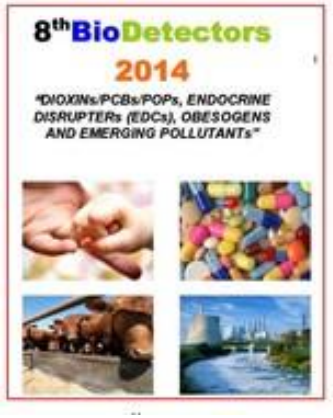


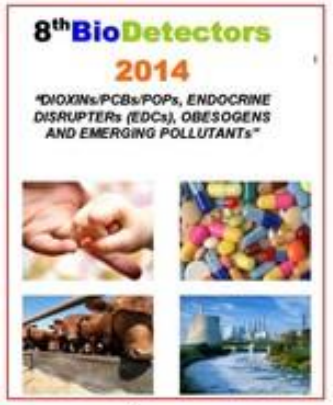
EDCs Alternatives & Bioassays

Functional cell-based bioassays to screen EDCs: from the substitution principle to LIFE-EDESIA

Stefano Lorenzetti

stefano.lorenzetti@iss.it

Dept. of Food Safety and Veterinary Public Health
Istituto Superiore di Sanità – ISS



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Critical aspects: MECHANISM vs MODE of action

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"Endocrine Active Substances / EASs"

"a substance having the inherent ability to interact or interfere with one or more components of the endocrine system resulting in a biological effect, but need not necessarily cause adverse effects."

EFSA Journal 2013;11(3):3132

"Endocrine Disruptors / EDs"

"An endocrine disrupter is 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."

WHO/IPCS 2002

In other words, "Endocrine Disruptors/EDs are Endocrine Active Substances/EASs causing adverse effects mediated by endocrine mechanisms"

Rovida, De Angelis, Lorenzetti. ALTEX 30, 2/13

"Endocrine activity" as a set of Modes of Actions (MoA)

….

endocrine activity as a collection of modes of action, potentially leading to adverse outcomes, rather than a (eco)toxicological hazard in itself.

EFSA Journal 2013;11(3):3132

Assessment of adversity is not unique to endocrine related effects. Scientific criteria for assessment of adversity have not been generally defined. In general, but not always, transient, inconsistent and minor fluctuations at the biochemical and molecular level may be considered adaptive, i.e. non-adverse.

Changes at the cell-, organ-, organism-, or (sub)population-level resulting in pathology or functional impairment *in vivo*, as well as altered timing of development, may be considered adverse. It is therefore difficult to propose ED-specific criteria for adversity and expert judgement in a weight-of-evidence approach is needed to assess substances for possible endocrine disrupting properties. EFSA Journal 2013;11(3):313

"Endocrine activity" *vs. Nuclear Receptor / NR activation:*

Searching for an adverse effect

In summary, **currently available definitions of "endocrine disrupter"** are **either** neutral in terms of specifying the toxicological relevance of the effects to be described, **or** they introduce the idea of adversity.

The former is in danger of being insufficiently discriminatory, **the latter** shifts the problem to defining **what adversity should mean in an endocrine context**, which could be too restrictive and not inclusive enough.

At the core of this dilemma is the fact that **"endocrine disruption" cannot presently be anchored to specific assay outcomes in a straightforward way.**

STATE OF THE ART ASSESSMENT OF ENDOCRINE DISRUPTERS
ec.europa.eu/environment/endocrine/.../summary_state_science.pdf

OECD conceptual framework (2002)

level 2: *in vitro* assays providing mechanistic data

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

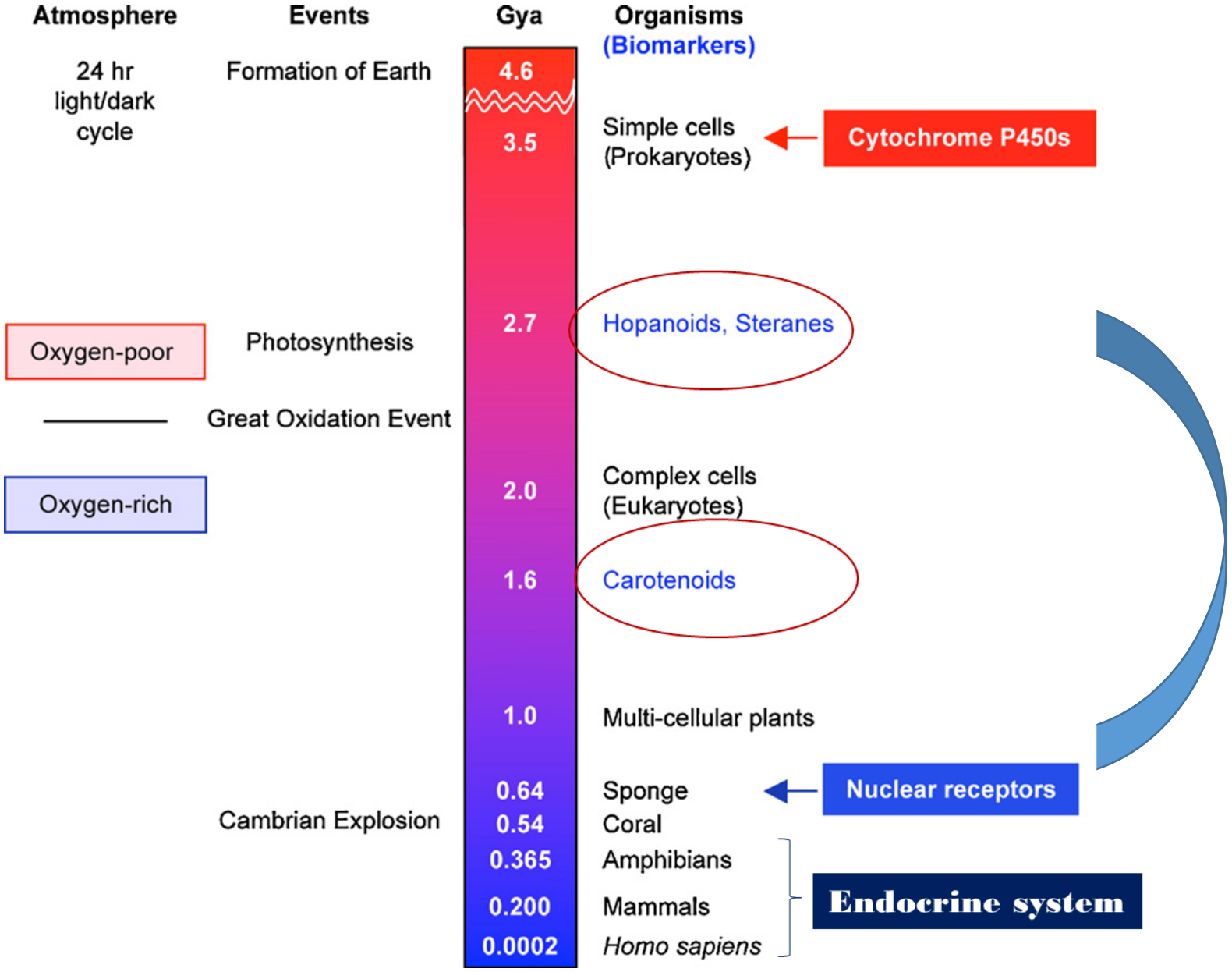
Level 1 Sorting & prioritization based upon existing information	•Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	•ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis *in vitro* •Aryl hydrocarbon receptor recognition/binding	•High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	•Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function	•Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
Level 4 *In vivo* assays providing data about multiple endocrine mechanisms and effects	•Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assays •Adult intact male assay	•Fish gonadal histopathology assay •Frog metamorphosis assay
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	•1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced)	•Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

ER, AR, TR, AhR binding affinity...

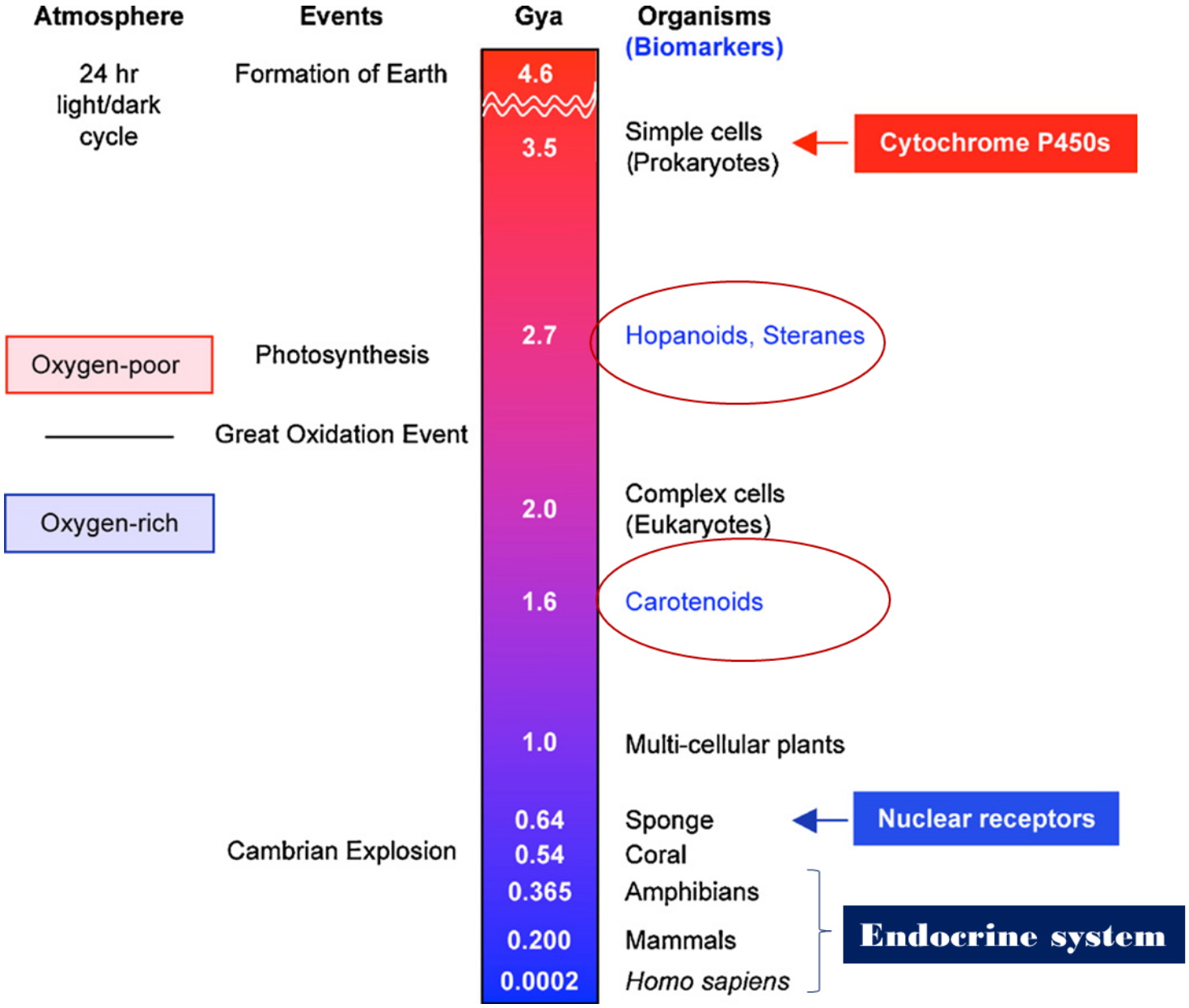
... and the endocrine activity of the other NRs (48 in humans) ?

other mechanisms of action ?

OECD conceptual framework (2002)



NR: an evolutionary perspectives



NUCLEAR RECEPTORS: WHAT THEY ARE?

DUAL FUNCTION PROTEINS

- **RECEPTORS** (not bound to the the plasma membrane)
- **TRANSCRIPTION FACTORS** (one the most abundant class in animals/metazoans)

✓ They regulate diverse functions, such as homeostasis, reproduction, development and metabolism (for a review, see Laudet and Gronemeyer, 2002).

✓ Nuclear (hormone) receptors / NRs function as **ligand-activated transcription factors**, and thus provide a direct link between signaling molecules that control these processes and transcriptional responses.

✓ A large number of nuclear receptors have been identified through sequence similarity to known receptors, but have **no identified natural ligands**, and are referred to as '**nuclear orphan receptors**'.

✓ As NRs bind small molecules that can easily be modified by **drug design**, and control functions associated with major diseases (e.g. cancer, osteoporosis and diabetes), they are promising **pharmacological targets**.

✓ The search for ligands for orphan receptors and the identification of novel signaling pathways has become a very active research field (Gustafsson, 1999; Kliewer et al., 1999).

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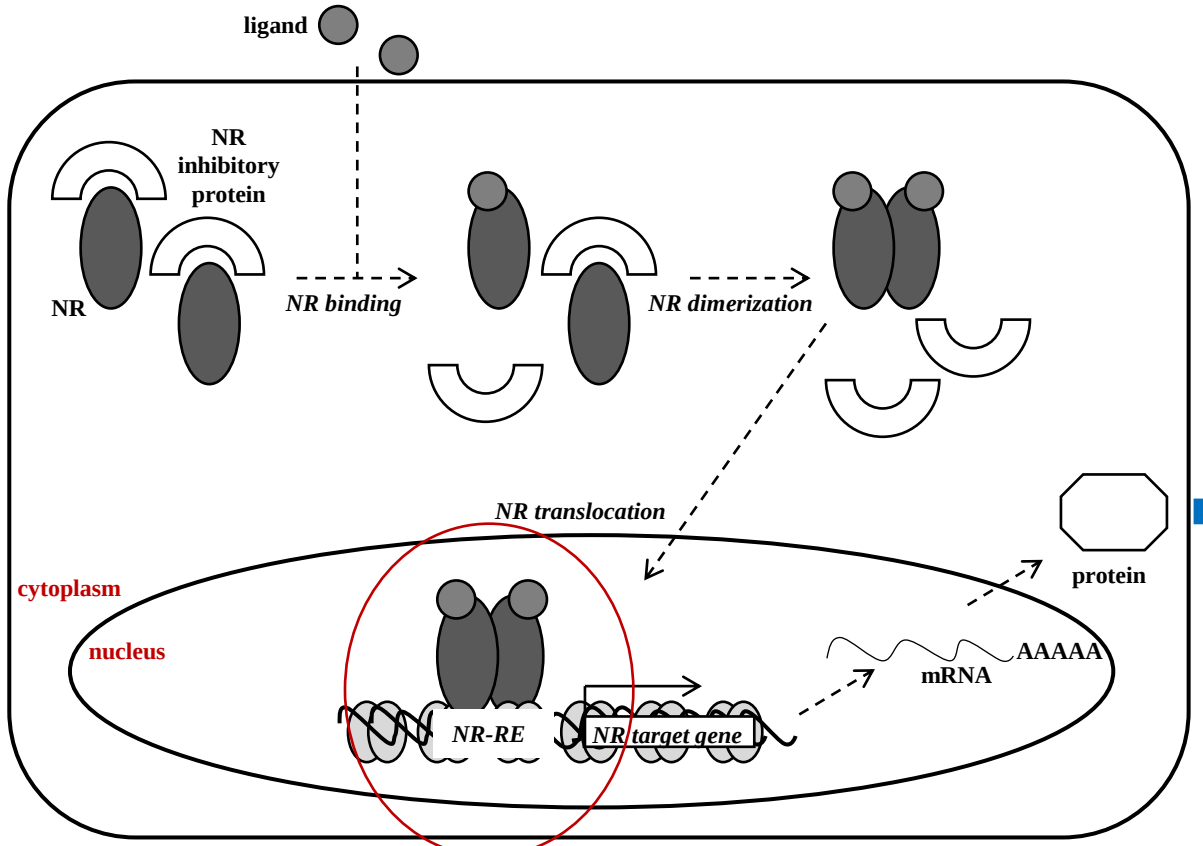
A TYPICAL SCHEME OF NR-MEDIATED SIGNALLING



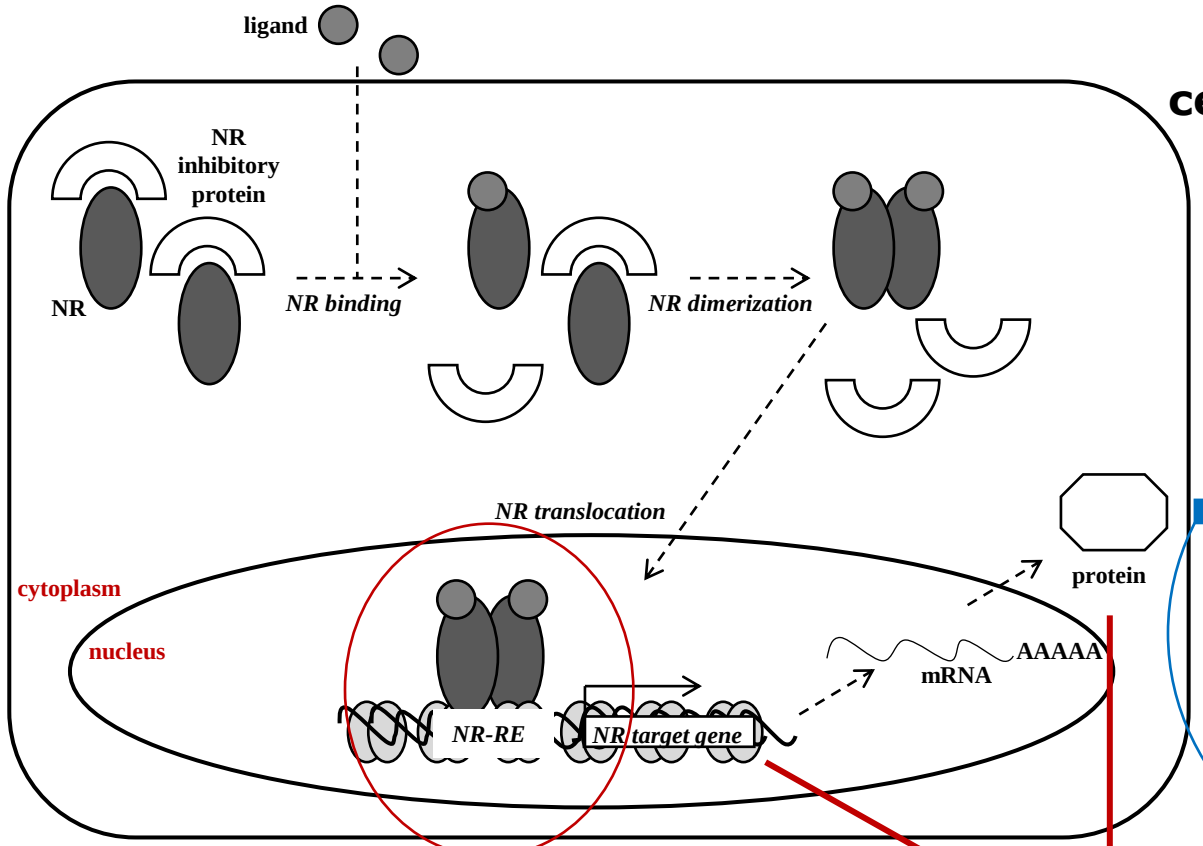
changed
cell function

transcriptional activation

Adapted from Lorenzetti and Narciso, 2012
DOI: 10.1039/9781849735353

A TYPICAL SCHEME OF NR-MEDIATED SIGNALLING

Which biomarkers are searched for ED-like activity ?



functional markers
cell-specific, clinically relevant endpoints

changed cell function

Part of a Mode of Action

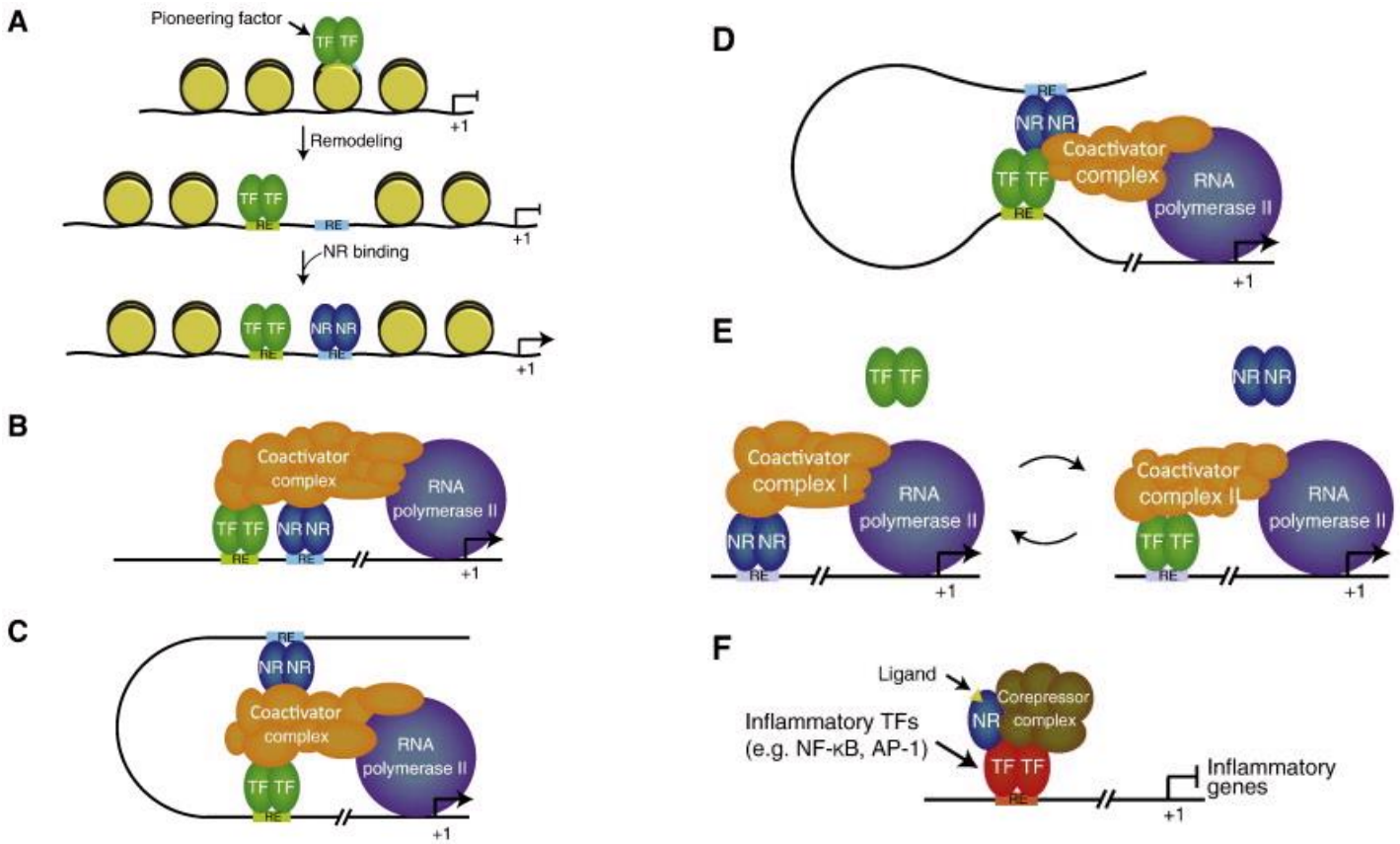
transcriptional activation

Part of a Mechanism of Action (incl. toxicogenomics)

molecular markers:
mRNA, miRNA, proteins, metabolites

Adapted from Lorenzetti and Narciso, 2012
DOI: 10.1039/9781849735353

NRs do not transactivate alone



- **Ligand-NR interaction is as important as the presence of dozens of transcriptional coregulators at the DNA binding site within the promoter of NR-target genes.**

  Indeed, the NR-dependent trascriptional activation (or repression) of NR-target genes depends on specific co-activators (or co-repressors) present within the nucleus of a specific cell in a well determined very short-time period.

NRs do not transactivate alone

- **Transactivation or gene reporter assays do not characterize an effect as adverse or not, but just indicate indirectly a ligand binding to a NR and, in turn, its activation in that particular cellular environment and on a specific promoter gene!**

- **Transactivation assay or, better, any gene expression profiling are indicative of a MECHANISM of Action and should be associated to a functional assay to search for a «realistic» MODE of action.**

- **Molecular interactions and/or gene expression modulation are not *per se* toxicological endpoints unless…**

Assessment of adversity is not unique to endocrine related effects. Scientific criteria for assessment of adversity have not been generally defined. In general, but not always, transient, inconsistent and minor fluctuations at the biochemical and molecular level may be considered adaptive, i.e. non-adverse.

Changes at the cell-, organ-, organism-, or (sub)population-level resulting in pathology or functional impairment *in vivo*, as well as altered timing of development, may be considered adverse. It is therefore difficult to propose ED-specific criteria for adversity and expert judgement in a weight-of-evidence approach is needed to assess substances for possible endocrine disrupting properties. EFSA Journal 2013;11(3):313

Gene expression (cDNA microarray) vs Functional biomarkers

cDNA microarray

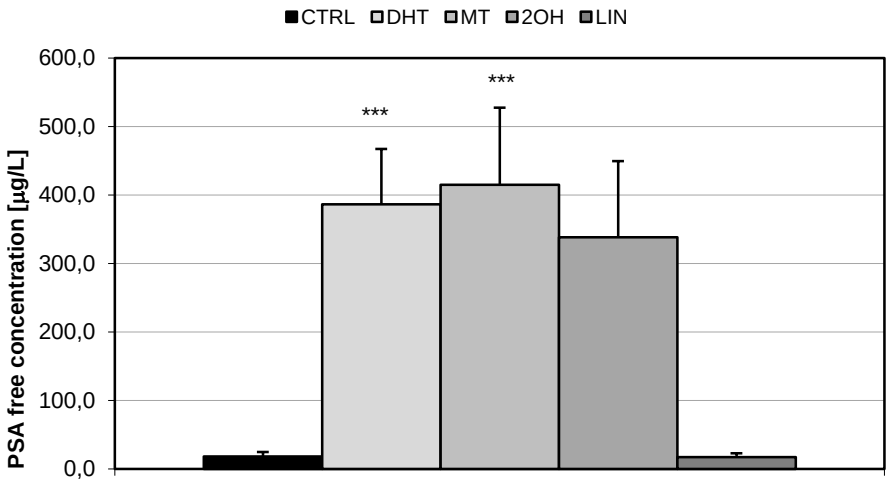
LNCaP, human epithelial cell line, treated with androgen receptor /AR agonist/antagonist: number of modulated genes

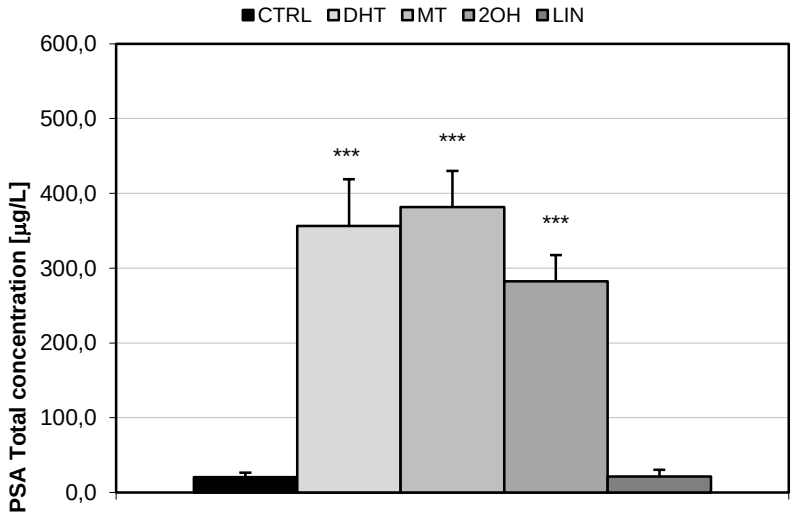
	Modulated genes	UP-regulated genes	DOWN-regulated genes
DHT	2369	1390	979
MT	2600	1442	1158
2OH-FTA	3995	2069	1926
LIN	304	139	165

Phenotypic anchoring

PSA secretion assay

(free PSA, upper panel; total PSA, bottom panel)





Lorenzetti *et al.*, *manuscript in preparation*

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Endocrine Disruptors *in silico / in vitro*
Evaluation and Substitution for Industrial Applications
LIFE12 ENV / IT / 000633

LIFE-EDESIA overview

www.iss.it/life

MAIN OBJECTIVES

- **to apply the substitution principle to Endocrine Disruptor Compounds (EDCs) of «equivalent concern », on the basis of**
  - **i) endocrine disruption effects,**
  - **ii) high production volume,**
  - **iii) widespread use and**
  - **iv) potential exposure of general population**

  as Substances of Very High Concern (SVHC), namely: phthalates, bisphenols and parabens;

- **to demonstrate a new, robust and cost-effective *in silico/in vitro* approach to evaluate suitable chemicals for replacing EDCs of equivalent concern, that can support the application of REACH legislative framework on the substitution principle environment;**

- **to demonstrate the feasibility of the substitution of EDCs considered in the project in industrial applications.**

WHERE WE MOVE FROM…

All substances including endocrine disruptors (EDCs) are subject to registration under REACH (Regulation (EC) No 1907/2006) when they are manufactured or imported into the EU in amounts of, or above, 1 tonnes *per* year. **However, substances with endocrine disrupting properties are subject to the authorisation procedure under REACH only if they are included in Annex XIV as Substances of Very High Concern (SVHC).**

Anyway, the test programme does not include specific tests for endocrine disrupting properties because there are no internationally agreed methodologies or criteria available for endocrine disrupting properties (ECHA Guidance for SVHC).

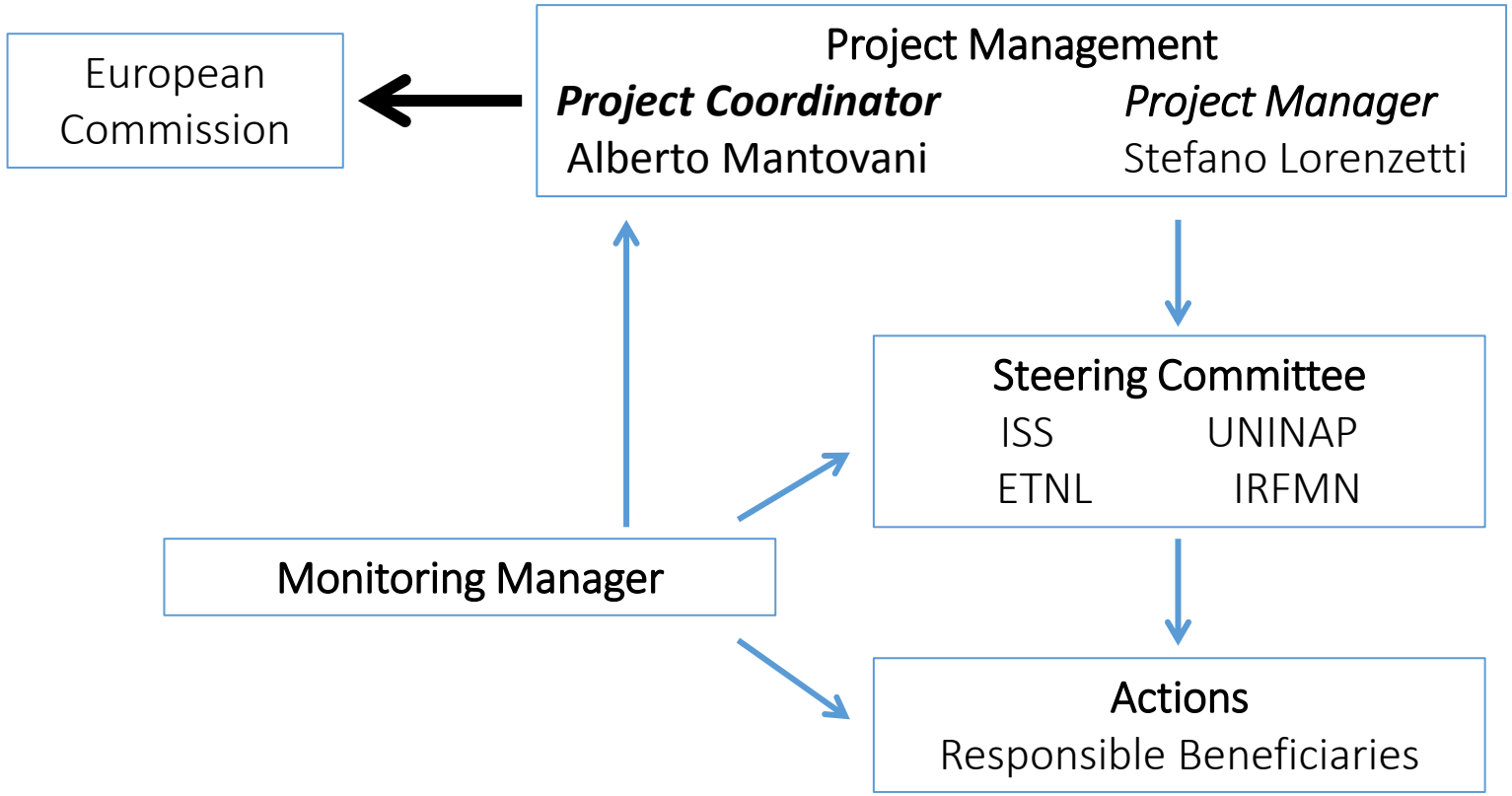
Substances with endocrine disrupting properties are considered as SVHC only on a case-by-case basis and only if scientific evidence and a weight of evidence approach indicate that they are of "equivalent concern" to CMR, PBT or vPvB substances ***(see definitions within REACH).***

Indeed, EU already adopted a Community Strategy for Endocrine Disrupters that contained, among the short-term actions, the establishment of a priority list of substances http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm for further evaluation of their role in endocrine disruption.

"SECONDARY" OBJECTIVES

- **-to identify potential substitutive chemicals, both on the basis of the state of the art and using available *in silico* approaches to identify new potential candidates;**
- **to perform a comparative assessment of the different potential substitutive chemicals using the *in silico* approach;**
- **to synthesize the selected substitutive chemicals;**
- **to validate the *in silico* results by a comparative assessment using *in vitro* methods identified previously;**
- **to create prototypes that use the substitutive chemicals, and to assess them for release of chemicals;**
- **to launch a demonstration and dissemination plan involving other projects (Life+, 7th FP) industry, regulators and consumer's organizations, so to present the project's approaches and outcomes as a support to the science-based implementation of the substitution principle.**

THE PROJECT SCAFFOLD



THE PROJECT PARTNERSHIP

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LIFE-EDESIA in ACTION(S) – 1 (www.iss.it/life)

A. Preparatory actions (if needed)
A1 Identification of potential substitutive chemicals in the literature

B. Implementation actions
B1 Identification of potential substitutive chemicals using *in silico* tools
B2 *In silico* validations
B3 Synthesis of the chemicals
B4 *In vitro* validation
B5 Prototyping and testing for industrial purposes in 3 application domains

C. Monitoring of the impact of the project actions (obligatory)
C1 Monitoring of the project impact

D. Communication and dissemination actions (obligatory)
D1 Communication and dissemination initiatives
D2 Interviews of stakeholders
D3 Web Portal
D4 Brochures, newsletters, layman's report, notice boards
D5 Seminars and workshops
D6 After LIFE communication plan

E. Project management and monitoring of the project progress (obligatory)
E1 Project management
E2 Project monitoring

THE CORE OF ACTION B4 AT A GLANCE

Cell aspecific endpoint:
Cell Viability
(MTS ASSAY)

Cell specific endpoint:
Functional Assay – Phenotypic anchoring

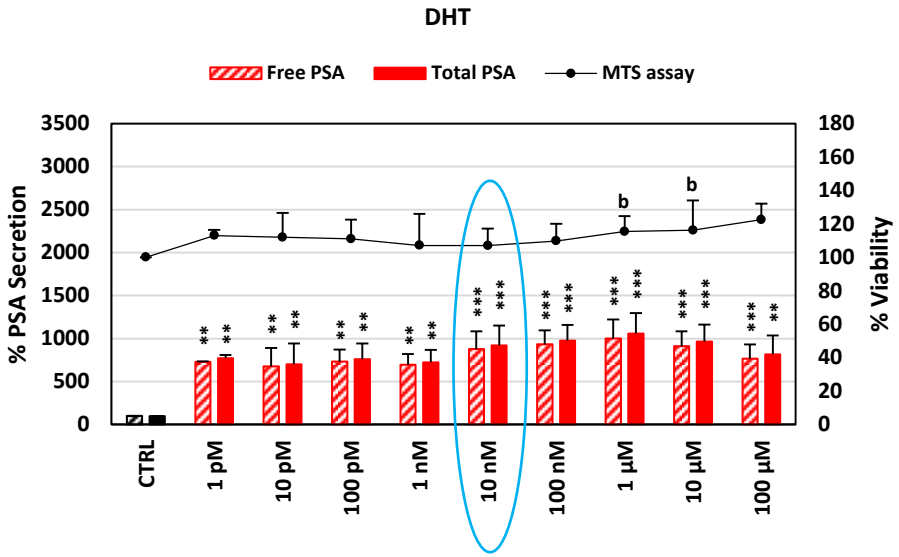
- Prostate (LNCaP): PSA secretion
- Trophoblast (BeWo): βhCG secretion
- Liver (HuH6): intracellular lipid accumulation and/or AFP secretion)

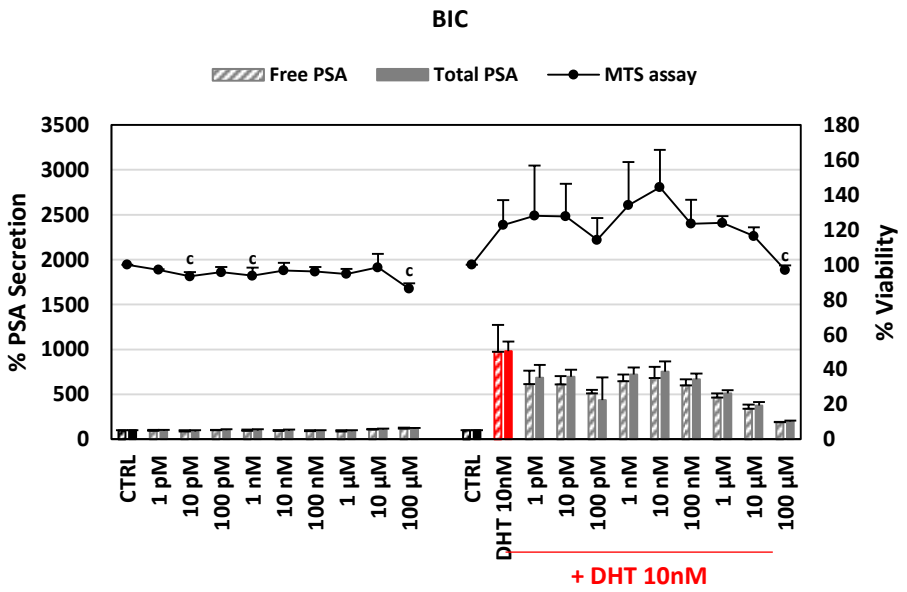
Molecular endpoint:
gene expression of Nuclear Receptors of interest
(qPCR)

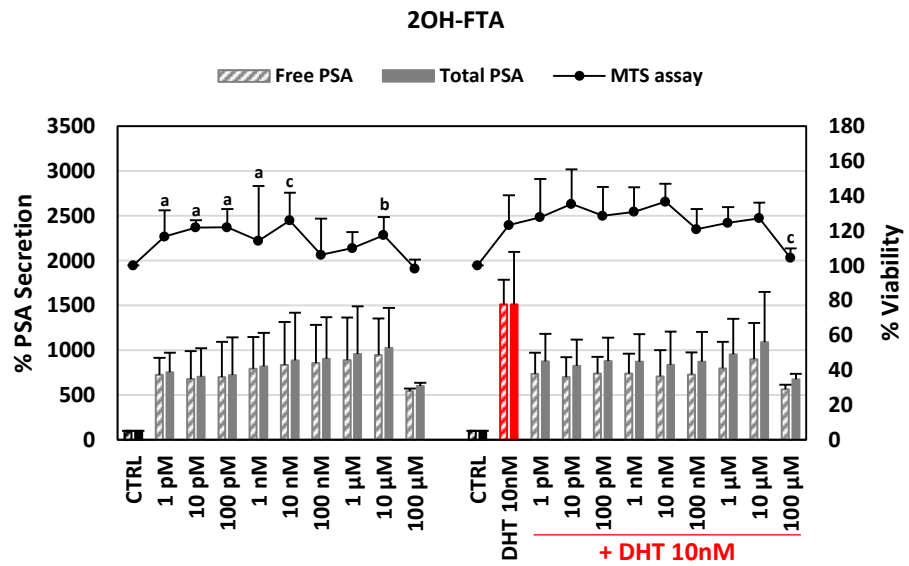
- **Lorenzetti *et al*., 2010. Reprod Toxicol, 30:25-35**
- **Kwiecińska *et al*., 2011. Pharmacol Rep, 63:1195-202 44**
- **Kawai *et al*., 2001. Hepatology, 33:676-691**
- **Mørck *et al*., 2010. Reprod Toxicol, 30:131-7**
- **Lorenzetti *et al*., 2011. Annals Ist Super Sanità, 47:429-**
- **Fujimura *et al*., 2009. J Appl Toxicol, 29:356-363**

Gene reporter assays
AR-, ER-, PPAR-gene reporter assays
(OECD and/or IHCP-JRC guidelines and/or protocols under the validation programme)

- Lorenzetti, Mantovani. 2014. Reproductive and Developmental Toxicity Testing: Issues for 3Rs Implementation, pp.330-47. In: Reducing, Refining and Replacing the Use of Animals in Toxicity Testing, Eds. D. Allen, MD Waters, RCS Publishing DOI:10.1039/9781849737920-00330
- http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/test-submission/tests-submitted-to-eurl-ecvam;
- http://www.oecd-ilibrary.org/environment/test-no-455-performance-based-test-guideline-for-stably-transfected-transactivation-in-vitro-assays-to-detect-estrogen-receptor-agonists_9789264185388-en.







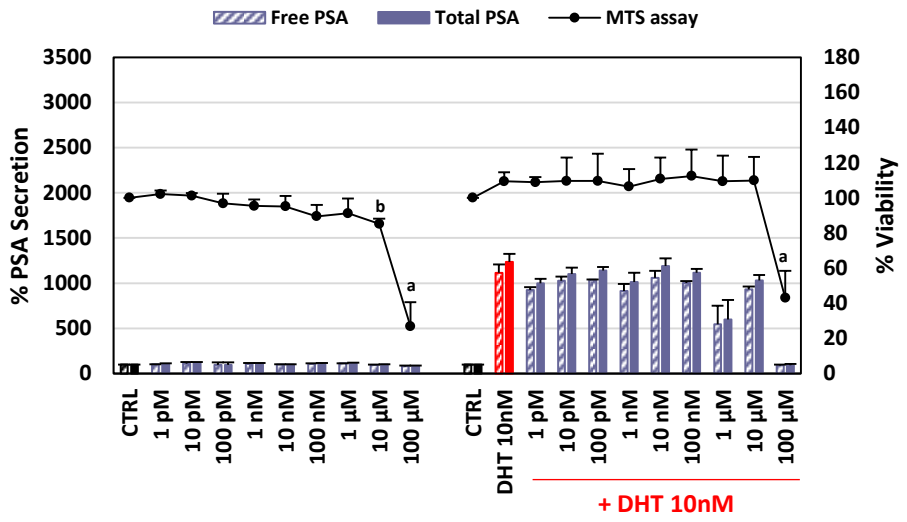
LNCaP: MTS + PSA secretion assay

72hrs treatment upon ON starvation

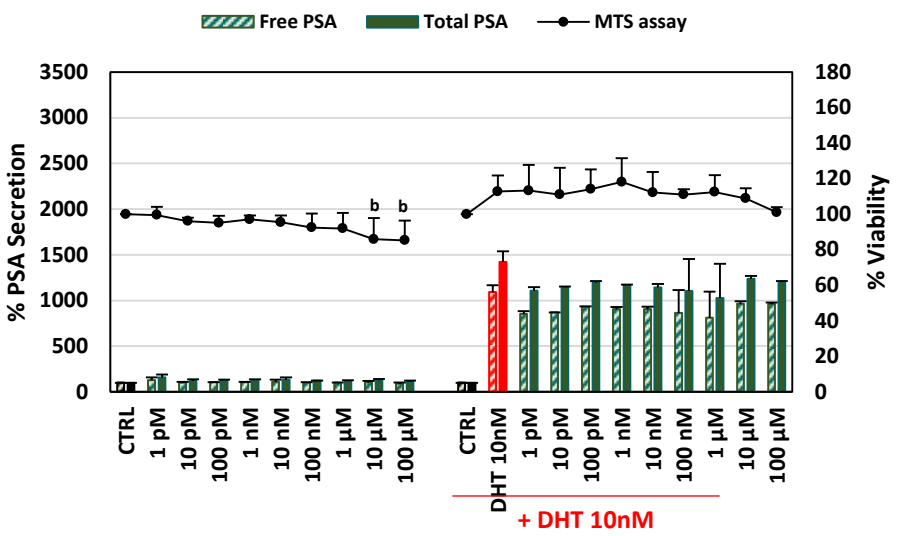
Lorenzetti *et al.* 2010, 2011
Marcoccia *et al.* 2014

LIFE-EDESIA project:
Action B4 – EXAMPLES: plasticizers

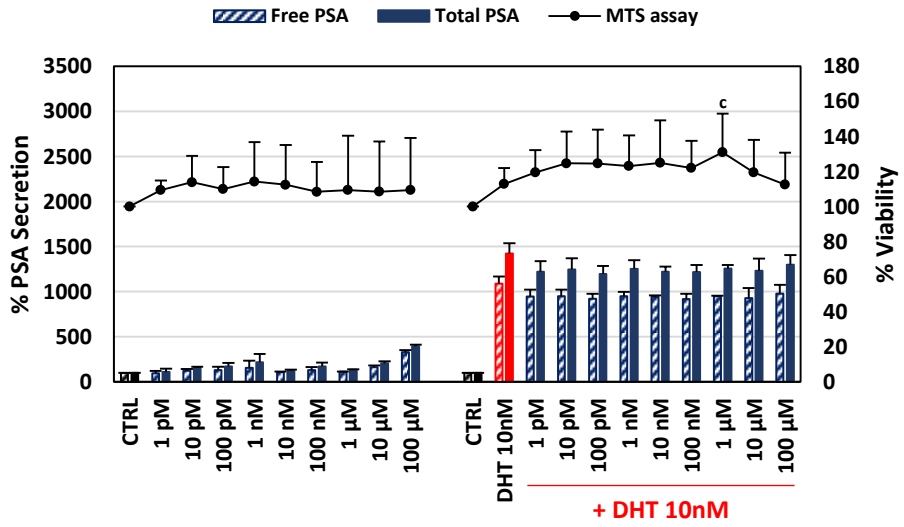
BPA



DEHP



DBP



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Marcoccia *et al.* 2014
unpublished data

LIFE-EDESIA project: ACKNOWLEDMENTS & CONTACTS

LIFE12 ENV/IT/000633

Daniele MARCOCCIA, Monica GIULIVO, Alberto MANTOVANI

http://www.iss.it/life

http://www.iss.it/life/index.php?lang=2

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Progetto Europeo LIFE-EDESIA

4.1 Beta :)

(IT) EN **Responsabile: Stefano Lorenzetti**

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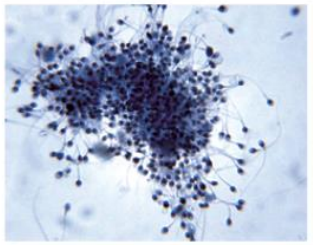

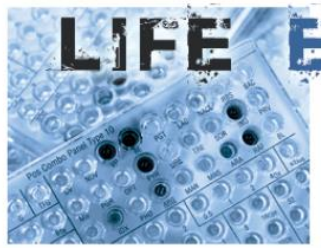

I contenuti più aggiornati

LIFE-EDESIA alla 8th BioDetectors Conference 2014

Il Dott. Lorenzetti esporrà le attività di LIFE- EDESIA con un intervento dal titolo: "Functional cell-based bioassays to screen EDCs from the substitution principle to LIFE-EDESIA".

8th BioDetectors Conference 2014
(25-26 Settembre 2014 - Torino)

Pubblicato il 24-07-2014 in Eventi , aggiornato al 24-07-2014 Leggi...

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