1. Publishable summary

Embryonic Stem cell-based Novel Alternative Testing Strategies

The aim of the ESNATS project is to develop a novel toxicity test platform based on embryonic stem cells (ESCs), in particular human ESC (hESCs), to streamline the drug development R&D process and evaluation of drug toxicity in clinical studies, reduce related costs and thus, to not only increase the safety of patients but also to reduce the number of animals due to earlier detection of adverse effects.

ESNATS addresses current shortcomings in toxicity testing:

- A major part of safety testing takes place late in the research and development cycle, implying protracted experimentation involving high numbers of animals and generating significant costs.
- Some *in vitro* assays rely on cells lines of malignant origin or primary cells that are hard to standardise and limited in terms of quantity, homogeneity and genetic diversity.
- Existing assay systems based on primary animal and human cell lines do not reliably represent the physiological situation of cells in native tissue.

To reach the project goals, a battery of toxicity tests is being developed using ESC lines subjected to standardised culture and differentiation protocols. Tests will cover hESCs in several stages of development as well as differentiated derivatives, including gamete and neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches will be used to determine predictive toxicoproteomics and toxicogenomic signatures. The individual tests will be integrated into an "all-in-one" testing strategy. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture will be developed and the test systems will be scaled up. In a later stage of the project, the predictivity, quality and reproducibility of the test strategy will be evaluated in a “proof of concept” study.

ESNATS is divided into four main research areas, each one representing a sub-project (SP). These SPs are complemented by central work packages (cWPs) which cover transversal scientific aspects of the project (see Figure 1).

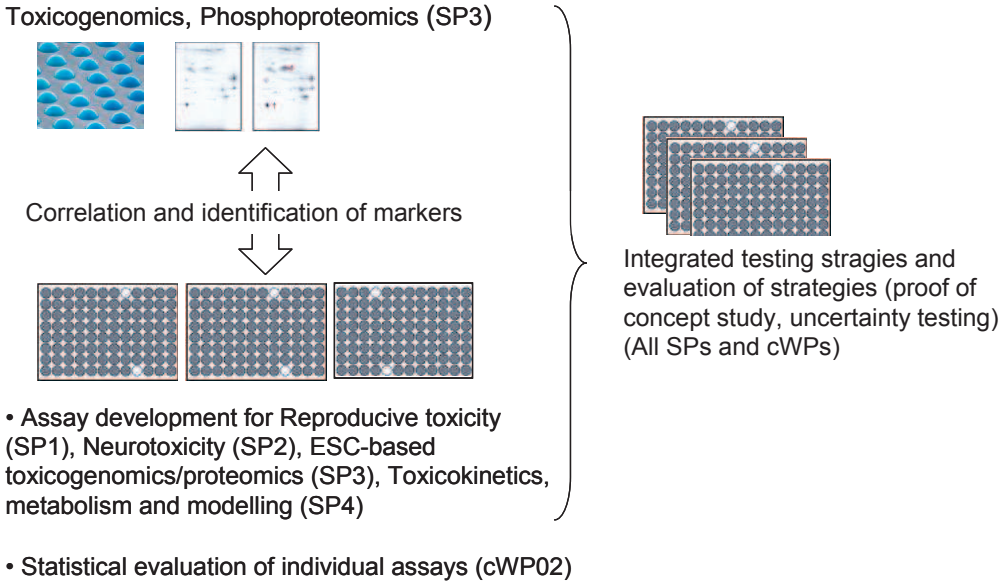


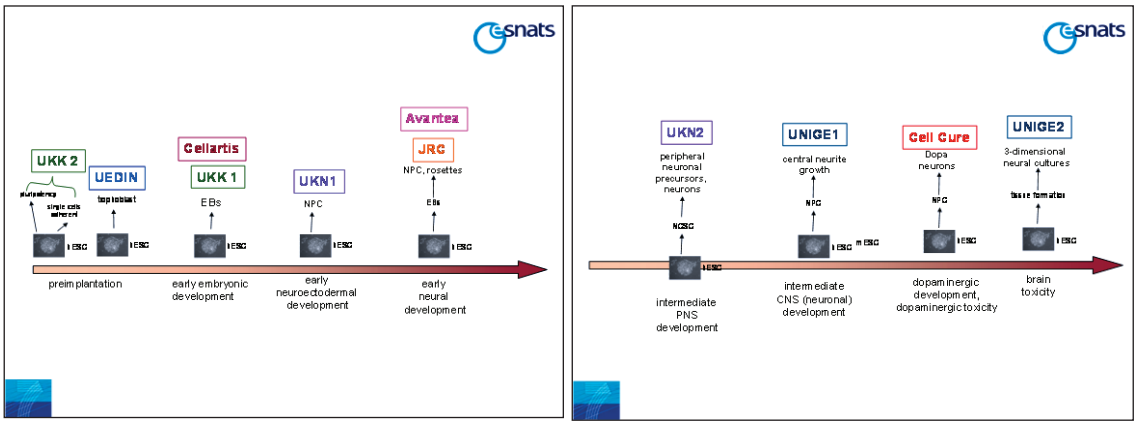
Figure 1 ESNATS main research areas

ESNATS objectives will be achieved in a five-year multidisciplinary collaboration of leading European researchers in alternative testing, toxicology, ESC research, genomics, modelling, and automation. The consortium also includes representatives from regulatory bodies, the pharmaceutical industry, and ethical advisors to provide guidance to ensure rapid applicability of the developed test systems.

In the third year of the project, a specific task force, the Strategy Working Group, was set up to define the overall test strategy to be applied in the last two years in the project. Further to the recommendations of the Strategy Working Group, it was decided to focus on the topic "prenatal toxicity with emphasis on the nervous system" examined in hES cells and cell types derived thereof. The spermatogenesis (murine model) and other murine-based models are therefore no longer part of the ESNATS test strategy. The list below presents the test systems corresponding to the focus of the ESNATS test strategy:

Partner	Test systems
UKK	- UKK1: Toxicity assessment in human embryonic development using H9 hES cells, feeder-free, critical window, exposure from day 0 or from day 10 - UKK2: Pre-implantation embryo toxicity based on hES cells; undifferentiated ES cells, pluripotency factors, first test the system, qPCR, Affymetrix-array
CELLARTIS	- Developmental toxicity assay using hES cells, feeder-free, early steps of development till germ layer - Presence and absence of bFGF
UEDIN	Preimplantation embryo toxicity based on hES cell trophoblast models
JRC	Toxicity assessment in human embryonic early neurogenesis/neural development using H9 hES cells
Avantea	Neural teratogenicity HUES1 line
CellCure	- hES cell-derived dopaminergic neurons - Assaying dopaminergic neurons for developmental toxicity - Assaying dopaminergic neurons for acute toxicity
UKN	Early and late developmental neurotoxicity of CNS and PNS cells: - UKN1: hESC –developmental toxicity during the generation of neuroectodermal cells (NEC) - UKN2: hESC –differentiation of neural crest stem cells (NCSC) to test toxicity on the developing peripheral nervous system
UNIGE	Neurotoxicity, two dimensional and three dimensional neural cultures: - UNIGE1: Human mature neurons (2D neurite extension, FACS analysis, regular tox tests; 3D histology, protein expression) - UNIGE 2: Human mature neurons 3D electrophysiology, +/- stimulation

Below is shown how these test systems cover “critical windows” during neural cell differentiation.



It was furthermore decided to include in this test strategy a “biomarker identification study” to be performed with the most advanced test systems. The aim of this study is to identify gene expression signatures by gene array analysis and to establish an algorithm that allows identification of compounds that act by a certain toxic mechanism or induce a specific phenotype in a pathway-based approach.

The further development and implementation of the suggested test strategy was then elaborated in a so called “Roadmap”, where the detailed planning of each step of the test strategy is described. Figure 2 shows an overview of the test strategy and its main phases.

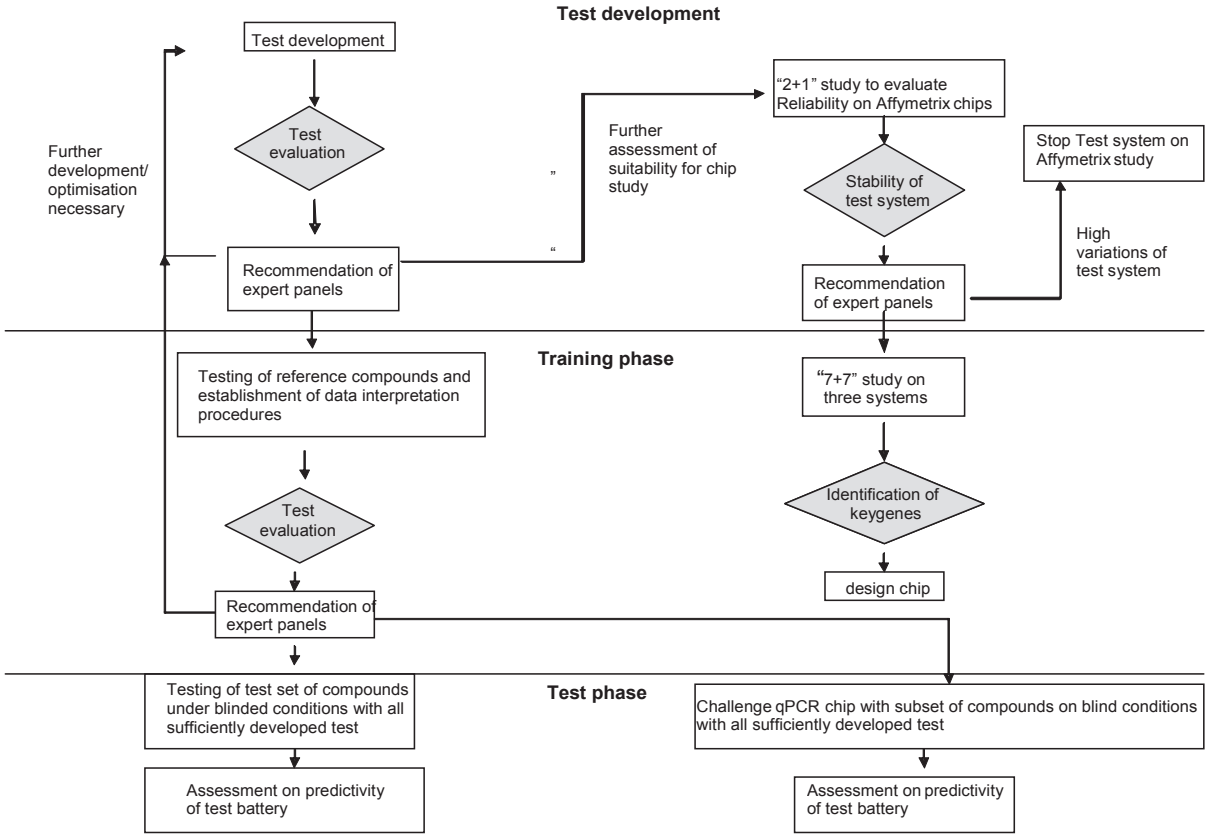


Figure 2 Overview of ESNATS test strategy

As shown in Figure 2, a test battery is developed to assess different aspects of prenatal toxicity such as functional impairments and changes in the differentiation capacity after exposure to well selected reference compounds. In parallel to this, a biomarker identification study is being carried out as mentioned earlier. The test battery and the gene array chip will then be challenged with compounds under blinded conditions and the predictivity of the tests will be assessed. Biostatistics will be employed to evaluate specificity, sensitivity and predictive capacity. PBPK modelling will allow the extrapolation of *in vitro* data to the *in vivo* situation.

Experimental design for toxicity tests participating in the battery approach is based on the following:

- Definition of the test method including its biological basis (test system) and a rationale for the relevance of the results produced such as the endpoints to be measured and a rationale or decision criteria for how the results are to be interpreted.
- Definition of the toxicity range of test compounds in the test system.
- Definition of basic characteristics of the test system and test method: dynamic range of the endpoint, detection limit, stability of the readout.
- Data on response characteristics of the endpoint.
- Data quality and statistical evaluation.

For the gene array study, the following experimental design has been agreed on:

- A ”2+1 study”, i.e. testing two positive and one negative compounds, should allow a first assessment whether the cell systems deliver reproducible and reasonable expression data. As positive control, methylmercury will be tested by all test developers participating in this study, as well as one compound to be chosen by the test developer individually based on the

relevance of the compound for the respective test system. As negative control, D-mannitol will be used by all test developers.

- Once the data from the "2+1 study" is confirmed, a "7+7 study" will start with those test systems who have delivered promising results in the "2+1 study". In the "7+7 study", seven positive and seven negative compounds that have been reported to be neurotoxic *in vivo* and to inhibit neurite outgrowth *in vitro* will be tested, allowing to identify a specific mode of action or inducing a specific phenotype.
- All experiments will be carried out in five biological replicates.
- Three concentrations will be tested: 1) solvent control, 2) IC10 and 3) IC10 x 0.25.
- Biomarkers identified in the different tests will then be assembled in a multiplex chip which will be challenged with compounds under blinded conditions as part of the overall testing strategy.

To assess the readiness of the ESNATS test systems, an evaluation was carried out during the period, both by the Steering Committee and by a specific Evaluation Group, composed of representatives of the project. The following evaluation criteria were applied:

- Availability of SOP
- Reliability of the test
- Acceptance criteria
- Negative and positive controls
- Non-specific controls (depending on system)
- Biological relevance of the test system

The conclusions from the two expert panels were that although significant results have been obtained, most of test systems would benefit from further optimisation in order to be included in the overall test battery. On the other hand, three test systems were considered ready to start in the gene array study: UKN1, UKK1 and JRC. These test systems have been suggested for the "2+1 study"; others might be included depending on results obtained. A new evaluation of test systems to be included in the test battery will be made in month 45.

In the next period of the project, 3-4 robust test systems covering different critical time windows of neuronal cell differentiation are trained with prenatal toxicants leading to the identification of a panel of marker genes covering a wider range of prenatal toxicity. Then, 7 positive and 7 negative compounds covering various toxicological mechanisms relevant for each particular time point defined by the test developers individually according to the suitability for the respective systems will be used in order to identify a range of potential marker genes. Further development of tests will also take place in order to fulfil recommendations of the expert panels and thus, to be ready for the participation in phase 3.

ESNATS partners

The ESNATS consortium is composed of the following organisations:

- Universität zu Köln – Universitätsklinikum (UKK)
- Commission of the European Communities – Directorate General Joint Research Centre JRC (JRC)
- University of Newcastle upon Tyne (UNEW) (until July 2010)
- Université de Genève (UNIGE)
- Forschungsgesellschaft für Arbeitsphysiologie und Arbeitschutz e.V. (IFADO)
- European Consensus Platform on 3R Alternatives to Animal Experimentation (ecopa)
- The Automation Partnership (Cambridge) Limited (TAP)
- OÜ Quretec (QURE)

- ProteoSys AG (PSY)
- Université de Liège (Ulg) (until October 2009)
- CELLARTIS AB (CELLARTIS)
- Cell Cure Neurosciences Ltd. (CELL CURE)
- Universität Konstanz (UKN)
- National Biological Standards Board (NIBSC)
- Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek – TNO (TNO)
- The University of Edinburgh (UEDIN)
- Vrije Universiteit Brussel (VUB)
- Technische Universität München Klinikum Rechts der Isar (TUM)
- ARTTIC (ARTTIC)
- The School of Pharmacy, University of London (ULSOP)
- N.V. Organon (Org)
- Läkemedelsverket / Medical Products Agency (MPA)
- H. Lundbeck A/S (Lundbeck)
- In Vitro Testing Industrial Platform (IVTIP)
- Bundesinstitut für Risikobewertung (BfR)
- Edinethics Ltd (Edinethics)
- Gottfried Wilhelm Leibniz Universität Hannover (LUH)
- F. Hoffmann-La Roche, Ltd. (Roche)
- Avantea srl (Avantea)

Contact details:

Prof Jürgen Hescheler
Universität zu Köln – Universitätsklinikum
Robert-Koch-Str. 39 50931 Cologne
GERMANY
Phone: +49-221-478 6960
E-mail: J.Hescheler@uni-koeln.de

ESNATS public website: www.esnats.eu