Abstract book

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National Center on Gene Therapy and Drugs
Based on RNA Technology – SPOKE 9

SPOKE 9 Congress

20-21 June 2024 **Milan**, Salone PIO XII

The Pharmacology of RNA Drugs: An Unmet Pharmacological Need Tackled by the National Centre of RNA Drugs.

Finanziato dall'Unione europea NextGenerationEU
Ministero dell'Università e della Ricerca
Italiadomani
UNIVERSITÀ DEGLI STUDI DI MILANO
RNA &GENETHERAPY

The Pharmacology of RNA Drugs: An Unmet Pharmacological Need Tackled by the National Centre of RNA Drugs.

Congress Organization Committee:
*Prof. **Paolo Ciana**, Prof. **Adriana Maggi**, Prof. **Paola Minghetti**,*
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Programme

Thursday 20th June 2024

8.30 – 9.30 **REGISTRATION**

9.30 – 9.50 ***Welcome by the authorities***

Prof. ***Elio Franzini,*** Rector of the University of Milan
Prof. ***Adriana Maggi,*** National Center for Gene Therapy and Drugs based on RNA Technology

9.50– 10:00 **Opening Remarks (1° Day):** *Prof.* ***Paolo Ciana***, University of Milan

Session: RNA Drug Pharmacokinetics
Chair: *Prof.* ***Cristiana Perrotta***, University of Milan

10.00 – 10:30 *Prof.* ***Tommaso Bellini,*** University of Milan
"Silencing of the R4496C mutation in the RYR2 gene: analysis of the sequence dependence of siRNA-mRNA hybridization by label-free biosensors".

10.30 – 11:00 *Dr.* ***Electra Brunialti,*** University of Milan
"Multidimensional reporter platform: a streamlined approach for profiling innovative drug effects".

11:00 – 11:15 *Prof.* ***Luisa Ottobrini,*** University Milan
"Development of non-invasive procedures for the study of RNA-based drugs biodistribution: preliminary *in vitro* results".

11.15 – 11.45 ***Coffee break***

Session: RNA Drug Pharmacodynamics
Chair: *Prof.* ***Massimiliano Pagani,*** University of Milan

11.45 – 12.15 *Dr.* ***Elisa Di Pasquale***, IRGB-CNR
"Assessment of biocompatibility of the allele-specific RyR2-mRNA silencing through CaP-U10 nanoparticle formulations in cardiac cellular models."

12.15 – 12:45 *Dr.* ***Mattia Toninelli,*** IFOM
"Charting the immune landscape and communication axes in metastatic colorectal cancer with spatial transcriptomics."

12.45 – 13:00 *Prof.* ***Simona Lodato,*** Humanitas University
"Investigating Neurodevelopmental Origins and RNA-based Therapeutic Interventions in C9ORF72 ALS Patient-Derived 3D Cortical Organoids".

13:00 – 14.30 ***Lunch & Poster session***

14.30 – 15.00 **Special guest:** *Prof.* ***Giorgio Valentini***, University of Milan
"An RNA Knowledge Graph to support AI-boosted research on the RNA world".

15.00 – 16:00 **Roundtable - the unmet pharmacological need in the design, discovery and development of RNA drugs**
Chair: *Dr.* ***Fulvio Ferrara***, CDI Centro Diagnostico Italiano S.p.A., CDI and *Prof.* ***Paolo Ciana***, University of Milan

Panelists: *Dr.* ***Giancarlo Aquino,*** Chiesi Farmaceutici S.p.A;
Dr. Franca Cattani, Dompé Farmaceutici S.p.A.;
Dr. ***Giandomenico Turchiano***, Astra Zeneca.

16.00 – 17.30 ***Coffee break and One-to-One meetings***

Friday **21st June 2024**

9.00 – 9.30 **REGISTRATION**

Session: Toxicology and Immunoreactivity of RNA drugs

Chair: *Prof.* ***Emanuela Corsini,*** University of Milan

9.30 – 10:00 *Prof.* ***Domenico Mavilio***, University of Milan
"*In vitro* characterization of the effect of nucleic acid therapeutics on immune cells".

10.00 – 10:30 Prof. ***Laura Rigon***, University of Padua
"Exploring ER Stress and UPR Pathway Activation as Biomarkers for Liver Toxicity in Liver-Targeted Gene Therap".

10.30 – 10:45 Prof. ***Marco Peviani,*** University of Pavia
"*In vivo* platform to investigate predictive biomarkers of the CNS-specific immune-toxicological effects induced by NA-loaded NPs".

10:45 – 11:10 *Coffee break*

11:10 – 11:20 *Welcome by the regulatory authority*

Dr. ***Emanuele Monti,*** CDA di AIFA, Consigliere Regione Lombardia

Session: Regulatory framework

Chair: *Prof.* ***Paola Minghetti,*** University of Milan

11:20 – 11:45 *Dr.* ***Sara Mucherino***, University of Naplee Federico II
"Outcome research as a key in the regulatory process: Cost-Effectiveness Of Treatment with Advanced Therapy Medicinal Products".

11:45 – 12:10 Dr. ***Sara Manellari***, University of Milan
"Nucleic acids as medicinal products: the regulatory landscape".

12:10 – 13.10 **Roundtable – Fast track to reduce toxicity and speed the market authorization of RNA drugs**

Chair: *Dr.* ***Roberto Orsenigo***, Novartis Farma S.p.A. and *Prof.* ***Paola Minghetti,*** University of Milan

Panelists: *Dr.* ***Laura Berardi***, Stevanato Group; *Dr.* ***Stefano Baila***, Eurofins Biolab Srl; *Dr.* ***Fabio Selis***, Thermo Fisher Scientific; *Dr.* ***Vincenzo Salvatore***, University of Insubria

13.10 – 15.00 *Lunch & One-To-One*

15.00– 15:15 *Concluding Remarks*
Prof. ***Paolo Ciana,*** University of Milan

Abstract summary

WP1 RNA Drug Pharmacokinetics

WP2 RNA Drug Pharmacodynamics

WP3 RNA Toxicology and Immunoreactivity of RNA drugs

WP4 RNA Regulatory framework

Reporter systems for immunomodulation: potential tools for studying anti-cancer vaccines

Alessia Panzeri[1] (presenting author), Electra Brunialti[1], Nicoletta Rizzi[2], Alessandro Villa[1], Clara Meda[1], Monica Rebecchi[1], Veronica Palmisano[1], Doga Tuna[1], Chiara Sfogliarini [3], Stefano Persano[4], Federico Arlati[4], Saverio Minucci[4], Elisabetta Vegeto[3], and Paolo Ciana[1]

[1]Department of Health Sciences, University of Milan, Milan, Italy;
[2]Animal Care Unit, University of Milan, Milan, Italy;
[3]Department of Pharmaceutical Science, University of Milan, Milan, Italy
[4]Department of Oncology and Hematology-Oncology, University of Milan, Milan, Italy

Vaccines are biological drugs designed to activate the immune system to recognize and combat non-self molecules, providing immunity. Historically, vaccines have been developed to prevent diseases caused by pathogens like viruses and bacteria. Recently, vaccines have also been developed for therapeutic purposes, such as cancer treatment1. Due to their intrinsic cytotoxic ability, vaccination presents an interesting alternative to classic chemotherapeutic agents that display high off-target toxicity. However, the efficacy and safety of new vaccines must be rigorously assessed to verify their potential use as drugs. A proper balance of the different immune components is critical for vaccines success[2]. The pro-inflammatory response plays a vital role in the initial activation of the immune system and in generating robust immunological memory. Conversely, the anti-inflammatory response is essential for resolving inflammation, ensuring a balance that promotes the vaccine's effectiveness without causing harmful side effects. Therefore, we propose a method to evaluate the potential efficacy and safety of new anticancer vaccines based on studying immune modulation. This strategy involves utilizing reporter systems that enable non-invasive analysis of drug effects through *in vivo* imaging on whole organisms and tissues, integrating pharmacokinetic, pharmacodynamic, and toxicological data into a single multidimensional output. For this purpose, we generated two reporter mouse models: one to study pro-inflammatory responses (the NFkB-Luc2 mouse3) and one to study anti-inflammatory modulation (the M2-Luc2 mouse). As an initial step, we assessed the responsiveness of the reporter mice to pro-inflammatory and anti-inflammatory stimuli (such as IL-4 [Fig. A] and LPS [Fig. B]) to validate the accuracy of the reporters in reflecting the expected immune modulation. Subsequently, these tools were employed to investigate the immunomodulation mediated by an mRNA anticancer vaccine targeting the RiDA protein, expressed on the surface of neoplastic cells[4]. Two distinct sequences of RiDA, each with a unique immune response profile *in vitro*, were utilized and compared against the lipid nanoparticle formulation. Longitudinal studies in reporter mice revealed differing effects of the two vaccines on critical immunomodulatory organs such as the liver and bone, confirming the diverse immunogenicity of the vaccine.

These findings suggest that studying immunomodulation mediated by vaccines using reporter systems is an efficient strategy for rapidly and effectively prioritizing the development of new vaccines, potentially accelerating the generation of new anticancer drug therapies.

References:

1. Kaczmarek, Mariusz, et al. "Cancer vaccine therapeutics: limitations and effectiveness—A literature review." Cells 12.17 (2023): 2159.
2. Zimmermann, Petra, and Nigel Curtis. "Factors that influence the immune response to vaccination." Clinical microbiology reviews 32.2 (2019): 10-1128.
3. Rizzi, Nicoletta, et al. "Identification of novel loci for the generation of reporter mice." Nucleic acids research 45.6 (2017): e37-e37.
4. Minucci, Saverio, et al. "Goat PRP14 (gPRP14) has xeno-antigenic properties and works as a vaccine in preclinical models of cancer." bioRxiv (2022): 2022-07.

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WP1 - RNA Drug Pharmacokinetics

Development of non-invasive procedures for the study of RNA-based drugs biodistribution: a pilot *in vitro* study.

Cannavale G[1], Martelli C[1], Sattin S[2], Bongianino R[3], Grisorio L[3], Catalucci D[4,5], Priori S[3,6], Ottobrini L[1,7].*

**presenting* author

[1]Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy.
[2]Department of Chemistry, University of Milan, Milan, Italy
[3]Molecular Cardiology, IRCCS Istituti Clinici Scientifici Maugeri, Pavia, Italy
[4]Humanitas Clinical & Research Center, IRCCS, Rozzano, MI 20090, Italy
[5]National Research Council, Institute of Genetic & Biomedical Research - UOS Milan, Milan 20139, Italy
[6]Department of Molecular Medicine, University of Pavia, Pavia, Italy
[7] Molecular Bioimaging and Physiology (IBFM), CNR, Milan, Italy

In the last years, research is focusing on the development of RNA-based drugs and the identification of the best procedures for their pharmacological profiling. Pharmacological studies in fact cannot be limited to the classical methods used for small molecules. Within *'CN3 - National Centre for Gene Therapy and Drugs based on RNA Technology'*, non-invasive imaging techniques have been proposed for this task.

As regards RNA-based drugs pharmacokinetics (PK), fluorescence imaging procedures will be tested to *in vivo* follow the fate of fluorescently labelled RNA-based drugs in animal models. However, before the *in vivo* application a detailed *in vitro* assessment of the pharmacological profile of the labelled compounds has to be set up.

In this context we propose an experimental procedure aimed at the optimization and evaluation of a fluorescently-labelled RNA-based drug to be used as a probe to study biodistribution and persistence of a therapeutic siRNA (U10) [1] for the mutated allele of the ryanodine receptor (RYR2) gene involved in the Catecholaminergic Polymorphic Ventricular Tachycardia, a life threatening arrythmia.
An engineered cell model expressing RYR2 wild-type and mutated alleles has been used to test the different molecules. Cy5.5 fluorophore was attached to 5'-end of the siRNAs and *in vitro* studies were aimed at checking if fluorophore could impair the silencing activity. The analyses showed that the labelling with Cy5.5 does not impair silencing efficiency and specificity. In detail, siRyR2-U10-Cy5.5 (20nM) was able to significantly reduce the mRNA levels of the mutated RYR2 allele to the 50%. Introducing a first set of stabilizing siRNA modification resulted in a significant decrease of siRNA efficiency and selectivity.

The same cell model has been used to test efficiency and specificity of the siRyR2-U10-Cy5.5 within a calcium-phosphate (CaP) nanoparticles-based formulation [2,3]. Both CaP-U10 and CaP-U10-Cy5.5 were able to selectively reduce the expression of the mutated allele, although with a higher dose (100nM). CaP-U10-Cy5.5 signal has been detected by IVIS-Spectrum/CT acquisition, demonstrating the possibility to detect the fluorescent signal.

In conclusion, our results show that siRNA labelling with Cy5.5 doesn't impair its activity, while allowing its non-invasive detection for *in vivo* pharmacological studies. The tests used herein will constitute the quality control core for each new formulated batch.

References:

1. Bongianino R, Denegri M, Mazzanti A, Lodola F, Vollero A, Boncompagni S, Fasciano S, Rizzo G, Mangione D, Barbaro S, Di Fonso A, Napolitano C, Auricchio A, Protasi F, Priori SG. Allele-Specific Silencing of Mutant mRNA Rescues Ultrastructural and Arrhythmic Phenotype in Mice Carriers of the R4496C Mutation in the Ryanodine Receptor Gene (RYR2). Circ Res. 2017 Aug 18;121(5):525-536. doi: 10.1161/CIRCRESAHA.117.310882.

2. Miragoli M, Ceriotti P, Iafisco M, Vacchiano M, Salvarani N, Alogna A, Carullo P, RamirezRodríguez GB, Patrício T, Esposti LD, Rossi F, Ravanetti F, Pinelli S, Alinovi R, Erreni M, Rossi S, Condorelli G, Post H, Tampieri A, Catalucci D. Inhalation of peptide-loaded nanoparticles improves heart failure. Sci Transl Med. 2018;10(424): eaan6205.[9]

3. Di Mauro V, Iafisco M, Salvarani N, Vacchiano M, Carullo P, Ramírez-Rodríguez GB, Patrício T, Tampieri A, Miragoli M, Catalucci D. Bioinspired negatively charged calcium phosphate nanocarriers for cardiac delivery of MicroRNAs. Nanomedicine. 2016;11(8):891-906. doi: 10.2217/nnm.16.26.

Development of workflows for the detection of RNA-based drugs in different biological matrices for pharmacokinetic studies

Alessandro Arcari, Matteo Giovarelli, Davide Cervia, Emilio Clementi, Cristiana Perrotta*

**Presenting author*

Department of Biomedical and Clinical Sciences, University of Milan

The current preclinical methods for obtaining biodistribution data on drugs rely on radiolabeling, liquid chromatography tandem-mass spectrometry (LC-MS/MS), or hybridization assays. While LC-MS/MS has emerged as a gold standard for the analysis of chemical drugs due to its high sensitivity, selectivity, and specificity, the use of this method for RNA therapeutics is changelling due to the unique properties of these molecules. Specifically, the high molecular weights, anionic nature, and chemical instability of phosphate linkages in RNA molecules often result in higher detection limits compared to small molecules.

This project aims to develop and optimize analytical techniques for the quantitative analysis of RNA drug distribution and activity. Our initial focus has been on a siRNA targeting a heterozygous mutation of the cardiac Ryanodine Receptor RYR2 gene, which causes catecholaminergic polymorphic ventricular tachycardia (CPVT), a leading cause of sudden death in apparently healthy individuals. A notable study by Bongianino et al. (1) demonstrated that a specific version (U-10) of a RYR2-targeted siRNA successfully downregulated mutated RYR2 expression at both the mRNA and protein levels.

In vivo studies have shown that the inhalation of drug-loaded Calcium Phosphate nanoparticles (CaPs) is a viable option for targeted lung-to-heart delivery, which has been found to effectively restore cardiac function in a mouse model of cardiomyopathy (2). In this context, our goal is to develop a workflow for the quantification of siRYR-U10 delivered through CaP nanoparticles in biological matrices following pulmonary administration in mouse and rat. We will perform quantitation analysis on plasma, lung, heart, liver and kidney matrices using various analytical approaches.

We are now designing and improving the protocols for the analysis of siRYR-U10 with high-throughput techniques as quantitative RT-PCR (qRT-PCR) and droplet digital PCR (ddPCR). Experiments have been designed to detect either the naked siRYR-U10 or the formulated version with CaPs. First, RNA has been tested at different concentrations – from micromolar to nanomolar - diluted in control human serum.

To optimize the RNA extraction process, we compared two different methods: one using silica membrane purification, the other employing cellulose magnetic beads. Our results indicate that the method based on beads outperformed the other, as it not only yielded higher RNA content, but also effectively mitigated matrix effects of the serum. Then the extracted RNA was reverse-transcribed and the cDNA was analyzed by qRT-PCR. After numerous attempts, we successfully validated the specificity of three different primer couples using a 735nM siRNA concentration in human serum. Subsequently, we were able to detect siRYRU10, but only in its formulated form and not in its naked state, using both qRT-PCR and ddPCR.

Our next steps will involve the further refining of the protocol and the detection of siRYR-U10 in cells and animal tissues for pharmacokinetic studies.

References:

1. Bongianino R, Denegri M, Mazzanti A, Lodola F, Vollero A, Boncompagni S, Fasciano S, Rizzo G, Mangione D, Barbaro S, Di Fonso A, Napolitano C, Auricchio A, Protasi F, Priori SG. Allele-Specific Silencing of Mutant mRNA Rescues Ultrastructural and Arrhythmic Phenotype in Mice Carriers of the R4496C Mutation in the Ryanodine Receptor Gene (RYR2). Circ Res. 2017
2. Miragoli M, Ceriotti P, Iafisco M, Vacchiano M, Salvarani N, Alogna A, Carullo P, Ramirez-Rodríguez G.B., Patrício T, Esposti L.D et al. Inhalation of Peptide-Loaded Nanoparticles Improves Heart Failure. Sci. Transl. Med. 2018

WP1 - RNA Drug Pharmacokinetics

Silencing of the R4496C mutation in the RYR2 gene: analysis of the sequence dependence of siRNA-mRNA hybridization by label-free biosensors

Luca Casiraghi, Thomas Carzaniga, Marco Buscaglia, Tommaso Bellini**

**Presenting author*

Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano

Small interfering RNAs (siRNAs) have emerged as powerful tools for targeted gene regulation in various biological processes. The gene-silencing effect of siRNA is known to vary strongly with the targeted position of the mRNA [1], and local mRNA structure is one of the main causes of such positional effect [2]. However, there are currently no reliable tools available to design siRNA sequences with predictable and finely tuned gene-silencing efficacy. In this framework, we investigated the specific binding between siRNA and target mRNA in the context of the R4496C mutation in the RYR2 gene [3], which has been associated with altered calcium channel activity and has been linked catecholaminergic polymorphic ventricular tachycardia (CPVT). We measured the strength, kinetics, and specificity of hybridization between 13 siRNA sequences differing by single-nucleotide shift in register, and two 60-mer fragments of mRNA with and without the R4496C mutation by optical label-free biosensors.

We employed the Reflective Phantom Interface (RPI) biosensor (Figure 1) that is based on a micro-array format, and the Surface Plasmon Resonance (SPR) biosensor, based on a microfluidic design. Both biosensors provide direct and real-time quantification of binding of siRNA molecules (in solution) with the surface-bound mRNA fragments (mutated and WT) without florescent and/or colorimetric markers. We find that, despite single-base differences between the siRNA candidates and between wild-type and mutated mRNA, the differences in binding parameters are significant and larger than expected on the basis of a naïve evaluation of hybridization free energy.
The analysis of the hybridization and dissociation kinetics enabled to identify selection criteria based on the prediction of the transient mRNA structures competing with siRNA binding. Through this analysis, we selected a short list of candidate siRNA that includes siRNA-U10, the one that gave the most effective effect in-vivo (Figure 1). The study revealed that the nucleotide composition and secondary structure of siRNA and mRNA play a major role in dictating its discriminatory behaviour towards the R4496C mutation. The use of the SPR and RPI biosensors proved instrumental in understanding the mechanisms underlying siRNA selectivity and specificity.

These findings represent a significant step towards the development of computational and experimental tools enabling the accurate design of siRNA with controlled gene-silencing effect.

References:
1. Luo et al., Biochem. Biophys. Res. Comm., 318:303–310 (2004), doi: 10.1016/j.bbrc.2004.04.027
2. Gredell et al., Biotech. Bioeng., 100:744–755, (2008), doi: 10.1002/bit.21798
3. Bongianino et al., Circ. Res., 121(5):525-5362017 (2017), doi: 10.1161/CIRCRESAHA.117.310882
4. Vanjur et al., Biophys. J., 119:989-1001 (2020), doi: 10.1016/j.bpj.2020.07.016
5. Zanchetta et al., Biosens. Bioelectron., 172:112751 (2021), doi: 10.1016/j.bios.2020.112751

Multidimensional reporter platform: a streamlined approach for profiling innovative drug effects

E. Brunialti[1], A. Panzeri[1], A. Villa[1], M. Garofalo 2, N. Rizzi3, C. Meda[1], M. Rebecchi[1], V. Palmisano1, D. Tuna1, R. Bongianino[3,4], J. Modica[5,6], L. Degli Esposti[7], M. Iafisco[7], Daniele Catalucci[5,6], S. G. Priori3,4 and Paolo Ciana[1]

[1]Department of Health Sciences, University of Milan, Milan, Italy; [2]Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy; [3]Molecular Cardiology, IRCCS Istituti Clinici Scientifici Maugeri, Pavia, Italy; [4]Department of Molecular Medicine, University of Pavia, Italy; [5]IRCCS Humanitas Research Hospital, Rozzano (Milan), Italy; [6]National Research Council- Institute of Genetic and Biomedical Research – Milan, Italy; [7]National Research Council– Institute of Science & Technology for Ceramics, Faenza, Italy;

Innovative drugs such as biopharmaceutical and RNA-based therapies, offer new opportunities to cope with unmet medical needs. However, the analysis of pharmacokinetics (PK), pharmacodynamics (PD), and safety required to transform a lead compound into an approved drug still remains a time- and costintensive process[1]. To address this challenge, we have developed a platform utilizing cutting-edge methods to efficiently profile the properties of pilot lead compounds. Our approach leverages the potential of reporter systems, enabling non-invasive analysis of drug effects on single cells, tissues, and whole organisms resulting in the combination of PK/PD, and toxic effects a in a unique measurement with multi-dimensional outputs[2]. The analysis focuses on the impact of potential new drugs on fundamental biological processes such as proliferation, apoptosis, oxidative stress, autophagy, and immune response, which are critical to pathophysiological and toxic mechanisms. The strategy can be applied to both *in vitro* and *in vivo* studies. For instance, using the approach in cellular systems enabled the assessment of off-target effects of siRNA designed to silence a mutant allele causing catecholaminergic polymorphic ventricular tachycardia and potential heart failure[3,4]. This approach allowed for the rapid identification of specific alterations in key pathogenetic pathways, helping to pinpoint the times and doses that warrant closer attention regarding potential side effects of siRNA and its formulation (Figure A). Similarly, conducting longitudinal *in vivo* studies on reporter mice demonstrated that specific bio-nanocarriers for the delivery of anticancer drugs or immunogenic oncolytic adenoviruses can achieve tumor-specific immune activity while minimizing off-target toxic effects (Figure B)[5].

Our data confirm the potential of this experimental approach to rapidly discriminate between the toxic and beneficial effects of candidate drugs. This method provides a comprehensive view of the drugs' effects and facilitates the identification of critical doses, timing, and target organs for focused drugspecific studies. We believe that implementing this broad first-round reporter assay will accelerate the prioritization of candidate drugs and their preclinical studies.

References: **1.** Cavagnaro, Joy A. "Preclinical safety evaluation of biotechnology-derived pharmaceuticals." Nature Reviews Drug Discovery 1.6 (2002): 469-475. **2.** Maggi, Adriana, and Paolo Ciana. "Reporter mice and drug discovery and development." Nature Reviews Drug Discovery 4.3 (2005): 249-255. **3.** Bongianino, Rossana, et al. "Allele-specific silencing of mutant mRNA rescues ultrastructural and arrhythmic phenotype in mice carriers of the R4496C mutation in the ryanodine receptor gene (RYR2)." Circulation research 121.5 (2017): 525-536. **4.** Miragoli, Michele, et al. "Inhalation of peptide-loaded nanoparticles improves heart failure." Science translational medicine 10.424 (2018): eaan6205. **5.** Garofalo, Mariangela, et al. "Extracellular vesicles enhance the targeted delivery of immunogenic oncolytic adenovirus and paclitaxel in immunocompetent mice." Journal of controlled release 294 (2019): 165-175.

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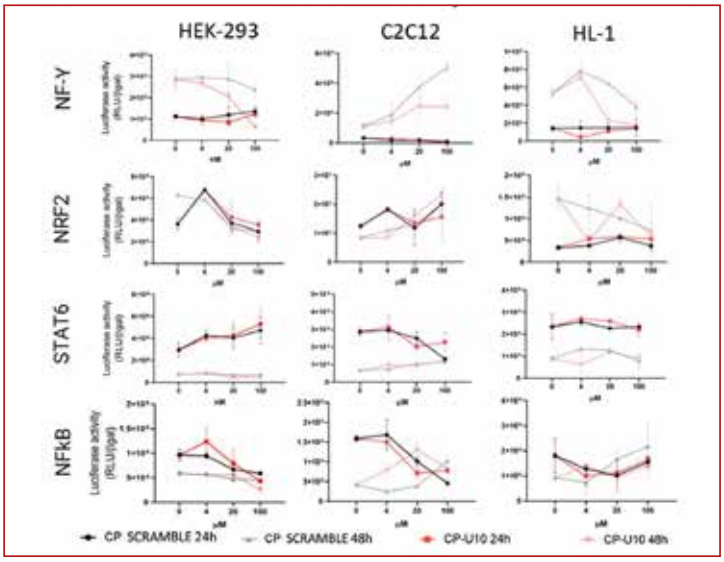

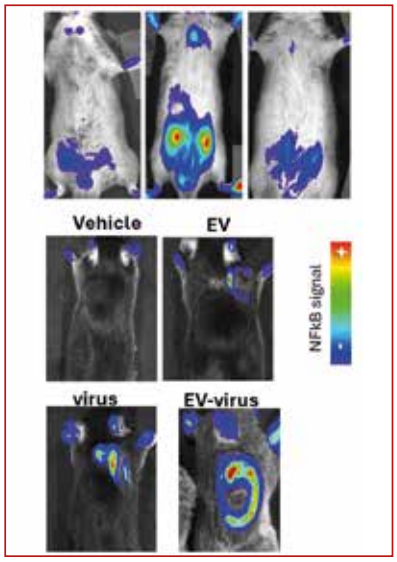

WP1 - RNA Drug Pharmacokinetics

Design and validation of a reporter mouse to study the modulation of autophagy and lysosomal pathway master regulators using *in vivo* imaging techniques

Electra Brunialti[1], Alessia Panzeri[1], Nicoletta Rizzi[2], Rita Pinto-Costa[3], Alessandro Villa[1], Clara Meda[1], Monica Rebecchi[1], Veronica Palmisano[1], Doga Tuna[1], Donato A. Di Monte[3], and Paolo Ciana[1]

[1]Department of Health Sciences, University of Milan, Milan, Italy;
[2]Animal Care Unit, University of Milan, Milan, Italy;
[3]German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany.

Dysregulation of autophagic and lysosomal pathways is associated with the onset of diseases such as cancer, metabolic disorders, and neurodegenerative diseases[1]. Consequently, researchers have explored pharmacological approaches to modulate these pathways as potential therapeutic targets. However, the physiological complexity of their functions has limited the development of selective modulators[2]. In this field, RNA-based therapies offer new opportunities to address these unmet medical needs, but their study and characterization can often be challenging due to the intrinsic properties of these molecules. Therefore, to support the development of new RNA-based drugs, we have taken advantage of the potential of reporter systems and developed a reporter system named CLEAROptimized. This system enables non-invasive analysis of drug effects on single cells, tissues, and whole organisms, of the master regulators of autophagic and lysosomal pathways, the transcription factors TFEB and TFE[3]. CLEAROptimized comprises a promoter with six coordinated lysosomal expression motifs identified through a bioinformatic analysis of the promoters of 128 TFEB-target genes and drives the expression of luciferase as a bioluminescent reporter and tdTomato as a fluorescent protein. We evaluated CLEARoptimized reporter's expression in response to starvation and treatment with various TFEB activators (Figure). To confirm the reporter's specificity, luciferase activity obtained in wild-type cells was compared with TFEB and TFE3 knockout cells, confirming that CLEAROptimized efficiently report the specific activity of both transcription factors. Therefore, the CLEAROptimized construct was utilized to generate the TFEB-luc2 reporter mouse, which is evaluable for detecting stimuli capable of activating TFEB *in vivo*, such as TFEB overexpression, food deprivation, and administration of TFEB pharmacological activators. Indeed, photon emission increases in the body regions and organs where TFEB activation was anticipated, as demonstrated by imaging, biochemical measurements, and realtime PCR on TFEB target genes. Overall, this study provides to the scientific community an innovative tool for studying autophagy and lysosomal pathway modulation across various biological levels, from individual cells to the entire organism[3].

References:
1. Steingrímsson E, et al. Melanocytes and the microphthalmia transcription factor network. Ann Rev Genet. 2004;38(1):365–411. **2.** Napolitano G, et al. TFEB at a glance. J Cell Sci. 2016 Jul 1;129(13):2475–2481. **3.** Brunialti E, et al. Design and validation of a reporter mouse to study the dynamic regulation of TFEB and TFE3 activity through *in vivo* imaging techniques. Autophagy. 2024 Mar 27:1-16.

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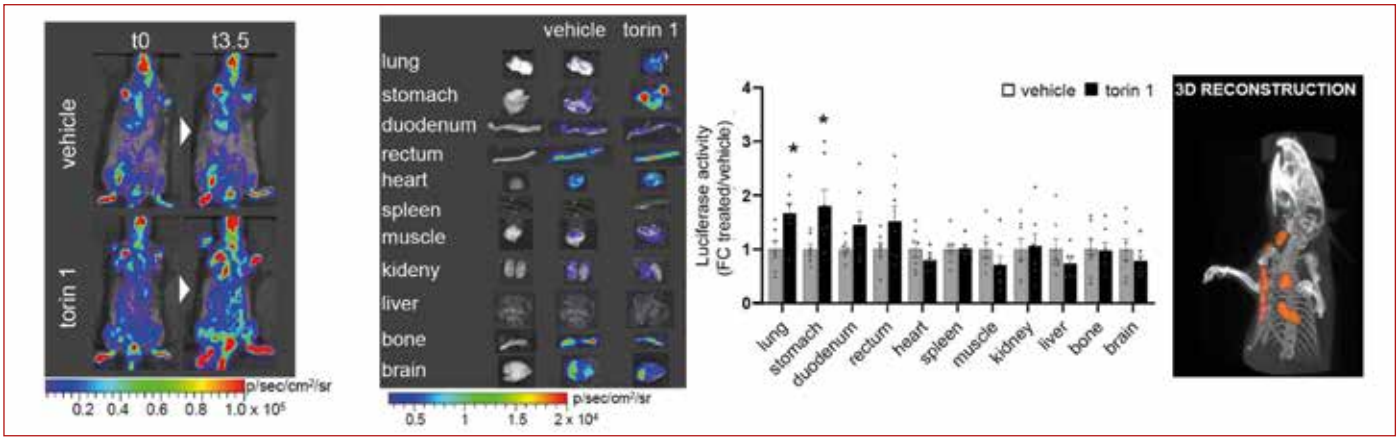

Application of human gastric organoids as a pre-clinical model to test RNA drug

Mariarita Brancaccio[1], Chiara Balzamo[1], Rosita Di Palma[1], Viviana Longo[1], Francesco Albano[1], Valeria Lucci[1], Paola De Cicco[2], Ester Pagano[2], Elena Amendola[3], Francesca Borrelli[2], Barbara Romano[2], Angelo Antonio Izzo[2], Geppino Falco[1].

[1]Dipartimento di Biologia, Università Degli Studi di Napoli Federico II, "Complesso di Monte Sant'Angelo", Via Vicinale Cupa Cintia, 21, 80126 Napoli-Italia
[2]Dipartimento di Farmacia, Università degli Studi di Napoli Federico II, Via Domenico Montesano, 49, 80131 Napoli-Italia
[3]CNR- IEOS - Istituto per l'Endocrinologia e l'Oncologia Sperimentale" Gaetano Salvatore", Via Sergio Pansini, 5, 80131 Napoli-Italia

Despite its global declines in incidence and mortality over the past several decades, gastric cancer (GC) is the 5thmost common malignancy and the 4th leading cause of cancer-related deaths, according to Global Cancer Statistics (GLOBOCAN) 2020 [1]. As a primary epithelial malignancy, the initiation of GC is a multistage process and it is generally associated with various risk factors [1], and some elements are related to its prognosis and survival [1]. Thanks to advances in therapeutic strategies, the incidence and mortality of GC has been decreasing gradually worldwide. However, certain challenges still exist in managing GC, such as the clinical applications of surgical treatment and chemotherapy. Considering this, it has become essential to generate pre-clinical models where new pharmacological therapies can be tested and validated to improve survival after GC diagnosis.

Therefore, our unit, in agreement with Spoke 9, has decided to deal with the development of a platform to evaluate the effectiveness of RNA/DNA drugs on preclinical models of GC. The 3D model of the organoid has proven to be a good model for drug screening, given the high similarity with tissue derived from it [2]. Our unit generates human gastric organoids (hGO) derived from healthy patients (or patients with non-cancer diseases) and patients with GC. The hGO generation procedure starts with mechanical and enzymatic digestion of the tissue and subsequent culturing of the primary cells. However, only adult stem cells (ASCs), located in the gastric mucosa can give rise to cultures of GO, which using growth factors and an extracellular matrix will self-organize to form 3D structures.

Our interest is the isolation and molecular characterization of ASCs, given their application in regenerative medicine. Through in silico studies, genes that colocalized with a known stemness marker, LGR5 [3], and localized on the cell membrane were chosen for analysis: AQP5, MUC6, PGC, and A4GNT. It was then decided to engineer the organoids by lentiviral transduction with their respective promoters to excise the positive populations and do molecular characterization. These results allow us to understand the molecular mechanisms after the use of the RNA drugs and how these can modulate the GC phenotype by the monitoring of the cytotypes validated in our models.

In conclusion, these data demonstrate how hGC can represent a pre-clinical model useful in the validation of new cutting-edge therapies.

References:
1. Yang WJ et al (2023). doi: 10.3748/wjg.v29.i16.2452.
2. de Poel E et al (2023) doi: 10.1016/j.jcf.2023.03.004.
3. Wang B et al (2016) doi: 10.1371/journal.pone.0168904.

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WP2 - RNA Drug Pharmacodynamics

Development of oligodendrocyte-based models to study the effect of anti-sense oligonucleotides

Juliana Helena Castro e Silva[1], Davide Marangon[1], Carolina Peri[3], Valentina Murtaj[3], Alessia Mauri[1], Stefano Raffaele[2], Marta Fumagalli[2], Paola Panina[3], Maria P. Abbracchio[1], Davide Lecca[1]*

*Presenting author

[1]Department of Pharmaceutical Sciences, Università degli Studi di Milano
[2]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano
[3]Neuroimmunology Unit, Institute of Experimental Neurology, San Raffaele Scientific Institute

Oligodendrocytes (OLs) are glial cells whose primary function is to produce myelin, a lipid structure that allows proper transmission and allows physical and metabolic support to neurons in the central nervous system (CNS). In several neurodegenerative diseases and chronic conditions, loss of OLs and myelin dysfunction have been described, while oligodendrocyte progenitor cells (OPCs) that promptly react to injury, fail to differentiate, and to produce new OLs.

Thus, supporting OPC maturation is a potential strategy to sustain OL functions and counteract neurodegeneration. In this project we aimed to develop different *in vitro* OL-based models and to adapt them to evaluate the effects of selected RNA lead compounds in relevant pathological conditions. Firstly, we have set up a model of OL differentiation failure exposing rat primary OPCs to a cocktail of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, and IFN-γ) abundant in several degenerating conditions.

Then, we have characterized iPSC-derived human OPCs in monocultures and OL-enriched 3D organoids treated with pro-myelinating drugs to identify a common transcriptomic signature associated to enhanced differentiation.

Finally, the murine immortalized cell line Oli-neu was selected for transient transfection experiments to easily introduce transgenes and reproduce genetic models of disease. These models will be used to test the effect of a morpholino-based antisense oligonucleotide (ASO) specifically designed to target the pathological expansion in the C9ORF72 transcript, the most common genetic mutation found in frontotemporal dementia and amyotrophic lateral sclerosis. This mutation, whose functions was mainly characterized in neurons, generates both toxic RNAs and dipeptide repeats (DPRs) that impair cellular processes such as autophagy and lysosomal functions. To reproduce the pathological alterations in our models, human OPCs and OL-enriched 3D organoids will be derived from mutated C9ORF72 patient iPSCs. In parallel, Oli-neu cell line will be transfected to express the mutated product of the C9ORF72 gene.

In these models we will assess DPR formation, markers of toxicity, inflammation, differentiation, myelination, and lysosomal functions after exposure to the selected ASO. Our results will allow to characterize the pathological consequences of C9ORF72 mutation in OLs and to evaluate both beneficial and side effects of the treatment with ASO.

Integrating different approaches for establishing a multi scale functional validation platformfor RNA-based drugs in the CNS (MULTIVAL)

Elia Ca[1,2], Bariselli S2,3, Borreca A[1,2], Fossati M[1,2,3], Leonzino M[1,2,3], Grassi R[2,3], Raya I[2,3], Franzoni D[2,3], Airi F[2,3], El Bouatmani M[2,5], Pozzi D[2,3], Ceccacci F[4], Rasile M[2,3], Lauranzano E[1,2], Rusconi R[2,3], Asselta R[2,3], Ottoboni L[6], Corti S[6], Matteoli M[2,3], Lodato S[2,3] and Malosio ML[1,2]

[1]Consiglio Nazionale delle Ricerche, Neuroscience Institute, Milan, URT c/o IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy
[2]IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy
[3]Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini 4, 20072 Pieve Emanuele, Milan, Italy.
[4]Consiglio Nazionale delle Ricerche, Institute for Biological Systems, Secondary Office of Rome-Reaction Mechanisms c/o Department of Chemistry, Sapienza University, P.le A. Moro 5, Rome, Italy
[5]Università di Milano-Bicocca, piazza dell'Ateneo Nuovo 1, 20126 Milan, Italy
[6]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, UOC Neurology; Milan, Italy

Abstract The task MULTIVAL within Spoke9 of the National Center for Gene Therapy and RNA Technology (CN3) aims to implement an integrated neuropharmacology plaotfrm for functional testing of RNA drugs.

Specifically, we will highlight the collaboration between the Institute of Neuroscience of the CNR and Humanitas University, focusing on their integrated neuropharmacology platform. This platform employs diverse approaches to facilitate functional testing of RNA drugs for central nervous system (CNS) diseases, with an emphasis on biomarker analysis and animal models to enrich our understanding of therapeutic outcomes within the CNS milieu. Additionally, it explores multimodal strategies to enhance the efficacy and safety assessment of RNA therapeutcs within the CNS context. For this purpose, we aim to generate a mouse model of Amyotrophic Lateral Sclerosis (ALS) based on adeno-associated viral vectors (AAV) to drive the expression of expanded G4C2 hexanucleotides of C9ORF72 gene in mouse brains of expanded G4C2 hexanucleotides of C9ORF72 gene. The later is one of the genetic variants of Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disease that results in the progressive death of motor neurons in affected patients.

Our approach is expected to drive the expression of a higher number of repeats than previsously published (Chew et al., 2019), more closely resembling the genetic condition of human patients. Furthermore, regarding the Antisense Oligonucleotide (ASO) 'MorB' (ASO-MorB), which is directed against the G4C2 hexanucleotide expansions of C9ORF72, we aim to test its pharmacodynamic properties through preclinical *in vitro*, and *ex vivo* models.
We will focus on delivery validation assays to detect its presence within cells and tissues and for functional evaluation.

WP2 - RNA Drug Pharmacodynamics

Assessment of biocompatibility of the allele-specific RyR2-mRNA silencing through CaP-U10 nanoparticle formulations in cardiac cellular models

Camilla Galli[1,2], Jessica Modica[1,2], Silvia Lucia Appleton[1,2], Lorenzo Degli Esposti[3], Marie-Louise Bang[1,2], Michele Iafisco[3], Vincenzo Lullo[4], Maria Strazzullo[4], Maria Rosaria Matarazzo[4], Elisa Di Pasquale[1,2], Daniele Catalucci[1,2]

[1]National Research Council - Institute of Genetic and Biomedical Research (IRGB) – Milan
[2]IRCCS Humanitas Research Hospital, Rozzano (Milan)
[3]National Research Council - Institute of Science, Technology and Sustainability for Ceramics (ISSMC) – Faenza
[4]National Research Council - Institute of Genetics and Biophysics Adriano Buzzati Traverso (IGB)- Napoli

The cardiac ryanodine receptor (RyR2) governs the release of Ca^{2+} from the sarcoplasmic reticulum (SR), which is essential for excitation-contraction coupling in the heart. Mutations in *RyR2 cause several cardiac diseases, such as inheritable cat*-echolaminergic polymorphic ventricular tachycardia (CPVT), and viral-based intervention to suppress the allele-specific p.R4496C mutant mRNA has shown therapeutic benefit in preclinical models.[1]

Based on these findings, a siRNA oligo targeting the mutant *RyR2* mRNA (U10) was developed as a novel RNA drug for the treatment of CPVT. Inhalable calcium phosphate nanoparticles (CaP NPs) carrying therapeutic biomolecules, such as peptides and small RNAs, have been developed and shown to be effective for enriched delivery of therapeutics to cardiomyocytes in the heart.[2,3,4] Accordingly, U10-loaded CaP NPs have been selected by Spoke 9 as the lead product to be evaluated. Our Cardio team is dedicated to assessing the developed CaP-U10 formulation for its biocompatibility and potential toxic effects in *in vitro* cardiac platforms. A first set of data was generated by testing the formulation in the HL-1 cardiomyocyte line, showing good levels of biocompatibility and no change in metabolic parameters. Subsequently, induced pluripotent stem cells-derived cardiomyocytes (iPSC-CMs) were adopted, which have a great potential for the study of cardiovascular diseases and have been extensively used as a platform for disease modeling, heart regeneration, and drug screening.

Therefore, our first objective was to determine potential toxic effects of siRNAloaded CaP NPs on WT hiPSC-CMs by checking cell viability, cell metabolism, and expression profile of stress-related genes, which are all important parameters for testing the biocompatibility of formulations. Preliminary results reveal that siRNA-CaP NPs are highly biocompatible and do not induce any adverse effects on cell vitality and metabolic activity. Further tests with iPSC-CMs carrying the *RyR2* mutation will be performed at a second stage.

Overall, we demonstrate here the suitability of our cardio platform for comprehensive biocompatibility and toxicity testing of novel drug formulations.

References:
1. Bongianino, Rossana, et al. "Allele-specific silencing of mutant mRNA rescues ultrastructural and arrhythmic phenotype in mice carriers of the R4496C mutation in the ryanodine receptor gene (RYR2)." Circulation research 121.5 (2017): 525-536.
2. Miragoli et al., "Inhalation of peptide-loaded nanoparticles improves heart failure" Science Translational Medicine 2018
3. Modica et al., "Nano-miR-133a Replacement Therapy Blunts Pressure Overload–Induced Heart Failure" Circulation 2021
4 Alogna et al., "Lung-to-Heart Nano-in-Micro Peptide Promotes Cardiac Recovery in a Pig Model of Chronic Heart Failure" Journal ofAmerica College of Cardiology 2024

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In vitro and *in vivo* testing of RNA delivery and translation efficiency by novel split-GFP based Delivery Sensors (SPLIDS)

Tito Calì[1], Caterina Peggion[2], Celeste Milani[2], Adamantia Deligiannopoulou[1] and Marisa Brini[2,3]

[1]Dept of Biomedical Sciences, Via Ugo Bassi 58b, University of Padova, 35131 Padova, Italy. tito.cali@unipd.it
[2]Dept of Biology, Via Ugo Bassi 58b, University of Padova, 35131 Padova, Italy. caterina.peggion@unipd.it
[3]Dept of Pharmaceutical and Pharmacological Sciences, Via Ugo Bassi 58b, University of Padova, 35131 Padova, Italy. marisa.brini@unipd.it

RNA-based therapies are regarded as the most attractive therapeutic target for personalized medicine strategies. They can broaden the range of druggable targets: by selecting the correct nucleotide sequence on the target RNA or by *in vitro* transcribing mRNA, it is in principle possible to target any selected genes by correcting/replacing defective proteins or immunizing for protein of interest. However, the efficiency of RNA delivery into the cytoplasm through overcoming the extracellular and intracellular barriers remains critical for successful RNA therapy.

In addition, escaping from the endosome and releasing RNAs into the cytoplasm in a non-toxic manner is a major technical problem. Various chemical modifications and the engineering of delivery formulations have been explored to solve challenges related to pharmacodynamics and pharmacokinetics.

Extensive effort is still directed to the development/improvement of effective and targeted delivery systems, and nanoparticles appear promising. However, the availability of a consistent platform for evaluating the effectiveness and specificity of nanoparticles or other vehicles is still a significant challenge.

Our aim is to develop a quantitative assay to detect RNAs efficiency delivery and translation *in vitro* and *in vivo*, by exploiting split-green fluorescent protein (splitGFP) technology. SplitGFP is composed of two nonfluorescent fragments, GFP1-10 and GFP11, able to reconstitute the GFP fluorescence upon selfcomplementation. We have generated reporters characterized by tunable enhancement of the fluorescence signal upon the complementation of tandem repeats of the β-strand 11 (1X, 3X, and 7X) and tested them both *in vitro* by complementation assays and *in vivo* in GFP1-10 stably expressing HeLa cell clones as well as in 3D cellular models, *i.e.* spheroids and in primary mouse adult fibroblasts obtained from Cre-Lox GFP1_10 mice.

So far, we have validated the approach by using expression plasmids, the next step will be to test the system by challenging our 2D and 3D cellular models with mRNA for GFP11 variants encapsulated in nanoparticles of different origin and compositions.

Behavioral effects and bio-distribution of MorB intraventricular brain delivery in WT mice

Bariselli S.[1,2], Borreca A.[2,3], Elia C.[2,3], El Bouatmani M.[1,2,4], Ottoboni L.[5], Corti S.[5], Malosio M.L.[2,3], Lodato S.[1,2], Matteoli* M.[1,2]*

[1]Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini 4,20072 Pieve Emanuele, Milan, Italy.
[2]IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy.
[3]Consiglio Nazionale delle Ricerche, Neuroscience Institute, Milan, URT c/o IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy.
[4]Università di Milano-Bicocca, piazza dell'Ateneo Nuovo 1, 20126 Milan, Italy.
[5]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, UOC Neurology; Milan, Italy.

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disorder characterized by a progressive loss of voluntary movements, leading to death. Overall, mutations in the C9orf72 gene account for 30-40% of the familial cases of ALS. Pathological G4C2 hexanucleotide expansions (> 35) in the C9orf72 genomic region between exon 1a-1b lead to protein aggregation, formation of RNA foci, and motor neuron degeneration.

Thus, one potential therapeutic approach to familiar ALS would be to inhibit the expression of the mutated C9orf72 sequence through antisense oligonucleotide (ASO) technology. Within the MULTIVAL platform, we evaluated the toxicity of intraventricular brain delivery of a Morpholino-based ASO, MorB, targeting the G4C2 expansion. We infused MorB (40 nmol) in the lateral ventricle of adolescent male and female C57Bl6/j WT mice. Between 3 to 8 weeks post-infusion, we conducted a longitudinal evaluation of the animal's behavior, which included general health assessment, ambulatory activity in the open field, motor skill learning in the accelerating rotarod task, and affective states during the elevated plusmaze test. At the end of the behavioral experiments, we collected tissues from these animals to evaluate the bio-distribution of MorB in the brain and peripheral organs.

Overall, we did not detect any change in the behavioral performance across all tests in MorBcompared to Sham-infused mice, excluding any toxic effects of MorB on the parameters analyzed. We will then evaluate MorB efficacy in the ALS mouse model we are currently developing in the MULTIVAL platform.

Investigating Neurodevelopmental Origins and RNA-based Therapeutic Interventions in C9ORF72 ALS Patient-Derived 3D Cortical Organoids

Franzone D.[1,2], Airi F.[1,2], Raya. I[1,2], Paulis M.[2,4], Ottoboni L.[5], Corti S.[5], Fossati M.[2,3], Leonzino M.[2,3], Lodato S.[1,2].

**Presenting author: Franzone Davide*

[1]Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini 4, 20072 Pieve Emanuele, Milan, Italy
[2]IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy
[3]Consiglio Nazionale delle Ricerche, Neuroscience Institute, Milan, URT c/o IRCCS Humanitas Research
[4]UOS Milan Unit, Istituto di Ricerca Genetica e Biomedica (IRGB), CNR, 20138 Milan, Italy
[5]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, UOC Neurology; Milan, Ital

Amyotrophic lateral sclerosis (ALS) onset spans between the 6th and 8th decade of life, aligning with most neurodegenerative diseases (NDD) that appear in late adulthood.

No direct evidence links ALS to early-life CNS disorders, but growing evidence suggests a developmental component in NDDs as recently demonstrated for Huntington's disease [1] and Spinal Muscular Atrophy (Faravelli, Rinchetti, Tambalo et al., *in revision*).
Supporting a developmental involvement in ALS etiology, several ALS-related genes are found to be crucial in neurodevelopment, as demonstrated in iPSC-derived neuronal progenitors bearing the C9ORF72 mutation. Impaired proliferation and accelerated neural differentiation are found in human 2D ALS models [2].

Embryonic cortical neurons from SOD1G93A mice exhibit early postnatal electrophysiological impairments resolved by P26, further suggesting that early neuronal dysfunction might be present and compensated before disease onset [3].
Based on this evidence, our project will leverage the study of the neurodevelopmental aberrations responsible for early cortical neurodevelopmental alterations that can trigger degeneration. Longterm culture of 3D cortical organoids [4] will be obtained from three independent iPSC lines derived from C9ORF72 patients, and their isogenic lines.

This model, already established in our lab, allows us to mimic the developmental trajectories of different neuronal and non-neuronal classes carrying the specific G4C2 repetitions.

We will longitudinally analyze their histological and cellular composition and characterize their activity patterns using calcium imaging and MEA recording to assess early functional circuit dynamics. In parallel, we aim to generate adeno-associated viral vectors (AAV) expressing expanded G4C2 repeats in cOrg derived from healthy controls.
This approach will help us dissect the temporal dynamics of G4C2 repeatsinduced phenotypes, allowing for precise AAV infection at multiple maturation stages in vitro.

As part of the MULTIVAL platform, we aim to use Antisense Oligonucleotide "MorB" against the G4C2 expansions in the C9ORF72 gene to mitigate neurodevelopmental alterations and address the potential therapeutic value of antisense approaches on ALS. Through transcriptional and functional profiling at single-cell resolution, we will identify MorB-responsive cell types in cOrgs and uncover molecular pathways linked to neurodevelopmental changes potentially corrected by MORB treatment.

References:

1. M. Barnat et al., "Huntington's disease alters human neurodevelopment," Science (80-.)., vol. 369, no. 6505, pp. 787–793, 2020.
2. E. Hendricks et al., "The C9ORF72 repeat expansion alters neurodevelopment," Cell Rep., vol. 42, no. 8, Aug. 2023.
3. J. Kim et al., "Changes in the excitability of neocortical neurons in a mouse model of amyotrophic lateral sclerosis are not specific to corticospinal neurons and are modulated by advancing disease," J. Neurosci., vol. 37, no. 37, pp. 9037–9053, 2017.
4. S. Velasco, B. Paulsen, and P. Arlotta, "Highly reproducible human brain organoids recapitulate cerebral cortex cellular diversity," Nature, vol. 570, no. 7762, pp. 523–527, 2019.

WP2 - RNA Drug Pharmacodynamics

REPorter system for RNA-based therapy detecting apoptosis and cellular stress in ORGanoid models - REP-ORG systems.

Valeria Pecce[1] PhD, Greta Pomanti[1], Gabriella Dobrowolny2 PhD, Alessandra Rossi[1] PhD, Ilenia Minicocci[1] PhD, Antonella Verrienti[1] PhD, Maurilio Sampaolesi[2-3], Cosimo Durante[1] MD PhD, Marcello Arca[1] MD.

[1]Department of Translational and Precision Medicine, Sapienza University of Rome, Policlinico Umberto I, Rome, Italy
[2]Department of Human anatomy, Histology, Forensic medicine and Orthopedics, Sapienza University of Rome, Rome, Italy
[3]Translational Cardiology Laboratory, Department of Development and Regeneration, Stem Cell Institute, Leuven, Belgium.

BACKGROUND

In recent years, the increasingly in-depth knowledge of the mechanisms linked to RNA has led to the development of new RNA-based drugs that directly regulate aberrant gene expression in diseases. The main types of therapeutics RNAs are those based on messenger RNA (mRNA), short interference RNA (siRNA), and RNA aptamers. Despite their great potential, using RNA molecules presents several limitations related to their multiple functions and structural malleability. This has driven the development of new methodologies to improve all systems used for these drugs' correct administration and functioning. In this study, we aimed to identify the off-target of RNA-based drugs. For this purpose, we exploited the reporter systems; they were used as models to standardize tests to analyze long-term effects on non-specific targets of RNA-based drugs, thereby enhancing the precision of RNA-based therapies.

METHODS and RESULTS

We generated reporter-based plasmids that express GFP and YFP fluorescent proteins under the control of consensus sequences recognized by the main transcriptional factors involved in inflammatory response (Nf-kB) and oxidative stress response (Nrf2). These constructs were then transiently transfected into several lines derived from different tissues, including myoblast (the C2C12 and C2SOD1 lines1), hepatocyte (HepG2 and HUH7), thyroid cell lines (Nthy-ori, K1, BCPAP). We verified the correct functioning of the constructs in 2D by inducing stress with hydrogen peroxide and analyzing the levels of Nf-kB and Nrf2 using Real-Time PCR and western blotting. After verifying the correct functioning of the 2D systems, we cultured transfected cells using the extracellular matrix to obtain a 3D culture. We obtained different structures depending on the cell lines, which were cultured for a long time. Following the verification of the formation of the organoids, we treated the structures obtained with different types of RNA, particularly two RNA-approved drugs (particularly the siRNA anti-ANGPTL32 and PCSK93) and two miRNAs derived from different research projects in our laboratory (the miR- 139-5p and the miR-335-5p). The aim is to monitor the effects of these molecules long-term up to the induction of cellular stress, repeating the treatments periodically, specifically for each RNA. Following the triggering of stress, the treated organoids were taken, and some targets implicated in the signaling pathways of the RNA target molecules were compared with the untreated ones through Real-Time PCR. (e.g. PPARa and SREBP1-c for the anti-ANGPTL3 and PCSK9 treatments showed substantial reduction following siRNA treatment; NIS for miR-335-5p and miR-139-5p appears to increase, as expected, following treatment).

CONCLUSIONS and FUTURE PERSPECTIVES

Our research has led to the development and validation of a long-term monitoring system for the adverse effects of RNA drugs. This system, combined with RNA-seq analysis, will allow us to identify aberrant expression on the cells and the off-targets of RNA-based therapies. We are currently conducting experiments with anti-RYR2 siRNA, further expanding the scope of our research.

References:

1. Martini, M., Dobrowolny, G., Aucello, M. & Musarò, A. Postmitotic Expression of SOD1G93A Gene Affects the Identity of Myogenic Cells and Inhibits Myoblasts Differentiation. Mediators Inflamm 2015, (2015).
2. Wang, J. et al. Targeting ANGPTL3 by GalNAc-conjugated siRNA ANGsiR10 lowers blood lipids with long-lasting and potent efficacy in mice and monkeys. Mol Ther Nucleic Acids 31, 68–77 (2023).
3. Enrico Mario Alessandro Fassi Andrea Citarella, M. A. E. G. M. L. L. C. L. A. S. & Grazioso, G. PCSK9 inhibitors: a patent review 2018-2023. Expert Opin Ther Pat 0, 1–17 (2024).

Charting the immune landscape and communication axes in metastatic colorectal cancer with spatial transcriptomics

Mattia Toninelli[*1,2], *Stefano Abbiati1*, *Lorenzo Drufuca*[1,3], *Arianna Di Napoli*[4], *Claudio Tripodo5*, *Grazisa Rossetti*[1] *and Massimiliano Pagani*[1,3]

[*]*Presenting Author*

[1]IFOM-ETS, Milan, Italy
[2]SEMM – European School of Molecular Medicine
[3]Università degli Studi di Milano, Milan, Italy
[4]Università di Roma "La Sapienza", Rome, Italy
[5]Università degli Studi di Palermo, Palermo, Italy

Colorectal cancer (CRC) is among malignancies with highest incidence and recurrence rates, meanwhile displaying inconsistent response to immunotherapy. Despite the wealth of studies carried out in CRC correlating the degree and type of immune infiltration with invasive potential, spatial relationships governing the interplay between immune and tumor cells in the CRC tumor microenvironment (TME) have been thus far explored at coarse-grained scale.

In this work, we employ spatial profiling technologies to investigate the cellular ecosystem of metastatic and non-metastatic CRC, providing a comprehensive picture of the spatial architecture of immune cell responses at play and elucidating their role in the evolution of an invasive cancer phenotype. In an effort to identify links between spatial determinants of the CRC ecosystem and the process of tumor dissemination, we assembled an initial retrospective cohort of primary tumor FFPE samples from CRC patients with either overt metastatic spread or without detectable metastases after surgery. Samples were processed for targeted spatial transcriptomics with the latest version of the CosMx™ SMI platform, profiling the expression of ~6,000 genes *in situ* resulting in a comprehensive collection of more than 200,000 single-cell transcriptomes coupled to their spatial location. Integrating spatial data with a curated dataset of existing large-scale scRNA-seq CRC cohorts facilitated cell type annotation and the identification of malignant cell states. Metastatic CRC displayed spatially-coordinated expression of gene programs linked to cellular invasion and inflammation, including markers of metastatization potential, with distinct usage of ligand-receptor interactions. By exploiting spatial information, we further linked these transcriptional states to communication events involving surrounding immune and stromal cell subtypes in the TME of invasive CRC.

We envision that further integration of this data across protein and imaging modalities could help gain deeper knowledge into actionable molecular mechanisms eliciting a pro-metastatic phenotype in primary CRC.

Formulation and characterization of fluorescent lipid nanoparticles: an optimized benchmark for the standardization of cellular uptake *in vitro* assay

Rossi R.[1], Perteghella S.[1], Buonocore D.[2], Sorrenti M.[1], Catenacci L.[1], Peviani M.[2], Bonferoni M.C.[1]*

*Sara Perteghella *Presenting author: email: sara.perteghella@unipv.it, tel +390382987165*

[1]Department of Drug Sciences, University of Pavia, Viale Taramelli, 12 - 27100 Pavia (PV), Italy
[2]Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Via Ferrata, 9 - 27100 Pavia (PV), Italy

Lipid nanoparticles (LNPs) are a well-known optimal drug delivery systems to encapsulate oligonucleotides and others bioactive compounds.
We developed and characterized LNPs based on two main lipids. Dimethyldioctadecylammonium bromide (DDA) and 2-hexyl-decanoic acid, 1,1'-[[(4-hydroxybutyl)imino] di-6,1-hexanediyl] ester (ALC-0315) as cationic and ionizable lipids, respectively. DDA was chosen to obtain cationic drug delivery systems considered as positive control; DDA-LNPs could be able to better interact with the plasmatic membrane of target cells but, on the other hand, many researchers demonstrated that positive charge could be responsible for cytotoxicity of LNPs. ALC-0315 is an ionizable lipid that is yet successfully used for RNA delivery in commercial formulations and is assumed as better tolerated standard.

We optimized the production process of LNPs by a microfluidic technique, an approach widely used to improve the process yield, the encapsulation efficiency, and the industrial scale up; in particular, a Design of Experiment (DoE) model was used to define the better production conditions in terms of lipid solution concentration, total flow rate and flow rate ratio between organic and aqueous solutions, with the aim of obtaining DDA and ALC LNPs with comparable dimensions.

For better tracking of the LNPs after interaction with the cells, fluorescent cholesterols (25-NBD and DHE) were added to the final formulations. This work should make available standard models of drug delivery systems for subsequent *in vitro* cellular assays. The first evaluation of these systems involved internalization tests in human microglial clone 3 HMC3 (HMC3-ATCC® CRL-3304™) cell line. In particular, we optimised a well-scann detection method, monitoring the fluorescence NPs probe with a high-performance microplate reader (SpectraMax iD5, Molecular Devices) that made possible to monitor the NPs internalisation at different time points. The obtained results, important for the immunotoxicological profile of NA-loaded NPs, showed that ionizable ALC-0315 lipid seemed to be able to enter cells such as cationic DDA lipid.

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References:

Roces CB, Lou G, Jain N, Abraham S, Thomas A, Halbert GW, Perrie Y. Manufacturing Considerations for the Development of Lipid Nanoparticles Using Microfluidics. Pharmaceutics 2020, 12, 1095. doi: 10.3390/pharmaceutics12111095
Kulkarni JA, Darjuan MM, Mercer JE, Chen S, van der Meel R, Thewalt JL, Tam YYC, Cullis PR. On the Formation and Morphology of Lipid Nanoparticles Containing Ionizable Cationic Lipids and siRNA. ACS Nano. 2018, 12, 4787-4795. doi: 10.1021/acsnano.8b01516.

Fine-tuning of efficient *in vitro* methods for evaluating the CNS-specific immunotoxicity of NA-loaded Nanoparticles

Buonocore D.[1],, Bonferoni M.C.[2] , Perteghella S.[2] , Rossi R.[2] , Cabras P[1], Spatafora M.G.[1], Gazzano A.[1], Peviani M.[1]*

**Presenting author, e-mail: daniela.buonocore@unipv.it phone: (+39)0382986390*

[1]Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Via Ferrata, 9 - 27100 Pavia (PV), Italy
[2]Department of Drug Sciences, University of Pavia, Viale Taramelli, 12 - 27100 Pavia (PV), Italy

In this work, based on well defined Standard Operating Procedures, we validated reliable assays to assess *in vitro* the tissue-specific immunotoxicological properties of NA-loaded NPs for gene delivery, with a focus on the Central Nervous System (CNS).
The experiments were performed using standard RNA delivery systems based on lipid nanoparticles (LNPs) containing cationic-DDA or ionizable-ALC-0315 lipids coupled with a fluorescent NBD-labeled Cholesterol. To model CNS-specific cell populations, we relied on different human cell lines used as platform to perform preliminary drug testing assessments and useful for potential future HTS/HCS campaigns. Firstly, we optimized the culture protocols for human SH-SY5Y neuroblast-like cells to achieve efficient and reproducible differentiation into mature neurons. This approach was designed to make subsequent applications possible, such as conditioning assays in co-culture systems together with HMC3 microglia-like cells, M03.13 glial (oligodendrocytic) hybrid cell line; CCF-STTG1 astrocytic-like cells.

Then, we developed an approach to evaluate at the same time NPs internalization and cells viability by measuring the fluorescence of NBD-labelled LNPs alongisde cell-permeable nuclear stains with a high-performance multi-mode microplate-based spectrofluorimeter by an optimised well-scan detection method. The results showed that both LNPs were able to enter cells within 24 hours of exposure and the cationinc DDA-based LNPs were more toxic, as expected. LNPs were then loaded with a GFP-expressing NA and used on HMC3 cell line. We leveraged multi-parametric flow cytometry to confirm and quantify NA (GFP) cargos expression, ROS production, measured by the CellROX™ fluorescent probe, and cellular viability, through Annexin-APC/ 7-amino-actinomycin D (7-AAD) staining.

Cells expressing fluorescent reporters induced upon activation of pro-inflammatory pathways (e.g. NFkB) could be instrumental to monitor the effects induced by exposure to NA-NPs or their components - carrier, cargo and/or biodegradation products. We selected the HMC3 cell line and stimulated it with LPS or IFNy as positive control. RT-qPCR analysis confirmed the upregulation of CCL2, CXCL10, IL1B and IL-6 gene expression. Increased release of the corresponding cytokines was detected also in the cell culture supernatant, analyzed by flow cytometry through a 13-plex multiparametric LEGENDplex™ (BioLegend®) assay suitable.y.

References:

Freitas NL, Gomes YCP, Souza FDS, Torres RC, Echevarria-Lima J, Leite ACCB, Lima MASD, Araújo AQC, Silva MTT, Espíndola OM. Lessons from the Cerebrospinal Fluid Analysis of HTLV-1-Infected Individuals: Biomarkers of Inflammation for HAM/TSP Development. Viruses 2022;14(10):2146, doi: 10.3390/v14102146.
Lepiarz I, Olajide O. The human microglia (HMC-3) as a cellular model of neuroinflammation. IBRO Reports 2019; 6 (Supplement), S92. ISSN 2451-8301, doi.org/10.1016/j.ibror.2019.07.299.
Shipley MM, Mangold CA, Szpara ML. Differentiation of the SH-SY5Y Human Neuroblastoma Cell Line. J Vis Exp 2016; 108, e53193, doi:10.3791/53193.
Xie H, Hu L, Li G. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chinese Medical Journal 2010; 123 (8), 1086–1092, doi:10.3760/cma.j.issn.0366-6999.2010.08.021.

In vivo platform to investigate predictive biomarkers of the CNS-specific immune-toxicological effects induced by NA-loaded NPs.

Peviani M.[1], Gazzano A.[1], Cabras P.[1], Spatafora M.G.[1], Travelli C.[2], Lanni C.[2], Bonferoni M.C.[2], Buonocore D.[1]*

**Presenting author, senior author.*

[1]Department of Drug Sciences, University of Pavia, Viale Taramelli, 12 - 27100 Pavia (PV), Italy
[2]Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Via Ferrata, 9 - 27100 Pavia (PV), Italy

The Central Nervous System (CNS) could be exposed to NPs or their byproducts as the result of direct-targeting, aimed at treating specific CNS-related disorders, or due to off-target biodistribution after administrations meant to treat non-CNS pathologies. The risks associated with potential CNS toxicity and the difficulty of sampling the brain prompt for finding reliable and robust predictive non-invasive biomarkers of toxicity and immune-system engagement. In this project, we implemented a preclinical platform to identify and validate biomarkers (measurable in the CSF or blood) that could be leveraged to define surrogate endpoints whenever the exposure to NA-loaded NPs or their byproducts affects the neuronal microenvironment.

To do this, we conducted the experiments in rodent models widely used for pharmacological/toxicological studies: i) the C57BL/6 mouse strain; ii) Sprague Dawley rats, employed to allow the longitudinal investigation of biomarkers in the CSF and/or blood withdrawn from live animals.

We adopted a method recently developed by our group to identify/isolate, through flow cytometry, the neurons, endothelial cells, astrocytes, oligodendrocytes, microglia, macrophages and infiltrating lymphocytes from the brain and spinal cord [1]. Through this approach, applied to transgenic animal models of Amyotrophic Lateral Sclerosis (exploited here as model of neurodegeneration and neuroinflammation) as well as to wild type animals treated with rhodamine-labeled NPs injected in the CSF through different routes of administration (as prototypical example of exposure to biodegradable NPs) we demonstrated that it is possible to monitor at the same time: i) the changes of the immunophenotypic profile of CNS-resident as well as CNS-infiltrating immune system cells upon direct exposure to NA-loaded NPs; ii) ROS production, changes in cell viability and upregulation of TSPO, a PET-traceable marker of microglia/macrophage activation [2], induced by NPs internalization; iii) the correlation of these changes with standard histological hallmarks of neurodegeneration and neuroinflammation analyzed in formalin/PFA fixed tissues. In parallel, we demonstrated the feasibility of isolating CSF and blood (and their cellular components) from the same animal to allow single-cell antigen
and RNAseq profiling.

So far, we have identified novel candidate biomarkers of neuroinflammation and we have established collaborations to fuel their clinical translatability.

References:

1. Molina Estevez, F. J., Mathews, T. D., Biffi, A., & Peviani, M. (2019). Simultaneous Flow Cytometric Characterization of Multiple Cell Types Retrieved from Mouse Brain/Spinal Cord Through Different Homogenization Methods. Journal of visualized experiments : JoVE, (153), 10.3791/60335. https://doi.org/10.3791/60335

2. Auriemma, R., Sponchioni, M., Capasso Palmiero, U., Rossino, G., Rossetti, A., Marsala, A., Collina, S., Sacchetti, A., Moscatelli, D., & Peviani, M. (2021). Synthesis and Characterization of a "Clickable" PBR28 TSPO-Selective Ligand Derivative Suitable for the Functionalization of Biodegradable Polymer Nanoparticles. Nanomaterials (Basel, Switzerland), 11(7), 1693. https://doi.org/10.3390/nano11071693

Xenograft models and fluorescence-based *in-vivo* assays: tools for understanding the efficiency and toxicity of potential novel drugs

Alberto DIANA, Luca DEL GIACCO(*)

**Presenting author.*

Department of BioSciences, University of Milan – Milan, Italy

Background

In drug discovery, zebrafish represent a versatile and reliable vertebrate model to reveal toxicity, as well as potential therapeutic properties of compounds, thanks to its unique possibility to establish in-vivo/low cost/high throughput/unbiased drug screening platforms[1]. Additionally, zebrafish embryo/larva xenotransplantation of cancer cells has the potential to be adopted as a preclinical tool to identify therapies for further evaluation[2]. Here, we show some representative examples of the employment of the zebrafish larva model to investigate cancer cells behavior in a whole, living organism, as well as to identify anticancer compounds and disclose potential synergistic effect. Also, being drug-induced kidney injury a serious safety issue in drug development[3], we propose a fluorescence platform to unveil drug toxicity and point-out drug ability to rescue chemical-induced kidney damage in search for novel treatments.

Results

1a. To test the metastasis potential of the SUM159PT and BT549 claudinlow breast cancer cells expressing different splicing isoforms of the NF-YA transcription factor, they were microinjected into the circulation of zebrafish embryos (Fig. 1). The clones KO for the long isoform (YAl-KO) showed less invasion potential suggesting that NF-YAl is involved in the metastatic process[4].

1b. MDA-MB-231 breast adenocarcinoma cells were grafted into the circulation of zebrafish embryos that were then exposed to different compounds or combinations (Fig. 2). The STM2457 + cisplatin and STM2457 + olaparib treatments exhibited a mutually reinforcing trend in comparison to each chemical administered alone, leading to a substantial reduction in the number and size of micrometastases[5].

2. Clearance of a FITC-labeled high-molecular weight dextran was used to measure glomerular filtration barrier permeability: Adriamycin (ADR)-exposed larvae (a model of podocyte damage) exhibited significant dextran loss, as shown by lower fluorescence intensity, indicative of failure of the renal function. Any given compound can be administered instead of ADR and its toxicity can be evaluated with this assay. The ADR-induced phenotype was rescued by the administration of the drug which revealed its efficacy in ameliorating kidney failure[under revision].

Conclusion

The methodologies here described are tailored to in-vivo assess the effectiveness of candidate drugs capable of improving the disease outcomes and for testing potential toxic effects.

References:

1. Cassar S, Adatto I, Freeman JL, Gamse JT, Iturria I, Lawrence C, Muriana A, Peterson RT, Van Cruchten S, Zon LI. Use of Zebrafish in Drug Discovery Toxicology. Chem Res Toxicol. 2020 Jan 21;33(1):95-118. doi: 10.1021/acs.chemrestox.9b00335. Epub 2019 Nov 16. PMID: 31625720; PMCID: PMC7162671.

2. Veinotte CJ, Dellaire G, Berman JN. Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. Dis Model Mech. 2014 Jul;7(7):745-54. doi: 10.1242/dmm.015784. PMID: 24973744; PMCID: PMC4073264.

3. Kato Y, Tonomura Y, Hanafusa H, Nishimura K, Fukushima T, Ueno M. Adult Zebrafish Model for Screening Drug-Induced Kidney Injury. Toxicol Sci. 2020 Apr 1;174(2):241-253. doi: 10.1093/toxsci/kfaa009. PMID: 32040193.

4. Londero M, Gallo A, Cattaneo C, Ghilardi A, Ronzio M, Del Giacco L, Mantovani R, Dolfini D. NF-YAl drives EMT in Claudinlow tumours. Cell Death Dis. 2023 Jan 28;14(1):65. doi: 10.1038/s41419-023-05591-9. PMID: 36707502; PMCID: PMC9883497.

5. Cesaro B, Iaiza A, Piscopo F, Tarullo M, Cesari E, Rotili D, Mai A, Diana A, Londero M, Del Giacco L, Masetti R, Di Leone A, Naro C, Masciarelli S, Fontemaggi G, Sette C, Fazi F, Fatica A. Enhancing sensitivity of triple-negative breast cancer to DNA-damaging therapy through chemical inhibition of the m6A methyltransferase METTL3. Cancer Commun (Lond). 2024 Feb;44(2):282-286. doi: 10.1002/cac2.12509. Epub 2023 Dec 15. PMID: 38102821; PMCID: PMC10876187.

WP3 - RNA Toxicology and Immunoreactivity of RNA drugs

In vivo identification of the possible immunotoxic potential of RNA drugs focusing on four specific stress pathways

Valeria Bettinsoli[1,2], Gloria Melzi[1], Sofia Pantaleoni[1], Irene Marchese[1], Marina Marinovich[1], Emanuela Corsini[1]

**Presenting author, senior author.*

[1]Department of Pharmacological and Biomolecular Science (DiSFeB) "Rodolfo Paoletti", Università degli Studi di Milano, Via Giuseppe Balzaretti 9, 20133, Milan, Italy
[2]Department of Pharmacy, Università degli Studi di Napoli Federico II, Via Domenico Montesano 49, 80131, Napoli, Italy

In the last few decades, the treatments of human disorders are focused on personalizing therapies. This evolution is unfolding due to the identification of the molecular target involved in the development of diseases with the consequent development of high specificity drugs as treatments disorder. The great power and flexibility of nucleic acids candidate these molecules in medicine with a wide range of possible applications, and the possible adverse eYects must be taken into consideration. This research aims to establish a platform by using new approach methods (NAMs) to evaluate the potential toxicity of RNA drugs, in view of their potential immunogenicity and oY-target eYects. To reach this goal, human peripheral blood mononuclear cells (PBMCs), obtained from buYy coats from healthy male and female donors were used and four toxicity pathways, known to be activated by several chemicals, were selected: I) oxidative stress, II) endoplasmic reticulum stress III) mitochondrial stress, and IV) autophagy. For each pathway, a specific positive control was used: tert-Butyl hydroperoxide (tBHP) for the oxidative stress, thapsigargin (TG) for endoplasmic reticulum stress, rotenone (Rot) for mitochondrial stress, and rapamycin (Rapa) for autophagy. siRNA RyR2 is the first RNA based drug tested, it acts through the silencing of the mutate gene target RyR2 that cause dominant catecholaminergic polymorphic ventricular tachycardia (CPVT); siRNA scramble is the negative control. Cell viability for positive controls, siRNA RyR2 and siRNA scramble was assessed through lactate dehydrogenase release and apoptosis. Furthermore RNA-seq was performed on the positive controls to generate information on target genes to be selected as representative of the main stress pathways and indicators of potential toxicity. The results obtained on the experiments performed on the positive controls allowed the selection of their maximum concentration that determines cell viability higher then 80%: 250 nM for tBHP, 2 µM for TG, 50 µM for Rot and Rapa. The experiments conducted on siRNA (target and scramble) allow the selection of the maximum viable concentration: 400 nM. The next steps will be focused on the study of other RNA drugs already selected: Morpholino ASO C9, mRNA vaccine anti-Rida, mRNA vaccine anti-Sars-Cov-2. In particular, through the Real-Time PCR, the modulation of the selected genes derived from the RNA-seq analysis will be analyzed in PBMCs exposed to the selected RNA drugs. The research has the potential to develop an *in vitro* method in the screening of safer RNA drugs allowing the selection of more promising drug candidates, thereby enhancing drug safety.

Keywords: RNA drugs; *in vitro*, safety assessments, toxicity pathways, NAMs.

Exploring ER Stress and UPR Pathway Activation as Biomarkers for Liver Toxicity in Liver-Targeted Gene Therapy

Laura Rigon[1,#], Carmine Gambino[1], Salvatore Piano[1], Paolo Angeli[1]

**Presenting author, senior author.*

[1]Unit of Internal Medicine and Hepatology (UIMH), Department of Medicine (DIMED), University of Padua, Italy

Liver-targeted gene therapy has shown great promise for treating various genetic diseases, including Spinal Muscular Atrophy (SMA) and haemophilia, by delivering genetic material to hepatocytes to produce therapeutic proteins [1,2]. However, the potential for liver toxicity associated with gene therapy remains a significant concern, and the activation of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) pathway are key factors to consider when evaluating safety and efficacy [3]. Indeed, this pathway has been implicated in other forms of liver toxicity, such as drug-induced liver injury [4].
Our study aims to determine whether liver-targeted gene therapy induces liver toxicity through the activation of ER stress and the UPR pathway. We aim to identify biomarkers associated with these stress responses to better understand and mitigate potential adverse effects.

To achieve this, we first developed Western blot analysis to evaluate the expression of key proteins involved in ER stress and UPR, and we are complementing this with RT-qPCR validation. After receiving regulatory approval in January and the necessary viral vectors in April 2024, we initiated our *in vitro* experiments. We are using HepG2 cells, a hepatocellular carcinoma-derived epithelial-like cell line, treated with adeno-associated viral vectors AAV8-CMV-mCherry for ubiquitous expression and AAV8-TBG-mCherry for hepatic targeting. We are assessing the activation of ER stress and UPR markers, such as BiP/GRP78, PERK, ATF6, IRE1a, CHOP, ATF4, and XBP1. Preliminary results indicate the induction of ER stress and alterations in all three branches of the UPR pathway, evidenced by the overexpression of various markers.

To confirm the results we will obtain from the *in vitro* study, we will test the identified molecules on wild-type mice treated with the same AAVs, providing valuable insights into the *in vivo* response to liver-targeted gene therapy and validating the potential biomarkers. Finally, the study will focus on paediatric patients with SMA undergoing liver-targeted gene therapy with Zolgensma. We will assess the biomarkers both before therapy and during follow-up to comprehensively understand the impact of gene therapy on liver health in these patients.

Ultimately, this study aims to offer a holistic understanding of the potential liver toxicity associated with liver-targeted gene therapy. By identifying reliable biomarkers, we will be able to improve patient monitoring and optimize therapeutic protocols, thereby enhancing clinical outcomes for patients undergoing liver-targeted gene therapy.

References:

1. Maestro S, Weber ND, Zabaleta N, Aldabe R, Gonzalez-Aseguinolaza G. Novel vectors and approaches for gene therapy in liver diseases. JHEP Rep. 2021 Apr 30;3(4):100300. doi: 10.1016/j.jhepr.2021.100300. PMID: 34159305; PMCID: PMC8203845.
2. Moscoso CG, Steer CJ. Liver targeted gene therapy: Insights into emerging therapies. Drug Discov Today Technol. 2019 Dec;34:9-19. doi: 10.1016/j.ddtec.2020.11.001. Epub 2020 Nov 22. PMID: 33357766.
3. Delire B, De Martin E, Meunier L, Larrey D, Horsmans Y. Immunotherapy and Gene Therapy: New Challenges in the Diagnosis and Management of Drug-Induced Liver Injury. Front Pharmacol. 2022 Jan 19;12:786174. doi: 10.3389/fphar.2021.786174. PMID: 35126126; PMCID: PMC8807695.
4. Pu S, Pan Y, Zhang Q, You T, Yue T, Zhang Y, Wang M. Endoplasmic Reticulum Stress and Mitochondrial Stress in Drug-Induced Liver Injury. Molecules. 2023 Apr 2;28(7):3160. doi: 10.3390/molecules28073160. PMID: 37049925; PMCID: PMC10095764.

WP3 - RNA Toxicology and Immunoreactivity of RNA drugs

In vitro characterization of the effect of nucleic acid therapeutics on immune cells

Letizia Venturini[1], Clara Di Vito[1,2], Lien Tran[3], Donatella Lattuada[1], Chiara Sfogliarini[3], Rossana Bongianino[4], Luca Grisorio[4], Teresa M. Formica[4], Elena Monica Borroni[1,2], Daniele Catalucci[2], Silvia G. Priori[4], Massimo Locati[1,2], Elisabetta Vegeto[3], Domenico Mavilio[1,2].

[1]Department of Medical Biotechnologies and Translational Medicine (BioMeTra), University of Milan, Milan, Italy;
[2]IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy;
[3]Department of Pharmaceutical Sciences, University of Milan, Milan, Italy;
[4]Molecular Cardiology, IRCCS Istituti Clinici Scientifici Maugeri, Pavia, Italy.

Nucleic acid (NA) therapeutics are synthetic or biologically-derived nucleic acids able to modulate gene expression, correct genetic mutations, or trigger immune responses. Among the most used in therapeutics, Antisense Oligonucleotides (ASOs), Small Interfering RNAs (siRNAs), MicroRNAs (miRNAs), Messenger RNAs (mRNAs) are numbered. NA-based drugs have been proposed as drugs and vaccines, with ongoing research aiming to broaden their use, improve delivery, and enhance safety. In the context of the PNRR-National Center for Gene Therapy and Drugs based on RNA, we focused on the optimization of assays aiming at investigate the biological and off-target effects of the NA-based molecules on immune cells.

To this aim, we set up several *in vitro* assays on peripheral blood mononuclear cells (PBMCs) and whole blood to assess by spectral flow cytometry: the phenotype, subsets frequencies, proliferation and survival of different immune cell populations, including T and B lymphocytes, natural killer cells, dendritic cells, monocytes, neutrophils, and macrophages,. We also optimized an assay evaluating calcium fluxes to study macrophage function and activation.

By using these assays, we recently test the siRNA U10 for the Ryanodine receptor 2 (*Ryr2*) gene, developed by Prof Priori's group, an NA-based drug aimed at treating patients affected by the dominant catecholaminergic polymorphic ventricular tachycardia caused by a specific *Ryr2* gene mutation. To improve *in vivo* its delivery and uptake, the siRNA U10 has been loaded in calcium phosphate nanoparticles (CaP). PBMCs have been cultured for 24-72 hours with different concentrations of siRNA-Cy5 (25-200 pmol/ml) loaded in CaPs. A CaP-siRNA scamble and CaP alone have been used as controls.

To evaluate the CaP-siRNA uptake, fluorescent (Cy5) siRNAs have also been used. Preliminary results demonstrate that siRNA-loaded CaPs do not affect cell survival and proliferation of both lymphocytes and monocytes at all siRNA concentration tested.
These preliminary data suggest that this therapeutic strategy should not have an immunomodulatory effect. To confirm this, future studies are ongoing to define the siRNA intracellular fate and possible changes in immune cell phenotype and subset frequencies.

Molecular mechanisms and gender differences in the immune reactivity to nucleic acid drugs

Hong Tran[*1], C. Sfogliarini[1], M.C. Cesta[2], F. Cattani[2], M. Allegretti[2], E. Borroni[3], M. Locati[3], E. Vegeto[1]*

**Presenting author: hong.tran@unimi.it*

[1]Department of Pharmaceutical Sciences, University of Milan, Milan, Italy
[2]Dompé Farmaceutici S.p.A., L'Aquila, Italy
[3]IRCCS Humanitas Research Hospital and Department of Medical Biotechnologies and Translational Medicine, University of Milan, Milan, Italy

Context: RNA therapies have emerged as promising tools for manipulating gene expression or producing therapeutic proteins, making them suitable for treating a wide range of pathologies with established genetic targets. However, the therapeutic use of RNA faces significant challenges, including poor pharmacological properties, challenging intracellular delivery and immune-related toxicity. Macrophages, key players in innate immunity, perform various effector and protective functions under the influence of physio-pathological signals.

The mechanisms underlying the immune response initiated by RNA molecules in macrophages remain unclear. It is known that endosomal toll-like receptors 7/8 (TLR7/8) detect RNA molecules and subsequently activate the immune response in macrophages, although the processes of RNA recognition by cellular sensors and the activation mechanisms of these sensors are not fully understood. Furthermore, there are evidence suggesting that sex differences in immune responses can influence disease outcomes and affect drug and vaccine efficacy. This study aims to investigate the macrophage response to nucleic acid molecules and sex dimorphism in TLR7/8 signaling. Objective: We first optimized the delivery systems for transferring exogenous nucleic acids into macrophages while avoiding cell toxicity. Thereafter, we aimed at defining macrophage activation and sexual dimorphism in TLR7/8 signaling pathway in response to the macrophage uptake of siRNA and mRNA molecules. Methods: Human and mouse primary macrophages were transfected with exogenous siRNAs and mRNAs. Analyses of cell viability, transfection efficiency and pro-inflammatory cytokine expression were performed. Results: We optimized an efficient delivery system of siRNA and mRNA molecules that do not induce immunotoxicity in macrophages, which instead showed a similar survival rate compared to non-treated cells up to 48h post-transfection.

Our preliminary data also indicate that the expression of the proinflammatory cytokine IL-6 is strongly induced shortly after transfection by exogenous nucleic acids. In parallel, we observed sexual differences in IL-6 induction by the TLR7 agonist (R848), which were reconciled with the sex-specific expression and activity of transcription factors, which will be discussed. Conclusions: Altogether, our findings indicate that mRNA and siRNA molecules can be studied *in vitro* to define the molecular mechanisms and the sexual dimorphism of immune toxicity. Our preliminary data also underscore the potential role of TLR7 signaling in the sexual dimorphism of the macrophage response to exogenous RNA therapies.

These results hold significant implications for drug development studies.

WP3 - RNA Toxicology and Immunoreactivity of RNA drugs

Comprehensive *in vitro* evaluation of mRNA-drugs off-target toxicity: mechanistic insights into autophagy, endoplasmic reticulum, oxidative, and mitochondrial stress

Gloria Melzi[1], *Valeria Bettinsoli*[1,2], *Valentina Galbiati*[1], *Marina Marinovich*[1], *Emanuela Corsini*[1]

[1]Department of Pharmacological and Biomolecular Science (DiSFeB) "Rodolfo Paoletti", Università degli Studi di Milano, Via Balzaretti 9, 20133, Milan, Italy.
[2]Department of Pharmacy, Università degli Studi di Napoli Federico II, Naples, Italy.

Nucleic acid-based therapeutics and vaccines are emerging as a promising new generation of drugs. Alongside their efficacy, it is crucial to evaluate their potential adverse effects, particularly regarding organ toxicity and immunotoxicity. This research aimed to develop an *in vitro* toxicological approach to assess possible off-target adverse effects of new RNA-drugs, developed within the *Centro Nazionale di Ricerca "Sviluppo di terapia genica e farmaci con tecnologia a RNA".*

The proposed approach involves evaluating the ability of selected mRNA-drugs to trigger specific toxicity pathways. These pathways offer a mechanistic understanding of how exposure to particular substances or stressors can result in toxicity. Toxicity pathways evaluation is essential in toxicological studies and risk assessments, helping scientists and regulators identify key events and biomarkers associated with adverse effects.

The toxicity pathways investigated included oxidative stress, endoplasmic reticulum stress, mitochondrial stress, and autophagy. Compounds were selected as positive controls for each pathway: tert-Butyl Hydroperoxide (tBHP) for oxidative stress, Thapsigargin (TG) for endoplasmic reticulum stress, Rotenone (Rot) for mitochondrial stress, and Rapamycin (Rapa) for autophagy. The toxicological evaluation was conducted on peripheral blood mononuclear cells (PBMC) obtained from anonymous buffy coats of five male and female healthy donors, sourced from Niguarda Hospital (Milan, Italy). PBMC were treated for 24 hours with the RNA-drugs and the four positive controls. Cytotoxicity was previously determined by lactate dehydrogenase release and apoptosis analysis. ELISA was performed to detect any secretion of Interleukin-8, Interleukin-6, Tumour Necrosis Factor α, and Interferon γ, while RT-PCR was performed to analyze the possible modulation of the main genes involved in the selected toxicity pathways following the treatment of PBMC with the RNA-drugs. Moreover, alkaline comet assay was conducted to exclude a possible genotoxic effect.

The results of these toxicity pathway analyses will be presented. Our approach offers the opportunity to screen new RNA-drugs for potential toxicity, assess possible gender effects and variability in responses, and identify markers that may be translatable to clinical settings.

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Validation of automated immunohistochemistry for markers of inflammatory cells in murine tissues: preliminary results

Andrea Cappelleri[1,2], Simone Canesi[1,2], Laura Sala[1,2], Patrizia Cristofori[3], Camilla Recordati[1,2]

[1]Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi – Italy.
[2]Mouse and Animal Pathology Laboratory, UniMi Foundation, Milan – Italy.
[3]Indipendent consultant

One of the main concerns regarding RNA-based therapy, is the triggering of undesired proinflammatory responses by the compound itself, or by the delivery vehicles.2,3 Our ability to predict any adverse effect of RNA-based therapeutics, relies on the possibility to study the adverse effects of the drugs in adequate preclinical animal models, through standardized and reproducible protocols. For this reason, our laboratory is currently involved in the process of validation of an automated immunohistochemical staining for markers of inflammatory cells in murine tissues.

We selected formalin-fixed paraffin embedded murine healthy lymphoid organs (submandibular lymph node, mesenteric lymph node, spleen, and thymus), for which pre-analytical variables (e.g. handling, fixation, storage conditions) were known. The following immunohistochemical markers were selected for validation: CD3 (T-cells), B220 (B-cells), Iba-1 (monocytes/macrophages), Ly-6G (neutrophils). The stainings were performed using the Thermo Scientific™ Autostainer 480S. The following parameters were assessed: precision (intra/inter-assay, and intra-instrument), sensitivity and specificity. The final acceptance criteria of the validation were based on the confirmed coefficient of variation (CV). More specifically, based on the current literature,[1] a value of CV ≤ 30% was considered acceptable for precision parameters. For acceptance of sensibility and specificity, all samples needed to be positive in the expected subcellular (e.g. for B220, membranous staining) and tissue (e.g. for B220 in lymph nodes, limited to follicles) locations. For each organ, the CV was calculated from the value of positive area normalized to total area.

Each organ was photographed by means of a Leica DM2500 microscope equipped with a Leica DFC310 FX camera, and the area of positivity normalized to total area was compared across 3 different runs of staining by means of QuPath software v0.5.1. The best threshold was identified for each organ and applied across different runs. Until now, we collected results for CD3 and B220.
None of the CV values exceeded 30%, which indicates acceptable level of consistency and reproducibility across runs. Further staining will be performed for the remaining markers. In the future, to assess the stability of the assay, we plan to test the same markers on unstained tissue sections stored for a relative long period of time after sectioning (e.g. 30 days).

References:
1. Baker GM, Murphy T, Block T, Nguyen D, Lynch FJ. Development and validation of an immunohistochemistry assay to assess glucocorticoid receptor expression for clinical trials of mifepristone in breast cancer. Cancer Manag Res. 2015;361–368.
2. Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. Chem Biol. 2012;19:60–71.
3. Sparmann A, Vogel J. RNA -based medicine: from molecular mechanisms to therapy. EMBO J. 2023;42:1–20.

WP3 - RNA Toxicology and Immunoreactivity of RNA drugs

Oxidative damage assessment in a static and dynamic lung organoid model: setting a platform for drug toxicity and safety evaluation.

Raquel Moll-Diaz[1,2], Paola Bisaccia[1,2], Luca Bonadies[2], Eugenio Baraldi[2], Maurizio Muraca[1,2], Michela Pozzobon[1,2]*

**Presenting author*

[1]Stem Cells and Regenerative Lab, Institute of Paediatric Research Città della Speranza, Padova, Italy.
[2]Department of Women and Children Health, University of Padova, Italy.

Introduction

Over the past years, 3D organoids have become an interesting *in vitro* model for the mimicking of the organ architecture and function, presenting applications ranging from disease modeling, toxicological studies to drug screening and safety evaluation [1].
Oxidative stress can be a common feature in several pulmonary pathologies, including COVID-19 [2].
In this study we developed a lung organoid model to mimic an oxidative damage in pulmonary diseases. The final aim of this work will be the creation of a platform to study potential toxicological effects of emerging RNA-based drug candidates in the lung environment.

Methods

Generation of mouse lung organoids (mLOs) from adult stem cells and use of the model to induce a lung oxidative injury by Rotenone and hydrogen peroxide (H_2O_2) administration. The 3D model was tested in a static and dynamic platform. As proof of principle, to test the model functionality, natural nanoparticles called extracellular vesicles (EVs) full of miRNA, were administered as a damage recovery tool. Immunofluorescence, gene expression analysis and viability assays were performed. Cellular death and proliferation were also assessed in the organoid cultures.

Results

Stemness markers like SFTPC, CC10 and P63 were present at early passages of the organoid cultures. This led to the generation of a mixed population of airway and alveolar organoids detected by the expression of MUC5AC, β-TUB IV, SFTPC and RAGE markers, at later timepoints of the protocol. Gene expression analysis confirmed it. On the other hand, the oxidative damage induced on mLOs was evidenced by the expression of 8-OXO-dG marker and a size reduction of organoids damaged on both oxidative conditions. Moreover, immunofluorescence analysis showed a rescue of the phenotype with EVs on the injured organoids.

However, qPCR analysis was only assessed under H_2O_2 condition and showed a modulation of *Tnf-α*, *Tgf-β*, *Sftpc*, *Muc5ac and Scgb1a1* genes, confirming the presence of a damage. Viability assays confirmed the vitality of the cultures in vitro and a reduced number of dead cells in cultures treated with EVs.

Conclusions and future perspectives

This ex vivo mLO model will provide new avenues to study several pulmonary diseases linked to oxidative stress insults and will serve as a safety evaluation platform to test emerging RNA-based drugs. In addition, ongoing co-culture systems with organoids will add value to the study due to the presence of the immunological compartment.
Therefore, a more accurate immuno-toxicological drug profiling will be provided.

References:

1. Clevers, H. (2016). Modeling development and disease with organoids. Cell, 165(7), 1586-1597.
2. de Las Heras, N., Martín Giménez, V. M., Ferder, L., Manucha, W., & Lahera, V. (2020). Implications of oxidative stress and potential role of mitochondrial dysfunction in COVID-19: therapeutic effects of vitamin D. Antioxidants, 9(9), 897.

ATMPs and hospital exemption: an appraisal of national regulatory frameworks through European academicians' point of view

Mudassir Khan[1] (Presenting author), Sara Manellari[2], Maria Cristina Vinci[3], Giulio Pompilio[3,4], Paola Minghetti[2]

[1]Pharmacological Biomolecular Sciences, Experimental and Clinical, University of Milan, Italy
[2]Department of Pharmaceutical Sciences, University of Milan, Italy
[3]Centro Cardiologico Monzino, IRCCS, Milan, Italy
[4]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy

Advanced therapy medicinal products (ATMPs) are medicines for human use that are based on genes, tissues or cells and they offer innovative opportunities for the treatment of diseases.
They are mainly ruled by Directive 2001/83/CE[1] and Regulation (EC) 1394/2007[2] and a marketing authorisation (MA) after a centralized procedure is mandatory for them[3].
To meet specific needs of patients, the European legislation also foresees the so-called "hospital exemption" for ATMPs, which are "prepared on a nonroutine basis and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner"[1].
In this context, the production, the management and the practical use of ATMPs on patients is ruled by each Member State (MS).

This study explores the regulatory frameworks of ATMPs and the practice of hospital exemption among different MSs. In this light, a 21-question survey was prepared by an internal expert panel and submitted to 97 European academicians working in the field of ATMP and hospital exemption, in 13 MSs to get insights into the production, regulatory requirements and clinical applications of ATMPs prepared under the hospital exemption rule in their MSs.

We obtained answers from 7 MSs, in most countries, ATMPs are commercialized with a MA. Regarding the hospital exemption rule, it has been pointed out that there is need for ameliorating clarity in regulatory guidelines, enhance collaboration between regulatory bodies and healthcare facilities and encourage early communication and transparency in related adverse events and their reporting mechanism. Moreover, most of respondents reported the presence of scientific labs in their universities specifically focused on research in somatic cell and gene therapies. However, to accelerate the translation of promising lab results into ATMPs administrable to patients, some improvements are needed, such as: reconsidering rules for cell-based product classification, streamline some expensive tests and extend criteria for ATMPs use to early access programs.

Certainly, the ATMP regulation is complex but, as explored by the survey, further improvements are needed, mostly for the hospital exemption rule. In particular, it is necessary a stronger collaboration between academic institutions and pharmaceutical industries and different strategies to accelerate the translation of ATMPs from bench to bedside.

References:
1. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use
2. Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004
3. Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency

WP4 - RNA Regulatory framework

Outcome research as a key in the regulatory process: Cost-Effectiveness Of Treatment with Advanced Therapy Medicinal Products

Marianna Serino[1,2], Sara Mucherino[1,2], Enrica Menditto[1,2], Valentina Orlando[1,2]

[1]CIRFF, Center of Pharmacoeconomics and Drug Utilization, University of Naples Federico II, Naples, ITALY
[2]Department of Pharmacy, University of Naples Federico II, Naples, ITALY

Introduction
Advanced Therapy Medicinal Products (ATMPs) are at the forefront of healthcare innovation, necessitating a thorough examination of their economic sustainability across different healthcare systems. The absence of a consolidated framework addressing their cost-effectiveness underscores the significance of this review, which aims to offer insights into the cost-effectiveness, cost-utility, and net monetary benefit of ATMPs.

Methods
Searches were conducted across PubMed, EMBASE, Web of Science, and ProQuest until December 18, 2023, utilizing a PICOS-based search strategy with MeSH Terms or Emtree and Boolean operators to refine parameters. Data extraction focused on country, treatment, comparators, willingness-to-pay (WTP) threshold, cost-effectiveness ratio, and affordability according to the WTP set.

Results
A systematic review identified 1,132 articles, resulting in 26 studies meeting inclusion criteria. Most economic evaluations of ATMPs were conducted in the US (29%; n=8). Among 17 studies on tisagenlecleucel, 64.7% indicated cost-effectiveness, primarily for DBLCL (60%) and ALL (75%), with a majority from the US. It was cost-effective under a $150,000/QALY threshold in the US and versus blinatumomab, but not in China for second/third-line treatment or in Ireland for pediatric/young adult ALL. Among 10 studies on axicabtagene ciloleucel, 54.4% found it cost-effective, particularly for DBLCL, being so in the US but not in China, with efficacy reliant on long-term outcomes. Other ATMPs like onasemnogene abeparvovec were generally cost- effective against nusinersen, excluding the Netherlands, and relmacabtagene autoleucel proved cost- effective for DLBCL in third-line settings with long-term benefits *(see Figure 1)*.

Conclusions
This review underscores the insufficient evidence on the cost-effectiveness of ATMPs across therapeutic indications. Yet, current data generally supports their cost-effectiveness in treating DLBCL and LLA. Tisagenlecleucel and axicabtagene appear promising for DLBCL treatment, particularly in second and third- line therapies relative to salvage chemotherapy, meeting a $150,000/QALY threshold from a US third-party payer's perspective. However, their cost-effectiveness diminishes below this threshold, although they still represent significant advancements in healthcare resource management for DLBCL patients.

Drug Utilization profiles of Advanced Therapy Medicinal Products: a Real World Evidence Study

Authors: Sara Mucherino[1,2], Marianna Serino[1,2], Maria Galdo[3], Enrica Menditto[1,2], Valentina Orlando[1,2]

Presenting author: Marianna Serino (email: marianna.serino@unina.it)

[1]CIRFF, Center of Pharmacoeconomics and Drug Utilization, University of Naples Federico II, Naples, ITALY
[2]Department of Pharmacy, University of Naples Federico II, Naples, ITALY
[3]UOSD Gestione Clinica del Farmaco - Responsible, Azienda Ospedaliera "Ospedali dei Colli", Naples, ITALY

Introduction

Advanced therapy medicinal products (ATMPs) represent the forefront of healthcare innovation. Despite the approval of the first ATMP in Italy in 2016, there is currently a lack of scientific evidence concerning the utilization patterns of ATMPs [1]. Study aim was to evaluate the drug utilization patterns among patients receiving ATMP treatments in Italy.

Methods

Retrospective study using data sourced from the Monitoring Registries of the Italian Medicine Agency, specifically the Drug Product Registry (DPR) containing information on dispensed treatments and clinical data for patients utilizing ATMPs in Campania Region (~6 million, 10% of the national population) and residents treated in a different Italian Region. Final cohort included individuals received at least one prescription for ATMP drugs in the Italian market between 2016 and 2023. We analyzed prescription patterns focusing on the index treatment, diagnoses, treatment interruptions, mortality rates and adverse events [2,3].

Results

Overall, 92 patients initiated ATMP treatments between 1 January 2016 and 1 September 2023. 21.6% received voretigene neparvovec, 25% onasemnogene abeparvovec, 22.8% tisagenlecleucel and 21.7% axicabtagene ciloleucel. The occurrence of adverse events was very low (1.1%), primarily associated with Autologous human corneal epithelial cells treatments. The overall mortality rate was 12%, affecting only two drugs: 28.6% tisagenlecleucel and 25.0% axicabtagene ciloleucel. Notably, nearly 90% of subjects completed their treatment without experiencing adverse events or mortality *(see Table 1)*.

Conclusions

This study underscores the low incidence of adverse events and mortality associated with ATMPs, highlighting their potential as a promising frontier for treating severe diseases that lack therapeutic alternatives.
The majority of patients completed their treatments without significant complications, suggesting that ATMPs could represent a groundbreaking advancement in the treatment of severe conditions.

These findings reinforce confidence in the safety and efficacy of ATMPs in real-world settings, opening new horizons for the future of advanced medicine.

References:

1. European Medicine Agency (EMA). Advanced therapy classification. Available from: https://www.ema.europa.eu/en/human-regulatory-overview/marketing-authorisation/advanced- therapies-marketing-authorisation/advanced-therapy-classification. Accessed on: 22nd May 2024
2. Mucherino, S., Rafaniello, C., Serino, M., Zinzi, A., Trama, U., Capuano, A., ... & Orlando, V. (2023). Drug Utilization and Measurement of Medication Adherence: A Real-World Study of Psoriasis in Italy. Pharmaceutics, 15(12), 2647.
3. Orlando, V., Coscioni, E., Guarino, I., Mucherino, S., Perrella, A., Trama, U., ... & Menditto, E. (2021). Drug- utilisation profiles and COVID-19. Scientific Reports, 11(1), 8913

Nucleic acids as medicinal products: the regulatory landscape

Sara Manellari[1], Umberto M. Musazzi[1], Paolo Rocco[1], Paola Minghetti[1]

[1]Department of Pharmaceutical Sciences, University of Milan, G. Colombo 71 - 20133, Milan, Italy.

Oligonucleotide-based medicinal products (ONMs) are a very heterogeneous class of medicines due to their different mechanisms of action, therapeutic indications, API production methods, and formulative features.

When delivered to the cell, ONMs can influence directly or indirectly the translation of proteins and, therefore,they are revolutionizing the prophylaxis and treatment of many human diseases. In this light, this work aims to analyze the features and discuss the challenges of ONMs authorized in the EU and US. The regulatory history of authorized ONMs was reviewed starting from regulatory portals. Until October 2023, 21 ONMs have beenauthorized by EMA and/or FDA websites. In the US, ONMs are assessed by the FDA under section 505 of the FD&C Act (21 USC 355).

In the EU, ONMs have been approved by EMA following a centralized procedure, under provisions of Regulation (EC) 726/2004. Moreover, the majority of ONMs (67%) are classified as orphan drugs by both EMA and FDA. From 1998, 11 ASOs have been examined by both Agencies, but only 3 of them are currently authorized in Europe and 7 in the US. Two products were withdrawn for commercial reasons in the US and/or in the EU. Until now, 1 aptamer is authorized only in the US. As far as siRNAs, following Onpattro® approved by the FDA in 2018, 5 additional products have been authorized in the last years.

Finally, mRNAs are available only as COVID-19 vaccines, approved in 2020 in both US and EU. From a quality point of view, ONMs are mainly produced by using chemical synthetic processes (e.g., solid phase methods), whereas mRNAs are produced by in vitro transcription (IVT).
Only Onpattro® and mRNA vaccines are formulated with lipid nanoparticles.
The overall results reveal how heterogeneous ONMs are in terms of API production methods and formulative features. Therefore, both EMA and FDA are still facing challenges in providing harmonized regulatory requirements and standards for such novel therapeutic classes [1].
A potential conceptual misalignment between the US and EU may emerge from the recent Reform of the EU pharmaceutical legislation, which seems to be aimed at classifying all ONMs as ATMPs [2].

However, grounding the regulatory classification on the nature of the API source may be counterproductive. Otherwise, the benefit/risk balance should be assessed both on their peculiar quality aspects related to the manufacturing process and on their efficacy and safety profiles related to the mechanism of action.

References:
1. Thakur S., et al., Front. Pharmacol. 13, 1006304, 2022.
2. European Commission proposals for the Pharmaceutical Regulation and Directive, https://health.ec.europa.eu/medicinal-products/pharmaceutical-strategy-europe/reform-eupharmaceutical-legislation_en, 2023.

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