Executive summary

Diseases of the arterial wall leading to acute arterial thrombosis and cardiovascular events are responsible for the majority of deaths in developed countries. Integrating an efficient transport mechanism using nanosystems, a stealth coating, a targeting and an active molecule has not yet been clinically validated in the field of atherosclerosis. Indeed, no specific nanoparticle-based system is approved for diagnosis or therapy in cardiovascular diseases. NanoAthero partners have patented and provided proof of the efficiency of different nanodelivery systems and ligands for use in targeted therapies. Due to the unique features of nanosystems, preliminary existing data and extensive expertise within the consortium, NanoAthero has focused on thrombus and plaque to transfer them into proof-of-concept clinical trials. NanoAthero aims Phase I clinical trials of nanosystems for targeted imaging and therapy of atherosclerotic diseases in humans: i) Nanosystems for enhanced local delivery and improved efficacy of drugs for athero-thrombotic therapy in humans. ii) Nanoimaging agents for non-invasive, molecular imaging of key pathological processes in vulnerable plaques and thrombus using clinical imaging.

A list of candidate nanosystems was established for A) molecular imaging and B) treatment of plaque, and for C) therapy of thrombus. Two imaging modalities were addressed for plaque imaging: MRI (iron oxide nanoparticles or gadolinium-loaded nanoparticles) and PET (68Ga labeled nanoparticles). Ga and Gd loaded nanosystems were based on micelles or lipid nanoparticles (liposomes or solid lipid nanoparticles). Successful drug-loading was achieved with liposomal nanosystems for plaque therapy. Two tPA-loaded nanocarriers were designed for thrombus therapy. All nanosystems were characterized and were evaluated for toxicity and efficacy. Ultrasmall SPIO (Dex-USPIOs) were found to accumulate in AS-plaques of balloon injured rabbits. The JURY device for pressure-controlled balloon injury in rabbits (bigger AS-animal model) was patent-protected and positively evaluated in a preclinical trial to induce homogenous AS-plaques in rabbits. Toxicological data for the nanosystems coming from the selection were acquired. In particular, toxicological regulatory studies aimed to prepare the IMPD documentation for clinical trials were performed. These studies were performed on two selected nanosystems, namely fucoidan (a SPECT imaging nanosystem proposed by P1 (INSERM) for the thrombus imaging, composed of fucoidan and 99Tc) and dextran-coated iron oxide nanoparticles (Dex-SPIONs) proposed by P7 (UKER) as an imaging nanosystem for plaque permeability.

Implementation of the protocol for a GMP production of fucoidan was achieved. Related CMC and IMPD documents were completed and submitted, receiving authorization to perform the clinical trials in France and The Netherlands In January and March 2018, respectively. We then produced the first batches of GMP 99mTc-Fucoidan in April 2018. Ethical Reports on the nanosystems in thrombus imaging were written. The synthesis of Dex-USPIOs was also successfully transferred in a GMP environment, optimizing production of quality controlled large scale batches in the liter range. All the analytical methods were validated according to ICH guidelines. Different technical batches were produced for toxicological studies.

In Phase I clinical trial, we showed delivery in human patients of pegylated liposomes into plaque macrophages. In patients undergoing surgical endarterectomy, we showed that liposomes (when infused intravenously) were delivered successfully into macrophages located deep in the atherosclerotic plaques (assessed after surgical removal of the plaque). The clinical trial of fucoidan for thrombosis imaging started in Amsterdam in June 2018. In 10 healthy controls, 99mTc-fucoidan was infused. Biodistribution was normal, tolerability was good and no adverse effects occurred in this Phase I clinical trial.

For dissemination, a final version of the Democs card game has been translated into French, Dutch and German, and distributed for playing with stakeholder and public groups. Three NanoAthero workshops have been organised, one in Barcelona, one in Rome and one in Genova. Each year, NanoAthero was also represented at the annual CLINAM conference trough notably the coordinator but also other partners who disseminated their result from the project at the conference. A graphic video has been produced for dissemination of the results and context of NanoAthero.

Summary description of the project context and the main objectives

Atherosclerosis is the buildup of a waxy plaque on the inside of blood vessels, and plaque deposits can block the flow of blood. Plaques can also rupture or crack open, causing the sudden formation of a blood clot (thrombosis). Atherosclerosis can cause a heart attack if it completely blocks the blood flow in the heart (coronary) arteries. It can cause a stroke if it completely blocks the brain (carotid) arteries. These diseases of the arterial wall leading to acute arterial thrombosis and cardiovascular events are responsible for the majority of deaths in developed countries. Integrating an efficient transport mechanism using nanosystems, a stealth coating, a targeting and an active molecule has not yet been clinically validated in the field of atherosclerosis. Indeed, no specific nanoparticle-based system is approved for diagnosis or therapy in cardiovascular diseases.

NanoAthero has the opportunity to deal with both diagnosis and therapy that will be addressed in pivotal interrelated projects on thrombus and plaque. The project aims to take profit of nanosystems that have been previously investigated and transfer them to proof-of-concept clinical trials. NanoAthero aims to demonstrate the clinical proof-of-concept and validation (Phase I clinical trial) of nanosystems for targeted imaging and therapy of atherosclerotic diseases in humans: i) Nanosystems for enhanced local delivery and improved efficacy of drugs for athero-thrombotic therapy in humans. ii) New nanoimaging agents for non-invasive, molecular imaging of key pathological processes in vulnerable plaques and thrombus using clinical imaging.

Sixteen partners from 10 countries created a large "Nanomedicine for Atherosclerosis" consortium with expertise from the design of nanosystems, preclinical and clinical validations, through toxicology, industrial development and production. Design and characterization of several nanosystems were designed in WP1. Establishment of preclinical proofs of concept of the efficiency of nanosystems was performed in WP2. NanoAthero supported and organized in WP3 the pharmaceutical grade production of injectable nanoproducts and the establishment in WP4 of toxicology data and risk assessment. WP5 was devoted to the proof-of-concept by Phase I clinical trials. Ethics in all steps was rigorously defined and managed in WP6. WP6 also included regulatory dossiers for clinical research in humans. Valorization and exploitation of the patented outcomes by companies within and/or outside the consortium was monitored in WP7, and partners also largely supported European dissemination and training. This innovative proposal was under tight and professional management & coordination (WP8).

WP1 was dedicated to the design (on the lab-scale) and physical and chemical characterization of nanosystems for molecular imaging and treatment of plaque atheroma, and for stroke therapy. Different nanosystems were examined using a variety of different technologies. The objectives of WP1 were 1) the lab-scale fabrication of these nanosystems; 2) their eventual surface functionalization to improve plaque targeting; 3) their loading or functionalization by contrast agents or active ingredients in order to confer them with imaging or therapeutic properties; and 4) their physical and chemical characterizations. The final objective of WP1 was to select optimal nanosystems. The selection was made in collaboration with clinical and other project partners, and was based on the results of the nanosystem characterization, the analysis and scale-up potential of their fabrication process, as well as the efficacy results obtained in WP2.

The aim of WP2 was the preclinical evaluation of all nanosystems that were designed, synthesized and characterized in WP1 by the WP1 partners. The evaluation consisted of six tasks with different durations. In accordance with the workflow of WP2, first the nanosystems had to enter a systematic, sequential validation process. This began with the in vitro cell culture models, and with perfused cell bifurcation systems later on. Meanwhile, the ex vivo validation set up was evaluated, represented by perfused arteries or carotids from different animal models. The in vivo efficacy studies for imaging strategies were performed on those nanosystems which had been positively evaluated. Nanosystems were injected into

atherosclerotic animal models for the detection of atherosclerosis by MR, PET or SPECT/CT imaging, once the imaging tools had been optimized. The measurements of biodistribution and pharmacokinetics were also performed on the positively evaluated therapeutic nanosystems.

WP3 was dedicated to the GMP (Good Manufacturing Practice) production of injectable products. The development of any new drug requires the definition of methods to characterize the manufacture and control of both the active drug substance and the drug product. In the context of the NanoAthero project, this corresponds to a nanomedicine capable of being delivered to the clinics in a suitable dosage form allowing its administration to human patients. The final objective of WP3 was to set up the GMP manufacturing process for the proposed nanosystems: 1-to complete the manufacturing process of the SPECT imaging nanosystem for the thrombus imaging, and to submit the documentation required to perform the related clinical trials. 2-to complete the activities related to production of the B22956/1 blood pool MRI agent. 3-to define and implement a manufacturing process of an imaging nanosystem for plaque permeability. 4-to complete manufacturing process development of a nanosystem for plaque imaging.

NanoAthero aimed to demonstrate initial clinical feasibility of nanosystems for targeted imaging of atherosclerotic disease in humans. In NanoAthero, the nanocarriers under R&D had to be suitable for proof-of-concept in patients. In order to support these aims, the main objective of WP4 has been to obtain toxicological data of selected nanosystems for thrombus and plaque imaging, thereby establishing the safety of subsequent clinical trials. A further objective of WP4 was a comprehensive hazard assessment of all the NanoAthero systems: an actual risk assessment and analysis procedures for each nanosystem, with a work plan for risk management and mitigation; an assessment of the compliance to GMP requirements for the manufacturing process of nanosystems; and a comprehensive data analysis of the full life cycle of nanosystems entering clinical trials.

For NanoAthero Phase I clinical trials, one of the objective was to conduct the feasibility study for delivery of GMP-produced Nanocort in human patients. Another goal was to evaluate the tolerance, dosimetry and SPECT imaging of 10 healthy control human subjects after injection of 99m-Fucoidan nanosystem. The third Phase I clinical trial was to evaluate the suitability of using an 'endogenous' HDL particles as nano-delivery systems. To this end, we assessed the local delivery of CER-001 labelled with 89-Zirconium, in atherosclerotic plaques. By comparing local delivery in atherosclerotic plaques (with 'leaky' vasa vasorum) to local delivery in tumors ('leaky' neovascularisation), an optimal insight can be obtained into the promise of apoAI-particles as local delivery vehicles in vivo in humans.

WP6 addressed the ethical and social factors of the NanoAthero project, particularly in that it involves novel nanosystems, animal experiments and Phase I clinical trials on human subjects. Ethical issues arise in several areas. Firstly the project entails preclinical experiments a) to induce atherosclerotic-like damage in mice and rabbits in order to test efficacy of the nanosystems to image and/or treat damaged cells, and b) in using animals to test for potential toxic and other harmful effects of the nanoparticles and their components. Secondly, an ethical judgment must be made whether the collective information obtained from the animal tests and laboratory studies is sufficiently indicative of likely human benefit, in terms of efficacy and acceptable risk, for preliminary trials in humans to be undertaken. Thirdly human participants in the clinical trials require appropriate information in understandable form for volunteers to give their informed consent to the trials. These require that ethical aspects be addressed suitably, that the work is done in an ethically acceptable manner, and with levels of harms and risks that are in proportion to the anticipated medical benefits. This includes addressing such questions as the appropriateness of the animal models, the '3Rs' principles for animal experiments (Replace, Refine, Reduce), and the informed consent of human subjects in any clinical trials. The NanoAthero project has sought to address these ethical and social factors by including in its consortium an expert in ethical issues and public engagement, Partner 16 Edinethics, to perform ethical assessment throughout the project in Work Package 6, with the assistance of an Ethical Advisory Board (EAB).

The scientific work of the project was accompanied by two non scientific WPs, WP7 on dissemination, exploitation and Intellectual Property Management and W8, dedicated to the coordination and Management of the project. Five types of activities were carried out in WP7 to raise the international and local awareness of NanoAthero, and to enable its findings to be applied: dissemination, engagement, teaching, IP management, exploitation and a website. 1) Dissemination of Results and key findings of the project: Targeting policy makers, the medical and scientific community, industry, public interests groups and the media, with their corresponding methods of outreach. 2) Stakeholder and Public Engagement: Engagement with relevant stakeholders (e.g. patients' groups), and the general public as the project's work develops, with the aim of raising awareness, allaying potential concerns, and also of providing a means to listen to and take account of lay and user perspectives. 3) Training: An integral part of the project was to train the next generation of researchers in the emerging fields of nano-enabled therapeutic and diagnostic systems, drawing on the multi-disciplinary expertise of the partners. 4) IP management and exploitation: To monitor IPR and business developments and opportunities during the course of the project and develop exploitation plans to provide the mechanisms for their uptake in the relevant medical and industrial contexts. 5) Website: A regularly updated, user-friendly website making available the project's publications and activities.

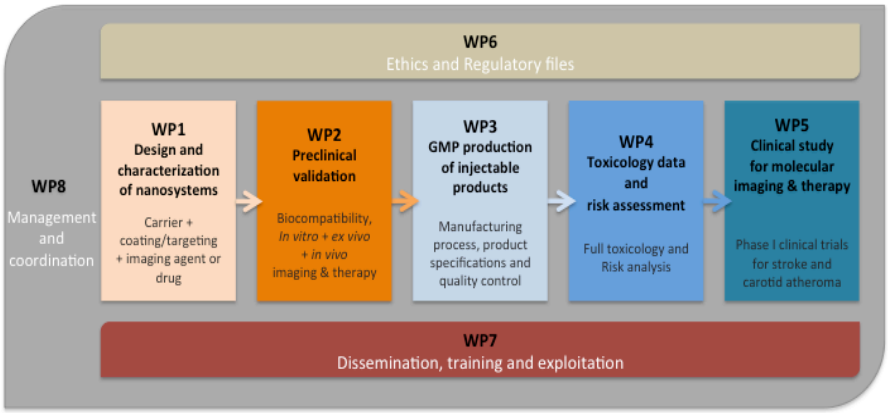
The objectives in WP8 for an effective management framework for the NanoAthero consortium were: 1) To act as the interface between the NanoAthero consortium and the European Commission; 2) To ensure that all actions are performed correctly and within the rules and regulations established by the European Commission, and in the Consortium Agreement, including financial and legal management 3) To ensure that the received funds are correctly distributed and accounted for; 4) To ensure the work and tasks are performed on time, within budget and to the highest quality and create an early warning system; 5) To keep each partner fully informed about the project status, scientific issues, the work planning (adjustments) and all other issues which are important and relevant to partners, in order to obtain maximum transparency for all involved and achieve synergy of the cooperation; 6) To ensure that all partners are informed of all important and impacting information that can influence the outcome of the project.

The goals of NanoAthero require a multidisciplinary approach, combining complementary skills and providing an interface between chemists, biologists, pharmaceutical scientists, imaging experts, clinicians, analysts, and ethicists. In order to realize these objectives, NanoAthero builds a high standard consortium throughout Europe, associating medical science of atherothrombotic diseases in humans, with cutting edge technology of nanocarriers in the cardiovascular system, enriched by ethicians and academic/private interactions. Such an association of medical, biological and technological high standards, to successfully put forward diagnostic and therapeutic nanocarriers in the field of human atherothrombotic diseases will provide a solid basis for a new nanosystem paradigm as a valid strategy to prevent and treat human atherosclerosis. Therefore, NanoAthero should have a high impact on the future development of nanotechnology, not only in atherothrombotic diseases but also in other pathologies comprising cancer and degenerative diseases. Moreover, the clinical demonstration of safety complying all EU regulations combined with efficacy of these nanocarriers in humans should prompt the pharmaceutical industry to embrace these novel approaches and provide the EU society with rational criteria to support further developments.

Description of the main S & T results/foregrounds.

Overall strategy of the workplan

Design and characterization of nano-systems was achieved in WP1 (Partners 1, 2, 5, 7, 8, 9, 10, 13, 15). Establishment of preclinical proofs of concept of the efficiency of nano-systems was done in WP2 (Partners 1, 2, 5, 6, 7, 8, 10, 13, 14, 15, 16). NanoAthero supported the GMP production of injectable nanoproducts for humans (WP3 - Partners 1, 2, 8, 10, 15), and the establishment of toxicology data and risk assessment (WP4 - Partners 1, 10, 12, 13, 14, 15, 16). WP4 provided toxicity analysis and risk analysis. WP5 was devoted to the proof-of-concept and clinical feasibility (Partners 1, 2, 4, 15, 16). Ethics in all steps were monitored in WP6 (Partners 1, 2, 3, 4, 8, 13, 15, 16). WP6 also included regulatory dossiers for clinical research in humans. Valorization and exploitation of the patented outcomes by companies within and/or outside the consortium were followed in WP7, and partners also supported European dissemination and training (WP7 - Partners 1, 3, 10, 11, 12, 13, 15, 16). This innovative proposal was under tight and professional management & coordination (WP8 - Partners 1, 3, 13, 15).



Through the 5.5 years, NanoAthero was in charge :
for thrombus of: i) a complete phase I for the imaging of thrombus with nuclear imaging (WP3-WP4-WP5, WP6) and ii) the design of a nanosystem for effective and safe thrombolytic therapy (WP1-WP2-WP3-WP4), **and for plaque**: i) the design and validation of a nanosystem for plaque treatment in two phase I trials (WP1-WP2-WP3-WP4, WP5, WP6), and ii) a phase I trial assessing the permeability of a nanosystem for treatment of vulnerable plaques (WP1-WP2-WP3-WP4-WP5, WP6).

Design and characterization of nanosystems in WP1

WP1 was dedicated to the design (on the lab-scale) and physical and chemical characterization of nanosystems for plaque imaging, plaque therapy, or tPA (tissue Plasminogen Activator) functionalization for thrombus therapy.

A list of candidate nanosystems was established for each category for A) molecular imaging and B) treatment of plaque, and for C) therapy of thrombus. Two imaging modalities were addressed for plaque imaging: MRI (iron oxide nanoparticles or gadolinium-loaded nanoparticles) and PET (68Ga labeled nanoparticles). Ga and Gd loaded nanosystems were based on micelles or lipid nanoparticles (liposomes or solid lipid nanoparticles). Successful drug-loading was achieved with liposomal nanosystems for plaque therapy. Two tPA-loaded nanocarriers were designed for thrombus therapy.

Different nanosystems were examined using a variety of different technologies. The objectives of WP1 were 1) the lab-scale fabrication of these nanosystems (Task 1.1); 2) their eventual surface functionalization to improve plaque targeting (Task 1.2); 3) their loading or functionalization by contrast agents or active ingredients in order to confer them with imaging or therapeutic properties (Tasks 1.3 and 1.4); and 4) their

physical and chemical characterizations (Task 1.5). The final objective of WP1 (Task 1.6) was to select optimal nanosystems. The selection was made in collaboration with clinical and other project partners, and was based on the results of the nanosystem characterization, the analysis and scale-up potential of their fabrication process, as well as the efficacy results obtained in WP2.

Polymeric nanoparticles (P1) can be described as core-shell nanoparticles with a hydrophobic core and a hydrophilic shell. They are synthesized by a redox radical emulsion polymerization reaction of isobutylcyanoacrylate (IBCA) in presence of a mixture of polysaccharides (dextran to stabilize the nanoparticles, fucoidan as targeting moiety and aminated dextran to interact with tPA). Stable solid spherical nanoparticles were obtained with an hydrodynamic diameter of 136 nm, a zeta potential value of – 5 mV and a fucoidan content of 1.6 % (w/w). They were radiolabeled with 99mTc and injected in mice for SPECT/CT imaging and tissue biodistribution. SPECT/CT profiles at 80 minutes showed a classical nanoparticle biodistribution (main accumulation in the bladder and liver, no significant accumulation in the lungs, the heart and the brain, and some activity in kidneys and urine). The tPA loading onto nanoparticles were done by physical adsorption between tPA and aminated dextran. More than 80% of the nanoparticles were loaded with tPA corresponding to 0.35 mg of protein per mg of nanoparticles. tPA was gradually released in vitro, in PBS buffer at 37°C under gentle agitation, from nanoparticles up to 54% at 90 min. These functionalized nanoparticles were able to bind P-selectin expressed by activated human platelets under venous shear rate. The thrombolysis efficiency of these nanoparticles was demonstrated in a mouse model of venous thrombosis by monitoring the platelet density with intravital microscopy. The functionalized nanoparticles generated a significant thrombus reduction with a tPA dose four time lower than the clinical dose. This work was published in 2018 in the high impact/visibility journal *Biomaterials*.

Liposomes (P5, P8) are vesicular structures composed of phospholipids that spontaneously self-assemble when dispersed in aqueous media due to their amphiphilic nature. The vesicular membrane enables the encapsulation of pharmacologically active ingredients, either in the membrane itself for lipophilic compounds, or in the aqueous core for hydrophilic compounds. Liposomes can be used to target atherosclerotic plaques and deliver drugs locally that interfere in inflammatory, proliferative or lipid modulatory pathways. Plaque macrophages are key cells in plaque progression and also in liposome uptake.

The main objective of the liposomes formulated by P5 was in the imaging of atherosclerotic plaques by MRI. Therefore the amphiphilic gadolinium chelate, B22286 (from P15), was successfully loaded into liposomes (P5) and characterized. B22286-liposomes showed an overall good stability and signal activity determined by MRI relaxometry studies. Further they were found to be non-toxic in WP2-related evaluations (with P7 and P14).

For targeting strategies, liposomes were conjugated (P5) with the globular domain of the protein adiponectin and fucoidan from P1. In early publications using fluorescence dyes and laser scanning microscopy, P5 could show that the globular domain of adiponectin accumulates in the fibrous cap area of atherosclerotic plaques in mice, and adiponectin coupled liposomes were found to accumulate in macrophages of the plaques.

Lipid nanoparticles (Lipidots™)(P10) can be described as nano-oil droplets stabilized by a mixture of surfactants (oil-in-water emulsion). They are composed of a lipid core, herein a mixture of soybean oil and a wax in which a lipophilic drug can be loaded, and a surfactant shell, containing a mixture of phospholipids and polyethylene glycol (PEG)-ylated surfactants.

Superparamagnetic iron oxide nanoparticles (SPIONs) consist of iron oxide core, commonly coated with organic shell. P7 developed several types of SPIONs with different coatings. Dextran T40-coated SPIONs have proven the most biocompatible formulation among SPIONs, showing no endothelial toxicity up to 400

µg/mL. These particles have been tested in MRI (P7) and assays (P14) confirmed their non-immunogenicity. These particles have been selected for further development as an atherosclerotic plaque permeability agent. Up to the present time, the synthesis of Dex-USPIO has been successfully upscaled and the nanoparticles can be reproducibly fabricated in the litre range. P7 succeeded in transferring the first part of the synthesis of Dex-USPIO to the GMP environment and to complete a full parametrization of the synthesis conditions.

In order to compare with SPIONS, which provide negative contrast in MRI, P13 also worked with Gd-based micellar formulations for positive contrast. Work with these particle nanosystems was conducted (as in the case of the SPIONS), with the aim of their being potential candidates as contrast agents for MRI of atherosclerotic plaque.

Physico-chemical characterizations (Task 1.5) of all the nanosystems produced in WP1 were performed and reported in deliverable D1.5 by P9 to ensure common procedures in the evaluation of the particles while performing a 1-year stability study. Results were reported in a consortium published paper (a 2nd paper focused on biological characterizations was accepted in 2018).

For the selection of nanosystems to pursue in WP3 ("scaled-up production") (Task 1.6), the medical profile of each nanosystem to develop (A. Nanosystem for thrombus therapy; B. Nanosystem for plaque imaging; C. Nanosystem for plaque therapy) was established by the clinicians and candidate nanoparticles were listed for each item (A, B, or C). In parallel, a list of the different criteria to take into consideration for the selection was drafted and organized in 6 categories (1- Physico-chemical properties; 2- Stability; 3- Biological properties; 4- Manufacturing process and materials; 5- Efficacy; 6- Toxicity). Data for each criteria were then collected (criteria 1-2-4 concerned WP1, criteria 3-5-6 were relevant from WP2).

Preclinical validation (WP2)

All nanosystems were characterized and were evaluated for toxicity and efficacy in WP2. The aim of WP2 was the preclinical evaluation of all nanosystems that were designed, synthesized and characterized in WP1 by the WP1 partners. The evaluation consisted of six tasks with different durations.

In accordance with the workflow of WP2, first the nanosystems had to enter a systematic, sequential validation process. This began with the in vitro cell culture models, and with perfused cell bifurcation systems later on. All particles were provided for in vitro cell culture and some for toxicity assays and the data were included in two collaborative papers. Meanwhile, the ex vivo validation set up was evaluated, represented by perfused arteries or carotids from different animal models. The in vivo efficacy studies for imaging strategies were performed on those nanosystems which had been positively evaluated. Nanosystems were injected into atherosclerotic animal models for the detection of atherosclerosis by MR, PET or SPECT/CT imaging, once the imaging tools had been optimized. The measurements of biodistribution and pharmacokinetics were also performed on the positively evaluated therapeutic nanosystems.

All particles were provided at first for in vitro cell culture assays performed by P7. The nanosystems were tested (a) in static cell cultures by real-time cell analysis system and live-cell microscopy; and (b) in perfused cell bifurcation systems under different hemodynamic conditions. The results of these standardized comparative in vitro studies were included in a collaborative paper published in Nanomedicine (J. Matuszak, J. Baumgartner, J. Zaloga, M. Juenet, A. Eduardo da Silva, D. Franke, G. Almer, I. Texier, D. Faivre, J. M. Metselaar, F. P. Navarro, C. Chauvierre, R. Prassl, L. Dézsi, R. Urbanics, C. Alexiou, H. Mangge, J. Szebeni, D. Letourneur, I. Cicha (NanoAthero Consortium), Nanoparticles for intravascular applications: physicochemical characterization and cytotoxicity testing, Nanomedicine 2016: 6(11), 597-616).

Further, studies of nanoparticle effects on endothelial cell migration, monocytic cell chemotaxis and monocytic cell recruitment by the endothelium under physiologic-like flow conditions were performed. A second publication was published (Matuszak et al 2018).

In vitro toxicity assays were performed by P14 during period 3, with promising outcomes for most of the evaluated nanosystems, including new Dex-USPIO nanosystems designed by P7 for the evaluation in the 4th period. According to the preclinical workflow, the next step was the evaluation of the nanosystems in perfused arteries/carotids (P7). Two papers were published, one containing the comparative in vitro studies on magnetic targeting of SPIONs (Matuszak J, Dörfler P, Zaloga J, Unterweger H, Lyer S, Dietel B, Alexiou C, Cicha I.Shell matters: Magnetic targeting of SPIONs and in vitro effects on endothelial and monocytic cell function. Clin Hemorheol Microcirc. 2015;61(2):259-77. doi: 10.3233/CH-151998.) and another, prepared and published during period 4, dedicated to the description of the human artery model (Janikowska A, Matuszak J, Lyer S, Schreiber E, Unterweger H, Zaloga J, Groll J, Alexiou C, Cicha I. A novel human artery model to assess the magnetic accumulation of SPIONs under flow conditions. Sci Rep. 2017 Feb 8;7:42314).

In the in vivo efficacy studies, different nanosystems for atherosclerotic plaque imaging and thrombus therapy were evaluated using different mouse models. P7 found that the newly characterized Dex-SPIONs have remarkably bio-inert and suitable for MRI. The first results were published in early 2017 (Unterweger H, Janko C, Schwarz M, Dézsi L, Urbanics R, Matuszak J, Őrfi E, Fülöp T, Bäuerle T, Szebeni J, Journé C, Boccaccini AR, Alexiou C, Lyer S, Cicha I. Non-immunogenic dextran-coated superparamagnetic iron oxide nanoparticles: a biocompatible, size-tunable contrast agent for magnetic resonance imaging. Int J Nanomedicine. 2017 Jul 24;12:5223-5238) and second in 2018 (Unterweger H, Dézsi L, Matuszak J, Janko C, Poettler M, Jordan J, Bäuerle T, Szebeni J, Fey T, Boccaccini AR, Alexiou C, Cicha I. Dextran-coated superparamagnetic iron oxide nanoparticles for magnetic resonance imaging: evaluation of size-dependent imaging properties, storage stability and safety. Int J Nanomedicine. 2018 Mar 28;13:1899-1915). The nanosystems are now in the process of GMP production.

For atherosclerotic plaque therapy (P8) three clinically relevant simvastatin nanomedicines, including high-density lipoprotein nanoparticles (S-HDL), PEGylated liposomes (S-Lipo), and polymeric micelles (S-PM), were evaluated in ApoE deficient mice, observing distinct biological behavior (Alaarg A, Senders ML, Varela-Moreira A, Pérez-Medina C, Zhao Y, Tang J, Fay F, Reiner T, Fayad ZA, Hennink WE, Metselaar JM, Mulder WJM, Storm G. A systematic comparison of clinically viable nanomedicines targeting HMG-CoA reductase in inflammatory atherosclerosis. J Control Release. 2017 Sep 28;262:47-57).

Concerning thrombus therapy, fucoidan-functionalized rt-PA-polymer nanosystems were formulated by P1. The thrombolysis efficiency was demonstrated in a mouse model of venous thrombosis by monitoring the platelet density with intravital microscopy. rt-PA (Actilyse) injection alone at a dose of 2.5 mg/kg decreased the thrombus density down to 59.0 %, which was not statistically different from the control group. At this dose, free rt-PA was thus not sufficient to generate a pronounced thrombolytic effect. Injection of rt-PA-fucoidan-polymer nanoparticles at a similar dose generated a significant thrombus reduction. The density decreased down to 29.5 % after 30 minutes. Furthermore, the treatment based on rt-PA-fucoidan-polymer nanoparticles was the only one to induce more that 70% of reduction (density <30%) in 4 mice out of 8. It further could be shown that the rt-PA-nanosystems showed similar thrombolytic effects like Actilyse but gained with a 4-times lower dose than the compound. The study supported the hypothesis that fucoidan-polymer nanoparticles improve the rt-PA efficiency and the results were published in period 4 (M Juenet, R Aid-Launais, B Li, ABerger, J Aerts, V Ollivier, A Nicoletti, D Letourneur and C Chauvierre, « Thrombolytic Therapy Based On Fucoidan-Functionalized Polymer Nanoparticles Targeting P-selectin". Biomaterials 156, 204-216 (2018)).

Previously, many efforts have been made to develop animal models that resemble human atherosclerosis as closely as possible, but the currently available animal models all have advantages and limitations. Within the foregoing animal studies of this project, an emerging problem was found with the ApoE mouse model in general, namely that the degree of atherosclerosis which is induced differs strongly even among inbred animals, which are equal in age, diet and environmental conditions. In humans, atherosclerotic plaques development, composition and characteristic also differ strongly from individual to individual. Therefore, a new method of automated balloon pressure control and retraction (JURY-system) was developed by P5 and

tested in a preliminary trial in rabbits. The main rabbit experiments indicated that, compared with manual control, the balloon catheter injury model with regulatory circuit pressure control and automatic retraction showed a superior homogeneity and reproducibility of the endothelial injury and the atherosclerotic plaque formation compared with controlling manually. The JURY technique is currently patent protected and planned for a further enhanced evaluation study in cooperation with P7.

The aim of the last task was to provide a new Magnetic Resonance Imaging (MRI) method dedicated to evaluating the response of plaque inflammation in patients who are under a therapeutic regimen using a nanomedical compound. The method proposed was the use of a Gd(III)-based blood pool agent (B22956/1) that binds albumin, which allows the evaluation of the local endothelial permeability. It could a compound able to stratify atherosclerotic lesions with respect of a different local endothelial permeability, morphology and composition of plaques.

GMP Production of injectable products (WP3)

WP3 was dedicated to the GMP (Good Manufacturing Practice) production of injectable products. The development of any new drug requires the definition of methods to characterize the manufacture and control of both the active drug substance and the drug product. In the context of the NanoAthero project, this corresponds to a nanomedicine capable of being delivered to the clinics in a suitable dosage form allowing its administration to human patients.

NanoAthero supported and organized in WP3 the pharmaceutical grade production of injectable nanoproducts. The final objective of WP3 was to set up the GMP manufacturing process for the proposed nanosystems.

The implementation of the protocol for a GMP production of fucoidan was achieved (Task 3.1). The SPECT imaging nanosystem, a nanostructure composed of fucoidan and 99Tc, was already preclinically-validated, and the GMP manufacturing process was successfully completed in WP3. All the materials, products and the flow-chart manufacturing process with the requirements of the GMP production were completed and validated. A first technical batch has been produced in February 2017 and sent to P15 (Bracco) to perform toxicology and regulatory studies.

With the overall results, the CMC (Chemistry, Manufacturing and Control) was achieved in October 2017 and the IMPD (Investigative Medicinal Product Dossier) documentation was submitted to French and Dutch authorities in November 2017 to obtain approval for the clinical trials. In January, the French agreement from ANSM was obtained. In March 2018, the Dutch agreement from IRB was obtained. The clinical batch was produced in April and the GMP Batch certificate and the Sponsor Batch release certificate were established in June 2018. The GMP Clinical Batch was delivered at the Bichat Hospital (France) and at Amsterdam Medical Center (Netherlands) in June 2018.

B22956/1 0.25 M formulation was completed and is now available (Task 3.2). The analytical characterization demonstrate that the final drug product meets all the required specifications and could be used for an eventual follow-up project. The B22956/1 is a blood pool MRI diagnostic product previously developed achieving the completion of Phase I for another clinical application (MRI coronography). A new GMP manufactured batch was proposed to undertake a new clinical pilot study in the NanoAthero project context on the B22956/1 diagnostic efficacy of atherosclerotic plaques in combination with the imaging procedure. A new API (Active Pharmaceutical Ingredient) batch (1.5 kg) was synthetized and analytically characterized; the compound meets all the required specifications. The batch provided complies with all the required specifications, assuring that the highest quality standards can be fulfilled to transfer the process easily into a future clinical use.

The synthesis of Dex-USPIOs was successfully transferred in a GMP environment, optimizing production of quality controlled large scale batches in the liter range (Task 3.3). During the last period of the NanoAthero project, P7 (UKER) produced the first lab-scale batches of Ultrasmall Superparamagnetic Iron Oxide Nanoparticles (USPIO), coated with cross-linked Dextran. The GLP production was increased in volume to allow synthesis of an amount of Dex-USPIO sufficient for regulatory preclinical studies. For the up-scaling of Dex-USPIO from the laboratory level to the production of larger batches, a dedicated minipilot reactor for cGMP/FDA compliant synthesis was prepared and adapted for synthesis in a cGMP-compliant environment. The synthesis of Dex-USPIOs was successfully transferred into the GMP environment and the nanoparticles were reproducibly fabricated in the litre range. The successfully produced "technical batches" were provided for toxicological studies. All the analytical methods were validated according to ICH guidelines.

Lipidots were produced In WP1 at the lab scale (formulations of 2 mL, 750 mg of lipids) by an ultrasonication process, using products chosen in Ph. Eur or comparable quality. We have developed the manufacturing process on a larger scale (formulations of 300 mL, 100 g of lipids) with a process that could be implemented for industrial application, namely High Pressure Homogenization (HPH). The "large scale" HPH process (150 mL, 56 g of particles produced/batch, 15 g of particles for purification step) was used to produce three batches. Nanoparticles were colloidally stable for more than 300 days when stored at 4°C. The particles displayed no toxicity. Critical Quality Attributes were defined for the F50-DOTA particles. Analytical methods were also developed for particle analysis and qualification, concerning their physical properties, stability, and chemical composition.

WP4 Toxicological data and risk assessment (WP4)

The main objective of WP4 has been to obtain toxicological data of selected nanosystems for thrombus and plaque imaging, thereby establishing the safety of subsequent clinical trials. A further objective of WP4 was a comprehensive hazard assessment of all the NanoAthero systems

Toxicological data for the nanosystems coming from the selection performed in WP1 and WP2 were acquired. In particular, toxicological regulatory studies aimed to prepare the IMPD documentation needed to ask for clinical trials were performed. These studies were executed on two selected nanosystems, namely fucoidan (a SPECT imaging nanosystem proposed by P1 (INSERM) for the thrombus imaging, composed of fucoidan and 99Tc) and dextran-coated iron oxide nanoparticles (Dex-SPIONs) proposed by P7 (UKER) as an imaging nanosystem for plaque permeability.

Task 4.1.1 was focused on the execution of toxicological regulatory studies aimed at preparing the Investigational Medicinal Product Dossiers (IMPD) needed for approval of clinical trials. These studies were performed on two selected nanosystems, namely fucoidan, a SPECT imaging nanosystem (composed of fucoidan and 99Tc), and Dextran-coated iron oxide nanoparticles (Dex-SPIONs). The main objectives have been achieved for both the nanostructures.

For fucoidan there were two studies. 1. A preliminary ascending dose toxicity study in rats was performed in order to identify the dose corresponding to the No Observed Adverse Effect Level (NOAEL) and to the Maximum Tolerated Dose (MTD). The main results showed that for the fucoidan extract formulation the NOAEL was 400 µg/kg, the MTD being higher than 400 µg/kg. 2. A GLP extended single dose toxicity study was performed in rats using three different doses: low (40 µg/kg), mid (250 µg/kg) and high (400 µg/kg) that confirmed the 400 µg/kg NOAEL value. There was no CARPA effect upon i.v. administration in pigs of fucoidan in doses of 1-10-100 µg/kg, in either repeated or single administration

There were two studies also for Dex-SPIONs. 1. A preliminary single dose toxicity study was performed in rats, with four different doses (10, 30, 60 and 100 mg Fe/kg). No clinical signs were observed at 10 and 30 mg Fe/kg, 2. An extended single dose toxicity study was performed with a 14-day recovery period to determine the possible toxicity after a single i.v. bolus No clinical signs were observed for 5 and 10 mg Fe/kg.

In vitro biocompatibility testing of nanoparticles were achieved, via collaborative efforts among WP4 and WP2 by P12. In vitro biocompatibility testing has been made of cell responses to P7 (UKER) iron oxide these nanosystems in static and dynamic settings, which is essential for selection of candidates for future in vivo studies. The pre-processing and stepwise analysis was as follows: 1. Data Pre-processing & Imputations. 2. Applying various schemes to create a standard set of data to enable & ensure reliable analysis. 3. Identifying, evaluating and testing the relevant statistical tools. 4. Applying various tools on each set, combining & verifying the results. Following these procedures, the data were analysed by combining various analysis schemes. These tools were supposed to provide a blockbuster for the full life cycle analysis. It could be an additional feature on top of all the clinical and environmental features, especially regarding human data.

Clinical study for molecular imaging and therapy (WP5)

WP5 was devoted to the proof-of-concept by Phase I clinical trials. For task 5.1 the objective was to conduct the feasibility study for delivery of GMP-produced Nanocort in human patients. In task 5.2 the goal was to evaluate the tolerance, dosimetry and SPECT imaging of 10 healthy control human subjects after injection of 99m-Fucoidan nanosystem. In task 5.3.1 the goal was to evaluate the suitability of using an 'endogenous' HDL particles as nano-delivery systems. To this end, we assessed the local delivery of CER-001 labelled with 89-Zirconium, in atherosclerotic plaques. By comparing local delivery in atherosclerotic plaques (with 'leaky' vasa vasorum) to local delivery in tumors ('leaky' neovascularisation), an optimal insight can be obtained into the promise of apoAI-particles as local delivery vehicles in vivo in humans. In task 5.3.2 the goal was to estimate the accessibility of plaques to nanoparticle delivery, using ultra-small paramagnetic iron particles (USPIOs), evaluated in patients with advanced atherosclerotic disease.

In patients undergoing surgical endarterectomy, we showed that liposomes (when infused intravenously) were delivered successfully into macrophages located deep in the atherosclerotic plaques (assessed after surgical removal of the plaque (Task 5.1). No severe adverse effects occurred. Using the pegylated liposomal platform, we were able to show that an intravenous administration of 'liposomes' results in delivery of the liposomes in macrophages residing in atherosclerotic plaques. We evaluated whether liposomal prednisolone, a widely respected anti-inflammatory agent, was able to reduce the inflammatory activity in atherosclerotic plaques.

Following convincing experimental data, fucoidan was elucidated as a potent 'binding agent' for P-selectin; the latter is abundantly present in the vulnerable arterial lesions, where active fibrin clot formation occurs. The IMPD (Investigative Medicinal Product Dossier) documentation, the Investigator's Brochure and the protocol were submitted to French and Dutch authorities in November 2017 to obtain approval for the clinical trials. In January, we obtained the French agreement from ANSM and the Dutch agreement from IRB in March 2018. We succeeded in producing the first batches of GMP 99mTc-Fucoidan in April 2018. The GMP Clinical Batch was delivered at the Bichat Hospital (France) and at Amsterdam Medical Center (Netherlands) in June 2018. The clinical trial started in Amsterdam at the end of June. We infused labelled Fucoidan, followed by whole-body scintigraphy and SPECT imaging on sequential time points during the first 24 hours after injection. In 10 healthy controls, 99mTc-fucoidan was infused (Task 5.2). Tolerability was good, no severe adverse effects occurred. hese data will provide us detailed insight into the suitability of the Fucoidan tracer for future use in patients with advanced atherosclerotic plaques and thrombosis.

Regarding choice of platforms, we also evaluated the endogenously available HDL particle as a potential nano-delivery platform task 5.3.1. To this end, we used an apoAI-reconstitute particle, CER-001, labelled with Zirconium. In patients with advanced atherosclerotic disease, we were able to show increased 'delivery' of the CER001 particle in those sites in the arterial wall, where atherosclerotic plaques were present. In comparison, lower signal for CER001 was observed at arterial sites without atherosclerotic plaques.

We established in task 5.3.2 a clinical protocol to quantitatively assess ultrasmall superparamagnetic iron-oxide (USPIO) nanoparticle delivery to carotid plaques in patients with atherosclerosis. We successfully assessed the relation between an MRI-based technique to estimate arterial wall permeability (dynamic contrast-enhanced MRI (DCE-MRI) and delivery of the USPIO nanoparticles in patients with advanced atherosclerotic disease in peripheral arteries.

Ethics and Regulartory files (WP6)

Ethics in all steps was rigorously defined and managed in WP6. WP6 also included regulatory dossiers for clinical research in humans. An essential part of the ethical overview of the work of the NanoAthero project has been for P 16 to follow the scientific development of the project throughout, interacting with partners and the project as a whole, to understand the ongoing results and assess the wider implications. P 16 has attended all the Steering Group and Consortium meetings, as well as some WP1/2 meetings, and visited most of the partner establishments. He has commented when appropriate on ethical implications, for example, when changes were proposed to a programme of animal experiments. Thus he advised that the use of additional animals to test the efficacy of the JURY device was ethically justified, because the device represents both a significant refinement to the balloon injury procedure in rabbits, and should lead to overall reductions in the numbers of animals required.

In parallel with the more general ethical advisory role, a key role of P 16 has been to visit all the facilities of the Partners performing experiments within NanoAthero. It has been very useful to compare the facilities and cultures of the various facilities in the project. The project documentation showed a commendable approach to the 3R's principles. The standard of care for the animals has been found to be consistently high. Animal experimentation always involves an ethical dilemma of whether the harm caused to the animal is justified by the benefit that might realistically be expected from the experiment. The induction of atherosclerosis is a significant harm not only in the direct sense that the animal will suffer pain and other distress, but also in the wider sense of respect for the integrity of the animal, in that it is being inflicted with a human disease that it would not normally suffer. The justification in the case of the NanoAthero project is the pressing need to address the disease atherosclerosis, that is the biggest single cause of death in the European population, and especially to offer a means of prediction and clinical intervention for people who are most vulnerable to a heart attack or stroke. For this balance of harms and benefits to be valid, however, the pre-clinical trials need to be as representative as possible of the likely human clinical situations in which imaging or treatment would be performed. It is clear that the current animal models for atherosclerosis have limitations, not least in having to induce the condition artificially in an animal that would not normally suffer from it, but also in size and species differences. For example, some doubt emerged over the ability of the ApoE mouse model to predict sufficiently the permeability of human plaques in a therapeutic context. But unless the animal models used would be clinically misleading, it is better to use them than to have no model at all.

In the current level of knowledge, and the detailed attention to both efficacy and risk aspects, the systems examined in NanoAthero were considered ethically to be a valid stepping stone towards clinical trials in humans. As more is understood from such human clinical trials, it should be possible to feed back the understanding into creating more refined animal models. Future projects in this field should also be prepared to consider 'organoids' as an adjunct to the proposed animal models.

P16 assessed the ethical case for two particular nanosystems, namely Fucoidan Tc-99m and Dex-USPIO for thrombus and plaque imaging, respectively. The evidence of likely efficacy, and the suite of toxicity tests and other risk assessment measures examined in WP4 and WP2 justified the animal experiments involved. Together with the capability for production of both systems under GMP conditions, the outcomes are considered sufficient, on ethical grounds, to present the case for clinical trials to the relevant authorities in the countries concerned. In the context of reported problems with current agents, the results with the Dex-USPIO system was particularly encouraging as a candidate MRI contrast agent for imaging human atherosclerosis patients.

Although the competence for authorising specific clinical trials resides in the relevant national authorities, the potential participants require appropriate information in understandable form to give their informed consent. Partner 16 discussed the French draft proposed clinical trial protocol (P2) and (P1), and made some useful recommendations to explain better the benefits of the study in the text of the documents given to potential trial participants. This led to a very fast agreement by the French Ethics Committee and by the French Regulatory Agency (less than two months) of the NanoAthero clinical dossier.

Dissemination, Training and Exploitation (WP7)

Dissemination: A State of the Art Session on Nanomedicine for Atherosclerosis and Cardiovascular Disease was held at each "**CLINAM Annual Summit**" (the annual conference on Nanomedicine, Targeted Delivery and Precision Medicine of the European Foundation for Clinical Nanomedicine, Basel, Switzerland) from 2014-2018. Several short presentations about the NanoAthero project were selected by the coordinator, followed by a well-animated debate with questions and statements from the audience. The annual CLINAM summit is the largest event in Europe on nanomedicine, the interdisciplinary participants include 30% of clinicians, a most relevant group for future application of the outcomes and also a mix of all stakeholders, including industry, policy makers and regulatory agencies. Therefore this event represented one of the key elements for the dissemination of the project.

In order to have a visible output of the project that can be used e.g. for dissemination also after the project we produced a short graphic video on NanoAthero's objectives and some of its results. The video is available on a dedicated **YouTube channel** and on the NanoAthero website. The video exists also with English subtitles for dissemination on social media. https://www.youtube.com/watch?v=eQ9dLpzcxtI

The website of NanoAthero includes general information on the project and its partners, and also a news section which was regularly updated with information on our workshops and meetings, and news in the media about the project, whenever possible enhanced with photographs. Under the section "achievements" we made available the publishable summaries of the periodic reports as well as a list of all of NanoAthero's publications. Video recordings of the presentations of some of the principal investigators of the project made at the CLINAM summit 2014-18 are available in the video section of the NanoAthero website.

Three one-day international workshops with external speakers have been organized by the project in the last five years on NanoAthero:

1. January 2017 in Barcelona under the title "Mutual understanding each other's projects in

Atherosclerosis to the benefit of patients" which was focused on seven EU-funded projects in progress in this field, exchanging the various approaches to Atherosclerosis, reaching out for co-operation between the different groups.

2. January 2018 in Rome under the title " Mutual understanding of projects in Atherosclerosis on international Level between Participants from Europe, USA and Asia". European projects in Atherosclerosis were presented in a comparison with relevant international studies. Experts from Korea and the USA were

invited to present their state of the art followed by presentations of the state of the art of NanoAthero, and with a follow-up discussion on this international level.

3. June 2018 in Genova under the title: "Pathways of Working within NanoAthero Successes and Hurdles & Partnering Reflections in Atherosclerosis Involved Groups". The young scientists of the NanoAthero project, having done the work in their laboratories, were invited to speak about their results and how they see the entire project's successes but also its hurdles. Three external senior scientists from Glasgow, Aberdeen and Bonn were also invited to give presentations on their own Atherosclerosis projects.

The three workshops that had been organized within the project's lifetime not only enriched the participants with more knowledge on what is currently going on in terms of research in our field but also gave the possibility to exchange informally and to network. Some of those new contacts have already enabled a co-operation or closer exchange between certain participants.

Stakeholder and public engagement: The public understanding and acceptability of novel nanosystems in medical treatments is a significant factor. While in general patients welcome new scientific developments with a realistic hope of addressing their condition, nanotechnologies remain largely unfamiliar to European citizens, and have aroused some public concerns as to potential risks to health and environment. These factors and the principles of Responsible Research and Innovation, to which EU research programmes are required to respond, point to a need for constructive and imaginative engagement with publics. The NanoAthero project has sought to address these ethical and social factors by including in its consortium an expert in ethical issues and public engagement, P 16 Edinethics, to perform ethical assessment throughout the project in Work Package 6, with the assistance of an Ethical Advisory Board (EAB), and to develop a public engagement tool in the form of a Democs card game in WP7.

The Democs card game has been created by Partner 16 as part of the dissemination of the project in WP7, to engage with both general publics and likely patients with both the science and the ethics of nanomedicine. The Democs concept was developed by Perry Walker of the New Economics Foundation in 2002, together with Partner 16 Edinethics and others. Since then, the concept (also called Decide) has been used extensively to engage lay publics at a grassroots level on a wide range of technological issues.

The game is played by groups of 6-8 people, and assumes no prior knowledge of the subject. To introduce the subject, case studies (Story Cards) are presented, which introduce imaginary individuals involved in or impacted by the technology, who have to make decisions in complex and realistic situations. Examples in this game include a heart attack patient, a stroke patient carer, a heart surgeon, the CEO of a biomedical start-up company, a member of an ethical committee, and so on. Information Cards then explain the basic scientific and medical basis of NanoAthero, why nanoparticles are being used, how they are tested, the need for animal experiments and clinical trials, etc. Issue Cards then explore the ethical and social dimensions, both positive and negative, which the group might wish to consider. Each group discusses the cards and gives both collective and individual opinions on questions on nanomedicine and the NanoAthero project. The outputs are in the form of consensus statements made by the group as a whole, their choices of cards to be discussed, and also individual opinions on which developments they would welcome, could 'live with', or would wish to reject, using their own words. Together these outputs provide valuable qualitative data which can be fed back to the task leader for analysis.

The cards were written in draft form, drawing upon information from the NanoAthero project partners, the ethical experience of Partner 16 and other expert sources. A pilot game was produced and trialled. From the feedback, the game content was finalised, designed and printed as boxed sets, first in English and then translated and printed in French, German and Dutch versions. The games were distribution to all partners and those who had expressed an interest in seeing and playing the game. The game has also been made available on the NanoAthero project website and the Edinethics website (Partner 16)

www.edinethics.co.uk, as a set of PDF and Word files, capable of being downloaded and printed out by users, under a Creative Commons licence. An adapted version will be added to the recently re-established collective website of downloadable Democs games, www.playdecide.eu, established in the EC FP7 DECIDE project. Partner 16has demonstrated the game at talks and poster displays at the Clinam European Clinical Nanomedicine conferences in Basel, 2013-2016, and presented the finished game and some of the results of playing the games at the CLINAM summit in 2018.

Several games have already been played in different languages with over 80 participants, including Edinburgh, Hereford, Paris, Erlangen, Aachen and Basel, and a preliminary analysis has been made of the results submitted to date. It is anticipated that further playing of the games will continue beyond the timescale of the NanoAthero project, since the game has been created to be a permanent free tool for public engagement on nanomedicine issues.

Training: A survey had been conducted at the start of the project among the partners on the needs for training. Most training exercises were then organized mutually between partners by exchanging staff from their labs for one or two days. This proved to be the most effective and interactive way of trainings, especially for the young researches involved in the project's work. We also proposed to NanoAthero members an official 4-day training course programme organized by P7 UKER on the topic "Endothelial compatibility of nanosystems: In vitro methods ". The training ran from Monday noon to Thursday noon and in order to assure an adequate setting. Two participants took part (from P9 and P1).

P11 CLINAM organized on the Sunday before the annual CLINAM conference in 2016 a "Youngsters day" for young researchers from the consortium with the aim to provide them with an overview of the current work in the field of nanomedicine in atherosclerosis and make them exchange and discuss on their work and results in the NanoAthero project. The session was chaired and animated by Prof Letourneur. The participants to that session were also granted by CLINAM a free access to the first day of the CLINAM conference 2016 in order to give the young researchers the possibility to benefit also from the other sessions of the conference and to network.

In 2015, P3 initiated a fellowship and external trainings program in order to allow exchange between the partners' laboratories and the learning of new techniques for young researchers:

Fellowship program: The fellowships are short-term visits to partners' laboratories and institutions aiming at strengthening collaboration within the consortium and supporting the exchange of specific expertise. The fellowships were open to students, post-docs and young clinicians from any NanoAthero partner. During the lifetime of the project one fellowship was granted to a young researcher from P1, who spent 2 weeks at P7's laboratory.

External trainings support: The objective was to support the training of early-stage researchers by helping to finance their participation in external training courses and workshops in the field of Nanomedicine, organised by institutions outside of the consortium. The maximum budget per support was 2000€.

A total of four training supports were granted during the project's lifetime, two for P13 and two for P8. The topics of the external training courses were the following: 1) Small Animal Imaging; 2) Risk management and analysis for medicinal products according to DIN EN ISO 14971:2013 3) Selecting the Right Enabling Technology at the Early Stage of Development; 4) Regulatory Landscape of Complex Drug Products

Intellectual Property Management and exploitation: The Consortium agreement laid down the principal terms of Intellectual Property (IP) including any excluded background and was signed by all partners before the start of the project. A project internal handbook to IP issues was edited by P3 and sent out to all

partners and stored on the extranet platform. The handbook gave some essential knowledge about IP issues and raised awareness on IP issues that might come up during the course of NanoAthero and that researchers are asked to pay attention to. The Intellectual Property (IP) of the project has been monitored throughout the project's lifetime, no IP issues were detected. The project has resulted in at least one patent submission (Jury Device by P5) but others might follow after the project's end.

In period 2, P3 organised an exploitation seminar via the ESIC service of the NMP programme in order to prepare the partners for the time when exploitable results will start coming in. The exploitation seminar took place in Milan directly after the consortium meeting on the 19th of January, with all partners present. In preparation for this exploitation seminar the ESIC expert asked the partners to provide information on the Key Exploitable Results (KER) of their work in the project, and provided the project partners with very useful templates to document these. Four partners identified KERs for their results and worked on them with their internal IP Management offices. The final exploitation plan of the project, lays down the different identified results of NanoAthero that will be followed up and exploited in a variety of ways after the project's lifetime. As an example, at the time of this report writing, several joint project submissions have been submitted by P1 and P7.

Potential impact and exploitation of results

The NanoAthero project aimed to demonstrate the clinical proof-of-concept and validation of nanosystems for targeted imaging of advanced atherosclerotic diseases in humans. In fact, in acute coronary syndrome and stroke, atherosclerotic plaque disruption with superimposed thrombosis, is the leading cause of morbidity and mortality, worldwide. New nano-imaging agents will allow non-invasive, molecular imaging of key pathological processes in vulnerable plaques using state-of-the-art multi-modality imaging. The nanosystems integrate a set of unique features: a carrier, a targeting moiety, an imaging agent and/or a drug. For imaging of thrombus and vulnerable atherosclerotic plaques, existing imaging techniques can be employed using nanosystems targeting key pathological events such as thrombus generation and increased inflammatory activity.

The potential of nanotechnology-based therapies to overcome the disadvantages of systemic drug administration has been well recognized in the field of oncology, but no specific nanoparticle-based system has yet been approved for diagnosis or therapy of cardiovascular diseases. In NanoAthero, we provided proof-of-concept for efficacy and systemic safety of Nano-delivery in patients. By bridging research centres and companies, NanoAthero creates a multidisciplinary environment for research, education and for transfering innovation and nanotechnology from "bench to bedside".

The main clinical outcomes of the NanoAthero project are nanosystems for diagnosis (imaging) or therapy, validated in Phase I clinical trials. This is a world "premiere" because no nanomedicine approved so far either in the US or in Europe is addressing the cardiovascular risk. Early diagnosis and efficient therapeutic treatment will significantly improve the health of EU-citizens and decrease the overall costs. It has also become clearer that not only plaques are the pathological targets in atherosclerosis but other tissues (e.g. lymphoid) can play an important role in the disease etiology. The use of targeted nanocarriers represent a major step towards targeted therapies in cardiovascular applications, and later on to some other major diseases like auto- and allo-immune diseases, infectious diseases, and cancer.

With the development and identification of different nanosystems of interest for cardiovascular applications, the Nano-Athero project enabled a decisive step towards the use of innovative nanosystems for improving cardiovascular disease diagnostics and therapy. In NanoAthero, we have constructed an imaging agent, fucoidan, with high affinity for sites of increased clot formation, facilitated by the interaction between Fucoidan and P-selectin. The IMPD (Investigative Medicinal Product Dossier) documentation, the Investigator's Brochure the study protocol, patient information leaflet and consent

form and CRF were submitted to French and Dutch authorities and authorizations were obtained to have the Phase I clinical trial with batches of the GMP grade Fucoidan. These data will provide detailed insight into the suitability of the Fucoidan tracer for future use in patients with advanced atherosclerotic plaques and thrombosis. This product is now considered for Phase II clinical trials expected to start in 2019.

Using fucoidan, we could also add to the functionalized nanoparticles a contrast agent to achieve the theranostic approach for preclinical and clinical investigations. We thus plan to exploit the drug-loaded nanoparticles coated with Fucoidan. The obtained preclinical study supports the hypothesis that fucoidan-nanoparticles improve the rt-PA efficiency. Possible targeted clinical applications could be for instance in sickle cell-related pain crises. During inflammation, P-selectin is expressed on the surface of activated platelets and of the endothelium resulting in (i) abnormal rolling and static adhesion of sickle erythrocytes to the vessel *in vitro*, (ii) the prompt adhesion of sickle erythrocytes to vessels and the development of vascular occlusion in transgenic mice with sickle cell disease, and (iii) the binding of activated platelets to neutrophils to form aggregates in a P-selectin-dependent manner in mice and humans with sickle cell disease. Furthermore, the microvascular blood flow is increased in patients with sickle cell disease with the administration of sufficient doses of heparin to block P-selectin. These data support the concept that to reduce the risk of vaso-occlusion, inflammation, and sickle cell-related pain crises P-selectin should be blocked.

In NanoAthero, we have demonstrated in human that liposomes can be used to increase local drug delivery into the atherosclerotic plaque. We showed delivery of GMP-pegylated liposomes into plaque macrophages. No adverse effects occurred. In patients undergoing surgical endarterectomy, we showed that liposomes infused intravenously were delivered successfully into macrophages located deep in the atherosclerotic plaques. We also showed in another NanoAthero clinical trial that HDL-like nanoparticles could have a high potential as delivery platform. HDL-cholesterol is a nano-particle, consisting of an liposomal layer carrying an apolipoprotein A-I. The apoAI has a high affinity for the membrane transporter ABCA-I which serves as a translocator for cholesterol and fosfolipids from the 'target cell' into the particle. When drugs can be 'hidden' inside the core of the HDL particle, these drugs selectively target the organs able to bind the HDL particle. These organs include ABCA1-carrying cells, predominantly the inflammatory cells (macrophages) and the liver cells. Interestingly, these promising findings of delivery of the HDL-nano-carrier platform into plaques also hold a promise for other highly frequent diseases, including cancer. Indeed, preclinical research revealed that HDL-particles establish delivery of their payload to tumor-cells (expressing ABCA1 on their surface), as well as to tumor-associated macrophages. Early experience of infusion of labeled HDL into patients with esophageal cancer confirmed the delivery of HDL into tumor locations. Further research should target the use of these safe HDL-like particles as local delivery agents in various disease states in humans, where increased compound delivery is hampered by potential systemic side effects. We expect to be able to continue the development of the lipid-based-platform as a non-toxic delivery platform in either cardiovascular or oncological areas.

Concerning imaging applications, we evaluated the potential of a routinely available imaging technique to predict local delivery of nanoparticles and to pre-identify patients, in whom the nanoparticles will have favorable local delivery. Using an MRI technique able to predict ‘arterial permeability’, we set out to correlate nanoparticle delivery to this DCE-MRI technique to preselect ‘hyperresponders’, introducing the option of future tailored therapy in cardiovascular nanomedicine. In this context, the analytical characterizations of B22956 formulation prepared in a non-GMP process for preclinical purposes only, demonstrate that the final drug product meets all the required specifications and could be used for a follow-up project. Another NanoAthero nanosystem, Dex-USPIO for magnetic resonance imaging was successfully applied in imaging AS-plaques in rabbits and is now upscaled in GMP-compliant environment for further clinical trials in screening plaque permeability. In humans the aim of the DEX-USPIOs would be to identify the permeability, so ‘at risk’ plaques for better patient screening, which would allow to forecast

whether the patient's plaque would be suited for therapy. The widely used USPIO Ferumoxytol has been removed from markets in the EU, which leaves an opportunity for the 'safer' USPIOs.

There is a also need for better and more reliable preclinical models. For reduction of animal experiments by increasing reliability of the results, constant pressure injury in preclinical athero-models was defined as a key issue. We showed that the manual method leads to inflation pressure variations during the passage of the catheter and this potentially introduces different degrees of injury severities. The new pressure controlled device named JURY has been designed to improve the balloon injury in the abdominal aortic vessel of New Zealand White rabbits (NZWR). This patented approach helps to generate more defined, reproducible atherosclerotic (AS)-plaques and prevents from varying degrees of injuries. Already performed JURY assisted balloon injuries in vivo (rabbits) indicated significant superiority compared to the manual injury procedure. Concerning clinical application, we could expect a machine-assisted endarterectomy to prevent endothelial damage as a further possibility for application. It will allow recording and monitoring of pressure and flow rate data during balloon thromboembolectomy in human.

Another important output from the whole NanoAthero consortium was the establishment of selection criteria for Nanosystems. Thanks to the elaborate experimental workflow that was developed within NanoAthero, the goal to identify potential candidate nanosystems for the imaging of plaques and for the visualization of thrombosis was achieved. We are convinced that upon clinical approval of these particles, radiolabelled fucoidan will contribute to improved in situ detection and evaluation of thrombosis, while Dex-USPIO could be a safe contrast agent to characterize atherosclerotic plaques, infarcted myocardium and aneurysms by MRI. In order to share our experiences and the methodologies used, the consortium published for the European Society of Cardiology guidelines on "From Design to the clinic practical guidelines for translating cardiovascular nanomedicine". Those guidelines could serve as a new reference for the selection of nanosystem in nanomedicine.

In NanoAthero, we have been able to validate safety and efficacy of several diagnostic or therapeutic nanoparticle platforms in healthy control subjects (Phase I) as requested in the original EC call. After 5.5 years of operation, the NanoAthero project has delivered a huge amount of scientific results (> 60 publications), which will serve for further exploitation. Furthermore, all partners have increased their expertise in Nanomedicine, educated students and future researchers/industrials and created a strong cooperation that will continue even after the end of the project. The exploitation on Lipid-based nanosystems, DEX-SPIONs and Fucoidan as well as the JURY assisted balloon system will also give opportunity to drive jointly those products to market authorization and use in patients. The final implementation of GMP processes of new nanosystems designed to propose novel strategies to improve imaging of cardiovascular pathologies could have a high impact on the future development of nanotechnology in atherothrombotic diseases. Moreover, the clinical demonstration of safety complying all EU regulations combined with efficacy of these nanocarriers in humans will prompt the pharmaceutical industry to embrace these novel approaches and provide the EU society with rational criteria to support further developments. In addition to industrial developments, we strongly believe that NanoAthero will benefit to the patients, reducing the burden on careers and strains on healthcare systems.