vesicles are also of interest being considered vectors for the delivery of RNA and other molecules. Of note, EVs are considered

Biotechnological medicines, such as viral vector and non-viral RNA/DNA delivery for cell-specific gene silencing, are emerging new strategies for the treatment of both inflammatory and infectious diseases, as well as for cancer. In parallel to treatment of disease by physiopathologic side, management of pain associated with inflammatory/neuropathic diseases, and cancer, represents a critical challenge. A "precision drug delivery" by displaying a better efficacy and safety profiles might represent novel approaches to target selective cellular/molecular pathways to treat different pathological conditions. Gene regulation by viral vectors are already used in therapy (i.e. COVID-19 vaccines), but their further wide application needs some immunological consideration. The efficacy of vectors based on viruses capable of commonly infecting human cells

can be negatively affected by the presence of a specific antiviral immune response pre-existing upon administration of the vector. This response involves the presence of neutralizing antibodies capable of blocking the vector itself, reducing the transduction efficiency and consequently the expression of the conveyed gene product. In the case of viral vector vaccines, this phenomenon leads to a reduced expression of the antigen against which the immunization is to be triggered, thus causing a reduced efficacy of the vaccine and affecting B and T cell mediated adaptive immune response.

How the Spoke will contribute to advance in this scenario. New strategies for the treatment of inflammatory, immune-related disorders and infectious diseases including viral and non-viral RNA/DNA delivery vectors for cell-specific engineering will be developed. An innovative circulating biomarkers discovery and analysis platform focusing on extracellular vesicles isolation, non-coding RNAs profiling and on immune factors, will be established. These approaches will provide a better efficacy and safety profiles aimed to develop "precision drug delivery" involved in selective cellular/molecular pathway for the treatments of different pathological conditions. **Spoke #5** will be organized in four main WPs. **WP5.1** focused on vaccines, prophylactic drugs design/development, and on immune responses profiling and vaccines efficacy/immunogenicity testing; in **WP5.2**, innovative strategies will be unleashed to combat novel bacteria and viral infections through novel technologies, such as phage engineering, Crisp-Cas system mediated viral genome degradation, and RNA/DNA aptamers design to block specific viral receptors; in **WP5.3** we will focus on biomarkers and therapeutic RNAs discovery in inflammatory, immune-related diseases and infections; finally, **WP5.4** will be fully dedicated to RNA/DNA drug design, delivery vectors and strategies, in order to skyrocket the translability of the results obtained in WP5.1, -5.3 and to enhance their TRL readiness.

In WP5.1, a platform for the production of novel viral vaccines based on "replication-deficient" Sendai virus vector will be developed (task 5.1.1). This platform will be paralleled by a vaccines immunogenicity and immune-response testing pipelines (e.g. high-thoughput pseudotype virus-based neutralization assays, Multicolor FACS, Multiplex Magpix, Nanostring) (task 5.5.1 and 5.1.4). Multiple platforms to generate prophylactic drugs will be developed (task 5.1.2 and 5.1.3). Moreover, a platform to generate vaccines to induce immune tolerance, thus quenching autoimmunity and driving the immune system to a resting or homeostatic state will be developed. In synergy with NC, this procedure with molecularly defined self-antigens will be assessed. **Spoke #7** will support all activities carried out in WP5.1 to identify further neo-antigens and sequences for the development of mRNA vaccines and mABs and to generate tolerogenic cells. WP2.5 will identify and apply non-conventional strategies to combat difficult-to-treat bacterial infections (e.g. antimicrobial-resistant bacteria) and novel viruses. Specific bacteriophages capable of destroying resistant bacteria will be identified together with innovative systems to target and kill infected cells using Genomics-Informed Drug Design (Gen-ID2) technology and aptamer design. One of the innovative technology developed in this WP finds a direct interaction with the **Spokes #1**, **and #2**. Cancer treatments often have debilitating side effects for patients. The main cause of the side effects is treatment failure to differentiate between cancer cells and healthy cells. The Genomics-Informed Drug Design (Gen-ID2) technology proposed by **Spoke #5** uses the RNA signature of the target cell types, allowing the selective expression of any transgenic protein in the target cell, including a kill gene. This approach also allows for the development of personalized treatment for cancers and potentially for genetic diseases (**Spokes #1**).

WP5.3, aimed to discover and analyze freely circulating or extracellular vesicles (EVs)-enclosed RNAs as biomarkers and therapeutic targets in immune-inflammatory diseases. **Spoke #5** will develop a platform for the analysis of novel blood-circulating non-coding RNA biomarkers across the entire **NC**. We will establish a core facility for sequencing analysis of circulating RNAs differentially associated with blood plasma components, thus enhancing the precision medicine approach of the **NC**. This core facility will initially focus on T1D (Task 5.3.1) and pulmonary fibrosis (Task 5.3.2) but will also provide support for other diseases [within **Spoke #5** and across different Spokes (e.g. **Spoke #2**)]. In collaboration with **Spoke #8**, we will analyse and develop EVs as potential therapeutic vectors for delivering of RNA molecules into cells of interest. As RNA-omics technologies will be used, big data from multiple sample sources is expected, **Spoke #5** will interact with the biocomputing infrastructures established in **Spoke #7** to facilitate -omics data analysis for the entire **NC**. Immune-based circulating biomarkers will be also assessed, thanks to the specific platform establishment outlined in WP1. **WP5.4** activities will be dedicated to RNA-based drugs and delivery methodologies development. RNA will be used to reprogram dendritic cells in the course of transplantation (graft vs host disease, GvHD) and/or autoimmunity. To this aim a new platform for cell-based therapy to control GVHD and autoimmune disorders (multiple sclerosis, type 1 diabetes) using GMP-certified mRNA-encoding FLIP to in vitro transfect monocytes/dendritic cells will be developed. In parallel, development of programmable viral and non-viral lipid-based RNA/DNA delivery systems (Task 5.4.4 and 5.4.4) will be outlined in order to boost the therapeutic development and delivery of targets identified in WP5.4 (Task 5.4.2) and WP3 (Task 5.3.1 and 2).

Mid-Long term outcomes of the Spoke. RNA therapy has many advantages over existing small molecule or monoclonal antibody-based therapies, including its potential to target previously undruggable gene/pathways as well as long-lasting effects in cells, the relatively simple design and manufacturing and time and cost effectiveness. In **Spoke #5** we will pursue research along these following aims: to develop vaccines, prophylactic drugs and innovative therapies against novel emerging viral pathogens; to generate vaccines, neutralizing monoclonal antibodies or new therapies against anti-microbial

resistant (AMR) bacteria; to discover novel RNA-based therapeutic targets and circulating biomarkers in autoimmune and inflammatory diseases; to engineer intelligent nanovesicles-delivering RNA-based drug to treat autoimmune/inflammatory diseases; to identify novel strategies to develop tolerogenic dendritic cells for a cell-based therapy to control GVHD and autoimmune disorders; to perform an in-depth analysis of immune responses to viral vectors; to identify disease-associated RNA biomarkers. The plan will be updated during the action lifetime and will include a sustainable roadmap which could be deployed over the years. These will include:

1. Biobanking and BioMolecular resources Research Infrastructure (e.g. a Bank of vectors containing the cDNA of specific miRNAs genes which will be maintained by one of the participating companies (to be defined, several have already expressed availability) and will be distributed also after the end of the project for at least additional five years.
2. Identification of potential end-users for these results, such as Research institutions and companies. Several of the participating companies have already express the willingness to continue to benefit from the results of the project also after the three-year programme. Other users have been identified in SME involved in the developments of diagnostics that need biological samples for kit validations, in pharma companies interested in the pharmacogenomics data, furthermore databases and biological samples including pseudoviruses and constructs will be essential in developing new concepts for rapid response to new outbreak situations.
3. The repository of biological samples will be connected to the networks and all possible income from the distribution of samples to institutions outside the consortium will be used for sustainability of the repository following the networks rules. The maintenance of databases will be at a public research institution that is part of the Italian network of public universities and the costs included in the local host facility.

Several activities in **Spoke #5** are fully supported by SME and pharma companies. In WP2 we will apply the innovative Genomics-Informed Drug Design (Gene-ID 2) platform (Task 5.2.2), which selectively recognize and degrade viral genomic RNA. Such platform can provide treatments for any disease that affects the genetic profile of the diseased tissue (**Spoke #1** and **#2**), thus its potential market size is considerable. The scientific collaboration with *Entropy Therapeutics*, a small US Start-Up that patented carriers to be used in the platform Genomics-Informed Drug Design (Gen-ID^2*)* has already guaranteed to grant the Consortium any patenting rights under favorable conditions, and to pursue the application development of the technology.

The innovative platform for in vitro transfection of monocytes/dendritic cells with mRNA to obtain tolerogenic cell is already covered by a specific patent (PCT/EP2017/051068); such activity will be further developed and boosted to the clinical translation with **BioNTech**. This new platform using GMP-certified, mRNA-encoding FLIP to in vitro transfect monocytes/dendritic cells with fully automated and scalable cell manufacturing procedures. mRNA will be provided by **BioNtech** by a free of charge collaboration. BioNtech will provide further material to UNIVR under signed Material Transfer agreement.

WP5.1 Novel mRNA Vaccines, prophylactic drugs design and immune response profiling	
General objectives: Development of an integrated vaccine and prophylactic drug design platform and immune responses analyses **Methodology:** Sendai Viral vectors design and engineering; mRNA mAb strategy design through screening of memory B cells, Mannose Binding Lectin-encoding mRNA design; tolerogenic innate immune cells design; immune markers, cytokines and pathways by FACS, Nanostring and multiplex, immunogenicity assessment by humoral and cell mediated response	
Task 5.1.1	**mRNA-vaccine technology and innovative viral-vectors design to prime antigen specific immune response (Task leader: Cusi, Montomoli-UNISI)**
TRL	**2→4**
	• Development and optimization of viral (Sendai virus based), and non-viral vectors for the targeted delivery of genetic material based on fully synthetic systems, namely lipid, polymeric and hybrid (lipid/inorganic nanoparticles) nanomaterials and on biogenic systems. • Efficacious and safe SARS-CoV-2 vaccine in animal model • Assessing vaccines immunogenicity
Task 5.1.2	**mRNA-encoded neutralizing monoclonal antibodies against viral pathogens (Task leader: Plebani- UNIPD)**
TRL	**2→6**
	• Development of a platform to produce mRNA encoding neutralizing antibodies against ZIKA virus and other emerging vector-borne viral pathogens to be used during pregnancy and in other fragile and immunologically compromised populations
Task 5.1.3.	**Innovative design and delivery of mRNA-encoding Mannose Binding Lectin (MBL) and optimized muteins (Garlanda- Humanitas)**
TRL	**2→6**
	• Development of a Mannose Binding Lectin-encoding mRNA approach for the therapy or prevention of COVID-19
Task 5.1.4	**Immune response profiling to vaccines and prophilactic drugs (Annunziato- UNIFI)**
TRL	**1→4**
	• Define how the immune response host mechanisms interfere with the viral and non-viral vectors administered, as well as with the prophylactic drug delivered, modulating their activity • Define the ability of RNA/DNA based vaccine to activate immune response against the target molecule

Task 5.1.5	**Tolerogenic Vaccine development (Brusatin – UNIPD)**
TRL	**1→4**
	• To use engineered biomaterials to combine the patient's own cells with TolDCs and introduce this cell mixture as a tolerogenic vaccine within a tolerogenic biomaterial
Deliverables WP5.1	D5.1 Design, cloning and production of genetically stable replication deficient Sendai virus vector for genetic material (12m); D5.2 Assays for characterization of pre-existing immune response versus viral vectors used for RNA and DNA delivery (12m); D5.3 Evaluation of vaccine safety (24m); D5.4 Analysis of the humoral immune response (24m); D5.5 Analysis of the cell mediated immune response (24m); D5.6 Development of nanoparticles to deliver MBL mRNA and MBL muteins mRNA (24m) D5.7 Assays to define the effect of viral and non-viral vectors on innate and adaptive immune response (24m); D5.8 Specific mRNA antibody clone identified and functionally validated in-vivo (24m); D5.9 mRNA-mAbs produced on the pilot scale (36m); D5.10 Development of mRNA-therapeutic approaches to deliver PTX3 (36m); D5.11 Evaluation of protection from disease, better from infection, in challenged mice (36m); D5.12 Assays to define eventually activation of immune response against the prophylactic drug (36m); D5.13 Assays to define efficacy of RNA/DNA based vaccine to activate immune response against the target molecule (36m); D5.14 Optimization of TolDCs preparation in-vivo and ex-vivo (36m); D5.15 Engineering injectable biomaterials to combine TolDCs with exosomes/ disease-antigens or autologous cells (36m); D5.16 In vivo tests of tolerogenic Vaccines in syngeneic mice (36m)
Milestones WP5.1	M5.1 Select the best candidate as mRNA vaccine (12m); M5.2 Isolation of antigen specific mAbs after high-throughput screening of memory B cells (12m); M5.3 Definition of immunogenicity/tolerance of different categories of proposed types vectors (12m); M5.4 Clarifying the molecular hallmarks of most effective TolDCs (12m); M5.5 Functional identification of neutralizing monoclonal antibodies (24m); M5.6 Definition of inflammatory pathways induced by different types of vectors (24m); M5.7 Report on the feasibility of mRNA-therapeutic approaches to deliver humoral innate molecules in infections (24m); M5.8 Provide a safe and efficacious vaccine (36m); M5.9 mRNA production of the selected antibody clones (36m); M5.10 Characterization of immune response induced against the target molecule of prophylactic drug (36m)
WP 5.2 - Innovative strategies to counteract novel antimicrobial resistant bacteria and viral infections	
General objectives. Generation of a pre-emptive therapy against multidrug resistant bacterial pathogens and novel emerging viruses **Methodology.** Bacteriophages library design; CRISPR-Cas system and Genomics-Informed Drug Design, RNA-DNA aptamers libraries design	
Task 5.2.1.	**Innovative phage library preparation to treat antibiotic-resistant bacteria (Task leader: Andreoni- UNIROMA2)**
TRL	**3→7**
	• Effectiveness of phages on multidrug-resistant bacteria • Development of a Phage therapy of antibiotic-resistant infections
Task 5.2.2.	**CRISPR-CAS mediated viral genomes degradation (Task leader: Novelli- UNIROMA2)**
TRL	**1→6**
	• Identify differences in miRNA signature of SARS-CoV-2-infected versus uninfected airway epithelial cells • Demonstrate selective expression and function of CISCAVIR gene circuit upon lipid nanoparticle delivery to infected cell • Demonstrate selective expression and function of CISCAVIR gene circuit upon lipid nanoparticle delivery to infected cell
Task 5.2.3	**RNA/DNA Aptamers (Task leader: Centanni- UNIMI)**
TRL	**1→7**
	• Proof-of-principle validation of the protocol developing aptamers • The preclinical development of metabolically stabilized aptamers binding to hACE2 and efficiently blocking SARS-CoV-2 infection. • Prototype aptamer identification
Deliverables WP5.2	D5.17 Cloning and production of phages active against multidrug-resistant bacteria (12m); D5.18 By identifying differences in the miRNA signature of SARS-CoV-2 infected airway epithelial cells versus uninfected ones (12m); D5.19 Assays for characterization of phages active against multidrug-resistant bacteria (24m); D5.20 Selected Aptamers identified (24m); D5.21 In vivo preclinical study with phages active against multidrug-resistant bacteria (36m); D5.22 By demonstrating the selective expression and function of the CISCAVIR gene circuit on the delivery of lipid nanoparticles to infected cells (36m); D5.23 Preclinical study started (36m)
Milestones WP5.2	M5.11 Select the best phages as candidate to treat antibiotic-resistant bacteria (12m); M5.12 To Identify and characterize the unique miRNA signatures of SARS-CoV-2-infected and non-infected airway cells. These signatures will serve as the foundation for the development of the unique circuitry that will selectively target SARS-CoV-2-infected cells (12m); M5.13 Identification of the molecular determinants of the infection (12m); M5.14 Provide a safe and efficacious phage library preparation (24m); M5.15 Optimization of CISCAVIR to express statistically significantly more CAS13d in SARS-CoV-2 infected versus uninfected cells (24m); M5.15 Selection of candidate aptamer leads (24m); M5.16 Definition of immunogenicity/tolerance of different categories of proposed phages (36m); M5.17 In SARS-CoV-2 infected cells, treatment with CISCAVIR results in a statistically significant reduced expression of viral mRNA and protein, as measured by RT-QPCR and quantitative near-IR Western blotting, respectively, and statistically significantly reduced infection, as measured by plaque assay (36m); M5.18 Aptamer formulation and preclinical ADMET study (36m)
WP 5.3 - Circulating RNA therapeutic target discovery and delivery strategies in autoimmune and inflammatory diseases	
General objectives. Development a combined workflow to discover, design and engineer mRNA/small RNAs-based delivering nanovesicles (NVs) to induce immune-tolerance and to treat autoimmune/inflammatory-based diseases. Identify novel RNA biomarkers and therapeutic targets. **Methodology.** High-throughput analysis of circulating RNAs differently associated with multiple plasma components. Design biogenic and synthetic nanomaterials to deliver RNA-based drugs and to check NVs-therapy immune responses	
Task 5.3.1	**High-throughput characterization and validation of circulating Small RNAs in patients with autoimmune and inflammatory diseases (Task leader: Dotta – UNISI)**
TRL	**4→8**
	• High-throughput analysis of circulating RNAs differently associated with multiple plasma components

	• Design biogenic and synthetic nanomaterials to deliver RNA-based drugs and to check NVs-therapy immune responses
Task 5.3.2	**Isolation and characterization of non-coding RNAs cargoed by extracellular vesicles for therapeutic target discovery (Task leaders: Bargagli/Ungaro- UNISI/UNINA)**
TRL	**4→8**
	• Discovery of altered circulating extracellular vesicles in the clinical setting of pulmonary fibrosis and to use them as biomarkers or to potentially investigate their role as druggable molecules to be moved forward in the clinical setting. • Validation of a RNAi therapeutic approach based on inhalable nanoparticles (iNPs) for local management of fibrosis-induced lung inflammation
Task 5.3.3	**RNAi therapeutics to target innate immune inflammation (Task leader: Gherardi- UNIPD)**
TRL	**3→5**
	• To develop an RNAi therapeutic approach to inhibit inflammasome in innate immune system
Task 5.3.4	**Smart nanostructured non-viral vectors for precision delivery of small RNAs to modulate autoimmune and inflammatory diseases (Task leader: Berti- UNIFI)**
TRL	**3→5**
	• Development and optimization of non-viral vectors for the targeted delivery of genetic material based on fully synthetic systems
Task 5.3.5	**Microimaging technologies (Task leader: Lupetti - UNISI)**
	• Ultrastructural characterization and stability evaluation of RNA/DNA delivery nanocarriers
Deliverables WP5.3	D5.24 Characterization of extracellular vesicles surface markers as potential druggable molecules (12m); D5.25 Four types of non-viral nanovectors (cubosomes/hexosomes, (co)polymer assemblies, soft systems loaded with magnetic nanoparticles, lipid/polymer nanohybrids) fully characterized for RNA delivery (12m); D5.26 Definition of small RNAs profiles associated to different plasma components (exosomes, ribonucleoproteins) in control subjects (24m); D5.27 Inhalable RNAi formulations validated in lab to progress in preclinical studies (24m); D5.27 Two types of non-viral nanovectors fully characterized for targeted RNA delivery (24m); D5.28 Selection of a set of small RNAs as circulating biomarkers or druggable therapeutic targets, altered in plasma od type 1 diabetic subjects (36m); D5.29 An inhalable RNAi prototype formulation to progress to GLP preclinical studies (Spoke #9) (36m); D5.30 Design of RNAi therapy to reduce lung inflammation (36m); D5.31 Fully characterized and efficient non-viral nanovector for targeted RNA delivery (36m); D.5.32 High resolution imaging and morphometry of exosomes from T-cells and beta cells (36m)
Milestones WP5.3	M5.19 Small RNA sequencing analysis of exosomes and ribonucleoprotein associated RNAs in control subjects (12m); M5.20 In-vitro evaluation of MCU as a potential target for therapies aimed at attenuating inflammatory responses (12m); M5.21 Efficiency results on the four types of nanovectors and selection of at least two nanovectors for further development (12m); M5.22 Small RNA sequencing analysis of exosomes and ribonucleoprotein associated RNAs in T1D subjects (24m); M5.23 Identification of extracellular vesicles markers in human lung fibroblast and biological fluids as potential target therapy (24m); M5.24 Ultrastructural quality controls of exosomes by advanced cryoTEM technologies (24m); M5.25 Production and in vitro validation of inhalable siRNA-loaded nanoparticles on human lung fibroblasts (36m); M5.26 Efficiency results on the two types of targeted nanovectors and selection of the most promising targeted type (36m)
WP5.4	**RNA-based drug design**
General objectives. New treatments to fight immune pathologies originating from enhanced immune reactivity against host self-antigens in the course of transplantation To identify and characterize non-coding RNA involved in different parasitic stages for detecting potential novel targets against CE. **Methodology.** The development of a new platform using GMP-certified, mRNA-encoding FLIP to in vitro transfect monocytes/dendritic cells with fully automated and scalable cell manufacturing procedures. Echinococcus as a "model parasite", the novel approaches developed could be adopted to control other parasitic infections	
Task 5.4.1.	**Design of RNA-based drugs to modulate immune system in autoimmune diseases (Task leader: Ugel-UNIVR)**
TRL	**3→7**
	• Characterizing the immunomodulatory properties of RNA-based transfection. • Developing therapeutic protocols to control in vivo GVHD progression based on engineered myeloid cells. • Validating a new platform for cell-based therapy to control GVHD
Task 5.4.2	**Identification and characterization of non-coding RNA involved in different parasitic stages (Task leader: Cringoli – UNINA)**
TRL	**3→5**
	• To identify and characterize non-coding RNA involved in different parasitic stages. • Detection potential novel targets against CE. • The novel approaches developed could be adopted to control other parasitic infections.
Task 5.4.3	**Smart lipid Nanocarriers design (Task leader: Magnani- UNISI)**
TRL	**2→5**
	• Development of a lipid-based RNA/DNA delivery platform technology that responds to an external stimulus • Development of a programmable lipid-based RNA/DNA delivery system with enhanced systemic circulation
Task 5.4.4	**Development of neuronal and glial cell-specific viral vectors for chronic pain treatment (Task leader. Nassini- UNIFI)**
TRL	**2→5**
	• Development of Viral vector for cell-specific gene silencing for the treatment of chronic pain • Development of methods for non-viral shRNA delivery
Deliverables WP5.4	D5.33 A genetic and functional map of FLIP-dependent RNA reprogramming of DCs (12m); D5.34 A new protocol to convert DCs into tolerogenic elements by RNA delivery (24m); D5.35 Characterization of non-coding RNA involved in eggs and cysts (24m); D5.35 Lipid-based nanocarriers that evade detection by the immune system (24m); D5.36 Development of cell-specific gene silencing technology by delivering RNA/DNA by viral/non-viral vectors (24m); D5.37 An innovative tolerogenic DC-based therapy to limit GvHD progression (36m); D5.38 New therapeutic tools RNA based for CE (36m); D5.39 Lipid-based

	nanocarriers with enhanced systemic circulation that are internalized by cells following stimulus input (36m); D5.40 Development of transgenic animal models of human painful diseases to test viral/non-viral vectors (36m)
Milestones WP5.4	M5.27 Mechanistic explanation and target identification of the tolerogenic properties of RNA-reprogrammed DCs (12m); M5.28 Preparation of stimuli responsive lipid nanocarriers containing biodegradable PEGylated lipids that can be cleaved by pH changes or light irradiation (12m); M5.29; Efficient RNA technology for in vitro gene silencing (12m); M5.30 Efficacy evaluation of RNA-reprogrammed DCs in mouse models of GvHD (24m); M5.31 Validation of non-coding RNA for diagnosis and development of new therapeutic tool for CE (24m); M5.32 Viral/non-viral vectors for tissue/cells specific RNA delivery (24m); M5.33 Design an effective protocol for in vitro RNA-based reprogramming of human DCs for clinical use (36m) M5.34 Demonstration of cellular uptake of viral and lipid-based nanonanocarriers (36m)

B.5.2 HORIZONTAL SPOKES

Spoke # 6: RNA Drug Development. Spoke leader CNR.

State of the art and unmet needs. Conventional pharmacological strategies rely on the ability of small molecules to target active sites of proteins: this drastically limits our therapeutic potential. Indeed, only ~1.5% of the human genome encodes for proteins and only 10-14% of proteins have druggable binding sites. The transformative force in therapeutics interventions nowadays is synthetic RNA either mimicking messenger RNA (mRNA) or targeting them by RNA-interference mechanisms or antisense oligonucleotides (ASO), that can effectively target mRNA of previously undruggable proteins and non-coding RNAs involved in regulatory circuits. Introns represent the largest group of non-coding transcribed sequences (~ 25% of the genome). In addition, a panoply of ncRNAs, originating from the pervasive transcription of the genome, is involved in all aspects of cell metabolism. However, as of 2021, only 11 RNA-based therapeutics have been approved by the FDA and/or the EMA including 4 therapeutic ASOs that target pre-mRNA splicing. In addition, ~ 40 RNA therapeutics are in phase II or III clinical development, but no lncRNA-based therapeutics have entered the clinic so far.
RNA technologies therefore emerge as disruptive therapeutic options, as small biotech, startups and academic groups can rapidly develop new and personalized RNA-based drugs. The development of these therapies, however, has been hampered so far by two main problems. The first one is the still incomplete understanding of the RNA regulatory circuits. Thus, for instance, post-transcriptional modifications (epi-transcriptome) and RNA folding (3D structure) may impact the interaction with the therapeutic molecules. The second hurdle is more technical and includes off-targets, mainly observed with siRNAs, and immunogenicity effects linked to ASO chemistries. Unproductive uptake is another important aspect and reflects the fact that >98% of ASO remain entrapped in intracellular endosomes, where they are pharmacologically inactive because they cannot access their targets or are degraded by lysosomes. Increasing productive uptake will drastically improve the efficacy of treatments and reduce undesired side effects such as off-target effects, toxicity and immunogenicity. Toxicity is actively investigated by biotech companies and for instance, Ionis Pharmaceutical has recently proved that cellular toxicity reflects the tendency of ASO to sequester and relocalize interacting factors, mainly RNA binding proteins (RBP), in unproductive complexes or sub-cellular compartments as nucleoli.

How the Spoke will contribute to advance in this scenario. With the goal to address the main hurdles detailed above, **Spoke #6** has brought together highly qualified scientists with complementary skills and scientific backgrounds to develop tools and strategies aimed at increasing the efficiency and specificity of RNA-based treatments and reduce cellular toxicity. This selected group of scientists includes: 1) molecular biologists active in the RNA field; 2) chemists that will develop innovative tools aimed at improving the specificity of RNA-base drugs; 3) physicists that will set up a new high-resolution imaging microscope to visualize the sub-cellular distribution of therapeutic molecules in living cells. The Spoke will take advantage of Oxford Nanopore Technology (ONT) to generate quantitative and qualitative (splicing and epi-transcriptome) RNA profiles. Many of these activities will take advantage of interactions with other horizontal Spokes.

Spoke 6 is organized in three WPs. WP1 aims to provide multimodal and multi-target approaches to RNA therapeutics by implementing innovative sequencing, imaging and RNA editing technologies coupled with tailored computational and AI analyses. Activities will benefit from the collaboration with **Spoke #7** and will support studies in WP2 and WP3 and in vertical Spokes. RNA editing has recently emerged as a promising therapeutic option and a handful of start-up companies are beginning to use RNA-editing systems. One task of WP1 is the development of programmable RNA editing tools to interfere with specific processes in cancer and genetic diseases and to provide bioinformatic tools to design guide RNAs.
A central task of WP1 is the implementation of direct RNA sequencing technologies towards RNA therapeutics design based on ncRNAs and epitranscriptional control of gene expression. Chemical modifications of newly-synthesized RNA molecules enriching the plain sequence of RNA with a multifaceted layer of functionalities that define the field of epitranscriptomics. Understanding how specific RNA modifications affect RNA-RNA and RNA-protein interactions, RNA subcellular distribution or even the interaction with therapeutic molecules is currently poorly explored. Yet, reverse-engineering these chemical modifications on RNA therapeutics may have disruptive consequences on this new class of drugs. A Technological Unit for DRS (Direct RNA Sequencing) on demand and interpretation of RNA modification profiles will be established to make these analyses more accessible to research groups in **Spokes #2, 3, 4, 5, 7 and 8**.
The third task is the Development of an optical microscopy platform to visualize and quantify efficacy of RNA therapeutics in live cells. This platform, developed in collaboration with Genoa Instruments, a spin-off company from the Istituto Italiano di Tecnologia (IIT), will integrate different imaging modules including fluorescence single-molecule analyses and will exploit AI-based computational approaches to provide high-throughput and high-information content abilities. This task

will provide invaluable insights into the subcellular distribution of target and therapeutic molecules guiding the development of protocols and technological approaches to improve productive uptake and reduce toxicity of treatments.
The goal of WP2 is the development of RNA-based and RNA-targeting molecules for therapeutic uses. This WP aims to design, synthesize and characterize molecules of different complexity with 'beyond-state-of-the art' properties in terms of: i) selectivity, both with respect to the cellular compartment and the desired RNA target ii) in vivo resistance iii) reduced immune response and iv) reduced toxicity. The activities will be developed in collaboration with two Italian Biotech Companies: **IRBM** and **Takis**. One major task is the Development of new RNA-targeting compounds (RIBOTAC, modified ASO, Stereopure Oligonucleotides, PNAs, and hybrid conjugates molecules). ASO modified at the sugar, at the base, at the phosphate linkage and all combinations of the above, will be synthesized and tested for folding, stability, nucleases resistance and interaction with cellular proteins. At the same time, we will study the efficacy of stereopure antisense oligonucleotides (SP-ASO) and we will implement the emerging RIBOTAC methodology to locally recruit endogenous latent ribonuclease (RNase L) to a specific transcript to selectively degrade RNA targets. We will also explore the possibility to use Peptide Nucleic Acids (PNA) that offer unique properties in terms of DNA/RNA recognition and manipulation. The objective is to obtain new PNA structures and nanosystems combining DNA/RNA binding with additional functions that can be programmed by design, leading to pharmacological applications in cancer. In addition, **IRBM** will leverage its screening capabilities and collection of compounds to identify hit molecules capable to bind and modulate therapeutically-relevant RNA target(s). These studies will be developed in collaboration with WP3 and with the **RNA production platform** of **NC** and will generate methodologies beneficial to vertical Spokes.
A great effort will be put on the development of innovative RNA Aptamers in the form of aptamer-drug conjugates/complexes to enhance specificity and decrease off-target effects of drugs. After a careful characterization and appropriate chemically modifications to enhance resistance in vivo, these molecules will be challenged in in vivo assays in collaboration with **vertical Spokes**, particularly **Spoke #2**.
Another qualifying and ambitious goal is the development of 'intelligent' RNA-based and inspired scaffolds, that can be monitored and modulated by exogenous, e.g. light, or endogenous, e.g. pH of the target cellular compartment, stimuli. These goals will be attained also by developing suitable DNA nanocarriers, designed to protect the therapeutic agents and improving their delivery to the desired target. Thanks to the functionalization with suitable chromophores and fluorescent base analogues, we plan to use light for monitoring the interaction with the biological targets, to follow the distribution of therapeutic RNA-based molecules in living cells (in collaboration with WP1) and to control molecular processes as mRNA translation. During this project, we intend to launch dedicated calls to implement additional competencies for the analysis of ASO metabolism. In particular, open calls for groups and biotech companies that develop tools to study ASO biophysical properties, monitor their subcellular distribution (i.e. by generating antibodies against ASO modifications) and impact pathological protein condensates, to systematically characterize their interaction with cellular proteins, and to identify small molecules able to modulate ASO release from late endosomes.
Finally, a major objective is the development of more efficient in vitro mRNA transcription and generation of modified mRNAs with higher efficiency of translation. This activity will be carried out in tight collaboration with **Spoke #8** and with **RNA production platform**. In close collaboration with groups in this Task and in **Spoke #8**, **Takis** Biotech will develop approaches for the production and delivery of optimised chemokine-expressing mRNAs (i.e CXCL9) to harness the immune system to fight cancer.
Thanks to the diversified expertise of the participants, WP2 can exploit complementary techniques (e.g. advanced organic synthesis, imaging, structural and molecular biology, molecular modelling) to develop new approaches/technologies in RNA-based therapies. The strong integration between research groups in addition to increasing the existing know-how in this field, will contribute to build a community of researchers, with intertwined skills and able to tackle the main challenges encountered during the development of RNA-drugs, from in-silico design to their use in vivo. In this spirit, WP2 will exploits the technological platforms developed in WP1, while WP3 will provide the necessary feedback on the most promising cellular targets. The development of methods and chemical modifications to improve productive uptakes, increase target selectivity and reduced off target and toxicity effects will benefit of scientific collaborations with vertical Spokes. At the same time, the know-how generated within this WP will certainly positively impact the activity of vertical Spokes and it is likely to generate patentable discoveries thus setting the bases of commercialization.
The overarching goal of WP3 is the development of molecules and strategies to target lncRNAs and alternative splicing events involved in heterogenous diseases, syndromes, and multi-systemic conditions currently lacking curative options. The knowhow and the products generated represent a launch pad towards their application in clinics and will impact well beyond the individual diseases initially targeted. Drugs will be developed in close collaboration with WP1 and WP2. Moreover, this WP will pursue the identification of novel RNA molecules with therapeutic potential. In this framework one task is the discovery and pre-clinical development of next-generation ncRNAs-based therapies. Methods for miRNA restoration will be set up in ex-vivo systems and represent a step toward innovative RNA-based therapies for leukemias, further developed in collaboration with **Spoke #10**. Along this line, this WP will finalize the discovery, characterization and development of a new toolbox of ncRNAs as i) biomarkers of epi-transcriptional defects in rRNA and t-RNA halves and ii) snoRNA targets

for therapies to correct translational defects in motor neuron diseases as Spinal Muscular Atrophy. This project will greatly benefit from activities in the **Spokes #3 and #7** and, in its later stages, in **Spoke #8**. Another task, carried out in collaboration with WP1 and 2, is the development of molecular tools to target circRNAs and ncRNAs, an activity that will assist the development of innovative RNA-based therapies for cancer. In particular, a CRISPR/Cas13 system will be developed to selectively target circRNAs while sparing the cognate mRNAs. At the same time, to improve the productive uptake of siRNAs against specific lncRNAs, different approaches will be tested including siRNA-ferritin-complexes. Targeting low-abundance chromatin-associated ncRNAs is a challenging task both in term of the accessibility of the target and for harmful effects resulting from the vast excess of therapeutic ASOs. In collaboration with the imaging and RNA sequencing groups in WP1 and with groups in WP2, we will pursue the development and optimization of ASO targeting telomere dysfunction-induced ncRNAs. ASO with different chemical modifications and designs, including HNA (hexitol in place of ribose), RIBOTAC, or a combination of them, will be tested in cultured cells bearing telomere dysfunction. We will determine biophysical properties, stability, cell distribution, nuclear target engagement and efficacy in DNA damage response (DDR) and impact on senescence markers. Finally, in collaboration with **Spoke #8** we will test selected molecules in animal models bearing telomere dysfunction.

In collaboration with **Spoke #7**, we will pursue the development of computational and experimental pipelines to target alternative splicing (AS). Based on RNA sequencing data generated within this WP and by WP1, computational pipelines will be developed to identify alternative splicing (AS) occurrences and predict their effects. Moreover, in collaboration with **Spoke #7**, computational approaches will be set up to design ASO to modulate splicing decisions either by hindering interaction with RBPs or by recruiting specific splicing factors on the pre-mRNA molecule (TOES). As an alternative approach, innovative Cas13-based systems for splicing manipulation will be set up. The efficacy of these methods will be tested on specific gene models such Lamin A and genes involved in tumour neo-angiogenesis. Another challenging task will be the characterization of the impact that chemical modifications or coupling to Aptamers may have on the activity and specificity of splicing regulating ASO. In particular, we will explore the effect of chemical modification for ASO conjugation with Listeria monocytogenes (Lmat) and how this coupling can be released in the intracellular environment.

WP 6.1 – Technological approaches for RNA therapeutics	
General objectives. This WP aims at providing a multimodal and multitarget approach to RNA therapeutics by sequencing, imaging and editing of RNAs coupled with tailored computational and artificial intelligence approaches. The aim is deciphering the complexity of human transcriptome and epitranscriptome by means of technologically advances approaches **Methodology.** Molecular and cell biology methods for RNA editing; Nanopore direct RNA Sequencing and Artificial intelligence analysis; confocal, image scanning, multiphoton, super resolved and label-free CIDS; Image scanning microscopy; fluorescence fluctuation spectroscopy; fluorescence lifetime assay to understand the chemical environment of the RNA therapeutic.	
Task 6.1.1	**Development of programmable RNA editing protocols (Task leader: Pesole - UNIBA)**
TRL	**2→5**
	• Deaminase-based RNA editing to correct pathological mutations (Collaboration Spke #7) • RNA editing to induce synthetic lethality in cancer (Collaboration Spoke #7) • Epigene-RNA Programmable Editing
Task 6.1.2	**Implementation of native RNA sequencing technology (Task leader: Nicassio- IIT)**
TRL	**2→5**
	• Transcriptome and Epitranscriptome profiles by Nanopore direct RNA Sequencing (Collaboration Spoke #7)
Task 6.1.3	**Development of an optical microscopy platform (Task leader: Diaspro- IIT)**
TRL	**1→7**
	• Development of MOMIX (Multimodal Optical Microscopy Image Correlation Sensing) microscope for evaluating the efficacy of RNA therapeutics approaches • High-Throughput Imaging and Spectroscopy Methods to Track RNA Therapeutics Activity in Living Cells
Deliverables WP 6.1.3	D.6.1.1 Benchmarked protocol for Nanopore direct RNA sequencing (M8); D.6.1.2 Validation of the image scanning microscopy architecture (M12); D.6.1.3 Realisation of the MOMIX architecture (M12); D.6.1.4 Molecular and bioinformatic characterization (coll. SP7-Biocomputing) of the effect of ADAR2 silencing (M14); D.6.1.5 Synthetic lethality by programmable RNA editing in cellular models (proof of principle) M16; D.6.1.6 Generation of guide RNAs and SNAP-tagged deaminases (Coll. Spoke #7) (M20); D.6.1.7 Atlas of Cancer Transcriptome by DRS (M20); D.6.1.8 In vitro toxicity and off-targets evaluation (M20); D.6.1.9 Database of synthetically lethal genetic pairs that can be targeted through RNA editing (SP 7) (M24); D.6.1.10 Integrating an AI computational core into MOMIX (M24); D.6.1.11 Fluorescence fluctuation spectroscopy module (M24); D.6.1.12 Characterization of cellular physiological properties following epigenetics remodeling (M26); D.6.1.13 Assessment of the SNAP-tag system, in vitro toxicity and off-targets evaluation (Spoke #7) (M28); D.6.1.14 Nanocompore tool for the detection of RNA modification (M28); D.6.1.15 Technological Unit to provide DRS applications on demand (M32); D.6.1.16 Fluorescence lifetime module (M36); D.6.1.17 Establishing an optimal optical microscopy protocol to follow ncRNA effects (M36) D.6.1.18Assessment of genome-wide alteration of RNA (processing, base modifications and RNA dynamics) following selected inactivation (M36).
Milestones WP 6.1.3	MS6.1.1 Construction of two reporter systems to quantify the efficiency of programmable RNA editing approaches (for A-to-I and C-to-U editing) (M4); MS6.1.2 Coordination with other spokes for the optimal usage of technological infrastructure and computational resource needed for Nanopore DRS and data analysis (M6); MS6.1.3 Design of the MOMIX architecture (M6); MS6.1.4 Selection of programmable RNA editing approaches and optimization of epigenetic technology (M8); MS6.1.5 Design of the image scanning microscopy architecture (M8); MS6.1.6 Integration of the AI module in Momix (M16); MS6.1.7 Characterization of cellular physiological properties following epigenetics remodeling (M24); MS6.1.8 Shared resource

	(website) to support the selection of RNA targets to induce synthetic lethality in cancer cells (M24); MS6.1.9 RNA editing correction in ADAR2 deficient cells, its assessment, in vitro toxicity and off-targets evaluation (M28); MS6.1.10 Assembly of constructs to target 12 synthetically lethal genetic pairs in tumor cells (M28); MS6.1.11 Preclinical applications of DRS on validating compounds or targets against RNA processing or modification pathways (M36); MS6.1.12 Stop tumor growth by RNA-editing triggered cell death in animal cancer models (in collaboration Spoke #2)(M36); MS6.1.13 Generation of statistically robust figures of merits to quantify the efficiency of a specific RNA therapeutics and RNA driven processes (M36)
WP 6.2 Development of RNA- based and RNA-targeting compounds for therapeutic uses	
General objectives. Design, synthesis, selection and characterization of compounds with 'beyond-state-of-the art' properties in terms of: i) selectivity ii) in vivo resistance iii) reduced immune response iv) binding strength to the target v) potentialities of control by external, e.g. light, or cellular stimuli. Optimization of a platform for mRNA therapeutics. **Methodology.** Advanced Organic Synthesis, Selex, Optical Spectroscopies, High-performance Computing, Structural and Molecular Biology, Nanodelivery, Nanosensing. RNA Biology, High-throughput screening.	
Task 6.2.1	**Development of new RNA-targeting compounds to modulate RNA function (Task leader: Sissi- UNIPD)**
TRL	**2→5**
	• Development of chemically modified ASOs targeting lncRNA as novel therapeutic agents • Development of RIBOTACs and Stereopure Oligonucleotides (SP-ASO) • Generation of Smart PNAs for biomedical applications • Development of RNA-based aptamers as innovative therapeutics and drug carriers • Identification of RNA-targeting compounds by High-Throughput Screening of a collection of > 300K small molecules
Task 6.2.2	**Fluorogenic / modulable RNA aptamers and synthetic DNA/RNA nanoscaffolds. Nano-carriers functionalized with aptamers. (Task leader: Improta-CNR)**
TRL	**1→5**
	• Fluorogenic and modulable Aptamers to monitor cellular RNA uptake, dynamics and interactions • Photo-modulable RNA aptamers with therapeutic potential • Development of nuclease-resistant RNA aptamer-based drugs for applications in cancer treatment. • DNA/RNA-based nanoscaffolds functionalized with aptamers to transport RNAs and ASO
Task 6.2.3	**New and more efficient methodology for the synthesis and formulation of chemically modified mRNA (Task leader: Nicosia- UNINA)**
TRL	**1→6**
	• Optimization of a platform for mRNA therapeutics • Development of a mRNA-based gene-therapy approach to improve albumin production in hepatic cells • Development of new vectors for the synthesis of mRNA with optimized 5'- and 3'- UTR regions and signal peptide for intra-tumoral expression and secretion of CXCL9
Deliverables WP 6.2	D6.2.1 Template to guide and inform an unbiased selection of small molecules targets (M4); D6.2.2 Synthesis and validation of PNA conjugates, aptamers targeting specific cell membrane proteins, DNA/RNA nanostructures decorated with different therapeutic cargos (M8); D6.2.3 multi-configurational quantum-chemistry based QM/MM model of an aptamer covalently linked to an organic chromophore (M8); D6.2.4 Development of aptamer-based delivery strategies (M10); D6.2.5 Achievement of an efficiency of modified mRNA in vitro transcription (at least 5mg/ml) (M12); D6.2.6 New *in vitro* transcription vectors for the synthesis of mRNA with optimized 5'- and 3'- UTR regions, codon usage and signal peptide (M12); D6.2.7 Production and characterization of modified ASO (M15); D6.2.8 An *in silico* library of Were-1 aptamer variants to visualize - modulate transcription optically (M14); D6.2.9 Improvement of aptamer thermal stability thought introduction of modified bases (M16); D6.2.10 drug-delivery capabilities of DNA/RNA scaffolds (M16); D6.2.11 In vitro study of LNP-Luc in HEPG2 cells (M16); D6.2.12 A report describing the development and validation of the assays for the screening of small molecules (M16); D6.2.13 4 multifunctional aptamer-decorated and drug loaded nanovectors validated for in vitro targeting (M18); D6.2.14 first generation RIBOTACs and comparison of PS-ASO and SP-ASO for protein binding (M24); D6.2.15 nanocarrier functionalized with antibodies (M24); D6.2.16 Identification of 5'UTR sequences with mRNA in vitro translation enhancing activity of at least 3 fold over wild-type (M24); D6.2.17 functionalizing the optimized lipidic nano formulations with tumor-specific monoclonal antibodies, by Takis biotech. (M24); D6.2.18 Development of nanoparticle-based RNA delivery strategies (SP 8) (M26); D6.2.19 A list of structures and scores of hits identified in the HTS for Target 1 is generated (M26); D6.2.20 joined drug-delivery and imaging capability of DNA/RNA scaffolds in cells (M26); D6.2.21 Characterization of the ASO-lncRNA complex (M28); D6.2.22 optimized PNA/peptide systems with respect to the selected target (M28).
Milestones WP 6.2	MS6.2.1 Development and chemical modification of DNA/RNA-based molecules (M8); MS6.2.2 Synthesis and purification of at least two conjugated PNA for selected targets for in vitro and structural studies (M12); MS6.2.3 Procedure for the synthesis of the multifunctional nano-vectors conjugated with aptamers and loaded with therapeutic RNA (M12); MS6.2.4 Modified nucleoside incorporation for improved mRNA translation and reduced innate immunity activation (M12); MS6.2.5 Generation of prototype cell-based primary assay for HTS (M12); MS6.2.6 Generation of innovative Vectors for mRNA expression (M12); Generation of a collection of 5'UTR RNA sequences with translation enhancement activity by rational design (M16) MS6.2.7 Optimized DNA/RNA scaffolds with imaging capabilities in vitro (M16); MS6.2.8 Setting up of an optimized and scalable purification process for the in vitro transcribed mRNA (M18); MS6.2.9 Analysis of aptamers treatments by targeted and untargeted omics approaches in vitro (M20); MS6.2.10 Miniaturization, automatisation and validation of assays for HTS (M20); MS6.2.11 Demonstration of LNPs-Alb induction of albumin synthesis in HEPG2 cells (M20); MS6.2.12 First generation of an *in silico* library of aptamer-chromophore models (M22); MS6.2.13 The compound collection (about 320K compounds) is screened at a single concentration (e.g. 10 uM) (M24); MS6.2.14 Cytotoxicity evaluation of aptamer-conjugated nanovectors on different cell lines, by MTT (or similar) assays and by biochemical evaluation of drugs' intracellular activities (M24); MS6.2.15 Construction of a library of short unbiased 5'UTR RNA sequences (M24); MS6.2.16 Preparation of the fluorescent aptamer (M24); MS6.2.17 Generation of IVT mRNA libraries encoding pools of innate immunity suppressive genes (M26); MS6.2.18 Transfer of the chemistry to other targets selected by other spokes (M28); MS6.2.19 ASO and PNA optimization (M28); MS6.2.20 Optimization of at least one PNA/peptide nanosystem for selected target for in vitro studies. Structural characterization and biophysical characterization (M28); MS6.2.21 Loading of the nanoparticle with the selected RNA aptamer (M28); MS6.2.22 Identification of a small molecule targeting human RNA via a Ribotac mechanism (M28); MS6.2.23 Demonstration of LNP-Alb induction of albumin synthesis in analbuminemic rats (M28); MS6.2.24 A set of *in silico* designed/predicted optically modulable aptamers with inhibitory activity to be prepared and investigated experimentally (M30); MS6.2.25 Correlation between therapeutic effect of aptamers-nanovectors and tumor-specific targeting in CDX models (in collaboration Spoke #8); MS 6.2.26

	Validation of light-dependent aptamers for the optical control of the protein interaction. (M32); MS6.2.27 RNA segments to increase translation efficiency in vivo (M32); MS6.2.28 Scale Up Synthesis of the best identified small molecule 'hit' for selected target (M32); MS6.2.29 Preliminary SAR studies are performed to increase potency and selectivity of the small compounds (M36); MS6.2.30 Experimentally tested light-dependent aptamers for the optical control of the interaction between inhibitor and target (M36); MS6.2.31 Identification of IVT-vectorized genes able to enhance reporter gene translation by inhibiting innate immune response to IVT mRNAs (M36).
WP 6.3 - Test-cases and identification of novel therapeutic RNA molecules	
General objectives. 1) Identifying new therapeutic ncRNAs impacting translation/epi-transcriptome and developing miRNA restoration approaches; 2) new strategies to target lncRNA; 3) Validation of tools developed in WP2 to target telomeric RNA and alternative splicing; 4) developing pipelines for splicing regulation together with Spoke #7; 5) collaboration with vertical spokes. **Methodology.** SELEX, genome wide splicing profiles, epi-transcriptome analysis in collaboration with WP1, cutting-edge positional sequencing, computational approaches in collaboration with Spoke #7, miRNA and siRNA cellular transfection, LC-MS/MS, ASO chemical modification in collaboration with WP2, know-how transfer to vertical spokes.	
Task 6.3.1	**Approaches for ncRNA restoration toward the development of innovative RNA-based therapies (Task leader: Viero- CNR)**
TRL	**2→5**
	• Next-generation of non-coding RNAs-based biomarkers and therapies for correcting epi-transcriptional and translational defects in genetic diseases (Collaboration with Spoke #8) • microRNA restoration approaches for RNA-based therapies (Collaboration with Spoke #8)
Task 6.3.2	**Development of targeting approaches specific for oncogenic lncRNAs and circRNAs (Task leader: Cimmino-CNR)**
TRL	**1→5**
	• Nanoferritins for small RNA-based targeting of oncogenic lncRNAs • Development of circular RNA targeting approaches: the T-LGLL model (Collaboration with Spoke #10)
Task 6.3.3	**Development and optimization of ASO targeting telomere dysfunction-induced ncRNA for the treatment of human telomere-driven diseases (Task leader: d'Adda di Fagagna - CNR)**
TRL	**2→5**
	• Development of innovative alternatives to ASO for targeting telomeric RNA in telomere-dysfunction diseases
Task 6.3.4	**Development of computational and experimental pipelines and innovative ASO to target alternative splicing (AS) (Task leader: Ghigna - CNR)**
TRL	**2→4**
	• SMART (Splicing Modulation by Advanced RNA Technologies): Splicing modulation by means of Bifunctional ASO (TOES) and Cas13 approaches • Targeting vascular restricted alternatively spliced isoforms by ASO conjugated to aptamers, L-Mat and other modification developed in WP2
Deliverables WP 6.3	D6.3.1 Panel of siRNAs against oncogenic lncRNAs (M4); D6.3.2 Splicing modulating ASO(s) for proof of principle experiments to the needs of the vertical Spokes #1-5 (M4); D6.3.3 Set up of miR-146b transfection in *ex-vivo* T-LGL (M8); D6.3.4 RNA-Seq identification of deregulated circRNA in T-LGL (M8); D6.3.5 ASO targeting binging sites for specific AS regulators, and TOES in vitro studies (M8); D6.3.6 ATLAS of epitranscriptional targets, identification phase in vivo models of SMA (2'O-Me sites) and computational pipeline for identification of snoRNA guiding epitranscriptional modifications in SMA (M12); D6.3.7 Stable siRNA-ferritin complexes (M12); D6.3.8 Testing ASO with different chemistries against DNA damage induced Telomeric RNA (M12); D6.3.9 Pipeline to retrieve RNA consensus sequences for specific RBP, (coll. Spoke #7) (M12); D6.3.10 Effect, localization and *in vitro* efficacy of Smart-biRNA to modulate splicing (M18); D6.3.11 Setup of the CRISPR/Cas13 system and design guide RNAs to target at least 10 selected circRNAs in vitro (M20); D6.3.12 Procedures for synthesis of the multifunctional aptamer-coniugated and ASO-loaded nanovector for splicing regulation (M20); D6.3.13 Transcriptomic changes upon miR-146b restoration in *ex-vivo*T-LGL (M22); D6.3.14 nc-RNA based strategies for validation phase to restore translational defects in *in cell* models of SMA (M24); D6.3.15 siRNAs ferritin complexes-repressing oncogenic lncRNAs in cancer cell (M24); D6.3.16 Testing anti-telomeric ASO with different designs (M24); M6.3.17 Smart-Cas expression vector for splicing regulation (M28); D6.3.18 Chemical conjugation of the linker with the ASOs to generate a Lmat-ASO (M32); D6.3.19 Functional readout of circRNA-silenced in T-LGLL-like cell (M36); D6.3.20 Study of biophysical properties of ASO targeting telomeric RNA (M36).
Milestones WP 6.3	MS6.3.1 Epitranscriptional target identification phase in vivo models of SMA (2'O-Me sites) (M8); MS6.3.2 ASO(s) effects on selected -splicing events are defined and validated by in vitro experiments (M8); MS6.3.3 Test and selection of effective siRNAs against lncRNAs (M8); MS6.3.4 Validation of splicing modulating ASO in *in vitro* experiments in ECs (M8); MS6.3.5 Identification of LNA-based ASO against telomeric RNA (M10); MS6.3.6 Development of siRNA-ferritin complex synthesis process (M12); MS6.3.7 Evaluation about catalogues of rRNA epitranscriptional target site – identification phase (2'O-Me sites and nc-RNAs) (M12); MS6.3.8 Smart-biRNA(s) effects on selected splicing events are defined and validated by in vitro experiments (M18); MS6.3.9 miR-146b restoration obtained by direct transfection in *ex-vivo* T-LGL (M18); MS6.3.10 Identification and validation of circRNA- dysregulated in T-LGLL subtypes (M18); MS6.3.11 Prioritization phase for tRNA-halves targets (M20); MS6.3.12 Chemical-physical characterization of specific siRNA-ferritin complex (M20); MS6.3.13 Synthesis of aptamers conjugated with splicing modulating ASO-loaded nanoparticles (M20); MS6.3.14 Design of Smart-Cas vector(s) for splicing modulation (M24); MS6.3.12 In vitro molecular assays for siRNA-ferritin silencing activity on lncRNAs (M24); MS6.3.15 Evaluation of the miR-146b restoration effects by transcriptomic, proteomic and functional analyses (M24); MS6.3.16 Setup of the CRISPR/Cas13 system for specific circRNA targeting in model cell lines (M24); MS6.3.17 Functional assays for modulation of cell tumorigenic properties upon siRNA-ferritin administration (M28); MS6.3.18 nc-RNA based strategies for validation phase in *in cell* models of SMA (M32); MS6.3.19 Determination of comparative efficacy among telomeric RNA targeting ASO (M30); MS6.3.20 Epitranscriptomic studies on the effects of selected protocol for splicing modulation (M34); MS6.3.21 Evaluation of pre-clinical phase of candidate biomarkers and nc-RNA based therapies in animal models (M36); MS6.3.22 Development of engineered carriers for targeted miR-146b delivery to *ex-vivo* T-LGL within a leukocyte pool and demonstration of the effective miR-146b restoration in the *ex-vivo* system (M36); MS6.3.23 *In vitro* validation of aptamer -ASO and Lmat-ASO (M36).

Spoke # 7: Biocomputing. Spoke leader UNIBA.

State of the field and unmet needs. The extraordinary technological advances that led to the development of novel, massively parallel and increasingly cost-effective high-throughput technologies (e.g. massive nucleotide sequencing, single cell analysis) have revolutionized biomedical research leading Biology in the era of "Big Data". The unprecedented ability to profile biological systems in a quantitative and accurate manner, however, is creating a pressing need to bridge the gap between high-throughput technological development and our ability for managing, analyzing, and integrating biological big data. Massively parallel assays are now routinely applied to investigate the mutational, transcriptional, epigenetic, and proteomic landscapes of healthy and pathological samples. Moreover, recent advances in single-cell techniques offer the unprecedented opportunity to disentangle tissue complexity and investigate cell identities and functions within tissues. Several are the gaps to be filled to optimize the capacity to transform collections of multi-layered data and patients' characteristics into models of cellular functions and diseases, and then into effective products for the healthcare market as, for example, new RNA-based therapeutic products and innovative approaches for gene therapy.
Concerning the exploitation of DNA- and RNA-based therapeutic approaches, no efficient algorithms for designing reliable guide RNAs for large-scale CRISPR-Cas and RNA-based approaches are available and the direct detection of nucleotide chemical modification through the analysis of third generation sequencing platforms is still at infancy. The prediction of 3D RNA folding is also still quite an immature field as computational modeling for RNA still suffers from significant limitations related to accurate conformational sampling and adequate potentials for extensive and reliable simulations. In addition, the difficulty of experimentally obtaining the structure of RNA and its complexes with biomolecules of therapeutic of interest, in particular, through the techniques of X-ray spectrometry or nuclear magnetic resonance (NMR), is still today one of the most limiting factors in the design and development of new RNA drugs. The improvement of computational methodologies for the accurate prediction of the RNA structure, and its complexes, represents today one of the most promising approaches to respond to this need in timescales that are competitive with those of drug development. Finally, a very relevant largely unmet issue is the development of computational approaches for AI-based drug discovery and multidimensional data integration (e.g. study of intracellular effects of RNA drugs exploiting 'omics and pathway analysis) cross-correlating molecular, clinical and personal lifestyle data. This requires a big effort in data and methods FAIRification to guarantee robustness and reproducibility of predicted inferences.
How the Spoke will contribute to advance in this scenario. Spoke #7 will provide a dedicated infrastructure, fully integrated in the framework developed by the National Center for HPC, Big Data and Quantum Computing, with a state of the art ecosystem of data and tools supporting the activities of **the vertical Spokes**. This integrated bioinformatic environment will play a dual role: on the one hand, providing data-driven hypothesis generating insights and testable hypotheses on novel classes of targets (such as unannotated genes or novel transcriptional isoforms), and on the other hand, our team will intervene in the fine tailoring of an RNA-based therapeutics, pinpointing to what ideal RNA sequence to be used as therapeutics (in siRNA, ASO and mRNA applications) in constant iteration between experimental and biocomputing predictions. The work will develop in 4 parallel WP. In WP7.1, a rich portfolio of data and tool resources will be implemented or suitably developed to establish a cutting-edge work environment in the computational infrastructure also implementing new algorithms for sequence and structural data analysis, to support the research activities of all other Spokes. These will include tools for genomic, transcriptomics, epitranscriptomics, epigenetic and proteomic analyses also at single cell resolution. WP7.2 will be devoted to the identification of neo-antigens for mRNA vaccinology carried out through large-scale epitranscriptomics profiling. An integrated computational pipeline will be created to identify Alternative Splicing (AS) from RNA-seq and infer integrating information of epitranscriptomic modifications detectable by Nanopore data analysis. AS and RNA editing (RE) provide novel potential targets for vaccines that may vastly outnumber mutational cancer neoantigens and may represent a largely unexplored pool of antigens in non-tumoral disease settings, including auto-immunity. WP7.2 also plans to develop a multiomics platform for the prediction of tumor neoantigens. The goal of WP7.3 is the development and application of informatics/computational methodologies to support the design and industrial development of RNA drugs in different therapeutic areas of the vertical Spokes. This will include a reiterated computational and experimental validation interplay aiming at the accurate prediction of the tertiary structure of native and chemically modified RNA and of their target. This can be achieved by optimizing Molecular Dynamics simulations, able to explore the conformational space in solution of native and chemically modified RNA and the interaction with small organic molecules, proteins, oligonucleotides and membrane systems. The integration with docking simulation data and Cryo-EM maps remarkably improves the accuracy and resolution of inferred functional-structural relationships. WP7.4 is dedicated to the development and application of data integration strategies focused on the reconstruction of regulatory networks also at single-cell resolution, the development of prioritization strategies for drug targets and actionable disease features, to patient stratification and identification of disease vulnerabilities.
The activities of **Spoke #7** will be fully integrated with those of the other vertical and horizontal Spokes. In particular, we plan to launch an internal call for "on-demand bioinformatics services" and "web-based bioinformatics platforms" to make the **NC** bioinformatics ecosystem fully compliant to project needs. Some relevant interactions have been already planned, e.g. with Spoke #6 for designing programmable RNA editing enzymes, with **Spokes #2** and **#3** for their validation in cancer and neurodegeneration, and with **Spoke #10** for optimizing biosafety in cell-based immunotherapy approaches. Concerning

the planned interactions with private companies a collaborative action with **Intesa San Paolo Innovation Center** has been planned for implementing a smart IT platform for genomic surveillance during viral outbreaks. The private Partner Intesa Sanpaolo will support this Spoke with Intesa Sanpaolo Innovation Center (ISPIC), an Intesa Sanpaolo subsidiary born with the mission of exploring and assimilating new future business models, with a strong focus on Circular Economy and Digital Transformation. In particular ISPIC has developed with the University of Milano and the Sacco Hospital a smart IT platform for SaRS-CoV-2 genomic surveillance. The idea is to implement the performances of this platform for other possible viral outbreaks and open it to the scientific community.

WP 7.1 – Setup and development of the bioinformatics environment for omics data analysis	
General objectives. To establish a secure and FAIR data oriented IT infrastructure adopting state-of-the-art computing and storage solutions and to provide production-ready and interoperable software solutions. Development of a complete bioinformatics framework for the identification of potential RNA-therapy targets in different disease models, also at the single cell level, and of a platform for high-throughput CRISPR screens. Development of a complete bioinformatics framework for the comparative genomics of emerging pathogens and of predictive methods for the identification of variants/strains of epidemiological interest. **Methodology.** We plan to establish a dedicated IT infrastructure at the datacenter ReCaS-Bari already hosting the ELIXIR-IT compute facility. New IT resources will be deployed that will be used to host both an on-demand and a web-based platform for the analysis of omics datasets. The underlying software environment will have a modular structure to allow scientists in this Spoke and from other Spokes to contribute new tools and data resources. The developed framework will adopt data security solutions and promote the FAIRification of data, according to the "as open as possible, as closed as necessary" paradigm. Novel algorithmic solutions will be developed and their implementation in an integrated suite of tools including CRISPR screenings and computational methods for the prioritization of epidemiologically relevant variants/lineages of emerging pathogens.	
Task 7.1.1	**Implementation of the Spoke computational environment with relevant data and tools (Task leader: Fosso-UNIBA)**
TRL	**6→9**
	• Hardware installation, testing and deployment • On-demand bioinformatics services platform • Web based bioinformatics platform
Task 7.1.2	**New algorithms and software for sequence and structural data analysis (Task leader: Pavesi-UNIMI)**
TRL	**3→4**
	• Development of a complete bioinformatics framework for the identification of potential RNA-therapy targets in different disease models, also at the single cell level • Development of a platform for high-throughput CRISPR screens
Task 7.1.3	**Smart platform for the genomic surveillance of emerging pathogens (Task leader: - Chiara-UNIMI)**
TRL	**4→9**
	• Development of an IT ecosystem for implementing algorithms and software for viral genomes identification, assembling and functional characterization (e.g. infectivity, immune escape and pathogenicity assessment) • Update of RNA vaccines formulation
Deliverables WP 7.1	D7.1 - Call for tenders for compute and storage resources (M6); D7.2 - First release of the documentation of the call for on-demand bioinformatics services (ODBS) and web-based bioinformatics platforms (WBB) (M6); D7.3 - First release of the ODBS platform on existing IT resources (M12); D7.4 - Open call for new ODBS and WBB applications from external contributors (M12); D7.5 - Implementation of state of the art methods and pipelines for sequence and structural data analysis (M12); D7.6 - Integration of a software suite for the analysis of transcriptome and epitranscriptome data also at single cell resolution (M18); D7.7 - First release of the WBB on existing IT resources (M18); D7.8-Implementation and initial testing of the novel algorithms for sequence and structural data analysis (M18); D7.9- Critical assessment of state of the art methods and pipelines; D7.10-Implementation of novel algorithms for pathogen comparative genomics and classification (M18); D7.11 - IT resources deployment and test (M24); D7.12 - IT resources in production (M24); D7.13 - Integration of tools developed or required by project partners in the ODBS and/or WBB (M24); D7.14-Implementation of novel algorithms for the prioritization of epidemiologically relevant variants/lineages (M24); D7.15 - Production release of the WBB and ODBS platforms (M30); D7.16 - Large-scale testing and final software implementation of the integrated computational analysis framework (M36); D7.17 - A platform for high-throughput CRISPR screens and computational approaches aimed at target identification and for the prediction of efficiency and safety (M36) D7.18 – Implementation of a smart IT platform for supporting genomic surveillance of viral outbreaks (M36); D7.19- Smart platform for the genomic surveillance of emerging pathogens (M36).
Milestones WP 7.1	M7.1 - Starting date of the call for tenders - IT platform (M1); M7.2 - Closing date of the call for tenders - IT platform (M6); M7.3 - First release of the on-line documentation of the ODBS and the WBB platforms with specifics about the activities and the requirements necessary to integrate new software units and software components into the platforms (M6); M7.4 - Open call preparation for ODBS and WBB (M6); M7.5 - Release of the living document describing the general design of the ODBS software architecture (M6); M7.6 - Release of the living document describing the general design of the WBB software architecture (M6); M7.7 - Design of a software suite for the analysis of transcriptome and epitranscriptome data also at single cell resolution (M12); M7.8 - Installation, configuration and testing of the WBB platform (M12); M7.9 - Installation, configuration and testing of the ODBS platform (M12); M7.10 - Final date for the delivery of compute and storage resources (M12); M7.11 - Technical and scientific evaluation of new dedicated services or bioinformatics tools for future integration in the ODBS and WBB platforms - collection of partner agreements; closing date of the call (M15); M7.12 - Production release of a software suite for the analysis of transcriptome and epitranscriptome data also at single cell resolution (M18) M7.13 - Evaluation of tools developed or requested by project partners for future integration in the ODBS and/or WBB platforms (M18); M7.14 - Installation and testing of compute and storage resources (M18); M7.15 - Integration and testing of the tools developed or requested by project partners in the ODBS and/or WBB platforms (M21); M7.16-Final implementation of novel algorithms for pathogen comparative genomics and classification (M21); M7.17 - Public release of the ODBS platform (M24); M7.18 - Public release of the WBB platform (M24); M7.19 - Medium size dataset for robust CRISPR target identification (other contributors (M24); M7.20 - Computational algorithms for CRISPR screen analysis (M24); M7.21 - Production release of the new tools in the ODBS and/or WBB platforms with documentation (M30); M7.22 – Design a smart IT platform for supporting genomic surveillance of viral outbreaks (M30); M7.23-Final implementation of novel software tools for the prioritization of epidemiologically relevant variants/lineages (M30); M7.24 - Final software implementation of the integrated computational

	analysis framework with documentation (M36); M7.25 - High-throughput CRISPR screen datasets and predictive algorithms (M36); M7.26-Release of the Smart platform for the genomic surveillance of emerging pathogens.
Milestones WP 7.2 Bioinformatics for the identification of neo-antigens for mRNA vaccinology	
General objectives. Development of computational workflows integrating short and long high-throughput sequencing read data for the identification of alternative-splicing and RNA-editing driven neo-antigens. Development of bioinformatics tools for profiling transient and non-transient RNA modifications through direct RNA sequencing data analysis. Development of novel methods for the identification of tumor MHC-I and MHC-II binding epitopes originated by somatic mutations. **Methodology.** Quality check and alignment of short and long reads for generating lists of known and novel splice junctions. Further read assembly to generate full-length transcripts and detect novel splice junctions. Screening of novel transcripts for putative proteins and their analysis by consolidated and novel algorithms for predicting neopeptides. Profiling of recoding RNA editing events for a list of novel putative proteins employed for neopeptides detection. Neopeptides validation by mass spectrometry. Directed RNA reads generated by the Oxford Nanopore technology will be base called using well consolidated software and quality checked. RNA modifications will be identified through the analysis of base-called 'error' features. The identification and prioritization of patient-specific tumor neoantigens through NGS profiling tumor/blood DNA and tumor RNA and neoantigen identification by machine-learning methods predicting the HLA-binding affinity of mutated epitopes.	
Task 7.2.1	**Development and applications of bioinformatics tools for the identification of alternative splicing or RNA editing driven neoantigens (Task leader: Pesole-UNIBA)**
TRL	**2→8**
	• Novel algorithms for neoantigens detections based on alternative splicing and RNA editing • Bioinformatics workflows for alternative splicing or RNA editing driven neoantigens
Task 7.2.2	**Development and applications of bioinformatics tools for epitranscriptomics profiling and the identification of RNA chemical modifications through direct RNA sequencing (Task leader: Picardi-UNIBA)**
TRL	**4→8**
	• Novel machine learning algorithms for detecting RNA chemical modifications • Optimized computational workflows for epitranscriptomics profiling
Task 7.2.3	**Development and applications of a multiomics platform for the prediction of tumor neoantigens (Task leader: Ceccarelli-UNINA)**
TRL	**3→5**
	• Computational platform for patient-specific tumor neoantigen prioritization
Deliverables WP 7.2	D7.20 Implementation of state of the art software for alternative splicing and RNA editing driven neoantigens (M6); D7.21 Implementation of state of the art software for RNA modification calling by direct RNA sequencing (M6); D7.22 Identification of alternative splicing and RNA editing driven neoantigens (M12); D7.23 Novel algorithms for detecting post-transcriptional RNA modifications by direct RNA sequencing (M12); D7.24 Optimized bioinformatics pipelines for RNA modifications by direct RNA sequencing (M24); D7.25 - Suite of tools for multiomics prediction of tumor neoantigens (M30)
Milestones WP 7.2	M7.26 Assessment of state of the art software for the identification of alternative splicing and RNA editing driven neoantigens (M3); M7.27 Assessment of state of the art software for RNA modification calling by direct RNA sequencing (M6); M7.28 List of predicted alternative splicing and RNA editing driven neoantigens (M12); M7.29 Novel software for detecting post-transcriptional RNA modifications by direct RNA sequencing (M12); M7.30 Optimized bioinformatics workflows and documentation for RNA modifications by direct RNA sequencing (M24); M7.31 Identification of patient-specific high quality tumor neontigens for a large cohort of patients in selected indications (M36).
WP 7.3 - Design and development of RNA drugs in different therapeutic areas	
General objectives. Development of computational approaches (i) to characterize RNA systems structure, dynamics and mechanism; (ii) to predict the interaction of small molecules/peptides with the RNA and to identify novel RNA-based drug candidates; (iii) to unravel the catalytic mechanism of RNA and RNA-based machines and to refine their cryo-EM structures. **Methodology.** Molecular simulations, based on force field and quantum mechanical methods, and machine learning methods to investigate the dynamical and catalytic properties of RNA/ RNA-based machines; Molecular docking and dynamics simulations to inspect the recognition pathways of small molecule, DNA/RNA oligos, and RNA-based drugs	
Task 7.3.1	**Molecular Dynamics simulations to explore the conformational changes of RNA and RNA-based machines (Task leader: Magistrato-CNR)**
TRL	**2→6**
	• Molecular dynamics protocols to infer secondary and tertiary structure of RNAs and their interactors. • Computational protocols for exploiting and refining of cryo-EM maps and for functional annotation of RNA-based machines components
Task 7.3.2	**RNA-protein /ligand molecular docking and dynamics simulations to identify new drug candidates (Task leader: Moro-UNIPD)**
TRL	**2→6**
	• Molecular docking and molecular dynamics simulation to inspecting of the structure and energetic profile of small molecules-RNA, protein–RNA and RNA–RNA complexes. • Recognition pathways of small molecules-RNA, protein–RNA, and RNA–RNA complexes using supervised molecular dynamics simulations.
Task 7.3.3	**RNA quantum enzymology and analysis of cryo-EM maps for resolving RNA structures and interactomes (Task leader: DeVivo-IIT)**
TRL	**3→6**
	• Quantum mechanical protocols to infer the mechanism of RNA-based catalysts • Computational protocols for the refinement of cryo-EM maps and improved functional annotation of specific components of RNA-based machines
Deliverables WP 7.3	D7.26 Quantum mechanical protocols to infer the mechanism of RNA-based catalysts (M12) D7.27 Molecular dynamics protocols to infer secondary and tertiary structure of natural and chemically modified RNA and potential interactors (M15); D7.28 Improved Force fields to assess the characterization of functional natural and chemically modified RNA molecules (ribozymes, riboswitches) and RNA-based machines (RNA polymerase, spliceosome, 3'End processing machinery, RNAse P

	and RNAse MRP) or proteins participating in RNA metabolism (Argonaute, Dicer) (M18); D7.29 Computational protocols for exploiting and refining of cryo-EM maps and improved functional annotation of specific components of the spliceosome and other RNA-based machines (M18); D7.30 New reliable computational protocol based on standard and enhanced sampling simulations to refine cryo-EM maps in conformationally heterogeneous regions solved at low resolution (M18); D7.31 Design and implementation of molecular docking in tandem with molecular dynamics simulation to inspecting the structure and energetic profile of small molecules-RNA, protein–RNA and RNA–RNA complexes (M18); D7.32 Recognition pathways of small molecules-RNA, protein–RNA, and RNA–RNA complexes using supervised molecular dynamics simulations (M18); D7.33 Setting up supervised molecular dynamics simulations (force field and supervision parameters, sampling strategies, trajectories clustering, and analysis) to trace and analyze the recognition pathways of the most promising RNA-based drug candidates (M18); D7.34 Computational protocols for the refinement of cryo-EM maps and improved functional annotation of specific components of RNA-based machines (M18); D7.35 Optimized molecular docking protocols (sampling methodologies and scoring functions) to accurately sample and rank docking poses of small molecules-RNA, peptides-RNA, protein-RNA, and RNA-RNA complexes(M24); D7.36 Selection of the most promising RNA-based drug candidates (M36)
Milestones WP 7.3	M7.32 Optimization of the force field parameters and development of a computational workflow for determining the stability and conformational space for RNA in native and chemically modified form, dissecting the molecular basis of gene expression/regulation and splicing alterations (M24); M7.33 Optimized computational workflow based on a hybrid molecular docking and dynamics approach for determining the structure and energetic profile of the possible complexes in solution and, in particular, inspecting the possible time-dependent recognition pathways among small molecules, protein, and DNA/RNA oligos, and RNA-based drug candidates (M18); M7.34 Optimized hybrid quantum mechanical and molecular mechanical codes and protocols, and software to study catalytic RNAs (M12); M7.35 Classical and Mixed quantum-classical methods optimized to address the challenging study of large RNA-based catalysts or proteins involved in RNA metabolism (M18); M7.36 Rational design and engineering of RNA-based enzymes to be used as gene editing tools. Unraveling the catalytic mechanism of RNA-based enzymes will aid in their engineering as gene editing tools and fore gene specific therapeutics (M30); M7.37 List of potential drug candidates to tackle pathogenesis related to RNA metabolism (cancer, neurodegeneration genetic diseases) (M36)

WP 7.4 – Computational Systems Biology and integrative data analysis

General objectives. Design and Development of cutting-edge Computational Biology and Artificial Intelligence technologies to support the research activities across the Spokes of the overall project. Development of reference models of gene regulatory network in disease settings and other biological context of interest to other tasks. Development of AI-based software tools to identify and prioritize targets of RNA drugs and predict their effects. Development of integrative tools leveraging complex multi-omics datasets to perform patient stratification and identification of disease vulnerabilities. Development of predictive approaches to characterize how mutations alter the network of genome-wide regulatory contacts of genes with their enhancers.

Methodology. The research activity will leverage state-of-the-art AI-based methods for Network Medicine, novel cutting edge Graph Representation Learning algorithms, Graph Neural Networks and Relational Learning algorithms to support target identification, RNA drug discovery and to construct a heterogeneous biomedical graph integrating massive data from public repositories with those generated during the project. We also plan to implement novel methods to predict how disease-associated large mutations rewire the regulatory contacts of genes expanding proprietary computational technologies based on Machine Learning, computer simulations and the Physics of Complex Systems to infer the 3D structure of chromosomes.

Task 7.4.1	**Implementation of integrated data analysis models for the reconstruction of regulatory networks (Task leader: Helmer-Citterich-Uniroma2)**
TRL	**3→7**
	• Software pipeline for inference of gene regulatory networks from publicly available data at scale • Reference regulatory networks in relevant biological contexts • Candidate targets for RNA-based therapeutics
Task 7.4.2	**Prioritization of drug targets and actionable disease features through Knowledge Graphs and Artificial Intelligence approaches (Task leader: Valentini-UNIMI)**
TRL	**3→6**
	• Construction of a Knowledge Graph for RNA drug analysis • Graph Representation Learning, Graph Neural Network, Relational learning and Transfer Learning methods for node label and edge prediction problems in heterogeneous graphs, tailored to RNA drug-target prediction • Software platform for the analysis of RNAd-Knowledge Graph to support RNA drug discovery
Task 7.4.3	**Development of network-based multi-omics data integration tools for patient stratification in cancer and other diseases (Task leader: Ceccarelli-UNINA)**
TRL	**3→5**
	• Software tools for multi-omics patient stratification
Task 7.4.4	**Dissecting the role of chromatin 3D architecture in genetic diseases for the design of personalized treatments (Task leader: Nicodemi-UNINA)**
TRL	**2→5**
	• Analysis of Hi-C and multiplexed microscopy data to infer and inform models • Development of structural models to understand gene activity at the single-cell level
Deliverables WP 7.4.4	D7.37 Curated database of publicly available gene expression and CHIP-seq data relevant for disease and biological contexts of interest to other tasks (M12); D7.38 First release the RNA-drug Knowledge Graph (RNAd-KG) (M12); D7.39 Analysis of Hi-C and multiplexed microscopy data (M18); D7.40 Candidate reference networks in biological contexts of interest (M24); D7.41 Deployment of the first release of the AI-based software platform for the analysis of RNAd-KG (M24); D7.42 Software workflow for integrative data analysis for the reconstruction of cellular regulatory networks (e.g. Adaptive Multiple Sparse Regression) (M30); D7.43 Design and implementation of graph kernel based methods and integrative message passing-based cross diffusion approaches for the integration of multi-omics data (M30); 7.44 Network-based multi-omics data integration tools for patient stratification and identification of disease vulnerabilities (M30); D7.45 Final release of the AI-based software platform for the analysis of RNAd-KG (M36); D7.46 Shortlist of prioritized drug targets and clinically actionable biological pathways (M36); D7.47 Development of structural models to understand gene activity and RNA transcription at the single-cell level (M36)
Milestones WP 7.4.4	M7.38 Design of AI Graph Representation Learning methods for RNA drug analysis and discovery (M12); M7.39 Selection of datasets of interest, based on the work of other tasks (M12); M7.40 Development of models from Hi-C data (M18); M7.41 Design of Graph Neural Network, Relational learning and Transfer Learning methods for RNA drug analysis and discovery (M24); M7.42 Curation of datasets of interest (M24); M7.43 Multi-omics homogeneous subtypes and disease vulnerabilities in pan-

	cancer studies (M24); M7.44 Software implementation of Adaptive Multiple Sparse Regression at scale (M30); M7.45 Novel small molecules or RNAs as candidate drugs targeting the RNA components of the investigated cellular systems (M36); M7.46 Finalized analysis of dataset of interest (M36); M7.47 Identification of molecular targets associated to specific diseases (M36); M7.48 Identification of novel RNAs as candidate drugs (M36); M7.49 Identification of structural models of chromosomes (M36)

Spoke # 8: Platform for DNA/RNA delivery. Spoke leader UNINA.

State of the field and unmet needs. A major obstacle in developing and applying nucleic acid (NA) therapeutics and gene therapies lies in the lack of efficient delivery technologies that, besides preserving their fragile structure in the biological environment, allow efficient delivery of the therapeutic cargo to the target of interest. The challenge for NA delivery resides in the dimensions of these macromolecules, their negative charge and their hydrophilic nature that prevent cell uptake. Viral technologies, preferentially applied to gene therapy, have been developed with alternate success. Viral gene therapies are often limited by pre-existing immunity, viral-induced immunogenicity, unwanted genomic integration, payload size constraints, the inability to re-dose, complications involved in upscaling, and expensive vector production. Also, most synthetic, chemical-based NA vehicles have suffered shortcomings in practical applications, from inefficient delivery to precision targeting, together with a trade-off between cargo protection before and unloading of nucleic acids after arriving at the destinations inside cells. Indeed, for a long-time, the first and foremost reason for clinical trial termination of RNA therapeutics has been the lack of efficiency.

For the real success of RNA therapeutics, a paradigm shift is needed to reshape the delivery strategies on interactions with the biological environment and regulatory constraints. The advances of chemical delivery strategies are at the base of the success of Patirisan, a drug approved for clinical use in 2018 that moved the field of RNA delivery towards lipid nanoparticles (LNP) as a suitable strategy to target hepatocytes. COVID-19 mRNA vaccines have finally proved the commercial potential of nanotechnology-based products as promising for treating a range of diseases. What is now needed are novel nano-technological and biological systems able to deliver RNA to organs different than the liver.

How the Spoke will contribute to advance in this scenario. The successful application of NA-based therapeutics requires an unprecedented interdisciplinary approach, including technical advancement in chemistry, nanotechnology and pharmaceutical technology, imaging and microscopy, molecular biology, and pharmacology. **Spoke # 8** has the ambition to respond to the current and future needs of NA delivery, collecting the top expertise available in Italy. The Spoke involves 106 participants from 10 institutions (UNINA, CNR, IIT, UNIBIC, UNIMI, UNIPA, UNIPI, UNIPD, UNITO, UNISA) selected based on their consolidated expertise in drug delivery technologies demonstrated by the massive impact of their publication track records, patent filing and transfer of knowledge to private companies. The strategic collaboration with 5 Private Companies (Antares, Chiesi, Dompé, Stevanato, Takis) will enable the Spoke to identify opportunities for the transfer of knowledge from the Academia to industries and develop applicable and scalable solutions.

The research infrastructures will be further expanded to meet the needs of this ambitious challenge and fill the technological gap toward international standards. We have the ambition to develop a novel generation of delivery technologies and speed up the translation of therapeutic concepts proposed in **NC** vertical Spokes in pharmaceutical products. We also aim to advance knowledge in pharmaceutical nanotechnologies by discovering novel functional materials, strategies of nanoparticle engineering toward precision delivery, the development of human-relevant models to assess the efficiency of delivery and bottom-up manufacturing technologies. Since we will cooperate closely with industrial partners considering the current regulatory aspects of investigational new drugs, we envisage removing specific roadblocks to nanomedicine product development and supporting/accelerating the clinical development of promising nanomedicine candidates.

Scientific plan. We plan to build a high-throughput discovery pipeline for NA delivery platforms with a research workflow facing the most challenging steps in the nanomedicine discovery pipeline. We plan to develop:

- delivery platforms providing a functional PoC of efficient transfection, safety and extrahepatic targeting and ready to be applied to a therapeutic concept (TRL 2-4);
- biological models recapitulating the disease complexity and reducing the use of animals to assess the safety and efficiency of delivery concepts (speed up of projects toward TRL 5 in close collaboration with **Spoke #9 and vertical Spokes**);
- novel technologies and tools to fabricate and characterize nanoplatforms and finished products fulfilling regulatory requirements (speed up of projects toward TRL 7).
- novel technologies based on omics sciences, nanoscopy and sensing to understand delivery platform properties fully.

We also foster platforms with a demonstrated therapeutic potential toward the clinic path (TRL 3-5);

The activities are organized in 3 WPs, each containing specific tasks and led by an expert in the field. In **WP 8.1**, we will generate a large library of cutting-edge delivery platforms based on non-viral and viral technologies. Novel synthetic materials (lipids, polymers) and nanoplatforms for efficient NA delivery to specific cell populations, novel concepts for the precision delivery of NA to a diseased site in the body (solid tumors, CNS) and surface-engineered viral delivery systems (AAV and oncolytic virus) will be developed. A significant effort will be made to identify novel precision platforms targeting cells (cancer cells, nervous cells, macrophages) and specific areas in the body (solid tumors, SNC, inflamed sites),

which will require developing novel methodologies for the surface engineering of nanoplatforms and close collaboration with **Spokes #6 and #7**. A cluster of activities will be devoted to biological vesicles (microalgal, protozoan and autologous vesicles), biological platforms (liver) and technologies for the skin delivery of NA. The quality-oriented bench preparation of nanoplatforms will be carried out under a constant control of the Critical Quality Attributes-CQA (size, polydispersity, shape, charge, structure, particle shape and morphology, physical and chemical stability, release kinetics) that are expected to affect efficacy, PK and biopharmaceutical properties. To this purpose, we will build specific quality check protocols to validate lab data and ensure the progression only for the compliant nanoplatforms. The delivery platforms developed in WP 8.1 will be available to the **vertical Spokes #1-5** to test if novel therapeutic concepts can become a product candidate. WP8.2 and WP8.3 can be seen as TRL accelerators covering two key aspects of the discovery pipeline of delivery platforms that is i) the development of in vitro models that recapitulate more closely the disease features and ii) the transfer of results from the lab bench to an industrial environment. In **WP 8.2**, we will focus on developing biological models recapitulating the disease complexity and reducing the use of animals to assess the efficiency of delivery with a specific focus on cancer models. We will generate 2D and 3D culture/co-culture of cancer cells to evaluate trafficking, transfection efficiency and cytotoxicity in close collaboration **with Spoke #2 and Spoke #9**. In embryo models will be considered preferentially to assess, besides toxicity and efficacy in a live organism without using rodents. The ability of nanoplatforms to escape macrophages and circulate in the blood will be carried out in zebrafish. Human organoids of intestinal cancer will allow us to investigate the behavior of nanoplatforms in a biological context recapitulating the human disease. A task is dedicated to applying omics in nanotechnology, assessing the effect of nanoplatforms on the proteomic and metabolomic profile of cells, and tools for live imaging of cells through nanoscopy. This strategy will be functional to reduce the use of animals to assess delivery efficiency, refine and select the most promising candidates developed in 8.1, and speed up the animal pre-clinical phase **in Spoke #9**. In **WP 8.3**, we will focus on the manufacturing processes of pharmaceutical prototypes meeting the quality requirements of IND in the early development phase and implementing industrially relevant technologies for nanoplatform fabrication. We will primarily focus on manufacturing technologies that accommodate scalable processes complying with GMP quality guidelines like microfluidic and supercritical fluids. For this purpose, we will consider as key issues the transition from small laboratory batch size to large industrial volumes and the selection of excipients required to produce high-quality pharmaceuticals. The production lines of nanoplatforms on gram-scale in GMP-like conditions available to **Spoke #8** participants will allow the progression of robust nanoprototypes toward preclinical animal studies **in Spoke #9 and vertical Spokes**, tackling the challenge of scale-up at a very early stage of development. To anticipate stability issues of the final products, we have planned activities around the evaluation of formulation-container compatibility and the possibility of formulating nanoprototypes as freeze-dried powders in collaboration with **Stevanato**, **Dompè** and **Chiesi**. To get proper control of the sample quality along their itinerary between labs, **Antares** will develop specific AI solutions and monitoring tools. AI will also be used to elaborate results in WP8.1 by linking the properties of coherent nanoprototype libraries to biological results.

WP 8.1 – Translational development of smart delivery platforms	
General objectives. To develop a novel generation of synthetic, biologically derived and bioinspired nanoplatforms for the precision delivery of nucleic acids up to a therapeutic PoC. To accelerate the translation of already developed platforms toward the clinic. To explore novel administration routes for nucleic acid therapies **Methodology.** Each task focuses on a specific cutting-edge delivery technology and intends to reach the delineated outcomes through a common workplan considering the bottlenecks in nanoplatform discovery pipelines (in-depth characterization, stability in complex media, satisfactory toxicity profile). Only compliant nanoplatforms will be further developed up to a therapeutical PoC	
Task 8.1.1	**Novel synthetic materials and nanoplatforms for NA delivery (Task leader: Caliceti-UNIPD)**
TRL	**2→4**
	• Synthesis of new functional biomaterials and tailored supramolecular biomimetic platforms for DNA/RNA delivery. • Tailor-made biopolymers able to integrate Carbon Nanodots, form interpolyeletrolyte complexes and decorate extracellular vesicles for the delivery of RNA/DNA. • Validation of lipid- and protein-based delivery platforms (CatCages, ProCages) for the stabilization and efficient delivery of siRNA. • Identification and structural characterization of new steroidal able to improve the RNA transfection efficiency by lipid nanoparticles. • Development of calcium phosphate-based nanoplatforms complexing nucleic acids.
Task 8.1.2	**Multifunctional synthetic nanoplatforms for precision delivery (Task leader: Quaglia-UNINA)**
TRL	**2→4**
	• Peptide-engineered nanoplatforms for the precision delivery of siRNA/chemotherapeutic combinations to triple negative breast cancer. • Nanoparticles targeting RNA to solid tumors and brain via multiple interactions and controlling innate immune reactions. • Polymer-coated lipid vesicles, cyclodextrin (CD)-based bioconjugates and nanosponges for the precision delivery of siRNA to solid tumors (breast cancer) and brain diseases. • Lipid nanovectors with specific tropisms towards macrophages subpopulations.

	• Polysaccharide-based nanoplatforms for the delivery of siRNA in the oral/locoregional treatment of colon cancer.
Task 8.1.3	**Engineered viral vectors (Task leader: Broccoli-CNR)**
TRL	**3→5**
	• Innovative genomic editing approaches on adeno-associated vectors (AAV) to achieve stable expression of the transgene in tissues relevant for gene therapy. • Isolation of new neurotropic AAV synthetic capsids with enhanced brain transduction for gene replacement therapy. • Oncolytic adenovirus multimodal "nanomachines" delivering shRNA and/or cDNA rewiring the tumor microenvironment.
Task 8.1.4	**Biological vesicles and platforms (Task leader: Martini-UNIPI)**
TRL	**3→5**
	• Camouflage of soft nanosystems and extracellular vesicles with b-cell derived membrane elements for miRNA delivery in Type-1 diabetes. • Generation of Leishmania tarentolae derived micro-vesicles and their potential to deliver RNA molecules to APCs. • PoC of the application of microalgal-derived extracellular vesicles as RNA delivery platform and scale-up of the manufacturing bioprocesses. • PoC of the ability of the oncological patient-derived extracellular vesicles (PDEVs) to deliver therapeutic RNA selectively to cancer tissues. • Generation of a RNA drug delivery system based on engineering of autologous serum EVs. • Assessment of explanted human livers (EHL) in ex vivo Normothermic Machine Perfusion as gene product delivery platform.
Task 8.1.5	**Skin delivery of nucleic acid therapies (Task leader: Netti-IIT)**
TRL	**3→5**
	• Development of biodegradable polymer microneedles made of microparticles and electroporation for the intradermal delivery of nucleic acids (Netti-IIT) • Set up and use of electroporation as non-viral delivery method for gene therapies (Takis)
Deliverables WP8.1	D8.1 Libraries of materials (3-5) able to self-assemble and increase the delivery efficiency of nucleic acids as compared to common methods (M12); D8.2 At least 50 synthetic nanoprototypes and vesicles prepared and characterized (M18); D8.3 Generation of Cas9 fusion proteins to promote integration of large donor DNAs (M18); D8.4 NA-loaded MNs functionality in an in vitro skin model (M18); D8.5 Proof-of-principle validation of the autologous clinical protocol for the tumor targeting using PDEVs: phase 1/2 clinical trial for safety and efficacy assessment of the protocol (M24); D8.6 Synthetic nanoprototypes tested for toxicity and transfection efficiency in cells (M24); D8.7 Validation of the new AAV9 display capsid library for high display peptide diversity and viral titers (M24); D8.8 A method for efficient production and loading of Lt-derived micro-vesicles, for medium scale production (M24); D8.9 Functional delivery upon including with a gene therapy platform in NMP (M24); D8.10 Selected prototypes validated in advanced preclinical models in WP8.2 and/or Spoke #9 (M30); D8.11 Therapeutic efficacy of AAVs with in vivo (M36); D8.12 Comparative analysis of the capside-engineered AAV vectors upon different intravascular routes in various mouse strains (M36); D8.13 Selection and formulation of the lead prototypes (M36).
Milestones WP8.1	MS8.1 Generation of building block libraries (M12); MS8.2 Development of technologies to efficiently decorate viruses with tumor-specific peptides to direct the immune response (M12); MS8.3 Identification of molecular and pharmacological modulators of cellular repair pathways to enhance targeted integration of large donor DNAs (M12); MS8.4 Development of technologies to efficiently decorate viruses with tumor-specific peptides to direct the immune response (M12); MS8.5 Preclinical assessment of PDEVs loaded with a known therapeutic siRNAs or mRNA (M12); MS8.6 NA-loaded MNs able to penetrate in the skin model and optically confirm of uniform spreading in around 2 h (M12); MS8.7 Delivery and functional assessment of gene therapeutics on prolonged NMP ex vivo platform (M18); MS8.8 Functional PoC of the delivery efficiency of synthetic nanoconstructs from novel materials (M24); MS8.9 Functional PoC of novel engineering strategies to achieve precision delivery (M24); MS8.10 Biodistribution and brain transduction efficiency of AAV-Se1/2, two novel engineered AAV9 capsids and generation of a new AAV9 capsids peptide library for in vivo directed evolution (M24); MS8.11 Generation of four different viruses for the delivery of shRNA to modify the epigenetic of the tumor cells (M24); MS8.12 Biodistribution of engineered EVs in injured and heathy organs (ex vivo in perfused organs (pig studies);, in vivo (mice models); (M24); MS8.13 Validation of the autologous clinical protocol using PDEVs (M24); MS8.14 NA loaded MPs suitable for microneedle manufacturing (M24); MS8.15 Therapeutic PoC of RNA-loaded microalgal EVs and myelin nanovesicles (M24); MS8.16 Proof-of-concept of therapeutic efficacy of the novel genome editing approaches in relevant models (M24); MS8.17 Vital and functional human explanted livers during 2 to 24 hrs of ex vivo NMP (M36); MS8.18 Proof of the NA activity by following specific cell functions triggering in the advanced skin model including macrophages as immune cells (M36); MS8.19 Validation of lead therapeutic platforms in animal models covered by Spoke #9 (M36); MS8.20 Selection of a panel of platforms suitable for proving a therapeutic concept developed in the vertical Spokes (M12, M24, M36).
WP 8.2 - Innovation in production and testing technologies	
General objectives. Build cancer models of increasing complexity and develop omics strategies to assess nanoplatform efficiency and safety. Provide a standardized experimental pipeline to evaluate efficacy and toxicity of delivery nanoplatforms for cancer treatment and speed up preclinical studies. Refine and select the most promising candidates from WP8.1 for further studies advancement. **Methodology.** We will develop cutting-edge biological models recapitulating disease features to evaluate the efficacy and toxicity of the nanoplatforms with a particular focus on cancer. We will apply omics sciences to bridge in vitro models and disease complexity and monitor efficacy and toxicity of treatments.	
Task 8.2.1.	**2D/3D cell cultures and organ-on-chip (Task leader: Borrelli-UNINA)**
TRL	**2→4**
	• Evaluation of delivery efficiency in a sequential three step process, i.e. single cell model, co-cultured cell model and organoids focusing on colorectal cancer. • Efficiency of RNA delivery systems in 2D, MultiCellular Tumour Spheroids and patients' derived Breast Cancer Organoids assessed by microfluidic-assisted single-cell transcriptomics.

	• Development of a skin-on-a-chip (SoC); platform for preliminary testing of skin delivery systems in a functional immune-competent human tissue model.
Task 8.2.2.	**In embryo models (Task leader: Argenton-UNIPD)**
TRL	**2→4**
	• Development of a zebrafish embryo platform to screen trafficking, payload-delivery efficiency and toxicity of nanoplatforms. • Development of chicken ChorioAllantoic Membrane (CAM)-based cancer models integrating cells stably deleted or overexpressing specific oncogenes or tumor suppressors.
Task 8.2.3	**High-throughput analysis linking in vitro models and disease features (Task leader: Campiglia-UNISA);**
TRL	**2→4**
	• Monitoring the efficacy and safety of novel delivery nanoplatforms using a metabolomics approach. • Evaluation of treatment efficacy, metabolism, safety and toxicity using combined mass spectrometry-based multi-omics approaches in patient-derived organoids. • Combination of state-of-the-art intelligent probes with optical nanoscopy to enable live functional imaging of nanoplatform cellular fate and downstream activity.
Deliverables WP8.2	D8.14 Culture of MCTS for 6 different breast cancer cell lines and computational pipeline for automatic evaluation of efficacy and toxicity from single-cell transcriptomics data (M12); D8.15 Characterization of single and co-cultured primary cells population (M12); D8.16 organ on chip platform designed to host HSE (M12); D8.17 Establishment of 2D, 3D/Spheroids cancer cell models with specific gene alterations (M12); D8.18 Establishment of cancer model (2D, 3D/Spheroids) (M18); D8.19 Development of novel novel super-resolution fluorescent probes and genetically-encoded fluorescent indicators (GEFI) (M18); D8.20 Identification, establishment and validation of translational relevant zebrafish models, mutated lines and human derived xenografts (M18); D8.21 Preliminary screening on RNA drug efficacy and toxicity on colon cancer organoids (M18); D8.22 Evaluation of delivery efficiency of nanoplatforms in MCTS and different breast cancer organoids (M24); D8.23 Organ on chip platform for hosting HSE-muscle tissue-lymph node axis (M24); D8.24 Toxicity profile and efficacy of nanoplatforms in 2D/3D cancer cells and CAM models (M24); D8.25 Mapping of spatial distribution of selected nanotherapeutics in primary tumor derived organoids (M24); D8.26 Evaluation of safety, cellular trafficking and efficacy of nanoparticles in 2D/3D (M24); D8.27 Efficacy of delivery platforms in patient-derived xenografts (PDX) in CAM (M36).
Milestones WP8.2	MS8.21 MS-based Metabolic profiling of at least 1 primary tumor organoid model (M12); MS8.22 Microfluidics platform for single cell sequencing (Drop-Seq) (M12); MS8.23 2D/3D cancer cell models and CAM-based tumor model established (M12); MS8.24 Set-up of a nanoscale imaging platform based on fluorescence microscopy and schemes based on colocalization/correlation/FRET (M15); MS8.25 Organ on chip platform for hosting immune competent HSE (M18); MS8.26 Identification of the lead prototypes in terms of antitumoral and anti-inflammatory efficacy and lack of cytotoxicity in 3D models (M24); MS8.27 Efficacy of at least 1 panel of nanoplatforms by MS-based multi-omics approaches (M24); MS8.28 Functional assessment of a panel of nanoplatforms in 2D/3D cancer cell models (M24); MS8.29 Organ on chip platform hosting HSE-muscle tissue-lymph node axis (M30); MS8.30 Completion of a horizontal pipeline from the nanocarrier design to the longitudinal analysis of efficacy in the zebrafish models (M30); MS8.31 Immune competent HSE on chip coupled with MNs array and protocol for direct and indirect detecting of released NA (M36); MS8.32 Assessment of at least 3 panel of nanotherapeutics in different organoid models (M36); MS8.33 Functional assessment of RNA based delivery systems in CAM based patient-derived xenografts (PDX) (M36).
WP 8.3 - Biological profile of delivery platforms	
General objectives. Production of pharmaceutical prototypes by microfluidic meeting the quality requirements of investigational new drugs. Novel technologies for nanoparticle fabrication working on industrial volumes. Development of sensing tools to assess the quality of samples along the research chain. AI-supported management of research data and sample flow. **Methodology.** We will tackle the issue of nanoplatform scale-up at a very early stage of development focusing on the technologies currently employed at the industrial level. Taking advantage of the Spoke industrial partners, we will develop tools to Track&Trace the outgoing samples throughout their itinerary in the CN3 labs and anticipate the critical quality issue of packaging compatibility.	
Task 8.3.1	**Nanoplatform production technologies (Task leader: Tirelli-IIT);**
TRL	**3→5**
	• Production of the best polymeric nanoplatform candidates by microfluidic technologies in pre-GMP. • Design, development, and validation of a modular microfluidic platform for the continuous production of polymeric and lipid nanoparticles. • Production of nano-sized liposomes and niosomes for nucleic acid encapsulation, using a continuous technology assisted by supercritical CO2 (SuperSomes).
Task 8.3.2	**Stability of the final products (Task leader: StevanatoGroup);**
TRL	**4→6**
	• Development of processes for the freeze-drying of nanoformulations and imaging methods to screen aggregation phenomena in liquid final products. • Stability of the final products and formulation-container interactions (Dompè).
Task 8.3.3	**Novel tools for pharmaceutical product development (Task leader: Antares)**
TRL	**4→6**
	• An AI-based strategy to understand how a nanoplatform modification can affect an outcome. • Nanosensors to detect RNA loading, carrier/target interactions and precision recognition in complex matrices along the research chain. • Methods to Track&Trace the research samples throughout their itinerary in the NC Spokes.
Deliverables D8.3	D8.28 Set up of proper analytical and testing technology for the assessment of container performance with aging and temperature (M6); D8.29 Stability results of the preformulations under mechanical and thermal stress conditions (M6); D8.30 Formulation of suitable phospholipid, and/or surfactant mixtures to produce SuperSomes (M12); D8.31 LNP production in pre-GMP (M12); D8.32 Hydrodynamic model of a microfluidic unit for the production of LNP processing industrial volumes (M12); D8.33 Design and manufacturing of a Human Machine Interface for samples and data monitoring, control and analysis (M12); D8.34 Results of preliminary studies on vial glass properties that can impact lyophilization process (M12); D8.35 Sensor for the recognition of DNA/RNA target down to pM level (M12); D8.36 Experimental validation in lab of the microfluidic unit processing industrial volumes (M18); D8.37 DoE on a model drug formulation (M18); D8.38 AI algorithms development (M18);

	D8.39 Nest and Tub solutions based on outcome of lyophilization studies (M18); D8.40 A glass forming equipment specifically dedicated to production of improved vials suitable for lyophilization (M18); D8.41 Optimization of SuperSomes manufacturing in operative conditions (M24); D8.42 Polymer-based nanodelivery platforms in pre-GMP (M24); D8.43 Experimental validation in lab of the purification unit processing industrial volumes (M24); D8.44 A coating technology to cover internal surface of syringes (M24); D8.45 Different sensors to check the samples status and conditions throughout their itinerary along the processing chain (M24); D8.46 Results of the interaction of lipid and polymer formulations with primary packaging (M24); D8.47 A dual chamber syringe container suitable for being used in lyophilization (M24); D8.48 Pre-GMP production of a panel of polymeric nanoprototypes for precision delivery of nucleic acid (M36); D8.49 Assembly of the production and purification platform and its parallelization for validation in industrial environment (M36); D8.50 Materials and components (i.e. plunger, barrel) to grant the container closure integrity in deep freezing conditions (M36); D8.51 Traceability, serialization and tracking platform, including check stations and necessary operator hardware and tools (M36); D8.52 Results of a model formulation stability in the best primary packaging (M36); D8.53 Validation of the new glass container (Vial) (M36).
Milestones MS8.3	MS8.34 Completion of stress test & preformulation studies (M6); MS8.35 Optimization of the rational design of recognition probes (M12); MS8.36 Standardized protocol for the production of LNP nanodelivery platform by microfluidics (M12); MS8.37 Results of the model simulations with realistic parameters (M12); MS8.38 DoE completed for glass forming optimization (M12); MS8.39 Model of the industrial microfluidic separation unit (M14); MS8.40 Construction of the industrial microfluidic production unit (M18); MS8.41 Key Critical Material Attributes identified and put in control on a model formulation (M18); MS8.42 New Nest and Tub solution developed (M18); MS8.43 Development of pilot tooling (plunger) (M18); MS8.44 A new glass forming machine (M24); MS8.45 Identification of the optimal SuperSomes operative conditions (M24); MS8.46 Standardized protocol for the synthesis of PMA nanoplatforms by microfluidics (M24); MS8.47 Interaction between primary packaging and nano formulations (M24); MS8.48 Construction of the industrial microfluidic separation unit (M24); MS8.49 A dual chamber syringe container suitable for being used in lyophilization processes (M24); MS8.50 Industrialization of the industrial microfluidic unit (M36); MS8.51 Completion of long term stability studies of nanoplatform formulations (M36); MS8.52 Track&trace tools and protocols ready (M36) MS8.53 AI-handled results linking nanoplatform input (properties) and output (efficacy) data.

Spoke # 9: From target to therapy: pharmacology, safety and regulatory competence center. Spoke leader UNIMi.
State of the field and unmet needs. Due to novelty of the fields of NA therapeutics, we have a very limited knowledge with regard to the methodologies necessary for the study of their pharmacokinetics, pharmacodynamics and toxicology. Thus, research must be addressed to the development of the tools necessary to assess NA-based drug biodistribution, metabolism and activity at desired and undesired targets; furthermore, in view of their known immunogenicity and potential for off target effects, novel paths should be generated for appropriate immune-toxicological evaluations.
Standardization of the PK, PD and toxicological analysis is essential for the clinical translation of RNA drugs: the process is now tailored case by case, complicated by their RNA biological instability, limited ability to cross biological barriers and presence of different categories of RNA-based therapies are available (those targeting nucleic acids like aON and siRNA, or proteins like aptamers or those that encode proteins, mRNA). Moreover, the long-term persistence of the drug effect (e.g. RNA vaccine, siRNA) is quite often opposed to the labile nature of these molecules in biological fluids due to their rapid catabolism. Thus, the standard analytical methods suitable for small molecules (liquid chromatography tandem mass spectrometry, LC-MS/MS) as well as methods specifically applied to the RNA drugs (northern blot, real-time PCR, Elisa, fluorescent probes) provide limited information for the determination of ADMET and their connection with PD parameters.
The ambition of the University of Milan is to build on its long term expertise in Biotecnology and Pharmacology to organize a Competence Center where the best academic groups from several Italian Universities (University Federico II of Naples, Rome, Padua, Pavia, Humanitas) and CNR will work in synergy with national and international Pharma, Diagnostic Companies and Contract Resesearch Organization to establish a unique Center devoted to the RNA-drugs development.
How the Spoke will contribute to advance in this scenario. The long term aim of **Spoke #9** is to take advantage of the most innovative methodologies for the **generation of novel protocols for a reproducible, rapid and safe ADMET and pharmacodynamics analysis of NA drugs**.
However, as a **contingency plan**, we will work in parallel at two levels: in **the shorter term aim** we will capitalize on our present expertise in regulatory sciences we will work according to EMA and FDA Guidelines on Bioanalytical, to develop and optimize analytical standard technique (liquid chromatography tandem mass spectrometry, LC-MS/MS) for the quantification and biodistribution of RNA therapeutics strategies to be applied in clinical studies in combination with preclinical distribution studies; furtheromore, we will take advance of the research carried out in the vertical Spokes to identify biomarkers suitable to verify the activity of the RNA-drug in use. Potential toxicity of RNA-drugs will be tested using both *in vitro* and *in vivo* methods that should be further implemented according to guidelines under GLP before submission for approval at EMA. **Sex differences and 3R approaches will be also considered.** In the spirit of GLP, to reduce the uncertainties all *in vitro* method-derived safety predictions, test methods and conditions under which data will generated in this WP will adhere to defined standards to ensure resulting data are rigorous and reproducible. All *in vitro* studies will be conducted in accord with the OECD Guidance document on Good In Vitro Method Practices (GIVIMP) (OECD (2018), "Good Cell Culture Practice (GCCP)", in Guidance Document on Good In Vitro Method Practices (GIVIMP), OECD Publishing, Paris, https://doi.org/10.1787/9789264304796-16-en.). A tailored based approach will be developed on a case-by-case, considering target expression, mechanism of action, literature review, indication, intended patient population and findings in pharmacological studies. Information gained in WP 9.1 and WP 9.2., and in the other Spokes, will be used to identify possible off targeted organs, and to develop *ad hoc in vitro* models. In addition, as current knowledge indicate that toxicity of RNA-based compounds is largely associated by their interaction with the immune

system, tasks 9.3.3 and 9.3.4 will specifically investigate this aspect applying a selection of available immunotoxicity assays and developing innovative *in vitro* assays to specifically detect adaptive and innate immune responses. Molecular constituents of RNA-drugs and vaccines will be investigated as a case-of-study representative of this new class of compounds. Similarly, pathways considered more relevant in the immune response to carriers involved in the project will be validated using standard NP with already known immunotoxicological behaviour based on different materials (lipid-based, polymer-based and inorganic nanoparticles) defined on the basis of a literature review. Indications of **Spoke #8** on materials of interest for the CN3 activities will be taken into account in this selection. Because the liver has emerged so far as the main target of gene therapy approaches, Task 9.3.2 will identify and validate biomarkers and assays for early detections of liver toxicity associated with gene therapy with the aim to develop a systemic approach for detecting and monitoring liver toxicity in clinical studies. A proof-of-concept study investigating liver toxicity associated with gene therapy compounds targeting the liver (lipid nanoparticles or viral vectors) will be performed in preclinical models (in vitro and in vivo) and in patients treated with gene therapy.

For the **long term aim**, **Spoke #9** we will test and optimize novel concepts aimed at speeding up the preclinical studies by combining the current steps required for ADMET studies by setting up new protocols based on a new rational flow: initial screening assays will study the effects of NA drugs on reporter systems of disease modifying pathways (DMPs): multiple biochemical signals will be measured in an array of imaging and biochemical reporter cells and reporter zebrafish embryos. Moreover, the effects produced by RNA drugs will be investigated with 2D or 3D cell cultures modelling the tissue complexity of several disease conditions. Comprehensive array of sequencing methods (i.e. single-cell RNA sequencing, analysis of epigenetic and epitranscriptomic marks, etc) will be applied to identify cell-specific RNA signatures associated with drug perturbations. Second and third generation sequencing experiments to evaluate targeted and genome-wide DNA and RNA methylation (BS-Seq, m6A-Seq, Nanopore-direct RNA-Sequencing), as well as chromatin accessibility (ATAC-Seq), will be carried out in *in vitro* and *in vivo* models generated for the study of RNA-based therapy. These technologies will provide for the first time the possibility to analyze, at the same time, the distribution of NA drugs and their activity inferred by data-driven integration of multi-dimensional molecular parameters, relative spatial locations of cells, and their precise molecular status upon perturbation. The pharmacodynamic data acquired with cellular and zebrafish models will drive the choice of the reporter mouse model carrying the correct surrogate marker for the *in vivo* imaging study of the biochemical pathway modulated by the NA drug. The combined PK/PD endpoint defined by these models will allow the identification of systemic on- and off-target effects in the whole organism. Modified RNA drugs with fluorescent chromophores activatable upon target recognition will be used in these combined PK/PD approach to link systemic drug effects with the specific binding to the target. The comprehensive picture of the drug activity (from systemic down to single cell level) generated by these studies, will be finally complemented with the standard efficacy study of the NA drug under investigation in specific disease models among the vast collection available within the Spoke. Indeed, cell lines, organoids and experimental animals available are covering all the relevant therapeutic areas identified by the Hub with the most relevant disease models and several clinically-significant endpoints have been already developed for most of them. Data management and analysis will be ensured at each step of the project by the collaboration with **Spoke #7** speeding up the quantitative analysis procedure. The data collected from WP 9.2 together with the PK and toxicological profile obtained from WP 9.1 and WP 9.3 will be the base for the definition of the relevant pharmacological parameters (route of administration, dose, therapeutic window etc.) needed for the compilation of the preclinical dossier to support an investigational new drug (IND) application and first-in-human clinical trial.

For all the work carried out in **Spoke #9**, the regulatory advice will be based on the existing European regulatory framework, but, considering the novelty of most topics of research, the research activities of WP 9.4 will include projects expressly focused on expanding the current regulatory knowledge and elaborating proposals to optimize the current regulatory requirements on specific product classes (e.g., ATMP). In addition, specific projects will be started to overcome possible criticisms in the classification, production, and characterization of proposed products.

The work to be done by **Spoke #9** will be carried out in very **strict collaboration with all the vertical Spokes**, in particular for designing the pharmacodynamics studies and the **orizontal Spokes** for drug chemical modification, delivery and AI application in particular to set up unsupervised methodologies for reporter activity detection by imaging. As better specified in the section A2, the support of the companies associated with the Spoke will be essential for the creation of protocols scalable for industrial use, the generation of appropriate tools for more efficient imaging, quality assessment, initial studies in GMP/GLP facilities and to facilitate the translation of the research products into standard, easily applicable technologies for RNA drug development.

Public-Private Partnerships will represent a key to success for **Spoke #9** as the collaboration with the affiliated Companies is considered indispensable achieve the aims of all WPs. The communication between **Spoke #9** and the Companies here mentioned is facilitated by a number of research programs established in the past years with UNIMI and other affiliated groups which have refined the terms of collaboration also with regard to protection rights. In particular, **IRBM SpA** with its tradition in the study of drug biodistribution and biotransformation and its novel laboratories created for the study of in vitro ADME of NA-based drugs will represent a major support to the work to be done in WP1.1 and 3.1, futrthermore, the

collaboration with IRBM will be seeked the industrialization of the research tools developed by the Spoke; the competence acquired by **Dompè** in the field of RNA vaccines immunotoxicology will be essential in task 9.3.3 to develop new methods and *in vivo* models for immuno-toxicological evaluations, and GLP toxicological studies; **Chiesi Farmaceutici** will contribute, also trough its collaboration with Moderna for Inc., to the identification of the RNA drugs most suitable of development and in the definition of the technologies for the study of RNA drug distribution with particular emphasis on the translation of dosage studies carried out in laboratory a imals into doses for uman use (tasks 9.1.1 and 9.1.2); **Eurofins Biolabs** as a world leader in testing and research services will support the research on the optimization of current analytical procedures as well as the search for innovative methodologies (tasks 9.1.1 and 9.1.2); **Bracco** will prove essential for the development of *in vivo* imaging farmaco-toxicological studies (task 9.1.5); in addition the large experience gained by CDI/Bracco in human genetic studies will be relevant in the identification and application of appropriate biomarkers to the PK and PD studies (task 9.1.4); **Antares vision** will assist in the design of quality control methodologies and smart data and tools for the digitalization of the process; in addition experience gained by Antare in the use of biological vescicles for the delivery of RNA drugs will be of interest to evaluate the PD efficiency of drugs delivered with such vehicles (tasks 9.1.1 and 9.1.2); It is expected that in the course of the three years program the Spoke will interact with most of the Companies currently present in the NC in particular for setting up TT operations at national and international level.

WP 9.1 – RNA drug pharmacokinetics	
General objectives. To better understand ADME properties of RNA-based drugs there is an increasing need to adapt current bioanalytical tools to achieve the necessary sensitivity to provide accurate quantification from various matrices, including biofluids such as plasma or serum. To this aim, in addition to standard analytical technique, a panel of non-invasive analysis for the assessment of NA-based drug PK, its interaction with the target and its modulatory activity on specific molecular processes will be developed and applied for preclinical and clinical investigations. **Methodology.** We will combine strategies to optimize the pharmacokinetic analyses with preclinical distribution studies. The analytical standard technique (liquid chromatography tandem mass spectrometry, LC-MS/MS) to be used in preclinical and clinical studies for the quantification and biodistribution of RNA therapeutics will be developed, optimized and validated according to EMA and FDA Guidelines on Bioanalytical.	
Task 9.1.1	**Define methodologies for preclinical and clinical studies of RNA drug absorption, distribution, metabolism and excretion (DADME) (Task leader: Clementi-UNIMI)**
TRL	**3→4**
	• Optimization of standard bioanalytical methods (mass spectrometry) of RNA drug types. • ADME of RNA drug types dependent on administration route. • Comparison of standard vs higher-throughput bioanalyses (ELISA and qRT-PCR). • PK of RNA drug types in human biofluids (Phase I study).
Task 9.1.2	**Define innovative methodologies for clinical studies of RNA drug ADME (LARD) (Task leader: Bellini-UNIMI)**
TRL	**4→5**
	• Selection of a panel of about 10 targets and their conjugation with the sensor surface and implementation of analytical tools for the detection of the RNA drugs based on the interaction with the targets. • Measure of binding strength and kinetics with the selected targets and definition of a protocol for the detection of RNA drugs. • Definition of a protocol for the detection of RNA drugs in simple fluid and in serum or cell extract with CN3 drugs.
Task 9.1.3	**Therapeutic Nucleic Acids TRACKing (ThERNAsTRACK) In common with WP2 (Task leader: Sattin-UNIMI)**
TRL	**3→5**
	• First fluorophore-drug conjugate for imaging- conjugation product with three additional probes (at least one suitable for BRET experiments). • QB with conjugation moiety (scaling up). • Synthesis of the selected conjugates. • Fluorescent probes for NP conjugation.
Task 9.1.4	**Multidimensional analysis of the RNA-based drug efficacy in animal models analysis of the RNA-based drug efficacy in animal models (MultiD-RNA) (Task leader: Ciana-UNIMI)**
TRL	**5→7**
	• Ministry authorization for animal experimentation related to the projects. • Set up the whole body in vivo/ex vivo imaging reporter assay with an AI-driven evaluation of the imaging output. • Proof of principle for the in vivo/ex vivo imaging output acute treatment time- and dose-response to well characterize NA-drugs. • Test of NA-drug candidate proposed by the Hub.
Task 9.1.5	**NA- based drug non-invasive IMAGing (NA-IMAGo) (Task leader: Ottobrini-UNIMI)**
TRL	**4→6**
	• Procedures for non-invasive monitoring of specific molecular processes involved in NA-based drugs' activity by optical imaging. • Procedure for non-invasive monitoring of NA PK by fluorescence imaging.

	• Procedures for non-invasive evaluation of NA-target interaction by activatable fluorescence imaging. • Procedures for the non-invasive evaluation of NA drug activity using endpoint imaging biomarkers and translational imaging strategies.
Deliverables WP 9.1	1 Protocols for LC-MS/MS (M9); D9.2 ADME by LC-MS/MS in vivo animal models (M20); D9.3 ADME by alternative methods in vivo animal models (M24); D9.4 Design for a phase I study (M36); D9.5 Report on the detection of a test RNA drug by the label-free detection of its binding to selected targets (M12); D9.6 Report on the detection of a test RNA drug by the detection of its binding to selected targets in serum and cell extract (M18); D9.7 NA-NIR probe conjugate (M6); D9.8 QB product (M12); D9.9 Best QB conjugate selected (M18); D9.10 Best conjugate synthesized (M24); D9.11 Responsive model system (M36); D9.12 Fluorescent probe for NPs conjugation (M24); D9.13 Request authorization for animal use (M1); D9.14 Report on set up of procedures for multiprobe acquisitions (M6); D9.15 Pilot multiprobe study (M18); D9.16 Report on molecular processes monitoring by non-invasive techniques during NA-based treatment (M24); D9.17 Report on molecular process monitoring in 3 other models and relative treatments (M36); D9.18 Procedures for the non-invasive evaluation of NA drug activity using endpoint imaging biomarkers and translational imaging strategies (D36) .
Milestones WP 9.1	M9.1 Authorization for the use of animals (M6); M9.2 realization probe conjugation (M6); M9.3 Test of samples of blinded drug concentrations (M12); M9.4 Label free detection of a test DNA drug in simple fluid matrix (M12); M9.5 Assay availability for ibridization and fluorescence assays (M20); M9.6 Caracterized synthesis of 2 compounds (M18); M9.7 Availability procedures to follow NA PK in vivo by fluorescence non-invasive imaging (M18); M9.8 Results from the non-invasive evaluation of molecular processes in relation to a NA-based drugs (M24);M9.9 Validated procedures to follow NA PK in vivo by fluorescence non-invasive imaging (M24); M9.10 Label free detection of two DNA drug developed by the HUB in their relevant fluid matrix and demonstrator design plans (M30)
WP 9.2 - RNA drug pharmacodynamics	
General objectives. To create a unique platform for PK and PD data of NA-drugs by measuring specific cellular functions (including, but not limited to, cell proliferation, inflammatory reaction, oxidative stress, autophagy, innate immunity, cell death) in a spatio-temporal dimension in cell arrays, zebrafish and rodent models. Using reporter systems, we will integrate cellular models, patient-derived organoid, cellular 3D co-culture systems and single cell omics technologies (comprising spatially resolved technologies), zebrafish and mice, enabling multimodality imaging (e.g. bioluminescence, PET, MRI) of surrogate markers of multiple biochemical pathways to combine PK and PD in a single readout. **Methodology.** We will work in close collaboration with pharmaceutical companies and regulatory bodies. RNA molecules with well described PK, PD and side effects will be utilized to set up and validate the methodologies proposed in the different WP s. After method validation, protocols for PD characterization (or combined PK/ PD for *in vivo* reporter assay) will be tailored for the therapeutic areas considered by the National Center. The implemented pipelines will be made available to the entire Hub to test the most promising candidates NA drugs developed by the Center. Demonstration of the protocol validity in an operational environment will bring the TRL of the protocols up to 7-8 and this will be reached in 22-32 months.	
Task 9.2.1	**Establishing Reporter systems to track RNA drug activity (Splids, MultiD-RNA, MULTIVAL) (Task leader: Brini-UNIPD)**
TRL	**3→6**
	• In vitro and in vivo testing RNA delivery and translation efficiency by novel split-GFP based Delivery Sensors. • Creation of reporter animals carrying surrogate reporters for in vivo detection of RNA drug activity (proliferation, inflammation, oxidative stress, apoptosis). • Generation of AAV and lentivirus based reporters.
Task 9.2.2	**Models to study of RNA drugs in cells and organoids (animal and human) (ORGAN-DREAM; GLIA; ARTBOARD; Cardio-test and iPSC; MULTIVAL; ORGANON (Task leader: Lodato-HUNIMED)**
TRL	**3→5**
	• Provide and establish new patient derived organoid models (PDO). • In vitro and in vivo neurodegenerative/demyelinating models. • Advancing RNA Therapeutics through Brain Organoids and Advanced Regulatory Deconvolution. • Patient and disease-oriented tool for pharmacodynamic testing of CNS drugs. • CARDIO-test in iPSC cells. • Drug interaction with intestinal mucosa. • Immortalized, patient-derived organoids, and ex vivo vascularized muscle engineered tissues from different disease types.
Task 9.2.3	**Identification and organization of models available for the study of RNA drugs therapeutic activity in vivo (Zebio; VEP-NAD; Vipe-NAD, MULTIVAL) (Task leader: Izzo-UNINA)**
TRL	**2→4**
	• Zebrafish engineering for drug screening. • Platform for in vivo efficacy and pharmacological dissection of nucleic acid-based drugs. • Multiscale functional validation of RNA drugs.
Deliverables WP 9.2	D9.1 Identification of the reporter systems to be applied (M3); D9.2 Repositories) of already available zebrafish DMPs' reporters (M4); D9.3 Library of in vitro systems available (M6); D9.4 Library of animal models available (M6); D9.5 Generation of AAV- and Lentiviruses based reporters (M6);D9.6 Reprogramming hiPSCs from patients (M12); D9.7 Control drug testing in organoids (M12); D9.8 Protocol to study RNAdrug activity by through high-throughput technology (M12); D9.9 Single-cell transcriptome analysis (M 12); D9.10 SLO-organoids establishment (M12); D9.11 Optimization of in utero electroporation-mediated delivery of AptaDir in embryonic brains of WT and disease mouse models (M24); D9.12 Measure the functionality of NVU following drug administration and the consequent multi-omic changes (M24): D9.13 Protocols for CRISPR/Cas9 genetically modified organoids to mimic specific genetic diseases (M24); D9.14 in-silico identification of putative in-cis regions; in-vivo characterization of the identified regions to generate new biosensors for Zebrafish (M24); D9.15 Report on the effects of reference effector molecules on cardiomyocytes structural and functional features (M8); D9.16 Report on effect of Remodelin and RNA AptaDiR on iPSC-CMs structural properties (M6); D9.17 Report on the effect of Remodelin and RNA AptaDiR on metabolic properties of iPSC-CMs (M12); D9.18 Report on the effects of testing material on cardiomyocytes structural and functional features (M36); D9.19 Report on the effect of Remodelin and RNA AptaDiR on functional parameters of iPSC-CMs (action potential properties, calcium transients and contractility) (M18); D9.20 Comparative protocols Preclinical information

	on efficacy and/or toxicity of a selected number (maximum three) of RNA therapeutics (M24)
Milestones WP 9.2	M9.1 Standardized procedures and guidelines for organoid line establishment (M12); M9.2 Robust and reproducible generation of neural cells from patients' iPSCs (M12); M9.3 Availability of SPLIDS for testing NAs therapeutics (M18); M9.4 Validated workflow for preclinical functional assessment of RNA therapeutic in iPSC-CMs (M18); M 9.5 Implementation and organization of research infrastructure, including a patients organoid biobank (18); M9.6 Availability of PDOs biobank across diseases relative to the vertical Spokes for pharmacological studies (M24); M9.7 Availability of validated reporter zebrafish for pharmacological studies (M24); M9.8 Whole brain activity network characterization following AptaDir administration in WT and disease mouse models (M36); M9.9 Tested workflow for the combined PK/PD study from in vitro to in vivo (M24); M 9.10 Preclinical information on PK PD toxicity of a maximum five RNA therapeutics using the novel platform generated (M36).
WP 9.3 - Toxicology and Immunoreactivity of RNA drugs	
General objectives. To establish a platform for appropriate toxicological evaluation of potential of NA-based drugs in view of their known immunogenicity and potential for off target effects. Typically, pre-clinical testing involves the evaluation of the immunotoxicity of the product, off target toxicity, exaggerated pharmacology, and the eventual species-specificity of the product. The goal is to provide consistent, accurate and timely information characterizing the potential side effects and toxicity associated with nucleic acid-based drugs. Emphasis will be put on the use of new approach methodologies to address potential adverse effects of nuclei acids-based drugs and identification of biomarkers translatable to clinic **Methodology.** As general approach, the potential undesirable (adverse) effects of NA-based drugs on organ toxicity (e.g. central nervous system, cardiovascular system, respiratory system, liver, etc.) and immunotoxicity will be investigated in appropriate in vitro and in vivo models. Knowledge generated in WP 9.1 and 9.2 will provide key information on possible target organs and will guide on the choice of the most appropriate models. the platform is to evolve these in-house assays towards a 'GLP-like' standard, in tight collaboration with WP 9.4.	
Task 9.3.1	**Tailored approaches for the study of RNA drugs undesirable effects on organ toxicity following single or repeated dose exposure (Task leader: Corsini-UNIMI)**
TRL	**3→5**
	• Classical toxicological evaluation of RNA-based drugs. (GenTox). • Identification of stress pathways triggered by RNA-based drugs (PathTox). • Development of GLP-like in vitro assays for organ-specific toxicology (VitroTox). • Development of GLP-like in vivo assays (VivoTox).
Task 9.3.2	**Generation of predictive models for the study of liver toxicology for RNA drugs and candidates for gene therapy (Task leader: Angeli-UNIPD)**
TRL	**3→7**
	• Evaluation of liver toxicity related to gene-therapy with a liver-targeted gene delivery (VecTox).
Task 9.3.3	**Characterization of innate immune and adaptive system cells response to the different components of vaccines in vitro and in vivo (Task leader: Locati-UNIMI)**
TRL	**3→4**
	• Development of GLP-like in vitro assays for immunotoxicology (ImmTox). • Innate immune system activation in response to RNA vaccine components (VaxTox). • Validation of a T cell activation assay (IMMUN). • testing RNA-base vaccines and drugs for specific activation of immune cells in SLO organoids (SLO_Organoids).
Task 9.3.4	**Characterization of immunotoxicological properties of nucleic acids-loaded nanoparticles in vitro and in vivo - (Task leader: Locati-UNIMI)**
TRL	**3→4**
	• Immunotoxicological properties of NPs for nucleic acids delivery (NanoTox).
Deliverables (D9.3.)	D9.1 Tox model design (M2); D9.2 Identification of biomarkers for liver toxicity after gene therapy (M3) D9.3 Protocol for a methodology for the identification of potential target organs for adverse effects (M3); D9.4 Definition of in vitro innovative protocols to assess off target toxicity (M6). D9.5 Definition of in vitro innovative protocols to assess off target toxicity (M6); D9.6 Preclinical development of liver toxicity integrated model (M6); D9.7 Toxicological study of 3 drugs (M 12); D9.4Development of innovative assays on specific immune pathways (M12); 9.8 Development of integrated model to assess RNA drug-induced cellular stress (M12); 9.9 Establishment of a standardized battery of in vitro models to assess off target toxicity (M15); D9.10 Integration of sex as a variable in preclinical toxicology on 3 drugs (M18); D9.11 Availability of novel readouts to predict possible RNA drug-induced cellular stress(M24); D9.12 Development of in vivo toxicity integrated model (24); Adaptation to toxicological studies reporter animals (M24); D9.13 Immune response to RNA-based vaccines (24); D9.14 Protocols for the study of NP toxicity integrated model (M24); D9.15 Study of immunereactions of drug n.1 developed by the Center (M24); D9.16 Immunotox of drug 2-4 developped by the center (M30); D9.17Establishment of a standardized battery of in vitro models to assess off target toxicity (M30); D9.18 Immunox of drug n.5 developped by the center (M36); D9.19 Testing the safety of liver selective or haematopietic selective therapeutic siRNA on chronic TLR7/8 and interferon 1 response;
Milestones (MS9.3.)	M9.1 PPP agreement for toxicological studies of RNA-based compounds (M12); M9.2 Innovative readouts to identify and quantify drug-induced cellular stress toxicity (M12); M9.3 Validated novel biomarkers to study liver toxicity (M12); M9.4 Establishment of a standardized battery of in vitro models to assess off target toxicity (M15); M9.5 Availability of GLP standard techniques for in vitro toxicology (18M); M9.6 Availability of GLP standards fo in vivo tox studies (M18); M9.7 Establishment of a standardized battery of in vitro models to assess off target toxicity (M36); M 9.8 In vivo toxicity integrated model (M39); Availability of an innovative preclinical toxicological model (M36)
WP 9.4 - Regulatory framework	
General objectives. To develop innovative regulatory strategies to speed up the development and success rate of innovative therapies with particular attention to those derived from university research, the benefit/risk balance assessment of biotechnological drugs, nanomedicines and ATMP and the quality standards without prejudice the economic sustainability (projects NANOCLARA, REGULATE); to investigate regulatory aspects related to the so-called Hospital exception for RNA-based biotechnological products and ATMPs (projects ORROP-AT, HEA-AT); to develop, through data science of real-world evidence (RWE), a predictive pharmacoeconomic model applied to place in the therapy and predict the real long-term budgetary impact to be key in	

regulatory processes (projects EBM OF ATMP, PREDICTIVE TO MAKE); to support research groups operating in other Spokes providing regulatory advice on the activated projects (projects ACORS, ROADMAP). **Methodology.** We will establish the Academical Center on Regulatory Sciences (ACORS), a *unicum* in the Italian context, aimed at combining the expertise in regulatory sciences and scientific and technological knowledge to support research activities and provide regulatory advice on aspects related to the quality pattern of innovative medicinal products, and preclinical and clinical studies. To maximize the interactions and supports to other Spokes, a ROADMAP project will provide regulatory support in the preparation of IMP and/or authorization dossier of products designed from the pharmacological entities discovered by the different Spokes (1-5). Using HTA methodology and Swot Analysis, pharmacoeconomic advice will facilitate the identification of the areas of greatest need for treatment, value for money and place in therapy for diseases covered by vertical Spokes.	
Task 9.4.1	**Innovative regulatory strategies to speed up development and increase success rate of innovative therapies (NANOCLARA, REGULATE) (Task leader: Minghetti-UNIMI)**
TRL	**2→9**
	• Review of existing literature on nanotechnology applied to the manufacturing of medicinal products and creation of a network of stakeholders and experts on nanomedicine products. • Reach a consensus (by DELPHI method) among stakeholders and create Regulatory Guideline on the Classification and Marketing Authorization of Nanomedicines. • Define critical quality attributes for RNA-drugs – comparative studies. • Create a regulatory guidance on the quality and comparability of RNA drugs.
Task 9.4.2	**Regulatory aspects related to the so-called Hospital exception for RNA-based biotech products and ATMPs (ORROP-AT, HEA-AT) (Task leader: Pompilio-UNIMI))**
TRL	**1→8**
	• Create an expert panel and stakeholders on RNA-based biotech products and ATMP. • Reach a consensus (by DELPHI method) among stakeholders on optimization and regulatory requirements for preclinical and clinical trials for RNAdrug authorization. • Investigate regulatory aspects related to the so-called Hospital exception for RNA-based biotech products and ATMP.
Task 9.4.3	**A predictive pharmacoeconomic model for the novel therapies and long-term budgetary impact EBM of ATMPS; PREDICTIVE) (Task leader: Orlando-UNINA)**
TRL	**3→6**
	• Technology assessment analysis of socioeconomic burden of disease focus on drug utilization, pharmacoeconomics and market access aspects for spokes 1-5 and benchmarking current healthcare models • SWOT analysis Spokes #1-5. • Find consensus on gaps and priorities about pharmaceutical legislation and pharmacoeconomic aspects of ATMPS. • Pharmacoeconomic and budget impact analysis Spokes #1-5. • Predictive algorithm to support decision making process and reimbursement models.
Task 9.4.4	**Support research groups operating in other Spokes providing regulatory advice on the activated projects (projects ACORS, ROADMAP) (Task leader: Minghetti-UNIMI)**
TRL	**3→7**
	• Creation of an advanced center for research in regulatory sciences. • Preparation of an IMP for products developed starting from the pharmacological entity identified by other Spokes members for treating generic disease. • Preparation of an authorization dossier for products developed starting from the pharmacological entity identified by other Spokes members for treating generic disease.
Deliverables (D9.4)	D9.1 Definition of Centre structure (M1); D9.2 Preparation of Constitution dossier of Centre (M3); D9.3 Website portal (M6); Technology assessment report of Genetic disease (M6); D9.4 Technology assessment report of cancer area (M6); IMP dossier (M8); authorization dossier (M8); D9.5 Technology assessment report of neurodegenerative area (M12); D9.6 Technology assessment report of metabolic & cardiovascular area (M12); D9.7 Technology assessment report of inflammatory and infectious area (M18); D9.8 Identification of essential requirements for accepting a hospital exception (M22); D9.9 Data on comparability of the quality pattern of RNA-Based nanomedicine products (M24); D9.10 workshop on the regulatory criticism in preclinical and clinical studies (M24); D9.11 Protocol on SWOT prototype on the ad-hoc tool (M18); D9.12 Audit with policy makers (M30); D9.13 press & media actions, videos (M30)
Milestones (MS9.4)	.1 Report on Critical Quality attributes of RNA-Based nanomedicine products (M11); M9.2 Consensus document on criticisms of preclinical and clinical studies on NA-based drugs (M26); M9.3 Regulatory guidance on quality and comparability of RNA-Based nanomedicine products in terms of quality pattern (M32); M9.4 Guideline on application of hospital exception (M36); M 9.5 Guideline on the Classification and Marketing Authorization of Nanomedicines (M36); M 9.6 Dissemination document (36); M9.7 software (M36)

Spoke # 10: Preclinical development, GMP manufacturing and clinical trials of GTMP. Spoke leader: OPBG.

State of the field and unmet needs. One of the main bottlenecks currently faced by academia in moving gene-modified somatic cells (GTMPs) projects to the clinic is the lack of capacity for undertaking process development, scale up and manufacturing under GMP conditions of these products; this frequently results in project dropout after the first proof-of-principle and high costs and long delays for the few survivors. The performance of Italian basic and pre-clinical research in the field of Advanced Therapy Medicinal Products (ATMPs) is outstanding and internationally recognized. Nonetheless, to keep pace with the rest of the world, Italian researchers need an enabling structure that allow them to advance those results into clinical proof-of-concept trials. An additional challenge for the Italian national health system is the high price of ATMPs. Until now, despite the high prices, the budget impact has been minimum as only few products reached the market

and most target rare or ultra-rare conditions. However, a rapid increase is expected in the coming years (US FDA – Food and Drug Administration – expects to approve 10 to 20 cell and gene therapy products a year by 2025) with a relevant impact on healthcare system budget. Italy, as national system, now is at a turning point, where it needs to decide whether to be among the net payers (those paying treatments developed elsewhere) or among the players called to govern and manage adequately the different challenges offered by the extraordinary biotechnological progress that is characterizing these years.
How the Spoke will contribute to advance in this scenario. The Spoke led by the IRCCS OPBG Rome in collaboration with Fondazione Telethon/Tiget, Tettamanti, CNR, UNICAMPANIA, UNICH will leverage on world-recognized scientific and technical expertise to further advance and bring to full effectiveness the translation of state-of-the-art pre-clinical research into clinic The Spoke led by the IRCCS OPBG Rome in collaboration with Fondazione Telethon/Tiget, Fondazione Tettamanti, CNR, UNICAMPANIA, UNIPD and UNICH will leverage on world-recognized scientific and technical expertise to further advance and bring to full effectiveness the translation of state-of-the-art pre-clinical research into clinic trials of novel GTMPs. This will be achieved through the execution of several pilot training projects, selected for their paradigmatic value, to address some key outstanding hurdles and to provide demonstration of potential solutions. Three of the Institutions affiliated to the Spoke, namely OPBG, Fondazione Telethon/Tiget and Fondazione Tettamanti have already in place several pre-clinical and clinical collaborations, often supported by other academic grants that have been obtained through submission to competitive calls. Moreover, these three institutions are all characterized by long-standing expertise in the field of gene therapy and genome editing for treatment of cancer and inherited disorders, which is widely recognized at the international level. Their relative qualification and expertise are largely complementary and instrumental to create synergies in the competitive field of GTMP. The research will be carried out with current, available facilities that will be further upgraded and scaled-up for serving the proposed national mission. Indeed, after an initial training and enabling phase, the results of the research will become ready to accept other projects from Italian Academic Institutions. It is expected that this critical mass of expertise and qualification will render Italy a highly competitive partner for international projects and for developing privileged partnership of collaboration in the field of personalized and precision medicine. Moreover, the **Spoke #10** is highly committed to establish and consolidate win-win collaborations with pharmaceutical/lifescience biocompanies. For examples, OPBG will collaborate with both Takis and IRBM biotech companies in projects aimed at discovering novel antigens that can be targeted by CAR T cells. The 2 biotech companies will screen antibodies directed against these targets and will perform the final antibody selection for CAR generation. The close collaboration between the research institute and the private partners will allow a rapid selection of CAR candidate for preclinical evaluation. On another side, other industrial partners such as Orgenesis, PBL and Stevanato will play a key role for implementing strategies of decentralized manufacturing, also thanks to the development and validation of a fully automated instrument for the manufacturing of GTMPs. The companies affiliated to the Spoke will substantially contribute to the activities that will be pursued by the **Spoke #10** and we will facilitate the transferability of research results to industry, increasing the value of the intellectual properties (IPs) owned by Italian research centers through the out-licensing of more mature technologies. These perspectives, coupled with the training of a new generation of scientists/biotechnologists/clinicians expert in emerging techniques, will advance the Country to a high biotechnology level, able to attract investors and developers from other Countries.
In WP 10.1, 10.2, 10.3, a strong collaborative effort will be pursued with **Spoke #7** to develop a comprehensive bioinformatics environment for the analysis of omic data to assess clonality of viral vector-transduced cell populations, with the aim to monitor the fate of individual gene-corrected cells in vivo, and to assess vector integration, and, thus, biosafety. **Spoke #10** will benefit from the bioinformatic infrastructure established by **Spoke #7** that will develop original methods and automated bioinformatic analysis workflows to rapidly process and annotate vector integration sites. In particular, the institutions participating into the 2 Spokes will collaborate for the optimization of NGS-based methods to explore the genome-wide distribution and the clonal architecture of transgene integration sites. Once sequencing data are generated, the application of rigorous bioinformatics analysis is key to the biological interpretation of the data. In order to better exploit the potential information available through these methods, **Spoke #7** and **#10** will collaborate in developing and optimizing bioinformatics tools and pipelines to analyze NGS clonality datasets. Transcriptomics, epitranscriptomics, epigenetic and proteomic analyses will be applied to GTMPs generated in **Spoke #10** for the deep characterization of signaling related to innovative drug products (DPs) and for predicting their effects after infusion in patients. scRNA-seq will be carried out on gene modified cells from DPs, as well as after their selection/isolation from biological samples of treated patients. In particular, although the activation process of normal T cells is well characterized, little is known about the activation of cells via the CAR signaling. Thanks to the collaboration between **Spoke #7** and **#10**, we will use flow-cytometry together with single-cell transcriptome profiling to characterize the starting material and CAR products either in the presence or in the absence of antigen-specific stimulation. The resulting molecular signature for CAR T-cell activation will be instrumental for future development of the DPs. Integration and interrogation of carefully selected omics data will also represent a powerful way to guide the assessment and optimization of clinical-grade CAR-T-cells, and inform future research into the underlying molecular processes.
Computational Systems Biology and integrative data analysis infrastructure generated by **Spoke #7** will provide

computational tools to **Spoke #10** activities with the goal of discovering novel targets for the immunotherapy approaches developed in **Spoke #10**. In particular, structure-modeling algorithms will be used to predict the best scFv configuration for CAR design, helping reduce the experimental validation of novel CAR T-cell products. In particular, combinational antigen recognition is the most logical way to improve the safety of cancer therapy. CAR T-cell therapy, combined with synthetic biology, protein engineering, and bioinformatics, will benefit from advanced computations to enhance tumor targeting specificity, also in the perspective of developing multi-specific products. **Spoke #10** expertise and infrastructures will be available across the NC to help the development of clinical grade GTMPs of interest for all the vertical Spokes (**#1-5**).

WP 10.1 – Development of innovative GTMPs based on novel targets/novel indications for oncologic disorders not covered by market-authorized ATMPs	
General objectives • Development of novel gene therapy products based on proprietary CARs for urgent clinical need diseases in the field of oncology • Pre-clinical validation of at least 5 different GTMPs to be employed in different clinical settings, including haematological malignancies and solid tumors. • GMP development of at least 3 innovative GTMPs • 3 clinical trials for the early clinical translation • Involvement of industrial patterns for the GMP development • Involvement of industrial patterns for the clinical/market development of the Spoke 10 GTMPs behind M36 of the current application. **Methodology.** Spoke 10's members involved in WP10.1 will pursue the pre-clinical development of at least 5 GTMPs, characterizing the mechanism of action, potency and biodistribution/toxicology properties of these innovative products performing *in vitro* and *in vivo* experiments. In parallel, taking advantage of the GMP facilities available in Spoke10, 3 GTMPs will be advanced to the generation of clinical grade products and the IMPDs formulation. Spoke 10 leaders will design and submit to the competent regulatory agency clinical trials for the early clinical evolution of at least 3 developed GTMPs. • At least 3 Phase I clinical trials will be started in the time frame of this application.	
Task 10.1.1	**CAR.CD7 T cells for T-ALL (Task leader: OPBG)**
TRL	**5→7**
	Phase I Clinical Trial
Deliverables WP 10.1.1	• IMPD submission (M12) • Clinical trial approval (M16) • First Patient treated (M20)
Milestones WP 10.1.1	• Validation of production of CD7 CAR T cells (M10) • Design and approval of the clinical trial in patients with relapsed/refractory T-cell acute lymphoblastic leukemia and lymphoblastic lymphoma (M16) • Start of the proof-of-concept clinical trial (M20)
Task 10.1.2	**CART-CD79B in adult/pediatric B-cell NHL (Task leader: Tettamanti)**
TRL	**3→6**
	Phase I Clinical Trial
Deliverables WP 10.1.2	• IMPD production (M24) • Clinical Trial Application submission (M28) • Clinical trial approval (M32) • First Patient treated (M36)
Milestones WP 10.1.2	• Preclinical activities completion (M12) • Protocol defined and Clinical Trial Application approval (M32) • Start of the phase I dose escalation study (M36)
Task 10.1.3	**Development of novel CAR approaches in refractory/resistant acute myeloid leukemia (Task leader: UNIPD)**
TRL	**3→5**
	Proof-of-concept data collection
Deliverables WP 10.1.3	• In vitro proof-of concept (POC) of efficacy (M4) • POC of efficacy in animal models (M12) • Pre-enquiry dossier submission (M18) • POC of safety/tolerability (M24) • GMP Vector production contract activation (M24)
Milestones WP 10.1.3	• CAR construct selection for clinical development (M3) • Advice from regulatory agency for planning a First-in-Human clinical trial (M18) • Start of chemistry, manufacturing and controls (CMC) activities with start of LV production (M30)
Task 10.1.4	**CAR.GD2 T cells in patients with Brain Tumors (Task leader: OPBG/Orgenesis)**
TRL	**5→7**
	Phase I/II Clinical Trial
Deliverables WP 10.1.4	• IMPD submission (M4) • Clinical trial approval (M8) • First Patient treated in Phase I (M12) • First Patient treated in Phase II (M24)

	• Development of a novel nasal-to-brain administration route for CAR T cells in CNS (M32-Orgenesis)
Milestones WP 10.1.4	• design and approval of the clinical trial in patients with brain tumors (M8) • start of the proof-of-concept clinical trial (M12) • Completion of Phase I and starting of Phase II (M24) • Establishment of a novel nasal-to-brain system for CAR T cell administration (M34)
Task 10.1.5	**Development of novel antibodies for CAR T cells (Task leader: Takis/OPBG)**
TRL	**2→5**
	Proof-of-concept data collection
Deliverables WP 10.1.5	• Identification of targets suitable for CAR-T generation (M4) • Generation of novel antibodies (M16) • Characterization of selected antibodies (M24) • Construct design for CAR generation (M32)
Milestones WP 10.1.5	• Selection of one or more available antibodies in Takis library (M8) • Identification of novel lead candidates for treatment of solid tumors (M18) • Final antibody selection for CAR generation (M30) • Selection of CAR candidate for preclinical evaluation (M34)
Task 10.1.6	**Discovery and validation of new targets for CAR T cell therapies (Task leader: IRBM/OPBG)**
TRL	**2→5**
	Proof-of-concept data collection
Deliverables WP 10.1.6	• Screening of potential new targets in haematological malignancies for CAR-T cell generation (M8) • Generation of novel antibodies (M16) • Screening of the novel Abs against healthy tissues and malignant cells (M24) • Construct design for CAR generation (M32)
Milestones WP 10.1.6	• Identification of novel lead candidates for treatment of haematologic malignancies (M12) • Final antibody selection for CAR generation (M18) • Selection of CAR candidate for preclinical evaluation (M36)
Milestones WP 10.2 Development of allogeneic GTMPs for treating patients with hematological malignancies	
General objectives • Pre-clinical validation of 4 different GTMPs to be employed in different clinical settings, including pediatric and adult patients, based on the use of allogenic effector cells. • GMP development of these 4 innovative GTMPs • 4 clinical trials for the early clinical translation • Involvement of industrial patterns for the GMP development of the GTMPs in Spoke10 pipeline • Involvement of industrial patterns for the clinical/market development of the Spoke 10 GTMPs behind M36 of the current application. **Methodology.** Spoke 10 Members will pursue the pre-clinical development of 4 GTMPs, characterizing the mechanism of action, potency and biodistribution/toxicology properties of the innovative products performing *in vitro* and *in vivo* experiments. In parallel, taking advantage of the GMP facilities available in Spoke10, the 4 GTMPs will be advanced to the generation of clinical grade products and the IMPDs formulation. Spoke 10 leaders will design and submit to the competent regulatory agency clinical trials for the early clinical evolution of at least 4 developed GTMPs. • At least 4 Phase I clinical trials will be started in the time frame of this application.	
Task 10.2.1	**Allogeneic CAR T cells in B-ALL and B-cell Lymphoma (Task leader: OPBG)**
TRL	**5→7**
	Phase I/II Clinical Trial
Deliverables WP 10.2.1	• IMPD submission (M4) • Clinical trial approval (M8) • First Patient treated in Phase I (M12) • First Patient treated in Phase II (M24)
Milestones WP 10.2.1	• validation of production of allogeneic, third-party CAR T cells (M2) • design and approval of the clinical trial in patients with relapsed/refractory B-cell acute lymphoblastic leukemia (M8) • start of the proof-of-concept clinical trial (M12)
Task 10.2.2	**Allogeneic CAR cells in Acute Myeloid Leukaemia (AML) (Task leader: OPBG/Tettamanti)**
TRL	**4→7**
	Phase I/II Clinical Trial
Deliverables WP 10.2.2	• Pre-clinical studies on CAR NK/CIK for AML (M8) • First IMPD submission for CAR NK (M18) • Second IMPD submission for CAR CIK (M22) • Clinical trial approval for CAR NK (M20) • Clinical trial approval for CAR CIK (M28) • First Patient treated CAR NK (M24) • First Patient treated in Phase II CAR NK (M32) • First Patient treated in Phase I CARCIK study (M32)
Milestones WP 10.2.2	• Collection of pre-clinical data (M12) • Validation of production of CAR NK cells and CAR CIK cells for AML (M16) • design and approval of the first clinical trial(s) in patients with relapsed/refractory AML (M20) • start of the proof-of-concept clinical trial(s) (M24)
Task 10.2.3	**Allogeneic CARCIK-CD19 in adults/pediatric B-cell NHL or CLL (Task leader: Tettamanti)**

TRL	**6→7**
	Phase I/II Clinical Trial
Deliverables WP 10.2.3	• Clinical Trial Application submission (M4) • Clinical trial approval (M8) • First Patient treated in Phase I (M12) • Completion of the dose escalation (M24) • Completion of the expansion phase (M36)
Milestones WP 10.2.3	• Protocol design and approval of the clinical trial application (M8) • Start of the proof-of-concept clinical trial (M12) • Completion of Phase I and starting of Phase II (M24)
WP 10.3 - Development and validation of innovative GMP manufacturing approaches in the field of GTMPs	
General objectives • First IMPD submission for CAR NK (M12) • Second IMPD submission for CAR CIK (M22) • Clinical trial approval for CAR NK (M16) • Clinical trial approval for CAR CIK (M28) • First Patient treated CAR NK (M20) • First Patient treated in Phase II CAR NK (M32) • First Patient treated in Phase I CARCIK study (M32) **Methodology.** To improve the scalability of the GTMPs manufacturing and in parallel to reduce the costs associated with the DP, several approaches will be taken in consideration in WP10.3, including the generation of nanoparticles to deliver transgenes, or DNA plasmid and RNA guides for the genome editing. Advanced approaches of large scale electroporation will be also considered for the purpose to manufacturing GTMPs. UD'A will advance the knowledge on the use of EV derived from CAR T cells characterizing their mechanism of action, potency and biodistribution/toxicology properties performing *in vitro* and *in vivo* experiments. • Development and validation of a fully automated instrument for the manufacturing of GTMPs.	
Task 10.3.1	**Nanotechnology-based tools to advanced genome editing (Task leader: CNR-NANOTEC/OPBG)**
TRL	**2→4**
	Proof-of-concept data collection
Deliverables WP 10.3.1	• Co-delivering of ssODNs and Cas9-gRNAs by Np for single gene targeting (M12) • Multiplex co-delivering of ssODNs and Cas9-gRNAs by Np for gene targeting (M24) • identifying modulators/interactors in CAR-T models (M36)
Milestones WP 10.3.1	• Development of non viral nanovectors for genome editing (M18) • Development of non viral nanovectors for multiplex genome editing (M32) • Non viral nanovector-based CRISPR/Cas9 genome wide screen (M36)
Task 10.3.2	**Set up of non-viral, DNA-based methods for CAR-T generation (Task leader: Takis/OPBG)**
TRL	**2→5**
	Proof-of-concept data collection
Deliverables WP 10.3.2	• Set up methods for plasmid DNA expressing CAR (M12) • Set up methods for linear DNA expressing CAR (M20) • Evaluation of electroporation technology to generate CAR-T in vivo/ex vivo (M24) • Combination of CAR-T and vaccines in preclinical models (M36)
Milestones WP 10.3.2	• Established SOP for CAR plasmid DNA preparation (M16) • Established SOP for CAR amplicon-based DNA preparation (M24) • In vivo efficacy data for CAR-T transduced through non viral approaches (M30) • Efficacy data of combo therapy in mouse models (M36)
Task 10.3.3	**Extracellular vesicles (EVs) from CAR-T cells in pancreatic adenocarcinoma (Task leader: UD'A)**
TRL	**2→5**
	Proof-of-concept data collection
Deliverables WP 10.3.3	• Ethical statement approval (M6) • CAR-T cells against pancreatic adenocarcinoma (M10) • CAR-EVs against pancreatic adenocarcinoma (M22) • First in vitro functional assay (M24) • first in vivo functional assay (M28)
Milestones WP 10.3.3	• Submission of documents to obtain ethical committee and animal use approval (M3) • CAR-T cell engineering (M12) • Isolation of CAR-EVs against pancreatic adenocarcinoma • Optimization of protocols for CAR-EVs functional evaluation (M20) • CAR-EVs in vitro and in vivo functional evaluations (M30)
Task 10.3.4	**Development and realization of a fully-automated closed system for the production of ATMPs (Task leader: PBL/OPBG)**
TRL	**5→7**
	First working approved prototype
Deliverables WP 10.3.4	• Facility Acceptance test (FAT) (M10) • Site Acceptance Test (SAT) of the prototype of the System, after installation within the GMP facility (M12) • Manufacture of at least two Advanced Medicinal Products using the protype of the System (M20) • AIFA inspection of the novel automated closed system for ATMP production (M36)

Milestones WP 10.3.4	• Definition of detailed technical specifications of the system (M14) • Approval of the Design of the System • Creation of the first prototype (M16) • Start of the tests on the prototype (M18) • Completion of all required tests on the prototype (M32)
Task 10.3.5	**Validation of the OMPUL system as point of care for the manufacturing of GTMPs (Task leader: Orgenesis/OPBG)**
TRL	**5→6**
	Regulatory approval
Deliverables WP 10.3.5	• Validation of SOP for the OMPUL use (M12) • AIFA inspection of the OMPUL system (M20) • Regulatory approvalI (M30)
Milestones WP 10.3.5	• validation of production of CD19 CAR T cells in the OMPUL system (M18) • Approval of the manufacturing approach by the national competent authority (M24) • design and approval of the clinical trial (M30)
Task 10.3.6	**Setup a high-throughput high-quality ATMP GMP manufacturing facility (Task leader: Stevanato)**
TRL	**5→7**
	Regulatory approval
Deliverables WP 10.3.6	• Development of an automated and new tech closed-loop bioprocessing equipment (M16) • Feasibility study #1 report (M16) • Feasibility study #2 report (M20) • Feasibility study #3 report (M24) • Improved GTMP manufacturing operational model (M28) • Improved GTMP manufacturing approach (M30)
Milestones WP 10.3.6	• Definition of detailed current ATMP manufacturing baseline operational model (M6) • Definition of ATMP needs and requirements based on therapeutic and capacity targets (M12) • Definition of criteria for and identification of three GTMP projects to be leveraged in feasibility studies (M14) • Launch of development activity plan for full-scale GTMP manufacturing facility implementation and for pre-qualification runs (M32)
WP 10.4 – Development of GTMPs for rare diseases in non-oncologic settings	
General objectives • Pre-clinical validation of 5 different GTMPs to be employed in different clinical settings, including rare diseases. • GMP development of these 5 innovative GTMPs • Five pivotal trials for the early clinical translation • Involvement of industrial patterns for the generation of GMP grade AAV vectors. • Involvement of industrial patterns for the clinical/market development of the Spoke 10 GTMPs behind M36 of the current application. **Methodology.** Spoke 10 Members will pursue the pre-clinical development of 5 GTMPs, characterizing the mechanism of action, potency and biodistribution/toxicology properties of the innovative products performing *in vitro* and *in vivo* experiments. In parallel, taking advantage of the GMP facilities available in Spoke10, the 5 GTMPs will be advanced to the generation of clinical grade products and the IMPDs formulation. Spoke 10 leaders will design and submit to the competent regulatory agency clinical trials for the early clinical evolution of at least 5 developed GTMPs. • At least 5 Phase I clinical trials will be started in the time frame of this application.	
Task 10.4.1	**Gene therapy for beta-thalassemia (Task leader: Telethon-Tiget/OPBG)**
TRL	**6→7**
	Phase II Clinical Trial
Deliverables WP 10.4.1	• SOP for manufacturing of the DPs (M8) • Technology transfer to GMP facility (M16) • Virus vector production (M20) • Toxicity and biodistrubiton data package (M20) • IMPD submission (M24) • Clinical trial approval (M30) • First Patient treated in Phase II (M32)
Milestones WP 10.4.1	• development of SOP for the DP manufacturing and release (M8) • GMP validation of DP production (M20) • Completion of GLP studies (M20) • design and approval of the clinical trial (M30) • start of the proof-of-concept clinical trial (M32)
Task 10.4.2	**Generation of an innovative platform for gene therapy approach for lysosomal storage disorders with bone involvement. (Task leader: Telethon-Tiget)**
TRL	**3→5**
	Proof-of-concept data collection
Deliverables WP 10.4.2	• Toxicity and biodistrubiton data package (M24) • IND filing (M28)
Milestones WP 10.4.2	• Start of GLP studies (M14) • Completion of GLP studies (M24) • IND evaluation from the regulatory agency (M30)

Task 10.4.3	***In vivo* intracerebral lentiviral gene therapy for metachromatic leukodystrophy (Task leader: prof. Magnani- Telethon-Tiget)**
TRL	**4→6**
	Phase I Clinical Trial
Deliverables WP 10.4.3	• Drug Product (DP) formulation development (M12) • Completion of pre-clinical study using DP in vitro (M18) and GLP tox/bio in NHP (M24) • ODD submission (M12) and ISS pre-submission (M18) • IMPD submission (M24) • First Patient treated (M36)
Milestones WP 10.4.3	• DP batch manufacturing and release (M20) • PoC of efficacy in MLD neurons/glia (M24) • PoC of safety/distribution in CNS of large animals (M32) • Design and approval of PhI/II clinical trial (M34) • Start of the PoC clinical trial and preliminary safety evaluation (M36)
Task 10.4.4	**Ex vivo expansion of genetically-engineered hematopoietic stem and progenitor cells (Task leader: Telethon-Tiget/Tettamanti)**
TRL	**4→6**
	Phase I Clinical Trial
Deliverables WP 10.4.4	• Toxicity and biodistrubiton data package (M22) • IND filing (M24) • First patient recruited in phase 1/2 study (M32)
Milestones WP 10.4.4	• Start of GLP studies (M5) • Completion of GLP studies (M22) • Start of clinical study (M30)
Task 10.4.5	**Gene therapy for inherited retinal disorders (Task leader: UNICA)**
TRL	**4→6**
	Phase I/II Clinical Trial
Deliverables WP 10.4.5	• Approval of natural history study (M3) • 1st year follow-up within Natural History Study (M15) • Toxicology Study report (M24) • 2nd year follow-up within Natural History Study (M27) • IMPD submission (M27) • Clinical Trial approval (M31) • First Patient treated in Phase I/II clinical trial (M32)
Milestones WP 10.4.5	• Completion of the Natural History Study designed to support definition of clinical biomarkers and to identify the best candidates for Clinical Trial (M28) • Completion of the Toxicology Study to support dose definition for Clinical Trial (M24) • Design and approval of the Clinical Trial (M31) • Start of the proof-of-concept phase I /II Clinical Trial (M32)
Task 10.4.6	**CD19 CAR-T for treatment of refractory systemic autoimmune disease (Task leader: Orgenesis/OPBG)**
TRL	**5→7**
	Phase I Clinical Trial
Deliverables WP 10.4.6	• IMPD submission (M16) • Clinical trial approval (M20) • First Patient treated (M24)
Milestones WP 10.4.6	• validation of production of CD19 CAR T cells from patients with auto-immune diseases (M12) • design and approval of the clinical trial in patients with refractory systemic autoimmune disease (M20) • start of the proof-of-concept clinical trial (M24)
Task 10.4.7	**cGMP grade plasmid manufacturing microbial facility to produce viral vectors AAV (Task leader: Innovavector)**
TRL	**4→8**
	Manufacture cGMP grade plasmids for AAV production
Deliverables WP 10.4.7	• Establishment of cGMP Microbial Fermentation Facility for plasmid production (M12) • Qualification of the Facility for plasmid production (M26) • Validation of Upstream Process for expansion of cell biomass and plasmid yield for Helper, Cis and GOI plasmids and establishment of cGMP Cell Banks (M30) • Validation of Downstream Purification for Plasmid (M32) • Establishment of Analytical Characterization and QC Release methods for Plasmids. (M36)
Milestones WP 10.4.7	• Rooms for cGMP plasmid production fully equipped and qualified (M26) • Plasmid production process in place (M30) • Quality check and analytical methods established and validated (M32) • Release of the first cGMP batch of in-house produced plasmids for AAV production (M36)

Refer to *Annex - Spoke list and description* for an overview on the spoke structure and objectives.

Monitoring of intermediate and final objectives

We have planned different responsibility levels for monitoring project progress. This refined monitoring of the programme will allow to evaluate if the different subprojects are in line with the expected plan during the intermediate phases of the activities that the participating institutions have included.

Spoke leaders. Spoke leaders will start monitoring research activities shortly after beginning the project (kick-off meeting for formative evaluation and integrative actions of the Spoke Committee) and throughout the project (process evaluation meetings every three months). Spoke leaders' monitoring of the activities will allow determining what is and is not working well so that adjustments can be made along the way. It will enable assessing what is actually carried-out compared to planned (technical milestones and research progress). Monitoring will allow research activities to:

1) implement rescue measures to get research activities back on track and remain accountable to the expected results the programme is aiming to achieve;
2) determine how funds should be distributed across the programme activities;
3) collect information that can be used in the evaluation process. The spoke leaders will have the task of monitoring these progress and will report to the hub eventual deviations from the plan.

Task leaders. The Task Leaders ensure that the participants are undertaking the research activities as outlined in the work plan and secure the smooth running of the project. The Task Leaders also provide that the exchange of knowledge and skills between participants occurs satisfactorily and monitor progress according to the scientific deliverables and milestones. For this purpose, each Task Leader organizes periodic task meetings and coordinates the activities with the Task Leaders of the Spokes. The Task Leaders will send periodic (each 6 months) and annual reports on scientific progress to the Spoke Committee. If any changes to the original plan need to be made, these will be first ratified by the task leader and then referred to the Spoke Committee.

Monitoring and refocusing the medicine pipeline

The success of a pipeline of drug development with an new technology (RNA based drugs) and a technology (gene therapy) that has a long history of successful application in Italy and has seen in recent years technological break-throughs allowing an exponential increase in applications and number of potentially treatable patients, requires an efficient cooperation between scientific projects aimed at the development of novel drugs and scientific projects improving the tools (modified RNAs, delivery tools, vectors for gene therapy, pharmacological assessment and biosafety). This was the rationale of building a center based on vertical (disease-oriented) and horizontal (technology-oriented) spokes and fostering their coordinated action. As far as disease-oriented spokes are concerned, success will rest on translating robust cell biology and animal studies on defined targets to the development of the novel drug, in a highly competitive, dynamic international panorama. For this purpose, the choice was to select a large number of perspective drug targets, based on robust preliminary data and qualification of the PI, thus committing to the task of the Center a roster of excellent scientists and exploring a spectrum of potential new drug, in which the quality of the research of the Italian groups could give a competitive advantage. However, it is clear that the drug development pipeline should be rapidly concentrated on the candidates with highest possibility of success. For this reason, also based on the recommendation of the Evaluation Committee of the proposal, budget has been refocused and a monitoring process clearly established for funnelling the research effort towards the most effective projects (in terms of advancement in TRL and perspective drug development):

- Budget assignments will be reconsidered yearly by the Board of Directors, based on the Report of Activity of the Spoke Committees, the evaluation of the Scientific Advisory Board and the recommendations of the Industrial Board.
- For horizontal Spokes, the Scientific Advisory Board and the Industrial Board will be requested to evaluate the scientific progress and improvement of existing technology; for vertical Spokes, they will be asked, in addition, to advise the selection of the most promising drug targets, in order to funnel funding and research activity into those projects.
- A proportionally greater emphasis, in terms of funding, has been placed on the RNA chemistry (#6) and Delivery (#8) Spokes, as well Pharmacology (#9) and Vector Development and Clinical Trials in Gene Therapy (#10), in order to sustain a strong technological backbone of the Center.
- For vertical Spokes, a significant portion of the potential grant budget (>14 M€) has been held within the Hub, to fund collaborative grants between vertical and horizontal Spokes for designing and testing in vitro or in animal studies new medicines or delivery tools.
- >13 M€ are held within the Hub, to fund Proof of Concept (POC) grants by Public and Private Entities to support the progress of RNA and gene therapy products to higher TRL and clinical application.

Contigency Plan

Considering the challenge of some tasks, an accurate contingency plan has been defined from the Spokes. The following table summarizes The main corrective actions to reduce the risks in the envisioned scenario.

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
1	Different mouse models are used in different labs.	M	M	SPOKE	An assessment of all the mice models available in partners' laboratory will be carried out.	An animal model will be chosen for a specific gene mutation or for a specific disease so that effects of treatments will be comparable among labs for different therapeutic RNAs.
1	Not all tasks are successful at all stages	H	M	SPOKE	1) Deploy a portofolio of tasks and targets; 2) Envisage high competition 3) Plan a two-step funding strategy	1) Mantain competitiveness high; 2) Prune the portfolio as needed; 3) Redistribute funding based on milestones met
1	Poor bio/pharmaceutical properties of nanomedicine	M	M	SPOKE	Deep analysis of chemico-physical properties of molecules to be delivered will help in designing the best formulative approach and material choice in order to fullfill the most appropriate composition and nanopreparation technologies (i.e. formation of complexes to enhance solubilty or ameliorating bioavailability)	Proper solutions will be applied aiming to increase stability and efficiency, namely with the exploitation of well-known technologies for gene material delivery, which could confer ameliorated features to gene delivery approach, as the use to cationic liposome or hybrid cationic systems as delivery systems, produced by following classical and well-known procedures for stabilization as lipoplexes formation.
1	Setting of production of nanomedicine by means of industrial technologies	M	M	SPOKE	Preformulation studies (i.e. solvent mixing conditions) and generally microfluidic technologies will be applied by pre-testing with laboratory equipments will be exploited in order to individuate the key parameters ranges enabling the obtainment of nanomedicine in pre-GMP environment	Optimization and re-setting of parameters such as the flow rate ratio, temperature, total flow rate will be re-considered and fully optimized to produce NMed with desired characteristics (size, composition, surface properites, drug loading) and ready to be scaled up in a GMP setting.
2	Too many novel identified targets during the screening of cancer mutations from patient's derived samples within WP1 will hamper subsequent development due to saturation of validation activities.	H	M	SPOKE	A priority assessment is forecast in order to drive the output of the discovery process to the validation step and even further to the regulatory activities in the framework of cooperation with Spoke 8.	Each set of novel targets will be evaluated at the end of each reporting period (four or six months). The evaluation process will be carried out by a group of experts from cooperating spokes under the coordination of the Steering committee.

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
2	Preclinical regulatory studies might require longer time to be executed due to the avilability of tumor models in animals.	H	M	AFFILIATED SPOKE	Synchronicity will be necessary to plan accurately the preparation of tumor animal models as soon as the novel therapeutics RNAs are synthesized.	Use of open calls to outsource specific studies on animal models in addition to those forecast within the participating centers.
2	Clinical translation in novel cancer therapies might be outside the limited time of the funding period	H	M	SPOKE	Innovative regulatory protocols includes the possibility of approaching direct intratumor delivery of appropriately formulated RNA therapeutics in order to accelerate clinical translation.	Innovative clinical studies protocols will be elaborated and discussed with regulatory authorities that take into account the possibility of fast track routes for RNA products formulated with already approved delivery systems.
3	Preclinical studies might require longer time to be executed due to the fact that neurodegenerative diseases are age-dependent and mice phenotypes are evident at 6-12 months of age.	H	M	SPOKE	Advance planning of breeding and of drafting regulatory documents so that mice are available at the right age as soon as the novel therapeutics RNAs are synthesized.	Use of mice models that present earlier phenotypes or pre-symptomathic markers (aggregates etc).
3	Many ncRNAs are potential drug candidates or drug targets	H	L	SPOKE	A priority assessment will be carried out taking into account next validation steps and route of delivery.	Focus on the most promising molecules given their sequence features, potential targets and how they will be delivered.
3	Different mouse models are used in different labs.	M	M	SPOKE	An assessment of all the mice models available in partners' laboratory will be carried out.	An animal model will be chosen for a specific gene mutation or for a specific disease so that effects of treatments will be comparable among labs for different therapeutic RNAs.
3	Temporary difficulties in carrying out a specific technique or data analysis by a single laboratory.	L	M	AFFILIATED SPOKE	An assessment of the ability to carry out the experiment and data analysis by other laboratories in the Spoke or in the CN.	Transfer the experiment to another laboratory within a collaborative agreement. Transfer of knowledge.
4	Not all tasks are successful at all stages	H	M	SPOKE	1) Deploy a portofolio of tasks and targets; 2) Envisage high competition 3) Plan a two-step funding strategy	1) Mantain competitiveness high; 2) Prune the portfolio as needed; 3) Redistribute funding based on milestones met

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
4	Progresses toward high TRL are limited	H	H	SPOKE	1) Have flagship projects with high starting TRL; 2) Include clinical scientists as PI; 3) Onboard big pharma	1) Redistribute more funding to projects closer to clinical development; 2) Mantain strong contact with biotech and pharma companies
4	Slow recruitment of personnel	M	M	HUB	1) Rely on permanent staff; 2) Distribute personnel funding on 3 years; 3) Ensure co-funding by participating Institutions	1) Promote local policies for accelerated recruitment
4	Lack of competencies within Institutions and research groups	M	M	HUB	1) Integrate horizontal with vertical Spokes; 2) Program Cascade Calls	1) Enable local institutions to issue Cascade Calls; 2) Monitor and promote H/V Spoke contamination
5	Different animal models used in different tasks and WPs may lead to differences in immune responses evaluation during pre-clinical stages thus limiting TRL advancements	M	L	SPOKE	Harmonization of animal models usage across multiple tasks and WPs	Assessment of immune response differences in different animal models in order to be take into consideration for TRL advancements
5	Too many candidate targets as potential novel drugs or biomarkers	H	M	SPOKE	Definition of a classification priority of the best candidates to be evaluated in validation or in pre-clinical stages. Classification priority will be pre-defined in concert with other WPs and Spokes	Most promising candidates will be validated and moved forward into the pre-clinical or clinical stages. Additional candidates will be maintained and putatively tested in future plans or evaluated in external project calls.
5	Identified vector-based delivering strategy is not appropriate for cargoed molecules	M	M	SPOKE	Selection of the best vector-corgoed molecules combination to be moved forward for production and in-vitro/preclinical validation.	Coordination with horizontal spokes in order to proceed with an alternative combination strategy
6	Delay in development of, or difficulties of vertical groups in accessing to technological units and consultancy	H	M	SPOKE	Spoke leader and WP leaders form a scientific committee responsible for management and proposing solutions	1) Prioritise activities of reasearch groups 2) prioritise access to technological units
6	Insufficient progress in attaining the main goals of the project	M	H	SPOKE	1) open calls to implement additional competencies. 2) A scientific advisory board 3) Plan a two-step funding strategy	1) Direct involvement of the companies associated with the spoke in devising innovative solutions 2) Redistribute funding based on milestones met

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
6	Not all tasks are successful at all stages	M	M	SPOKE	1) Deploy a portofolio of tasks and targets; 2) Plan a two-step funding strategy	1) Prune the portfolio as needed; 2) Redistribute funding based on milestones met
7	Limited accuracy of the predictive tools based on biocomputing apporaches	M	M	SPOKE	Use of extended datasets to carry out the analysis and /or generation of ad-hoc data to improve the reliability of predictive tools and provide more robust training sets for ML/AI approaches.	Optimize the prediction of neo-antigens, RNA drugs and regulatory networks reconstruction
7	Limited computational efficiency of algorithms and software implemented in the dedicated ICT facility	M	M	SPOKE	Adoption of new generation computer processors, high performance computing and/or GPU cards to increase the comptational efficiency of inmplemented tools	Increased efficiency of the Bioinformatics ecosystem designed to support the activities of all other spokes of the National center on gene therapy and RNA drugs.
7	Lack of effective and efficient algorithm solution to address specific computational issues	M	M	SPOKE	Extended literature mining to gather an updated state of the art and/or interaction witj top international scientists working on the specific subject of interest.	Use of open calls to outsource specific bioinformatics solutions needed to achieve the planned goals.
8	Too many platforms are developed in WP 8.1 to test a therapeutical concept from vertical spokes	H	M	SPOKE	Platform prioritization based on the quality profile and number/type of biological models tested	Report on results achieved will be examined and best candidate platform selected after a meeting between PI and the vertical spoke leader involved
8	The development of preclinical models in WP 8.2 and platforms in 8.1 is not temporally aligned	H	L	SPOKE	The interdisciplinary discovery pipeline in wp 8.1 and interactions with spoke 9 garantees that each platform proceeds to preclinical phase	PI will communicate to the task leader that a lack of biological experiments can hinder project advance. Task leader informs spoke leader who facilitate project progression in the appropriate task in spoke 9.
8	Best performing nanoplatform candidates cannot be produced according to industrial processes and quality requirements	L	M	AFFILIATED SPOKE	Support from external collaborations of the pharmaceutical companies in the center	Cooperation with companies is requested by spoke leader. In case no effective solution is found, an external call is activated by the spoke
8	Unavailability of sufficient patient-derived samples to build models in WP8.2	M	H	SPOKE	Interaction with hospitals and owners of cell banks	The PI solicit intervantion from the spoke to interact with hospital and cell bank owners and get the appropriate spacimen

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
9	Long term project:generation of innovative methods to combine PK-PD studies too ambitious	M	H	SPOKE	The mitigation strategy is to pursue several lines of study in parallel and closely follow the results obtained by each research line in order to select in progress the most promising pathways	PPP will be created as well as research within the spoke will be done in order to ensure the possibility to develop NA-Based drugs using current technologies
9	Tasks not performing as expected	H	L	AFFILIATED SPOKE	The mitigation strategy is to incentivate collaboration among tasks by periodic meetings and create a reporting system enabling the rapid identification of the underperforming tasks in order to try to identify the reasons causing the delay and find a solution in other groups inside the CN or outside	The PI will solicit specific interactions with groups which could help in overcome the obstacles created
9	The regulamentary authorities will not approve the innovative methodologies set up and proposed	H	M	SPOKE	There is no mitigation strategy to be taken other than creating a cultural environment supported by the rigour of the data and quality of pubblications that will prove the advantages of the methodology selected	-
9	Research overlap	M	M	SPOKE	The probabilities of duplication in the research to be done within the spoke are limited by the fact that the groups participating were selected for their diversified background, however the problems of unnecessary duplications will be avoided by periodic reporting on the research results	The PI will organize periodic group meetings aimed at following closely the outcome of the researchand to facilitate communication and synergies among the participating groups.
10	Preclinical regulatory studies might require longer time to be executed due to the avilability of tumor models in animals.	H	M	AFFILIATED SPOKE	Synchronicity will be necessary to plan accurately the preparation of tumor animal models as soon as the novel therapeutics RNAs are synthesized.	Use of open calls to outsource specific studies on animal models in addition to those forecast within the participating centers.
10	Clinical translation in novel cancer therapies might be outside the limited time of the funding period	H	M	SPOKE	Innovative regulatory protocols includes the possibility of approaching direct intratumor delivery of appropriately formulated RNA therapeutics in order to accelerate clinical translation.	-
10	Large number of clinical trials in the field of GTMPs	M	H	SPOKE	Three members of Spoke10 have developed large and very well-documented experience in the clinical translation of GTMPs.	OPBG, FT and Telethon will drive and promote not only the clinical translation of several GTMPs already developed by theirself, but also will support the other members in the clinical development of novel products in the time-frame of this application.

Spoke N°	Scenario	Probability	Impact	Responsible	Preparation	Response
10	Large investment in infrastructure to promote GTMPs at national level	M	M	SPOKE	Infrastructure investments have been fractioned between Spoke 10 members taking in consideration the facilities already present at national level and the strategy ensuring their strengthening to provide the ability of the NC to handle the large number of innovative GTMP manufacturing also behind the timeframe of this application.	The budget allocation of the infrastructure investments will be closely monitored by Spoke 10 leader and by the Hub to promptly act in case of delay of their application. Spoke 10 leader and Hub have designed a specific contingency plan for this point, already described in Section B.4, for the corrective actions planned to overcome any issues related to infrastructure investiments.

Contigency Plan: The infrastructure investement in the field of Gene Therapy of this programme is a key feature of the NC. In particular, it will allow to generate a high capability in Italian Institutions for conducting, in close collaboration with Industrial parterns, innovative programs on GTMPs to be leaveraged from low to high TRL, reaching first patients on the national level, and then worldwide patients thanks to the program of technology transfer and feseability studies that will be activated by the Industrial pattern. For these reasons, an accurate contingency plan has been defined by the Spoke 10 leader to monitor the investements, applying the following cardinal priniples:
1) Allocate: the budget repartition has been decided taking into account the predicted ability of each Spoke 10 member's to implement their existing infrastructures with more updated and *state-of-the-art* instruments and facilities;
2) Monitor: the milestones relative to the infrastructure implementation will be closely monitored by the Spoke 10 leader to early identify risks and issues that could be associated with a delay in the facility refurbishing;
3) Report: any delay in the investements and infrastructure generations will be promptly outlined to the Hub leader to disclose information regarding performance and provide transparency to enable effective governance and decision-making of budget reallocation.

B.6 PROMOTION OF EQUAL GENDER OPPORTUNITIES

The **NC** acknowledges the gender equality issue as one of the general challenges of the RRNP and supports the objectives of the EU Gender Equality Strategy 2020-2025[1] – focused on the achievement of a Union of Equality.

Gender balance in research teams

The **NC** aims at maintaining a representative gender balance in the overall organizational structure of the Hub as well as in each Spoke, based on an equal opportunity policy during the recruitment phase. All the organizations participating in this **NC** are already committed to gender equality via their respective HR policies. Excellence will be the foremost selection criterion for new team members and initial assessment will be irrespective of gender.
After the EU Gender Equality Strategy 2020-2025 announced the ambition for a Gender Equality Plan (GEP) as a requirement for organizations participating to EU Research and Innovation (R&I) projects, in December 2020 the Council of the European Union in its conclusions on the New European Research Area (ERA)[2] also called on the Member States to adopt renewed focus on gender equality, through tools as the GEP and integration of the gender dimension into R&I. Considering that all organizations participating to this National Center are already involved in collaborative international projects, they have already developed/are in the process of developing their own Gender Equality Plan – which represents a mandatory requirement to participate to EU R&I projects (e.g. Horizon Europe). The GEP normally addresses the following areas, using specific measures and targets: i) work-life balance and organizational culture; ii) gender balance in leadership and decision making; iii) gender equality in recruitment and career progression; iv) integration of the gender dimension into research and teaching content.

Specific Actions

During the implementation of **NC**, the participating organizations will carry-out their activities and align their policies following the EU guidelines. Examples of main specific measures are listed below:

[1] Gender equality strategy 2020-2025 (link)
[2] Conclusions on the New European Research Area (ERA), December 2020 (link)

- *Actions to shape a more inclusive organizational culture.* Organizations participating to the **NC** will implement policies to ensure an open and inclusive working environment, the visibility of women in the organization and externally. Specific measures will be parental leave policies, flexible working time arrangements and support for caring responsibilities.
- *Actions to ensure equality in decision making organisms.* All the decision-making bodies of the **NC** take into account gender balance. Measures to ensure that women can take on and stay in leadership positions can include providing decision-makers with targeted gender training, adapting processes for selection and appointment of staff on committees, ensuring gender balance through gender quotas, and making committee membership more transparent.
- *Actions to ensure equality in recruitment.* Organizations participating in the **NC** will be encouraged to critically review the selection procedures and remedying any biases to ensure that women and men get equal chances to develop and advance their careers. Following the EU Gender Equality Strategy, organizations will establish recruitment code of conduct and they will proactively identify women in underrepresented fields and consider organization-wide workload planning models. Finally, the retrospective recruitment analysis will follow the principles of the Gender Equality Strategy 2020-2025.

Gender neutrality in the context of the research

The objective, scientific content and outcomes of the research conducted within the framework of the 10 Spokes included in this NC project are gender-neutral. No assumption related to sex difference was made to determine the methodologies and targeted outcomes of the project which will be addressed equally to men and women.

The NC gender equality: Permanent Staff from Universities and Research Centers overseen by MUR

Considering permanent staff from Universities and Research Centers, the percentage of females is overall 37%. However, among researchers who have obtained doctorates for less than 10 years, the percentage of women rises to 49%. Therefore, new hires expected will allow to increase the current percentage of women. Furthermore, all the 32 institutions involved (excluding Private Companies) have a Gender Balance Plan or are implementing it. Furthermore, among the eligibility requirements for private companies there is the adoption, or commitment to adopt in the first year of the project, of a *Gender Balance* and a *Gender Equality Plan*, in analogy to the Gender Equality Plan (GEP), a prerequisite for all Horizon Europe projects.

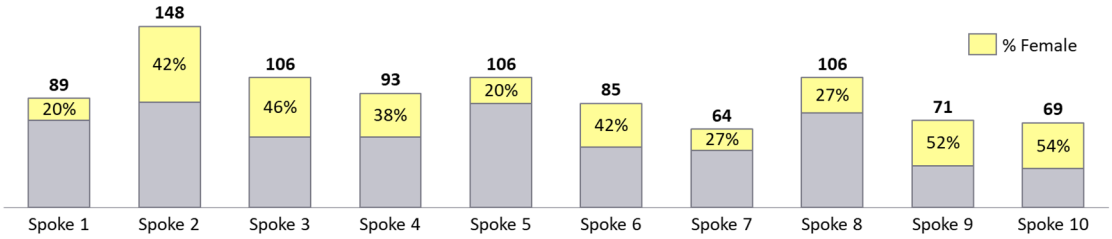


Figure B. 7- Percentage of females structured staff for Universities and Research Centers

B.7 INVOLVEMENT OF SCHOLARS WHO HAVE OBTAINED THEIR PHDS FOR NO MORE THAN 10 YEARS AND ATTRACTION FROM OTHER EU AND NON-EU COUNTRIES

The **NC** was built aiming at having a significant part of scientists who got their PhD by less than 10 years. The aim was that approximately 30% of the personnel involved in the **NC** was represented by young scholars. Therefore, in describing their features, research qualification and critical mass, the institutions were required to select for the **NC** young experts with an excellent track record. In several Institutions, however, many young scientists were not available for **NC** as they were already involved in/paid by other projects and were therefore not eligible to participate. The level of involvement of young researchers in the different Spokes ranges from 20% to 50% with an average of young scientists of 27% in the whole **NC**. This critical mass will be surely improved during project development thanks to the recruitment plan. In fact, new researchers (RTDA) will be hired to work in the **NC**. The recruited researchers need to have got their PhD less than 10 years (preferentially much less than 10 years), and this will enrich the young scientists component of the **NC** critical mass.

The **NC** initiative can be attractive for young scientists as its overall objectives are timely and represent global and international priorities. Having the possibility of doing research in the thematic areas of the **NC,** Spokes will be attractive for both national and international scientists. In addition, the **NC** will leverage on several actions to increase international mobility and attract young talented scientists from abroad. To encourage the attraction of young talents **from other EU and non-EU countries** and extend the competences useful to advance research activities planned in **NC**, **a starting research grant** (Start-up Package) **will be attributed**.

By applying and co-funding EU mobility projects such as for example Marie Slodowska Curie Actions of the Horizon Europe workplan, the **NC** will create a dynamic and exciting environment that will be useful to enhance the scientific profile and create experts in the novel technologies.
Applicant researchers for the projects to be implemented within the 10 Spokes of this **NC** will be selected on the basis of their potential for scientific excellence and their availability to work in a multicultural environment. Recruitment will be transparent, open and equal following the guidelines outlined in the European Charter for Researchers and the Code of Conduct for the Recruitment of Researchers. To ensure that the procedure is transparent, open and equal for all applicants and the selection of researchers follow the recruitment criteria of scientific excellence, a multi-step application procedure will be set-up. In order to comply with the criteria as set in the Call, the **NC** will advertise the job opportunities on the web site of the NC as well as on the web site of each Institution involved. Additionally, the job opportunities will be advertised on international website and scientific journals. Finally, the NC will strive to provide equal opportunities and will monitor gender balance and the alignment with the target fixed by the Call.

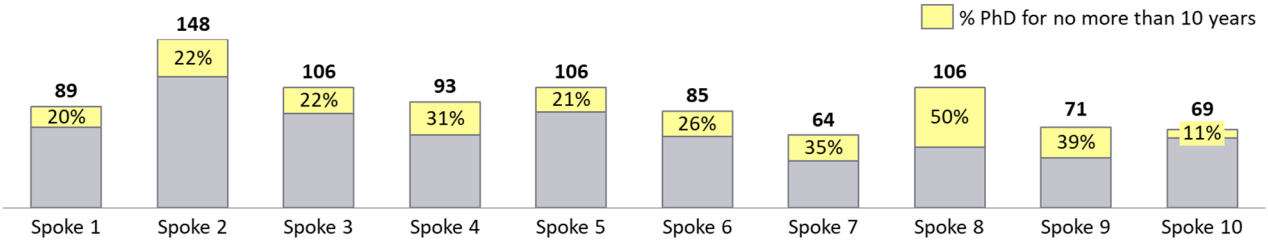


Figure B. 8 - Percentage of PhD for no more than 10 years in Universities and Research Centers

B.8 INVOLVEMENT OF PRIVATE PARTNERS AND INTELLECTUAL PROPERTY POLICY

The research activities carried out in each Spoke belonging to the **NC** for Gene Therapy and Drugs based on RNA Technology are oriented and directed towards a long-term strategy for further expansion of the concepts beyond the research and academic world, to boost the advantages of the concepts developed within their own sector of utilization and beyond, by seeking for technology transfer to enable commercial exploitation in collaborative manner.
Gene therapy approaches and RNA-targeted therapies are indeed very promising, but particularly the latter is at an early stage of development. This is why the involvement of the Private Partners is extremely useful in order to find new treatments on unmet medical need. It will be important to make sure to have those new treatments ready to be used for any eligible patients. Consequently, significant efforts will be devoted to registration path, technology transfer, compliance and production. More in details: i) 50% of Spokes (1-5) have been focused on specific unmet medical needs for selected diseases ii) 50% of Spokes (6-10) have been focused on activities, platforms and processes that enable those treatments to be available to patients as soon as possible. The ambition of this programme is to leverage successful results on selected field of human health to address faster other unmet medical needs.

In this direction, there was no alternative to access "state of art" know how than exploiting all available resources wherever they are, either belonging to public entities or to private companies. All relevant private companies have been contacted and have been offered the opportunity to join the birth of the Foundation. So far, the following companies have accepted to join (we will briefly describe their differential contribution to the programme.

To successfully and safely involve private partners, we foresee the following actions:

- Stakeholders' engagement and networking. The objective of this action is to develop a "Public-private co-creative environment" trying to accelerate the acceptation of the innovative developed concepts from public and private stakeholders along the value chain in order to advance in terms of TRL (demonstration and validation activities) and achieve commercial roll-out. Both **NC** (through the Living Lab) and the Spokes will contribute to the creation of an ecosystem where researchers from universities and private actors will have the chance to cooperate and cross-pollinate. As to research projects, an extensive interaction between academic and industrial partners has already occurred during the initial planning of the objectives and organization in the Spokes of the **NC** and also in the revision of the initial proposal, leading to a number of coordinated activities for a common series of objectives and tasks. This is apparent in the more detailed presentation of tasks, deliverables and milestones that is included in the present, revised proposal. We foresee, however, that working together and sharing scientific data and visions in the consolidated "co-creative environment" will foster new research development and translational opportunities that we can only partially sketch today. The success of the **NC** will rely not only in pursuing the scientific objectives of the current proposal, but also in proceeding from those and rapidly designing and developing new applications, in a continuous science-to-drug pipeline made successful by the common effort of public and private partners. With this view, the governing bodies and operation mechanisms of the **NC** have been designed to guarantee a central role of private partner in the **NC**, thus making an initiative originating from the University Ministry, and with Ministry-

controlled entities obligatorily representing the majority of participants, a truly equal enterprise of public science-oriented entities and private partners capable of successfully bringing to market the scientific developments. Specifically, at the Spoke level, representatives of private companies will be part of the Spoke committee, that receives and evaluates the activity reports of the funded groups, the deliverables and the attainment of the scientific milestones. At the Hub level, the Board of Directors will include the largest admitted participation of private entities. We thus foresee a very significant contribution of private companies in the strategic planning and decision making of the **NC**. Moreover, all companies participating in the Hub will form the Industrial Board, that will advise the Board of Directors in all major decisions, and in particular on those concerning technology transfer and support of spin-offs and new biotechs originating from the activity of the **NC**.

- Promoting an entrepreneurial mindset among researchers. Within the scope of this **NC**, the open science principles will be followed, corresponding to open physical or digital access to data or other results needed for validation of conclusions scientific publications. Nonetheless, protection of key research results will be one of the priorities of the exploitation and valorization strategy. Workshops and sessions will be organized to provide participants of the project with entrepreneurship education, more specifically on how to manage intellectual property, interact with industry and understand go-to-market related activities. More specifically, this action will focus on i) the identification of background and foreground IP; ii) first thoughts on the exploitation of project results; iii) define a roadmap for valorization and exploitation of the research outcomes and identification of the most suitable protection means (IPR). In this context, the foundation of a new Ph.D. programme focused on training young scientists in the KETs of the **NC**, and in the technology transfer in this area, with industrial partners in the teaching staff, will contribute to building a human capital with strong scientific background and entrepreneurship mindset. At the same time, training, in the form of a Tech Academy, will be carried out by the **NC** also towards the industrial system (senior and junior staff of industries, startuppers, finance institutions) to allow the rapid transfer of scientific know-how to the business community.
- Trial definition workshops in order to maximize feedback of private parties, will be set up to define new trials to test and generate feedback for the developments within the **NC**. In these workshops, the goal is to establish a feedback loop where research bodies will have the chance to receive feedback directly on the concept and discuss the validation approaches. These workshops, and an annual meeting of the whole **NC**, will act as conferences at which information can be shared. In addition, a continuously updated intranet website will rapidly communicate the scientific data and the tools developed by the research activity of the Spokes. A public section of the website will serve the important function of disseminating to a wider public the results of the activity and their social and economic implications.

The programme of the research center will be supported by contributions from the Founders of the Foundation for 4.85 M€ of which around 3M€ will be from Private Companies.

The NC is an unprecedented common enterprise of a large part of the Italian scientific and academic community and national and international private companies for exploiting, in terms of new drug development, the advances in the fields of gene therapy and RNA therapeutics. It is thus a unique opportunity, in a very promising area of biomedical technology transfer, for strengthening biotechnology as a successful business area in Italy, where it has not expanded proportionally to the quality of fundamental science. Part of the delay in biomedical technology transfer may be attributable to two causes: the relatively short experience of universities and public research entities in terms of IP protection and spin-off creation in this field and the occasional, and overall limited collaboration with private partners. The NC, also with the aim of guaranteeing the sustainability of its activities beyond the funding period, will place a major effort on these aspects. Within the Hub, a Technology Transfer Office (TTO) will be operative, that, by regulating the IP matters generated by the research activity of the NC, will also establish common criteria and policies among public universities, with a strong benefit for the Italian academic system and the researchers operating in public research institutions. Also the private companies will have a strong benefit, as they will participate in developing a coherent and clear scheme of interaction and IP exploitation with the highly differentiated panorama of public research. In particular, we envision that the following models will be employed:

1) Academic partners develop and own IP (e.g,. identification of RNA targets, development of RNA delivery methods, improvement of viral vectors, etc.). In this case, CN3, through its TTO:
   i) Assists the academic partner in filing the IP.
   ii) Assists the academic partner in the search for investors. Priority will be given to industrial partners internal to the consortium, who have early access to the discoveries. CN3 organises dedicated events with internal and external companies and VCs to catalyse their interaction with the academic partners and foster their interest.
   iii) Assists in the development of spin-off companies. A preferred model is the creation of new start-ups supported by industrial partners and with shared ownership including the academic partners. Other models can be envisaged (e.g. direct industrial support to early research and development in the academic laboratory).

iv) Provides facilitated access by the start-up to CN3 common research platforms and facilities (e.g. production of viral vectors or RNA, or access to delivery technologies).

2) Academic and industrial partners identify priority areas of common interest in which to develop joint activities (e.g. identification of an unmet therapeutic need and design of a strategy to achieve the development of a novel therapy). In this case, the academic and industrial partner agree to develop a novel activity that is funded by the industrial partner and is carried out in the academic laboratory. In this case, CN3, through its TTO:
   i) Catalyses this interaction by promoting the specific expertise of the academic partner and matching it to the industrial interest.
   ii) Assists the academic partner in the establishment of the agreement (which can include CN3 as a third party)
   iii) Provides facilitated access by the joint initiative to CN3 common research platforms and facilities (e.g. production of viral vectors or RNA, or access to delivery technologies).

B.9 QUALITATIVE-QUANTITATIVE INDICATORS PROPOSED FOR MONITORING ACTIVITIES AND FOR EX-POST EVALUATION

Key Performance Indicators need to be established at the beginning and monitored through the funded project period and beyond, thereby allowing for an evaluation of the impact of the project on competitiveness of the sector on sustainability of results. The Spokes and the affiliated entities will monitor progress of KPIs for the whole duration of the programme and will support the Hub in the collection of relevant information. A preliminary set of relevant KPIs is listed in the table below. The list will be integrated at the second stage of this proposal.

Program indicators
Scientific publications on peer-reviewed international journals
No. of scientific events organized
No. of researchers recruited
No. of laboratories with unique expertise on RNA therapeutic created
No. of shared protocols for testing RNA products in small and large animal models
No. of RNA products scaled-up for production for large animal experimentation
No of RNA products scaled-up for production for clinical trials with human
No. of partnerships with private companies established to increase manufacturing capacity of mRNA/ncRNA therapeutics
No. of shared guidelines for pharmacological assessment generated per RNA product
No. of public stakeholders (outside the National Center) involved in the activities
No. of private stakeholders (outside the National Center) involved in the activities
No. of patents generated

Additionally, the table below indicates examples of long-term indicators that will support the NC in monitoring the economic and social potential impact of the projects at regional, national and European level.

Long-term impact indicators
No. experimentations of RNA products on large animal models kicked off
No. experimentations of RNA products on clinical trials kicked off
No. of therapies based on the RNA products developed within this project
No. of new projects (EU/national) generated from the National Center activities
No. of new jobs created
No. of spin-off companies generated from research activities
No. of additional PhD and post-docs attracted in the biotech sector
Amount of additional private/public investments attracted during the project

C. PROGRAM IMPACT

The development of advanced gene therapy medicinal products (AGTMPs) and the booming field of coding (mRNA) and non-coding RNA (ncRNA) therapeutics offer today unprecedented opportunities for the long-term management and even the cure of life-threatening or highly invalidating diseases, especially in areas of high-unmet medical need, including cancer, cardiovascular diseases and rare inherited diseases. These advanced therapies are driving an exciting and revolutionary new paradigm in science and healthcare. The benefits of the proposed **NC** for advancing preclinical research, promoting advanced studies of proof-of-concept and facilitating validation, manufacture and clinical application of AGTMPs and RNA therapeutics in Italy would not only progress innovation and science, but also add to the growth of the economy of the Country and improve patient health.

In particular, the close and fruitful interactions of disease-focused Spokes, Spokes involved in strategies for DNA and RNA precise delivery and a Spoke directly addressing preclinical development, GMP manufacturing and clinical trials of AGTMPs will enable robust and effective translation of research results from pre-clinical phases to proof-of-concept, early clinical testing stages. Particularly, the disease-focused Spokes will provide relevant advancements in preclinical research in the diseases of high social impact (genetic, neurodegenerative, metabolic/cardiovascular, inflammatory/infectious and cancer). In this context, the implementation of an existing gene therapy consortium, which will service and funnel the AGTMP applications of the whole nationwide new **NC**, as well as the construction of a new platform for high quality production of RNAs to be used in clinical settings, will provide the Country with the complete array of competences and infrastructures needed to be efficient and competitive in this fundamental new medical, social and economic challenge.

The transformative potential of advanced therapies has been fuelling an impressive increase in investment from private enterprises. In 2020, according to the most recent data by the Alliance for Regenerative Medicine (ARM), worldwide the sector raised nearly US$ 20 billion, for a total of more than US$ 50 billion in the last 4 years (2017-2020). A fruitful interaction between Academia, advanced research institutes and enterprises, in the frame of a win-win model of interaction, can ultimately promote an unprecedented expansion of these breakthrough therapies in Italy, where a remarkable critical mass of outstanding scientists in the field is present since many years, but has operated in a non-coordinated manner with a series of bottlenecks that limited the interaction with firms and the exploitation of existing scientific research. At the same time, there has been a tremendous acceleration in the development of RNA therapeutics, which has culminated in the approval of two COVID-19 mRNA vaccines in 2020, pioneered by Moderna (mRNA-1273 (*1*)) and Pfizer/BioNTech (BNT162b2 (*2*)), and of 9 non-coding RNA (ncRNA) products just in the last three years. The perceived relevance of the RNA-based therapeutic modality and market analysis are concordant in indicating that RNA therapeutics are destined to progressively expand their impact in clinical medicine in the next decade.

The overall purpose of this **NC** is to foster scientific and technological developments. This will be achieved by:

- Coordinating and expanding the scientific and technological capacities of Italian Universities and Research Centers in this area.
- Fostering the interaction of these research and development centers with pharmaceutical companies and the private sector.
- Promoting the specific development of individual gene therapy and RNA-based pharmaceutics toward clinical applications.
- Building a technological infrastructure that make these key enabling technologies easily accessible to the whole country.
- Fostering the digitalization and the accessibility of data allowing the use of artificial intelligence in research, clinical application and production of new therapeutics.
- Providing a teaching and training support to scientific and health institutions and to industries nationwide.

THE ECONOMIC IMPACT OF GENE THERAPY AND RNA MEDICINES

The economic impact of gene therapy is an extremely important aspect to take into consideration. To date, the costs of gene therapies are very high, owing to the expenses associated to the complex and prolonged research & development (R&D), to the Good Laboratory Practices (GLP) studies required for the preclinical toxicological analysis, and to the costs of manufacturing into extremely specialized GMP factories. However, gene therapy can be considered a game-changing technology simultaneously producing a significant cost offset by replacing expensive existing long-term treatments (such as enzyme replacement therapy or regular red blood cells transfusions) and decreasing hospitalizations and adverse clinical events, which also have a high social and economic cost. To assess the full financial impact, these long-term savings should be included in affordability considerations.

AGTMPs are expected to reach the market at an increasing pace, paralleled by an increasingly high demand for gene therapy approaches by the patients. In the United States, the FDA expects to approve 10 to 20 cell and gene therapy products a year by 2025 (Deloitte, "2020 Global life sciences sector outlook," 2020). However, in the absence of a national structure able to meet this medical need, the costs for the national health system in Italy will be exceedingly high. Therefore, investments that reduce the costs for the Health System and exploit the scientific knowledge keeping the revenues in our country is thus needed. Moreover, as the case of the BluebirdBio drug product Zynteglo® clearly shows, some ATGMPs may end up being rejected by industries and left out of the market, and therefore inaccessible to patients. This is mainly related to the high costs of development and production and the small number of patients, such as in the case of rare diseases, that make unprofitable private investments for those therapies. These unmet medical needs raise relevant ethical concerns that cannot be left unaddressed. Therefore, early-stage public investments may play the important role of allowing the technological development and the accumulation of dynamic capabilities needed for the medical treatment of rare, neglected diseases.

Curative therapies, as gene therapy, must be considered better off than conventional treatments, as they have the potential to halt the need for long-term chronic treatments, thus providing long-term improvement in quality of life. The main challenge is how to make these 21st century breakthroughs available quickly, widely, and sustainable for the health system. This goal is currently pursued by several other European Countries and, thanks to a strong national investment, some of these countries are now leading this area. Indeed, at the end of 2020, there were more than 1200 clinical trials testing ATMPs worldwide, among which 380 involving European clinical sites. In Europe, over the period 1 January 2014 - 30 June 2019, the UK (112), Spain (102), and France (101) have initiated the highest number of new ATMP clinical trials, followed by Germany (83) and Italy (66) (Alliance for Regenerative Medicine, "2020: Growth & Resilience in Regenerative Medicine" 2021). A relevant and instructive example is represented by the Cell and Gene Therapy Catapult initiative in the UK. This is an independent innovation and technology organization, composed of a team of experts covering all aspects of advanced therapies, and committed to the advancement of cell and gene therapies. Thanks to the collaboration with academia, industry and health care providers, Catapult develops new technology and innovation that led to an increase of the manufacturing capacity in the industry by 48% (from 7,970 m^2 in 2019 to 11,756 m^2 in 2020) (Cell and gene therapy Catapult, "Cell and gene therapy GMP manufacturing in the UK: Capability and capacity analysis"). Notably, yet the facilities for gene therapy manufacturing in the UK are booked up to 95% of their capacity.

After a decade of promise, RNA medicines have now come of age. Academic investigators and pharmaceutical companies entering the field today are benefitting from the clinical and regulatory pathways pioneered by a handful of leader companies (e.g. Ionis, Dicerna and others) that, over the last years, have spearheaded the field. This significantly simplifies clinical development, as new RNA medicines can take advantage of already developed technologies in terms of chemical RNA modifications, delivery methods pharmacological assessment, as well as simplified interaction with regulatory authorities. Both Universities and the private sector in Italy, however, still need to significantly catch up in this field. This will be one of the main goals of this **NC.**

The potential economic impact of RNA medicines is enormous not only for the economic relief from the burden of currently untreatable diseases, but also for the market value of the newly developed therapeutics. Current estimates indicate that there are more than 500 companies actively developing RNA therapeutics and vaccines, with over 4000 live clinical trials. This activity currently attracts over 1500 investors and has ended up in 101 fund raises in 2020 and over 96 just in the first 6 months of 2021, totalling more than $1.65 billion raised by the RNA biotech only by mid-2021 (source: https://www.lsxleaders.com/rna-leaders-world-congress).

For mRNAs, after the success of the two major COVID-19 vaccines (namely Comirnaty and Spikevax) in 2020, the global market is expected to grow from $46.7 billion in 2021 to $101.3 billion by 2026, at a compound annual growth rate (CAGR) of 16.8% for the period of 2021-2026 (source: Research and Markets; https://www.researchandmarkets.com/reports/5441159/mrna-therapeutics-and-global-markets-2021-2026#tag-pos-1).
The antisense and RNAi therapeutics market is equally rising globally, especially considering that, of the 13 currently approved ncRNA drugs, 11 have gained clinical approval just in the last 5 years. In 2021, this market was estimated globally at $1.2 billion, with an estimate to raise to $1.8 billion by 2025, with a market forecast to grow at CAGR of 11%

(Research and Markets; https://www.researchandmarkets.com/reports/5345452/antisense-and-rnai-therapeutics-global-market#relc0-4748172).

These transactions are now almost exclusive to North America (United States and Canada), with only a few countries in Europe (United Kingdom, Germany in particular) and Asia (China, Singapore) also playing a role. A core objective of our **NC** is to coordinate a collective effort in Italy by the public sector (Universities, Research Center) and pharma companies/biotech to generate intellectual property and/or products around RNA therapeutics. This will permit Italian science and biotech industries to access this large and expanding market in an active rather than in a passive manner (as it has largely been so far for the approved ncRNA products as well as for the COVID mRNA vaccines).

Overall, the proposed structure of the **NC** will increase the number of early-phase clinical trials and AGTMPs developed in Italy, driving the overall research on RNA from average initial TRL2/3 to an estimated final average of TRL5/6. The **NC** will also facilitate the transferability of research results to industry, increasing the value of the intellectual property owned by Italian research Centers through the out-licensing of more mature technologies. These perspectives, coupled with the training of a new generation of scientists/biotechnologists/clinicians expert in emerging techniques, will advance the Country to a high biotechnology level, which will be able to attract investors and developers from other Countries, thus further boosting the development of the biotech sector in a virtuous circle of amplification.

SYNERGIES WITH OTHER INVESTMENTS ENVISAGED BY THE NC WITH OTHER NATIONAL AND REGIONAL PROGRAMMATIC FRAMEWORKS

The Research Program displays a complementarity with other Investments envisaged by Mission 4, Component 2 of the NRRP and specifically:

- **Investment 1.3 - Creation of Enlarged Partnerships extended to Universities, Research Centres, Enterprises and funding basic research projects**. We envisage synergies with the research projects on "topic 6 - Diagnostics and innovative therapies for precision medicine". NC involvement in research topics such as omics sciences (Spoke #8 and #9), drug-delivery systems (Spoke #8), bioinformatic platform and machine learning tools (Spoke #7) and the acquired knowledge in these field could represent a strategic advantage for the incoming consortium. We can also play a relevant role in the "topic 12-Neuroscience and neuropharmacology" and "8- Effects and challenges of aging". NC research on brain diseases, moving from a one-size-fits-all approach that may lead to clinical trials failures into patient-tailored and deeply acting cures (Spoke #3) and the development of novel precision nanoplatforms for brain delivery of therapeutics (Spoke #8) can generate a strategic alliance and osmosis of knowledge with the incoming consortium. Furthermore, NC activity around Spoke #3 can contribute to discovering novel therapeutical targets in neurodegenerative diseases associated with aging and an understanding of the underlying biological processes. Finally, in the context of "topic 13-Emerging infective diseases", the development of vaccines, prophylactic drugs and innovative therapies against novel emerging viral pathogens or anti-microbial resistant (AMR) bacteria (Spoke #5) can be again strategic to the mutual growth of knowledge.
- **Investment 2.2 of the NRRP aims at supporting research and innovation projects within the framework of so-called European Partnerships, financed by the Horizon Europe program**. European Partnership are initiatives in which the EU and private and/or public partners commit to jointly support the development and implementation of a program of research and innovation activities, including market, regulatory or policy uptake. Similarly, to the National Centers, Europe Partnerships aim to create a medium to long-term vision for research and innovation activities, in order to overcome fragmentation of research efforts and create a critical mass of investments. While one of the pillars composing the priorities of the Horizon Europe Program is Health, there is no institutionalized Partnership, among the 11 that have been launched by the European Commission, specifically focusing on the development of therapies or procedures of pharmacological interest, thus allowing for the development of a complementarity between the National Center and the European Partnerships on the specific topic of research.
- **Investment 3.3 – Novel innovative doctoral programmes promoting industrial innovation and employment**. **NC** is creating an academia-private company ecosystem in training that will foster students in a path toward pharmaceutical companies. The **industrial-doctoral training programme** of **NC** that goes beyond the 'classical division of sciences', enables the doctoral candidates (DCs) to become ready to respond to the present and future challenges in the discovery area of RNA therapeutics and gene therapy. Furthermore, with the **PharmaTech Academy**, **NC** proposes a new training paradigm based on the concept of Learning by Doing, where University and Private Companies closely collaborate in the definition of the professional profile of the next generation of operators in the pharmaceutical arena.

Importantly, the proposed NC fits perfectly into the priorities of the **Ministry of Health– PNRS – mission 4**, stating specifically that life science research should focus on the so called "red biotech", including in particular AGTMPs. Moreover, as already highlighted, the Center will address other relevant priorities of the PNRR, thanks to the innovative delivery strategies developed, to the collaboration with Institutions that are pioneering innovative nanotechnologies and to the integration of advanced bioinformatic technologies and of advanced digitalization of the system.

At the national level, the **National Research Program (NRP)** 2021-2027, approved by the MUR, is a multiannual framework programming tool guiding research policies and investments in Italy, encouraging an effective coordination among national and European entities to reinforce competitiveness of the Italian research system at a European and at a global level. The NRP calls for, among other priorities, the improvement of pre-competitive innovation and bidirectional exchanges among State Universities, national Research Centers and other stakeholders, including in the field of pharmaceutical and pharmacological technology. More specifically, the NRP provides incentives and career awards for scientists who generate innovation, promoting an entrepreneurial culture in universities and research centers and facilitating the flow of information and skills between the stakeholders in the research environment. The NRP is thus aligned with the Research Program in encouraging investments in sectors with high economic risk and favoring start-ups, biotech and large companies' investments in highly innovative sectors. Moreover, the significant scale of the Research Program carried out by the National Center can provide an added value to the implementation of similar activities, thus improving the overall competitiveness of the players in the sector.

The research activities proposed in **NC complies also with NRP (National Research Plan 2021-2027) objectives**

expressed in:

- **5.1.2 Pharmaceutical and pharmacological technologies** by i.) sponsoring the translation of basic research into industrial research (5.1.2 -4); ii.) creating the critical mass useful for the production of innovative processes and products (5.1.2 -5); iii.) potentiating experimental models for human pathologies (Articulation 4), iv.) improving the drug discovery process (Articulation 6) and v.) creating innovative therapies (Articulation 7);
- **5.1.4 Technologies for health** by i.) developing innovative experimental models suitable for preclinical research (Articulation 5); ii.) using novel sensors and reporters for rapid screening (Articulation 6); iii.) applying nanotechnologies in drug delivery and pharmacodynamics (Articulation 9 and 11); iv.) using in silico biology for the identification of targets and molecular docking (Articulation 10).

Finally, the Expression of interest by the President of Area Science Park (Trieste) to collaborate and support the activities of the NC by acting as a reference infrastructure for the center, ensures the synergy within the **National Infrastructural Research Plan 2021-2027** and specifically with the CERIC-ERIC Infrastructure- in the frame of the project Pathogen Readiness Platform (PRP).

In addition, our proposed **NC** will have a strong complementarity with the already mentioned implementation initiatives active in Europe. It will be fully aligned to the call that the European Innovation Council is about to launch on Emerging Technologies in Cell and Gene therapy. In particular, it will include two aims dedicated to manufacturing: i) advancing cell therapy manufacturing and products to a clinical stage; ii) improving gene therapy manufacturing processes and production. Indeed, the proposed Center will have a strong power of attraction for developers aimed at testing their innovative solutions, thus expanding the impact of the initiative by the MoH. The win-win interaction with small and large enterprises involved in the gene therapy field and participating to the Spoke will guarantee a smooth advancement of the most innovative and promising approaches to the highest phases of clinical testing and, ultimately, to the marketing.

IMPACT OF THE RESEARCH AND DEVELOPMENT (R&D) ACTIVITIES OF THE NC

Gene therapy: an area of consolidated Italian expertise, with rapidly expanding applications

Currently, gene therapy is still experimental, but several approaches are rapidly moving into the clinical practice, with an increasing number of products approved for the market. AGTMPs have already demonstrated outstanding results in the treatment of patients with B-cell lymphoproliferative disorders, multiple myeloma, and rare inherited disorders such as severe combined immunodeficiency due to adenosine-deaminase (ADA) deficiency, spinal muscular atrophy (SMA), inherited retinal dystrophy. To date, the majority of AGTMPs has been marketed with a narrow license indication and, in the case of CAR T cell products, often for use in second or subsequent lines of treatment. However, the approach is broad, with a curative potential for diseases caused by recessive genetic disorders (including, but not limited to, cystic fibrosis, haemophilia, muscular dystrophy, beta-hemoglobinopathies), non-communicable diseases, cancer and certain viral infections, such as AIDS. For many of these disorders, AGTMPs represent the only potentially curative option in an otherwise clinical hopeless scenario, or the strategy for improving patient's quality of life. In addition, conventional therapies are associated with relevant costs for the national economy (cf. also later). Gene therapies offer the potential for short, 'one-off' treatments leading to lifelong benefits. The promise of successful treatment with gene therapy could therefore positively affect millions of lives.

The gene therapy R&D projects in this **NC** will tackle several cancers, including some with highly unmet medical needs (i.e. T-cell lymphoproliferative disorders, brain tumours and acute myeloid leukaemia - AML) and hereditary disorders, including beta-hemoglobinopathies, congenital retinopathies and lysosomal storage disorders (LSD). Moreover, there will be a specific project aimed at evaluating the efficacy of CD19-targeting CAR T cells in severe/refractory B-cell mediated autoimmune disease. A wide array of technologies will be developed, optimized and exploited, including more conventional viral vectors (adeno-, adeno associated-, retro-and lenti-viruses), non-viral systems (transposons), innovative genome-editing technologies and pioneering delivery systems, including nanotechnologies and intracerebral *in vivo* delivery of therapeutic genes. In addition, thanks to the synergistic collaboration with some biotech companies, including Orgenenesis, PBL, Stevanato, a point-of-care model of GTMP manufacturing will be developed with the goal of rendering the approach more widely accessible and more affordable form the economical point of view. In the field of cancer treatment using genetically modified cells redirected against targets expressed on tumour cells, we will use not only the classical autologous T cell platform, but also other cells of the innate immunity, including NK cells and cytokine-induced killer (CIK) cells, the manufacturing of which has been already optimized by institutions contributing to **Spoke #10.** CNR and Takis will contribute, together with research entities such as OPG and Fondazione Tettamanti, to set up non-viral, DNA-based methods for CAR-T generation, thus promoting the development of innovative strategies for gene modification of somatic cells based on the use of nanotechnology and electroporation of DNA.

In the field of cancer, CAR T-cell therapies are providing new hope for patients. These complex medicinal products are an

example of personalized advanced therapies, tailor-made for every patient: specific immune cells are isolated from the patient blood and genetically modified to recognize and attack the cancer cells after being returned to the patient. The first two of these highly anticipated advanced therapies – Novartis's Kymriah (tisagenlecleucel) and Gilead's Yescarta (axicabtagen ciloleucel), both approved in non-Hodgkin B-cell lymphomas in 2018 – have been characterized by demonstrated success and are good examples of the new possibilities that personalized medication can bring, especially for vulnerable patients who often have no time to lose. Despite the success obtained in B-cell malignancies, the development of CAR T-cell approaches for other extremely refractory hematological malignancies is lagging, in particular for T-cell ALL/lymphoma and for acute myeloid malignancies. In Italy, more than 2100 adults are diagnosed with AML and T-cell lymphoproliferative disorders every year, and 70-80% of the patients have a refractory disease [source: AIRTUM registry]. Unfortunately, this unmet medical need is reported to affect also the paediatric population, with 20% of the children with T-ALL and 30-40% with AML displaying a resistant and often incurable disease (*3, 4*). After the failure of first-line conventional approaches, very few innovative treatments are currently available for these patients. Moreover, even for those patients that can be cured with conventional therapies, the burden of the long-term *sequelae* related to the treatment is extremely relevant, this observation being especially pertinent to the paediatric population, whose life expectancy is naturally longer. Although the life expectancy difference between childhood cancer survivors and people without a history of cancer has progressively narrowed over the years, people diagnosed to be affected by neoplasia in the 1990s still have 9.2 fewer years of life expectancy (*5*). Moreover, a relevant proportion of cancer survivors suffer from secondary cancers, cardiac and pulmonary events, endocrine and fertility dysfunctions leading to a significant burden, also in terms of medical assistance. The development of approaches to precision medicine like CAR T cells could therefore offer more effective and less toxic curative options for these patients, producing a wide advantage for the national healthcare system. The synergy between some prestigious Institutions participating in the project (Ospedale Pediatrico Bambino Gesù and Fondazione Tettamanti that already have well-established and long-standing collaboration) with a widely recognized qualifications and expertise in the field has the strong potential to advance such a possibility from an already consolidated preclinical stage to an advanced phase of clinical testing, with the possibility of offering such approach also to patients suffering from different types of brain tumors targeting the disial-ganglioside GD2. Overall, all these projects will lead to an expected improvement from an initial TRL of 4/5 to a final TRL of 7, as defined by the NIH creteria. NC activities will be further developed behind the timeframe of this application, thanks to the collaboration with the industrial patterns that will allow to reach further TRL 8/9. In particular, the program of project implementation behind the third year will become already at M5-22 with specific technology transfer of products from the Privates to the Academic centers for the clinical development, and vice-versa for products already developed by the Accademia and that will be leveraged to the industrial level by the Private Members. Moreover, at M1-24 it has been planned the start of feseability studies from the Private partners, allowing them to allarge the deep knowledge on the GTMPs developed in **Spoke #10**, and to define their interest to start the development of the products to TRL 8/9. In this respect, the participation to the **Spoke #10** of large international pharmaceutical companies, such as Novartis and Sanofi, is vital, since they have the experience, strength and know-how for guaranteeing the sustained fruitful translation at the patient's bedside of this therapy also at the end of the project.

In this project, we will test a few innovative approaches for cancer CAR T-cell therapy. One of these will be based on the use of allogeneic cells to overcome one of the obstacles that still limit the applicability and the success of conventional CAR T-cell therapy, namely the generation of a sufficient number of fully-functioning effector elements able to provide clinical benefit to patients, with an expected final TRL of 6-7. One of the components of the Consortium at the University of Chieti will also conduct initial preclinical studies aimed at developing novel approach based on the infusion of extracellular vesicles (EVs) released by CAR-T cells in pancreatic adenocarcinoma. As the use of EVs derived from CAR T-cells has been already tested and shown to be effective in preclinical models of breast, lung and ovarian carcinoma, these preclinical findings may represent the basis for further investigating if this approach can become in the future a strategy to complement cell-based approaches of CAR cell therapy. Another member of the Spoke at the University of Padova will invest efforts for identifying suitable innovative targets for developing CAR T-cells able to tackle the unmet medical need of refractory/resistant AML. In both cases, we expect that the current TRL2/3 will be raised to TRL5 at the end of the project, this opening the door for subsequent development of innovative CAR T-cell therapies. The presence in the spoke of Takis and IRBM will contribute to further promoting the identification of novel targets suitable to be attacked by CAR T cells for patients with AML.

In the field of genetic diseases, hemoglobinopathies represent an extremely relevant group of disorders with significant epidemiologic relevance and burden of assistance for the Italian national health system. Sickle-cell disease and beta-thalassaemia are two of the most common genetic disorders attributable to mutations in a single gene. Both conditions affect the production of beta-globin, a component of haemoglobin. Each year, 60,000 people are diagnosed worldwide with a severe form of beta-thalassemia, and 300,000 are diagnosed with sickle-cell disease. In Italy, about 7,000 patients are diagnosed with beta-thalassemia and need lifelong, regular red blood cell transfusions, as well as regular iron chelation therapy to avoid iron overload. Allogeneic hematopoietic stem-cell transplantation is a potentially curative therapy for beta-thalassemia, with the best outcomes being reported in patients younger than 14 years of age who have an HLA-identical

donor. However, hematopoietic stem-cell (HSC) transplantation is limited by a lack of suitable, unaffected, histocompatible donors, and by the risk of transplantation-related death, graft failure, and graft-versus-host disease.

Being caused by mutations in the beta-globin gene (HBB) that either reduce (beta +) or abrogate (beta 0) production of functional beta-globin, beta-thalassemia represents an ideal model of disease amenable of genetic correction through gene therapy. Gene addition therapy has shown its curative potential in beta-thalassemia and, recently, an optimized manufacturing process was shown to promote the achievement of transfusion independence in 90% of patients enrolled into a phase III multicenter clinical trial coordinated by the PI of one of the institutions of **Spoke #10** (*6*). However, industries are struggling due to the high costs of development and manufacturing, and in August 2021, the Company (i.e. BluebirdBio) that sponsored the trial decided to discontinue the commercial activities in Europe, despite having received conditional marketing authorization from the European Commission. This decision clearly demonstrates that, should patients have affordable access to treatment, a fair balance among the costs of drug development and manufacturing, the return on investment for shareholders, and the pricing of cell therapy and gene therapy products is necessary. Moreover, and more importantly, this decision left a large unmet medical need, in particular in Italy, which is the Country with, by far, the highest number of patients with beta-thalassemia in Europe. The development of an academia-driven initiative aimed to enhance efficacy and reduce interpatient variability of the outcome by optimizing manufacturing and reducing costs would therefore have a clear and immediate benefit for the patients' community and the national health system. Taking advantage of a new proprietary improved and shortened HSC gene transfer protocol, using a lentiviral vector already tested in a phase I/II clinical trial (GLOBE, (*7*)) by the members of **Spoke #10** (Telethon/Tiget and Ospedale Pediatrico Bambino Gesù) the advanced clinical testing of the approach will be feasible. The conduction of a clinical trial in these two Institutions will allow further validating the results obtained in the GLOBE phase I/II clinical trial, raising the overall TRL of such project from 6 to 7. In addition, once validated, the OMPUL system proposed by Orgenesis can be instrumental in the perspective of supporting a point-of-care model for the manufacturing of HSC genetically transduced with the vector carrying the beta chain of hemoglobin.

Besides the efficacy of gene addition therapy, another promising and potentially curative approach, namely gene editing, could change the natural history of patients with hemoglobinopathies. In this regard, as a tool of great promise for the treatment of inherited disorders, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system has captured public imagination with its precision and versatility. A landmark advancement has been the international, multicenter study, in which OPBG participated, that reported a remarkable level of functional correction of the disease phenotype in two transfusion-dependent patients with either beta-thalassemia or sickle cell disease by alleviating the severe anaemia and associated medical problems caused by these disorders (*8*). In that study, the CRISPR-Cas9 technology was used to disrupt a portion of the erythroid-control element that governs the expression of BCL11A, a gene that regulates the switch from the production of foetal haemoglobin before birth to the production of adult haemoglobin after birth. In comparison to the approaches of gene addition, gene editing has the potential advantages of being associated with a lower risk of insertional mutagenesis and with reduced costs of manufacturing. This extraordinary therapeutic opportunity will be rapidly pursued, thanks to the expertise already available in the participating teams. Indeed, Telethon/Tiget has pioneered the use of engineered zinc-finger nucleases for genome editing of human HSC and T cells for the correction of inherited immunodeficiencies, OPBG is developing a virus-free platform based on a CRISPR/Cas nuclease system for the correction of specific mutated regions of the beta-globin gene and the consortium will further expand these studies by leveraging on optimized RNA-based delivery, lipid nanoparticle transfection and including DNA break-free editors (base and prime editors). In addition, a protocol is being developed using base editors to develop a clinical-grade protocol for the reversion of pathogenic mutations in patient-derived HSCs. It is reasonable to predict that thanks to the synergy of the efforts between the two institutions, it will be possible to move from an initial TRL of 3 to an expected final TRL of 6. Notably, as a paradigmatic example of collaboration between the Spokes of this project, **Spoke #7** and **#10** will develop a comprehensive bioinformatics environment for the analysis of omic data to assess clonality of viral vector-transduced cell populations, with the aim to monitor the fate of individual gene-corrected cells in vivo, and to assess vector integration, and, thus, biosafety.

In the field of genetic diseases, along the project we will also tackle another group of diseases with an unmet medical need, collectively called lysosomal storage disorders (LSD). These rare, monogenic disorders encompass more than 50 distinct conditions caused by defects in various aspects of lysosomal function leading to waste accumulation and severe organ damage. Neurodegeneration and/or demyelination are the hallmark of approximately 70% LSDs. Current treatments are unsatisfactory and unable to cure CNS and skeletal abnormalities. Gene therapy represents a promising approach for the treatment of CNS manifestations in LSDs, as it has the potential to provide a permanent source of the deficient enzyme, either by transplantation of gene-corrected cells or by direct injection of vectors. Based on the expertise of the research teams in this **NC** (*9, 10*), an innovative platform approach will be validated, based on "simultaneous and parallel" development of AGTMP for 3 LSDs with bone involvement and high unmet need (MPSIVA, MPSIVB and alpha-mannosidase). The goal is to propose to EU and Italian regulatory authorities a joint development roadmap of 3 AGTMPs for 3 different diseases, sharing the same: a) therapeutic mechanism of action (enzyme over-expression and local cross-

correction by hematopoietic cells), b) lentiviral vector design, c) manufacturing process d) non-clinical GLP studies (3R principles). Therefore, for this project, we envisage an increase from the initial TRL of 3 to an expected final TRL of 5. Another potential approach for these disorders is represented by the direct injection of vectors in the CNS of affected patients to induce the local production of the defective enzyme. Indeed, intracerebral *in vivo* delivery of therapeutic genes by lentiviral vectors could rapidly provide robust and widespread distribution of therapeutic molecules across the blood brain barrier. Metachromatic leukodystrophy (MLD) is a severe form of LSD characterized by demyelination and neural degeneration. Gene therapy has proven an effective treatment for early-diagnosed patients, but is not effective in already symptomatic patients due to the long time required to exert its therapeutic effects. Clinical projects within the **NC** are aimed to develop the first-of-its-kind application of intracerebral delivery of lentiviral vectors encoding the defective enzyme, thanks to the experience developed by one of the group participating to the spoke 10, namely Telethon/Tiget, in collaboration with another participant to the Spoke, the San Raffaele University (*11*).

Finally, an R&D effort will aim to develop a gene therapy cure for a high-impact disorder, Stargardt disease (STGD1, an autosomal recessive inherited retinal disease caused by biallelic mutations in the ATP-binding cassette transporter subfamily A4 gene (ABCA4). STGD1 is one of the most common genetic inherited retinal diseases, accounting for 12% of IRD-related blindness. To date, no curative treatment exists for STGD1, leaving these patients without any possibility of restoring their visual abilities. Gene therapy thus promises a new curative option for STGD1. Most of the ongoing clinical trials are based on adeno-associated viral (AAV) vectors, with low immunogenicity and long-term transgene expression after a single administration shown. The most successful example of this approach is Luxturna, an AAV-based gene therapy recently approved for an ocular disease (FDA approves hereditary blindness gene therapy (*12*). Thanks to the specific expertise of one of the Institutions participating to the project (Vanvitelli University of Naples) and to the collaboration with the small biotech company InnovaVector, an innovative approach using AAV is in an advanced phase of preclinical study (*13*). Along the proposed project, we will complete the potency, toxicity and biodistribution GLP studies and the GMP technology transfer of the approach, leading to the conduction of a phase I clinical trial. Therefore, we will promote an increase from the initial TRL of 4 to a final of 6.

As already mentioned, *ex vivo* gene therapy based on gene addition or genome editing of HSCs has shown promising results in clinical trials. However, genetic engineering to high levels and in large scale remains challenging and the development of strategies to enable the efficient *ex vivo* expansion of HSCs may open major new opportunities to gene therapy. Faster hematopoietic recovery from myeloablative conditioning, especially in patients whose autologous stem cell collection is challenging, would reduce the requirement for myeloablative conditioning by infusing mega-doses of genetically engineered HSC. Moreover, next-generation gene editing technologies would be enabled, increasing the yield of edited cells and allowing selection and expansion of cells carrying the intended genetic outcome. UM171, a small molecule with properties of HSC agonist, supports prolonged *ex vivo* culture, has shown promising results in cord blood transplantation (*14*) and pre-clinical gene therapy models (*15*). We will leverage the expertise of one of the Institutions participating to the project, Telethon, to develop an innovative HSC expansion/transduction process, transfer this process to GMP manufacturing (in collaboration with ExCellThera, the owners of UM171) and apply it to a phase I/II clinical trial for a genetic model disease, osteopetrosis (*16*). Thanks to the close collaborations between the members of the Consortium, following proof-of-concept data, the process will be available to Spoke members for broader applications. During the project, we envisage that the actual TRL4 will reach TRL6.

B-cell mediated auto-immune disorders sometimes have a very severe course and are refractory to conventional immune-suppressive therapies. Recently, a seminal case published in the New England Journal of Medicine of a patient with systemic erythematosus lupus that has greatly benefited from treatment with CD19-targeting cells has been reported, providing the rationale for testing the safety and long-term efficacy approach in patients with this and other forms of B-cell mediated auto-immune disorders. Orgenesis will promote the conduction of a study based on the decentralized manufacturing of CD19-targeting CAR T cells in these diseases, working in close collaboration with research institutions participating to the **Spoke #10**.

In summary, **Spoke #10** will launch the pre-clinical/GMP development of at least 15 GTMPs, and for 12 of them an early clinical evaluation has been already planned in the period of this application. The collaboration and close interaction among research institution and industrial partners provides the best conditions for both translating the therapeutic approaches rapidly on a large scale and for giving further continuity over time to the activities that will be developed during the 3-year duration of the project.

RNA based medicines: the new frontier of drug development

The booming field of coding and non-coding RNA therapeutics: setting the stage

There are four categories of RNA therapeutics that are the focus of this **NC**, one of which is based on protein-coding mRNAs, while the other three on non-coding RNAs. Non-coding RNAs (ncRNAs) include: i) antisense oligonucleotides (ASOs) that pair with and inhibit mRNAs, miRNAs and lncRNAs; ii) positive effectors of the RNAi pathway (siRNAs,

miRNAs); iii) conformational RNAs that target proteins (aptamers). In addition to the 13 ncRNA therapeutics that have already received clinical approval from the regulatory authorities (EMA, FDA), there are currently (January 2022) at least other 20 RNA products in phase II or III of clinical development (*17*).

Messenger RNAs (mRNAs). The development of an mRNA-based vaccine for COVID-19 took just 63 days from release of the virus sequence to first dosing in humans, leading to accelerated clinical trials and ultimately billions of doses manufactured. The new mRNA vaccines (mRNA-1273 (*1*) and BNT162b2 (*2*)) have demonstrated efficacies in the high 90s, with minimal side effects, and have been manufactured by two different RNA technology companies to date, with others adding further production capacity. The mRNA-based vaccine technology offers tremendous opportunities for other infectious diseases and as a rapid response to new pathogen treats given the relative ease of adapting mRNA vaccine manufacturing to new RNA sequences. In addition to vaccines, the mRNA technology, coupled with the possibility of delivering RNA in vivo using nanocarriers (cf. later) permits the development of CRISPR/Cas9 gene editing systems amenable to *in vivo* administration. The first clinical trial for *in vivo* gene editing in the liver using and RNA payload within lipid nanoparticles has been already completed in 2021 (*28*).

ASOs against mRNAs, miRNAs and lncRNAs. ASOs are 17-22 nt long oligonucleotides that are chemically modified to decrease degradation by endogenous nucleases and improve cellular uptake. Pairing with the antisense mRNAs, miRNAs or lncRNAs is followed by inhibition of target function. To date, 9 ASOs have received regulatory approval in Europe and the USA. The first of these, *fomivirsen*, was a 21-mer ASO against cytomegalovirus (CMV) for the treatment of CMV retinitis; its commercialisation ended in 2002 due to the development of more effective antivirals. Three of the approved ASOs have a specific cellular mRNA target (*mipomersen* against apolipoprotein B [ApoB], *inotersen* against transthyretin and *volanesorsen* against ApoC3). The remaining five ASOs are alternative splicing modulators of the dystrophin (*eteplirsen*, *golodirsen*, *viltolarsen* and *casimersen*) or the SMN2 (*nusinersen*) pre-mRNAs.

RNA interference (RNAi) therapeutics (miRNAs and siRNAs). The development of technologies to administer synthetic siRNAs and miRNAs to mammalian cells has progressively opened a vast series of therapeutic opportunities. The first clinically approved siRNA was *patisiran* in 2018, against the transthyretin mRNA for hereditary transthyretin amyloidosis (HTT-amyloid) (*18*). This was followed by *givosiran*, an siRNA against the delta aminolevulinic acid synthase 1 (ALAS1) mRNA for acute hepatic porphyria (*19*); *inclisiran*, an siRNA against PCSK9 for familiar hypercholesterolaemia (*20*); and *lumasiran*, an siRNA against the hydroxyacid oxidase 1 (HAO1) mRNA for primary hyperoxaluria type 1 (*21*). As siRNAs needs to interact with the cellular RISC complex to exert their function, they usually tolerate fewer chemical modifications than ASOs, in particular in their guide strand (the one that pairs with the mRNA targets). The success of these therapeutic siRNAs is largely due to innovations for their efficient delivery to the liver, in particular the conjugation of the siRNA passenger strand with N-acetylgalactosamine (GalNAc). No miRNA has so far reached clinical approval, yet these molecules have significant therapeutic appeal for several reasons. First, miRNAs are naturally occurring molecules, characterized by a precise and physiological mechanism of action. Second, by targeting several different mRNAs (in contrast to ASOs/siRNAs with a single target) they can elicit complex yet specific phenotypes (e.g. cell proliferation or transdifferentiation). Third, the human miRNAome has relatively low complexity (2654 human mature sequences annotated in miRBase v.22 (*22*)), therefore a synthetic human miRNA mimic library can be screened with relative ease for therapeutic leads.

Aptamers. The SELEX (systematic evolution of ligands by exponential enrichment) method developed in the 90s permits selection of RNAs that specifically bind proteins or small organic molecules, starting from libraries of oligoribonucleotides with random sequence (*23-25*). The selected RNA molecules (aptamers), exploit the secondary structure of nucleic acids rather than sequence complementarity for binding (*26*). Unlike antibodies, aptamers are easier to obtain, non-immunogenic and chemically modifiable to increase stability. *Pegaptanib*, a 28-mer modified RNA aptamer conjugated with two polyethylene glycol molecules and binding the 165aa isoform of VEGF, was the first aptamer to reach clinical approval in 2004 (*27*). To date, 8 other RNA aptamers and 5 DNA aptamers have undergone clinical trials (*26*). SELEX remains of interest to generate RNAs that bind tissue-specific target molecules for use in combination with delivery systems to achieve cell/organ targeting.

The impact of the National Center for RNA therapy R&D in Italy

One of the defining objectives of this **NC** is to coordinate and expand the expertise of laboratories from Italian Universities and Research Centers and to connect their activities with those of the private biotech sector. This will ultimately bridge the gap that exists between Italy and other advanced countries (in particular, USA, Germany and United Kingdom) in the area of RNA therapeutics and will bring Italy at the forefront of technological development and generation of specific RNA products.

With an aim to generate impact at a systemic level in R&D, this **NC** will operate along two main paths. The first path aims to foster interaction between existing expertise in Italian laboratories and re-deploy this expertise towards the specific field of RNA therapies. The second path has the goal to generate technological advancements and thus overcome some of the

hurdles that the field of RNA therapeutics still suffers.

Coordination and expansion of RNA therapeutic R&D activities at the national level

Italian laboratories have advanced competences in several fields of RNA therapy R&D, ranging from target identification, delivery strategies, generation of proof-of-concept evidence of therapeutic value in animal models, pharmacological studies, and pharmacological and regulatory aspects of pre-clinical development. However, this expertise is scattered across the country and lacks proper coordination and specific focus towards this specific area.

This **NC** will provide support and expand activities that are already well established in Italy and are instrumental to the development of RNA therapies and provide access to these activities to external users from the public and private sector.

The currently available expertise that needs expansion and re-focusing includes:

Facilities for bioinformatics analysis for target RNA product identification and RNA design

We expect significant support from bioinformatic capabilities in different areas of RNA therapeutic development. In particular, analysis of complex transcriptomics datasets, computational microRNA target identification, evaluation of high throughput screening data and image analysis are all areas that will be coordinated within this **NC** through an essential input from the **Spoke #7** with the aim to covey the current, scattered expertise towards proper data-driven RNA identification and design. An additional area that will require essential bioinformatic support is therapeutic mRNA design to predict the pharmacodynamic profile of mRNA-based products sufficient to achieve "first-pass" fabrication success. This will be provided by the **Spoke #9**. For these mRNA products, a reliable prediction of potency and immune reactivity will be computed as far as the extent of innate immunity response induced by the RNA product is concerned. Finally, the **Spoke #7** will exploit bioinformatics capability for the design of CRISPR guides for effective gene editing, in conjunction with the analysis of the epitranscriptome for site accessibility. Support for ncRNA target identification, generation of aptamers, identification of phenotypes triggered by lncRNA modification and similar activities will be provided by the **Spoke #3**.

Facilities for therapeutic RNA lead identification by high throughput screenings

This **NC** will leverage expertise throughout the consortium and in particular from the **Spoke #6** to coordinate and expand access to facilities for robotic high throughput screening, providing availability of whole genome siRNA, microRNA and anti-microRNA GapmeR locked nucleic acid (LNA) libraries. Whole genome screenings will be used for both RNA lead identification upon development of suitable cellular assays (for example, microRNAs for cell proliferation and tissue regeneration, or siRNAs targeting specific cancer-associated genes) or for pathway identification (for example, transcriptome and epitranscriptome modulation by specific mRNAs).

Pharmacological assessment of RNA drugs

An essential component of the National Center and, in particular, of the **Spoke #9**, will be the design of shared protocols for the assessment of the pharmacological properties of the RNA products under development. This will include studies in established cell lines and primary cells; 3D tissue and organoids in vitro, including studies in patients' cells from hiPSC; studies in animal models for toxicology, immunotoxicology, and pharmacological properties of RNA drugs (pharmacodynamics and pharmacokinetics - PK/PD). Standardisation of these activities will provide common guidelines for more rapid product development and facilitate the interaction with regulatory bodies.

Testing in large animal models

A common bottleneck in the development of both small molecules and biologics is the availability of testing in large animals, which is an essential component of the efficacy and safety package required by regulatory authorities but is underdeveloped in Italy. Our **NC** aims to develop shared protocols for large animal testing and promote access of its investigators to the existing large animal facilities for testing for both pharmacological properties and efficacy of novel RNA drugs.

Testing in large animals is also essential to achieve organ-specific delivery of RNA products. As targeting of organs or specific cell types other than the liver is still not attainable after systemic RNA administration (cf. also later), one possibility to achieve organ specificity is administration using catheterization. For example, the heart can be reached by direct intramyocardial injection through percutaneous trans-endocardial delivery via the left ventricular cavity, by intracoronary administration through antegrade coronary artery catheterisation or by retrograde administration via the coronary sinus (*29*). Analogous approaches can be devised for the kidney, liver, pancreas and other organs, in addition to tumour specific delivery for solid cancers. Testing such conditions requires large animals for proper body size, anatomy and physiology.

The indicators of impact for this part of the coordination activities include:

- Creation of a structured network of expert laboratories, each one providing unique expertise in one aspect of the field of RNA therapeutic development. These include national facilities for RNA bioinformatics; facilities for high

throughput screenings genetic leads from RNAi and mRNA libraries; shared protocols for testing of RNA products in small and large animal models.

- Generation of shared guidelines for pharmacological assessment of RNA products, including criteria for PK/PD, toxicology and immunotoxicology, biodistribution and safety.
- Provision of access to this network of Facilities and sets of guidelines and protocols to external users from the public and private sector.
- Coordination of activities with the Italian biotech private sector (cf. later).
- Provision of interaction with Venture Capital companies at the national and international level (cf. later).

Development of novel, key enabling technologies for RNA therapies

Besides fostering coordination and promoting expansion of the current expertise of Italian laboratories in the field of RNA therapeutics, this **NC** will specifically promote activities in some of the key areas that still need significant improvement for RNA therapies to become more broadly and successfully applicable.

Manufacturing of RNA therapeutics

Manufacture of nucleic acid therapeutics is currently complex, expensive and resource intensive, limiting large-scale production of these medicines. The discovery and development of RNA-based products is still subject to the same, existing limitations chronically afflicting biologics, namely a difficult access to GMP material for clinical trials, by which RNA scientists trying to develop an innovative product are limited to using laboratory-grade materials in pre-clinical studies.

This **NC** aims to increase markedly the number of RNA products that can be designed, developed, and produced every year, reducing their costs and increasing equitable access and to create a self-sustaining network of manufacturing facilities providing a sustainable source of GMP material to progress these newly developed therapies for large animal experimentation and, eventually, clinical trials in humans. This can be achieved by new developments in solution-phase synthesis, which offer exciting opportunities to scale up (especially for guide RNAs for CRISPR/Cas9 gene silencing (*30*)), improve efficiency and reduce organic solvent use and the creation of improved platforms for conjugation of targeting ligands for organ specific delivery (cf. also later). The improvement in manufacturing capability at the national level would have a tremendous impact on the development of RNA medicines and would permit Italian R&D to progress towards clinical application without depending on the manufacturing capability of a few production companies in other countries, which impose high costs and long waiting lists.

Chemical modifications of RNA

The original idea of therapeutic ASOs dates back three decades (*31*), with over 100 Phase 1 studies performed and 25% reaching Phase 2/3 (*32, 33*). Clinical success, however, was only rendered possible by the progressive identification of chemical modifications that improve pharmacological properties along with reducing immune recognition. Classic DNA ASOs undergo DNA:RNA pairing but are then relatively rapidly degraded by RNAse H in a few minutes. This problem can be circumvented by substituting non-bonding oxygen atoms in the phosphate groups of ASOs with sulphur atoms (phosphorothioates, PS) or introducing an alkyl group in the 2' position of the sugar molecule of nucleotides (2'-O-methyl- and 2'-O- methoxy-ethyl-nucleotides; 2'-OMe and 2'-MOE respectively (*34, 35*)). The most effective modification is the inclusion of RNA nucleotides in which the ribose moiety contains an extra bond connecting the 2' oxygen and 4' carbon (2'-*O*,4'-*C* methylene bridge) (*36*). These locked nucleic acid (LNA) nucleotides confer remarkable thermal stability against enzymatic degradation while maintaining base-pairing specificity (*37*) and are often used in a steric block approach (GapmeRs). This arrangement improves ASO stability while still permitting RNAse H recruitment to the central DNA-RNA duplex for degradation. In contrast to mRNA inhibition, exon skipping does not require RNAse H and the ASO design mostly aims to improve stability. In *nusinersen* this this achieved by using 2-MOE nucleotides, while the other four approved ASOs for Duchenne muscular dystrophy are formed of phosphorodiamidate morpholino oligomers, in which the nitrogen bases are connected to a morpholino ring rather than to the deoxyribose ring and a phoshorodiamidic bond substitutes the natural phosphodiester bond.

In the case of mRNAs (including for Cas9), in vitro transcription (IVT) or chemical synthesis with the inclusion with pseudouridine (Ψ), N1-methylpseudouridine (m1Ψ), 5-methylcytidine (m5C), 5-hydroxymethylcytosine (5hmC), 5-methyluridine (m5U), and 2-thiouridine (s2U) can suppress the intrinsic innate immune response recognition, which may degrade exogenous mRNA and suppress its translation (*30*).

Collectively, these chemical modifications have permitted successful clinical translation of ncRNA and mRNA therapies. However, the current situation is not optimal yet, as modified ASOs still are fraught with residual adverse effects, while mRNA and RNAi therapeutics can still elicit the innate immune response. While this can be beneficial for vaccination, it still creates problems for applications other than those involving the immune system stimulation.

Based on these considerations, this **NC** will foster joint efforts towards the development of safer, more effective and

scalable solutions for the production of RNA therapeutics with improved pharmacological profile. This activity will also generate new intellectual property (IP). This will constitute an important requisite to attract additional interest from the private sector at the international level, especially considering that most of the modifications currently used for ASO and mRNA production are now already off-patent.

Development of effective nanocarriers for in vivo RNA delivery

A major challenge in the field remains the efficiency with which nucleic acid molecules cross cellular membranes. Accordingly, the foremost reason for the termination of clinical RNA trials so far has been lack of efficacy (*17*). Whether delivered as naked nucleic acid, ligand–oligonucleotide conjugates or via various nanocarriers, all RNAs enter cells by endocytosis and need to exit endocytic vesicles to gain access to the inner cellular compartments (*38-40*). An effective delivery tool therefore needs to provide stability against nuclease degradation while promoting cellular uptake and RNA escape from endosomes. An ideal system should also provide cell specificity, elicit minimal immune activation and be cost-effective and scalable for manufacturing. No current delivery tool fulfils all these requirements.

ASOs with a phosphorothioate backbone can be taken up by cells as naked nucleic acids as they can associate with different molecules on the plasma membrane (*41, 42*). However, natural nucleic acids with a phosphodiester backbone or single-stranded ASOs with a morpholino or peptide nucleic acid backbone (e.g. siRNAs, miRNAs) require either conjugation with a ligand or incorporation into a carrier complex for efficient cellular uptake.

One of the core activities of the **Spoke #8** will be to foster the generation of innovative mRNA and ncRNA administration vehicles. A breakthrough in this area has been the development of the Stable Nucleic Acid Lipid Particle (SNALP) technology, which is based on (a) the use of ionisable cationic lipids which are positively charged at acidic pH and complex tightly with negatively charged RNA, while becoming neutral at physiological pH, and (b) the development of methods to use these molecules to form small lipid nanoparticles (LNPs) that entrap the RNA (*43*). Patisiran, the first siRNA therapeutic to reach clinical approval in 2018 (*18*), as well as the two COVID-19 vaccines pioneered by Moderna (mRNA-1273 (*1*)) and Pfizer/BioNTech (BNT162b2 (*2*)) for SARS-CoV-2 Spike mRNA, are all formulated in LNPs. There is ample possibility of improving the efficiency of SNALP formulation, especially through the identification of novel ionisable lipids. This is an area of rapid development, and our **NC** aims to play a central role in it, especially considering the potential for innovation and IP generation.

Other RNA carriers that will be comparatively assessed in our coordinated activities will include new synthetic or natural polymers, dendrimers and different inorganic carriers for ASO and RNAi therapeutics delivery (*44*). Another interesting area of current investigation is exosomes (*45*). These endogenously produced 30-100 nm diameter vesicles are spontaneously released by cells and participate in intercellular communication by transferring bioactive material, including small nucleic acids (*46*). Methods under development include the enrichment of selected miRNAs into exosomes (*47*) and the inclusion of specific ligands within exosome membranes for cell targeting (*48*). Again, the potential for IP in this area is very high.

Novel approaches for cell and organ targeting

An ideal ncRNA therapeutic should fulfil the mandate of the so-called Ehrlich's magic bullet, namely be administered systemically to then reach the target organ in a specific manner. Thus, a breakthrough technology for naked ncRNA therapies has been the development of RNA conjugates carrying a trimer of GalNAc, which avidly binds the cellular asialoglycoprotein receptor (ASGPR) (*49*). As this receptor is expressed on hepatocyte plasma membrane, this strategy also permits specific liver targeting. Of the 4 currently approved siRNAs, 3 are GalNac-conjugates, while at least 10 other GalNac-conjugated ASOs and siRNAs are in late phase clinical development for liver-directed therapy (*17*). Unfortunately, no other targeting system for any other organ still exist or has progressed towards clinical application. After intravenous injection, polymer:RNA or lipid:RNA complexes (including LNPs) are phagocytosed by mononuclear cells in the liver, spleen, lymph nodes and bone marrow (*50*). A first step towards tissue-specificity is therefore to reduce interaction with phagocytic cells and increase circulatory half-life, for example by PEG surface modification of LNPs and other nanocarriers to provide a hydrophilic shield (*51*). Active cellular targeting could be achieved through inclusion, in the LNPs or other nanocarriers, of ligands that bind specifically expressed surface molecules. These ligands can include peptides (for example, identified through phage display panning techniques), molecules binding cell type specific receptors, antibodies, or substrates for cell-specific enzymes. In the cancer setting, nanosized particles may accumulate in tumour tissue due to enhanced vascular permeability and retention (EPR effect) (*52*).

Based on these considerations, one of the important areas of impact for this **NC** will be the development of innovative strategies for organ targeting amenable to effective clinical translation (**Spoke #8**). This will be achieved by the coordination of activities at the national level, in particular by fostering interaction of experts in ligand identification for specific cell types (including specific tumour cells), experts in ligand generation or in the selection of RNA aptamers through the SELEX technology and experts in RNA therapeutic development and in animal models for specific application. This will be a defining area for this **NC**, as this kind of activities require cross-disciplinary competence and coordination.

The impact indicators for this part of the coordination activities include:

- Partnership with private pharma or biotech to improve the manufacturing capability of mRNA/ncRNA therapeutics.
- Development of novel modification of mRNA and ncRNAs, with consequent IP generation.
- Development of novel platforms for in vivo administration of RNA products (e.g novel ionisable lipids, polymer-lipid formulations, biological carriers).
- Development of strategies for specific organ- or tumour-targeting upon RNA/carrier systemic administration.
- Attraction of private partners and co-development of patented products through the generation of start-ups or licensing.

RNA products

Besides coordinating RNA therapies at the national level, by providing RNA investigators with advanced technological support and promoting technology transfer activities to industry (cf. later), this **NC** will also directly support development of a series of RNA products. A non-exhaustive list of products that will be developed along with their status of current development and that at the end of the project is summarized below.

In the *cancer therapy* field, the **Spoke #2** has identified a set of miRNAs involved in the development of acquired resistance to target therapy in melanoma and has validated these mRNAs as therapeutic tools when delivered in using LNPs both in vitro or in vivo (TRL 4). These LNP-miRNAs will be progressed towards clinical application, first by testing them in vivo in tumour xenograft immunocompetent mouse models and then validating them in regulatory preclinical studies, prior to IND filing (TRL 5/6). In another project, autoassembling ferritin cages loaded with a specific ncRNA against an endogenous lncRNA overexpressed in acute myelogenous leukaemia will be progressed towards clinical application. A GMP-compliant batch of the vector has already been produced at a dedicated start-up company for completion of regulatory preclinical studies (TRL5), including ADME in non-human primates, and will be progressed towards starting of clinical phase I (TRL6) at the end of the project.

Several projects will focus on *mRNA vaccination* for cancer and infectious diseases. **Spoke #2** has studied the antigenic properties of hRidA as a tumour associated antigen and is now developing an mRNA vaccine (TRL4), to be progressed towards phase I/II clinical studies (TRL5/6). In another project, the same Spoke will develop chimeric RNAs formed of an aptamer selected against specific tumour associated antigens coupled with RNAi molecules (miRNAs, siRNAs) exerting anti-metastatic function (TRL3/4 to be progressed to TRL5). One of the projects coordinated by **Spoke #4** will be based on a novel pipeline for antigen discovery, which has been used to identify cardiac antigens driving the immune responses leading to heart failure. Three novel antigens, when administered in a tolerizing vaccine protocol, induce immunosuppressive Treg cells and thus pre-emptively reduce the severity of cardiac disease. The technology, which is now at TRL4, will be brought to IND approval (TRL5) through a spin-off company. The **Spoke #5** will adopt the mRNA vaccine technology to prime antigen specific immune response and develop mRNA-encoding neutralizing monoclonal antibodies against viral and bacterial pathogens. The same Spoke will implement a combined workflow to discover, design and engineer mRNA/small RNAs-based delivering nanovesicles to induce immune-tolerance and to treat autoimmune/inflammatory-based diseases. Several projects will be brought from current TRL3/4 to clinical testing (TRL5/6).

In the field of *neurodegenerative disorders*, **Spoke #3** will take advantage of its expertise in the ncRNA field to develop new programmable linear and circular non-coding RNAs to modify gene expression *in vivo* as a therapeutic strategy for Alzheimer's disease (AD). The same Spoke will also aim to develop new RNA-based strategies for therapy of Parkinson's Disease (PD). These include mRNA vaccines, a set of lncRNAs to activate endogenous neuroprotective pathways and antagomiRs. Finally, a third focus in neurodegeneration will be amyotrophic lateral sclerosis (ALS), for which the Spoke will investigate the role of linear and circular non-coding RNAs. In all three cases, activity will first be developed in mouse models and neurons differentiated *in vitro* from patient-derived iPSCs (TRL3/4) to then progress towards advanced pre-clinical development (TRL5). A common theme for various groups in the Spoke will also be targeting neuroinflammation, which will be achieved using siRNAs, piRNAs and RNAzymes.

Several investigators are interested in developing RNA therapies for cardiovascular and metabolic disorders. In particular, scientists associated with **Spoke #4** at the University of Trieste are developing innovative strategies for cardiac regeneration. This project is based on the past identification of microRNAs that stimulate endogenous cardiomyocyte proliferation. Pre-clinical studies have already demonstrated the efficacy of one microRNA in both mice and large animals (TRL4). Through an already established start-up company, this microRNA will be formulated in LNPs and progressed toward a phase I/II clinical trial for cardiac regeneration after myocardial infarction in patients (TRL5/6). Another group is interested in inhibiting oncostatin M using siRNAs, as knock down of this protein in myeloid cells rescues progenitor

cell traffic, normalizes myelopoiesis, and improves vascular response to ischemia in diabetes. This project is at TRL3/4 and will be progressed to TRL5 through a university spin-off. Other investigators in the same Spoke have identified a series of genes that are involved in muscle atrophy occurring in several diseases, including cancer, diabetes, cardiac and renal failure, unhealthy aging, and infections. RNA inhibitors of these genes are currently being used in animal models of cachexia, sarcopenia and disuse atrophy (TRL4) and will be progressed toward clinical application (TRL5). Other investigators aim to develop an RNAi therapy for dominant catecholaminergic polymorphic ventricular tachycardia (CPVT), a hereditary cardiomyopathy due to defects of the RyR2 gene. This innovative therapy will be based on siRNAs targeting three common polymorphisms located in the coding region of the gene (this strategy allows the development of therapies common for most patients, rather than requiring personalized therapies for each individual mutation). The project is TRL3/4 at this moment and will be brought forward towards clinical translation (TRL5). Finally, **Spoke #8** will be interested in the delivery of other miRNAs and siRNAs and base-editing components using nanoparticles. Different projects are currently at the early lead candidate definition (TRL1/2), others are already at TRL3/4 and will be carried forward towards the clinic (TRL5).

Impact indicators for this part of the activities of the **NC** include:

- Number of projects progressing from basic research activity (TRL1-3) to pre-clinical studies (TRL3-4);
- Number of filings for Investigational New Drug (IND) approval (TRL5);
- Number of initiated or fully planned phase I/II clinical trials (TRL6);
- Parameters of academic success, such as number of publications in top ranking scientific journals and indicators of international recognition in the field;
- Number of students, PhDs and post-docs that will undergo a career in the national biotech sector;
- Amount of additional external funding attracted by academic projects stemmed from these studies.

CONSTRUCTION OF A NATIONAL INFRASTRUCTURE FOR GENE THERAPY AND GMP PRODUCTION OF RNA DRUGS

The gene therapy infrastructure

Italian research in the field of gene therapy has been world leading, this being confirmed by the evidence that 3 out of the first 5 AGTMPs approved in Europe had been developed in our Country. However, the lack of an enabling infrastructure to boost translation limits Italian international competitiveness and ability to exploit the know-how generated in academia. Moreover, the lack of manufacturing capacity is a major roadblock for the sector while chemicals and biologics do not suffer the same limitation. Interestingly, gene, cell & tissue-based therapeutic developers worldwide are over 1000, of which one fifth (around 200) based in Europe. Nonetheless, at the beginning of 2020, the ATMPs companies operating in Italy were only 8, half of those based in Switzerland or The Netherlands, a quarter of Germany, 1 in 7 in the UK. Moreover, as of mid-2020, only 23 sites in Italy were authorized by AIFA for the production of ATMPs, of which 9 for the production of gene therapies but only 2 of them in an academic setting, whereas at global level this is one of the fastest growing sectors. Due to these limitations, Italian academia struggles to reach the clinical proof-of-concept which is a key step to enable collaboration with industry and foster the social and economic impact of the knowledge accumulated through research.

The creation of a **NC** with homogeneous manufacturing processes has the potential to overcome some relevant challenges. Indeed, it would leverage the already distributed infrastructures and expertise of the most advanced GMP facilities, as those provided by the Ospedale Pediatrico Bambino Gesù/ Fondazione Tettamanti, to provide robust potency assays for AGTMPs and supplies of new materials such as cells and vectors and to reduce the heterogeneity of products by establishing standardized procedures and common best practices.

In accordance with the national strategies and the existing initiatives, **Spoke #10**, besides operating in R&D, will be designed on 3 layers, which include:

- facilities for cell process and assays development, vector manufacturing and pre-clinical studies;
- existing and updated cell factories authorized for manufacturing of gene therapies using somatic cells;
- service Center and school for advanced studies.

Building on the expertise and facilities provided by the Institutions participating in **Spoke #10**, the existing facilities will be refurbished to increase capability. Ultimately, this will permit the support of users in the process development required to move from lab-grade protocols through optimization and scale-up to GMP-compatible processes ready for tech transfer to an authorized GMP cell factory and adequate for early-stage clinical trial. Once fully operational, the Spoke will be able to support 5 to 10 different projects per year, spanning the entire variety of provided services. Overall, once fully operational, we envisage to reach an estimate of production capability of up to 250-300 gene therapy drug products per year, available

to the other members of the **NC**, distributed over the entire Country. In particular, this capability will be reached thanks to several strategies that will be pursued in parallel: 1) a significant infrastructure investement is planned to enlarge the existing facilities already present in the NC; 2) two Private partners (PBL and Stevanato) will be involved in the optimization of the manufacturing phase for the generation of a fully-automated chain for GTMP production, that could be compatible with the concomitant manufacturing of multiple products in one single clean room; 3) the development of "mobile" Cell Processing infrastructure by Orgenesis srl, also involved in Spoke 10. The Orgenesis point-of-care platform will overcome conventional processing challenges by enabling high-quality, sterile, scalable onsite processing of GTMPs. Production infrastructure will corroborated by OMPUL (Orgenesis Mobile Processing Unit and Lab technology). It shortens implementation time from 18-24 to 3-6 months, offering cost-effective production and enabling local scalability by connecting additional OMPULs. In particular, one OMPUL can provide 80 batches of a standard CAR-T per year, as opposed to a standard clean room that can provide 20 to 30 batches. The cost of production is also minimized by use of closed systems and lack of complex logistics. The Ompul placement in Italy will be programmed at OPBG site, as Pilot study, and then its location will be further offered to other Spoke 10 members.

This structure will largely increase the accessibility of these innovative treatments, reducing the inter-regional differences and mitigating the sanitary migration. A centralized office will be set up to coordinate the Spoke activities. The Service Center will offer to both manufacturing sites and academic developers a wide range of services, including regulatory expertise and clinical operations support. Moreover, the Service Center will manage the peer review process to select the development projects that will get access to spoke.

The RNA production Facility

Despite the recent success of ncRNA and mRNA medicines, the current RNA production and purification process is still in its infancy and requires an evolution in accordance with the rapid progress offered by the research carried out in the field of RNA chemistry, formulation and delivery to optimise the biological activity of the drug. Therefore, the Facility will be supported by basic research on RNA synthesis, formulation and delivery (the 'RNA technology platform') as well as the necessary tools for the preclinical in vitro and in vivo testing of its biological activity. To this end the Facility will be equipped with state-of-the-art instrumentation, capable of making the manufacturing processes "agile" but at the same time robust and reproducible, with the recruitment of highly qualified personnel with in-depth knowledge of RNA chemistry, biochemistry, synthesis, purification, formulation and quality control. For the production of material for clinical studies, the Facility will be equipped with a quality system that is approved by the regulatory agencies (AIFA, EMA) and will have a Qualified Person (QP) with European and US cGMP guidelines and requirements expertise that is authorized to release materials for clinical use.

Through the successful implementation of the Facility, a number of objectives will be achieved, including:

- the rapid and efficient implementation of basic and translational research as well as clinical studies, thus accelerating the discovery of novel RNA drugs;
- the generation of IP, 'know how' and proprietary reagents and processes;
- the creation of an efficient network of research groups and small and medium-sized enterprises.

The Facility, which will serve the research groups of the Center, will develop collaborations with the main national and international research structures in the field of life sciences. The Facility will also be complementary to the 'Biotecnopolo Sienese Foundation' and, in particular, to the Antipandemic Hub project, with which it will develop a national network of manufacturing plants. This activity will be finalised to achieve the necessary synergies to intercept the needs of research and development aimed to generate new drugs against emerging infectious diseases of primary importance.

Based on the above, the Facility will have a dual mission: on the one hand it will accelerate the development of novel RNA drugs by the different research groups in the Network, possibly generating a financial return to be agreed according to a 'success-based' model. Successful development and marketing of an RNA drug may generate annual incomes over €100-200M, with significant return to the Facility. On the other hand, the Facility will offer a manufacturing service of RNA molecules produced in accordance with the rules imposed by the Italian, European and US Medicines Agency (AIFA, EMA, FDA) to be used for genetic vaccination or for gene therapy and/or personalized therapy. It must be noted that, at present, there is a limited GMP RNA production capability worldwide, with a consistent and growing demand for manufacturing sites especially in EU, since over half of global manufacturers are in the US due to the high volume of active clinical studies being conducted there. Therefore, we envisage that the offer of a GMP RNA production service to Companies and/or Big pharma involved in the production of biotechnological molecules will have a significant added value (eg: over 30%). Based on our previous experience in biologics and vaccine manufacturing we assume as a conservative figure an annual income over €20M after the first three years from the implementation of the Facility. Lastly, as a further source of income, we may count on the licensing of the internally developed IP, 'know how' and proprietary reagents.

With €8.460M income, the biotech pharma confirms it relevance in the pharmaceutical industry sector (data from Farmindustria Reviews). As a consequence, investments of pharma industry in R&D and manufacturing for biotech product development are increasing annually and represents a continuous opportunity for the Facility to establish partnerships. Therefore, we will also focus on the establishment of a network with small and medium-sized enterprises that will invest in the Facility.

Based on the first three years of activities of the Facility, the business plan will be updated considering the evolution of the market and the development of internal capabilities. Particularly, the business plan will consider the strengths of the Facility due to lower cost localization in Italy vs. US and EU countries (mainly UK) and its highly qualified and integrated Manufacturing and R&D profile, as well as the weakness such as the risks linked to a novel and rapidly evolving field. These analyses will have the objective of directing the efforts of the Facility toward the most effective implementation and positioning strategy for higher probability of success.

Sustainability beyond the PNRR funding period

The NC is conceived with a time perspective that goes beyond the funding period of the PNRR. In this respect, also the choice of the legal entity (the Foundation, a government-supervised long-term institutional body, with complex governing bodies and formal requirements, instead of the simpler Consortium of collaborating public and private partners) highlights the commitment to extend and expand the activities of the Center through time after the funding period.

Besides the long-term goal of providing the bases for national competitiveness in this crucial area of the new pharmacology aimed at multiple, individual drug targets, the NC has also designed a plan for economical consolidation beyond the funding period. Independently of the possibility of the public and private entities grouped in the Spokes to apply for future funding opportunities, several intrinsic properties of the NC will allow guarantee of income generation to be employed for the future activities:

- commitment to long-term funding (yearly fee) by public and private entities participating in the Foundation;
- valorization of the IP generated by research activity. The NC will include in the bylaw of the Foundation the principles of exploiting the IP generated by the research activity and will locate in the Hub a joint Office for managing the licensing of internally developed IP, know-how and proprietary reagents. In the case of startups stemming from protected IP and knowledge, the NC will hold company shares and participate in the governance.
- Service by the two large Facilities developed by the NC: the RNA production facility and the implementation of the Gene Therapy network. The former will offer a manufacturing service of RNA molecules produced under the rules imposed by the Italian, European and US Medicines Agency (AIFA, EMA, FDA) to be used for genetic vaccination or gene therapy and/or personalized therapy. In particular, the RNA platform will develop a business model in relation to the growing demand and evolution of the RNA drug market, ensuring sustainability beyond PNRR-funded activities. As to the Gene Therapy infrastructure, besides the R&D activity and the clinical trials within the Center, it will offer manufacturing sites and academic developers a wide range of services, including regulatory expertise and clinical operations.
- The Center will operate as a training hub, organizing advanced programs for medical and health professionals, biotechnologists, engineers and business people. On the one hand, this will further support the transfer of the KET to the Italian health system and the business community. On the other hand, this will represent a source of revenue for the Center. Training will also be operated in digital transformation, which represents a common best practice of the whole Center (from advanced biocomputing for R&D to GMP data filing and regulatory compliance).

DIGITAL TRANSFORMATION

In developing the most relevant expertise and cutting-edge technologies at the service of patients with unmet medical needs, the National Center will also pursue another relevant priority of the PNRR, namely investing on the digital transition of the processes. In particular, advanced bioinformatics will have two main applications, first as a crucial facilitating approach in R&D applications, i.e. for designing and testing new drugs, and, second, as an instrument for the safe and efficient use of the newly developed therapies.

Regarding clinical applications, the core rule for any good manufacturing practice regulation relies on maintaining proper documentation and records. The documentation is needed as a detailed record of the actions undertaken by a manufacturing site in the past, and of those ongoing. As such, it also provides an instrument for planning the actions to be undertaken in

the future. Effective documentation enhances the visibility of the quality assurance system. Therefore, the migration from a paper-based quality management system to a digital platform is required to maintain high quality standards in highly performing GMP facilities and RNA production platform. Similarly, the production is more reliable, reproducible and less amenable to mistakes when more automated production processes are in place. The proper accumulation and classification of data allows the use of artificial intelligence methods aiming at the reiterative continuous improvements of the processes.

For all these considerations, National Center, we aim to develop a progressive digitalization and automatization of processes, both in production and in quality control. Indeed, manual processes are the biggest threat to compliance with GMP and RNA production platform requirements. Nowadays, almost all the processes in academic GMP facilities are paper-based, despite inefficiencies created by the burdening associated to paperwork and slow manual processes. Moreover, other issues, although not immediately evident, contribute to increase the costs of managing a GMP system and RNA production platform using only a paper-based approach, including:

- There is no efficient means of consolidating data within a paper-based system.
- Data that are manually collected from and entered into paper documents are inherently limited.
- Information contained in manually compiled files is time-consuming to retrieve during audits and inspections, if retrievable at all.
- Manually tracking, approving, and recording GMP and RNA related documents increases the likelihood of misunderstandings and human errors, all of which slow the working flow.
- Manual reviews of the documentation required for GMP and RNA compliance tend to delay drug product releases.
- Training is harder to provide and even harder to track efficiently.

For most companies subject to regulatory requirements, proof of compliance has always been associated with boxes of paper records. Paper documents - and later PDFs, which can be equally as problematic to extract data from - have historically been hallmarks of compliance. These are the main reasons why the future of regulatory compliance lies in digital data connectivity and big data analysis. Life-sciences companies that have implemented paperless solutions are showing that it is possible to extend digital advantages to the daily work of a GMP and RNA production facilities. For example, a cloud-based batch record system could enable to reduce the GMP-related quality reviews from six hours per batch record to 20–30 minutes per record and may increase employee efficiency and expedite drug product release.

Digital systems that automatically contextualize data can provide the Qualified Person (i.e. the responsible person for a GMP production site for AGTMP and RNA medicine) with information to take better decisions and provide recommendations in real time. Other compliance advantages that can be achieved by digitally connecting quality data and processes include:

- Audit-ready documentation. A digital quality management system (QMS) can maintain the GMP facility updated in real-time
- A digitized QMS that automatically distributes reminders about important tasks like document approvals and late training tasks sustains the progress of quality.
- Additionally, it helps to guarantee that quality processes are always headed in the right direction.
- Comprehensive training. Automation helps ensure training is appropriately administered, completed, and recorded.
- Smoother interactions with regulators. Digital records can be easily retrieved during an inspection, providing inspectors with faster access to complete, correct information.
- Enhanced trending and tracking. Digitization affords real-time reporting capabilities that empower decision-makers to act quickly with the knowledge that their information is accurate and up to date.
- The ability to identify mistakes and see bottlenecks quickly brings quality issues to the fore sooner so they can be addressed with speed and precision.
- The possibility to identify in advance undesirable outcome. Indeed, artificial intelligence allows to forecast outcome based on past track records, thus managing proactively processes and experiments.

The goal of digitization in **NC** is not only to reach a fully digitized system in the involved GMP and GLP facilities, but also to interconnect the facilities with Hospital Pharmacy and Clinical Sites. Fully digitalized processes enable companies and research units to collect, connect, and contextualize the metrics, data, and insights needed by both manufacturing and quality teams. It puts the facilities, Hospitals and Clinical Sites in a better position for sharing processes that directly correlate to compliance, such as document revisions, training, sampling and testing, and review and release. It is very clear that the proposed digitalization strategy resolves many issues because connected, contextualized data represents the regulatory future.

One of the bottlenecks that commonly limit the translation of laboratory research towards both clinical experimentation and/or commercial exploitation of products is the lack of proper infrastructure and professional expertise that could support and facilitate these processes. This is one of the key issues that will be addressed by our **NC.**

5.1 Facilitating the path from laboratory to clinical application and commercial exploitation

The development of RNA therapeutics and gene therapy approaches leads from laboratory studies and their validation in animal models is very strong in the academic environment, including in several Universities and Research Centers in Italy. The transition from laboratory research to commercial exploitation, however, requires significant funding and facilitation. The steps that traditionally are followed by successful biotech initiatives involve (in order): 1) interaction of the scientist(s) with the technology transfer office (TTO) of the Institution where they operate; 2) analysis of the market, intellectual property (IP) space and freedom to operate; 3) filing of a patent; 3) identification of initial business angels or public body support; 4) incorporation of a start-up/spin-off; 5) upon a successful continuation of activities, funding by Venture Capital investors and expansion of the company; 6) exit through acquisition by larger biotech of pharma companies. All these steps are generally underdeveloped and underfunded in Italy, and experienced human resources to assist scientists in this endeavour and provide suitable links with international investors are missing.

Our **NC** is aimed to overcome some of the limitations in this area by creating, at the national level, an inter-institutional tech transfer committee (TTC), which will help in identifying opportunities, potential partners and in defining general strategies to avoid a major risk of the IP, namely fragmentation, which would diminish its social and economic impact.

Such a model in which public research institutions and private companies are facilitated in their interactions recapitulates what already occurs in other countries, in which public-private partnerships are essential for the success of similar initiative. For example, in the United Kingdom, the Leap Program funded by Wellcome Trust in conjunction with CEPI R3 in 2021 has started funding initiatives with $60M aimed to overcome the current limitations to the manufacturing of GMP RNA-based biologics. In particular, the programme funds automated biofundries for the manufacturing of therapeutic mRNAs, which can be rapidly deployed to produce vaccines and other therapeutic mRNAs. Always in the United Kingdom, the Nucleic Acid Therapy Accelerator (NATA) was founded by the UK Research Innovation (UKRI) initiative of the Medical Research Council (MRC) with a mission to accelerate the development of nucleic acid therapeutics by building partnership with industry and academia (https://www.natahub.org). Besides its own hub in Harwell, in 2021 NATA have issues a call to support international consortia including academia and SMEs to tackle to main challenges in RNA therapeutics development, one aimed to solve the problems related to in vivo delivery of nucleic acids (£6M of MRC UKRI funding), and the second, to be issued in 2022, to solve the problems related to manufacture of synthetic nucleic acids.

With our **NC**, we aim to provide similar support to competitive applications from our academic stakeholders in the fields of priority outlined in this application. Besides the intrinsic value of generating a similar support model for the valorisation of academic research – a model that can later be of inspiration for other analogous initiatives -, we expect that this will lead to the creation of entrepreneurial activities in Italy through the creation of start-up technological companies, having the capacity to attract highly trained personnel and supported by international venture capital funding.

This strategy will also significantly contribute to the continuation and stabilisation of the network beyond the duration of the funding period. This will be achieved via the definition of specific "packages", not necessarily anchored to individual Institutions, governed through inter-institutional agreements: we believe that this will result in a higher overall value, than the output generated by single group.

A critical component required for success of RNA drug development, similar to other biotech products, is an early understanding of the requirements for clinical studies. This appears more problematic than with small molecule drugs, as the path towards clinical application is newer for biotech products and, in particular for RNAs, there is still limited information on the specific interaction of chemically modified RNA payloads and their nanosized carriers with human organs. Therefore, it appears important for RNA investigators and start-ups to engage early with Regulators. This discussion necessarily needs to be at the international level, as the interest of investors for start-up activities/early biotech in this area, similar to other biotech areas, looks at a global market.

Based on these considerations, our **NC** aims to provide support to RNA investigators and start-ups for early access to discussion with relevant regulators from AIFA, EMA and FDA. An inter-institutional Unit at the Hub level will coordinate requests, provide expert comments and organise contacts with regulatory authorities when appropriate. Of interest, the current dialogue with Regulators is bi-directional with investigators, hence an early engagement is pivotal in driving development of R&D activities. In addition, the Hub, based on the assessment of the Scientific Advisory Board and the Industrial Board, will directly manage a special funding scheme to support Proof of Principle project, Tech Transfer to industries and creation of innovative start-up and spin-offs.

The indicators of impact for this part of the activities include:

- Coordination of TTO offices from Universities and Research Centers for RNA therapeutics, to assist RNA scientists in generating intellectual property and finding venture/biotech investment
- Number of filed PCT and patents for specific RNA molecules with therapeutic potential and for new RNA technologies.
- Number of patents licensed to private companies. While an accumulation of revenues is unlikely in the initial time frame of the **NC**, we aim to lay down a licensing strategy that can allow a stream of revenues in the long term and/or provide the IP protection to incorporate new private biotech companies nurturing the national landscape in this sector.
- Number of start-up companies stemmed from protected IP and knowledge generated by **NC**.
- Amount of private investments in innovative start-ups in the RNA therapy field, including attraction of international venture capital investment into Italian companies.
- Establishment of a core coordination Unit within the National Center to provide regulatory advice and catalyse early contact with regulatory authorities at national, EMA and FDA levels.
- Creation of a stable network of activities, including institutional support, governed through inter-institutional agreements, able to sustain activities beyond the duration of the funding period.

To get those goals the National Center will coordinate and support the activities of the Spokes with an integrated array of services aiming at fostering the social and economic impact trough the exploitation of the know-how and the capabilities generated. In particular, the institutions involved in the National Center will have access to:

1. Technology transfer office to promote and manage the exploitation of scientific knowledge accumulated;
2. Central regulatory affairs office aiming at supporting the Spokes in legal issues related to clinical trials;
3. Training center for development & use of emerging advanced therapies (cf. later).

Ultimately, the main goal of those activities is to support a smoother translation of therapies to real, cost-effective, reproducible and consistent commercial AGTMPs and RNA-based products.

TRAINING

Advanced, post-graduate training is also included in the proposed project. It will provide advanced academic training to research and clinical students and generate a novel training ecosystem in partnership with private companies. These activities will capitalize on existing capabilities of academic staff available in the members of the Consortium (Universities and Research Institutions and hospitals accredited for AGTMPs and advanced cell therapies) and scientists and managers from leading pharma and biotech companies.

Activities of our **NC** will include:

- A novel PhD programme devoted to training in excellent science covering all disciplines necessary to create innovation in the field of RNA therapeutics and gene therapy.
- The PharmaTech Academy educating the next generation of young professionals in the pharmaceutical arena as a new training concept in close collaboration with private companies.
- The organisation of advanced training programs for medical and health professionals to meet the need of new specialists, such as the physician-scientist (physician-researcher with in-depth training in the biology/pharmacology of advanced therapies), the pharmacist-biotechnologist (with expertise in AGTMP manufacturing processes), research nurses and healthcare personnel trained in the use of AGTMP;
- Provision of both formal teaching and practical training within the network.

The major and immediate impact of the training programme will be the emergence of new generation of young researchers educated in the fields of chemistry, biotechnology, precision medicine, nanomedicine, pharmacology and medicine with expertise in the main pillars of emergent scientific areas for the development of gene therapies and RNA therapeutics. The industrial and biotech companies, being an integral part of the network, will provide both an immediate benefit by strengthening students' skills and competences and a long-term benefit by improving their employability. Overall **NC** will impact on the PhD students careers by: i) developing a high impact, original and independent research line and career development plan as the basis for future applications; ii) increasing the potential for successful starting grant applications and cross-sector mobility; iii) acquisition of high impact technical expertise and publication record; iv) promoting fruitful interactions with industrial partners active in the field of pharmaceutical research and applied nanotechnologies; v) enlarging collaboration network and improving collaboration skills; vi) enhancing communication, dissemination and presentation abilities; vii) expanding the entrepreneurial capability to protect and commercialize IP, transferring knowledge and

technology for the benefit of the economy and society. NC impact on PharmaTech Academy students will foster students' careers through: i) an experiential approach and direct contact with the world of the pharmaceutical companies; ii) skills to manage processes, work in teams, and effective communication; iii) handling of real face challenges offered by the companies involved; iv) consciousness on the abilities required to position as highly-specialized professional profiles in the pharma companies (e.g. discovery pipelines, analytics, cGxP, Quality-by-design, Regulatory Affairs -development, registration, market approval, manufacturing, distribution and post-marketing surveillance- of Advanced Therapy Medicine and RNA-based medicine). Furthermore, thanks to the advanced training and qualification of researchers and clinical operators in this emerging area of science and health, provided through collaboration among universities, qualified hospitals and the existing GMP facilities, there will be a clear improvement to the shortage of well-trained engineers and production and quality control personnel who understand the manufacturing processes and are capable of developing automated/robotic methods and common platforms.

The education and training of health professionals, regulators, health-technology assessment (HTA) experts, and payers is another extremely important aspect in the expansion and establishment of AGTMPs: to date, 60% of ATMPs is developed by research Centers and hospitals – none of them with any commercial capability. There are difficult trade-offs in HTA between meeting the customary demands for head-to-head comparators and what is ethically possible, and between long-term follow-up requirements and costs.

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