

# PROJECT FINAL REPORT

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SaveMe

**A Modular Active Nano-Platform for Advanced Cancer Management: Core Nanosystems, Tumor Targeting and Penetration, Molecular Imaging & Degradome based Therapy**

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## Executive summary

Cancer is the second most common cause of death in the EU a figure that is expected to rise due to the ageing European population. Although significant advances are being made in the fight against the disease, cancer remains a key public health concern and a tremendous burden on European societies<sup>1</sup>. Cancer cost the EU €126 billion in 2009, with health care accounting for €51.0 billion (40%). Productivity losses because of early death cost €42.6 billion and lost working days €9.43 billion. Informal care cost €23.2 billion.<sup>2</sup> SaveMe project addressed major urgent needs for cancer diagnosis and treatment, by exploiting partners' expertise, vast experience and most recent research achievements.

SaveMe designed, developed and validated novel **modular nanosystems platform integrating advanced functionalized nano-core particles and active agents**. SaveMe validated the new platform with a proof of concept in **PANCREATIC CANCER**: Pancreatic cancer has the highest one-year mortality rate of any cancer and is Europe's sixth deadliest cancer<sup>3</sup>. The overall five-year survival rate is 4%, and was not improved during the last 25 years.<sup>4</sup> Most pancreatic tumors are detected late, at metastatic stage and 85% are unresectable at the time of detection.<sup>5</sup> The consortium led by Tel-Aviv University (Israel) included 20 partners from Germany, Italy, UK, Spain, Sweden, Belgium, Austria and Russia. The work was organized within five main technical Workpackages. The dissemination activities included participation in scientific conferences and numerous publications in the scientific journals.

In WP1, innovative nanocarriers (NCs) were fabricated from a variety of materials including small magnetic MRI-enabling metal cation-doped maghemite ( $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>)-based nanoparticles (NPs), non-toxic PLGA-*b*-PEG-NH<sub>2</sub> polymeric and hybrid magnetic functional nanocarriers (f-NCs), protein-based NPs made of recombinant or natural human serum albumin (HSA), **PDMAEMA and PMAAc** polymer-based f-NCs synthesized by an innovative cross-linking click-chemistry process, including various functional linkers, and biodegradable hybrid PLGA-based f-NCs. The most promising NCs underwent successful scale-up and decoration with human pancreatic tumor targeting species (somatostatin analogues and/or tPA (tissue plasminogen activator) and with biologically active payloads for therapy (siRNA for gene silencing and with Fab anti-MMP antibodies). In WP2, two approaches were applied to develop targeting moieties for pancreatic cancer: Somatostatin analogs and Galectin-1 based targeting moieties to allow imaging of tumor and to serve as a therapeutic vehicle for siRNA delivery and targeted protein-expression silencing. Sixteen novel somatostatin analogue peptides have been designed and synthesized and most had high binding affinity at least to one or several SSTRs. *In vivo*, functionalized NCs were found to demonstrate selective uptake by the tumor compared to normal tissues. Galectins, including Gal-1, are upregulated in PaCa-cells and in pancreatic cancer precursor lesions and are not expressed in adjacent normal tissues. NCs functionalized with the tPA-peptide showed up to 90% binding to cells as assessed by fluorescence. In WP3, novel non-classic drugs based on cancer degradomics were developed. Two parallel strategies have been used for the development of new therapeutic warheads to be loaded onto the SaveMe NCs, namely small interfering RNAs (siRNAs) and function-blocking antibody fragments (Fabs). Key genes were selected by bioinformatics search and included MMP-7, MMP-14, S100P and PLK-1 as principal targets. A major technological advance of the project was the development of NCs that can carry siRNAs into cells with high efficiency and low toxicity. These oxidized CAN-maghemite NCs effectively delivered siRNAs against MMP-7 and S100P into human pancreatic carcinoma cells *in vitro*, resulting in strong suppression of the specific target genes. The strongest apoptosis induction was achieved with NP-mediated delivery of siRNAs against polo-like kinase-1 (PLK-1). The second therapeutic strategy was the successful development of novel Fabs that specifically target MMP7 and MMP-14, that retain their function-blocking effectiveness *in vitro* when coupled to NPs, indicating their potential for *in vivo* preclinical testing. In WP4, *in-silico* and *in-vitro/in vivo* safety and efficacy analysis were performed. In the *in silico* studies, two physiologically based pharmacokinetic (PBPK) models, flow restricted (perfusion rate limited) and membrane-limited models, were developed in order to understand and predict biodistribution of NPs in different organs with time. *In vivo* studies in mice were conducted to support the results of *in-vitro* tests. Acute toxicity experiments demonstrated that positively charged NCs induced embolism when injected in high doses, while negatively charged ones were much safer. High doses of all types of NCs injected *i.v.* accumulated in the liver. Biodistribution studies demonstrated that small <100 nm NS accumulated in tumour significantly better than larger >100 nm NS. In WP5, the consortium developed an imaging-based diagnostic tool based on targeted NCs for the early diagnosis of pancreatic cancer. One of the particles showed promising imaging capabilities, and allowed the visualization of the tumour *in vivo* by SPECT with acceptable contrast up to 48 hours after administration. In parallel, a reconstruction process based on Monte-Carlo simulations to achieve better contrast images was developed. This development was paralleled by the development of a miniaturized control unit coupled to a hand-held gamma camera. CAN-maghemite NCs uploaded with siRNA against PLK-1 can stop tumor progression as demonstrated in initial set of *in vivo* experiments in mice bearing SU.86.86 eGFP tumors.

In conclusion, SaveMe's research and development outputs have a tremendous potential to be successfully translated into clinical practice, thus providing more precise and efficient detection, diagnosis and treatment of pancreatic cancer.

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<sup>1</sup> Eurostat 2008

<sup>2</sup> Ramon Luengo-Fernandez, et al., Economic burden of cancer across the European Union: a population-based cost analysis. The Lancet Oncology, Volume 14, Issue 12, Pages 1165 - 1174, November 2013

<sup>3</sup> Michaud DS. 2004. Epidemiology of pancreatic cancer Minerva Chir. Apr; 59(2):99-111

<sup>4</sup> Jemal A, et al., Cancer statistics, 2004. CA Cancer J Clin 2004; 54:8.

<sup>5</sup> Francis, I., Role of CT and MR in detection and Cancer Imaging, 4, 10-14 (2003)

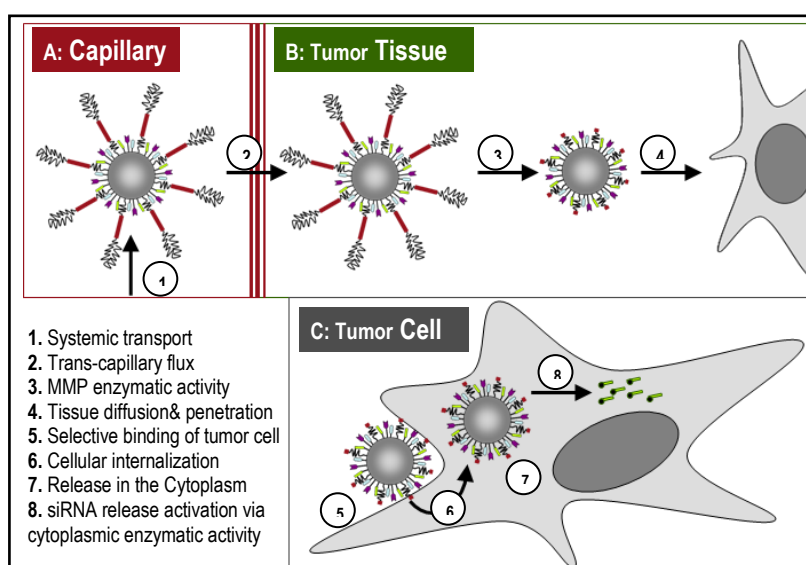
## Summary description of project context and objectives

The overall goal of the **SaveMe project** is to develop novel nanoparticles that can be used to deliver imaging agents or therapeutic drugs to pancreatic cancers.

**Why pancreatic cancer?** It is one of the most deadly cancers with only 4% of patients surviving 5 years after diagnosis, and the prognosis has not changed for several decades despite improvements in the treatment of many other forms of cancer.

**Why nanoparticles?** Nanoparticles are particles of any composition (they can be organic or inorganic) that are in the size range of 1-100 nanometers (ie a billionth of a meter to 100 billionths of a meter). To put this in perspective, a typical human cell might be about 30 micrometers in diameter, a bacterium 1-2 micrometers – so these are less than one-tenth the size of a bacterium. This gives them some interesting properties and allows chemists and cancer biologists to put interesting things inside them and on their surfaces.

Cancer is the second most common cause of death in the EU– a figure that is expected to rise due to the ageing European population. Although significant advances are being made in the fight against the disease, cancer remains a key public health concern and a tremendous burden on European societies<sup>6</sup>. Cancer cost the EU €126 billion in 2009, with health care accounting for €51.0 billion (40%). Productivity losses because of early death cost €42.6 billion and lost working days €9.43 billion. Informal care cost €23.2 billion.<sup>7</sup> Thus, SaveMe project is addressing major urgent needs for cancer diagnosis and treatment, by exploiting partners' expertise, vast experience and most recent research achievements. SaveMe proposes the design and development of novel **modular nanosystems platform integrating advanced functionalized nano-core particles and active agents**. The modular platform will enable the design of diverse active nanosystems for diagnostic or therapeutic applications as defined by their active agent compositions: ligating only crucial active agents, at the optimized composition per application, while excluding other agents to minimize toxicity risks.



**A schematic presentation of tumor perfusion and drug delivery by SaveME's active agents**

### The objectives

*SaveMe main goal focused on the development of brand new generic nanosystems, which act as advanced molecular imaging agents or as delivery vehicles of highly potent non-classic drugs, for cancer diagnosis, guided surgery and therapy. The consortium will study, test, select and optimize the proposed nanosystems using primarily in-silico and in-vitro advanced models, leading to the final in vivo step with a minimal animals study.*

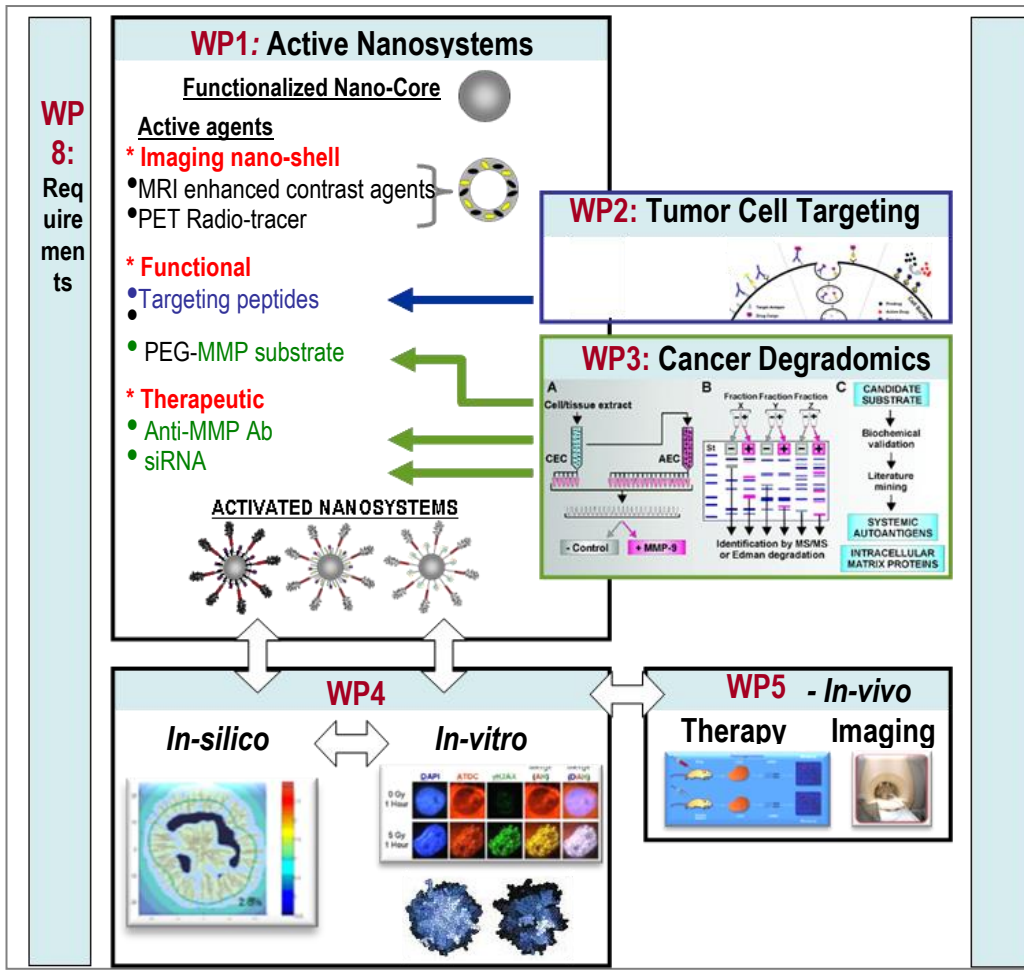
1. Requirements and Specifications (R&S) per regulatory guidelines and constrains
2. Development of generic nano-core platform activated for molecular imaging and drug delivery
3. Cancer selective targeting and diffusion active agents
4. Novel non-classic drugs (nucleic acids and antibodies) and personalized therapy based on cancer Degradomics

<sup>6</sup> Eurostat 2008

<sup>7</sup> Ramon Luengo-Fernandez, et al., Economic burden of cancer across the European Union: a population-based cost analysis. The Lancet Oncology, Volume 14, Issue 12, Pages 1165 - 1174, November 2013

5. *In-silico* & *in-vitro* analysis: safety & efficacy of various active imaging and therapeutic nanosystems
6. *In-vivo* proof of concept – minimally required studies of selected prototypes



**Project layout**















**Expected breakthroughs were:**

- (1) Superior active nanosystems for targeted molecular MR, PET sequential and simultaneous imaging, SPECT and gamma camera, enabling highly sensitive diagnosis and guided surgery;
- (2) Active non-classic anti-tumor antibodies and nucleic acid based therapies based on cancer degradome / "protease web" studies, enabling efficient therapy by the inhibition of tumor cell growth, invasion and metastasis;
- (3) Profiling individualized degradome can be implemented for personalized therapy;
- (4) Proven concept of the designed nanosystems activated with targeting peptides, PEG –MMP-substrate-PEG agent, enabling targeted diagnosis, and targeted therapy.

**The consortium** included partners from European countries, Israel and Russia:

No.	Organization name	Acronym	Country	website	
1.	Tel Aviv University Prof. Louis Shenkman-Coordinator; Prof. Ehud Gazit	TAU	IL	<a href="http://www.tau.ac.il">www.tau.ac.il</a>	
2.	Wilhelminenspital Prof. Peter Knöll Prof. Siroos Mirzaei	WSP	AT	<a href="http://www.wienkav.at/kav/wil/">www.wienkav.at/kav/wil/</a>	

3.	<b>UniversitätsKlinikum Heidelberg</b> Prof. Hans-Ulrich Kauczor Dr. Klaus Felix	<b>UKH</b>	DE	<a href="http://www.uni-heidelberg.de/">www.uni-heidelberg.de/</a>	
4.	<b>Bar-Ilan University</b> Prof. Jean-Paul Lellouche Prof Shulamith Michaeli	<b>BIU</b>	IL	<a href="http://www.biu.ac.il">www.biu.ac.il</a>	
5.	<b>University of Bologna</b> Prof. Mauro Comes Franchini	<b>UNIBO</b>	IT	<a href="http://www.eng.unibo.it">www.eng.unibo.it</a>	 ALMA MATER STUDIORUM UNIVERSITÀ DI BOLOGNA
6.	<b>Johann Wolfgang Goethe-Universität</b> Prof. Dr. Joerg Kreuter	<b>GU</b>	DE	<a href="http://www.uni-frankfurt.de">www.uni-frankfurt.de</a>	
7.	<b>Nanosystem LTD</b> Dr. Svetlana Gelperina	<b>NAN</b>	RU	<a href="http://www.nanosystema.ru/en/">http://www.nanosystema.ru/en/</a>	
8.	<b>CIDETEC</b> Dr. Iraidia Loinaz	<b>CID</b>	ES	<a href="http://www.cidetec.es/">www.cidetec.es/</a>	
9.	<b>CIC biomaGUNE</b> Dr. Jordi Llop Dr. Torsten Reese	<b>CIC</b>	ES	<a href="http://www.cicbiomagune.es">www.cicbiomagune.es</a>	
	<b>COLOROBBIA</b> -terminated	<b>COL</b>	IT		
10.	<b>Sheba Medical Center</b> Prof. Arie Orenstein	<b>SHEBA</b>	IL	<a href="http://www.eng.sheba.co.il">www.eng.sheba.co.il</a>	
11.	<b>University of East Anglia Studies, School of Biological Sciences</b> Prof. Dylan Edwards	<b>UEA</b>	UK	<a href="http://www.uea.ac.uk">www.uea.ac.uk</a>	
12.	<b>Technischen Universität München - Klinikum rechts der Isar</b> Prof. Dr. rer. nat. Achim Krüger	<b>TUM</b>	DE	<a href="http://www.med.tu-muenchen.de/sprache/englisch/index.php">www.med.tu-muenchen.de/sprache/englisch/index.php</a>	
13.	<b>Katholiek University of Leuven</b> Prof. Ghislain Opendakker	<b>KUL</b>	BE	<a href="http://www.kuleuven.be">www.kuleuven.be</a>	
14.	<b>Weizmann institute of Science</b> Prof. Irit Sagi	<b>WEIZMANN</b>	IL	<a href="http://www.weizmann.ac.il">www.weizmann.ac.il</a>	



15.	<b>Institute of Bioorganic Chemistry, Russian Academy of Sciences</b> Prof. Sergej Zavriev Prof. Alexander M. Sapozhnikov	<b>IBCh</b>	RU	<a href="http://www.ibch.ru/en">www.ibch.ru/en</a>	
	<b>nHance Technologies Limited-terminated</b>	<b>nHance</b>	UK		
16.	<b>Filarete Servizi S.r.l</b> Prof. Paolo Milani	<b>FS</b>	IT	<a href="http://www.fondazionefilarete.com">www.fondazionefilarete.com</a>	 FILARETE SERVIZI
	<b>GE Healthcare-terminated</b>	<b>GE</b>	SW	<a href="http://www.gehealthcare.com">www.gehealthcare.com</a>	
17.	<b>OSMDAN Ltd.</b> Dr. Pnina Dan	<b>OSM</b>	IL	<a href="http://www.osmdan.com">www.osmdan.com</a>	
18.	<b>BENCAR Ltd</b> Prof. Bengt Langstrom	<b>Bencar</b>	SE	<a href="http://bencar.nu/?page_id=5">http://bencar.nu/?page_id=5</a>	
19.	<b>Ascend Technologies Ltd</b> Dr. Victor Popov	<b>ASC</b>	UK	<a href="http://www.ascendtechnologies.co.uk/">http://www.ascendtechnologies.co.uk/</a>	
20.	<b>COLOROBIA Consulting</b> Dr. Laura Niccolai	<b>COLOR</b>		<a href="http://www.colorobbia.it">www.colorobbia.it</a>	

Project website address: <http://fp7-saveme.com/>

Logo



## A description of the main S&T results/foregrounds

### (WP1) Functional nanosystems, based on a generic core platform, for targeted imaging and therapy BIU

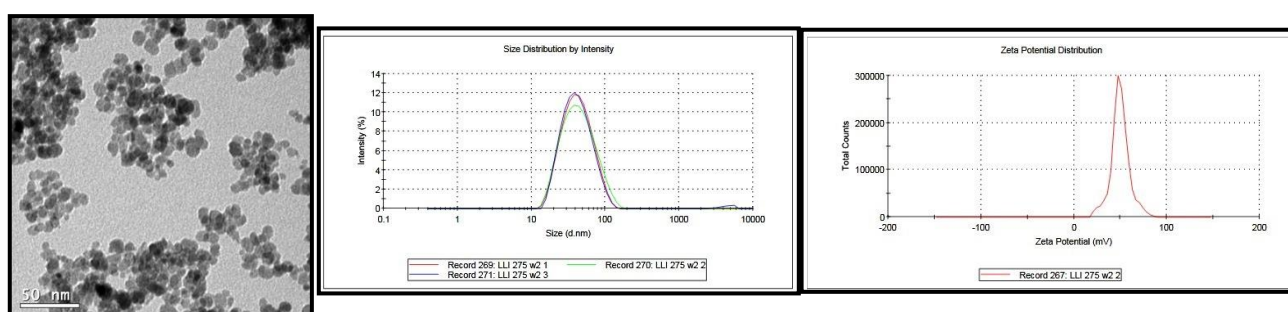
SaveMe main goals of **WP1** activities (see **WP title** above) were multiple and serially set-up from a less to a more complex design of **a wide range of 25-150 nm-sized generic polymeric nanoscale carrier particles (NCs)**. Appropriate surface linking functionalities present on such generic core polymeric nanocarriers (polyNH<sub>2</sub> and/or polyCOOH for example) enabled the covalent attachment of (i) targeting species for **human pancreatic cancer** diseases (tPA peptides/somatostatin peptide analogues), (ii) MRI enabling nanoparticles such as innovative CAN [Cerium(IV) Ammonium Nitrate] **Ce(NH<sub>4</sub>)<sub>2</sub>(NO<sub>3</sub>)<sub>6</sub>]-stabilized maghemite nanoparticles (NPs) or Gd(III)/PET/SPECT component (<sup>68</sup>Ga(III) cations)-based organic complexes (NOTA, DOTA), (iii) PEG-containing MMP substrates (for targeting of human pancreatic cancers), facilitating and/or stabilizing species (penetratin, PEG polymeric chains) and (iv) **therapeutic anti-MPscFv metallo bodies**.**

Moreover selected MRI-active maghemite-based NCs mentioned above have been also functionalized by polyethyleneimine (PEI) polymers (chemically derivatized for NCs toxicity mitigation) to be decorated by therapeutic siRNA sequences designed for **gene silencing** of specific genes specifically expressed in human pancreatic cancers.

Such novel generic core and/or core composite polymeric nanosystems have been specifically designed and screened for optimal nanomaterial properties (NCs average size and low size dispersivity, functionality level, water compatibility, minimal aggregation level) towards corresponding fully biocompatible non-toxic delivery vehicles of highly potent non-classic drugs for cancer diagnosis, guided surgery and therapy. Main polymers used for this NCs fabrication purpose have been designed as non-toxic biodegradable and biocompatible polymers classified as **GRAS (Generally Recognized As Safe)** like PLGA (poly[lactic-co-glycolic acid] co-polymer), PEG (polyethyleneglycol) derivatives including PLGA hybrids, recombinant human serum albumin (rHSA), and polyacrylates/derivatized polyacrylates (PAs, PAs, PLGAs/PEG-PLGAs, PEGs, rHSA).

In this context of WP1 activities, various nanofabrication methodologies for functional NCs have been explored successfully, including **polyacrylate (PAs) Huisgen “Click” cycloadditions** (Intramolecular polymer single chain cross-linking/collapse), NCs **nanoprecipitation** (solvent deposition) with oil/water emulsion, and **desolvation** methods (fabrication of PLGA-COOH/PLGA-PEG-COOH polymeric particulate systems) including *hybrid or non-hybrid core/mixed forms* that have been systematically and fully characterized before any *in vitro/in vivo* biological test. More specifically, a large number of attractive project scientific outputs have been achieved, opening wide avenues of future R&D studies in the field of polymeric, and magnetic/non-magnetic composite delivery NCs that have been shortly summarized below:

**BIU Partner:** The BIU team developed an innovative way to (i) control NPs aggregation based on charge repulsion, and (ii) enable covalent attachment of various polymers (25 kDa branched PEI polymers - *b*-PEI<sub>25</sub>, mixed *b*-PEI<sub>25</sub>-hyaluronic & alginic acids), and small bifunctional ligands (diamines for Kaiser testing) via **unique lanthanide cationic complex M(III/IV)Ln (M: lanthanide metal) doping** of the surface of ultra-small maghemite NPs. Specifically designed statistical methods using Design Of Experiments (**DoE**) software also lead to the delivery of effective M(III/IV)-doped maghemite NPs (Figure 1 below) that can act as strong Lewis acid NPs vs. any organic Lewis base organic species for its attachment without using any bifunctional linker. Toxicity mitigated 6.5-7.5 nm-sized *b*-PEI<sub>25</sub> and oxidized *b*-PEI<sub>25</sub>-M(III/IV)-doped maghemite NPs disclosed very strong gene silencing capabilities (siRNA technology, *in vitro* tests, on-going work for *in vivo* tests in selected therapeutic models) with a total lack of toxicity observed for *in vivo* tests (siRNA delivery/siRNA technology).



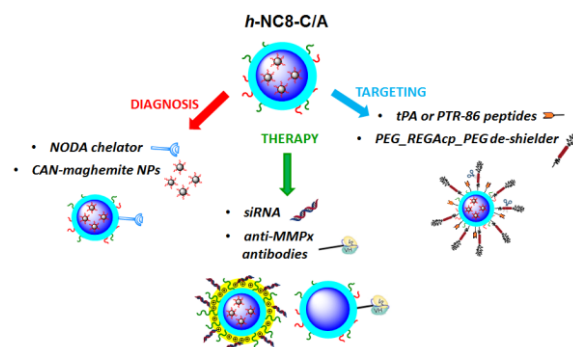
**Figure 1.1:** (Left) TEM microphotograph of illustrative CAN-maghemite NPs (Ce-Fe<sub>2</sub>O<sub>3</sub> NPs); (centre) DLS measurements of Ce-Fe<sub>2</sub>O<sub>3</sub> NPs; & (right)  $\zeta$  potential measurement of same particulate system

In addition, this specific metal cation doping step has been extended successfully to **PET-enabling <sup>68</sup>Ga(III) cation doping**, which provided first known dual MRI and PET-enabling imaging NPs without using any <sup>68</sup>Ga(III) cation-complexing species such as DOTA/NOTA.

**UNIBO Partner:** Main goals of this partner focused on the design, formation, and characterization of multifunctional hybrid polymeric nanoparticles (PNPs, Figure 2) loaded with BIU magnetic nanoparticles (CAN-maghemite NPs). PNPs have been made of biodegradable and biocompatible polymers, *i.e.*, **Food and Drug Administration (FDA)-approved poly[lactic-co-glycolic acid]-co-polyethyleneglycol (PLGA-b-PEGs)**. Moreover, NCs surface functionalities have been exploited for 2<sup>nd</sup> step decoration of resulting nanosystems with several different moieties for therapeutic, tumour targeting and imaging purposes as mentioned above in the introductory part.

Particularly UNIBO firstly developed a **double phase transfer protocol** for the synthesis of lipophilic CAN-maghemite nanoparticles entrapped into a PLGA-*b*-PEG-NH<sub>2</sub>/COOH matrix. The presence of two orthogonal functional groups (amino and carboxylic acid) in the outer shell represents a great advantage for the subsequent decoration of such nanosystems with active moieties, meaning pancreatic tumour targeting PTR-86 (SST analogues) or tPA peptides and NODA chelator as binding sites for PET imaging radioisotopes (<sup>68</sup>Ga(III)). Moreover, UNIBO also developed relating polymeric multi-functionalized nanosystems that possessed an hybrid outer shell comprising targeting, imaging species as well as a **MMP-sensitive de-shielding PEG-REGAcP-PEG agent**.

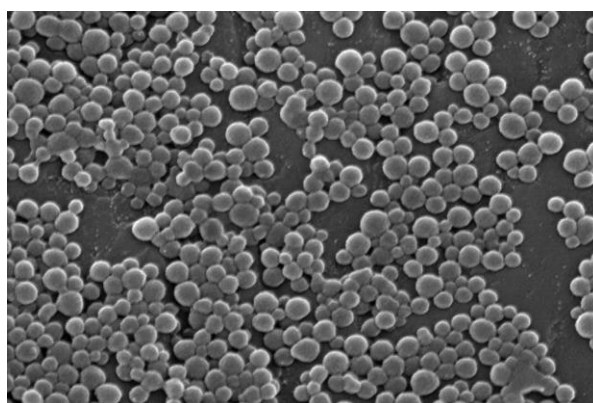




**Figure 1.2:** Schematic representation of UNIBO-developed nanosystems and their imaging/therapy-driven purposes

Therapeutic moieties such as monoclonal antibodies (anti-MMPx) or PEI-captured siRNAs for gene silencing have been also successfully developed was investigated (polyethylene imine (PEI)-coated nanosystem **h-NC8-PEI** for the incorporation of siRNA therapeutic moieties).

**GU Partner:** The GU partner focused its activity on the fabrication and characterization of **human serum albumin/recombinant serum albumin (HSA/rHSA) nanoparticles** for diagnosis and therapy of pancreatic cancer including stability studies for *optimal long-term storage and stability* (Fig. 3, use of cryo-protectors). Magnetically responsive composites have been also prepared by incorporating formerly described BIU CAN-maghemite NPs reaching impressive iron loading levels of around 6,000  $\mu\text{g}$  iron/100 mg recombinant rHSA with an incorporation efficiency of above 92% (h-rNC-9 NC). Toxicological tests showed that all these functional NCs are non-toxic. For PET/SPECT medical imaging needs, organic chelating species for  $^{68}\text{Ga}$  (DOTA and NOTA) have been successfully attached covalently onto the **h-rNC-9** NC surface and tested *in vivo* when using a new somatostatin analogue (PTR-58) as a tumour targeting species.



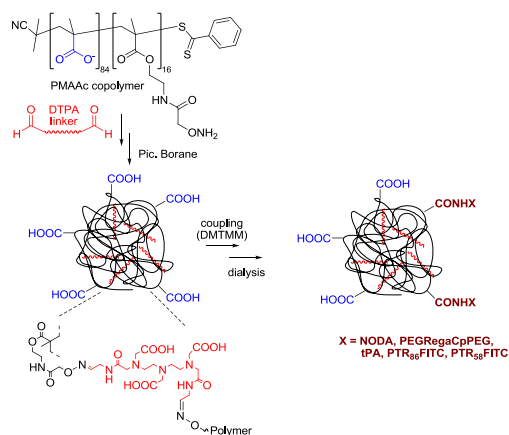
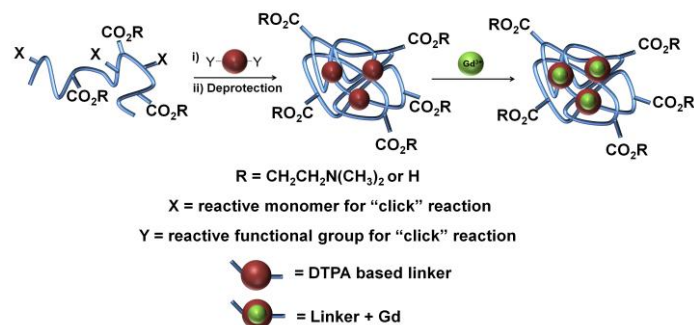
**Figure 1.3:** SEM image of 150-200 nm-sized rHSA NPs obtained when using EtOH 96% (v/v) as a non-solvent

Interestingly, the cellular uptake of resulting **targeted rHSA-based NPs** was found to be higher than the non-specific uptake of similar but non-targeted rHSA NPs (CLSM characterization).

**COL Partner:** The COL partner carried out **scale-up feasibility evaluations** of most promising intermediates synthetic pathways selected by nanofabrication WP1 partners BIU, CID, NAN, GU, & UNIBO while focusing on processes that lead to NCs systems fulfilling relevant colloidal/thermodynamic stability criteria. Consortium selected **CID** NPs as one of the most promising functional NCs system and has been successfully scaled-up with suitable procedures for pre-pilot production of: 1) trials for the scale-up of the last step of precursors synthesis: PMAAc-based copolymer deprotection and linker deprotection; 2) synthesis of single chain PMMAc-based nanoparticles (SCPNS), aiming at the optimization of several scale-up parameters: agreement with lab-scale production, automation, reproducibility and optimization of processes for large scale production aiming at time-saving industrial pathways.

Therefore, COL successfully attained **effective lab-scale production** and **good reproducibility** of intermediate steps, being able to implement full automation of the production pathway with feasibility assessment of dialysis method that can be employed in industrial development lines. In addition, a preliminary study on the scale-up possibilities of the rHSA-based nanosized system developed by partner **GU** was also successfully conducted. Several leads were focused and outlined as future inputs for an industrial scale-up.

**CID partner:** CIDETEC successfully designed, fabricated and characterized various single chain polymeric NCs (SCNPs) arising from cross-linking of single chain polymers nanoparticles using “click” chemistry. These nanocarriers (NCs) were based on both **poly(*N,N*-dimethylaminoethyl) methacrylate (PDMAEMA)** and **polymethacrylic acid (PMAAc)** polymers while and for polymer chain collapse NCs formation, various bi-functional linkers have been used, *i.e.* for example, one that contained a diethylenetriaminepenta-acetic acid (DTPA) species to chelate Gd<sup>3+</sup> cations for MRI applications (Figure 1.4).



**Figure 1.4:** Illustrative examples of the general preparation (*left*) of **polymeric PMAAc SCNPs** based on a copolymer of both methacrylic acid and alkyloxy-amine derivative as monomers (*right*) - Cross-linking executed by slow addition of a DTPA-type bis-aldehyde together with simultaneous incorporation of a NODA complexing unit (amide coupling – PET imaging)

As expected for tumour targeting and imaging activities involving such polymeric SCNPs, selected SCNPs have been covalently decorated by SST analogues (PTR 3207-86) for pancreatic tumour targeting and/or maleimide-NODA-GA for PET imaging applications. In addition, positively charged and pegylated **PDMAEMA-based SCNPs** have been successfully used in *in vitro* siRNA-based gene silencing applications, thus demonstrating their high therapy potential for further *in vivo* testing due also to lack of toxicity of such polymeric SCNPs. This partner also produced one specifically consortium-selected mass-scaled (1.0 g level) negatively charged polymethacrylic acid (PMAAc)-based single chain polymer SCNPs that was functionalized with chelating agents, targeting peptides and/or therapeutic agents for final *in vivo* tests (“consortium final joint experiment”).

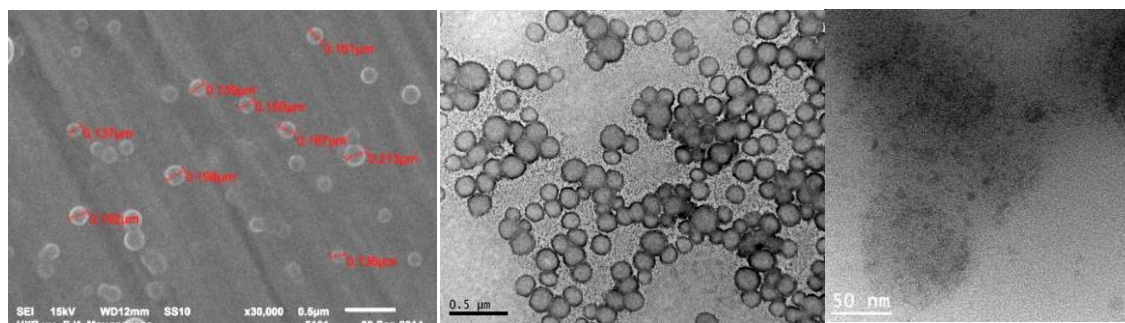
In this context, various types of functional polymeric SCNPs have been designed and fabricated for delivery to other consortium partners for evaluation of effectiveness: (i) PMAAc NPs functionalized with NODA and/or targeting/penetrating agents (PTR, tPA, and PEG-MMP-substrate-PEG), (ii) PMAAc NPs functionalized with fluorescently labeled tumor targeting agents (PTR) and PEG-MMP-substrate-PEG (de-shielding reagent sensitive to MMPs enzyme gradient), (iii) rhodamine and fluorescein-labeled PMAAc and PDMAEMA SCNPs for *in vivo* toxicity studies including bio-distribution, and (iv) finally positively charged PDMAEMA SCNPs, - (PDMAEMA-PEG(750)-PTR-FITC and PDMAEMA-PEG(750)-NODA-FITC) *in situ* loaded with siRNAs for therapeutic purposes (siRNAs delivery/gene silencing technology).

**NAN Partner:** Main research activities of the NAN partner emphasized the chemical design and fabrication of biocompatible biodegradable **hybrid PLGA-based nanocarriers (NCs)** – Such NAN activities focused on the use of both biodegradable and biocompatible copolymers of **lactic and glycolic acids (PLGA)**. Resulting PLGA-based nanocarriers (NCs) thus provided a high versatility for NCs functionalization with all types of therapeutic and targeting moieties mentioned above towards fully active nanoscaled theranostic delivery systems.

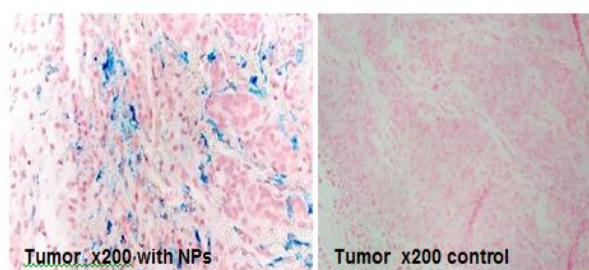
This partner tested the two main most effective nanofabrication approaches: (i) developing hybrid core-shell NCs comprised of the PLGA nanoparticulate core and an additional polyanionic shell, and (ii) developing hybrid functional NCs comprising both this same PLGA phase and a 2<sup>nd</sup> polycarboxylated oligomeric lactic acid one (fabrication of **polyCOOH PLGA/OLA NCs**). Interestingly, both types of functional NCs might be readily prepared by easily scalable processes, *i.e.*, nanoprecipitation and

high-pressure homogenization (COL partner input/cooperation input). The existing functional polyCOOH (high level of functionalization in contrast to common PLGA NPs) shell quite effectively enabled an easy introduction of the whole set of pancreatic tumor targeting moieties mentioned above (PTR: somatostatin analogue) and tPA (modified tissue plasminogen activator) via the use of specifically designed PEG linkers.

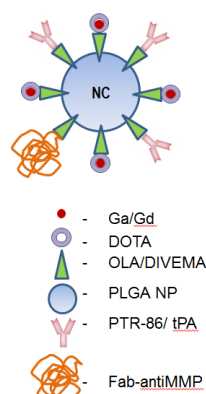
As a main output of this nanofabrication activity including toxicity testing, such novel non-toxic **PLGA/OLA NPs** appeared to be optimal due to their full biodegradability to physiological metabolites. Same non-toxic **PLGA/OLA NCs** have been also successfully modified by (i) Fab-antiMMP antibodies used as therapeutic agents, (ii) MRI-enabling CAN-maghemite (Ce-Fe<sub>2</sub>O<sub>3</sub>) NPs (strong T<sub>2</sub>\* contrast agent), and specific complexone-type species for effective <sup>67/68</sup>Ga(III) complexation (tumor-driven PET imaging applications, Figure 5).



**Figure 1.5: Hybrid PLGA/OLA NPs:** (a) unmodified ones (SEM); (b) modified with Ga(III) incorporation (TEM), (c) modified with incorporated iron oxide CAN-maghemite NPs (TEM)



*In vivo* uptake of vectorized hybrid NC in orthotopic pancreatic tumor in mice: Prussian blue staining



Schematic presentation of hybrid PLGA-based nanocarriers

**BENCAR Partner:** This partner specifically optimised NCs labelling with PET-enabling radioactive short-lived <sup>68</sup>Ga(III) cations using a specifically developed BENCAR microfluidics platform exploiting both anion exchange purification and cation concentration processes while kept within an Mini-clean room to enable the production of <sup>68</sup>Ga(III) solutions of very high specific radioactivity. For example, selected BIU-originated maghemite-based NPs doped with various different lanthanide metal cations of the type [ML<sub>n</sub>, L ligand]<sup>n+</sup> have been successfully investigated with regard to **direct <sup>68</sup>Ga(III) labeling** without using any Ga(III) cation complexing species.

## (WP2) Pancreatic cancer selective targeting and diffusion active agents SHEBA

### Somatostatin analogs as a targeting moiety for pancreatic cancer:

The major goal of this study was to develop novel cancer-targeting peptides with enhanced tumor specificity and selectivity based on the human somatostatin receptor specific carriers (ligands). These ligands conjugated to nano-structures with imaging contrast or therapeutic agents are being used as a platform for advanced detection/treatment of pancreatic cancer.

**Synthesis:** Sixteen novel fluorescent somatostatin analogues (PTRs) were synthesized by solid-phase peptide synthesis (SPPS). Backbone cyclization was used to achieve constrained conformation of the peptide. This cyclization included cysteine moiety and GlyS2 building unit bearing thiol functional group which was also synthesized. Purification method on



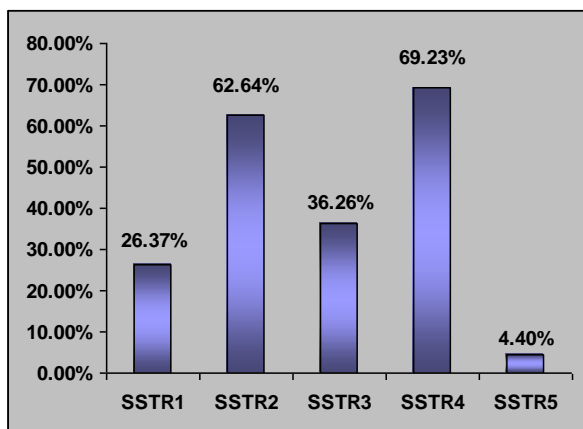
preparative HPLC was developed to each one of the peptides. All the analogues were characterized by analytical RP-HPLC and mass spectrometry.

A batch of 410 milligrams of fluorescent PTR 3207-86 was synthesized, purified and delivered to SaveMe partners. Robust protocols for the synthesis and purification of the somatostatin analogs have been established. Additionally, two leading analogs (PTR 86 and PTR 58) were conjugated to DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) isotope chelators for in vivo SPECT imaging.

**Definition of SSTRs patterns in human tissue sections from pancreatic cancer patients:**

The expression pattern of SSTRs in human pancreatic cancer tissues was studied by IHC using specific SSTR primary polyclonal antibodies (anti SSTR 1-5). A comprehensive database of expression and distribution of SSTR subtypes from 100 human pancreatic cancer patients was completed. The results indicate that SSTR4 is the leading subtype, and SSTR5 is the lowest expressed subtype. Overall, SSTR 2 and 4 are the most prevalent throughout the pancreatic carcinoma samples. SSTR2 is present in stage 2 or 3 at 62.6% of all patients, and SSTR4 is present in stage 2 or 3 at 69.2% of all patients. Moreover, 81.3% of all cases express SSTR2 or 4 or both.

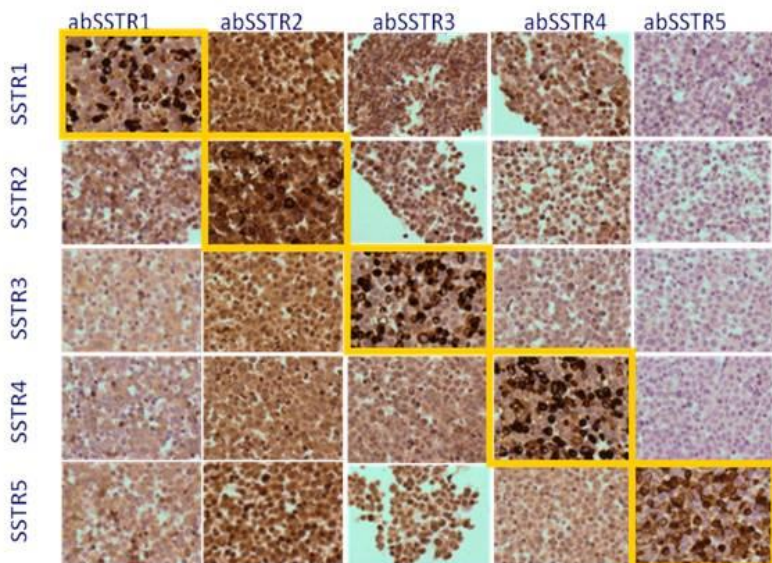
The results of statistical analysis are depicted in figure below:



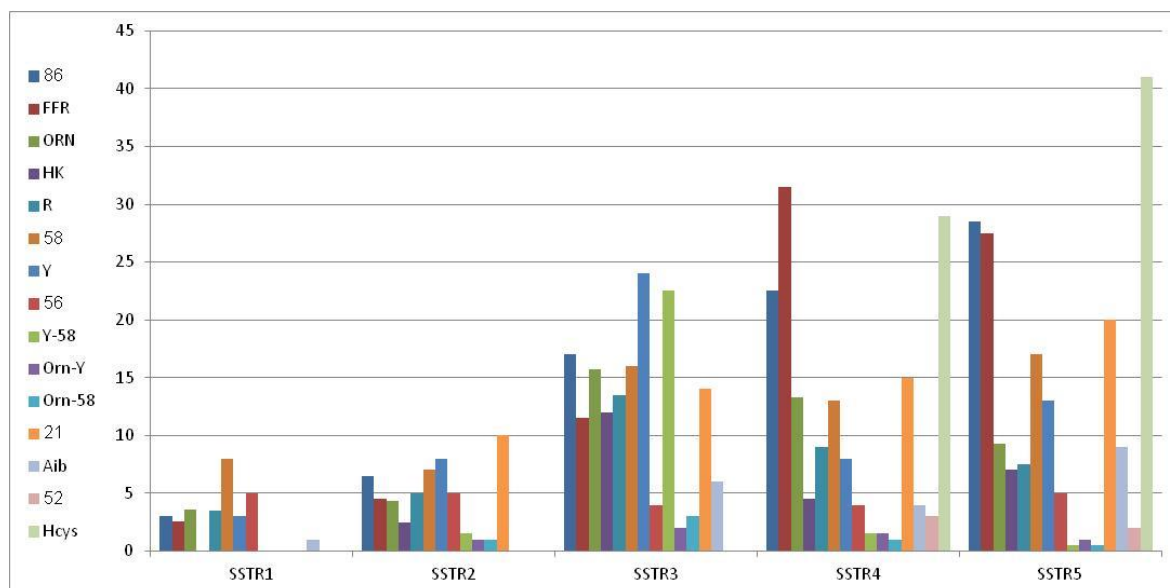
**Assessing specific binding efficiency using advanced in vitro model**

Advanced cellular model, based on genetic engineered human embryonic kidney (HEK) cells was developed. Human SSTRs have been expressed in these cells by transient transfection. Each SSTR was expressed separately, creating 5 different cell lines, using human gene transcript. This model has been used for verification of developed PTRs and their specific affinity to the different SSTR subtypes for personalized medicine applications.

Expression of appropriate receptors was validated by RT-PCR and IHC, demonstrating efficient transfection and specific over-expression of all 5 human SSTRs.



Quantitative assessment of binding affinity of all novel PTRs has been performed by FACS in this transfected HEK cell model. All PTRs were found to have multi-receptor affinity (similarly to human native somatostatin), with different degree of efficiency.

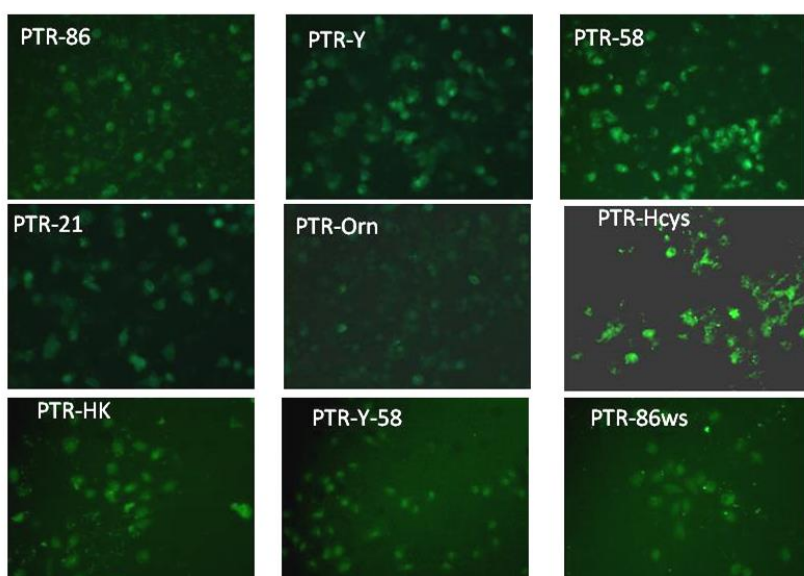


**Figure 2.1** Summary of specific binding affinity (percentage of PTR-positive cells in total transfected cells) with of newly developed PTRs to SST 1-5 human receptors, in advanced model of transfected HEK cells, determined by FACS.

### Internalization assays in human pancreatic cancer cell lines :

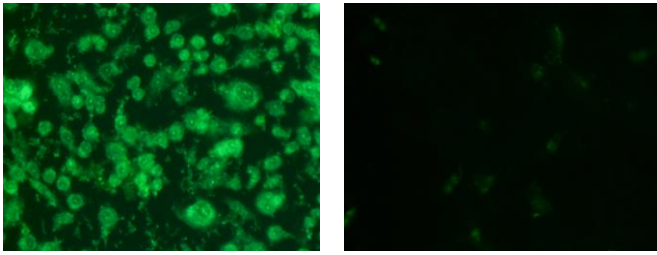
**PTRs:** Novel PTRs were tested in selected human pancreatic cancer cell lines using fluorescence microscopy. Most of the agents demonstrated efficient intracellular internalization on one or several cell lines. Generally, the intensity of the fluorescent signal was dependent on concentration and time of incubation.

#### In vitro activity of PTRs in panc-1 cells



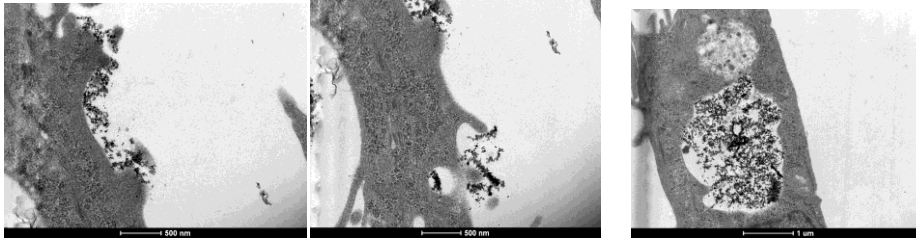
Based on in vitro testing, 9 out of 16 novel PTR analogs have been chosen for assessment of in vivo bio-distribution and tumor targeting efficiency in panc-1 animal model.

**PTR-NPs:** Novel PTRs were tested in selected human pancreatic cancer cell lines using fluorescence microscopy. Most of the agents demonstrated efficient intracellular internalization in one or several cell lines. Generally, the intensity of the fluorescent signal was dependent on concentration and time of incubation. The figure below demonstrates a typical example of intensive fluorescence staining indicating efficient intracellular internalization and accumulation of PTR-targeted NPs, compared to non-targeted (control) NPs.



**Figure 2.2** PDMAEMA based NPs (CIDETEK sample 24) binding and internalization into human pancreatic carcinoma cells (left), compared to NPs without PTR (right).

Receptor-mediated endocytosis of PTR-NPs into human pancreatic carcinoma cells was demonstrated using TEM technique.



**Figure 2.3** Can-maghemite (BIU, LLI-279) PTR-NPs binding and internalization into BxPc human pancreatic carcinoma cells via receptor-mediated endocytosis. i) NPs adhesive to cell surface (left), ii) Pseudopodes creation and endosome formation with NPs clodure and internalization into the cell (middle) iii) Merging of small endosomes into mega-vacuole full of NPs.

In summary, all PTR-functionalized NPs showed efficient intracellular internalization in vitro, in contrast to control (non targeted) NPs. Three types of functionalized NPs (NAN, CID, GU) were found to demonstrate selective uptake by the tumor, compared to normal tissues, in vivo.

### Galectin-1 based targeting moieties

Four peptides derived from human tPA were selected as potential ligands to the receptor Gal-1 which is highly expressed intracellular and on the cell surface in human PaCa cell lines and PDAC-tissue.

<p><b>Peptide 1</b>  <i>Name:</i> h-NC8-tPA-1 (AA 137-164)  <i>tPA:</i> 137-164, ID Nr 17-04-12  <i>Volume:</i> 4 mL  <i>Solvent:</i> PBS 0.01 M  <i>DLS analysis:</i> <b>d = 89.07 ± 1.16nm</b>            PDI = 0.113 ± 0.004            Zpot = -16.1 mV            pH = 7.4</p>	<p><b>Peptide 3</b>  <i>Name:</i> h-NC8-tPA-3 (AA 198-224)  <i>tPA:</i> 198-224, ID Nr 17-06-12  <i>Volume:</i> 4 mL  <i>Solvent:</i> PBS 0.01 M  <i>DLS analysis:</i> <b>d = 89.38 ± 0.14nm</b>            PDI = 0.138 ± 0.01            Zpot = -9.17 mV            pH = 7.4</p>
<p><b>Peptide 2</b>  <i>Name:</i> h-NC8-tPA-2 (AA 91-118)  <i>tPA:</i> 91-118, ID Nr 1679-04-12  <i>Volume:</i> 4 mL  <i>Solvent:</i> PBS 0.01 M  <i>DLS analysis:</i> <b>d = 87.36 ± 0.33nm</b>            PDI = 0.126 ± 0.01            Zpot = -2.91 mV            pH = 7.4</p>	<p><b>Peptide 4</b>  <i>Name:</i> h-NC8-tPA-4 (AA 476-484)  <i>tPA:</i> 476-484, ID Nr 17-07-12  <i>Volume:</i> 4 mL  <i>Solvent:</i> PBS 0.01 M  <i>DLS analysis:</i> <b>d = 93.26 ± 0.71nm</b>            PDI = 0.139 ± 0.01            Zpot = -13.3 mV            pH = 7.4</p>

Quantitative analysis of Gal/ tPA was done using micro scale thermophoresis technique (MST). The affinity constants between the tested tPA peptides 1-4 and galectins were assessed. Affinity constants (KD) of tPA peptide-1 and galectin-1 are presented and compared to the value (4.92  $\mu$ M) of recombinant human tPA protein to galectin-1; lower binding (-) stronger binding (+).

peptide	label	$K_D$ [ $\mu$ M]	fold difference
rec. h tPA protein	N-Fluorescein	4.92	1
P1	N-Fluorescein	141	- 28.7
P1	C-Fluorescein	nd	nd
P1 (Ser-Lac 3Ac)	C-Fluorescein	0.189	+ 26.0
P1 (Ser-Lac 4Ac)	C-Fluorescein	0.257	+ 19.1
P1 (Ser-Gal)	C-TAMRA	0.662	+ 7.4

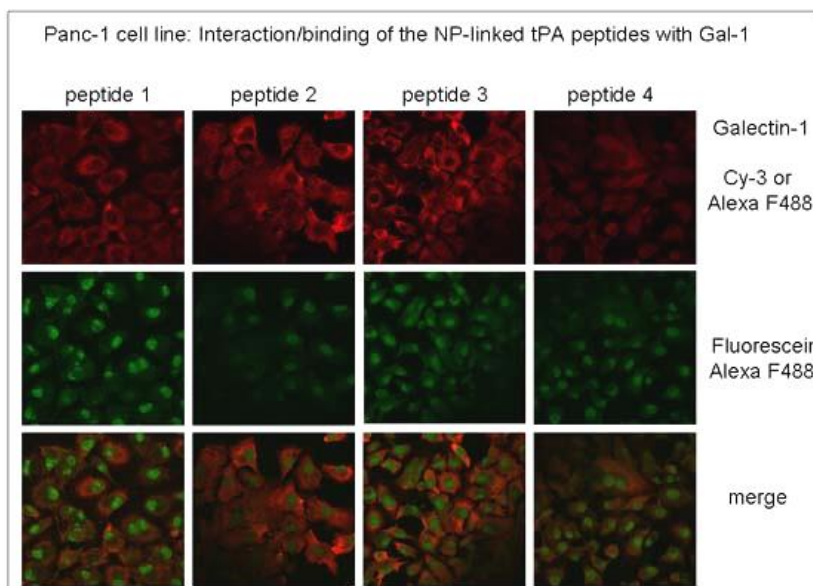


tPA peptide-1 was chosen as the most promising ligand for targeting galectins-1,-3,-4 on pancreatic cancer cells surface.

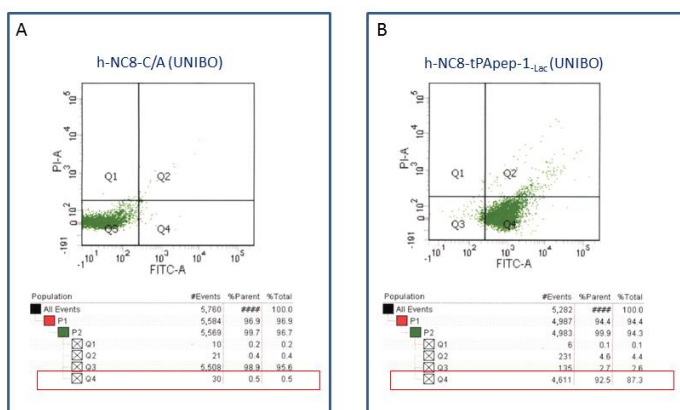
**tPA – NCs** complexes were successfully developed, characterized, optimized and tested for their internalization properties into human PaCa cell lines using fluorescence microscopy, electron microscopy and quantitative FACS methods.

Interaction/binding of the NP-linked tPA peptides with Gal-1 (in vitro investigation):

Human pancreatic tumor cell lines Panc-1 and Su.86.86, were seeded on collagen-coated glass plates (1.0 cm diameter) in 6 well culture plates ( $1 \times 10^6$  cells/well) allowing to grow under treatment with concentrations of 100 nM, 200 nM and 400 nM of fluorescence-linked peptides for 24h. Subsequently immunofluorescence (IF) for Gal-1 (Cy3 when fluorescein or Alexa F488 when TAMRA-labelled tPA peptides-NPs were used). The ligand/receptor-complex formation (tPA-Peptid-NP/Gal-1) was assessed by IF and confocal microscopy.



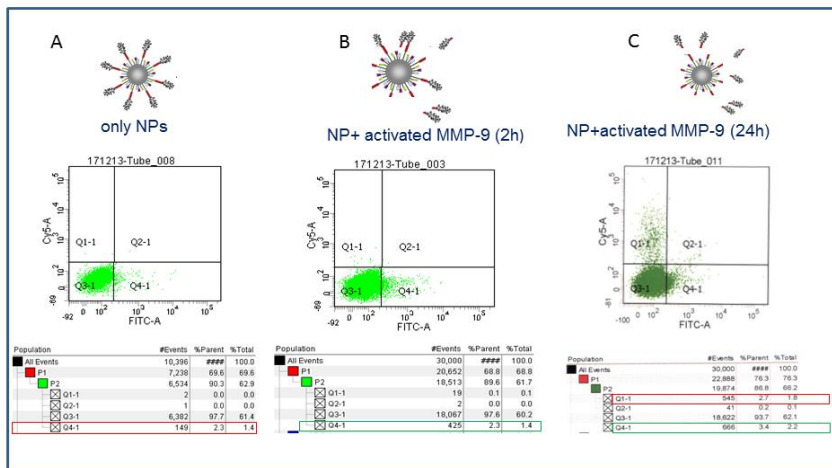
**Figure 2.4.** Colocalization of fluorescence labeled tPA peptides decorated nanoparticles and galectin 1 in the Panc-1 cell line.



**Figure 2.5** Quantitative FACS analysis demonstrated that while only a minor fraction (0.5%) of non-targeted h-NC 8-C/A were bound to PaCa cells (A), the tPA-peptide-1Lac functionalized NCs showed up to 92.5% binding (B). The highest binding efficiency was reached after 6 hours incubation.

**Deshielding effect:** In vitro binding efficiency of three modalities was tested in human PaCa cells:

- 1: The hNC8-tPA-peptide-1Lac
- 2: The hNC8-tPA-peptide-1LacPEG-REGAcp-PEG
- 3: The hNC8-tPA-peptide-1LacPEG-REGAcp-PEG, exposed to activated MMP-9



**Figure 2.6** Binding of NPs (h-NC8-tPA1Lac-PEG-REGAcP-PEG) to Panc-1 cells NP [1.24  $\mu$ M]; Incubation time 2 hrs + proMMP-9 and its activator (cdMMP-3)

The results revealed that NP with outer shell decoration (hNC8-tPA-peptide-1LacPEG-REGAcP-PEG) bind poorly (2.3%) of the gated cells. A short (2h) preincubation of such particles with MMP-9 shows no enhancing effect. An extended MMP9 preincubation step for 24 h slightly elevated the binding (3.4%) of the NPs. The pilot study here demonstrated that an extended preactivation phase (deshielding step) is necessary for better binding of the particles to cells as measured by FACS.

### (WP3) Novel non-classic drugs based on cancer degradomics UEA

The central concept of the SaveMe project has been to develop nanoparticles (NPs) that will: 1) be large particles as a result of having an outer "shield" of polyethylene glycol (PEG), which will give them long half-lives in the circulation; 2) be able to be "deshielded" by enzymes present in the tumour environment, exposing their ability to interact with pancreatic cancer cells; and 3) be able to deliver a "payload" to the tumour, which may be either a contrast agent to enhance imaging or a therapeutic molecule to suppress the growth of the cancer. Linked to these aspirations, it is important that these novel particles do not themselves have negative effects on tissues that may provoke inflammation or promote the metastatic spread of the tumour.

To achieve these goals, the SaveMe project has developed and adapted a number of experimental model systems for evaluating the NPs, the targets that they are designed to hit, and the therapeutic agents that they will deliver. We also set up a mouse model of spontaneous pancreatic cancer, the KPC mouse (Cre-Kras<sup>G12D</sup>-p53<sup>R172H</sup>) which allowed us to study events during the development of disease in a way that most closely resembles the situation in patients. We review below the progress we have made using systems for studying pancreatic cancer in mice and for preclinical testing of NPs or therapies in mice.

A key aspect of our work has been the focus on extracellular enzymes known as **matrix metalloproteinases (MMPs)**. The MMPs are a family of 24 enzymes in humans which are found at high levels in many types of cancer, including pancreatic cancers. Several of the research groups involved in the SaveMe project are world leaders in the study of MMPs. In the past, MMPs were thought to be primarily responsible for breaking down the extracellular matrix (ECM) molecules that are essential for tissue structure and organization, allowing cancer cells to invade through tissue barriers and metastasize to secondary sites in the body. This view led to the development of broad-spectrum synthetic MMP inhibitors as anti-cancer agents in the 1990's. However these agents failed in the clinic, and we now know that one of the reasons is that MMPs in fact have many different roles in cancer biology, and indeed some MMPs have protective functions. After almost a decade of pre-clinical research and clinical trials, the development of these inhibitors was suspended, and subsequently it was recognized that broad-spectrum MMP inhibitors can induce liver metastasis in mice. One of the MMPs that has both pro- and anti-tumour actions is **MMP-9**, otherwise known as **Gelatinase-B**. Originally a prime target for the synthetic MMP inhibitors, in the SaveMe project we have used our knowledge of MMP-9 to take advantage of its elevated levels in tumours to provide the **de-shielding mechanism** for the NPs. In this context we do not aim to block the function of MMP-9, but instead use it as a tool to unmask the NPs and make them locally effective in the tumour environment. But MMPs can also be legitimate anti-cancer targets, and within the SaveMe project we have worked to identify key MMPs that promote the growth or spread of pancreatic cancers and develop specific ways to block them. **Key target MMPs are MMP-7 and MMP-14**, and we review below some of the evidence that we have gathered about them and the novel blocking agents we have developed.

Two parallel strategies have been used for the development of new therapeutic warheads to be loaded onto the SaveMe NPs, namely **small interfering RNAs (siRNAs)** and **function-blocking antibody fragments (Fabs)**. We have also taken advantage of the wealth of data that is publicly available to carry out **bioinformatic** searches of gene expression databases for pancreatic

cancer, to identify novel potential targets whose inhibition may lead to therapeutic benefit. Several pancreatic cancer-associated genes emerged from this approach, with one gene, **polo-like kinase-1 (PLK-1)** showing great promise from our results.

## **A: Development of In Vivo models and test systems for evaluation of NPs and therapeutic strategies**

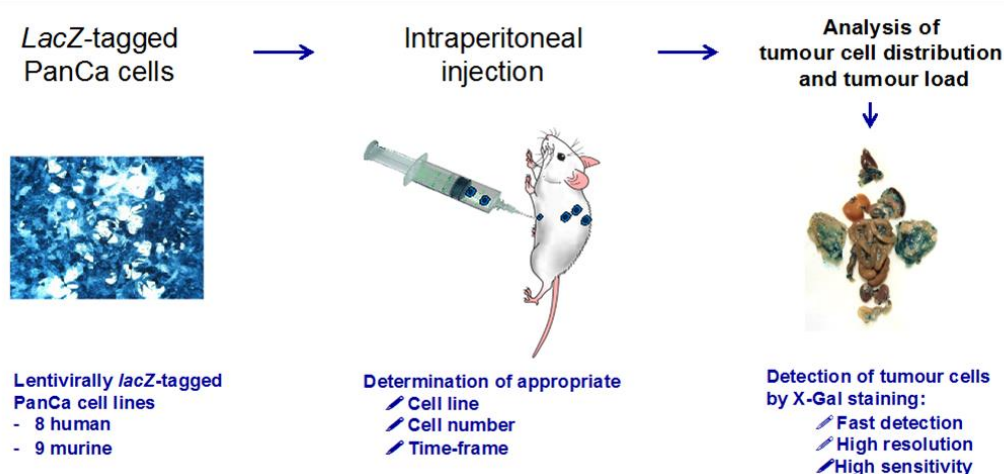
### **A1. The Air-Pouch Assay**

One of the challenges with new chemical formulations, particularly involving particles the size of NPs, is the effect that they may have on provoking an inflammatory reaction following administration. This can have severe adverse consequences and influence experimental findings, as well as ultimate clinical utility. As a quality control measure it is therefore important to verify that NPs developed by the SaveMe consortium do not provoke inflammation. As a reference system for NP evaluation, we optimized the **intra-dermal air pouch model for immunological evaluation of nanoparticles**, to allow pre-clinical evaluation of the effects of NPs on innate immune responses. This test works by creating a small air pouch under the skin of a mouse, into which the NP formulations are injected. Subsequently the levels of inflammatory cells (e.g. neutrophils) that have migrated into the air pouch can be quantified easily. Using this system we were able to test the range of NP types developed by the consortium, which were used subsequently for imaging and therapy evaluation studies.

### **A2. The intraperitoneal (IP) pancreatic cancer model for rapid testing of NPs and therapies.**

An essential task in the SaveMe project was to have a rapid model system to test the newly developed nanoparticles for their therapeutic potential and to identify any possible unwanted cancer-promoting actions. Such a model was required to: 1) allow fast and efficient proof-of-principle testing of the newly identified therapeutic targets against pancreatic carcinoma in vivo; and 2) allow identification of potential un-wanted treatment-related side-effects, especially unwanted metastasis to the liver. We developed a rapid intraperitoneal model to address these two prerequisites.

For fast and efficient screening, an intraperitoneal *xenograft* model was developed that allows quantification of therapy success using a very simple strategy: The pancreatic tumor cell lines under investigation were all genetically tagged with a bacterial *lacZ* gene allowing straightforward detection of tumor cells, even at the single cell level, by indigo blue, a coloured product from the *lacZ* substrate X-gal, which we use to a) visualize the tumor cells in different organs, and b) quantify tumor load spectrometrically in a cost- and time-efficient manner. The experimental set-up for establishment of this model is outlined in the figure below. We have used this model to provide proof-of-principle for the validity of the newly identified target PLK-1 as we could quantify the experimental therapeutic effect of PLK-1 inhibition (see section D below).



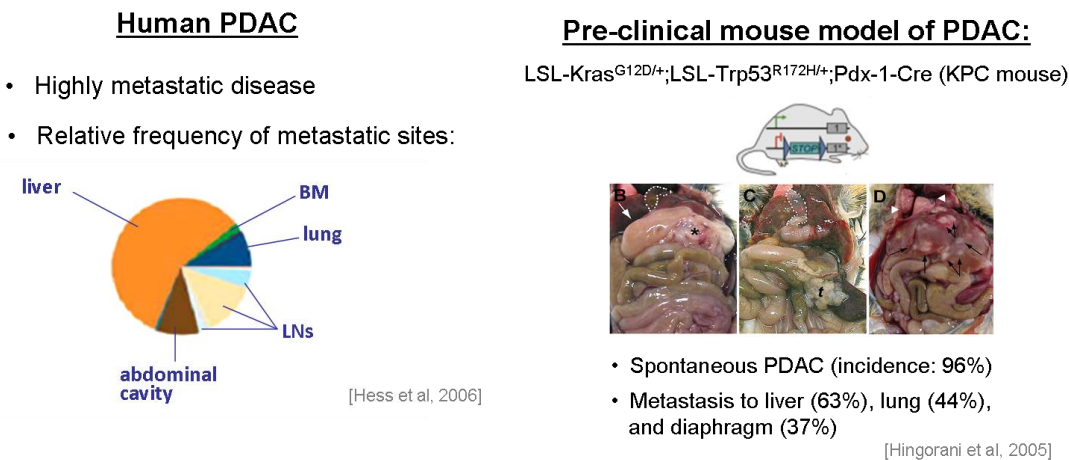
**Figure 3.1.** Schematic overview of the experimental setup used for implementation of an i.p. model of pancreatic cancer.

*LacZ*-tagging of tumor cells was also employed in this project to elucidate potential treatment-associated side effects in terms of metastasis: Mice were pre-treated with the newly developed nano-agents and then challenged with *lacZ*-tagged tumor cells which we could thus precisely quantify. Using this approach we could demonstrate that the newly developed NPs are, at least in our mouse models, promising for further development as they did not induce pro-metastatic side effects at this stage.

### **A3. The conditional KPC pancreatic cancer mouse model (Cre-Kras<sup>G12D</sup>-p53<sup>R172H</sup>)**

We also introduced a mouse model that spontaneously develops pancreatic cancer for the purposes of the SaveMe project, as it can be used to address more complex, therapy-related questions of the use of the newly developed NPs, as well as helping to analyse molecular events during disease development and progression. This well-established pre-clinical mouse model is based on pancreas-specific activation of the Ras oncogene which in combination with a mutated form of the tumor-suppressor p53 leads to spontaneous development of pancreatic tumours in these mice. Disease progression in the KPC model closely mirrors that in human patients in terms of anatomy, histology, as well as frequent metastasis to the liver (see Figure below). The

(pre-) metastatic niche in the liver of pancreatic cancer patients is of great interest for studies on the devastatingly high metastatic efficiency of pancreatic cancer on the one hand and the potential role of novel therapeutics in interfering with these processes on the other hand. We have used the KPC model to elucidate cellular composition and pro-metastatic gene expression as well as the cell signalling milieu in the (pre-) metastatic liver of pancreatic cancer. Additionally, we evaluated and confirmed the feasibility of the concept of MMP9-dependent de-shielding of nanoparticles under the complex *in vivo* settings in the KPC model (see section...below).

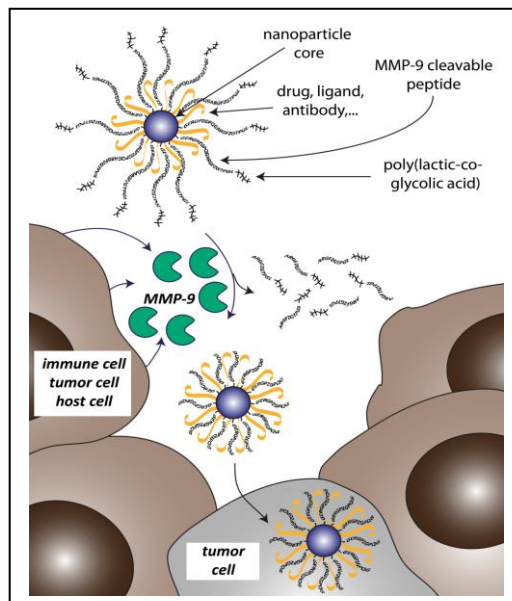


**Figure 3.2. Comparison of human pancreatic cancer metastasis to the KPC model.**

## **B: Matrix Metalloproteinases (MMPs) as tools and targets in the SaveMe project**

### **B1. Gelatinase B/MMP-9 and NP de-shielding**

The presence and activity of proteolytic enzymes in samples of pancreatic cancer was evaluated, in particular with an eye on the development of protease-modifiable nanoparticle drugs. Elevated expression of several proteases, and in particular of **gelatinases** (gelatinase A/MMP-2 and gelatinase B/MMP-9), is associated with inflammation, also in tumor micro-environments. Peritumoral inflammation is a hallmark of cancer, also in pancreatic adenocarcinoma. We evaluated the presence of gelatinases in cancer cell lines, tissues of a mouse model for pancreatic cancer and serum samples from patients with pancreatic cancer. By using quantitative gelatin zymography, a reverse-degradomics technique by which we can quantify simultaneously various proteases, we were able to show that both MMP-2 and MMP-9 are present in pancreatic cancer and that both are increasingly induced over time during pancreatic cancer development.



This fact was exploited conceptually by MMP-targeted drug delivery. Within the SaveMe project, we aimed to use the presence of enzymatically active MMP-9 in the tumor microenvironment to potentiate targeted delivery and infiltration of nanodrugs into the tumor tissue of pancreatic cancer patients. For this purpose an optimal substrate for MMP-9 was designed. Based on the degradation pattern of collagen II by MMP-9 and on degradomics analysis of synthetic peptides at the Rega Institute, an optimal cleavable peptide (CP), named RegaCP, was generated. Next, a Polyethyleneglycol-RegaCP-Polyethyleneglycol (PEG-RegaCP-PEG) chemical structure was synthesized and attached to different nanoparticles generated during this project. Incubation experiments with active MMP-9 indicated that RegaCP was indeed degraded after PEGylation and after covalent attachment to the nanoparticles. In parallel, a reverse-degradomics platform, based on capillary electrophoresis, was optimized to directly evaluate the degradation efficiency of the RegaCP peptide in complex samples such as cell cultures and tissues. Within the consortium, the biochemical, biophysical and functional characteristics of zymogen and activated forms of MMP-9 monomers and multimers were also investigated. This study led to the discovery of trimeric MMP-9 and the seminal finding that this trimeric form is differentially regulated by TIMP-1, compared to monomeric proMMP-9. This reflects in a significantly different TIMP-1 inhibition profile on MMP-9-induced angiogenesis by proMMP-9 monomers compared to proMMP-9 trimers.



These findings indicate a different function for proMMP-9 trimers and open new ways for selective targeting of monomeric or trimeric MMP-9 in disease.

## B2. Profiling the repertoire of MMPs and other proteases in pancreatic cancer and cell lines.

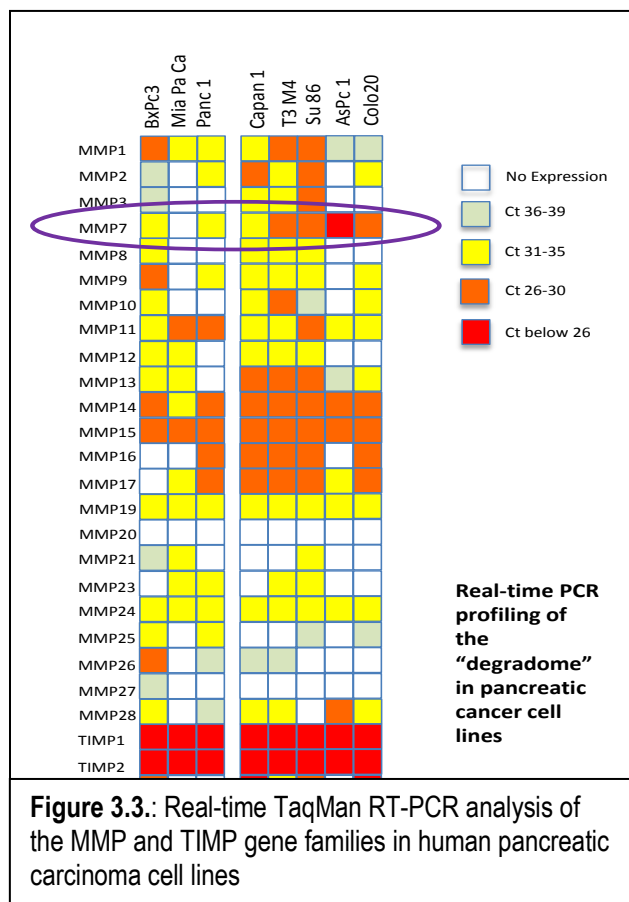
To complement the protein analyses described above we used quantitative real-time RT-PCR to analyze the expression of the entire “degradome” – the full repertoire of 588 proteases of 5 catalytic classes in the human genome – in human pancreatic cancer cell lines. The patterns of expression of the MMP family and their four natural tissue inhibitors (TIMPs) is shown here (the darker the colour representing higher levels of gene expression), highlighting that one family member, MMP-7, shows increased expression in cell lines from metastases of pancreatic cancer versus primary tumours.

This observation is supported by analysis of other expression databases, and along with published evidence indicating a tumour promoting role for MMP-7 in pancreatic cancer, it contributed to selection of MMP-7 as a prime candidate for therapeutic targeting, which will be discussed in a subsequent section.

## C: Bioinformatic analysis of pancreatic cancer databases for potential therapeutic targets

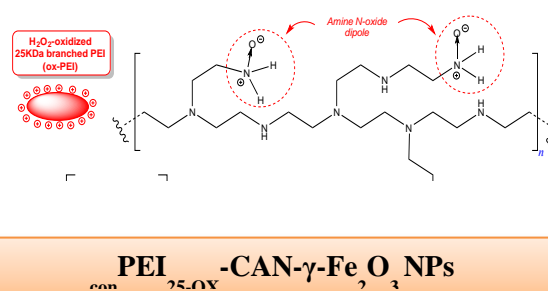
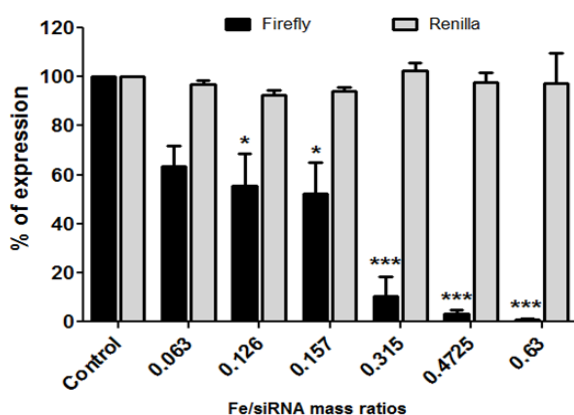
We carried out **bioinformatic analyses** of expression datasets in publications that analysed pancreatic cancer tissues and cell lines. Data were extracted from 17 papers in which protein and RNA expression were determined using a variety of platforms, including commercial and custom arrays, mass spectrometry, immunohistochemistry and real time qRT-PCR. We assembled an interrogatable Access database that allowed integration of all published datasets on pancreatic cancer tissues and cell lines, as well as the degradome profiling data by TaqMan qRT-PCR from studies discussed above.

The bioinformatics analyses identified a cast of genes that show dysregulation in pancreatic cancer. These are candidates to explore functions by siRNA knockdown. Key genes that have emerged are: **S100P, FN1, THBS2, PNLIPRP1, CLPS, CPA2, AMY1A, VCAN, POSTN** and **PLK-1**. Among the degradome genes, 3 genes stand out from the comparisons of the human pancreatic cancer cell lines, namely **MMP7, MMP14** and **ADAMTS4**

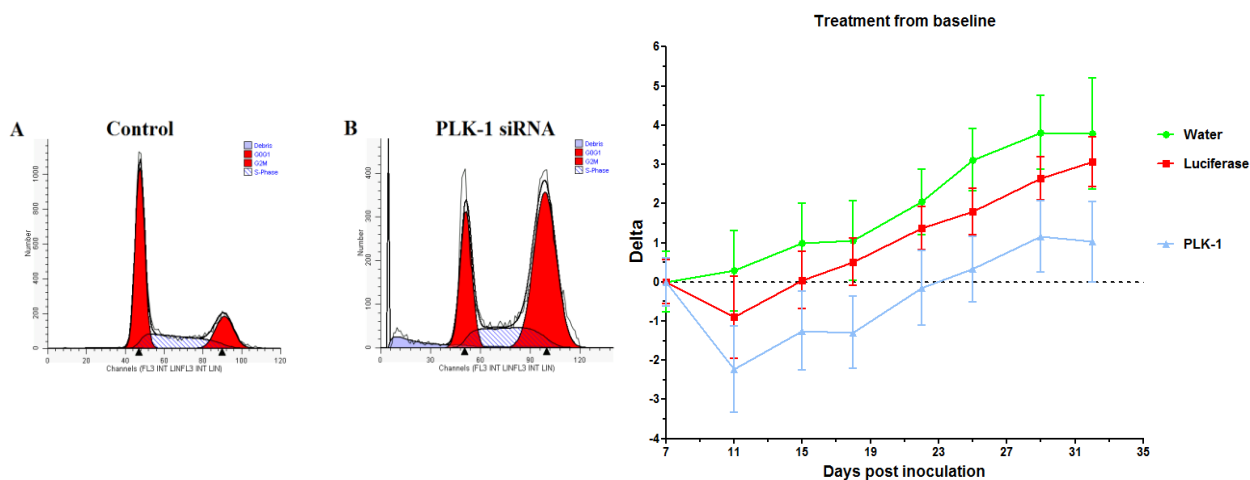


## D: Targeting pancreatic cancer by NP-mediated delivery of siRNAs

One of the major advances achieved by the project is the development of NPs that can carry siRNAs into cells with high efficiency and low toxicity. The effectiveness of these oxidized CAN-maghemite NPs ( ${}_{\text{con}}\text{PEI}_{25\text{-OX}}\text{-CAN-}\gamma\text{-Fe}_2\text{O}_3$ ) was initially demonstrated using NPs carrying siRNAs against Firefly luciferase, which showed dose-dependent suppression compared to control Renilla luciferase in a dual reporter assay system.



We subsequently showed that NPs with this formulation carrying siRNAs against two principal pancreatic cancer target genes, MMP7 and S100P, effectively silenced the genes and showed modest induction of apoptosis in cultured cells. However the most spectacular results have been seen with delivery of siRNAs against PLK-1, a regulator of the mitotic machinery. These NPs caused prominent cell cycle arrest *in vitro* (left panel) and suppressed growth *in vivo* in the ip pancreatic cancer model discussed in section A (right panel).



These data demonstrate proof-of-principle of NP-mediated siRNA suppression of pancreatic cancer growth *in vivo*, indicating promise for future development.

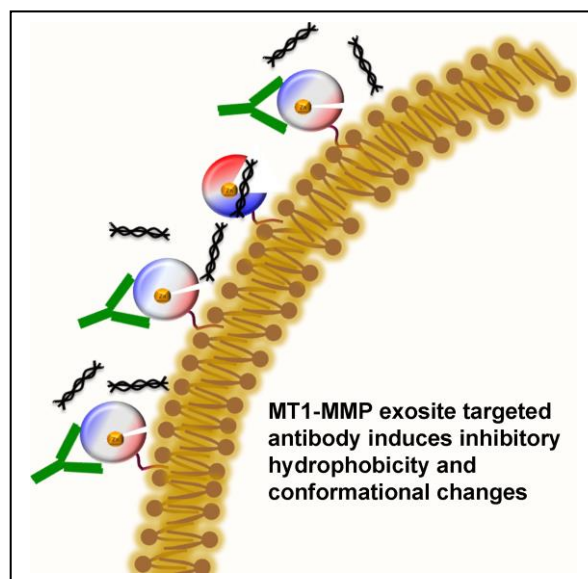
### E: Novel therapies based on function-blocking antibodies

Our goal was to develop a novel inhibition strategy for cancer therapeutics based on the key degradome-related molecules discussed above. We selected MMP-7 and -14 as principal targets for pancreatic cancer, but we also have developed function-blocking antibody fragments (Fabs) that are specific for MMP-2 and -9 during the course of the project. We also developed a highly selective protein-based inhibitor targeting ADAM17 (TACE). The design of these molecules involved consideration of the optimal site of inhibition, targeting a singular activity (e.g. catalytic) vs. multifunctional inhibition (inhibiting the activity of various functional domains), and substrate localization - extracellular, membrane-bound or intracellular.

The Fab fragments were purified and produced in high scale. Their activity was tested *in vitro*, *in situ* and *in vivo*. Furthermore, the newly developed nanosystems, were conjugated with the Fab agents and their inhibitory *in vitro* activity was characterized.

Particularly exciting for the SaveMe project is the production of inhibitory anti-MMP7 fab (clone 192). Using functional cell sorting analysis we found that the newly designed anti MMP-7 antibody kills pancreatic adenocarcinoma and pancreatic neuroendocrine tumor cells (PanNET). The treated cells showed changes in morphology with increasing concentrations of the mAb treatment. The anti-MMP7 mAb was shown to induce cell death via apoptosis in a dose-dependent fashion. Structural characterization (at atomic level) and *in vitro* functional analyses revealed that anti-MMP7 binds the activated conformation of MMP7 by binding the catalytic cleft of the enzyme.

Fab fragments of antibodies targeted against MMP7, MMP9 and MMP14 have been conjugated to NPs, and these conjugated NPs have been shown to retain inhibitory activity. These new conjugated NPs are being evaluated in preclinical disease models, as in the previous section.

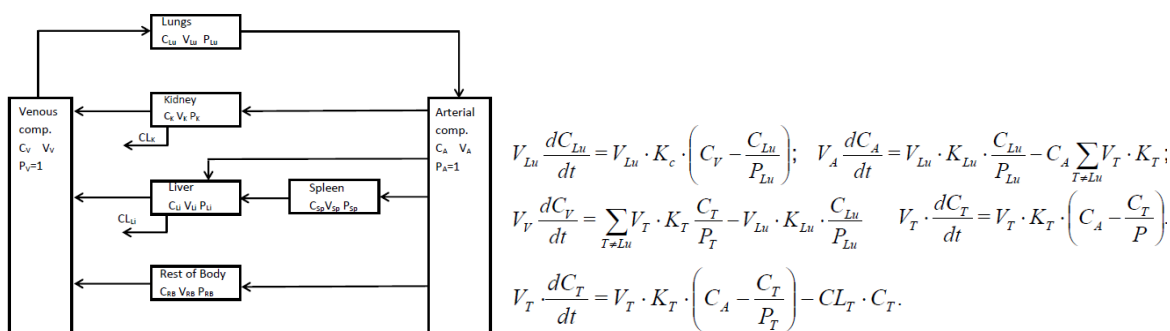




**(WP4) In-silico & in-vitro safety and efficacy analysis: Study various active imaging and therapeutic nanosystems IBCh**

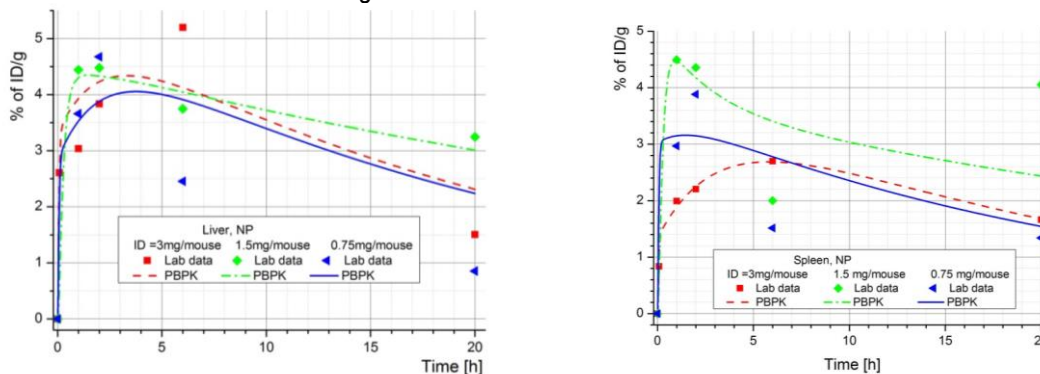
**In-silico modelling of NS biodistribution, toxicity and tumour growth.**

Two different physiologically based pharmacokinetic (PBPK) models and software tools were developed (a blood flow restricted (perfusion rate limited, PL, and a membrane-limited, ML, model) for biodistribution of NS in living organisms. The model equations are based on principles of mass conservation, fluid flow, and biochemistry. The model parameters, e.g., partition, diffusion and clearance coefficients for different organs or tissue systems are determined through calibration using data on concentration change of NS with time in plasma and organs. It was found that the permeability of the blood capillary membrane has significant effect on the transport of 200-400 nm NS between blood and tissue. The ML model would be more accurate than PL model in predicting the accumulation of NS with large binding affinities, amplified by multivalent interactions. These NS will accumulate in the first cells they encounter after extravasation. One of the ML models developed in SaveMe project is shown in Figure 4.1 together with the balance equations for lungs (Lu), arterial (A) and venous (V) compartment as well as any other non-clearing (T) and clearing (CL<sub>T</sub>) tissue/organ:



**Figure 4.1:** Membrane-limited PBPK model developed in SaveMe project and some of the equations used.

For ML PBPK models additional parameter, K [1/min], which represents the diffusion coefficient for individual tissue/organ, appears in the equations. This parameter is not known and the model determines this parameter for each tissue/organ, therefore providing additional accuracy and information on the particular organ. Comparison of the model results with laboratory data for different initial doses can be seen in Figure 4.2.



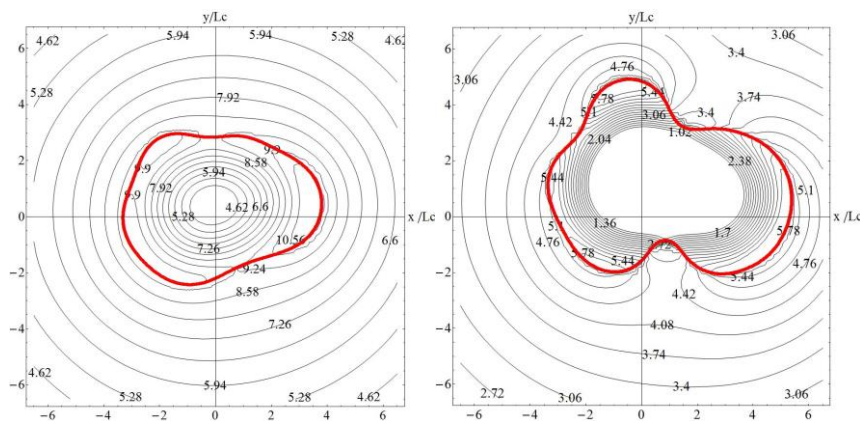
**Figure 4.2:** The laboratory data and results obtained by the membrane-limited PBPK model for concentration of rhodamine B fluorescence-labelled nanoparticles (NPs) with three different initial doses in liver and spleen.

The PBPK models can also be used to conclude on the toxicity of nanosystems based on *in-vitro* toxicity results.

A tumour growth model and a software tool were developed which determined the sharp interface between the tumour and the surrounding tissue. The model starts with a certain size and shape of the tumour which determine its growth depending on the parameters/conditions. The model is based on the CFD equations for diffusion of nutrients and Darcy’s law for cell movement. A number of constitutive equations provide the required links between blood-tissue transfer rate and concentrations in blood and tissue; cell velocity depending on pressure (constitutive equation based on Darcy’s law); Laplace-Young interface condition linking interface pressure, surface tension related to cell-to-cell adhesive forces and local total curvature; and several other constitutive equations related to characteristic tumour cell mitosis rate and tumour cell apoptosis rate. The cell-to-cell pressure is solved in the tumour as well as the pressure in the surrounding tissue to increase accuracy. The application domain includes the presence of necrotic core in viable tumour tissue and surrounding healthy tissue. The system of equations is non-linear and represents a moving interface problem. The numerical implementation has been based on the boundary element method. The

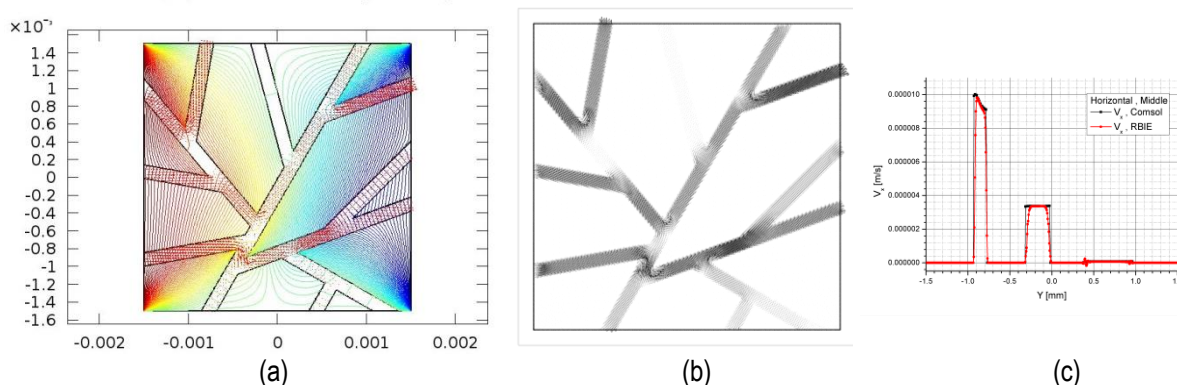
model can include the effect of therapeutic drug through the characteristic tumour cell mitosis rate and the tumour cell apoptosis rate. In Figure 4.3 the shape and size of the tumour can be seen, together with the distribution of the normalized pressure field after 5 and 16 days.

A model for transport and uptake of nanosystems in tissue was developed based on the CFD equations for dual porosity approach. The field variables in the model are fluid pressure for the flow, which is described by the Darcy law, and the solute concentration for the transport, which is calculated using the advection-diffusion equation. According to the dual porosity approach the tissue is considered to consist of two overlapping porous systems representing the interstitial and the vascular space.



**Figure 4.3:** Distribution of the normalized pressure field after 5 days (left figure) and 16 days (right figure)

The flow and the transport models consider that there is exchange of fluid and solute through the walls of the blood vessels, respectively. This exchange is taken into account in the model via an exchange term in the equations which depends on the difference in pressures inside the blood vessels and in the surrounding interstitial space, for flow, and on the difference in concentrations of species inside the blood vessels and in the surrounding interstitial space, for transport. The model and software tool is developed in-house and is calibrated and validated using COMSOL Multiphysics software platform. Figure 4.4 shows the comparison of the flow through tissue with a number of blood vessels obtained with the developed RBIE model – computer code and the commercial software COMSOL Multiphysics. Figure 4.4 shows the comparison of the velocities obtained with both computer codes with a good agreement in a profile which is defined through the middle of the domain.



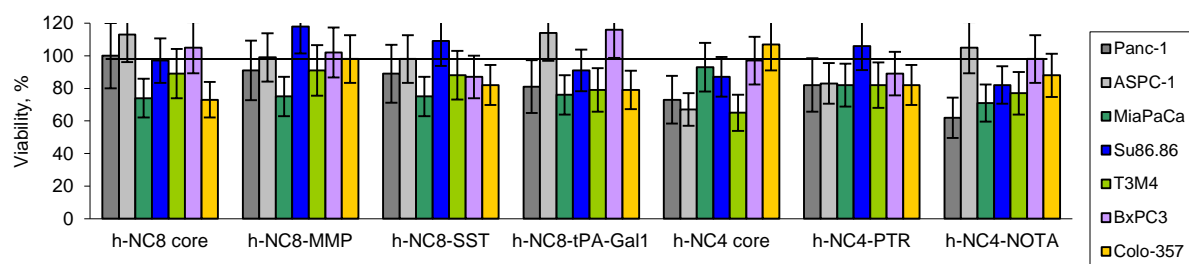
**Figure 4.4:** Comparison of the flow through tissue with a number of blood vessels obtained with the developed RBIE model – computer code and the commercial software COMSOL Multiphysics

### **lin-vitro safety and efficacy analysis: Study of various active imaging and therapeutic nanosystems**

*In-vitro* safety and efficacy analysis was done to find the parameters which can predict the possible toxicity of NS *in-vitro* aiming a selection of the best candidate for the development of a therapeutic delivery vehicle. These tests included the estimation of toxicity against cancer cell lines and biocompatibility with normal epithelial and blood cells. All the NS were routinely tested using MTT assay (toxicity against epithelial cells), blood cells (biocompatibility studies), traffic inside epithelial and macrophage cells using 2D and MCTS cultures (delivery of NS cargo). The results of *in-vitro* tests were verified by *in-vivo* experiments in mice in acute and chronic models.

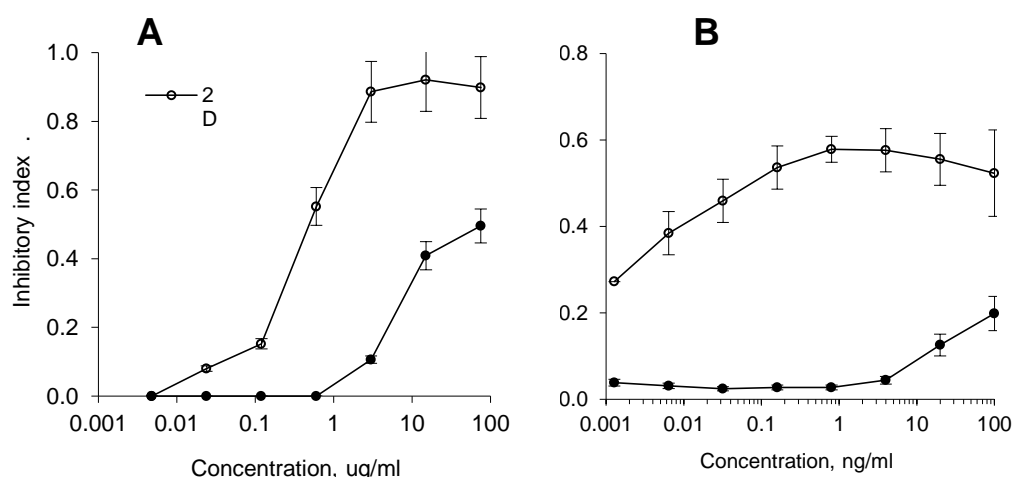
*In-vitro* tests using pancreatic and non-pancreatic (kidney, liver, skin) cell lines do not predict acute *in-vivo* toxicity of core NS but are sensitive to therapeutic moieties.

Incubation of core NS with epithelial cells, both pancreatic and non-pancreatic, demonstrated a low to moderate NS toxicity at high doses. Decoration of NS with PEG, targeting peptides, chelating moiety DOTA or NOTA did not affect core NP toxicity (Fig. 4.5).



**Figure 4.5.** Toxicity of h-NC8 or h-NC4 core NS and their analogues decorated with targeting moieties SST, PTR, tPA, Gal1; anti MMP antibodies; or chelating agent NOTA against epithelial cells lines estimated by MTT assay. NS were added at 100 ug/ml.

At the same time both flat and multicellular 3- dimensional (3D) cultures of cells were sensitive to anticancer drugs (Fig. 4.6). These tests are recommended only for the estimation of specific activity of therapeutic moiety such sRNA or low molecule drugs.



**Figure 4.6** Resistance of 2D and 3D BxPC-3 cultures to doxorubicin (A) and gemcitabine (B).

#### In-vitro platelet activation predicts an in-vivo risk of embolism

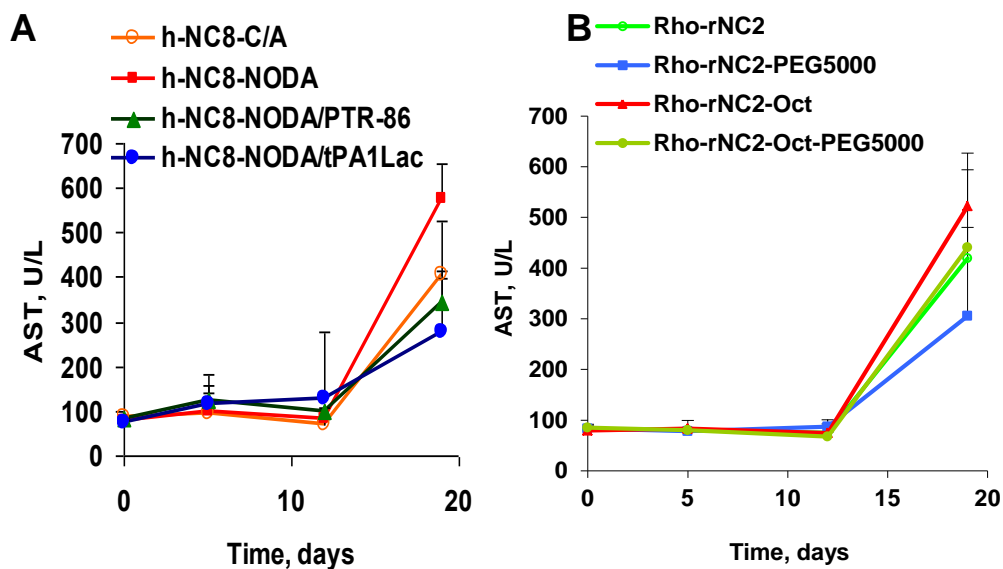
To verify the effect found *in-vitro* on platelet activation by positively but not negatively charged NS, mice were injected with high doses of NS and monitored for weight loss and mortality. All positively charged NS induced dose-dependent weight loss and mortality which directly correlated with platelet activation (Table 4-1).

Tumour therapy requires long term injection of NS. To estimate possible chronic toxicity mice were injected multiple times with medium doses of negatively charged NS. Liver and kidney enzymes were monitored over time. The only parameter – liver aspartate aminotransferase - was increased after 2 weeks of NS injections (Fig. 4-7) while all others were in a normal range showing relative safety of NS.

**Table 4.1.** Relation between positive charge and lethal dose of NS.

N	Types of NPs	Type and decoration	Z-potential	LD50, mg/kg	PL, %
1	rHSA	Rho-rNC2	negative	>300	7.6
		Rho-rNC2-PEG5000		>300	7.0
		Rho-rNC2-Oct		>300	7.3
		Rho-rNC2-Oct-PEG5000		>300	8.0
2	PLGA	<i>h</i> -NC18	negative	>300	7.5
		<i>h</i> -NC18-Rho6G-PEG		>300	7.3
		<i>h</i> -NC18-Rho6G-PEG-oct		>300	7.5
		<i>h</i> -NC8-C/A		>300	8.1
3	PLGA-PEG-maghemite	<i>h</i> -NC8-NODA	negative	>300	7.8

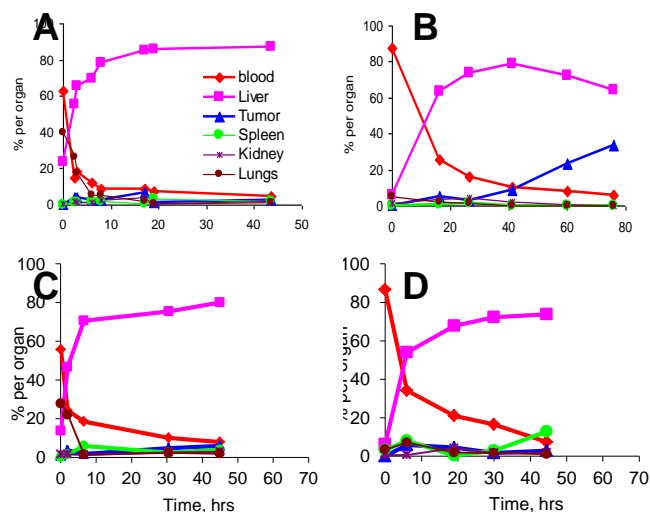
		h-NC8-NODA/PTR-86		>300	6.8
		h-NC8-NODA/tPA1Lac		>300	7.8
4	pMAA-PEG	NC12-PEG 2000	negative	58	95
5	DMAEMA-PEG	NC13-PEG 2000	positive	174	20
6	Maghemite	injPAMAM/PEI25-CAN- $\gamma$ -Fe2O3	positive	20	95
		injPEI25-CAN- $\gamma$ -Fe2O3		20	95
		H2O2conPEI25-CAN- $\gamma$ -Fe2O3		12	95



**Figure 4.7.** Chronic toxicity of NS. Mice were injected with 10 mg/kg of core h-NC8 or rNC2 NS as well as decorated with NODA, PTR, tPA1Lac, PEG, Oct, Rho every second day, total dose 100 mg/kg of weight. Aspartate aminotransferase blood level was the only parameter increased as a result of therapy. Decoration did not affect chronic toxicity.

#### NS <100 nm are accumulated in tumours better than larger NS

Biodistribution studies demonstrated that 60-70% NS were retained by liver after 24 h (Fig. 4.8, A). Smaller than 100 nm NS were accumulated 6-10 times more in tumours than large NS (>150 nm) (Fig. 4.8, B). Decoration with PEG did increase blood circulation of PLA 200 nm NS and led to a higher retention of NS in spleen but not in tumours (Fig.W4-9, B-C) possibly because of the large size of NS.



**Figure 4.8.** Biodistribution of NS in mammary tumour bearing ASn mice. A-B. 450 nm acrolein NS (A) and small 20 nm quantum dots (B) are differently accumulated in tumours. C-D. Effect of PEGylation on biodistribution of PLA (C) and PLA-PEG (D) NS.

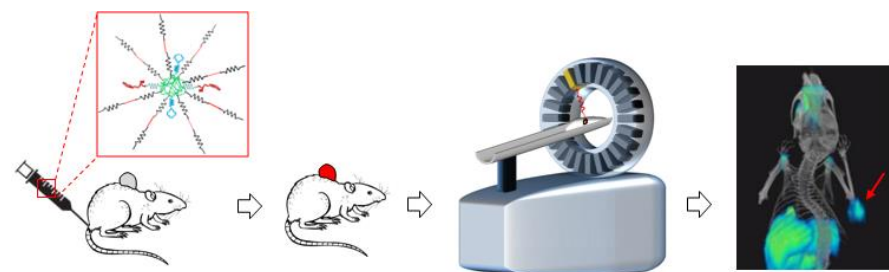


In vitro studies demonstrated a different intracellular traffic of positively and negatively charged NS. NS<sub>pos</sub> were accumulated presumably in mitochondria while NS<sub>neg</sub> co-localized with lysosomes. Overloading of cells with NS leads to their redistribution between many organelles (lysosomes, mitochondria, Golgi apparatus, cytoplasm) which is a prerequisite to siRNA delivery into tumour cells.

### (WP5) In-vivo proof of concept – minimally required studies of selected prototypes UKH

One of the ultimate goals of the project was the development of an imaging-based diagnostic tool suitable for the early diagnosis of pancreatic cancer; in other words, a multi-decorated nanoparticle (NP), capable to accumulate selectively or preferentially in the tumour, and containing a label suitable for the non-invasive detection of the NP loci after administration into a living organism (see Figure 5.1).

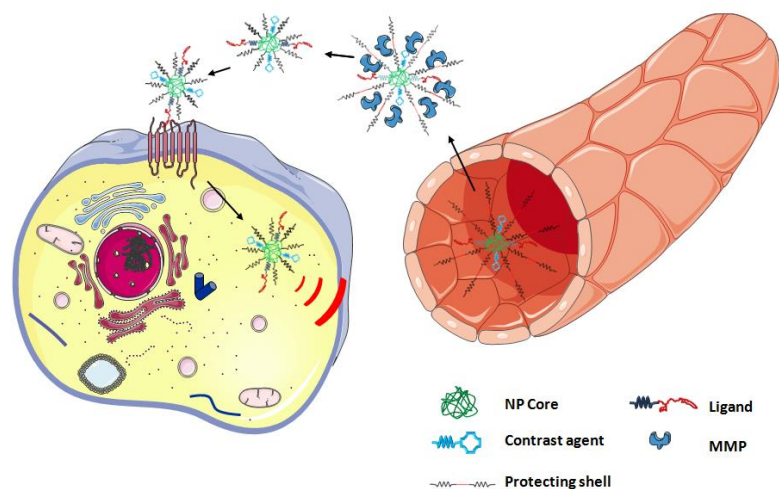
The mechanism by which NPs reach tumour is well established: Tumour growth is usually accompanied by the formation of new vessels. If this process occurs fast, the neo-vasculature has defects and is more permeable, allowing large molecules and NPs to move from the blood stream into the tumour interstitium. Additionally, tumours usually have a deficient lymphatic drainage, contributing to a longer residence time of the NPs in the tumour. Overall, this process is known as Enhanced Permeability and Retention (EPR) effect. The residence time of the NPs in the tumour can be even prolonged by incorporation of a molecule (so-called *ligand* or *targeting moiety*) on the surface of the NPs, able to specifically interact with a receptor which is over-expressed in tumour cells.



**Figure 5.1.** Schematic representation of the use of NPs as diagnostic tools. Multidecorated NPs bearing a contrast agent are administered into living organisms. After selective/preferential accumulation in the tumour, images are obtained using dedicated cameras and the tumour can be visualized.

time in blood. However, if NPs become larger in size, internalization into tumour cells might be compromised. To overcome this problem, a radically novel strategy was assessed in this project. The process consisted of functionalizing the NPs with PEG, attached to the NP via a cleavable linker. The presence of the PEG in the outer shell should prolong circulation time (and prevent sequestration by the RES), while the linker, which was a substrate of a matrix metalloproteinase (MMP) over-expressed in cancer cells, should enable particle down-sizing in the tumour to favour cell internalization (this process is schematized in Figure 5.2).

Five different particles, two different ligand types (one ligand of somatostatin receptors -SSTR, one ligand of Galectin, tPA-1)



**Figure 5.2.** General scheme of the diagnostic strategy: NPs, which have a long circulation time thanks to the presence of PEG attached to the NP via a linker (MMP substrate), accumulate in the tumour interstitium by EPR. Once there, the MMPs cleave the linker; NPs become smaller, and the presence of the ligand enables interaction with receptors over-expressed in cancer cells, leading to potential internalization. The contrast agent (which is attached to the NP) finally enables localization of the NPs *in vivo* using imaging techniques.

One of the main problems associated to the use of NPs as diagnostic tools is the bioavailability, or residence time of the NPs in the bloodstream. Usually, if NPs are small they are rapidly filtered by the kidneys and cleared from the body via urine. If NPs are large, they can be sequestered by the Reticulo-endothelial system (RES, mainly liver, pancreas and lungs). Decoration of the NPs with different polymers, such as polyethylene glycol (PEG), has proven to prevent aggregation and to prolong circulation

time in blood. However, if NPs become larger in size, internalization into tumour cells might be compromised. To overcome this problem, a radically novel strategy was assessed in this project. The process consisted of functionalizing the NPs with PEG, attached to the NP via a cleavable linker. The presence of the PEG in the outer shell should prolong circulation time (and prevent sequestration by the RES), while the linker, which was a substrate of a matrix metalloproteinase (MMP) over-expressed in cancer cells, should enable particle down-sizing in the tumour to favour cell internalization (this process is schematized in Figure 5.2).

Five different particles, two different ligand types (one ligand of somatostatin receptors -SSTR, one ligand of Galectin, tPA-1) and two different imaging modalities (Single Photon Emission Computerized Tomography-SPECT and Magnetic Resonance Imaging-MRI) were available in the toolbox of the consortium. Additionally, MRI was available at two different field strengths: 11.7 T and 1.5T, the latter chosen in order to be representative of the diagnostic capabilities for the general public, as scanners with this field strength are available in most clinical institutions worldwide.

The most promising particle core/s, ligand/s and imaging modality/ies were selected in a first step. With that aim, the 5 different NP cores containing both (either one or another) ligand and containing tracers to enable visualization using SPECT-gamma camera or MRI were administered to mice bearing PANC-1 (human pancreatic carcinoma) tumours, and images were obtained at different time points after administration. Non targeted particles were used as controls, to evaluate the

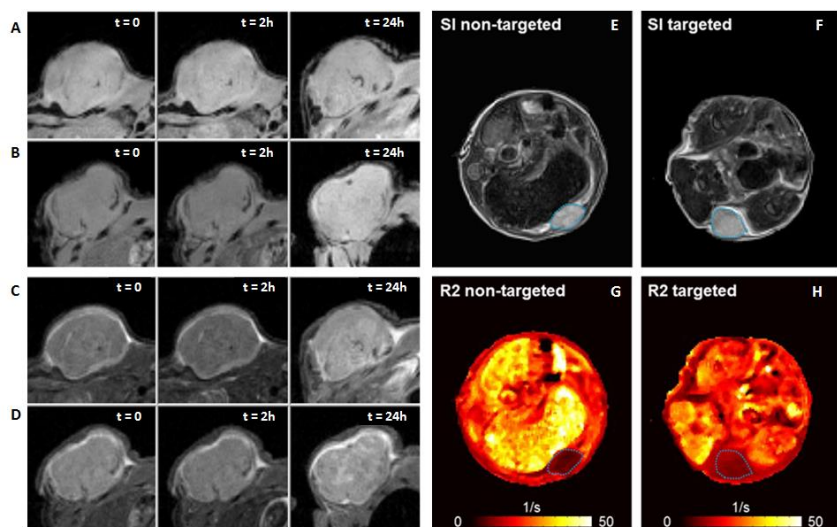
effect of the ligand. At this stage, the attachment of the PEG-MMP substrate to the NPs was skipped.

11.7T MRI experiments showed a rapid uptake of the NPs in the liver. Signal changes within the tumour were difficult to detect in the first 2h after NP administration, but significant diffuse signal alterations were observed at 24h (Figure 3A-D). These results highlight the excellent relaxation properties of the NPs and indicate that the particles are able to penetrate the experimental tumour slowly in low concentrations. Also, the images permitted the visualization of heterogeneity in the tumour. However, no differences between targeted and non-targeted agents could be observed.

1.5T experiments showed that for the non-targeted NPs, only the liver presented a significant signal intensity drop caused by the NPs' presence (Figure 3E). On the contrary, the targeted NPs showed a loss in signal intensity in tumour, liver, and kidneys,

but not in the muscle (Figure 5.3F). The signal drops for each region of interest (ROI) were, however, not significant. R2 relaxation rates (quantitative method, with increase of R2 rates representing increased uptake of particles) were measured before MNP administration and 90 min after administration. The kidneys expressed a slightly increased but not significantly different relaxation rate before and after NP administration. The liver and for one of the NPs also the tumour, showed a significant R2-increase due to the non-targeted MNP influence on the spin-spin-relaxation (Figures 3G-H). These results are in good agreement with the signal intensities measured for the mice injected with non-targeted NPs (Figure 5.3).

SPECT-computerized tomography images (computerized tomography used for anatomical localization of the radioactivity) showed in general terms an increase in the tumour-to-muscle ratio over time, irrespective of the NP type and the nature of the targeting moiety (Figure 4). Most of the NPs accumulated in the liver even at short

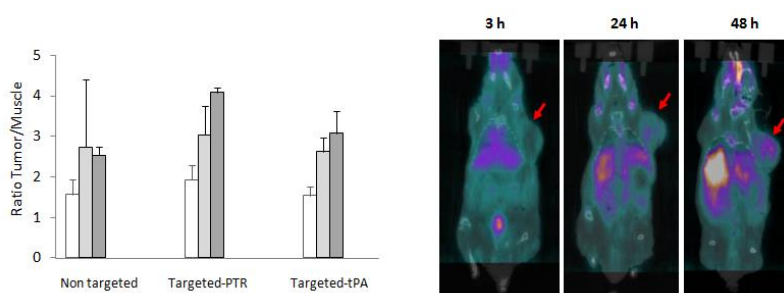


**Figure 5.3.** Representative zoomed T1 (A,B) and T2 (C,D)-weighted images (11.7T) showing the temporal evolution of MNPs uptake before (left), 2 (middle) and 24 hours after injection (right) of NPs. Representative slices of mouse MRI-datasets 90 min after MNP administration. (E, F) Conventional signal intensity images obtained at 1.5T; (C,D) Spin-spin-relaxation rate (R2). E and G were acquired after an injection on non-targeted nanoparticles; F and H were acquired after an injection of targeted nanoparticles. The sc tumors' outer boundaries are indicated in light blue. The tumour expressed a significantly higher R2 in case of the injection of targeted nanoparticles compared to the non-targeted nanoparticle injection.

times after administration, and no significant differences were observed between targeted and non-targeted NPs (Figure 5.4). However, determination of the tumour-to-muscle ratio was quite straightforward, enabling the visualization of tracer uptake within the tumour at late time points after administration (48 hours). Hence, this was selected as the imaging modality of choice for subsequent experiments.

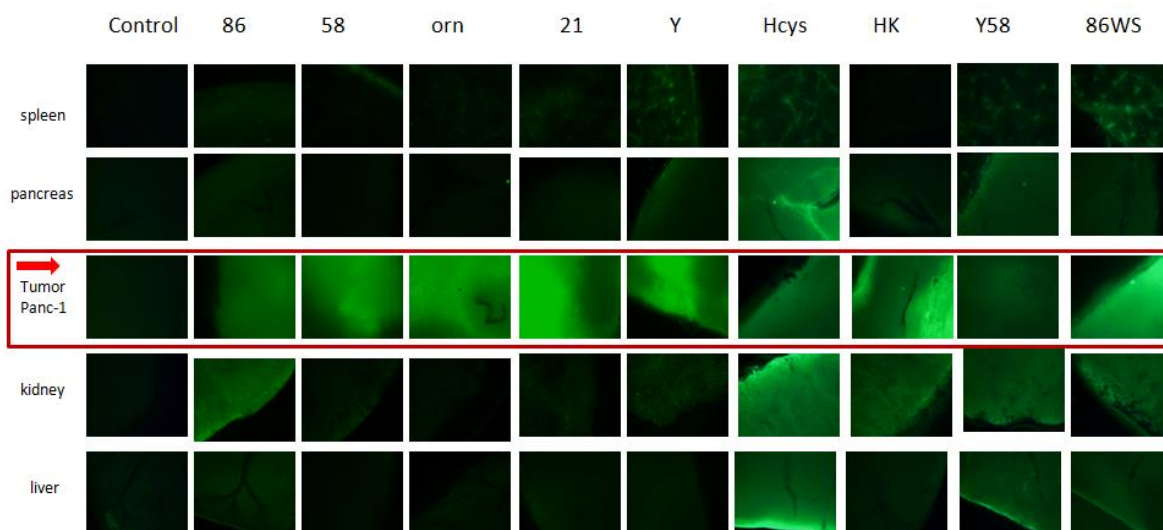
Among the 5 types of NPs, two of them were selected for decoration using the PEG-MMP substrate and further investigation. Finally, both targeting moieties (SSTR and Galectin-1 ligands) were also selected, but exploration for the pursuit of more selective SSTR ligands was continued.

In this regard, an exhaustive search was performed in order to find more selective SSTR ligands. The ligands were synthesized and labelled with a fluorescent moiety, and the accumulation in different organs was determined after intra-venous administration into mice bearing subcutaneous PANC-1 tumours using fluorescence microscopy. The results, depicted in Figure 5.5, show that several of the candidates presented a high accumulation in the tumour, while accumulation in other organs (spleen, pancreas, kidneys and liver) was low. Among all candidates, PTR-58 was the most promising one, and this somatostatin analogue was selected for subsequent *in vivo* investigations. As shown in Figure 5.5, accumulation of PTR-58 in the spleen, pancreas, kidney and liver is negligible, while accumulation in the tumour is significant.



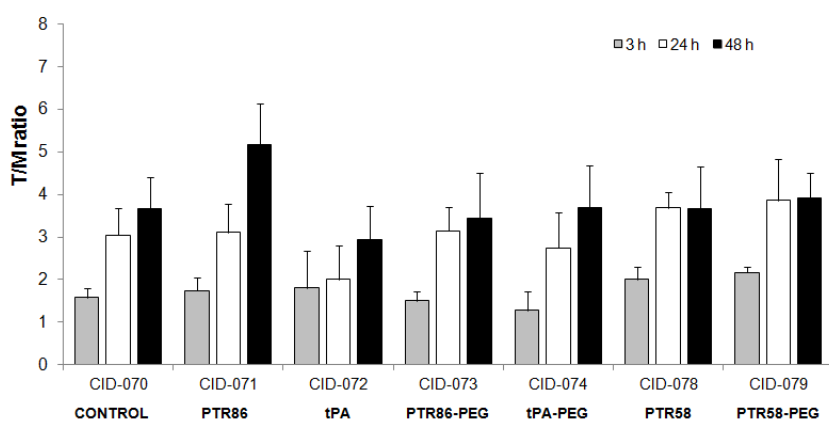
**Figure 5.4.** Tumour-to-tissue ratios and representative SPECT images for non-targeted and targeted NPs incorporating the two ligands available.





**Figure 5.5.** Signal intensity in the different organs (namely, spleen, pancreas, tumour, kidneys and liver) for the different SSTR ligands assayed. PTR-58 was selected for subsequent *in vivo* investigations using nuclear imaging.

The two selected NP cores were decorated simultaneously with the ligand (either PTR-58 or tPA1) and the PEG moiety anchored to the NPs via the cleavable linker. All NPs were investigated in mice bearing PANC-1 tumours at different time points after administration, using SPECT as the imaging modality.



**Figure 5.6.** Tumour-to-muscle ratios obtained for the different NPs at different time points after administration into mice bearing sub-cutaneous PANC-1

SPECT images (Figure 5.6 for results of tumour-to-tissue ratios) showed that all NPs, irrespective of the ligand and the presence of the PEG-cleavable linker, showed increased tumour-to-muscle ratios with time. One of the combinations, constituted by a polymeric NP with PTR-58 (CID-071 in the Figure), showed significant differences with respect to the control NP, and in fact this NP also showed the highest capacity for tumour visualization, resulting in images with correct tumour contrast. Despite these NPs cannot be considered appropriate diagnostic tools, they showed improved capacity for the visualization of the tumour. Still, fine tuning of the NP properties (e.g. size, surface charge, loading of the ligand, etc) might be

required for it to become a diagnostic tool in the pre-clinical setting. Interestingly, the imaging modality (SPECT) enabled the quantification of the amount of NP accumulated in the tumour in absolute terms; hence, a prediction of the therapeutic efficacy might be performed if NPs would be intended for therapeutic purposes.

In parallel to the work carried out for the development of new contrast agents for the early diagnosis of pancreatic cancer, and

moving towards the clinical application of the technology, another activity was conducted in the frame of the project. Indeed, the clinical need to improve detection and to observe in real-time if lesions are correctly removed leads to the need for small, handheld gamma cameras. The hand-held gamma camera used in this project has a square shape with 65 mm side length and 180 mm length, and a total weight of 800 g (Figure 5.7). The sides of the camera are shielded with 3 mm lead, and the device is optimized for the most common nuclide in nuclear medicine,  $^{99m}\text{Tc}$ , but can be used for energies from 50 till 250keV.

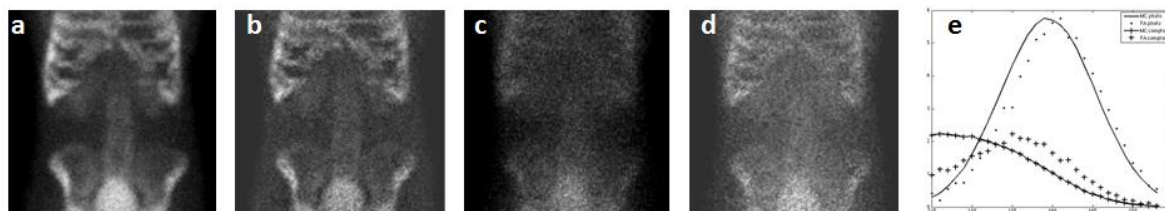
Although the handheld gamma camera is relatively small, the control unit to monitor and visualize the images is a standard PC. The preliminary clinical data obtained by sentinel lymph node measurements showed that a smaller control unit would improve the handling of the imaging system dramatically. Hence, an open-source hardware was used to develop a control unit, which was controlled using a touch-screen (with the logo of the project in Figure 5.7).

One of the main problems associated to nuclear imaging is the poor resolution obtained and the low signal-to-noise, especially when the uptake of the contrast agent in the area of interest is low, when there is a high unspecific uptake or when there are issues associated



**Figure 5.7.** Hand-held gamma camera used in the frame of this project.

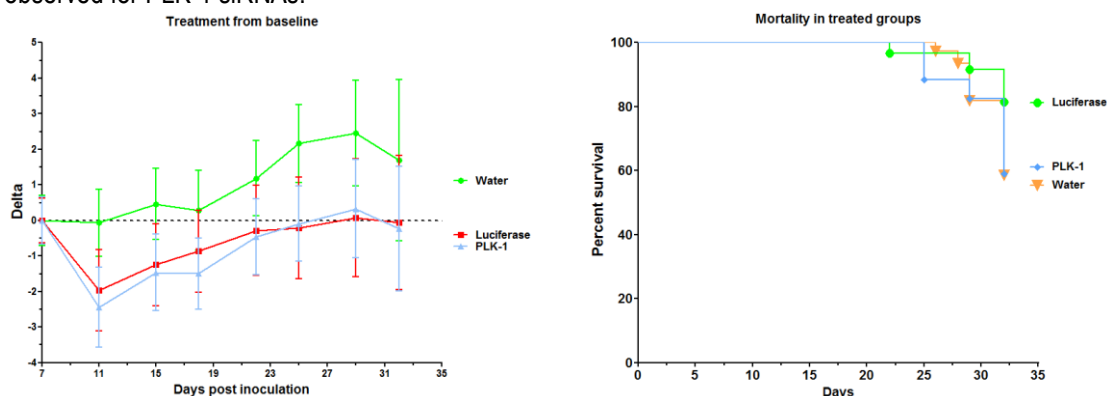
with scattering of the gamma photons. Here, in parallel with other activities conducted in the frame of the project, factor analysis was used to optimize the scatter correction for both imaging modalities, planar and SPECT studies. Since scattered photons degrade the image quality in nuclear medicine, many attempts to correct this effect have been performed in the past. Factor Analysis for scatter correction assumes that the spectrum of the photons detected in a specific pixel can be decomposed into a photo-peak (the signal of interest) and a Compton spectrum (originated by scattering of the gamma rays, not of interest). Previously, a number of spectral images were acquired and used for the analysis procedure. Here, a novel methodology based in subdivision of the energy windows was developed. As a result of the analysis, two images (photopeak, scatter) and two factor curves, representing the corresponding energy spectrum, can be obtained; the overall result is that images with higher contrast can be obtained. Despite this methodology has not been applied in the context of the project, it can be applied to any nuclear imaging study and hence may become a powerful tool for future application of radiolabelled NPs.



**Figure 5.8.** Illustration of the application of the new method for image processing, applied to a  $^{99m}\text{Tc}$  bone study. (a) MC-simulation: true photo peak image; (b) MC simulation: photopeak image obtained by factor analysis; (c) MC simulation: true scatter image; (d) MC simulation: scatter image obtained by factor analysis; (e) Comparison of factor curved with spectra obtained by factor analysis.

Partner BIU selected the best therapeutic siRNA candidate- PLK-1 (see data from WP3). **SU.86.86 eGFP** cell line from were used for *in vivo* therapy study by intraperitoneal NSs delivery (**MagRET- . conPEI<sub>25</sub>-OX-CAN- $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>**)

The experiment is still in progress. The eGFP can be clearly seen by the Maestro machine. The results (**Figure 5.21**) demonstrate a clear effect as compared to water injection, but at this point the protection with NS-siRNA is similar to the one observed for PLK-1 siRNAs.



From the experiment presented we can conclude that MagRET/PLK-1 siRNA can stop tumor progression and that the IP injection of both tumor and NPs is a valid model to examine the efficacy of the nano-carriers that we developed during the funding period. We are optimistic about these results but they need to be repeated several times before we can reach a firm conclusion.

## Potential impact

SaveMe project was dedicated to the development and optimization of novel active nanotechnology systems for advanced imaging and therapeutic options for solid cancer, providing intracellular drug delivery of novel non-classic drugs. The proposed novel drugs designed based on extensive degradomics analysis, thus potentially defining a therapeutic profile per pancreatic cancer and per patient, increasing the efficiency of the treatment and reducing side effects. Proper toxicity studies were performed following standard *in-vitro* protocols and guideline, including using the developed *in-silico* modeling for estimation and prediction of toxicity related effects, evaluation of biocompatibility, biodegradability, along with efficacy features.

This project's research and development outputs have a tremendous potential to be successfully translated into clinical practice, thus providing more precise and efficient detection and diagnosis of pancreatic cancer in its earliest stages, more accurate staging, post-therapy monitoring and potent inhibition of tumor and metastasis, combined with much lower side effects, resulting in better healthcare for the citizens along with reducing the cost of healthcare. The SaveMe technological platform can be implemented for other cancers by activating the nanosystems with the specific functional particles. Implementing a core concept based on highly innovative scientific concepts, SaveMe has a high positive impact on the competitiveness of the European healthcare industry in world markets.

## Scientific impact

The novel modular platform of targeted nanosystems for imaging diagnosis and guided surgery, and for therapeutics is a breakthrough in cancer. The innovative nanotechnologies functionalized nanosystems together with *in-silico*, *in-vitro* and minimally *in-vivo* tests for pancreatic cancer will serve as a background for further applications in different solid cancer therapies and diagnostics. The proposed modular active nanosystems platform can be implemented for the management of various solid tumors, as per their tumor-specific receptors- SSTR and Gal-1/tPA selective expression.

### Breakthroughs:

- (1) Superior active nanosystems for targeted molecular MR, PET imaging, SPECT and gamma camera, enabling highly sensitive diagnosis and guided surgery;
- (2) Active non-classic anti-tumor antibodies and siRNA- based therapies, based on cancer and degradome / "protease web" studies, enabling efficient therapy by the inhibition of tumor cell growth, invasion and metastasis;
- (3) Profiling individualized degradome can be implemented for personalized therapy;
- (4) Proven concept of the designed nanosystems activated with targeting peptides, PEG –MMP-substrate-PEG agent, enabling targeted diagnosis, and targeted therapy.

**Early detection of cancer through screening:** The European Commission is continuing its efforts to protect the health of European citizens by taking concrete actions based on sound scientific evidence. It is active in working to identify and promote good practice in cancer related prevention, diagnosis, treatment and care. Cancer is not only a national but a European health challenge. By sharing knowledge, capacity and expertise in cancer prevention and control, the EU will be able to more effectively tackle and combat cancer across the Union. SaveMe proposed platform of nanosystems for solid tumors diagnostics and therapeutics is totally in line with this EU policy and activities<sup>8</sup>, in providing effective treatment that would bring significant health and economic benefits to all EC countries, and at the same time disease prevention that will actively target high risk groups and individuals.

## Reinforcing the competitiveness of European industry

Nanotechnology is emerging as one of the most promising and rapidly expanding fields of R&D. It is currently the focus of intense development in the field of **nanomedicine - the application of nanotechnology to achieve breakthroughs in healthcare**.

**Targeted diagnostics and therapy in solid cancer** is emerging as one of the most promising tools for cancer treatment. SaveMe proposed a leading research partially based on partners achievements and successful preliminary results aiming at a nanosystems platform capable of binding targeting moieties, enhanced contrast agents for imaging, therapeutic agents and intracellular drug delivery, for solid cancer with a proof of concept for pancreatic cancer. The realization of this project results will effectively contribute, to **the transformation of the European medicine, pharmacology, medical imaging and nanotechnology related industries from a resource-intensive to a knowledge-intensive phase, which will further contribute to the EU WW position in these fields**. This project is part of the chain in this transformation, introducing high added value technologies and products in a **sustainable manner**. The outputs of the project will increase European scientific and technological qualities, thus preventing relocation of European science to other areas worldwide, and at the same time creating industrial and employment growth within Europe.

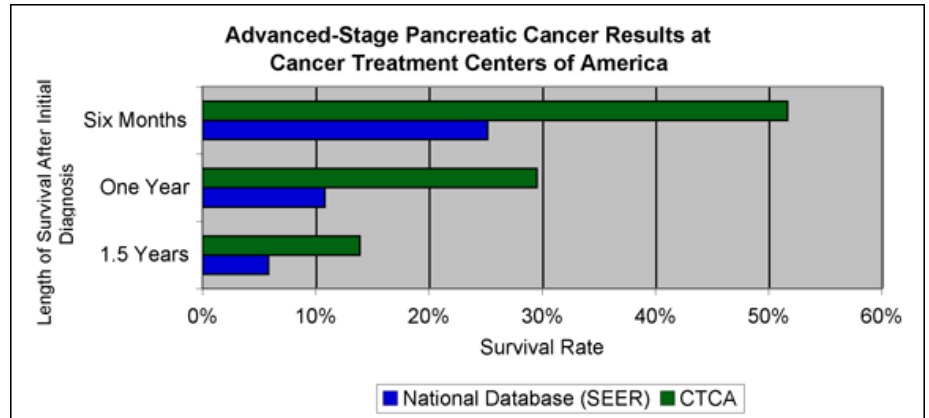
<sup>8</sup> [http://ec.europa.eu/health-eu/health\\_problems/cancer/index\\_en.htm](http://ec.europa.eu/health-eu/health_problems/cancer/index_en.htm)

Socio-Economic impact

• **Solid tumor and PANCREATIC CANCER**

The proposed modular active nanosystems platform can be implemented for the management of **various solid tumors**, via their selective expression<sup>9</sup>. As a test case, SaveMe developed and validated the new platform for **PANCREATIC CANCER**: Pancreatic cancer has the highest one-year mortality rate of any cancer and is Europe's sixth deadliest cancer<sup>10</sup>. The overall five-year survival rate is 4%, and was not improved during the last 25 years<sup>11</sup>. Most pancreatic tumors are detected late, at metastatic stage and 85% are unresectable at the time of detection<sup>12,13</sup>. This is due, in part, to the limitation of current imaging systems in diagnostic accuracy, particularly in determining resectability and evaluation of sub-cm nodal and metastatic disease. Molecular imaging will **greatly reduce the need for animal tissue sampling or human biopsy** when studying the progression of a disease or the follow-up of a gene therapy. It has the advantage of being non-invasive, and because it can be repeated many times, it provides both spatial and temporal dimensions to the understanding of the gene expression of the disease or therapy.

**The superior performance of the SaveMe platform is expected to improve imaging, thus enabling earlier detection and more accurate staging and post-therapy monitoring. Furthermore, MMPs selective and potent inhibition, which blocks tumor metastasis, will be a key therapeutic approach for pancreatic cancer, thus having a tremendous impact on the European citizens' health care and quality of life.**

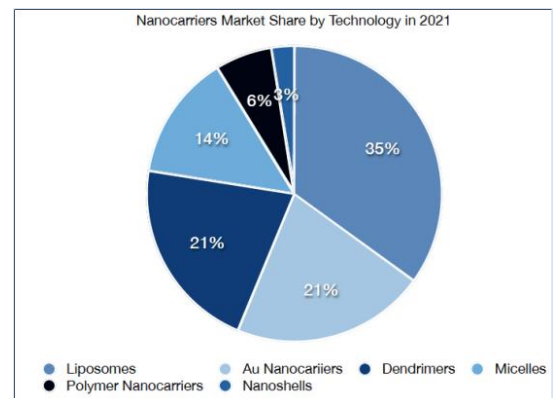


• **Market estimates-**

**Nanocarriers as MRI and PET tracers**

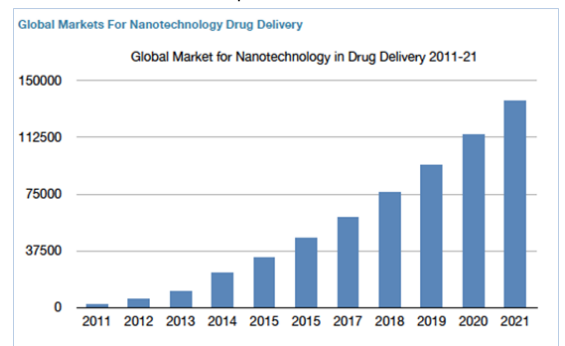
Nanocarriers-enabled tracers with novel targeting cancer-targeting moieties labeled for dual imaging will target the MRI and PET contrast agents markets.

MRI contrasting agents and PET radiopharmaceuticals show tremendous growth potential and help to balance the effects of market saturation and price erosion in the overall contrast media and radiopharmaceuticals market. Companies that overcome the pricing threats and introduce cost-effective and enhanced imaging agents are well poised for success<sup>14</sup>. Contrast media gain increased acceptance as their role in improving diagnostic accuracy is being realized. The market for medical imaging contrast media reached **\$1.41 billion** in 2004 and anticipated to increase to **\$4.72 billion by 2014**<sup>15,16</sup>. Active-imaging nanosystems, to be developed in this project, are expected to become a significant player in the contrast agents market. The radio pharmaceuticals market generated revenues of **\$ 443 million in 2004** with an anticipated increase to **\$ 811 million by 2014**.



**Nanocarriers for drug delivery market:**

- Annual sales WW 2011: Abraxane \$426 million, Doxil \$402 million, Ambisome \$457 million. All 3 are non-targeted nanoparticles;
- Total market size of Nanotechnology (includes nanocrystals and nanocarriers) in 2021, \$136B. Nanocarriers represent 40% of that market
- At present, there are about 30 main drug delivery products on the market. Overall income is around US\$33 billion with an annual



<sup>9</sup> Pelosi G. et al., A subset of high-grade pulmonary neuroendocrine carcinomas shows up-regulation of matrix metalloproteinase-7 associated with nuclear beta-catenin immunoreactivity, independent of EGFR and HER-2 gene amplification or expression; Virchows Arch. 2005 Dec;447(6):969-77. Epub 2005 Sep 13.

<sup>10</sup> Michaud DS. 2004. Epidemiology of pancreatic cancer Minerva Chir. Apr; 59(2):99-111

<sup>11</sup> Jemal A, et al., Cancer statistics, 2004. CA Cancer J Clin 2004; 54:8.

<sup>12</sup> Francis, I., Cancer Imaging, 4, 10-14 (2003)

<sup>13</sup> www.cancercenter.com/.../survival-outcomes.cfm

<sup>14</sup> Frost & Sullivan: <http://healthcare.frost.com>

2.1 <sup>15</sup> Frost & Sullivan; North American Contrast Media Markets; July 11, 2007 151 Pages - Pub ID: MC1521546

<sup>16</sup> [http://www.biotechsystems.com/breakingmarketnews/good\\_market\\_growth\\_for\\_contrast\\_agents.asp](http://www.biotechsystems.com/breakingmarketnews/good_market_growth_for_contrast_agents.asp)



growth of 15% (based on global product revenue). Liposomes and gold nanocarriers account for 45% of the total addressable market. Liposomes will offer the largest addressable market (\$15 billion) in 2021 while gold nanocarriers will see the highest compound annual growth rate (CAGR)—53.8%—in the next decade.<sup>17</sup>

- **Health care costs:** Healthcare costs now accounts for more than 10% of GDP in Europe<sup>18</sup>. An ageing population, with higher levels of costly chronic conditions, will place an increasing strain on healthcare systems at a time when budgets are under pressure. Healthy citizens reduce the strain on healthcare systems and boost economic growth by staying active for longer. A recent OECD study<sup>19</sup> concludes that just a one-year improvement in a population's life expectancy could contribute to a 4% increase in output. Devoting resources to prevention, screening, treatment and care can reduce chronic diseases and improve people's well-being, cutting costs in the long run. Although generally effective in slowing the progression of the disease and prolonging survival, traditional cancer treatments have significant limitations. The cost for cancer diagnostics and treatment could be dramatically reduced by the development of more effective targeted therapeutic modalities. The economic impact of **PANCREATIC CANCER** is also of great concern. In a recent analysis using a prevalence approach method by Wilson and Lightwood<sup>20</sup>, the direct medical care costs in the US were estimated to be **\$881 million annually**.

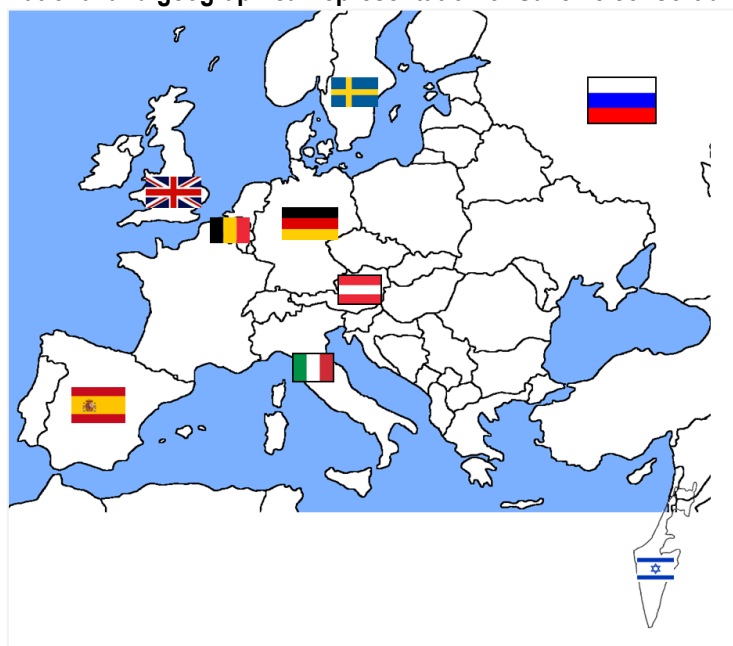
- **Employment:**

SaveMe is a cross-disciplinary research area, which promotes collaborations between nanochemists, physicists, biochemists, biologists, imaging and modeling experts, and clinicians. Thus it represents a promising field for growth and sustainable employment in Europe, since it is a high-added value industrial area. MRI and PET nanosized agents for diagnosis and follow up solid cancer treatment could become powerful tools alongside or even over the existing methods and hence SaveMe will contribute to increase job opportunities and competitiveness in nanochemistry, nanobiology diagnostics and industry sectors. Moreover, the technologies developed in the project will have an added value in improving working conditions of the imaging and medical staff.

### European added value

The challenging objectives of this project demanded a multidisciplinary, international collaboration of biology, biochemistry, nano-chemistry, imaging, oncology, and physics as well as training, exploitation, dissemination and project management. These disciplines were chosen from different countries to establish a consortium of great expertise. The participants will benefit from different approaches in Europe. The consortium is formed from **20 organizations** from **9 different countries**: **7 member states: DE, UK, IT, BE, SE, ES and AT**; **one associated state: IL**, and one ICPC partner: **RU**. It is obvious that the research work cannot be achieved at a national level, as research expertise gathered in this European project **is critical for its success**. The success of the project is highly dependent on this expert consortium. This challenging research needs **European cooperation**, since the specific knowledge and expertise of the different partners from different countries is essential, and it cannot be performed at the level of a single country.

**The national and geographical representation of SaveMe consortium**



<sup>17</sup> Research and Markets Report. Nanotechnology for Drug Delivery. February 2012ID: 2059352

<sup>18</sup> WHO Global Health Expenditure Database, Eurostat, MedTech Europe calculations

<sup>19</sup> [http://www.nber.org/papers/w8587.pdf?new\\_window=1](http://www.nber.org/papers/w8587.pdf?new_window=1)

<sup>20</sup> [http://www.medscape.com/viewarticle/409001\\_2](http://www.medscape.com/viewarticle/409001_2)

## Main dissemination activities and exploitation of results

### Dissemination

SaveMe consortium has allocated substantial efforts and resources for the purpose of dissemination of the project results within the scientific community and to the general public. The main efforts and dissemination activities are presented below:

### Scientific Articles published

1. **UEA**- Decock J, Thirkettle S, Wagstaff L, Edwards DR. Matrix metalloproteinases: protective roles in cancer. *J Cell Mol Med* 2011; 15(6):1254-65.
2. **UEA** -Wagstaff L, Kelwick R, Decock J, Edwards DR. The roles of ADAMTS metalloproteinases in tumorigenesis and metastasis. *Front Biosci* 2011; 16:1861-72.
3. **UNIBO, BIU, CIC, FS**: Erica Locatelli, Lorena Passoni, Liron Limor Israel, Maria Naddaka, Andrea Pucci, Torsten Reese, Larraitz Gil, Vanessa Gómez-Vallejo, Paolo Milani, Michela Mattioli, Jordi Llop, Jean Paul Lellouche, Mauro Comes Franchini. Biocompatible nanocomposite for PET/MRI hybrid imaging of pancreatic cancer. *International Journal of Nanomedicine*, 2012;7, 6021-6033.
4. **UNIBO**: Mauro Comes Franchini, Erica Locatelli. Biodegradable PLGA-b-PEG polymeric nanoparticles: synthesis, properties and nanomedical applications as drug delivery system. *Journal of Nanoparticles Research*, 2012;14, 1316-1324.
5. **UNIBO**: Erica Locatelli, Francesca Broggi, Jessica Ponti, Patrick Marmorato, Stefano Lena, Mauro Comes Franchini. Lipophilic Silver Nanoparticles and Their Polymeric Entrapment into Targeted-PEG-based micelles for the treatment of Glioblastoma. *Advanced Healthcare Materials*, 2012: 1, 342-347.
6. **KUL**: Jennifer Vandooren, Nathalie Geurts, Erik Martens, Philippe E Van den Steen, Ghislain Opdenakker. Zymography methods for visualizing hydrolytic enzymes. *Nature Methods*, 2013;10, 211-220.
7. **BIU**: [Liron L. Israel](#), [Emmanuel Lellouche](#), [Ron S. Kenett](#), [Omer Green](#), [Shulamit Michaeli](#) and [Jean-Paul Lellouche](#). Ce<sup>3/4+</sup> cation-functionalized maghemite nanoparticles towards siRNA-mediated gene silencing. *J. Mater. Chem. B*, 2014,2, 6215-6225 (DOI: 10.1039/C4TB00634H)
8. **BIU (IBCh, GU, FS)**: Liron L Israel, Elena I Kovalenko, Anna A Boyko, Alexander M Sapozhnikov, Ina Rosenberger, Jörg Kreuter, Lorena Passoni and Jean-Paul Lellouche. Towards hybrid biocompatible magnetic rHuman serum albumin-based nanoparticles: use of ultra- small (CeL<sub>n</sub>)<sup>3/4+</sup> cation-doped maghemite nanoparticles as functional shell. *Nanotechnology*, 2015, 26, 045601 (DOI: 10.1088/0957-4484/26/4/045601)
9. **UEA**: Thirkettle S, Decock J, Arnold H, Pennington C, Edwards DR. Matrix metalloproteinase-8 (collagenase-2) induces the expression of interleukins-6 and -8 in breast cancer cells. *JBC*, 2013.
10. **UEA**: Kelwick R, Edwards DR. The ADAMTS ( A Disintegrin and Metalloproteinase with Thrombospondin motifs) family. *Genome Biology*, 2013.
11. **WSP**: Siroos Mirzaei, Peter Knoll. Additional lesions detected in therapeutic scans with 177Lu-DOTATATE reflect higher affinity of 177Lu-DOTATATE for somatostatin receptors. *Oncology*, 2011;80, 326-329.
12. **WSP**: Siroos Mirzaei, Peter Knoll. Easy to use online software package for internal dose assessment after radionuclide treatment in clinical routine. *Clinical Nuclear Medicine*, 2013.
13. **UNIBO**: Mauro Comes Franchini, Erica Locatelli, Andrea Pucci. "Click chemistry on the surface of PLGA-b-PEG polymeric nanoparticles: a novel targetable fluorescent imaging nanocarrier". *Journal of nanoparticles Research*, 15: 1818-1823, 2013.
14. **KUL (BIU, GU, UNIBO, CID, UEA, TAU)**: Jennifer Vandooren, Nele Berghmans, Chris Dillen, Ilse Van Aelst, Isabelle Ronsse, Liron Limor Israel, Ina Rosenberger, Jörg Kreuter, Jean-Paul Lellouche, Shulamit Michaeli, Erica Locatelli, Mauro Comes Franchini, Miren K. Aiertza, Laura Sanchez-Abella, Iraidia Loinaz, Dylan R. Edwards, Louis Shenkman and Ghislain Opdenakker. Intradermal air pouch leukocytosis as an in vivo test for nanoparticles. *International Journal of Nanomedicine* 8: 4745 – 4756, 2013.
15. **KUL**: Jennifer Vandooren, Philippe E. Van den Steen and Ghislain Opdenakker. 2013. Biochemistry and molecular biology of Gelatinase B or Matrix Metalloproteinase-9 (MMP-9): the next decade. *Critical Reviews in Biochemistry and Molecular Biology*. 48:222-272.
16. **KUL**: Rocco Rossano, Marilena Larocca, Lea Riviello, Maria Gabriella Coniglio, Jennifer Vandooren, Grazia Maria Liuzzi, Ghislain Opdenakker and Paolo Riccio. 2014. Heterogeneity of serum gelatinases MMP-2 and MMP-9 isoforms and charge variants. *Journal of Cellular and Molecular Medicine*. 18:242-252.
17. **KUL**: Ghulam Mohammad, Jennifer Vandooren, Mohammad M. Siddiquei, Erik Martens, Ahmed M. Abu El-Asrar and Ghislain Opdenakker. 2014. Functional links between gelatinase B/matrix metalloproteinase-9 and prominin-1/CD133 in diabetic retinal vasculopathy and neuropathy. *Progress in Retinal and Eye Research*. 43:76-91.
18. **KUL, WEIZMANN**: Jennifer Vandooren, Benjamin Born, Inna Solomonov, Ewa Zajac, Radka Saldova, Michael Senske, Estefanía Ugarte-Berzal, Erik Martens, Philippe E. Van den Steen, Jo Van Damme, Angeles García-Pardo, Matheus



- Froeyen, Elena I. Deryugina, James P. Quigley, Søren K Moestrup, Pauline M. Rudd, Irit Sagi and Ghislain Opendakker. 2015 Circular trimers of gelatinase B/ matrix metalloproteinase-9 constitute a distinct population of functional enzyme molecules differentially regulated by tissue inhibitor of metalloproteinases-1. *Biochemical Journal*. 465:259-270
19. **KUL:** Jennifer Vandooren, Jo Van Damme and Ghislain Opendakker. 2014 On the structure and functions of gelatinase B/matrix metalloproteinase-9 in neuroinflammation. *Progress in Brain Research*. 214:193-206.
  20. **BIU, IBCh, GU, FS;** Israel LL, Kovalenko EI, Boyko AA, Sapozhnikov AM, Rosenberger I, Kreuter J, Passoni L, Lellouche JP Towards hybrid biocompatible magnetic rHuman serum albumin-based nanoparticles: use of ultra- small (CeLn)<sub>3/4</sub>+cation-doped maghemite nanoparticles as functional shell. *Nanotechnology*, **2015**, *26*, 045601.
  21. **KUL, FS, BIU, UNIBO, GU:** Christian Schmithals, Jennifer Vandooren, Silvia Bianchessi, Paolo Milani, Erica Locatelli, Liron L. Israel, Frank Hübner, Michela Matteoli, Jean-Paul Lellouche, M. Comes Franchini, Lorena Passoni, Eugenio Scanziani, Ghislain Opendakker, Albrecht Piiper, Jörg Kreute. rPhysico-chemical and toxicological characterization of iron-containing albumin nanoparticles as platforms for medical imaging. Ina Rosenberger,. *J. Controlled Release*, **2014**, *194*, 130-137.
  22. **IBCh:** A. Zubareva, T. S. Shcherbinina, V. P. Varlamov, E. V. Svirshchevskaya. Intracellular Sorting of Differently Charged Chitosan Derivatives and Chitosan-Based Nanoparticles. *NanoScale*. 2015. Waiting for DOI:
  23. **IBCh** Ekaterina Myrsikova, Maria Grechikhina, Alexander Prokhorov, Nadezhda Osipove, Ina Rosenberger, Klaus Felix, Elena Svirshchevskaya, Quiescent and non-quiescent multicellular tumor spheroids formed by pancreatic cells. *NanoScale* (submitted).
  24. **TUM** Seubert B, Grünwald B, Kobuch J, Cui H, Schelter F, Schaten S, Siveke JT, Lim NH, Nagase H, Simonavicius N, Heikenwalder M, Reinheckel T, Sleeman JP, Janssen KP, Knolle PA, Krüger A. *Tissue inhibitor of metalloproteinases (TIMP)-1 creates a premetastatic niche in the liver through SDF-1/CXCR4-dependent neutrophil recruitment in mice*. *Hepatology*. 2015 Jan; 61(1):238-48.
  25. **TUM** Grünwald B, Schlage P, Krüger A, auf dem Keller U. MMPs:From Genomics to Degradomics. Chapter in Irit Sagi's Book. *In press*
  26. **TUM** Berchtold S, Grünwald B, Krüger A, Reithmeier A, Hähl T, Cheng T, Feuchtinger A, Born D, Erkan M, Kleeff J, Esposito I. *Collagen type V promotes the malignant phenotype of pancreatic ductal adenocarcinoma*. *Cancer Lett*. 2015 Jan 28;356(2 Pt B):721-32.
  27. **TUM** Seubert B, Cui H, Simonavicius N, Honert K, Schäfer S, Reuning U, Heikenwalder M, Mari B, Krüger A. Tetraspanin CD63 acts as a pro-metastatic factor via  $\beta$ -catenin stabilization. *Int J Cancer*. 2014 Oct 29.

#### **Popular press:**

1. Louis Shenkman, Mauro Comes Franchini. A Modular Nanosystems Platform for Advanced Cancer Management: Nano-vehicles; Tumor Targeting and Penetration Agents; Molecular Imaging, Degradome based Therapy. *Chimica & Industria*, June 2012, 110-113.
2. Louis Shenkman, Mauro Comes Franchini. A Modular Nanosystems Platform for Advanced Cancer Management: Nano-vehicles; Tumor Targeting and Penetration Agents; Molecular Imaging, Degradome based Therapy. *Nanotech-Italy*, 2013.
3. A modular nanosystems platform for advanced cancer management. Louis Shenckman, M. Comes Franchini, *Chimica e Industria*, 2012, N. 16, Anno 94, 64-67.
4. Save-Me the newsletter paper from EU. 2013. A Modular Nanosystems Platform for Advanced Cancer Management: Nano-vehicles; Tumor Targeting and Penetration Agents; Molecular Imaging, Degradome based Therapy. Louis Shenkmana and Mauro Comes Franchinib
5. 2013 Web edition. UniBo english magazine <http://www.unibo.it/en/research/projects-and-initiatives/Unibo-Projects-under-7th-Framework-Programme/cooperation-1/nanosciences-nanotechnologies-materials-and-new-product-technologies-nmp/saveme>. Title: A Modular Nanosystems Platform for Advanced Cancer Management: Nano-vehicles; Tumor Targeting and Penetration Agents; Molecular Imaging, Degradome based Therapy.

#### **Project Brochure:**

OSM has developed and produced the project brochure. The Project Brochure was designed as a dissemination tool for the project. The brochure contains a general description of the project. The informative brochure promotes and describes the vision of the project, main objectives and outcomes as well as the project layout. The brochure also contains information about the project partners and their contact info. The brochure can be downloaded via the SaveMe website domain and as prints during relevant conferences, meetings and workshops.

#### **Website construction and maintenance**

The SaveMe's website (<http://fp7-saveme.com/>). contains two main sections (1) The public section which aims to introduce the consortium, project concept, objectives and vision; (2) The partners' restricted section which offers an access to project records, e.g. submitted documents, deliverables and reports.