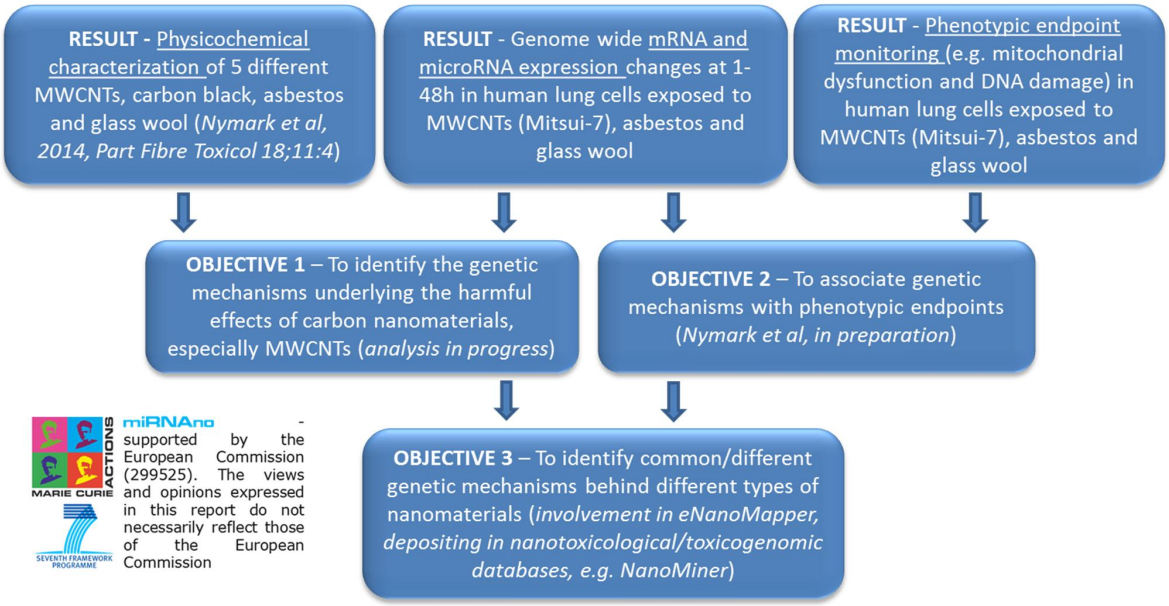
1. Final Publishable Summary Report

The *miRNAno* project set out to identify the molecular mechanisms underlying experimentally observed harmful effects of multi-walled carbon nanotubes (MWCNT), with special emphasis on the involvement of microRNAs (miRNAs, thereof the acronym of the project miRNA+nano). MWCNTs are being used to increasing extents in various nano-based technologies, such as paints, plastic and rubber composites, and lithium-batteries in mobile phones and laptops. Future MWCNT-based technology is expected to have a tremendous impact on the development in several technological areas, including therapeutics, electronics and construction materials. Thus, human exposure, especially in occupational settings, where MWCNT may be handled in a free volatile and inhalable state, can be foreseen. Certain types of MWCNT have consistently been shown to induce both inflammatory and carcinogenic effects in rodents. Knowledge on the mechanisms underlying the harmful effects of MWCNT is a necessary foundation for reliable risk assessment of these nanomaterials. Furthermore, the wide extent of various types of MWCNTs calls for extensive knowledge about the physicochemical characteristics associated with the observed adverse cellular responses. Therefore, one of the objectives of the project was also to, in parallel with the mechanistic studies, characterize the precise physicochemical properties of the tested materials in the specific experimental settings that were used. In this project, the use of well-known and well-studied positive and negative fibre controls enabled the identification of harmful effects with human relevance even though the experiments were performed *in vitro*, which is in general criticized for its ability to resemble the actual *in vivo* situation. On the other hand, the development of relevant *in vitro* methods for toxicological studies and screening methods is being emphasized today together with computational *in silico* methods as a contribution to alternative testing.

For the analysis of the physicochemical properties, five MWCNTs were studied for their size, agglomeration, sedimentation rate and ability to induce free radicals, both in cell-free systems and in the presence of cells. These physicochemical characteristics were correlated with the cytotoxicity of the materials to human bronchial epithelial cells (BEAS-2B cells). A spherical carbon nanomaterial (Printex 90 carbon black), asbestos (crocidolite) and glass wool (MMVF10) were also studied as control materials. Asbestos is a well-known and well-studied human carcinogen to which certain MWCNTs have been compared, due to their similar shape, but also to their similar effects in both animals and cells. Insulation glass wool is also a similarly shaped fibre comparable to asbestos and MWCNTs, but is well-known to be non-carcinogenic in humans, based on several decades of epidemiological research (IARC *Monograph 81*, 2002). The five MWCNTs included two long, needle- (or rod-) like variants, one long, tangled variant and two short purified and non-purified variants. The results indicated that the two similarly shaped long, rod-like MWCNTs were the most cytotoxic of all five MWCNTs (as determined by Trypan Blue assay) and also generated a unique, yet unidentified free radical. The generation of this radical correlated dose-dependently with the cytotoxicity of the materials. One of these MWCNTs is the so called Mitsui-7 variant, which has repeatedly been found to induce asbestos-like effects in various experimental settings, including both animals and human cells. These results were presented as posters at two international conferences (the 10th International Particle Toxicology Conference and the 11th International Conference of Environmental Mutagens), invited for oral presentation at a third international conference (the International Conference on Monitoring and Surveillance of Asbestos-Related Diseases) and published in Particle & Fibre Toxicology 2014 (see below).
For the mechanistic studies toxicogenomic methods were used to characterize genome wide expression changes in mRNA and miRNA expression over a period of two days in the same human bronchial cells that were used for the physicochemical studies. miRNAs are involved in most, if not all, biological processes in the cell and have been shown to provide an additional level of understanding of toxicological effects. miRNAs may furthermore provide promising biomarkers of exposure, since they are more stable than proteins and gene transcripts (mRNAs), which is why special emphasis was put on identifying miRNAs associated with MWCNT exposure. Profiling transcriptional changes on several levels also gives an added robustness to the results. In parallel with the expression studies, phenotypical endpoints, such as mitochondrial dysfunction, DNA damage and cell cycle distribution were studied to enable correlation of genetic mechanisms with observable phenotypic changes. For these analyses, the well-studied long, rod-like MWCNT, Mitsui-7, was chosen as a representative of harmful MWCNTs, together with the positive (asbestos) and negative (glass wool) controls. Low, biologically relevant doses were used based on calculations from inhalation experiments in published studies. Samples were taken as a long time series, including six time points over the two days of exposure. Significant results were obtained from the

correlation between MWCNT-induced mitochondrial dysfunction and mRNA/miRNA expression. Part of the results were presented at the NanoTox 2014 meeting in Turkey, Antalya and a manuscript has been prepared for submission (see below). Further results are expected from these analyses, describing e.g. genetic mechanisms underlying MWCNT-induced DNA damage in human bronchial cells.



Overview of the main results obtained in *miRNAno* and how they met the objectives of the project.

Besides shedding light on the molecular mechanisms underlying the harmful effects of MWCNT and thus contributing to the knowledge needed for proper regulation of these materials, the results of this project are expected to contribute to the development of computational infrastructures for toxicological data management of engineered nanomaterials. The *miRNAno* project generated relevant interest in the EU funded eNanoMapper project (www.enanomapper.net) in which the objective is precisely the above mentioned type of knowledge management. Furthermore, the published data will be deposited in public databases specifically designed for nanotoxicogenomic data (NanoMiner, http://nanominer.cs.tut.fi/) to provide a basis for meta-analysis on data sets from different research groups. This type of coordinated data management is expected to contribute significantly to the so called 3Rs, i.e. the refinement, reduction and ultimately the replacement of animal testing, which also this project will contribute to.

Furthermore, the results on the free radical formation by MWCNTs pointed out the usefulness of electron spin resonance (ESR) spectrometry for characterization of the nanomaterials' behavior in experimental conditions. The use of this method has increased within the field of nanotoxicology during the past 2 years.

Finally, the results obtained in *miRNAno* can also be expected to support the understanding of the molecular mechanisms underlying the development of asbestos-related lung cancer. Five to seven percent of all lung cancers worldwide have been estimated to be associated with an occupational exposure to asbestos and knowledge on the genetic signatures in these tumors may be relevant both for therapeutic and medico-legal reasons.

On a larger scale the results from this project are expected to contribute to appropriate precautions regarding the health effects of MWCNTs, especially of the Mitsui-7 variant, for which the full toxicological effects are currently unclear. In 2008 IARC had chosen carbon-based nanomaterials for review with high priority and this year (2014), specifically carbon nanotubes were listed to be reviewed in the next set of IARC *Monographs*, during 2015-2019, which will provide recommendations for policy-makers, government officials, industrial consultants and researchers regarding the health effects and carcinogenicity of industrially used MWCNTs.

Appropriate regulations on engineered nanomaterials will not only prevent the spreading of harmful materials, which may have devastating consequences several decades later due to the long latency period of the development of cancer, but also support the development of safe new technologies associated with large socio-economic impact.