New INDIGO NPP1
FINAL REPORT

August 2012

NanoLINEN is an international collaborative project funded by EC – FP7

New INDIGO NPP1
Reporting template – Final Report

1. Identification of project and participants

PROJECT FULL TITLE: NANOTOXICOLOGY LINK BETWEEN INDIA AND EUROPEAN NATIONS

Project acronym: NanoLINEN

Project starting date: November 2010

Period covered: November 2010 to August 2012

Date of submission: August 2012

Project coordinators writing the report:

Prof. Dr. Bensu Karahalil (European Coordinator)

Prof. Dr. Alok Dhawan (Indian Coordinator)

Name, title and organization of the representative of the project's coordinators:

- Gazi University, Faculty of Pharmacy, Department of Toxicology
  Tel: +903122124699
  Fax: +903122222326
  Email: bensuka@gmail.com
- CSIR-Indian Institute of Toxicology Research, Nanomaterial Toxicology Group
  Tel: +91-522-2230749
  Fax: +91-522-2628227
  Email: dhawanalok@hotmail.com; alokdhawan@iitr.res.in

Identification of project participants

(1) GU, Ankara/Turkey

(2) IITR, Lucknow/India

(3) MUI, Innsbruck/Austria

(4) UDC, A Coruna/Spain

(5) INSA, Porto/Portugal

(6) NCOD, Amsterdam/Netherlands

(7) UBA, Dessau, Rosslau/Germany

(8) CEA, Paris/France

(9) SR, Roma/Italy (unofficial partner)

(10) MU, Manchester/England (unofficial partner)

COUNTRY	NAME	INSTITUTE	E-MAIL
AUSTRIA	Dietmar FUCHS	Innsbruck Medical University, Division of Biological Chemistry Biocenter/**INNSBRUCK**	Dietmar.Fuchs@i-med.ac.at
FRANCE	Nathalie Herlin-BOIME	French Atomic Energy Commission (CEA), SPAM-LFP/**PARIS**	nathalie.herlin@cea.fr
	Marie CARRIE	Laboratoire Lesion des acides nucleiques CEA Grenoble/**GRENOBLE**	marie.carriere@cea.fr
GERMANY	Heidi BECKER	Federal Environment Agency, Section II 1.2 Toxicology, Health-related Environment Monitoring/**BERLIN**	Heidi.Becker@uba.de
	Heinz MORINSKE		
INDIA	Alok DHAWAN*	Indian Institute of Toxicology Research, Nanomaterial Toxicology Group/**LUCKNOW**	dhawanalok@hotmail.com
	Rishi SHANKER	Indian Institute of Toxicology Research, Environmental Toxicology Group/**LUCKNOW**	rishi@iitr.res.in
	K. C. GUPTA	Indian Institute of Toxicology Research, Director/**LUCKNOW**	kcgupta@iitr.res.in
NETHERLANDS	Pieter van BROEKHUIZEN	IVAM UvA, Nanotechnology and Chemical Risks/**AMSTERDAM**	pvbroekhuizen@ivam.uva.nl
	Gert van der LAAN	Netherlands Center for Occupational Diseases (NCOD), Coronel Institute, Academic Medical Center/**AMSTERDAM**	g.vanderlaan@amc.uva.nl
PORTUGAL	João Paulo TEIXEIRA	National Institute of Health, Environmental Health Department/**PORTO**	jpft12@gmail.com
	Carla COSTA	National Institute of Health, Environmental Health Department/**PORTO**	cstcosta@gmail.com
SPAIN	Blanca LAFFON	University of A Coruña, Toxicology Unit/**A CORUNA**	blaffon@udc.es
	Juan Fernandezt TAJES	University of A Coruña, Cell and Molecular Biology Dept.)/ **A CORUNA**	jfernandezt@udc.es
	Vanessa VALDIGLESIAS	University of A Coruña, Toxicology Unit/**A CORUNA**	vvaldiglesias@udc.es
TURKEY	Bensu KARAHALIL**	Gazi University Faculty of Pharmacy, Department of Toxicology/**ANKARA**	bensuka@gmail.com
	Ali Esat KARAKAYA		alikarakaya@tr.net
	Ayse Basak ENGIN		abengin@gmail.com
	Erdem COSKUN		erdemcos@gmail.com
ITALY (unofficial partner)	Stefano BONASSI	IRCCS San Raffaele Pisana/**ROMA**	Stefano.bonassi@sanraffaele.it
UK (unofficial partner)	Diana ANDERSON	Biomedical Sciences, School of Life Sciences University of Bradford/**UK**	d.anderson1@bradford.ac.uk

* Indian Coordinator
** European Coordinator

2. Publishable summary

2.1. Short description of activities and significant results

NanoLINEN project partners have achieved all their prospected objectives for this term. Among them, the significant results of the project are listed:

2.1.1. WORKSHOPS

a. *Kick off Meeting* (8-9 November 2010): A successful meeting among the partners to discuss details of the project and to create a strategy plan has been held in Istanbul and the project has been kicked off.

b. *1st Workshop of NanoLINEN – International Symposium on The Safe Use of Nanomaterials & Workshop on Nanomaterial Safety: Status, Procedures, Policy and Ethical Concerns* (3-5 February 2011): This meeting was successfully held in Lucknow organized by the Indian partners, chaired by Prof. Dr. Alok Dhawan.

c. *2nd Workshop of NanoLINEN – Nanomaterials: Risk perception and early warning systems* (25-26 May 2011)*:* This one has been organized by both German and Dutch partners in Berlin and chaired by Dr. Heidi Becker, Dr. Gert van der Laan and Dr. Pieter van Broekhuizen.

2.1.2. EXCHANGES between LABORATORIES

The list below represents the exchange between partners.

FROM	TO	NAME	PERIOD
Portugal	India	**Carla Costa** (PhD Student)	2011-February
Spain	India	**Vanessa Valdiglesias** (PhD Student)	2011-February
Portugal	Spain	**Joao Paulo Teixeira** (Senior Scientist)	2011-May
Portugal	Spain	**Carla Costa** (PhD Student)	2011-May
France	Spain	**Marie Carriere** (Senior Scientist)	2011-May
France	Spain	**Nathalie Herlin** (Senior Scientist)	2011-May
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2011-April-May

Portugal	Spain	**Joao Paulo Teixeira** (Senior Scientist)	2012-April
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2012-January
Spain	Portugal	**Blanca Laffon** (Senior Scientist)	2012-January
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2012-March
Spain	Portugal	**Blanca Laffon** (Senior Scientist)	2012-March
Spain	Portugal	**Juan F. Tajes** (Senior Scientist)	2012-March

2.1.3. SCIENTIFIC PROJECTS

- Validation study on TiO2 nanoparticles toxicity: Effects on genetic stability, DNA damage, immunological status, and mutation frequency in vitro.
    - This project has been launched at the workshop in India for validation of standardized testing systems for the assessment of TiO_2 toxicity.
    - ……..
    - ……..
    - ……
    - ……..

2.1.4. FURHER EU PROJECT PROPOSALS

- During the executive meetings at the workshops, a lot of discussions have been made for submitting a new EU project proposal on this field. Also for this purpose, many scientific connections have been established with the distinguished scientists in this area.
- In all our meetings, it is discussed to be a part of an EU-FP7 project (a middle size or large) and serious steps have been taken. The next call of EU projects NMP.2012.1.3-1 is thought to be a good opportunity for giving proposals. It is decided to make our team stronger by joining our forces with other groups working in nanotoxicology area in Europe.
- A new application has been made to New-INDIGO as a research part of this project, project number given by the system INDIGO-DBT2-034. However, this proposal has been rejected. (MAYBE WE CAN CRITICISE THEM OF THEIR CHOICE)
- ……
- ……

3. Work progress and achievements during the period

3.1. Overall objectives

NanoLINEN (NANOTOXICOLOGY LINK BETWEEN INDIA AND EUROPEAN NATIONS) is aimed at establishing strong scientific links between the EU and India in the emerging area of nanotoxicology to initiate interdisciplinary collaborative studies.

NanoLINEN will help to investigate the potential environmental and human health risks associated with nanotechnology.

NanoLINEN leads to trans-national networks in this upcoming area using the following strategy:

3.1.1. Research visits of senior and junior scientists

NanoLINEN puts strong emphasis on the mobility of scientists between the participating laboratories to facilitate interaction for developing a meaningful and active collaborative research program.

3.1.2. Organizing workshops

To create awareness and understanding of this new area, NanoLINEN will organize workshops with academia, industry, regulatory agencies and civil society in different participating countries. Travel grants will be provided for young NT researchers from India and EU.

The themes of the "The New-INDIGO WORKSHOPS ON NANOTOXICOLOGY" are:

1. Nanomaterial Safety: Status, Procedures, Policy and Ethical Concerns.
2. Nanomaterials: Risk perception and early warning systems
3. Biomarkers & Human Exposure to Nanoparticles
4. Physico-chemical aspects, toxicity and in-vitro methods
5. Biomarkers in vivo and in vitro
6. Nanosafety Congress – Turkey

The workshops have provided a platform to improve the understanding of the new technology and increase the awareness on possible associated risks. A network of multidisciplinary Nanotoxicology-scientists has started to be set up among the different countries and students will be trained in this important area. This also helps to evolve coherent and loose networks among the different countries as well as generating trained man power in this important area. Participation in the 'public nanodialogue' is assured by giving the floor to an interaction between the civil society, academia and industry. This may generate consensus and trust for Nanotoxicology and prevent adverse reactions as was experienced with the Genetically Modified (GM) foods. The structured brainstorming at the workshops have guided the development of future projects and allowed setting out strategies to effectively pool and manage the limited resources.

Also, some of the workshops included laboratory work apart from lectures, which will make NanoLINEN stand out among others for the initiative taken to develop trained human resource in this important field.

3.1.3. Development of new projects

It is envisaged that new scientific collaborative projects are developed targeted on the assessment of occupational and consumer exposure to NPs. These approaches could predict outcomes of human exposures to NMs in various settings.

3.1.4. Development, establishment and validation of tests and biomarkers for NM safety

It is proposed to develop, establish and validate tests and biomarkers for NM safety at cellular and molecular level. Inter and intra laboratory validation of tests for assessing health risks of NMs shall be accomplished by exploiting the diverse expertise of the scientists and infrastructure available with the partners.

3.1.5. Providing contribution to the preparation of international guidelines for NM safety

The data gathered via the workshops and research visits of scientists between EU and India will be declared as a report ("New INDIGO-NanoLINEN Group Report on Nanotoxicology"), two times, in October 2011 (current Mid-Term Report) and October 2012 (Final Report). The project is expected to improve our understanding about interaction of NMs with biological systems and thus contribute to international guidelines for NM safety/toxicity.

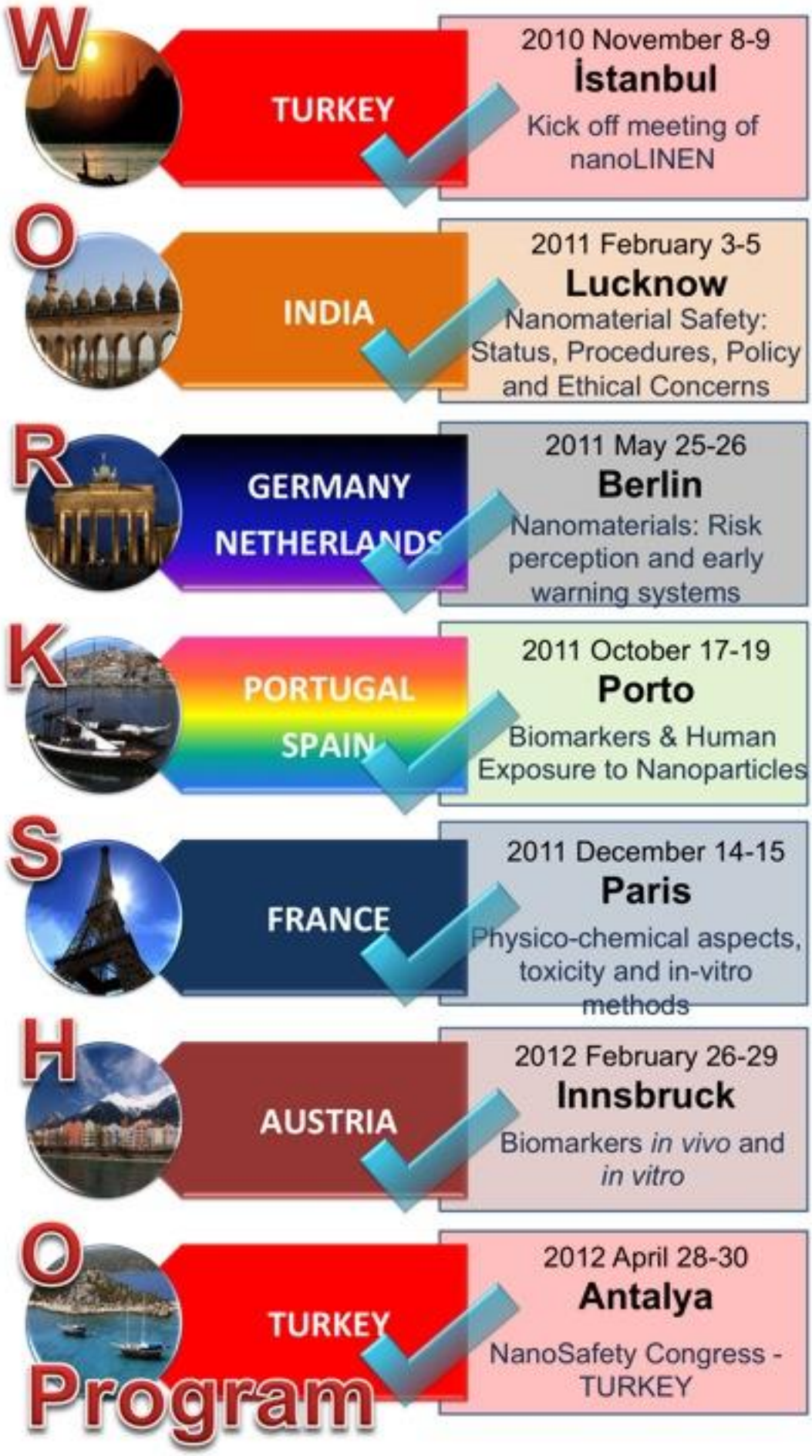
3.2. Project activities that have been foreseen:

1. All the workshops, which have been planned at the beginning of the project, has been completed successfully.
2. A collaborative effort to validate useful biomarkers to assess toxicity profiles of commonly used engineered nanoparticles.
3. Future connections have been established between other groups who works with nanoparticle toxicology and fruitful collaborations has been initiated.
4. Many student and senior exchanges has been made between the partner laboratories.

3.3. Work progress and achievements:

3.3.1. A summary of activities

3.3.1.1. Workshops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TURKEY
2010 November 8-9
İstanbul
Kick off meeting of nanoLINEN
O
INDIA
2011 February 3-5
Lucknow
Nanomaterial Safety: Status, Procedures, Policy and Ethical Concerns
R
GERMANY
NETHERLANDS
2011 May 25-26
Berlin
Nanomaterials: Risk perception and early warning systems
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PORTUGAL
SPAIN
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Biomarkers & Human Exposure to Nanoparticles
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Physico-chemical aspects, toxicity and in-vitro methods
H
AUSTRIA
2012 February 26-29
Innsbruck
Biomarkers in vivo and in vitro
O
TURKEY
2012 April 28-30
Antalya
NanoSafety Congress - TURKEY
Program

3.3.1.1.1. Kick off Meeting:

A kickoff meeting was successfully convened at Istanbul by the EU Coordinator – Turkey, where all the partners participated and decided the future course of action.

The kick off meeting in Istanbul was very fruitful in the meaning of setting up the links between the partners and deciding the high priority duties of the project. The following questions were tried to be answered in the meeting;

- Format for the workshops? Is it only supposed to be a workshop for the project team, or is strategic persons invited as well (stakeholders: from academia, industry, governments etc)?

Rationale: If stakeholders are invited as well, who takes care for identifying them, and inviting them?

- Is there a maximum size for the workshops?

Rationale: the larger the group, the more difficult it is to involve all participants actively in the discussions. Therefore it might be considered to set a maximum to the participation

- Who is supposed to take part in the brainstorming?

Rationale: a successful brainstorming needs a thorough preparation and a thorough steering. If a workshop is supposed to be a mix of participants, this might work contra productive. Therefore it might be considered to organize separate, but in timing connected meetings: one with an open workshop followed by a brainstorm meeting only for the project team.

- What is the supposed location of the workshops?

Rationale: it is supposed that the national teams will organize a workshop on the theme. But it might be wise not to organize this workshop in their own country, but instead in the country where discussion on the specific topic is the most needed, or the country from where it is expected to get the most participants. This would set also a claim on the organizing country, for organizational activities.

- Is participation of all partners in all workshops obligatory?

Rationale: not all topics belong to the core business of the specific partner, and as a consequence their input might be minimal. What are ideas concerning this point?

- What is the role for students in the workshops?

Rationale: do we try to involve potential students already in the workshops. And what students (nationality, discipline) do we like to involve? To involve students would mean a significant effort already before the workshop.

- Reporting of the workshops?

Rationale: who is responsible for reporting the results? Are workshops supposed to lead to a result, or are they supposed to be part of the capacity building activities, just leading to more educated, more aware participants.

3.3.1.1.2. International Symposium on the Safe Use of Nanomaterials & Workshop on Nanomaterial Safety: Status, Procedures, Policy and Ethical Concerns:

This 1st workshop (combined with the conference) of NanoLINEN group was organised in Lucknow, India by the CSIR-Indian institute of Toxicology Research from February 1-6, 2011. In the conference participants from 15 different countries and the scientists underscore the possible adverse effects exerted by the nanomaterials. The challenges in the nanoparticle toxicity studies were also addressed in the conference and it was emphasized that a systematic study design using different assays can give the relevant information about the toxicological properties of the nanomaterials. Selected articles from the conference were published in a special issue of the ***Journal of Biomedical Nanotechnology (volume 7, number 1, February 2011)***.

The purpose of the conference was to create awareness about the toxicological concern of nanomaterials. A "Workshop on the Methods in Nanomaterial Toxicology" was also conducted to validate the protocols used in the nanoparticles toxicity studies. A laboratory manual of validated protocols was prepared, so that all the partners can establish and use the protocols in their laboratories. Selected articles from this conference have also been published in a special issue of the Journal of Biomedical Nanotechnology (volume 7, number 1, February 2011). A guidance document for the safe use of nanomaterials in the laboratories was also published to minimize the exposure of nanoparticles to researcher and the environment. The genotoxic potential of titanium dioxide (TiO_2) nanoparticles in human liver cells as well as the further consequences aroused by DNA damage, such as oxidative stress, apoptosis; mitochondrial dysfunctions were also investigated. However, in *Escherichia coli*, the metal oxide nanoparticles (Zinc oxide, ZnO; TiO2) were found to induce the oxidative stress and DNA damage which leads to the reduced viability of the *E. coli.* The official website (http://www.nanolinen.org/) for the project was also prepared and the relevant information can obtain from this website.

3.3.1.1.3. Nanomaterials: Risk Perception and Early Warning Systems:

In the month of May 2011, another international conference and workshop was convened at Berlin, Germany, where the main concern was focused towards the risk possessed by the nanomaterials and the need for the guidelines to mitigate the risk. The International Workshop on Risk Perception and Early Warning Systems on Nanomaterials was organized by the Umwelt Bundes Amt in close collaboration with the Netherlands Centre for Occupational Diseases.

For logistic and efficiency reasons it was decided to combine the Workshops on Risk Perception and Early Warning Systems on Nanomaterials instead of organizing two separate workshops with overlapping participants. Therefore, German and Dutch partners hosted 2 separate themes:

Risk Perceptions of Nanomaterials (hosted by the Germany team):

The Berlin Workshop on Risk Perception focussed on differences and approaches towards risk perception on Nanomaterials of the public and government agencies in Europe and India. In the Workshop on Early warning systems medical surveillance of workers with engineered nanoparticles to monitor possible adverse health effects was discussed, especially the use of biomarkers of effect, their feasibility and ethical and legal considerations.

Early Warning Systems (hosted by the Netherlands team):

Medical surveillance of workers with engineered nanoparticles might be used to monitor possible adverse health effects. The possibilities of the use of biomarkers of effect, their feasibility, ethical and legal considerations have been discussed in this part of the workshop.

3.3.1.1.4. Biomarkers & Human Exposure to Nanoparticles

JP should write a paragraph for here.

3.3.1.1.5. Physico-chemical aspects, toxicity and in-vitro methods

Marie and Nathalie should write a paragraph for here.

3.3.1.1.6. Biomarkers in vivo and in vitro

Dietmar should write a paragraph for here.

3.3.1.1.7. Nanosafety Congress – Turkey

The "Nanosafety Congress-Turkey" was held in Antalya on April 26-28, 2012. The congress was organized by NanoLINEN team with contribution of two of the largest FP7 projects on nanosafety field, MARINA and NanoValid. As nanotechnology is one of the most emerging sciences with the amazing potential applications for the human society, the safety assessment of those magical particles have the right to get the highest concern. Therefore, during the congress, we have invited young and senior scientist all over the world to refresh their knowledge and catch up with the latest developments in this field. The recent progresses on the nanosafety field was discussed in this congress under the workshop entitled "*Workshop on the safety assessment of nanomaterials: New paradigms*". Also, a separate workshop on genotoxicity tests was organized as part of the congress to overview the developments in this area.

During the three days of the congress, we brought together scientific and regulative leaders from all over the World, as well as young scientists and newcomers to the field of nanotechnology especially from Turkey, in order to lead lively and interactive debates.

Overall, with more than 100 participants from 18 different countries of the world have followed over 30 poster presentations and 35 lectures. Many collaborations has been initiated by the help of scientific and social atmosphere of the successful meeting in Antalya.

3.3.1.2. Exchanges

Junior and Senior exchanges between the partners are listed below.

FROM	TO	NAME	PERIOD
Portugal	India	**Carla Costa** (PhD Student)	2011-February
Spain	India	**Vanessa Valdiglesias** (PhD Student)	2011-February
Portugal	Spain	**Joao Paulo Teixeira** (Senior Scientist)	2011-May
Portugal	Spain	**Carla Costa** (PhD Student)	2011-May
France	Spain	**Marie Carriere** (Senior Scientist)	2011-May
France	Spain	**Nathalie Herlin** (Senior Scientist)	2011-May
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2011-April-May
Portugal	Spain	**Joao Paulo Teixeira** (Senior Scientist)	2012-April
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2012-January
Spain	Portugal	**Blanca Laffon** (Senior Scientist)	2012-January
Spain	Portugal	**Vanessa Valdiglesias** (PhD Student)	2012-March
Spain	Portugal	**Blanca Laffon** (Senior Scientist)	2012-March
Spain	Portugal	**Juan F. Tajes** (Senior Scientist)	2012-March

According to this table, below is given the outcomes of the exchanges from partners' experiences;

Portugal → India & Spain → India

From February 7th to March 3rd, Carla Costa (Portuguese team partner) and Vanessa Valdiglesias (Spanish team partner) had the opportunity to work with the Indian Nanomaterial Toxicology Group.

During this period, they learned and performed experiments with metal oxide nanoparticles to assess their toxicity. This involved techniques such as nanomaterials characterization (DLS), nanoparticles uptake (flow cytometry), cytotoxicity tests (MTT, NRU, LDH, MMP, AnnexinV/PI double staining by flow cytometry), genotoxicity assays (MN evaluation by flow cytometry). Additional techniques related to oxidative stress and protein expression were also carried out (ROS quantification and Western blot, respectively). All these assays were performed in vitro using cell lines of human origin. This training provided the necessary preparation and technical support to improve research in nanomaterial toxicology in their respective laboratories.

Spain → Portugal

The Spanish partner Vanessa Valdiglesias spent two months (from March 21 to May 19, 2011) in the Portuguese National Institute of Health, the research centre of the Portuguese team. During this time, she carried out the study of the effects of different nanoparticles on human cells, continuing the work that was started in the previous stay in India. Specifically, Vanessa together with Carla Costa (Portugal) established in Portugal different techniques that were learnt in Dr. Dhawan's laboratory: the MTT assay, the NRU assay and the evaluation of nanoparticles cellular uptake by flow cytometry. Besides, these assays were performed with several metal oxide nanoparticles, such as zinc oxide and titanium dioxide, in order to evaluate their cytotoxic effects on human neuronal cells.

In January 16 and 17, 2012, Blanca Laffon and Vanessa Valdiglesias, from the Spanish team, visited the Portuguese team at the National Institute of Health (Porto). They were discussing about the possibility of starting a new work together to evaluate the toxicity of nanomaterials. Some important issues included in the discussion were the type of nanomaterial to be tested (on the basis of their applications), the most suitable assays to be performed and which group could be in charge of them, the coordination and timeline, etc. The scientific discussion was very fruitful and they also straightened their relationship.

The Portuguese team, leaded by Dr. Joao Paulo Teixeira, received a visit of three members of the Spanish team, namely Drs. Blanca Laffon, Juan Fernández-Tajes and Vanessa Valdiglesias, in March 13-16, 2012. They maintained fruitful discussions on the new research work they are starting together and with the collaboration of the company Nanogap, involved in the production of nanomaterials from different chemical origins and with a variety of industrial, medical and consumer applications. They established the criteria for selecting the new materials to be tested regarding their toxicity, mainly focused on cytogenetic and molecular events. They also evaluated the possibility to apply to new calls for projects to the European FP7, and also to Spanish and Portuguese governments. This meeting was very useful for advancing towards new perspectives in the nanotoxicological field.

France → Spain & Portugal → Spain

In May 10-13, 2011, the French partners Nathalie Herlin and Marie Carriere, and the Portuguese partners Joao Paulo Teixeira and Carla Costa were paying a visit to the Spanish

team at the University of A Coruña. They spent some time discussing several issues related to the evaluation of cytotoxicity and genotoxicity of nanoparticles in established cell cultures, and to the optimization of protocols for the suitable dispersion of nanoparticles in the different culture media, in order to obtain dispersions as stable as possible for carrying out the cell culture treatments. They also maintained discussions on the strategies to be followed for testing the ecotoxicity of nanomaterials in the marine environment: in vitro and in vivo studies, selection of species, dispersion of nanomaterials in the natural seawater, etc.

During this visit partners straightened their relationship, and it was a really fruitful knowledge exchange.

Portugal → Spain

The Spanish team, headed by Dr. Blanca Laffon, received a visit of Portuguese partner Dr. Joao Paulo Teixeira in April 16-18, 2012. The purpose of this visit was to analyse jointly the results obtained in the round robin tests performed in the frame of the NanoLINEN consortium, aimed to standardize several cytotoxicity and genotoxicity tests to be used in the evaluation of nanomaterials. They discussed some important issues on the data analyses, the reliability of the tests used and their suitability to be employed in interlaboratory studies, in order to obtain comparable and reproducible results.

3.3.1.3. Scientific Projects

One of the main aims of the project was to development, establish and validate tests and biomarkers for NM safety.

For this reason, at the kick off meeting, a validation study has been launched by the help of Dr. Stefano Bonassi from Italy. Below are the details of the validation study.

WORK PROGRAM FOR VALIDATION STUDY:

PLEASE MODIFY WHERE NECESSARY

Title: *Validation study on TiO2 nanoparticles toxicity: Effects on genetic stability, DNA damage, immunological status, and mutation frequency in vitro.*

Introduction:

Nanotechnology is a rapidly growing converging technology bringing a growing amount of nanotech-based products on the market. This is associated with potential environmental and occupational health risks. The manufacturing, trade, use and disposal of nanoproducts may lead to worker exposure and environmental emissions of nanoparticles while the extent and the potential effects are still uncertain. Due to limited knowledge on the toxic effects of nanoparticles, there is a need to undertake studies in this new area.

Therefore, the “NanoLINEN – Nanotoxicology Link between India and European Nations” project has been proposed to New-Indigo Networking Pilot Program with contribution of 7 European countries and India. The project is funded by for a 2 years period and a kick-off meeting has been held in Istanbul by the participation of all partners in 8-9 November 2010.

As it was proposed on the project, one of the primary goals of the NanoLINEN is to develop, establish, and validate tests and biomarkers for nanoparticles (NP) safety at cellular and molecular level. This issue was discussed during the kick-off meeting. It was agreed that, inter- and intra-laboratory validation of tests for assessing health risks of NP shall be

accomplished by exploiting the diverse expertise of the scientists and infrastructure available with the partners.

At the end of the discussions, all the partners agreed to launch a validation study in order to find useful biomarkers for the assessment of the toxicity profile of some most commonly used NP. This will be one of the main steps for achieving our ultimate goal; to develop robust risk assessment methodologies which will be useful and comprehensible for the occupational and environmental health care in the production, use and disposal (life-cycle) of nano-products, while bringing a precautionary approach into practice.

Participants:

AUSTRIA:	Dietmar FUCHS
FRANCE:	Nathalie Herlin-BOIME, Marie CARRIE
INDIA:	Alok DHAWAN (Indian Coordinator), Rishi SHANKER
ITALY:	Stefano BONASSI*
NETHERLANDS:	Pieter van BROEKHUIZEN, Gert van der LAAN
PORTUGAL:	João Paulo TEIXEIRA, Carla COSTA
SPAIN:	Blanca LAFFON, Juan Fernandezt TAJES, Vanessa VALDIGLESIAS
TURKEY:	Bensu KARAHALIL (EU Coordinator), Ayse Basak ENGIN, Erdem COSKUN, Ali Esat KARAKAYA

*Stefano Bonassi representing Italy is not a legal partner of NanoLINEN group, however a partner of the Validation Study.

The Round-Robin based approach of the study;

The human health risks posed by human exposure to NP are not clear yet. Moreover, there is a big debate on whether methodologies currently applied to toxicological assessment can be applied with the same efficiency to nano-sized particles. Therefore, a big need is raising for standardized methods of exposure characterisation. The most suitable validation methodology for our project seems to be that used in "round-robin" studies. In experimental methodology, a round robin test is an inter-laboratory test (measurement, analysis, or experiment) performed independently several times. This can involve multiple independent scientists performing the test with the use of the same method and same material synthesized in different equipment, or a variety of methods and equipment.

A round robin program is a Measurement Systems Analysis technique which uses Analysis of Variance (ANOVA) random effects model to assess a measurement system. The main principle of a round-robin study is that the robustness of an assay is demonstrated when all participants in a round-robin get the same answer using the same protocol. This method will allow the team to validate the robustness of biomarkers to be used for assessing toxicity profiles of NP in those challenging cases where scientific evidence is not interpreted in the same way by the scientific community. The originality of the project consists in the involvement of different areas of expertise represented by participating laboratories. This methodology will help to evolve newer and more sensitive 'round robin' validated test systems using different approaches to toxicity assessment of NP for safer working conditions and consumer products.

TiO2 nanoparticles as the candidate particle

For the validation test, all the partners agreed upon the use of TiO2 nanoparticles to assess the toxicity profile sizes.

The sizes of TiO2 nanoparticles are;

1. 10 nm (will be distributed by French partners on the Berlin Meeting)

2. 25 nm (will be distributed by French partners on the Berlin Meeting)

The reasons to choose these particles are;

1. French partners have those particles in their stock

2. Enough data are published on literature

The French partners have checked if their laboratory could synthesize different sizes of TiO2 nanoparticles, and identify the characteristics of the synthesized nanoparticles (deadline was May 15). The Indian team also checked the availability of TiO2 nanoparticles by Sigma or other commercial companies such as Degussa (deadline was May 15).

Some samples have been distributed among the partners by CEA. These samples of nanoparticles have been dispersed using the same procedure in the various laboratories of the network. Moreover, their toxicity is under test using the same procedure. Some specific tests will also be done according to the speciality of the partner.

Assays selected for toxicity testing of TiO2 nanoparticles

Regarding to the validation of biomarkers proposed to evaluate the toxic effect of exposure to TiO2 nanoparticles, separate studies are proposed for each biomarker. However, some common features must be taken into consideration to select the assays to be validated:

1. Candidate tests should provide information about the genotoxicity, cytotoxicity and immunomodulatory effects of the nanoparticles,

2. The expertise and the equipment of the partners in their laboratories

For the reasons listed above, the following tests are selected for the toxicity testing of TiO2 nanoparticles:

1. Micronucleus (MN) Assay: The MN Assay is an extensively used genotoxicity test in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile. MN assay is also successfully applied to identify dietary and genetic factors that have a significant impact on genome stability. The high reliability and low cost of this technique, has contributed to the worldwide success and adoption of this biomarker for in vitro and in vivo studies of genome damage (Ref: An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Bonassi S., et al. Carcinogenesis. 2007 Mar; 28(3):625-31)

2. Comet Assay: The comet assay is a genotoxicity test which is widely accepted as a simple, sensitive and rapid tool for assessing DNA damage and repair in individual eukaryotic as well as some prokaryotic cells, and it has increasingly found application in diverse fields ranging from genetic toxicology to human epidemiology (Ref: Alok & Anderdson. 2009. Issues in Toxicology Series, The Comet Assay in Toxicology, RSC Publishing)

3. Lactate Dehydrogenase Assay: Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors

4. MTT Assay: The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

5. Immunomodulatory Activity Determination Assays

Workload shared by the partners:

The partners volunteered to participate to the assays are as follows;

ASSAY	Cell Line	Coordinator	Participators
MN	THP1 (Both for Flow and CBMN)	Erdem Coskun/ Alok Dhawan	Spain / Portugal / France / Italy / India / Turkey
Comet	THP1	Blanca Laffon	Portugal / Spain / France / India / Italy / Turkey
LDH	THP1	Marie Carrie	Austria / Spain / Portugal / France / India
MTT	THP1	Joao Paulo Teixeira	Austria / Spain / Portugal / France / India / Turkey
IM	THP1	Dietmar Fuchs	Austria / Italy / India

Netherlands and Germany will provide technical consultancy during the whole study on regulatory issues and on the possible delivery of guidelines concerning the use of genotoxicity and immunological assays for the screening of the toxicity profile of specific nanoparticles.

Organization of the validation study

1. The whole validation study will be coordinated by Stefano Bonassi (IRCCS San Raffaele Pisana, Rome) including the general organisation as well as collection and analysing of the final data.

2. Different study coordinators have been selected for each assay specific validation study.

Selection of the biological material as SHSY5Y cell lines:

All partners agreed about the disadvantages of collecting samples from exposed human populations. Therefore it was decided that, all assays would be validated using a selected number of cell lines to reduce the sources of heterogeneity among the participating laboratories. The SHSY5H cell lines were selected as the most appropriate biological material for every test. The reasons to select SHSY5H cell lines were;

1. To have a uniform cell line for each assay

2. Easy to harvest

Steps of the study

1. Standardisation between participating laboratories:

Since most of these assays are affected by inter- and even intra-laboratory heterogeneity, a first step of standardization between participating labs is required. Before starting to analyze the effects of TiO2 nanoparticles, a well known challenge compound for each assay will be used by all the laboratories in this first step.

Specific standardization protocols will be developed before testing NP to be sure that all laboratories measure the effect in a homogenous way:

1) Collect and evaluate protocols from participating laboratories

2) Choose a common assay protocol

3) Verify and adjust procedures by evaluating the same specimen in all participating labs (a different challenge for different studies can be used for this purpose, e.g., ionizing radiation for mutational assays)

4) Finalize the protocol to be used for the toxicity study.

However, due to the limited time of the project, all the partners agreed to start the validation study as soon as possible, therefore, for the standardization effort, minimum time will be spent.

2. Validation of the tests

A steering committee composed by coordinators of each assay develop the protocol for the study. Main steps of the protocol include;

1) Exposure issues, such as;

a. Who will prepare the TiO2 nanoparticles (French)

b. According to which method,

c. The characterization method (TEM / XRD / BET)

d. The number and the amount of exposure doses required to create a dose response curve

2) Cell lines:

a. Different assay may require different cell lines so a common line will be selected for every assay (THP1 has been selected) and

b. Circulated among participant laboratories.

3) Exposure conditions, such as

a. The protocol how to treat cells with nanos.

4) Agreement on statistical criteria to evaluate the degree of concordance among laboratories, to establish the extent of the effect observed, and to shape the dose-response relationship.

Once the protocol has been finalized and agreed within all partners, TiO2 doses and cell cultures will be distributed for the experimental phase.

3. Collecting the results
    a. Final results will be centralized by the coordinating centre in Rome.
    b. Statistical analysis of results will be performed in the same centre,
    c. The results will be shared with all the partners.
4. Publishing the results

After the end of the analysis, a writing committee will prepare a report to be discussed and approved by the NanoLINEN partners. A publication strategy will be chosen.

PLEASE INSERT HERE PUBLISHED MATERIALS FROM THE VALIDATION STUDY!!!

3.1.2. Significant results and progress towards objectives

The workshops were endeavouring to harmonise the test methods used in the laboratories of different partners as well as to create the awareness and understanding of nanotoxicology. At the end of "Workshop on the Methods in Nanomaterial Toxicology" a laboratory manual of validated protocols were prepared for all the partners so that it can be used by all the participating laboratories. As an example, the protocol prepared in India is used by all the partners during the Validation Study of testing methods for TiO_2 toxicity. A guideline for the safe use of nanomaterials in the laboratories was also prepared to minimize the exposure to researcher and the environment. The guideline also provides the assistance on the proper handling and disposal of the nanomaterials for the personnel involved in the activities that entail the handling of the nanomaterials.

PLEASE ADD HERE OUTCOMES OF YOUR WORKSHOPS OR ANY OTHER PROGRESS TOWARDS THE OBJECTIVES

As an outcome of the NanoSafety Congress-TURKEY, it was decided to have the biggest annual meeting of Nanotoxicology in the world, **NanoTOX-7 (7th International Nanotoxicology Congress),** in Antalya/Turkey at April 23-26, 2014 with an expected participation of 600 scientists all over the world.

All the other results and progresses have been stated at the previous chapters.

4. Deviations from proposal/work plan

There have been no deviations from the prospected plan.

(MAYBE SHOULD WE TALK ABOUT DISSAPEAR OF GERMANY HERE as a deviation???)

However, there have been a few positive changes/additions compared to the original proposal.

- At the original proposal, French and Spanish partners were not organising a workshop. However, after the discussions at the executive meetings, it is decided that, both partners will also organize a workshop. According to this plan, Spanish partners

decided to organize the workshop together with the Portuguese partner. As well as the Spanish team, French team, in addition to the initial proposal, is organizing in Paris a workshop devoted to "Nanoparticles : physico-chemical aspects and toxicity".

- Two important groups have joined their forces with our group:

1. Prof. Dr. Stefano BONASSI (IRCCS San Raffaele Pisana)

Dr. Bonassi has started collaboration with our group from the beginning of the project launch at the "Kick off Meeting" in Istanbul. As a distinguished scientist in the field of Molecular Epidemiology, his experience on validating standardized testing protocols will be very useful for our study. He has coordinated some very important validation projects, e.g. for Chromosomal Aberration and Micronucleus tests. Dr. Bonassi will be the coordinator for interpreting the results which will be collected from Validation Study for testing TiO_2 toxicity.

2. Prof. Dr. Diane ANDERSON (University of Bradford)

Dr. Anderson is an expert in Human Biomonitoring and Cancer. Previously, she had another EU project called UKIERI with Indian Partners (Dr. Alok Dhawan). Recently, at the India Workshop of NanoLINEN in conjunction with an Indo-UK project (under the UKIERI programme) led to the publication of a Special Issue of the Journal of Biomedical Nanotechnology 7 (1) 1-228, 2011. This included papers from several partners of the NanoLINEN project and also the scientists involved in the UKIERI project. The joint workshop led to the formation of a new interest group of scientists from different countries willing to collaborate on various facets of nanomaterial safety/toxicity issues. Therefore, the nanoLINEN group agreed on collaboration with Dr. Anderson. Recently, she is trying to finalize an EU project proposal as the coordinator in collaboration with our group.

5. Dissemination activities in the period in question (including list of publications and patents where applicable)

An interview about the project's progress, outreach, output and expected impact was done and uploaded as featured Cooperation Story. Also an opinion article by Prof. Alok Dhawan was published by the **Science and Development Network** (http://www.scidev.net/en/new-technologies/nanotechnology/opinions/address-risk-of-nanotech-toxicity-1.html)

To achieve the objective of this project an International conference on the safe use of nanomaterials (SUN 2011) and the workshop on the methods used in nanomaterial toxicology was conducted to create awareness about nanotoxicity and to validate the protocols used in it. The toxicity data on the commonly used metal oxide nanoparticles (ZnO and TiO2) was discussed with the scientific community. A guidance document for the safe handling of nanomaterials in the laboratories was also prepared and published in the Journal of Biomedical Nanotechnology [7(1): 218-224, 2011]. Apart from these activities, as a model organism, bacteria were used to study the uptake of nanomaterial using flow cytometry. The effects (genotoxicity and cytotoxicity) of metal oxide nanoparticles on the bacterial cell were also measured. The genotoxic potential of TiO2 nanoparticles and the further consequences aroused by it, such as oxidative stress, apoptosis; mitochondrial dysfunctions were also investigated in human liver cells.

ANYTHING ELSE TO ADD HERE???

List of publications by NanoLINEN partners
(PLEASE PROVIDE YOUR PUBLICATIONS FROM NOVEMBER 2010 to Now)

1. Ritesh K. Shukla, Ashutosh Kumar, Deepak Gurbani, Alok K. Pandey, Shashi S. Singh and Alok Dhawan, TiO_2 Nanoparticles Induce Oxidative DNA Damage and Apoptosis in Human Liver Cells. *Nanotoxicology*, (2011), Article in press.
2. Ashutosh Kumar, Alok K. Pandey, Shashi S. Singh, Rishi Shanker and Alok Dhawan, Engineered ZnO and TiO(2) Nanoparticles Induce Oxidative Stress and DNA Damage Leading to Reduced Viability of *Escherichia coli*. *Free radical biology & medicine*, doi: 10.1016/j.freeradbiomed.2011.08.025. (2011)
3. Alok Dhawan, Alok K. Pandey and Vyom Sharma, Toxicity Assessment of Engineered Nanomaterials: Resolving the Challenges. *Journal of Biomedical Nanotechnology*. 7, 6-7 (2011)
4. Marcel Jenny, Sebastian Schroecksnadel, and Dietmar Fuchs, Testing for Immunomodulatory Properties of Nanoparticles. *Journal of Biomedical Nanotechnology*. 7, 11-12 (2011)
5. Pieter van Broekhuizen, Dealing with Uncertainties in the Nanotech Workplace Practice: Making the Precautionary Approach Operational. *Journal of Biomedical Nanotechnology*. 7, 15-17 (2011)
6. Gert van der Laan, Tracing New Occupational Diseases in Nano-Workers. *Journal of Biomedical Nanotechnology*. 7, 18 (2011)
7. M. L. Jugan, S. Barillet, A. Simon-Deckers, S. Sauvaigo, T. Douki, N. Herlin, and M. Carrière, Cytotoxic and Genotoxic Impact of TiO_2 Nanoparticles on A549 Cells. *Journal of Biomedical Nanotechnology*. 7, 22-23 (2011)
8. Axelle Casanova, Marie Carriere, and Nathalie Herlin-Boime, Dispersion of Aeroxil P25 TiO_2 Nanoparticle in Media of Biological Interest for the Culture of Eukaryotic Cells. *Journal of Biomedical Nanotechnology*. 7, 24-25 (2011)
9. Ritu Goyal, S. K. Tripathi, S. Tyagi, A. Sharma, P. Kumar, K. Ravi Ram, D. K. Chowdhuri, Y. Shukla, and K. C. Gupta, *In Vitro* and *In Vivo* Evaluation of Linear Polyethylenimine Nanoparticles. *Journal of Biomedical Nanotechnology*. 7, 52-53 (2011)
10. Ashutosh Kumar, Alok Dhawan, and Rishi Shanker, The Need for Novel Approaches in Ecotoxicity of Engineered Nanomaterials. *Journal of Biomedical Nanotechnology*. 7, 79-80 (2011)
11. Shailendra K. Gupta, Alok Dhawan, and Rishi Shanker, In Silico Approaches: Prediction of Biological Targets for Fullerene Derivatives. *Journal of Biomedical Nanotechnology*. 7, 91-92 (2011)
12. Vyom Sharma, Diana Anderson, and Alok Dhawan, Zinc Oxide Nanoparticles Induce Oxidative Stress and Genotoxicity in Human Liver Cells (HepG2). *Journal of Biomedical Nanotechnology*. 7, 98-99 (2011)
13. Ritesh K. Shukla, Ashutosh Kumar, Alok K. Pandey, Shashi S. Singh, and Alok Dhawan, Titanium Dioxide Nanoparticles Induce Oxidative Stress-Mediated Apoptosis in Human Keratinocytes Cells. Journal of Biomedical Nanotechnology. 7, 100-101 (2011)
14. Ashutosh Kumar, Alok K. Pandey, Shashi S. Singh, Rishi Shanker, and Alok Dhawan, Cellular Response to Metal Oxide Nanoparticles in Bacteria. *Journal of Biomedical Nanotechnology*. 7, 102-103 (2011)
15. Deepak Gurbani, Ritesh K. Shukla, Alok K. Pandey, and Alok Dhawan, Stable Metal Oxide nanoparticle Formulation for Toxicity Studies. *Journal of Biomedical Nanotechnology*. 7, 104-105 (2011)

16. N. V. Srikanth Vallabani, Sandeep Mittal, Ritesh K. Shukla, Alok K. Pandey, Sanjay R. Dhakate, Renu Pasricha, and Alok Dhawan. Toxicity of Graphene in Normal Human Lung Cells (BEAS-2B). *Journal of Biomedical Nanotechnology*. 7, 106-107 (2011)
17. Sandeep Mittal, Vyom Sharma, N. V. Srikanth Vallabani, Swati Kulshrestha, Alok Dhawan, and Alok K. Pandey, Toxicity Evaluation of Carbon Nanotubes in Normal Human Bronchial Epithelial Cells. *Journal of Biomedical Nanotechnology*. 7, 108-109 (2011)
18. Priyanka Khare, Madhavi Sonane, Rakesh Pandey, Shakir Ali, Kailash C. Gupta, and Aruna Satish, Adverse Effects of TiO_2 and ZnO Nanoparticles in Soil Nematode, Caenorhabditis elegans. *Journal of Biomedical Nanotechnology*. 7, 116-117 (2011)
19. Gulshan Singh, Poornima Vajpayee, Imrana Khatoon, Anurag Jyoti, Alok Dhawan, K. C. Gupta, and Rishi Shanker, Chromium Oxide Nano-Particles Induce Stress in Bacteria: Probing Cell Viability. *Journal of Biomedical Nanotechnology*. 7, 166-167 (2011)
20. Imrana Khatoon, Poornima Vajpayee, Gulshan Singh, Alok K. Pandey, Alok Dhawan, K. C. Gupta, and Rishi Shanker, Determination of Internalization of Chromium Oxide Nano-Particles in Escherichia coli by Flow Cytometry. *Journal of Biomedical Nanotechnology*. 7, 168-169 (2011)
21. Anurag Jyoti, Surinder P. Singh, Madhu Yashpal, Premendra D. Dwivedi, and Rishi Shanker, Rapid Detection of Enterotoxigenic Escherichia coli Gene Using Bio-Conjugated Gold Nano-Particles. *Journal of Biomedical Nanotechnology*. 7, 170-171 (2011)
22. Lokesh Baweja, Deepak Gurbani, Rishi Shanker, Alok K. Pandey, V. Subramanian, and Alok Dhawan, C_{60}-Fullerene Binds with the ATP Binding Domain of Human DNA Topoiosmerase II Alpha. *Journal of Biomedical Nanotechnology*. 7, 177-178 (2011)
23. Shishir Kumar Gupta, Lokesh Baweja, Deepak Gurbani, Alok K. Pandey, and Alok Dhawan, Interaction of C_{60} Fullerene with the Proteins Involved in DNA Mismatch Repair Pathway. *Journal of Biomedical Nanotechnology*. 7, 179-180 (2011)
24. Madhulika Singh, Priyanka Bhatnagar, Amit K. Srivastava, Pradeep Kumar, Yogeshwer Shukla, and Kailash C. Gupta, Enhancement of Cancer Chemosensitization Potential of Cisplatin by Tea Polyphenols Poly(lactide-co-glycolide) Nanoparticles. *Journal of Biomedical Nanotechnology*. 7, 202 (2011)
25. Alok Dhawan, Rishi Shanker, Blanca Laffon, Juan Fernandez Tajes, Dietmar Fuchs, Gert van der Laan, Pieter van Broekhuizen, Heidi Becker, Heinz-Jorn Moriske, Joao P. F. Teixeira, Marie Carriere, Nathalie Herlin-Boime, Ayse Basak Engin, Erdem Coskun, and Bensu Karahalil, NanoLINEN: Nanotoxicology Link Between India and European Nations. *Journal of Biomedical Nanotechnology*. 7, 203-204 (2011)
26. Poornima Vajpayee, Imrana Khatoon, Chandra Bali Patel, Gulshan Singh, Kailash Chand Gupta, and Rishi Shanker, Adverse Effects of Chromium Oxide Nano-Particles on Seed Germination and Growth in *Triticum aestivum L*. *Journal of Biomedical Nanotechnology*. 7, 205-206 (2011)
27. Sebastian Schroecksnadel, Marcel Jenny, and Dietmar Fuchs, Myelomonocytic THP-1 Cells for In Vitro Testing of Immunomodulatory Properties of Nanoparticles. *Journal of Biomedical Nanotechnology*. 7, 209-210 (2011)
28. Alok Dhawan, Rishi Shanker, Mukul Das, and Kailash C. Gupta. Guidance for Safe Handling of Nanomaterials. *Journal of Biomedical Nanotechnology*. 7, 218-224 (2011)

6. Project management

Overall, the project has been managed just according to the strategy plan created in Kick Off meeting in Istanbul. Many fruitful collaborations have been established bilaterally and for

other EU project with many additional distinguished scientists on the field of Toxicology and Nanosafety. On this aspect, besides the scientific data communication, the NanoLINEN Workshops were tremendously important for creating new collaborations and networks.

Also, exchanges of scientists between the partners have been managed successfully during the project. As an Indian participant, CSIR- Indian Institute of Toxicology Research, Lucknow was instrumental in establishment and transfer of the different techniques used in the nanoparticles toxicity studies.

PLEASE CONTRIBUTE HERE.

7. Financial report (PLEASE REVISE!!!)

Table A: Estimate of costs for the period		
Cost category	**Amount**	**Description of major cost items**
AUSTRIA		
Travel costs	*€ 5450.74*	Istanbul (D.Fuchs): travel € 361.61, hotel € 264.54 Lucknow (D.Fuchs, M.Pfurtscheller, S.Schröcksnadel): travel, visa, € 3722.83 Berlin (D.Fuchs): travel € 285,78, hotel € 237.00 Porto (D.Fuchs, M.Jenny): travel € 578,98
Workshop costs	*€ 409.22*	Registration fees, Lucknow (S.Schröcksnadel, M.Gleinser): € 409.22
Other costs	*€ 2000.00*	Overhead-costs, Innsbruck Medical University
FRANCE		
Travel costs	*€ 6500*	Travel and accommodation expenses
GERMANY		
Travel costs	---	No data received from partner yet
Workshop costs	---	No data received from partner yet
INDIA		
Travel costs	*€ / Rs 2.00Lakhs*	Travel of Indian scientists to EU for kick off meeting in Istanbul and Berlin meeting.
Workshop costs	*€/ Rs5.00 Lakhs*	Local hospitality of scientists from EU counties
Equipment costs (Indian participants only)	*€/ RsNIL*	Not sanctioned
Personnel costs (Indian participants only)	*€/Rs 1.65 Lakhs*	One project fellow has been hired

Other costs	*€ /Rs 5.60Lakhs*	Consumables, overheads and and contingency expenditure.
NETHERLANDS		
Travel costs	*€ 3.500*	Travel costs GL and PB to the kick-off meeting and the meeting in Lucknow
Workshop costs	*€ 33.000*	Organization of the Berlin Workshop May 26 and reimbursement of travel costs and hotel accommodation invited speakers
PORTUGAL		
Travel costs	*9960€*	Kick off meeting at Istambul (Turkey) (1 people) Symposium and Workshop at Lucknow (India) (2 people) Project meeting Coruna (Spain) (2 people) Workshop at Berlin (Germany) (3 people) Project meeting Saclay, Paris (France) (2 people) Project meeting Istambul (Turkey) (1People) Project meeting Amesterdam (Netherlands) (1 People)
SPAIN		
Travel costs	*12047.72€*	Kick off meeting at Istanbul (Turkey) (2 people) Symposium and Workshop at Lucknow (India) (3 people) Stay of 1 researcher for 2 months at Porto (Portugal, INSA laboratories) Workshop at Berlin (Germany) (2 people)
TURKEY		
Travel costs	*11131 €*	Kick off Meeting at Istanbul Workshop at Lucknow (India) (3 people travel) Workshop at Berlin (Germany) (3 people travel+accommodation) Workshop and Congress in Porto (Portugal) (3 people travel+acc) Workshop in Paris (France) (4 people travel+acc) Workshop in Innsbruck (Austria) (4 people travel+acc)
Workshop +Congress	*7140 €*	Nanosafety Congress - Turkey
TOTAL COSTS		

Financial statements

Each project partner has also shared their ***detailed*** budgetary information with their national regulations and procedures and reported their expenses to their national funding agency.

NOTE:

This report is truncated due to the message size, as it will be too many MBs for sending via e-mail. The detailed report, which includes all the visual and printed materials, should be requested from the EU coordinator of the project via airmail;

Prof. Dr. Bensu KARAHALIL,
Gazi University, Faculty of Pharmacy, Department of Toxicology
06330/Ankara-TURKEY
e-mail: **bensuka@gmail.com**