International Symposium on

Alternative *in vitro* methods to characterize the role of Endocrine Active Substances (EASs) in hormone-targeted tissues

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Istituto Superiore di Sanità — ISS
*Aula Pocchiari*

Rome, Italy
Welcome address

Dear Colleagues,

it is a great pleasure to welcome you at the CAAT-IPAM-ISS Symposium on “Alternative in vitro methods to characterize the role of Endocrine Active Substances (EASs) in hormone-targeted tissues”, jointly organised by the Department of Veterinary Public Health and Food Safety and the Department of Environment and Primary Prevention, whose activities include the use of alternative in vitro methods to investigate key issues of the role of EASs in endocrine-regulated targets.

The interest in alternative in vitro methods for toxicity testing has raised in the last years within the scientific community and has been moved forward also by the increasing societal demand on the reduction of animal use coupled with the requirements in high quality evaluations of chemical safety. In particular, the processes of screening and prioritization of chemicals may be strongly supported by an integrated use of alternative (either in silico or in vitro) methods.

To this end, the European Commission recently adopted new legislative tools aimed to improve the use of alternative methods as the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH, EC No 1907/2006), the Revised Directive on the Protection of Animals used for Scientific Purposes (EU 63/2010) and the new Cosmetics Regulation (EC No 1223/2009). In all of them, the 3Rs (Refinement, Reduction, Replacement) principle is emphasized in many articles as part of the new toxicological requirements.

Therefore, we are grateful to the Center for Alternatives to Animal Testing (CAAT) Europe and the Italian Platform on Alternative Methods (IPAM) for their efforts to promote and disseminate alternative methods and to contribute to the successful organization of this Symposium.

Along with a pleasant stay in Rome, we wish all participants a fruitful exchange of ideas.

Umberto Agrimi, Head of the Department of Veterinary Public Health and Food Safety
Loredana Musmeci, Head of the Department of Environment and Primary Prevention
Aims of the Symposium

This Symposium is aimed to emphasize the role of alternative methods in search for potential Endocrine Disrupting Chemicals (EDCs) or Endocrine Active Substances (EASs).

Under the REACH Regulation, EAS identification is one of the main concern since within the definition of Substances of Very High Concern (SVHC, Article 57 of Regulation (EC) No 1907/2006 ) are included substances which are i) Carcinogenic, Mutagenic or toxic to Reproduction (CMR), ii) Persistent, Bioaccumulative and Toxic (PBT) or very Persistent and very Bioaccumulative (vPvB), and iii) identified, on a case-by-case basis, from scientific evidence as causing probable serious effects to human health or environment of an equivalent level of concern as those above (e.g. endocrine disrupters).

Nowadays, EASs are challenging classical concepts in toxicology, due to their suggested “low dose effects” and/or “non monotonic dose responses”, leading, for these reasons, innovative approaches in risk assessment.

The Symposium programme is divided in four sessions aimed to give an overview of the state-of-art in EAS investigation by alternative methods, highlighting the academic, regulatory and industrial points of view as well presenting critical issues in human targets of endocrine disrupters and their metabolic fate.
In particular, recent advances in the field of biokinetics as well as in the characterization of new molecular and cellular biomarkers of different reproductive- and hormone-targeted tissues will be presented by invited international experts.

A general discussion among all participants at the end of the symposium is essential part of the program.

Isabella De Angelis, Department of Environment and Primary Prevention
Stefano Lorenzetti, Department of Veterinary Public Health and Food Safety

Acknowledgements

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Scientific Committee

Isabella De Angelis  Istituto Superiore di Sanità - ISS, Rome, Italy
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Simonetta Gemma  Istituto Superiore di Sanità - ISS, Rome, Italy
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Scientific Secretariat

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This event has been supported by unrestricted grants of:
INTRODUCTIVE SESSION
Chairpersons: Isabella de Angelis (ISS-Rome) and Stefano Lorenzetti (ISS-Rome)

8.50-9.10 Thomas Hartung (CAAT-Baltimore)
Endocrine disruption as the pilot of mapping the human toxome

9.10-9.30 Costanza Rovida (CAAT-Konstanz)
Implementation of regulatory issues

9.30-9.50 Alberto Mantovani (ISS-Rome)
Endocrine Active Substances / EASs: understanding modes of action for risk assessment

9.50-10.10 Johann Steinkellner (EFSA-Parma)
Exploration of alternative methods for toxicity assessment of pesticide metabolites

10.10-10.30 Serena Cinelli (RTC-Pomezia, Rome)
Improving test methods in the spirit of the 3Rs; the point of view of a contract research organization

SESSION 1: EASs in reproductive-targeted tissues
Chairpersons: Simonetta Gemma (ISS-Rome) and Marcello Spanò (ENEA-Rome)

11.00-11.20 Stefano Lorenzetti (ISS-Rome)
A prostate perspective on male fertility and EASs: from toxicogenomics to phenotypic anchoring

11.20-11.40 Marcello Spanò (ENEA-Rome)
Human sperm (epi)genetic biomarkers to assess the impact of EASs on male reproductive function

11.40-12.00 Luana Paulesu (Univ. Siena-Siena)
In vitro effects of EASs in human placenta
SESSION 2: EASs in different hormone-targeted tissues
Chairpersons: Alberto Mantovani (ISS-Rome) and Paolo Marzullo (Univ. Novara-Novara)

12.00-12.20 Igor Bendik-Falconnier (DSM-Basel)
Endocrine active nutrients explored in human bone cell cultures

12.20-12.40 Robert A Smith (Univ. Glasgow-Glasgow)
The use of cell models in determining neuronal responses to EASs

12.40-13.00 Arti Ahluwalia (Univ. Pisa-Pisa)
Dynamic in-vitro organ models of metabolism

13.00-13.30 General discussion

13.30-14.30 Lunch

SESSION 3: EASs and kinetics
Chairpersons: Thomas Hartung (CAAT-Baltimore) and Emanuela Testai (ISS-Rome)

14.30-14.50 Emanuela Testai (ISS-Rome)
The role of biokinetics in in vitro tests and in the interpretation of results

14.50-15.10 Frédéric Yves Bois (Univ. Compiègne-Compiègne)
Physiologically-based modeling of ovarian steroid hormones synthesis for EASs’ health risk assessment

15.10-15.30 Daniel R. Dietrich (CAAT-Konstanz)
EASs contra human & environmental health: relevant or playground for merchants of doom?

15.30-16.00 General discussion
INTRODUCTIVE SESSION
ENDOCRINE DISRUPTION AS THE PILOTE OF MAPPING THE HUMAN TOXOME

The US National Academies / National Research Council report from 2007 "Toxicity Testing in the 21st Century: A vision and a strategy" has created an atmosphere of departure in the US. It suggests moving away from traditional (animal) testing to modern technologies based on pathways of toxicity. These pathways of toxicity could be modeled in relatively simple cell tests, which can be run by robots. The goal is to develop a public database for such pathways, the Human Toxome, to enable scientific collaboration and exchange.

There is a continuously growing awareness about Tox-21c in all stakeholder groups. It was first embraced by scientists and in the US. Most importantly, the US agencies followed fast on the 2007 NAS/NRC report: the Tox-21 alliance in 2008 (paper in Science first-authored by now NIH head Francis Collins, EPA made it their chemical testing paradigm in 2009, FDA followed most evidently with the Science article by FDA head Margaret Hamburg in 2011). Chemical and consumer product industry got engaged, e.g. with the Human Toxicology Project Consortium. In Europe, all this is rather delayed, with some adaptation of the vocabulary but not necessarily grasping the new approach. This is not alternative methods under a new name. However, interest is lately increasing strongly in Europe.

Tox-21c suggests moving to a new resolution, i.e. pathways of toxicity. The problem is that the respective science is only emerging. What will be needed is the Human Toxome as the comprehensive pathway list, an annotation of cell types, species, toxicant classes and hazards to these pathways, an integration of information in systems toxicology approaches, the in-vitro-in-vivo-extrapolation by reversed dosimetry and finally making sense of the data, most probably in a probabilistic way. The NIH is funding since September 2011 by a transformative research grant The Human Toxome project led by CAAT. The project involves US EPA ToxCast, the Hamner Institute, Agilent and several members of the Tox-21c panel. The new approach is shaped around pro-estrogenic endocrine disruption as a test case.

Early on, the need for quality assurance for the new approaches as a sparring partner for their development and implementation has been noted. Formal validation as developed for the first generation of alternative methods can only partially serve this purpose. For this reason, the Evidence-based Toxicology Collaboration (EBTC) was created in the US and Europe in 2011 and 2012, respectively. This collaboration of representatives from agencies, industry, academia and stakeholder groups aims to develop tools of Evidence-based Medicine for toxicology. The secretariat is run by CAAT, the first conference was held in early 2012 hosted by US EPA and working groups have started to address pertinent issues and methodologies. All together, Tox-21c and its implementation activities including the Human Toxome and the EBTC promise a credible approach to revamp regulatory toxicology.

References
Costanza Rovida is scientific Officer at the Center for Alternatives to Animal Testing in Europe (CAAT-Europe) and project manager for REACH Mastery. She took a degree in Chemistry with specialisation in Analytical Chemistry in 1989. After a period focussed on the optimisation of analytical techniques in the area of food and environmental analyses, she moved to a pharmaceutical Industry where she learned about method validation plus toxicology and drug efficacy. Convinced that in vivo methods are not the right scientific answer to our toxicological questions, in the period 2005-2008 she worked at ECVAM (European Centre for Validation of Alternative Methods) where she gained experience of alternative methods applied in the area of skin and respiratory sensitisation and she had the opportunity to participate in the working groups organised by the European Commission for the implementation of REACH, the latest Regulation on the evaluation and authorisation of chemical substances. She was one of the pioneer members of CAAT Europe and since 2009 she has strived for the application of in vitro methods for regulatory purposes. Publications on this topic are many, including a publication on Nature (Hartung, T. and Rovida, C. Opinion. Chemical regulators have overreached. 2009. Nature, 460). She is engaged in many scientific Committees that are active in the field of alternative methods, by organizing symposia and workshops to disseminate the 3R strategies. She is also still very much involved in the REACH process by following clients to be compliant with all the provisions of that Regulation.

IMPLEMENTATION OF REGULATORY ISSUES

The term “endocrine disrupter” (ED), was introduced in the early 1990s and later defined as (WHO, 2002): “… an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.” Possibility of perturbation of the endocrine system has been considered for a long time, since both the uterothrophic bioassay (OECD TG 440) and the anti-androgenic screening test (OECD TG 441) were originated in the 30’s.

In the latest 20 years, concern about endocrine disruptors is definitely increasing, and in the European Union there are at the moment three important Regulation that specifically ask to address this effect. The first one was probably REACH (Regulation 1907/2006) which includes the endocrine disruptor activity as a condition to include a substance in the authorization list. In addition, both Regulation 1107/2009 for Plant Protection Products and proposed new Regulation for Biocidal products ask that approved substances should not have endocrine disruptor activity and for the definition both refers to Article 57(f) of REACH, which does not provide a clear definition for getting to a unique and generally approved decision. Regulation 1272/2008 (CLP) adds the endocrine disruptor activity in Annex II regarding the description of the procedure to assign the classification as reproductive toxicant in the area where “weight of evidence” is described.

Even if endocrine disruptor activity is generally recognized as a serious effect, there is no agreement about the methods that should be applied for the assessment and when the activity for a substance is universally recognized, as the case for Bisphenol A, the discussion is still open for the definition of the no effect dose.

One of the problem is that endocrine disruption is not a toxicological endpoint in itself, but rather a class of modes and mechanisms of action. There are a battery of in vitro tests available that must be used for initial screening, but in the end, confirmation in an intact organism is always required, i.e. in vivo in rats, even though it is well recognized that endocrine activity is very different among species.

State of the art in this topic has been recently presented in a document issued by an expert panel charged by the European Commission. Regardless the ambiguous conclusion of this report, there is an urgent need to define a set of methods for this class of substances. Within the scope of REACH, 18 substances have been now selected due to their concern of being endocrine disruptors. Decision is expected in two years.
Alberto Mantovani (AM, 1956, DVM, MSc) is currently director of the Food and Veterinary Toxicology Unit at the Italian National Health Institute (ISS). The main research topics of the Unit are endocrine disrupters (ED), natural bioactive substances, trace elements and nanotoxicology; the research activities pivot on translating mechanistic research into risk (and risk-to-benefit) assessment. AM co-authored about 50 scientific papers on toxicology and risk assessment in the 2006-12 period: he co-ordinated the pilot national project on ED (2000-2003), the research area IV (cross-cutting technologies) of the EU project ReProTect (2005-10) on non-animal testing strategies for reproductive toxicants and the national PREVIENI project (2008-11) on the development and use of biomarkers to assess the impact of ED exposure. Since 2003 AM is an expert of the European Food Safety Authority (EFSA): member of FEEDAP Panel (substances used in feeds) on 2003-12, member of PPR panel (pesticides) since July 2012 and expert collaborating to EFSA opinions on non-animal testing, bisphenol A and TTC-thresholds of toxicological concerns.

ENDOCRINE ACTIVE SUBSTANCES: UNDERSTANDING PATHWAYS FOR RISK ASSESSMENT

Alberto Mantovani, Stefano Lorenzetti, Cinzia La Rocca, Laura Narciso, Sabrina Tait.

Endocrine disrupters (ED, see the dedicated ISS web area, http://www.iss.it/inte) are natural or man-made chemicals in diet and/or environment able to cause adverse effects in exposed organisms or their progeny by altering endocrine homeostasis. In a broader sense, endocrine active substances (EAS) include all compounds modulating endocrine functions; thus, EAS may also exert beneficial or protective effects. The so-called “phytoestrogens” are a recognized example of EAS that may exert beneficial or adverse effects depending on the exposure level, lifestage, sex as well as on the interactions with other EAS (see the EDID database at http://www.iss.it/inte). The endocrine system is a network regulating most body functions: the pre- and post-natal lifestages are particularly sensitive, and long-term, persistent effects may result from early exposures to EAS. From the toxicologist standpoint, rather than an effect per se endocrine disruption is a group of modes of action highly relevant to risk assessment due to their complexity and serious long-term (even trans-generation) impact. As a consequence, the assessment of ED (and EAS) would likely progress together with the development of new testing approaches that exploit system biology and integrate pathways into the classical hazard characterization. Moreover, up to now the toxicology of ED has largely pivoted around the effects on fertility, reproductive development and thyroid, whereas comparatively limited attention has been paid till now to other relevant aspects, such as the neuroendocrine and endocrine-immune interfaces and especially the impact on metabolic programming, which might represent a field of considerable public health significance. The development of conceptual frameworks supported by robust scientific is also needed to deal with long-debated issues in ED/EAS assessment, such as: i) the relevance of low-dose effects and non-monotonic responses (an EAS eliciting different hits with partly overlapping dose-response relationships ?) or ii) combined exposures to different EAS (is there something more than dose addition ?). We present two examples of published ISS studies providing mechanistic insights into risk assessment issues, namely, the use of transcriptomics to characterize components of mixtures and the use of clinically relevant biomarkers to characterize EAS potentially interfering with the same pathways.

Acknowledgement: the abstract has been elaborated within the frame of the Ministry of Health grant “Reach: endocrine disrupters”
Between 1994 and 2002 I have worked in the Department of Environmental Toxicology of the Institute for Cancer Research of the University of Vienna in the field of genetic toxicology (e.g. gene mutation assays with *Salmonella typhimurium*, chromosomal aberration and micronucleus tests with plant cells, cultivated hepatic cells in rats and primary human lymphocytes, Comet Assays with a variety of cell types, spectrometric and biochemical enzyme measurement methods and determination of GST genotyping of humans (e.g. with PCR). I have been teaching in the field of genetic toxicology at the University of Vienna. I have obtained a PhD in the field of anticarcinogenesis/antimutagenesis in humans. I hold a master degree in toxicology and am a Eurotox registered member of the Austrian Society of Toxicology.

From 2002 to 2007 I have worked as a toxicologist in the field of health effects and environmental effects of “Existing Chemicals”, “New Chemicals” and Pesticides (Regulation 67/548/EEC and adaptations thereof, Directive 91/414/EC), Risk Assessment of “Existing Chemicals” (Regulation 793/93/EEC) and have contributed to the implementation of REACH.

Between 2007 and 2009 I have worked at the European Food Safety Authority (EFSA) as a toxicologist responsible for human health risk assessment in the field of peer review of plant protection products under Directive 91/414/EC.

Since 2009 I am working as a toxicologist in EFSA’s Pesticide Unit in support of EFSAs Panel on Plant Protection Products and their Residues (PPR). I have been involved, beyond many other activities in support of pesticide risk assessment, for instance in the drafting of scientific opinions/guidance documents on neurotoxicity of deltamethrin, dermal absorption, exposure assessment for workers, operators, bystanders and residents, cumulative risk assessment of pesticides and the toxicological relevance of pesticide metabolites.

**EXPLORATION OF ALTERNATIVE METHODS FOR TOXICITY ASSESSMENT OF PESTICIDE METABOLITES**

The framework of evaluation and authorisation of chemical plant protection products and the active substances they contain in the EU is laid down in Regulation (EC) No 1107/2009 and appertaining regulations. Assessment of the risk for the consumer requiring also the identification of metabolites and of degradates of the active substances is a major part of this process.

One of the outcomes of the evaluation of an application for use of an active substance on a crop is the establishment of two residue definitions, one for monitoring and one for dietary risk assessment. While the residue definition for monitoring has regulatory purposes for the enforcement of the MRLs (Maximum Residue Levels) and must reflect analytical practicalities, the residue definition for dietary risk assessment may be wider, as its purpose is to assess consumer safety, and it should therefore include all metabolites and degradates of toxicological relevance.

However, in practice only the toxicological properties of the active substance are investigated through the range of toxicological studies while only very limited information about the toxicological properties of metabolites and degradates is available in the majority of cases. In this context it is notable that subjecting all metabolites to the full testing scheme applied for active substances appears not feasible due to the sheer number of metabolites identified in many cases. In addition this would lead to a significant increase in the use of test animals in the field of pesticide risk assessment.

Therefore EFSA’s PPR Panel has developed a scientific opinion in which alternative testing methods were explored in regard to their applicability for testing of pesticide metabolites.

The opinion identifies the Threshold of Toxicological Concern (TTC) concept as an appropriate screening tool for assessment of toxicity of metabolites and identified three critical steps in its application which are 1) estimation of the metabolite level, 2) evaluation of genotoxicity alerts and 3) detection of potentially neurotoxic metabolites. A TTC concept for acute exposures was also established based on pesticide active substances to which an Acute Referenced Dose (ARfD) has been allocated. Assessment schemes both for chronic and acute dietary risk assessment are presented in the opinion using a combination of the TTC approach with QSAR models/read across and targeted testing.

In addition to proposals for assessment of metabolites the opinion also describes how the proposed assessment tools could be utilised for assessment of differential toxicity of pesticide isomers.

The results obtained from the work on this opinion will be the basis for the development of a guidance document for toxicity assessment of metabolites and isomers.
Dr. Serena Cinelli is Associate Scientific Director at Research Toxicology Centre (RTC), a Contract Research Organisation specialised in non-clinical safety studies, located in Pomezia (Rome), Italy. She has more than 25 years experience in genetic and in vitro toxicology testing for industry and she is author of numerous toxicology reports submitted to international regulatory authorities. Dr. Cinelli also acts as Contract Professor in Environmental Mutagenesis and is author of numerous peer reviewed publications.

**IMPROVING TEST METHODS IN THE SPIRIT OF THE 3RS; THE POINT OF VIEW OF A CONTRACT RESEARCH ORGANIZATION**

Serena Cinelli, Germano Oberto, Isabella Andreini

A multiple-level tiered approach to identify and characterize the hazards of Endocrine Active Substances (EAS) is suggested by the most important international organisations which provide guidance for industry for the safety assessment of chemicals. Both OECD and EPA indicate that non-clinical development should include a screening phase in which in silico, in vitro and in vivo assays are performed to provide mechanistic data for hazard identification, followed by an in vivo testing phase to better characterize the identified risk.

Based on current evidence, scientists and regulators acknowledge that stand alone in vitro methods are not sufficient to reliably predict in vivo effects due to the complicated nature of hormonal systems. Nevertheless several of the available in vitro methods can provide extremely important data to clarify in vivo mechanisms of action of EAS and can be used as alternatives to in vivo assays traditionally employed for single endocrine mechanism and effect (e.g. Uterotrophic assay).

In order to obtain an effective reduction of animal use with the application of alternative in vitro methods, it is necessary to satisfy different steps, such as a successful validation process, inclusion into regulatory requirements, acceptance by industry and wide application in non-clinical Contract Research Organizations (CROs).

The role of CRO is increasingly important in application of alternative methods since the general trend of industry is to contract out most of the non-clinical development. In this respect CRO is the ideal candidate not only to run but also to validate new alternative methods since understanding the needs of industry and regulatory framework becomes a key factor.

However, one strong hurdle for the CRO is the balance between the immediate investments and the delayed validation project payback. The CRO must keep in mind that industry may have a conservative approach driven by the risk aversion: fear that results from alternative in vitro approaches might be not readily accepted by regulatory authorities as the ones from in vivo conventional studies.

Different scenarios will be presented by the author with possible situations and proposed solutions to manage safety assessment programs, aware that a complete fulfilment of the 3Rs philosophy is not possible, but a wise strategy of in vitro testing selection and expert data interpretation can help to reduce and refine animal testing.
SESSION 1:
EASs IN REPRODUCTIVE TARGET TISSUES
Stefano Lorenzetti, graduated in Biological Sciences (1991) and in Human Nutrition Sciences (2003), is employed since 2006 at the Dpt. of Food Safety and Veterinary Public Health of the Italian National Health Institute - ISS (Istituto Superiore di Sanità) in Rome. He has been lecturer (2006-09) in Molecular Biology and Toxicology at the Faculty of Medicine of University Tor Vergata of Rome at the post-graduated course of Sciences of Nutrition. Since January 2012, he has been appointed as a member of the Italian national expert group of “Alternative methods to animal experimentation”.

He participated to different international project on the hazard and risk characterization of environmental and dietary contaminants such as the EU Integrated Project ReProTect (“Development of a novel approach in hazard and risk assessment or reproductive toxicity by a combination and application of in vitro, tissue and sensor technologies”) to develop an integrated in vitro approach linking toxicogenomics to clinical biomarkers (phenotypic anchoring).

His research is focused on the set up of in vitro alternative methods to animal experimentation to study the role of endocrine disruptors/EDCs or Endocrine Active Substances/EASs such as bioactive compounds of plant origin (e.g. polyphenols) and environmental and dietary contaminants (e.g., plasticizers, pesticides and biocides).

A PROSTATE PERSPECTIVE ON MALE FERTILITY AND Endocrine Active Substances: FROM TOXICOGENOMICS TO PHENOTYPIC ANCHORING

Stefano Lorenzetti

Although prostate function is critical for male fertility, in reproductive toxicology it is still an overlooked target (1). Indeed, LNCaP cell line may represent an alternative in vitro method of the human prostate epithelium to screen bioactive chemicals affecting male fertility (1-3): within the EU Integrated Project ReProTect, LNCaP cell line has been used as a cellular model to investigate androgen receptor (AR)-dependent signaling to perform toxicogenomic studies of (anti)androgen-like chemicals (1-5) and a cell-based bioassay was employed to provide a phenotypic anchoring to gene expression profiling data (4). The selected cell-based, cell-specific, clinically used biomarker of effect has been the Prostate-Specific Antigen (PSA) monitored as secreted protein. Besides to be a supportive tool for the toxicogenomic approach, the PSA secretion assay has been thus implemented as an independent tool to investigate prostate-mediated effects on male reproduction (4).

References.
Notes
Marcello Spanò (MS) is senior scientist at the Toxicology lab, Unit of Radiobiology and Human Health, ENEA (Italian National agency for New technologies, Energy and sustainable economic development). His main interest is represented by the study of sperm DNA/chromatin (epi) genetic integrity after environmental exposures (endocrine interferers, ionizing and not ionizing electromagnetic radiation). MS has more than 25 year experience in the field of automated cytology development, use and application of methods to detect damage in mammalian germ cells induced by radiation and chemicals, also exploring the impact of sperm DNA damage on human reproductive capabilities. MS has been involved in EU projects on reproductive toxicology since 1991 and co-authored more than 130 peer review papers and book chapters.

**HUMAN SPERM (EPI)GENETIC BIOMARKERS TO ASSESS THE IMPACT OF ENDOCRINE ACTIVE SUBSTANCES ON MALE REPRODUCTIVE FUNCTION**

*Marcello Spanò, Eugenia Cordelli and Francesca Pacchierotti*

Infertility is a common disorder affecting about 15% of all couples trying to conceive and subfertility has become a relevant growing problem in affluent countries. Temporal and spatial trends toward decreasing semen quality, increasing rates of reproductive tract abnormalities, and a well documented worldwide testicular cancer incidence increase pointed to possible environmental causes of male reproductive system impairment. Even if it is difficult to disentangle the responsibility of endocrine stressors from other lifestyle factors potentially impairing human fertility, it is believed that a high level of environmental endocrine active substances (EASs), especially during fetal life, may disturb the hormone control of male urogenital tract organogenesis with lifelong consequences. From a toxicological point of view spermatogenesis is expected to be vulnerable to reproductive toxicants because of the large number of cell divisions and cell differentiation processes continuously occurring throughout the whole reproductive life. In addition, the final stages of gamete differentiation in male mammals are sensitive targets of DNA-reactive chemicals because they are repair deficient. Human biomonitoring studies of South African and Mexican populations living in endemic malarias areas have consistently indicated impaired semen quality associated with high levels of environmental DDT-exposure. There are limited but sound epidemiological data indicating adverse effects of EASs on human sperm DNA. Low-level exposure to PCB-congeners apparently interferes with sperm chromatin integrity (and sperm motility as well) in humans — findings that are consistent with experimental studies. The need to establish alternative methods modelling mammalian spermatogenesis is an issue of high priority. In this context, direct in vitro assays on spermatozoa addressing motility and genetic damage are relevant since they can detect effects on terminally differentiated male gametes. It has recently been proposed that EASs exposure can affect sperm chromatin integrity by a mechanism involving epigenetic changes to the paternal genome. Rodents exposed in-utero to the fungicide vinclozolin or the insecticide methoxychlor showed abnormal DNA methylation patterns in spermatozoa and impaired male fertility. Once established, these epigenetic changes may be permanent and thus paternally passed to subsequent generations. Following human population studies showing an association between EASs exposures and altered global methylation levels in blood lymphocytes, biomonitoring studies are in progress to determine sperm DNA methylation patterns in relation to EASs exposures. It is probable that in the near future, reproductive risk assessment from EASs exposure could benefit from more affordable high-throughput technologies providing a more complete evaluation of the possible genetic and epigenetic effects.
IN VITRO EFFECT OF EASs IN HUMAN PLACENTA

Luana Paulesu, Nicoletta Bechi, Roberta Romagnoli, Francesca Ietta

Endocrine disrupter chemicals (EDCs) are environmental pollutants of agricultural or industrial origin which may influence human reproductive health. These compounds are able to interfere with the delicate balance of the endocrine system by mimicking the action of the steroid hormones. They can also be transferred from the mother to the embryo and cause reproductive and developmental toxicity. We investigated the effect of selected EDCs on in vitro models of human placenta. In particular we examined para-nonylphenol (p-NP) and Bisphenol A (BPA). The choriocarcinoma BeWo cell line and the HTR-8/Sv-neo cells were used to identify chemical lethal concentration able to reduce 50% of cell viability (cell toxicity) and the chorionic villous explants from fresh human placenta were used to perform functional studies of chemicals at non-toxic but environmentally relevant concentrations. Vehicle-treated cultures were used as negative controls.

We found toxicity of the chemicals tested at concentrations in the order of µM. Lower non-toxic concentrations, in the order of nM, were able to interfere with the hormone (β-human chorionic gonadotropin, β-hCG) secretion as well as inducing trophoblast differentiation and apoptosis.

These results raise concern about maternal exposure to environmental factors and the potential involvement of these chemicals in pregnancy disorders.
SESSION 2:
EASs IN DIFFERENT HORMONE TARGET TISSUES
Igor Bendik-Falconnier obtained his Bio II diploma in molecular genetics and his Ph.D. in cell biology at the University of Basel, Switzerland. Subsequently he spent his post-doc and associate professor years at the Burnham Institute, La Jolla and the Sidney Kimmel Cancer Center, La Jolla, California. Back in Switzerland he was a deputy group leader at the department of research (ZLF) at the medical faculty of Basel, where he was awarded several grants to explore cancer formation. In 1998, he started his career in industry at Hoffmann-La Roche Ltd, later in Roche Vitamins, Basel, and explored the efficacy of natural health–beneficial food ingredients. In the VHF department, he led the bone biology group. During his career, he has successfully trained and supervised a number of diploma and Ph.D. students. Since the merger with DSM in October 2003, he has continued in his role as a senior scientist to support and lead different teams in nutritional research with the focus on vitamins and nutraceuticals. During his industrial commitment he has led different research teams and was responsible for different cellular and molecular research technologies. Currently, he is heading the biostatistics and bioinformatics group at DSM Nutritional Products, Ltd.

ENDOCRINE ACTIVE NUTRIENTS EXPLORED IN HUMAN BONE CELL CULTURES

Many natural food-borne compound classes interact with the human body in an endocrine hormone-like manner. These nutrients fulfill important physiological roles mediated through healthy nutrition. An example is genistein, the major soy isoflavone, which is well known to be active in bone tissue, a common target for endocrine hormones. DSM has explored the function of genistein in bone cell biology using available omics and primary cell culture technologies. DSM has develop geniVida™, the nature-identical aglycone, as a product for postmenopausal bone health in the field of nutrition. This presentation will cover the use of cell culture technology for the characterization of an "endocrine active nutrient" in a given biological concept and the strategy to develop it as a nutraceutical for postmenopausal bone health. Besides efficacy, also the industrial toolbox for testing of endocrine disruption properties and the regulatory framework will be discussed.
DO CELL MODELS OFFER REALISTIC ALTERNATIVES FOR DETERMINING POTENTIAL NEURONAL RESPONSES TO ENDOCRINE ACTIVE SUBSTANCES?

The complexity of the human nervous system, comprised of a multitude of interacting neural and non-neuronal cell populations, not to mention the plasticity changes occurring during its development and throughout adult life, presents a difficult task when attempting to determine perturbation following potential toxic insults. This is particularly so when employing in vitro approaches. Emphasis has focussed on monitoring a number of neuronal-specific endpoints rather than merely reporting general cytotoxic responses leading to cell death, for example quantifying changes in neural and glial specific marker proteins, including receptor expression, analysing effects on neurite outgrowth, functional competency and synaptogenesis. Primary neurons, mainly from rodent species, have been successfully cultured from numerous regions of the brain, spinal cord and peripheral systems, and have been widely applied to toxicological studies for many years (Smith, 2009). A number of transformed neural lines, including those established from human tumours such as SH-SY5Y cells, have also been routinely used both in immature and differentiated states with promising outcomes in screening programmes. During the last decade exciting developments in molecular biology have resulted in the generation of human embryonic neural stem cell lines, derived from umbilical blood (Buzanska et al., 2010), and recently induced pluripotent stem cell (transduced from adult dermal fibroblasts) have become available (Kumar et al., 2012). Although issues of variability and high costs may limit their current usefulness, such technologies have potential as future in vitro model systems for toxicological assessments. The pros and cons of utilising these in characterising the role of endocrine active substances relevant to neural tissues will be discussed.

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**DYNAMIC IN-VITRO ORGAN MODELS OF METABOLISM**

Unraveling the complexity of inter-organ and inter-tissue cross talk *in vivo* is a complex and challenging task. Intelligent *in vitro* models able to recapitulate the physiological interactions between tissues in the body connected by the bloodstream have enormous potential as they enable detailed studies on specific two-way or higher order organ-organ and tissue interactions. These models are the first step towards building an integrated picture of systemic signaling in physiological or pathological conditions. Using a scaled down fluidic system, we have developed *in vitro* models of endogenous metabolism and toxicity through ingestion composed of 3-tissue connected cultures. The metabolic model represents the central abdominal region and is composed of hepatocytes, endothelial cells and visceral adipose tissue. Closely paralleling *in vivo* nutrient balance, in normoglycemic media the model is able to maintain homeostatic glucose and lipid equilibrium. Moreover, it expresses systemic inflammation as well as endothelial specific stress in the presence of hyperglycemic and hypoinsulinemic conditions. A similar model of nanomaterial ingestion, developed within the "InLiveTox" project, is composed of an epithelial barrier and downstream endothelial cells and hepatocytes. Exposure of the epithelial barrier causes downstream inflammation and stress even in the absence of nanoparticles, indicating that the model can be used to study multiple pathways and systemic responses to metabolites or drugs.
SESSION 3:
EASs AND KINETICS
The expertise of Dr. Testai is focused on mammalian toxicology, toxicokinetics and risk assessment. She has been involved not only in research but also in regulatory activities in the area of risk assessment for human health associated to exposure to natural and synthetic chemicals with different field of application, as outlined below.

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**THE ROLE OF BIOKINETICS IN IN VITRO TESTS AND IN THE INTERPRETATION OF RESULTS**

When developing testing strategies, kinetics is considered the crucial body of information for the design and performance of toxicological tests and for toxicity data interpretation. Indeed, following exposure to a chemical, its uptake, bioavailability and biotransformation determine the actual *in vivo* target dose which is one of the most relevant parameter in quantitative risk assessment. A typical example is the oral pharmacokinetics for bisphenol A (BPA), showing <1% of total BPA in the unconjugated and biologically active form in serum from experimental animals and humans at peak levels, due to a relevant pre-systemic detoxication, when compared with the higher values attained after parenteral administration. Despite the above consideration is even more relevant for alternative/non animal testing strategy, *in vitro* biokinetics is generally neglected: it is a matter of fact that the nominal applied concentration is generally associated to observed effects, rather than the actual level of cell exposure. This causes a high level of uncertainty in the *in vitro* concentration-effect relationship, it has been considered one of the major causes for *in vitro/in vivo* differences and make it difficult to translate an *in vitro* concentration into an *in vivo* target dose (*in vitro–in vivo* extrapolation). The actual intracellular concentration may be affected both by abiotic processes (i.e. interactions with medium/plate, chemical instability) or by interaction with cells (i.e. transport across the membranes, biotransformation to reactive/inactive metabolites, bioaccumulation) altering *in vitro* bioavailability after acute and even more after repeated treatments. Physiologically based toxicokinetic (PBTK) models can be developed for the integration of dynamic and kinetic data produced from *in vitro* methods into a biologically meaningful framework for the extrapolation to *in vivo* conditions. This approach could make possible to derive a NOEC in *in vitro* experimental models from which extrapolate the corresponding *in vivo* dose. Examples will be discussed, evidencing the impact of kinetic behaviour on the effects of chemicals including endocrine active substances.

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Frédéric Y. Bois is an internationally known expert in quantitative toxicology. He was trained and worked at Harvard University, UCSF, UC Berkeley, the Lawrence Berkeley Laboratory, INSERM and INERIS. He is currently Professor at the Compiègne Technology University and Research Director at the Institut National de l’Environnement Industriel et des Risques (INERIS). He has coordinated and participated to international research projects in the areas of statistics and mathematical modelling for pharmacology, toxicology, epidemiology, and health risk assessment (funders: US-FDA, US-NIH, US-EPA, US-OSHA, European Commission, INSERM, French Ministry of Research). Recipient of the American Statistical Association "Outstanding Statistical Application Award" and of the French Epidaure Prize for Environmental Health Research.

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2009-: Professor, Chair of Mathematical Modelling for Systems Toxicology, UTC and INERIS, France.
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1987-90: Post-Doctoral Researcher Biologist, UC San Francisco and UC Berkeley, USA.
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SCIENTIFIC COMMUNICATIONS
Cheng S., Bois F., Environmental Health Perspectives, in press.

PHYSIOLOGICALLY-BASED MODELING OF OVARIAN STEROID HORMONES SYNTHESIS FOR ENDOCRINE ACTIVE SUBSTANCES' HEALTH RISK ASSESSMENT
Frédéric Y. Bois and Nadia Quignot

A finely tuned balance between estrogens and androgens controls reproductive functions, and the last step of steroidogenesis plays a key role in maintaining that balance. Environmental toxicants are a serious health concern, and numerous studies have been devoted to studying the effects of endocrine active substances (EASs). The effects of EASs on steroidogenic enzymes may influence steroid output and thus lead to reproductive toxicity. We first review the most significant quantitative modeling efforts aimed at predicting the EASs effects. Those examples clearly show that a tight coupling of systems toxicology with quantitative in vitro to in vivo extrapolation through physiological pharmacokinetic modeling is required for improving predictive capacity. Our most recent work is an illustration of that approach: to predict hormonal balance disruption on the basis of data on aromatase activity and mRNA levels modulation obtained in vitro on granulosa cells, we developed a mathematical model for the last gonadal steps of the sex steroids synthesis pathway. The model can simulate the ovarian synthesis and secretion of estrone, estradiol, androstenedione, and testosterone, and their response to endocrine disruption. The model is able to predict ovarian sex steroid concentrations under normal estrous cycle in female rat, and the ovarian estradiol concentrations in adult female rats exposed for 6 hours to the parent compound and the metabolites of atrazine, bisphenol A, methoxychlor, and vinclozolin. Results are presented and discussed in the framework of the next generation of risk assessment for EASs.
EAS’S CONTRA HUMAN & ENVIRONMENTAL HEALTH: RELEVANT OR PLAYGROUND FOR MERCHANTS OF DOOM?

Endocrine Active Substances (EAS) have been made responsible for increased incidences of nearly all human diseases imaginable and especially for the demise of populations of certain species. Moreover, unfounded suggestions such as the “low-dose hypothesis” or “no-threshold hypothesis” for adverse effects have provided for a media and research hype that created an atmosphere of biased and emotional science that has all the ingredients that current “merchants of doom” in science need to thrive without having to prove any of the hypotheses or suggestions put forth. The latter is exactly contrary to what is needed, namely a through assessment of facts in a bigger context, a factual and critical assessment of what mechanistic toxicology, hazard characterization and risk assessment can achieve to properly estimate the dangers of EAS. Moreover, this assessment needs to be compared to available epidemiological data for each of the diseases within the context of verified EAS exposure to ensure that predictions from in vitro and in vivo mechanistic assessments are realistic and target the population(s) at highest risk. While the environmental impact of EAS appears to restricted to locations of highest contamination, e.g. sewage treatment plant effluents and thus can be remediated by technical improvement of sewage treatment, more generalized adverse effects of EAS in wildlife has not been convincingly documented to allow causal relationships between presumed exposure and population effects. Similarly, although in vitro assays would predict a potential for EAS to interact with human endocrine receptors and associated signal transduction pathways and some in vivo experiments purport the existence of transgenerational effects at low doses of EAS, or effects on development of endocrine organs, fertility or fecundity at high doses of EAS in surrogate animals, so far very little of the latter evidence has been found to withstand scrutiny with regard to scientific quality. On the contrary, real-life exposures with EAS, e.g. BPA in humans specifically dosed demonstrate that absence of biologically available and thus active form of BPA and therefore the absence of potential endocrine activity. Corroborating the latter findings, an extremely thorough epidemiological study from Denmark annihilated the association of inhibited sperm counts in Danish men with exposure to EAS; despite that the latter was purported to be the case even in top journals for nearly two decades. These examples demonstrate 2 main issues: 1) only a rigorous scientific approach will provide analytical results that allow any predictions of risk in humans; 2) most epidemiological studies provide for wrong associations of exposure to EAS and adverse effects primarily due to faulty design, too small cohorts and especially due to biased, politically motivated or financially driven approaches at the outset.