# Publishable Summary for 22HLT04 MetrINo
# Metrology for innovative nanotherapeutics

## Overview

Europe is facing significant healthcare challenges driven by an ageing population coupled with an increase in chronic diseases like cancer, diabetes, heart disease, and brain conditions. These conditions require diverse and complex treatments, which increase healthcare costs. Nanomedicine and nano-enabled medical devices therapeutics (defined here as nanotherapeutics) are vital for tackling health and societal challenges, providing versatile technical solutions. This project responds to the immediate metrological needs expressed by industry, regulatory agencies, and policymakers to develop and validate traceable measurement methods and reference materials (RMs) candidates for the assessment of the critical quality attributes of nanotherapeutics. The project focuses on clinical formulations, including synthetic lipid-based nanotherapeutics, lipid nanoparticles (LNPs) for RNA delivery and liposomes, and metal oxide nanoparticles (MONPs) used for localised cancer treatment, gene therapy, vaccines (e.g. COVID-19) or as contrast agents. By providing fit for purpose methodologies, standardised methods and RM candidates to regulators and industrial stakeholders, the project is supporting their clinical translation, providing more efficacious nanotherapeutics with fewer side effects to improve the patient's quality of life and enhancing the competitiveness of the European health technology industry.

## Need

Nanotechnologies offer improved treatment of diseases through the effective targeting of therapeutic agents. Approved clinical formulations include (i) liposomes for cancer treatment, (ii) lipid-based nanoparticles for vaccination (COVID-19) and gene therapy, and (iii) metal oxide nanoparticles for local tumour treatment or as contrast agents. In their strategic paper, 'Regulatory Science to 2025', the European Medical Agency (EMA) states that, with the approval of innovative nanotherapeutics, it is crucial to 'develop and standardise new testing methods related to the quality and safety assessment of nanomedicines', to reach an understanding of 'the critical quality attributes of a given product and the relationship between those and its biological activity and in-vivo behaviour'. Recently, the European Commission's Joint Research Centre (JRC) identified general priorities for method development and standardisation of nanotherapeutics, including methods to measure: (i) surface properties, (ii) stability and kinetic properties in biological media, and (iii) uptake, absorption, and distribution in cells and tissues.

Industrial stakeholders have expressed similar measurement needs, specifically associated to different classes of nanotherapeutics. For example, stakeholders identified that MONP reference materials and fit-for purpose methodologies for their synthesis and characterisation are lacking. Improved methodological approaches are needed to measure (i) the particle size and morphology of small and not spherical particles, (ii) the amount, homogeneity, and functionalisation of the nanoparticle surface and (iii) for the detection of nanoparticles *in vivo*.

For liposomes and LNPs, standardised methods are needed (i) to ensure the integrity of lipid-based products, (ii) to characterise their physico-chemical properties, (iii) to understand their behaviour in complex biological medium, and (iv) finally to follow nanoparticle biodistribution and biotransformation *in vivo*.

## Objectives

This project aims to develop traceable measurement methods for the characterisation of innovative nanotherapeutics and associated RM candidates, focusing on synthetic lipid-based nanoparticles, e.g., liposomes and LNPs-RNA, and MONPs. The specific objectives are to:



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1. Develop RM candidates for synthetic lipid-based and metal oxide nanoparticles and to measure their chemical composition, particle size distribution, polydispersity and particle concentration with a controlled uncertainty using existing methods.
2. Develop and harmonise traceable methodologies for measuring: a) particle size and concentration of small (<10 nm) nanoparticles and agglomerated or aggregated non-spherical objects, b) lipid composition and structural integrity of lipid-based nanoparticles, and c) nanoparticle surface properties, including the amount, chemical composition, and homogeneity of the surface coating.
3. Develop and harmonise robust and efficient sample preparation methods for the measurements of the nanoparticle stability and their biotransformation in biological matrices, and to minimise matrix interferences. Fractionation methods will be used, including size exclusion chromatography (SEC), field flow fractionation (AF4) and ultracentrifugation (UC) that are used to separate nanoparticles from free proteins in plasma, reach a demonstrated recovery of the analyte of interest above 70 %, as required by ISO/TS 21362:2018 and CEN/TS 17273:2018. Additionally, to evaluate if repeatability/reproducibility target identified in ISO/TS 21362:2018 is fit for purpose.
4. Improve correlative methods and model tissue phantoms to measure nanoparticle uptake and distribution in single cells and tissues associated to their biodistribution profile, safety, and efficacy. This includes using a combination of complementary techniques (e.g., MS, single cell ICP-MS, EM, Raman- and fluorescence-based approaches) with the aim to reduce the uncertainty in the detection, localisation, and quantification of nanoparticles in single cells and tissues. The developed methods, RM candidates and model tissue phantoms to be consolidated through the performance of an interlaboratory comparison.
5. Facilitate the take up of the technology and measurement infrastructure developed in the project by the measurement supply chain (NMIs, accredited laboratories), standards developing organisations (e.g., CEN/TC 352-Nanotechnologies, ASTM E56, ISO TC 229), VAMAS TWA 2 and TWA 34 and end users (e.g., the pharmaceutical industry, regulators, clinicians, nanomedical product manufacturers, nanomedicine research community).

**Progress beyond the state of the art and results**

*Objective 1: RM candidates for synthetic lipid-based and metal oxide nanoparticles.*

In this project, NMIs are collaborating with industrial stakeholders to develop a library of RM candidates representative of nanotherapeutics. The library includes:

(i) Citrate and dextran coated iron oxide ($Fe_xO_y$) and hafnium oxide ($HfO_2$) formulations characterised by small particle sizes (constituent particle core<10 nm) or aggregated sub-populations;

(ii) Onpattro-like LNPs and Moderna-like LNPs encapsulating mRNA and siRNA;

(iii) PEGylated and not PEGylated liposomes loaded by different active pharmaceutical ingredients (doxorubicin, siRNA, mRNA) and with different surface charges;

(iv) Fluorescent multi-element nanoparticles such as NaYF4 and $NaGd_xY_{1-x}F_4$ doped with Yb(III) and Er(III) as model systems for correlating and comparing elemental and optical methods for measuring nanoparticle (NP) uptake and distribution in single cells and tissues on the same formulations;

(v) Amine-modified mesoporous silica nanoparticles (~100-150 nm); as model systems for the exploration of the measurement capabilities of certain surface-analytical methods

Stability studies of first NP samples have been started and are running to provide the timely basis for NP selection and storage conditions. The most promising test materials and candidate RM will be selected for stability and homogeneity testing. At the end of the project, a roadmap towards certification and commercialisation of these materials will be derived. All materials produced in the project will be used to advance the methodological state-of-the-art for measurement.

*Objective 2: Methods for measuring a) particle size and concentration of small nanoparticles and of agglomerated or aggregated non-spherical objects, b) lipid composition and structural integrity of lipid-based nanoparticles, c) nanoparticle surface properties*

The project is progressing beyond the state of the art in this objective by validating the use of traceable methodologies, including small-angle X-ray scattering (SAXS) and atomic force microscopy-scanning electron

microscopy -energy-dispersive X-ray spectroscopy (AFM-SEM-EDX) hybrid microscopy approach, for the measurement of particle size and concentration of $Fe_xO_y$ and $HfO_2$ NPs (<10 nm) and their aggregates, also advancing the approaches for data analysis to reduce the associated measurement uncertainty. Additionally, the project is providing a structured tier approach for the determination of the lipid composition, drug loading and for the structural properties of lipid-based formulations including (i) bulk techniques, e.g. advanced mass spectrometry (MS), calorimetry, capillary electrophoresis, Raman spectroscopy, SAXS, and (ii) innovative emerging particle-by-particle measurements such as cryo-time of flight secondary ion mass spectrometry (cryo-TOF-SIMS) and cryo-transmission electron microscopy (cryo-TEM), by evaluating the sensitivity, precision and robustness of selected protocols and performing a preliminary uncertainty budget determination. First samples for metal-oxide and lipid nanoparticles have been characterised using these techniques. Under this objective, the project will also evaluate the applicability of emerging methodologies for the characterisation of particle surface chemistry and coating, including the quantitative determination of the average coating mass by differential scanning calorimetry and thermogravimetric analysis coupled to MS, and the particle-by-particle coating homogeneity by cryo-TOF-SIMS.

*Objective 3: Sample preparation methods for the measurements of the nanoparticle stability and their biotransformation in biological matrices, and to minimise matrix interferences*

The work on the development of fractionation methods for selected NPs is well progressing: participants are optimising the established separation methods, including MD-AF4 and SEC, for the characterisation and separation of $HfO_2$, $Fe_xO_y$, liposomes or LNPs-RNA from complex biological media (serum, plasma and blood). Notably, optimised separation methods employing MD-AF4 have enabled efficient separation of LNPs/liposomes from matrices, bypassing the needs for invasive extraction procedures. Current efforts focus on MONP separation optimisation, analysis in biological matrix, and nanoparticle stability evaluation. Encouraging progress is evident in the development of fractionation methods for NPs, achieving target recovery thresholds required by available standards (ISO/TS 21362:2018 and CEN/TS 17273:2018) and ensuring comparability across institutes despite differing hardware and methodologies.

*Objective 4: Methods and model tissue phantoms to measure nanoparticle uptake and distribution in single cells and tissues*

The project is developing tissue phantoms systems based on gelatine and hydrogel technologies that are spiked with a known amount of $HfO_2$, $Fe_xO_y$, fluorescent multi-element NPs, and fluorescent LNPs-RNA. Those materials serve altogether as RM candidates for particle localisation, quantification and uptake in solid tissues. The tissue phantoms under development will be used for evaluating method sensitivity, precision and robustness, as well as develop standard operating procedures (SOPs) for a range of complementary methods to identify, localise and quantify NPs correlatively, and providing the basis of an external (future) ILC for the evaluation of the associated measurement uncertainty.

**Outcomes and Impact**

The project has actively engaged its community by hosting notable events like co-sponsoring "International Standardisation Roadmap for Nanomedicine" and organising the first METRINO workshop during the NME23 conference. These gatherings fostered collaboration among various stakeholders, including industry professionals, characterisation experts, standardisation committees, European Pharmacopoeia representatives, and metrology institutes to discuss key concerns related to metrology, standardisation in nanomedicine and the need for a coordinated approach. As an outcome of community feedback gathered during these engagements, Metrino is focusing on educational actions such as expanding accessible resources through their digital communication channels and organising webinars like 'Standardisation and Validation made simple'. By targeting professionals, students, and lab technicians with this disseminated knowledge on advanced metrology techniques, standardisation processes, best practices, and common pitfalls in nanomedicine development, Metrino is dedicated to fostering a more unified and collaborative effort within the industry. To support this endeavor, Metrino has designed and launched (in early March 2023) a dynamic website (https://metrino.eu/), which provides not only the traditional description of the project's vision, missions, expected outcomes, results, publications, and deliverables but also features a dynamic list of news and events. Additionally, it offers a wide range of useful resources covering topics such as Nanomedicine, Regulatory Guidelines, key definitions, Metrology for Nanomedicine, SOPs, and Standards, as well as Reference Materials for Nanomedicine. Furthermore, Metrino is actively leveraging social media channels created for the project to reach and engage with the broader nanomedicine and metrology communities. In summary, Metrino has

started to demonstrate unwavering commitment to education and collaboration in the field of nanomedicine and metrology, providing accessible resources and fostering exchanges among industry stakeholders.

*Outcomes for industrial and other user communities*

This project's work and results on the development of reference materials and validated protocols will support industry in the design, characterisation, regulatory evaluation, and clinical translation of innovative nanotherapeutics. Such standardised methods will also provide knowledge about the structure-function relationship of the nanomaterial. This knowledge will provide confidence for regulatory acceptance and improving quality control methods. The understanding gained will be directly exploited by the industrial participants involved in the project in their exchange with regulatory agencies and by adopting the validated protocols for the characterisation of their formulations under development for their release. The uptake of the developed protocols and RM candidates, and alignment with industry beyond the consortium has been promoted through early engagement with additional industrial end users belonging to (i) the pharmaceutical industry and the medical device industry, (ii) SMEs that are members of the main European nanomedicine associations and (ii) instrument providers. Importantly, the ETPN (European Nanomedicine Technology Platform) is leading the impact work package, addressing the whole European nanomedicine community, and benefitting from its well-established network and methodology- for community building and common strategic thinking. All target groups of stakeholders have been invited to join the Stakeholder Advisory Board. Additionally, to promote the uptake of the project's results by these communities, the consortium has invited these to two workshops organised by the consortium i) NME23 Satellite event MetrINo workshop "Advancing on characterisation and standardisation methods: the regulatory needs and the industrial perspective"(Liverpool, June 22 2023) and ii) The international standardisation roadmap for nanomedicine workshop (Paris, 13 November 2023). The consortium will continue to engage them in future events, including workshops, webinars and dedicated events.

*Outcomes for the metrology and scientific communities*

The project is expanding the measurement capabilities of the NMIs involved in the consortium to provide preclinical, regulatory, and quality control services at the highest metrological level. Collaborations between NMIs, academia and industrial companies within the consortium is supporting the use of the created metrological infrastructure. The project additionally supports academia and several on-going EU projects by (i) providing SOPs, guidelines and templates for data reporting for less mature measurement methods and (ii) moving more advanced methodologies towards standardisation. To promote the uptake of the project's results by this community, the project is organising in-person workshops, webinars and high-level meetings between stakeholders all along the value chain of nanomedicine translation to the clinic.

*Outcomes for relevant standards*

The consortium is actively disseminating the outcomes of the project to national standardisation organisations, to standardisation committees, e.g., ISO/TC 229, CEN/TC 352 and ASTM E56, to the VAMAS Steering Committee, and to the Nano working party of the EDQM. Moreover, the consortium is closely in contact with the CCQM Task Group on Particle Metrology. Metrino is initiating targeted networking actions and sharing information on specific initiatives among the different stakeholders belonging to existing metrology and standardisation networks, as already done in the "The international standardisation roadmap for nanomedicine workshop" organised in Paris, the 13rd November 2023. The project actively collaborates in drafting three ongoing standardisation projects focused on lipid-based NPs in ASTM E56.08 and ASTME56.02. Furthermore, the project will initiate a nanomedicine roadmap with CEN/TC 352, and two new standardisation projects on MONPs and inorganic NPs, based on the validation of methods responding to Objective 2 and Objective 3. Finally, the RM candidates, model tissue phantoms, and results of the internal inter laboratory comparison (ILC) responding to Objective 4 will serve as a basis to initiate an external ILC study under VAMAS TWA 40 - Synthetic Biomaterials, that will run beyond the lifetime of the project.

*Longer-term economic, social and environmental impacts*

This project actively supports the translation of European nanomedical innovation to clinical application. It reinforces and extends cooperation of the European nanomedicine community to worldwide measurement experts, innovators, and regulators. It leverages previous public and private European investment in nanomedicine, notably the hundreds of million Euros committed by the EU in their research and innovation programmes on nanotechnology for the last 15 years and will accelerate and optimise the translation of innovation into advanced and safer therapies, available to European citizens as globally. By reducing the risks

of the nanotherapeutic development process, this project will actively contribute to the local creation and development of innovative European SMEs. This is an impactful response to the need expressed by EMA of more collaboration across the European regulatory landscape to improve the innovation environment and enhance patient access to new medicines in line with the EMA Strategic Roadmap to 2025. The project is strengthening the position of Europe in determining international policy, regulation, and the direction of standards committees related to medicinal products. Such leadership will contribute to the development of European and international standards directly relevant to EU centred activities. The project will support the work of EMA and European national and international regulators within the pharmaceutical sector, while also creating a framework of global cooperation across stakeholders that will boost confidence in innovation, attracting investment. The project aims to reinforce the cooperation of NMIs with EMA, the U.S. Food and Drug Administration (FDA), and other international regulators in the pharmaceutical sector and support the translation of such cooperation to standard bodies and measurement expert communities in Europe and the world. The project outcomes will help ensure that Europe sustainably and enduringly engages in the medical innovation process and that European citizens and economies will benefit from these innovations. In summary, this project represents a unique chance to ensure Europe is a global leader in nanomedicine, leading to value creation, both in cost savings for the healthcare systems and job creation notably through the creation of specialised SMEs, benefiting from a whole nanomedicine-friendly ecosystem.

**List of publications**

n/a

This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

<table>
<tr><td colspan="2">Project start date and duration:</td><td>01 June 2023, 36 months</td></tr>
<tr><td colspan="3">Coordinator: Robin Schürmann, PTB Tel: + 49 30 3481 7114 E-mail: robin.schuermann@ptb.de<br>Project website address: http://www.metrino.eu/</td></tr>
<tr><td>Internal Beneficiaries:<br>1. PTB, Germany<br>2. BAM, Germany<br>3. INRIM, Italy<br>4. LNE, France<br>5. RISE, Sweden<br>6. SMD, Belgium<br>7. TUBITAK, Türkiye</td><td>External Beneficiaries:<br>8. CEA, France<br>9. Curadigm, France<br>10. ETPN, France<br>11. Nanobiotix, France<br>12. nanoPET, Germany<br>13. OGSA, Italy<br>14. OVGU, Germany<br>15. SINTEF, Norway<br>16. UGent, Belgium<br>17. UniPV, Italy</td><td>Unfunded Beneficiaries:<br>-</td></tr>
<tr><td colspan="3">Associated Partners:<br>18. Empa, Switzerland<br>19. LGC, United Kingdom<br>20. NPL, United Kingdom</td></tr>
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