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1. Final Publishable Summary Report

1.1 Executive Summary

Embryonic Stem cell-based Novel Alternative Testing Strategies

The aim of the ESNATS project, an integrated project funded under FP7, was 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 normally used to these aims, thanks to earlier detection of adverse effects.

In doing so, ESNATS has addressed 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 cell lines of malignant origin or primary cells that are hard to standardise and that are 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 human physiological situation of cells in native tissue.

In a five and a half-year multidisciplinary collaboration of leading European researchers in alternative testing, toxicology, ESC research, genomics, modelling, and automation, as well as representatives from regulatory bodies, the pharmaceutical industry and ethical advisors, the ESNATS partners have developed a battery of toxicity tests using ESC lines subjected to standardised culture and differentiation protocols. Specific focus has been put on reproductive toxicity, and more precisely on **prenatal developmental toxicity with emphasis on the nervous system**. The developed tests cover hESCs in several stages of development as well as differentiated derivatives, including neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches have been used to determine predictive toxicoproteomics and –toxicogenomics signatures. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture have been developed. In the last phase of the project, the predictivity and quality of the test strategy has been evaluated in a "proof of concept" biomarker and test battery study. This proof of concept study demonstrated that compounds causing developmental neurotoxicity can be identified in these ESNATS systems. The test systems are now ready for entering a more formal evaluation, possibly even starting the pre-validation procedure.

Further projects should be initiated to study a broader range of chemicals and to optimise the test systems. It has become clear that stem cell based *in vitro* systems will become an accurate, fast and cost-effective tool for identification of toxic compounds in the broad field of developmental toxicity. This will be a major contribution to human safety.

1.2   Description of the Project Context and Objectives

The aim of the ESNATS project was 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 normally used to these aims, thanks to earlier detection of adverse effects.

ESNATS has addressed 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 cell lines of malignant origin or primary cells that are hard to standardise and that are 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 human physiological situation of cells in native tissue.

Specific focus was put on reproductive toxicity, and more precisely on **prenatal developmental toxicity with emphasis on the nervous system**. Reproductive toxicity testing is one of the most challenging and expensive fields of toxicology. A large fraction of the animals required in drug development and in the context of REACH will be used in the area of reproductive toxicity to fulfill the respective testing requirements (Seiler et al., 2011; Krug et al., 2013). Hundreds of animals are needed for testing of a single compound. Reproductive toxicity testing includes evaluation of effects on the fertilisation process, spermatogenesis, oogenesis but also compromised embryo-foetal development. Currently, animal tests for developmental toxicity follow OECD guidelines 414 (2-generation study), 426 (developmental neurotoxicity) or others. These tests analyse for example the numbers of embryo-foetal deaths, altered weight, anatomical and behavioral abnormalities. They require exposure and analysis of animals over long periods. For example according to OECD 426, exposure is performed during gestation and lactation and the offspring has to be analysed for neurological, histological, neurochemical and behavioral alterations. These complex *in vivo* tests are too laborious and expensive to allow the required testing for thousands of chemicals (Krug et al., 2013), and might also not well reflect the human situation because of inter-species variation. Therefore, there is a general agreement that reliable, faster and more accurate *in vitro* tests of developmental toxicity are urgently needed.

To reach the project goals, the ESNATS partners have developed a battery of toxicity tests using ESC lines subjected to standardised culture and differentiation protocols. Tests cover ESCs in several stages of development as well as differentiated derivatives, including neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches have been used to determine predictive toxicoproteomics and –toxicogenomics signatures. The individual tests have been integrated into an "all-in-one" testing strategy. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture have been developed. In the last phase of the project, the predictivity, quality and reproducibility of the test strategy has been evaluated in a "proof of concept" biomarker and test battery study.

Figure 1 gives an overview of the overall test strategy employed in ESNATS: the main steps in the biomarker study, the test battery and how they are linked together.
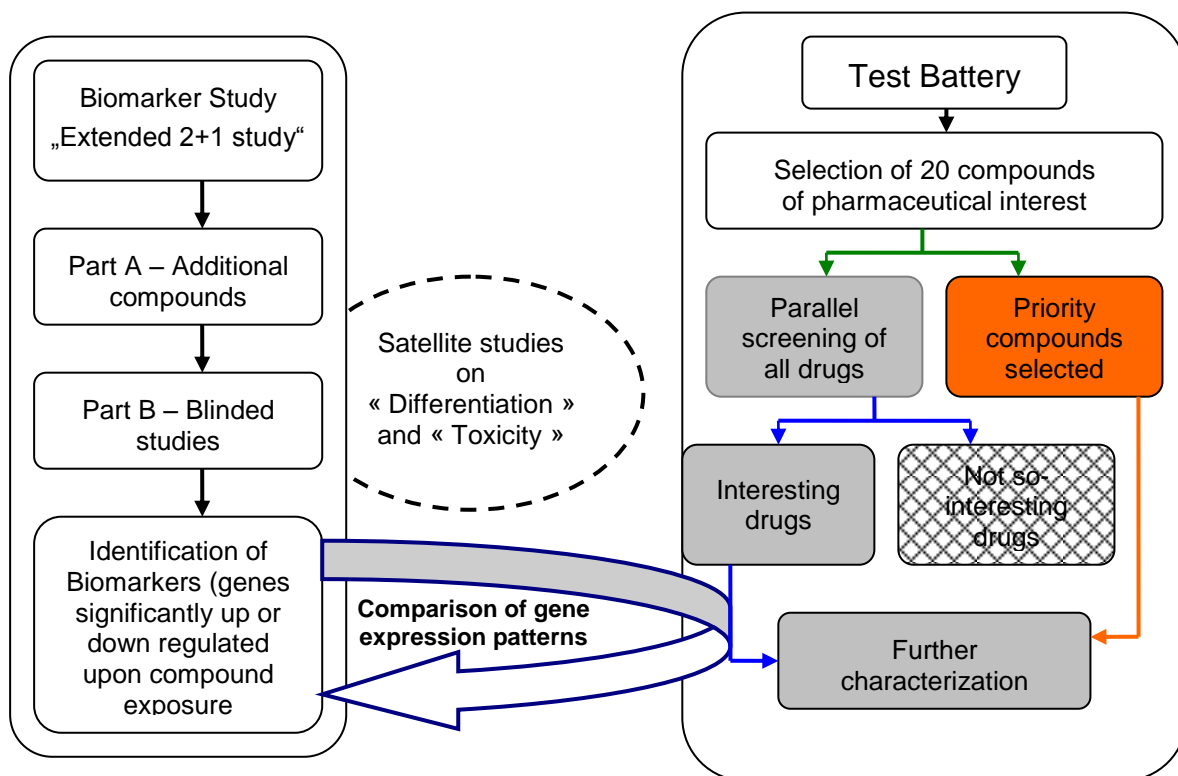
Figure 1: Overview of ESNATS test strategy

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

1.3 Description of the main S&T Results/Foregrounds

1.3.1 Summary

The primary goal of ESNATS was the development of a set of toxicology assays by applying primarily human stem cells in four areas of research:

1. Reproductive toxicity

2. Neurotoxicity

3. Embryonic stem cell (ESC) based toxicogenomic and toxicoproteomic signature

4. Toxicokinetics, metabolism and modeling

The project was divided into two phases as shown in Figure 2. During the first three project years, work was focused on the development and optimisation of assays in these specific areas. During the last two and a half years, the consortium has brought together a selection of the different assays developed in a test battery, and focused on the elaboration of the testing strategies. Tests have been run under blind conditions with the reference substances in order to evaluate sensitivity and specificity of the individual test systems used in the testing strategy.
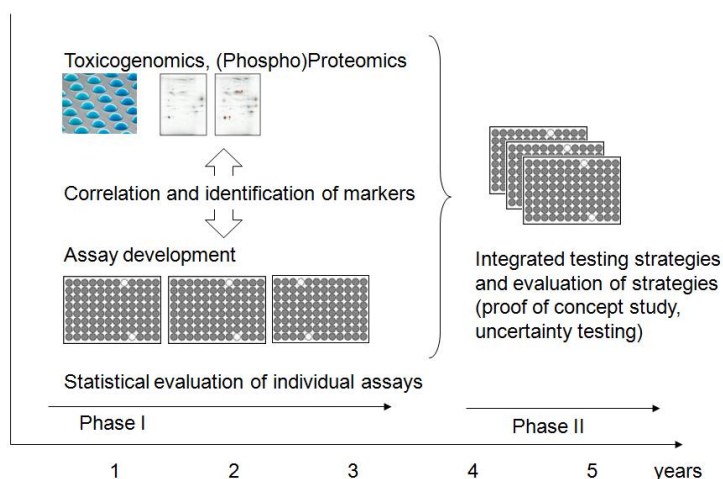

Figure 2: The ESNATS work phases

During the first three years, 14 new, complementary tests were developed, but only 5 were evaluated in depth in the last two years representing the different stages of differentiation and with the challenge of combining them in an effective strateg. The test protocols were developed and optimised, the prediction model arranged and now they are ready for entering a more formal evaluation, possibly even starting the pre-validation procedure.

The work done has demonstrated the possibility of applying human ESC (hESC) in a reproducible way giving indications that hESC-derived systems for the assessment of developmental neuronal toxicity could be a potential alternative approach to animal based testing assays, if not even a major improvement in the predictive capacity. This goal was achieved through the optimisation and standardisation of the procedure for handling global transcriptomics data.

The limit in the development of those tests was the low number of chemicals with known developmental neurotoxicity (DNT) properties. For sure this list needs further expansion for future activity and validation of the strategy. Development of test systems toward 3D and higher complexity for long term and chronic exposures must represent the future challenge. *In vitro* results must be correlated to *in vivo* adverse outcomes with better prediction of adverse effects *in vivo* based on the tested compound omics signatures.

The ESNATS project was not only scientific work. Ethics of using hESC was also considered with some interesting deliverables, such as the creation of a card game that may stimulate a constructive discussion.

Training and dissemination were also important parts of the work, with the very interesting experience of summer schools dedicated to the students participating in ESNATS, and the organisation of several conferences, amongst which the final conference in Linz, which attracted about 300 European and international participants.

In the five and a half years of the project, over 100 papers were published in peer reviewed journals under the umbrella of ESNATS (see list in chapter 2.1.1 "List of Scientific Publications"). Many more will come, as the work done still bears fruit.

1.3.2 Progress and main results

Preparatory work: Selection of a list of chemicals with known activity, definition of a common nomenclature, protocols

A prerequisite for the development of a toxicity test is the selection of a list of chemicals with known activity to challenge the new tests and measure their relevance and predictivity. This step was really complex as there is no clear indication about the toxicant effect of chemical in humans, apart from few exceptions. This issue is shared in most areas of in vitro method development (Leist et al., 2012), whose development is generally based on results from animal models. Eventually, some possible mechanisms were considered with a final agreement on only 10 chemicals which are known to be neurotoxicant during development in humans (Kadereit et al., 2012). Those are not enough for a full validation, but they represent a very good starting point for method development also considering that this list includes chemicals that can affect neuro behavior.

Beyond the development of new tests, minor tasks were performed like the definition of nomenclature which was lacking harmonisation, plus the description of all the details of the protocols such as medium change step, cell replating and so on. These minor issues seem irrelevant but on the other hand they may have a tremendous impact in the development of a robust assay.

Novel *in vitro* toxicity test systems

Significant effort has been devoted to the development of *in vitro* test systems. During the first three years, 14 new, complementary tests were developed. They have been named after the main involved institutions and are shown below in the order of the different developmental stages they cover (**Error! Reference source not found.**).
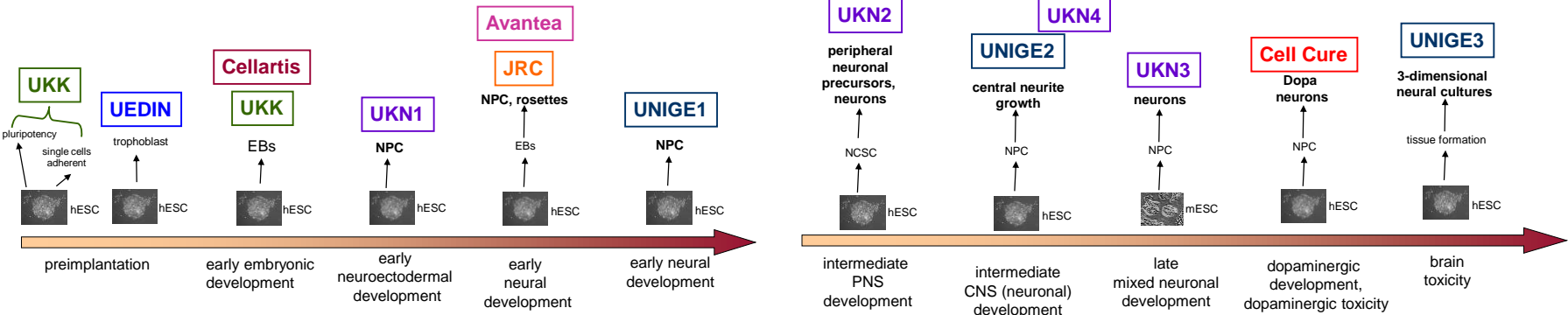
Figure 3: Overview of in vitro test systems that were available at the end of the first phase of the ESNATS project.

Just to further illustrate one of the test systems as an example, the assay developed by Avantea that tests prenatal neural teratogenicity is presented in Figure 4. It is based on the differentiation of hESC into neural rosettes which are peculiar radial structures that develop *in vitro* during neural differentiation. The *in vitro* model reproduces the formation of the neural tube that *in vivo* is the origin of central and peripheral nervous systems. During the course of the project the designed test has been challenged with many compounds known to be neurotoxic and teratogenic. This test system has consistently demonstrated the ability to respond to toxic challenges with cellular and molecular changes similar to those observed *in vivo*. Therefore this *in vitro* method represents a promising alternative test for the detection of human early neural developmental toxicity (Colleoni et al., 2011, Colleoni et al., 2012).

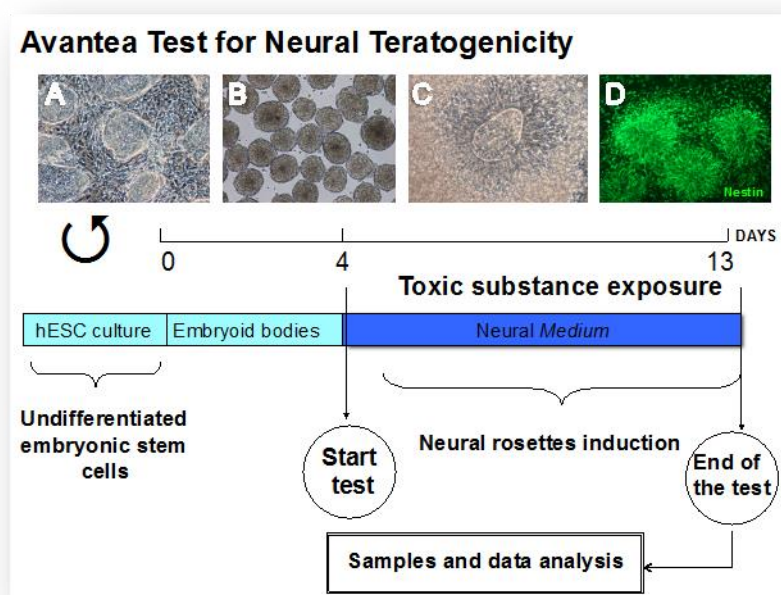The figure below illustrates the scheme of the test.

Figure 4: Schematic illustration of Avantea test for neural teratogenicity. hESCs are cultured undifferentiated (A), then hESCs colonies are detached from the feeder layer and embryoid bodies (EBs) are generated (B). After 4 days of culture in suspension EBs are plated in neural medium to induce the formation of the neural rosettes (C) expressing typical neural markers (i.e. nestin, D). Toxicant exposure is done during the neural rosettes generation, for 8 days. Afterwards cells are analysed by immunohistochemistry and/or recovered for transcriptomic studies by RT-PCR and microarrays.

Test systems forming the "ESNATS test battery"

To streamline the overall strategy towards the eventual accomplishment of a meaningful result, during the 3rd project year, the ESNATS consortium had decided to concentrate its efforts on prenatal toxicity with emphasis on the nervous system, and how to most efficiently feed suitable tests concertedly into the approach, observing the coverage of all critical windows of neuronal cell differentiation.

To do so, five test systems out of the available 14 test systems recapitulating different critical periods of human early neuronal development have been chosen to be part of the "ESNATS test battery": UKK, UKN1, JRC, UNIGE1 and UKN2 (Krug et al., 2013). The criteria for the selection of these test systems out of the available tests that covered the relevant human development stages have been:

- Availability of SOP
- Reliability and robustness of the test system
- Acceptance criteria

- Negative and positive controls
- Non-specific controls (depending on system)
- Biological relevance of the test system

All other test systems should support the core efforts of the project by producing data complementary to the primary focus of work.

Experimental design for toxicity tests participating in the battery approach has been 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
- Capacity of testing at least 20-30 compounds

Figure 5 provides an overview of the five selected test systems. The five methods are focused on the following developmental phases:

UKK recapitulates the multi-lineage differentiation of human embryonic stem cells into ecto-, meso- and endoderm. UKN1 represents the stage of neuroectodermal induction leading to the formation of neural ectodermal progenitor cells. JRC models formation of the neural tube during early neurogenesis by the formation of neural rosettes. UNIGE1 recapitulates the transition from neural precursor cells to mature neurons. It focuses on the maturation of post-mitotic neurons and the outgrowth of neurites. UKN2 uses neural crest cells generated from hESC and examines their functional properties.
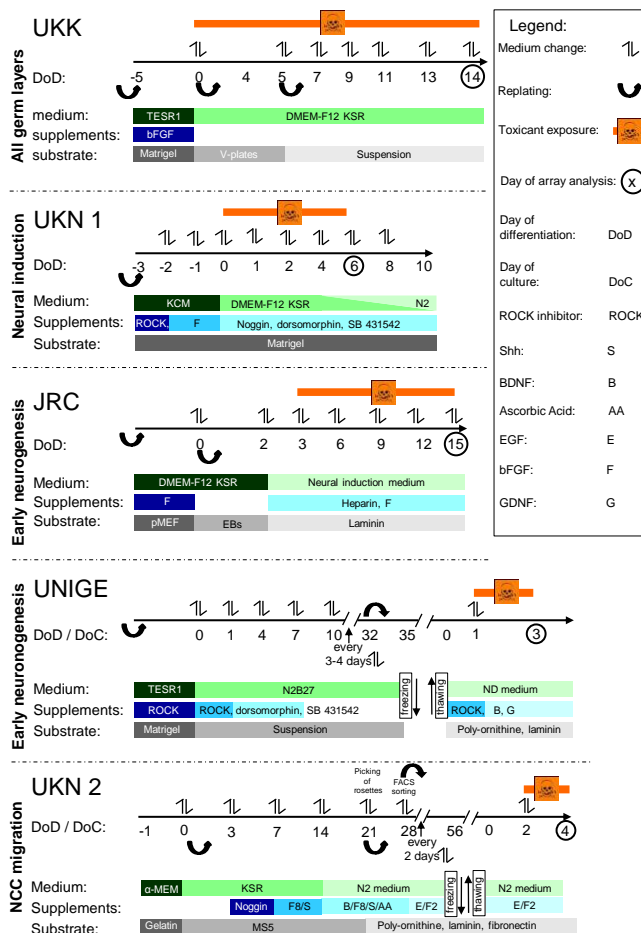

Figure 5: Overview over the novel FP 7 ESNATS test systems for developmental neurotoxicity. The five test systems cover different periods and processes relevant to early embryonic/neuronal development, as indicated to the left. The time arrows indicate when cells were re-plated, medium was exchanged, toxicants were added and when analysis was performed.

Standard operation procedures of all test systems are available (Krug et al., 2013). To consider metabolism, the *in vitro* systems have been combined with cultivated human hepatocytes. It has been demonstrated that inclusion of hepatocytes may enhance toxicity by more than 100-fold or strongly reduce toxic effects in the target cells depending on the type of test compound. To identify *in vivo* relevant test compound concentrations, techniques of modeling have been improved by integrating metabolic, PBPK and spatial-temporal tissue models (Hoehme et al., 2010; Zeigerer et al., 2012). All test systems have been established in close cooperation with pharmaceutical companies and with regulatory authorities. The starting cells of the novel FP7 ESNATS test systems are either neuronal precursor cells or embryonic stem cells (hESC). As far as hESC are involved, pilot experiments have been successfully performed to establish test systems also on the basis of induced pluripotency stem cells (iPSC).

Specific signatures identify DNT compounds

The ESNATS consortium had also decided to set up a specific "biomarker study" which would complement the all-in-one test battery by focusing on gene expression analysis to establish an algorithm that allows identifying compounds that act by a certain toxic mechanism or induce a specific phenotype in a pathway-based approach.

The five selected novel ESNATS test systems were exposed to two classes of compounds known to cause developmental neurotoxicity (DNT). The goal was to explore whether the assays discriminated between different toxicants, and how data should be interpreted. Valproic acid (VPA) and related compounds cause neural tube defects while the human neurotoxicity of methylmercury (MeHg) has been well documented due to catastrophic endemies caused by contaminated food (the Minamata disease).

Analysing the gene expression alterations induced by both test compounds allowed a clear differentiation from negative control compounds (here: mannitol) and from each other (Figure 6).
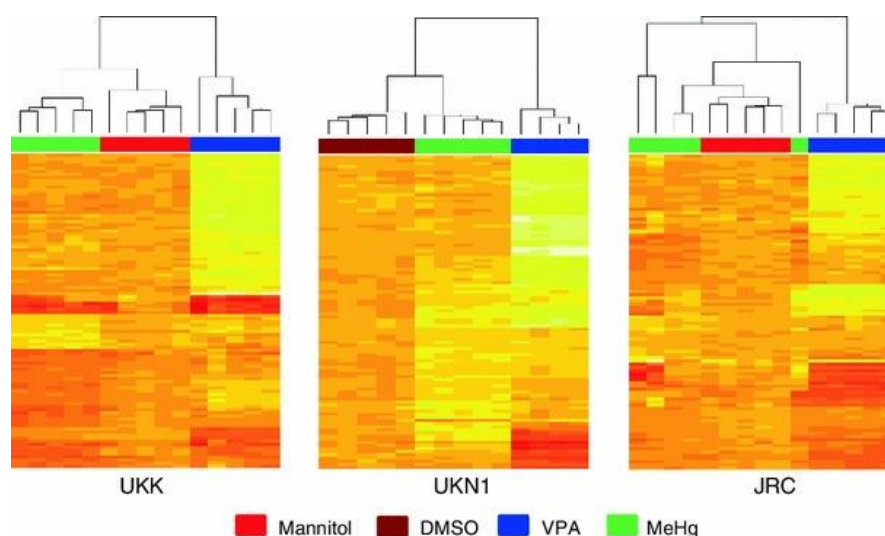

Figure 6: Heatmap analysis of gene expression alterations in the FP7 ESNATS test systems. Valproic acid (VPA) and methylmercury (MeHg) are representatives of two classes of compounds known to cause developmental neurotoxicity in humans. Gene expression alterations induced by both compounds are clearly distinct and also differ from those of a negative control compound.

This success encouraged the ESNATS consortium to perform a blinded classification study using six compounds acting either by 'valproic acid like mechanisms' (histone deacetylase inhibitors) or by mechanisms similar to methylmercury. Classifiers could be established that clearly differentiate the DNT compounds from their solvent controls. This is remarkable, considering that simpler cell systems,

such as fibroblasts or even neuronal cell lines do not allow a sufficient distinction. Genome-wide analyses also made clear that our current categories of DNT, e.g. HDAC inhibitors, mercurials, kinase inhibitors, etc., may not be sufficient to correctly describe the influence of chemicals on the developing central nervous system. Most probably, extended analyses will lead to novel categories and classification systems. The ESNATS proof of concept study clearly demonstrates the importance of cell systems that recapitulate critical processes of human development. Exposure to test compounds *in vitro* must be performed exactly during time windows when such developmental steps take place. In this case stress response pathways and adverse outcome pathways (AOP) have been derived from the deregulated genes. For both compound classes AOPs associated with disturbed neuronal development are now available.

Gene Expression Toxicity Atlas

To make the biomarker study results more conveniently available for the public, we have set up a "ESNATS Gene Expression Toxicity Atlas". This is an interactive web based resource where everybody can browse the results of the gene expression and related *in vitro* test data associated with the biomarker study. The website is available at https://www.quretec.com/esnats/.

This resource is a combination of several components. First, before microarray experiments were made on the test systems it was necessary to find the reasonable benchmark doses. For this, cell viability tests were performed with every system and compound, and a statistical algorithm to find the doses was developed. In our atlas we have all the raw data from these experiments and we have als o implemented the algorithm. So it is possible to interactively to draw the dose response curves and calculate the benchmark doses. For expression data we have made data available in a series of interfaces that closely follow the analysis in the article. There is possibility to draw the principal component analysis plots, cluster the gene expression patterns, identify differentially expressed genes and browse the GO annotations for these lists. A screenshot of the resource can be seen in Figure 7.
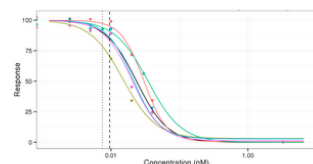
Figure 7: A screenshot of ESNATS Gene Expression Toxicity Atlas

Hepatocyte *in vitro* systems to study metabolism: stem cell derived hepatocytes as an alternative to primary hepatocytes

The ideal *in vitro* system must include metabolism as it is well known that in many cases the effect of a chemical is generated by any of the metabolites, rather than the chemical itself.

One of the goals of the ESNATS project was therefore to integrate functional metabolizing systems into newly developed *in vitro* testing strategies for the toxicological study of substances affecting the nervous system and the reproductive system. Since the liver is the main organ for xenobiotic biotransformation, *in vitro* models properly reflecting the human *in vivo* situation are preferably liver-derived. Several primary hepatocyte-based *in vitro* models were already available, but they undergo progressive dedifferentiation with loss of specific functionality over time. Different animal cell-based models have been proposed. Yet, the correlation between animal-based liver safety studies and humans is less than 60%, demonstrating that the available animal-based models for assessing liver toxicity are not representative for the human situation. Hence, the pharmaceutical industry urgently needs novel, preferably *in vitro* test systems that are more suitable to predict adverse liver responses in humans. Since primary human hepatocytes are very scarce and their large-scale *in vitro* use is hampered by their inability to proliferate in culture, other cell sources need to be explored. As such, stem cells were considered to be suitable as they have the ability for self-renewal and the potential for multi-lineage differentiation. In this context, the 3D cell culture of pluripotent stem cell-derived hepatocytes was challenged by IFADO with many chemicals demonstrating that metabolic activity is present, even though with limitations. Furthermore, the VUB-group's primarily focus was on human skin-derived precursor cells (hSKP). These cells acquired, upon *in vitro* exposure to hepatogenic

growth factors, specific features of hepatic progenitor cells as well as typical characteristics of adult hepatocytes (hSKP-HPC). Although these hSKP-derived hepatic cells were not yet fully functional hepatocytes, they were able to respond to exposure to acetaminophen, a well-known hepatotoxicant, in a comparable way as cultured primary human hepatocytes (Figure 8). As such, hSKP-derived hepatic cells might represent a suitable early preclinical model for *in vitro* hepatotoxicity testing of new chemical entities. Future research will involve the analysis of more chemical substances that cause acute liver failure or other types of liver damage including steatosis, cholestasis, ...
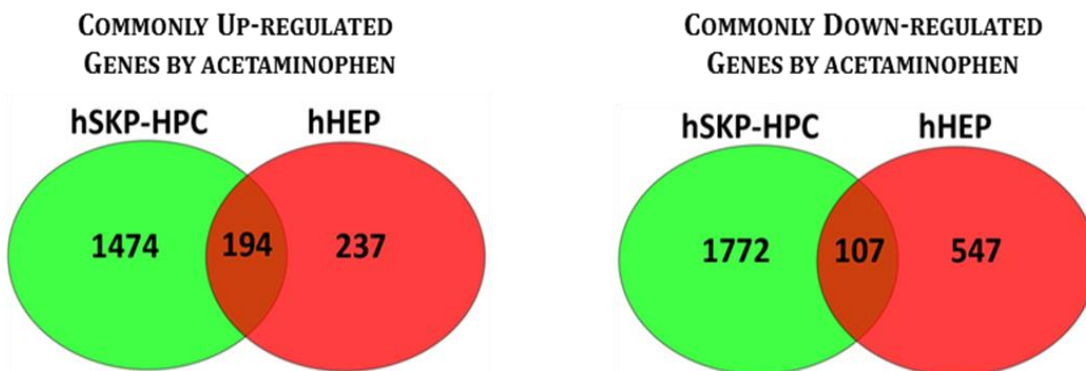
Figure 8: Comparison of up and down regulated genes in hSKP-HPC (hSKP-derived hepatic cells) and hHEP (primary human hepatocytes) exposed to sub-cytotoxic concentrations of acetaminophen.

In vitro-in vivo extrapolation of prenatal (neuro)toxicity assay data by PBPK modeling

A simple good *in vitro* model must be able to predict the possible effect of a specific chemical on a cell system, but this data should be correlated to the real effect of that chemical on a whole human organism, i.e. the understanding of the human relevance of the *in vitro* model. This evaluation is complex as it is not only a matter of transposing two concentrations, but several effects should be considered including absorption, metabolism, distribution and excretion (ADME).

Confidence in new *in vitro* test systems can be built by showing that toxicity in these systems is observed in the same concentration range as *in vivo*. However, *in vivo* toxic exposure levels in published animal studies or human epidemiological studies are often reported as doses. The corresponding concentration in the target tissue is seldom measured. Besides, different fractions of compounds may be freely available to cause toxicity due to differences in protein and lipid composition between *in vitro* medium and plasma or extracellular fluids *in vivo* (

Figure **9**).

Figure 9: *In vitro-in vivo* extrapolation of toxicity assay data by PBPK modeling

In the ESNATS project, TNO used physiologically based pharmacokinetic (PBPK) models to facilitate the comparison between *in vitro* and *in vivo* observations of toxicity. First, animal and/or human PBPK models for the test compounds were built based on available kinetic information, or published models were implemented.

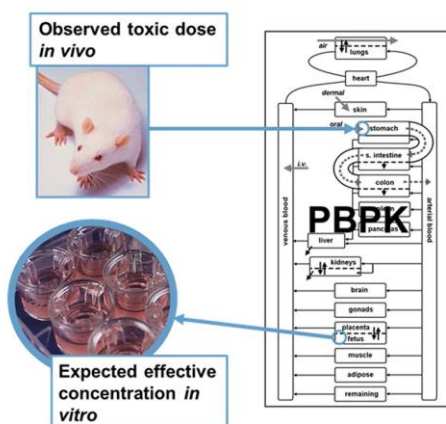These models were used to predict the target tissue concentrations that would be reached by doses at which relevant symptoms of prenatal neurotoxicity were observed *in vivo*. Then, differences in protein binding and lipid partitioning between *in vitro* and *in vivo* were corrected. This strategy helped to

1. define relevant concentrations at which to test the compounds in our test battery;

2. explain differences between test systems in our battery;

3. establish good correlations in general between *in vitro* and *in vivo* toxic concentrations.

The same models may be used in the opposite direction: to extrapolate *in vitro* concentrations to human toxic doses. This "reverse dosimetry" approach is required in the ultimate replacement of animal studies by *in vitro* test systems in human risk assessment.

Pluripotent stem cell-derived engineered neural tissues

Neurons can be developed from pluripotent stem cells (PSC) in both 2D and 3D models (Lancaster et al., 2013). The 3D culture can resemble central nervous system (CNS) organoids and is called cell-derived engineered neural tissues (ENT) to indicate that it is a highly sophisticated system without being a real tissue.

The protocol is relatively easy and the PSC-derived neural stem cells can be stored in a freezer, making the management of the system quite easy (Preyant-Seauve et al., 2009). Neurospheres are plated on air-liquid interface with a nanofilm of medium on a semipermeable membrane that separates the cells from neural induction medium. After 24 hours the system is homogeneous and after two weeks in culture it is very similar to foetus neural tissue and they are ready for further experiments, for example by measuring electrophysiology. Storing is also possible, even though neurons disappear after some time.

There is the possibility of preparing different types of ENTs, by changing the type of starting material, tuning the timing of neurosphere culture, including of cell fate modulating compounds (growth factors, peptides, small molecules) and/or including other cell types.

This model was challenged to study glioblastoma (Nayernia et al., 2013) and CMV (Cytomegalovirus) infection of the foetal brain with encouraging results, even though other experiment with other tumors were not useful, as for example no real interaction was measured.

In conclusion, ENT may have many biomedical applications and it can be applied to study human brain development, but also pathophysiology research of human CNS disease (glioblastoma invasion; CMV infection) with evident positive impact on drug development and toxicology.

Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of hESCs

The disaster caused by Thalidomide in the late 50s is well known and the causality studied in depth. Thalidomide is the typical example of the failure of the animal model during the development of a drug. Thalidomide is a hypnosedative drug that was discovered causing retardation of limb growth (dysmelia) during human embryogenesis. It was first introduced on the market in in 1954 and distributed in many countries all over the world. The first child affected by thalidomide damage to the ears was born on December 25, 1956, in Australia, but before being completely withdrawn from the market, the number of affected new born children were enormous, being almost 3,000 only in Germany. Most of the animal tests for Thalidomide are negative, hindering the real toxicity on humans and therefore, this tragedy was the consequence of the poor predictivity of animal models for human beings.

There are different phases from basic research to marketing of a new drug. Drug discovery and early development is relatively fast and inexpensive. The problems start with the beginning of the pre-

clinical phases, when testing on animals the new drug candidate is mandatory. Among 5000 compounds starting the pre-clinical phase, only 5 enter the clinical phase and one is marketed in the end. From the economical point of view, 900 million euro are spent for drug development and 75% of this amount is spent on projects that fail, most of it due to the weakness of the animal model. Of course this is not generally true. For example, penicillin protects both mice and humans from staphylococcal infections, but the majority has different outcomes like for example corticosteroids which are widely teratogenic in animals but not in humans.

Back to the example of Thalidomide, understanding the mechanism of action may help elucidating the reason why the animal model was not effective and moreover, generating the possibility for testing new drugs *in vitro* to elucidate the possible effect on the development of the foetus during pregnancy.

The gene expression during hESC differentiation was therefore studied in relation to thalidomide exposure. Details of the protocol are reported elsewhere (Meganathan et al., 2012). LC-MS analysis performed during the first 14 days of hESC ddifferentiation showed expression of nerve cell development related proteins and loss of pluripotency related proteins. When exposed to sub-toxic concentration of Thalidomide, a clear and reproducible effect was recorded in a concentration dependent manner with strong perturbation genes associated to heart, limb development and WNT signalling.

It was discovered that thalidomide suppresses the glutathione transferase genes and nucleocytoplasmatic transporter with consequence interference in the nucleocytoplasmic trafficking pathways. Another important conclusion is that Thalidomide inhibits the expression of the glutathione S-transferases of the alpha class (GSTA), which has a very important activity in protecting the cell against the damage caused by ROS (Reactive Oxygen Species).

Automating scale up of stem cell production and cell banking

One of the ESNATS work packages delivered recommendations, concepts and qualification of assays. The recommended approaches suitable for the expansion of cells using automation were provided which concluded that feeder-free systems could be adapted for automation. However, achieving this for the preservation and expansion of undifferentiated cells in a robust and reproducible manner, would require optimisation of factors such as the method of cell dissociation and the seeding density of the cells. Concept papers were also produced that defined the equipment for cell culture automation and for the cryopreservation of differentiated cells, in the form of a functional description and a requirements specification.

It is important for predictive toxicity assays in industrial settings that the methods are reproducible, robust and reliable. To this end, Cellectis evaluated the embryo toxicity assay with several different culture conditions and different cell lines, including feeder-free cell lines in multiwall plate format. Moreover, Cellectis developed a proof of concept of a feeder-free reporter cell line, with a green fluorescent protein (GFP) tagged actin. This has potential to be used in predictive embryotoxicity, as well as neurotoxicity high throughput readouts. The availability of industrialized stem cell production and culturing, together with quick and easy endpoints using GFP-tagged proteins of interest, simplifies the readout procedure substantially, and allows larger scale experiments to be carried out.

Identification & validation of epigenetic biomarkers of human embryonic stem cell response to oxidative stress inducing compounds

The utility of human embryonic stem cells (hESC) as a source of cells for therapy and screening is offset by the challenges of controlling cell growth, differentiation and functionality and genomic stability. Epigenetic modifications of DNA and chromatin are critical regulators of gene expression and DNA repair during development, tissue homeostasis, disease and injury, for which natural or environmentally induced oxidative stress is a key determinant. The objective of UEDIN's research has been to identify & validate novel genes to predict the effect of oxidative stress on the epigenome of undifferentiated hESC and cell commitment to self-renewal or differentiation. UEDIN's accomplishments include:

- Characterisation of the prevalence and distribution of DNA hydroxymethylcytosine (hMC) in pluripotent and derivative stem cells in developing mouse embryos and human cells (Figure 10) and tissues (Ruzov et al., 20111; Cell Research 21:1332).
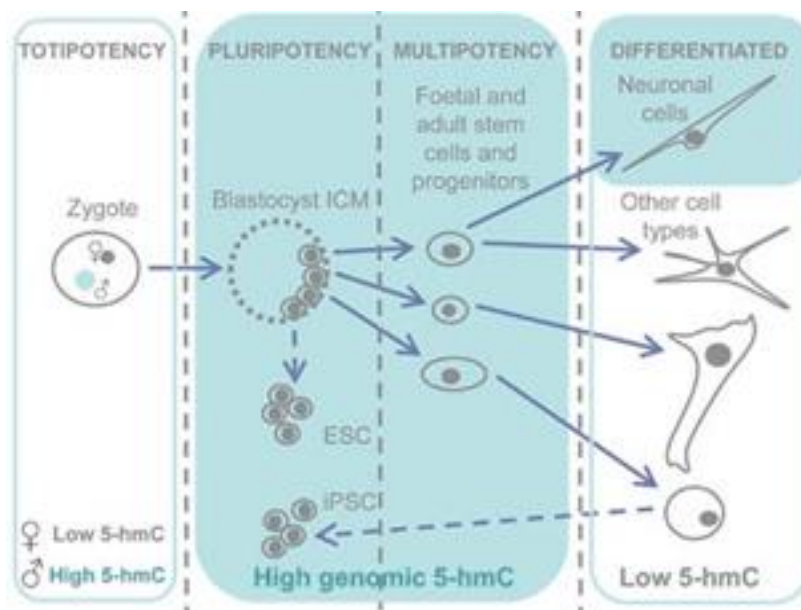-

Figure 10: Characterisation of the prevalence and distribution of DNA hydroxymethylcytosine (hMC) in pluripotent and derivative stem cells in developing mouse embryos and human cells

- Identification of genes expressed in undifferentiated hESC with a unique and conserved pattern of methylation of gene associated sequences (CpG islands) as compared with adult tissues. This includes members of gene families responsible for DNA hydroxy-methylation, histone demethylation, transcriptional activation and repression and protein folding.
- Demonstration that interference with the expression of 7 of these novel genes induces pluripotent stem cell differentiation concurrent with loss of DNA hydroxymethylation. Conversely, UEDIN have discovered that some of these genes augment the frequency with which a pluripotent stem cell phenotype can be induced when combined with established factors.
- Evaluation of the interaction of these genes with transcription factors regarded as core mediators of pluripotent stem cell renewal.
- Characterisation of the consequences of sub-cytotoxic exposures of undifferentiated hESC to diverse test compounds which induce oxidative stress (Azacytidine, Cadmium Chloride, Sodium Arsenate) under normal and hypoxic atmospheric conditions as assessed by effects on lineage-specific and novel gene expression, DNA hydroxymethylation and methylation, cell differentiation, Reactive Oxygen Species (ROS) production and expression of genetic modulators thereof.
- Demonstration of the capacity of selected genes to protect against the differentiation inducing effects of oxidative stress inducing compounds.
- Assessment of the extent to which another oxidative stress-inducing compound, Valproic Acid, recapitulates the effects of the aforementioned test-compounds and conditions. UEDIN's research has identified new genes that: 1) regulate undifferentiated hESC renewal, 2) regulate and are regulated by epigenetic modifications which determine this phenotype, and 3) are sensitive to and protective against compound induced oxidative stress.

The ESNATS ethical guidelines and ethics Democs card game

The ESNATS project did not only comprise scientific work for the development of novel hESC-based toxicity tests, but did also cover work on the ethics associated with the use of hESC for such purposes. Throughout the project duration, ethical issues associated with the work undertaken in ESNATS have been monitored.

In particular, amongst others, the ESNATS partner in charge of ethical issues, Edinethics Ltd., wrote public ethical guidelines on the use of hESC for toxicity testing.

Edinethics also created a "Democs Card Game". This Card Game aims at engaging lay publics on the issues of the use of hESCs and their derivatives to test potential new medicines for toxic side-effects, as an alternative to testing them on animals.

A hard copy of the game has been distributed to all ESNATS partners as well as EC DG Research, the European Group on Ethics, and the Environment, Public Health and Food Safety of the European Parliament, and the European Medicines Agency. A downloadable version of the game will be available on the ESNATS website.

The primary purpose of the game is to get the public to play the game and think about the issues at stake. But it also produces qualitative and semi-quantitative information, which can be analysed. To this end whenever games are played the dealer is asked to send the results back to Edinethics. If enough games are eventually played for a meaningful analysis, this would be done subsequent to ESNATS.

1.4   Potential impact

1.4.1   Major impact

Human stem cell-based *in vitro* test systems have been established in ESNATS that recapitulate relevant processes of the developing human central nervous system. A proof of concept study demonstrated that compounds causing developmental neurotoxicity can be identified in these systems. Further projects should be initiated to study a broader range of chemicals and to optimise the test systems. **It has become clear that stem cell based *in vitro* systems will become an accurate, fast and cost-effective tool for identification of toxic compounds in the broad field of developmental toxicity. This will be a major contribution to human safety.**

It is clear that the ESNATS assays have been scientifically validated and reproduced across a number of research laboratories as documented by the several papers that have been published in refereed journals. However, at the present time, none of the assays have yet entered the EURL ECVAM Validation process that is a prerequisite for regulatory acceptance. However, the published ESNATS assays can already be used by companies for preliminary screening in early drug development for "in-house" use for prioritisation during lead compound optimisation, to select the candidate compounds and early screen-out of compounds predicted to show undesirable toxicity profiles. For those chemicals which have already shown toxicity *in vivo*, the *in vitro* techniques can be helpful in unraveling the mode of action and to improve the confidence in read-across based on chemical similarity by adding information based on biological similarity.

At the moment, the use of *in vitro* systems is generally very limited probably because of a lack of precise transposition of the final endpoint to the *in vivo* situations and of adequate description of the uncertainty associated with the testing strategy employed.

The potential use of the ESNATS test systems should be improved in the future for example with further development of 3D models and by replacing immortal cell lines with iPSCs and hepatocytes with full metabolic activity, plus providing cells that represent healthy or diseased humans.

Another step for the acceptance of *in vitro* methods is represented by the change in mind set that should be focused on the definition of adversity at molecular level. The problem is that at cellular level there is always an effect and the clear distinction with what can cause adversity in an organism is not trivial. To make things more difficult, it should be considered that for example in developmental tests, the cells themselves are changing. It is true also that the problem of defining adversity is exactly the same in *in vivo* tests, when in many cases there is no defined endpoints but only the opinion of the operators.

1.4.2   Future directions

Deeper understanding of the test systems

One of the reasons for the success of the ESNATS test systems is that a relatively high effort has been invested to guarantee that the *in vitro* systems recapitulate relevant processes of human central nervous system development. Should the consortium have chosen an approach with easier already available cell systems and a screening of hundreds of compounds, this approach would most probably have failed. Nevertheless, an even deeper understanding of the established test systems is urgently needed. For example, neuronal differentiation in the ESNATS test systems is characterized by tightly coordinated waves of gene expression (Schulz, 2009; Zimmer, 2011; Gaspar 2012). This feature of the differentiating stem cells recapitulates expression waves of the developing central nervous systems *in vivo*. Complex modeling and systems biology approaches will be needed to understand how such 'waves of development' are coordinated and how they can be disturbed by toxic compounds. It is also critical to understand how these disturbances are linked to adverse effects *in vivo*. This leads to a critical aspect of EU funding policy. In previous projects, funding has been limited to human *in vitro* cell systems. However, to achieve a better understanding of the *in vivo* relevance of 'developmental waves' *in vitro* it should be possible to compare them to the *in vivo* situation. *In vivo*

data are also required to understand how disturbance of 'developmental waves' are linked to adverse effects. Such an understanding could be achieved by comparing developing mouse *in vitro* systems to mouse *in vivo* data. This would help to better interpret data of the corresponding human *in vitro* systems, such as those established by ESNATS. Therefore, future research programs aimed at improving human safety assessment and replacing animal experiments would benefit from inclusion of well justified supplementary research in rodents and rodent cells, besides human cell systems, in order to guarantee that the *in vitro* systems indeed recapitulate the most critical steps i*n vivo*.

Reducing complexity and modeling.

A central result of ESNATS is that DNT compounds cause specific patterns of gene expression alterations in the novel FP7 ESNATS test systems of developmental toxicity (Krug et al., 2013). To interpret these patterns, software for identification of overrepresented biological motifs is usually applied. One result of the ESNATS project is that identification of the transcription factors responsible for the compound induced gene expression alterations is an efficient strategy to reduce complexity. While some transcription factors indicate a general stress response, others seem to be linked to more specific toxic processes. In future, a close cooperation between experimentalists, biostatisticians and modelers is required to decipher the complex expression patterns and understand their relationship to adverse effects *in vivo*.

Compound screening and validation studies.

A final goal of *in vitro* test systems development is the determination of sensitivity and specificity after analysis of large numbers of positive and negative compounds. However, on the way to this ultimate goal pitfalls should be avoided. One is to initiate large screening programs too early. First, we have to answer the question whether the battery of available *in vitro* systems covers a sufficient number of mechanisms and processes relevant for *in vivo* toxicity. In the FP7 ESNATS test systems this has been shown for only two classes of DNT compounds, namely the valproic acid and methylmercury type of compounds. It is difficult to predict whether these *in vitro* systems cover already all relevant mechanisms of developmental neurotoxicity. Considering the high complexity of the CNS this seems rather unlikely. Therefore, a stepwise strategy of optimization seems to be most promising. First, further compounds with known developmental neurotoxicity but acting by other mechanisms than valproic acid and methylmercury should be tested. It will be particularly relevant for further progress, if compounds can be identified that trigger new patterns of toxicity in the so far established FP7 ESNATS *in vitro* systems. In this case the critical *in vivo* mechanisms leading to toxicity must be identified. In the future, also the question has to be addressed whether there are mechanism of toxicity that are not sufficiently represented in the available *in vitro* systems. Possibly additional cell systems or improved *in vitro* techniques have to be established. Only when this process will have been convincingly accomplished, large studies for determination of sensitivity and specificity and formal validation studies will make sense. Successful establishment of *in vitro* systems can only be an iterative process with many cycles of improvement and comparisons to processes in more complex settings (gold standards). The classical gold standard of the past have been rodent *in vivo* studies. It remains to be seen whether human cell-based 3D tissues may not be more suitable and reliable as far as human prediction is concerned.

Table 1: Achievements of ESNATS and future directions

Achievements of the ESNATS consortium	Future perspectives
● Novel *in vitro* systems have been established that recapitulate critical processes of human central nervous system development; standardization of test systems and sufficient reproducibility have been accomplished	● Analyses of broader sets of test compounds must show if all *in vivo* relevant processes of DNT are represented; eventually optimizations will be required; hESC based systems may be replaced by iPSC technologies
● Procedures of handling of genome wide-complex data have been optimized and standardized: normalization based on optimized frozen RMA, cluster identification, recognition of biological motifs, stability analyses and identification of overrepresented transcription factors	● Future studies will have to identify the most efficient and accurate techniques of complexity reduction; e.g. are transcription factor based classification systems superior over gene based classifiers?
● Classifiers for identification of DNT compounds are available; a blinded classification study correctly differentiated DNT compounds from negative controls	● Current text books do not adequately categorize DNT compounds. Novel more accurate classification systems of DNT and DT have to be developed
● The human hepatic metabolism has been included by cultures of primary human hepatocytes and culture medium transfer. Improved techniques of metabolic modeling are available.	● Besides the available 'medium transfer techniques' more direct technologies of metabolite transfer to the target cells are needed, eventually based on the 'body-on-a-chip' principle
● PBPK based techniques for analyses of *in vivo* relevant concentration are available; *in vitro-in vivo* extrapolation to the prenatal situation is possible. PBPK modeling has been integrated into spatial-temporal models.	● The precision of *in vitro-in vivo* extrapolation of test compound concentrations must be improved and confirmed, including *in vivo* analyses of test compound and metabolite concentrations as well as the possibility to predict *in vivo* concentrations by modeling
● The basic principles of concentration and time resolved compound effects are understood; e.g. unspecific toxicity associated signatures (such as downregulation of metabolic functions) can be differentiated from specific events of dysregulated neuronal development. 'Waves of development' *in vitro* show a high degree of similarity to the *in vivo* situation.	● Control mechanisms of 'waves of development' and their susceptibility to chemicals still has to be understood an modeled; a causal understanding of disturbed expression waves and adverse effects *in vivo* still has to be established.

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