Alternative methods to animal testing: the LIFE-EDESIA *in silico-in vitro* approach to Endocrine Disruptors

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OUTLINE

- ✓ **Why alternatives to animal testing**
- ✓ **Endocrine Disruption and adverse effects**
- ✓ **Endocrine Disruptor (ED)-screening: mechanism-based *versus* effect-based**
- ✓ **The *in vitro* LIFE-EDESIA approach:**
  computational prioritization
  plus
  ***in vitro* testing by ED-dependent, biomarker-based, cell-specific bioassays**

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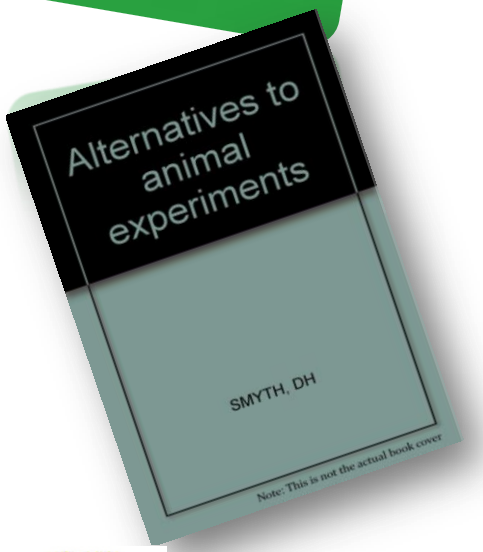
THE 3Rs PRINCIPLE or WHY ALTERNATIVES ?



Russell & Burch - *The Principles of Human Experimental Technique*
Methuen ed, London, 1959 - http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc

REPLACEMENT
Methods which avoid or replace the use of animals

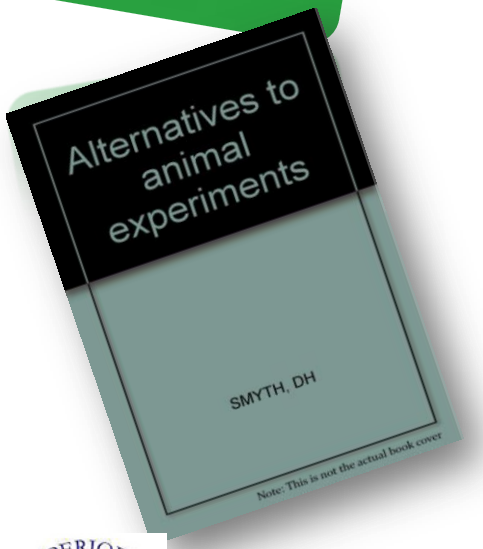
REDUCTION
Methods which minimise the number of animals used *per* experiment

REFINEMENT
Methods which minimise suffering and improve animal welfare

- ✓ **The principles of the 3Rs (Replacement, Reduction and Refinement) were developed over 50 years ago as a framework for human and animal research.**
- ✓ **They have subsequently become embedded in national and international legislation regulating the use of animals in scientific procedures.**

David Henry Smyth - *Alternatives to animal experiments*
Scolar Press [for] the Research Defence Society, 1978 -

WHY ALTERNATIVE METHODS TO ANIMAL TESTING ?



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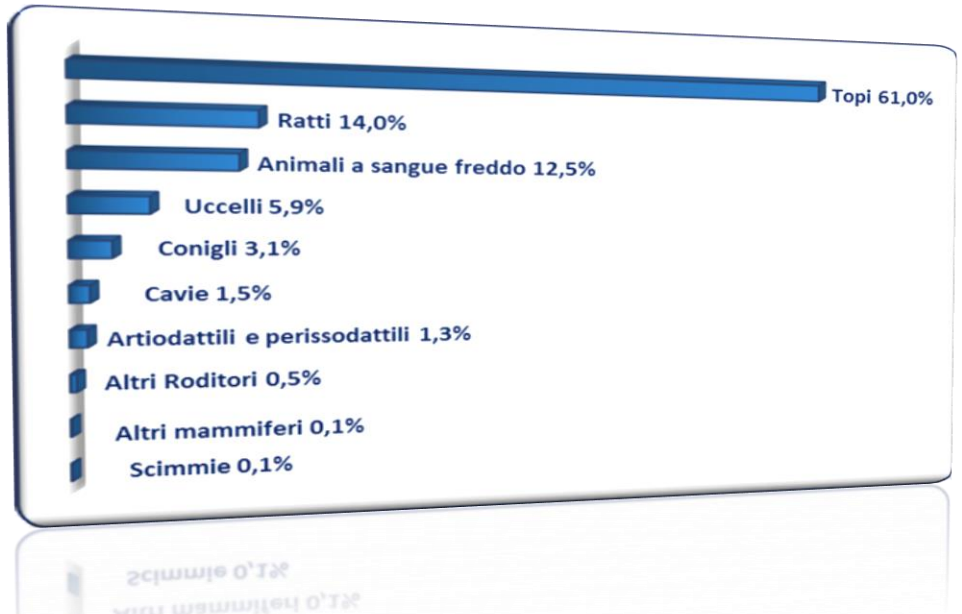
WHY ALTERNATIVE METHODS TO ANIMAL TESTING ?

- ✓ **Biological research (46,1%)**
- ✓ **Research and development in human and veterinary medicine (21,7%)**
- ✓ **Quality controls of pharmacological products (13,9%)**
- ✓ **Assessment of toxicological effects (8,8%)**
- ✓ **Education and training (1,6%)**



Rodents 75%

About 11,5 million animals used*

**** EU data 2013***

Courtesy of Isabella de Angelis (IPAM)

WHY ALTERNATIVE METHODS TO ANIMAL TESTING FOR ENDOCRINE DISRUPTION ?

➢ EU regulatory framework

The EU has introduced specific legislative obligations aimed at ***phasing out endocrine disruptors*** in **water** (*Water Framework Directive 2000/60/EC),* **industrial chemicals** (*REACH Regulation 2006/1907/EC, Food Contact Materials Regulation 2011/10/EU* and following amendments, …), **plant protection products** (*Plant Protection Products Regulation 2009/1107/EC*) and **biocides** (*Biocidal Products Regulation 2012/528/EU*).

- Importantly, EU regulations strongly recommended **the use of *in vitro* alternative** (to animal experimentation) **methods**, at least as a prioritizing screening approach to identify ***endocrine disrupting properties of Endocrine Active Substances*** (**EAS**).

➢ REACH Regulation

- In REACH, Endocrine Disrupting Chemicals (EDCs) are considered of **similar regulatory concern as *Substances of Very High Concern (SVHC)***.
- REACH also calls for the **progressive substitution *of*** the most dangerous chemicals (referred to as ***SVHC***) ***when suitable alternatives have been identified***.

WHY ALTERNATIVE METHODS TO ANIMAL TESTING FOR ENDOCRINE DISRUPTION ?

REACH Regulation
1907/2006/CE

case-by-case

ENDOCRINE DISRUPTORS
as SVHC - Art. 57f e ss.,
138.7; All. II – 12.6

equivalent concern to:
CMR, , PBT, vPvB
(Art. 57 a-e)

ANNEX XIV
Substances of Very High Concern - SVHC

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ANNEX XIV
Substances of Very High Concern - SVHC

- Endocrine Active Substance / EAS : "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 J. 2013; 11(3):3132
- Endocrine Disruptor / ED : "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 – Weybridge definition
- "EDs are EASs causing adverse effects mediated by endocrine mechanisms"
  Rovida C, De Angelis I, Lorenzetti S. ALTEX 30, 2/13

- ✓ …, 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.
- ✓ WHAT ADVERSITY SHOULD MEAN IN AN ENDOCRINE CONTEXT
- ✓ 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

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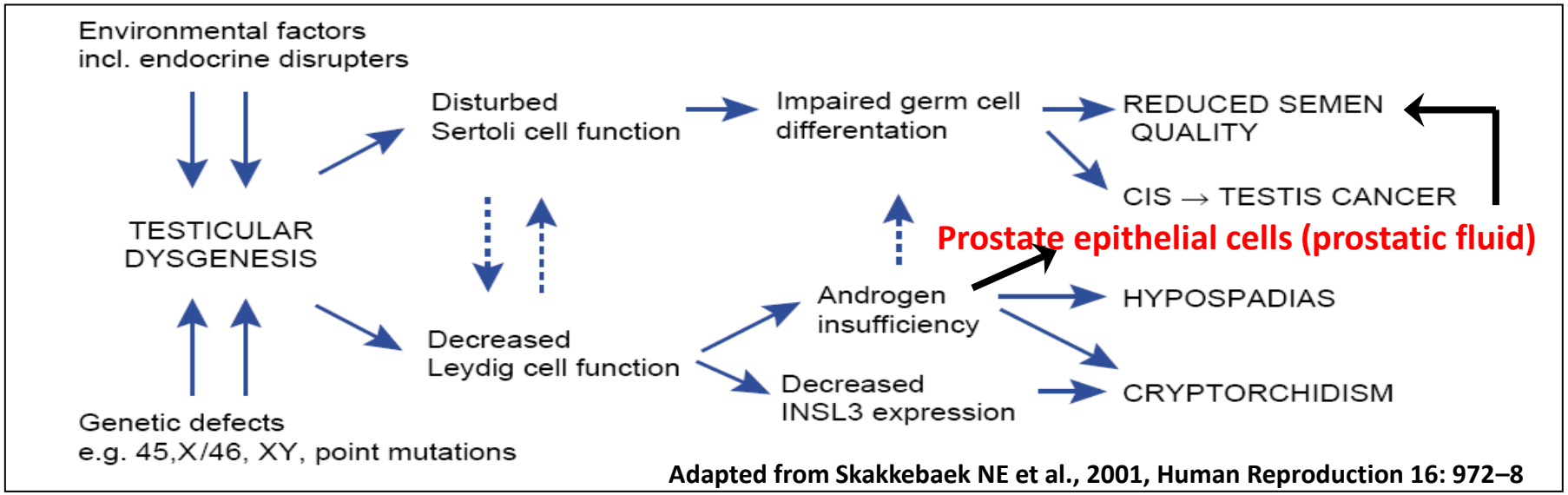
ED-related adverse health effects: TDS syndrome

➢ Testicular Dysgenesis Syndrome (TDS) in humans

exposure *in utero* to environmental factors (anti-androgenic compounds) in Western Europe and USA are responsible of male infertility and associated-diseases/malformations.

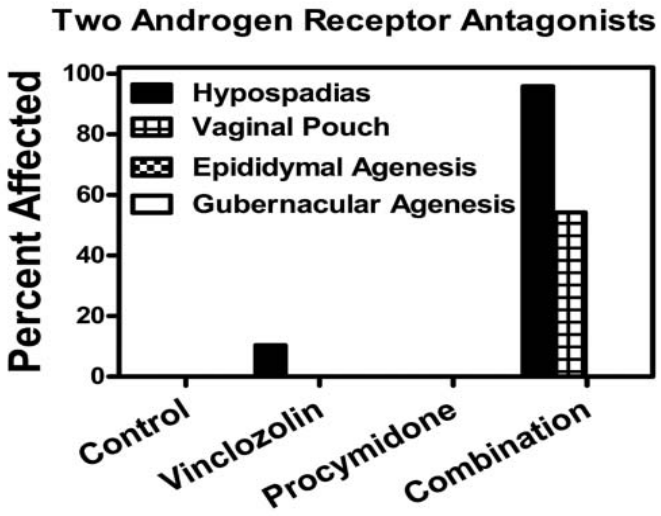


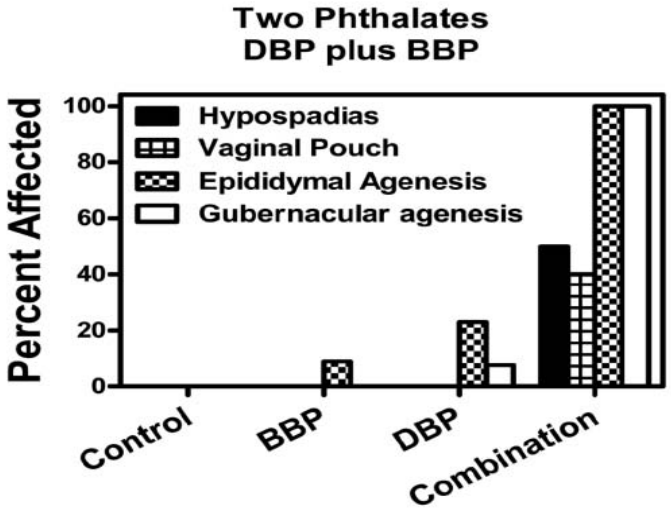
Adapted from Skakkebaek NE et al., 2001, Human Reproduction 16: 972–8

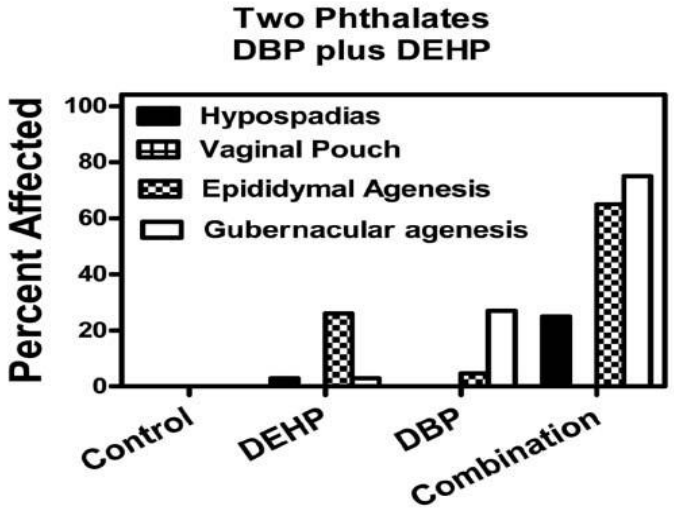
➢ or «phthalate syndrome» in experimental rodents

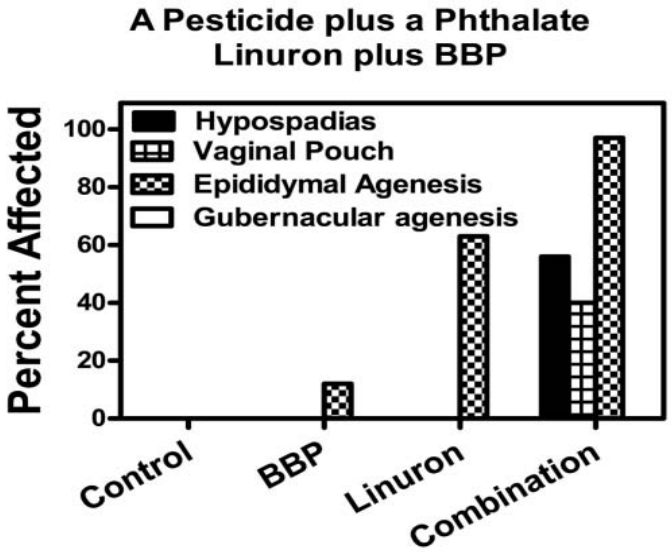
Fisher, Reproduction 2004
Sharpe and Skakkebaek, Fertil Steril. 2008
Martinez-Arguelles *et al*., JSBMB 2013

ED-related adverse health effects: what a mixture!









«Phthalate syndrome» (rodent TDS)
and pesticides:
the role of EDC mixtures

a cumulative, dose addictive effects
of different anti-androgens
(binary combinations)

Rider *et al*. Toxicol. Pathol. 2009

ED-related adverse health effects: obesity pandemics

➢ Obesogenic EDCs (including BPA and DEHP)
in experimental *in vivo* models and in humans (?)

Heindel *et al. Environmental Health* (2015) 14:54
DOI 10.1186/s12940-015-0042-7

ENVIRONMENTAL HEALTH

COMMENTARY Open Access

CrossMark

Parma consensus statement on metabolic disruptors

Heindel *et al.*, Env. Health 2015, and refs therein

Grün and Blumberg, Endocrinology 2006

Summary and conclusions

The Parma workshop helped to focus this emerging field by developing an overarching hypothesis for the role of environmental chemicals in the current worldwide epidemics of obesity, diabetes and related metabolic diseases. We hope that the consensus statements will aid in expanding understanding of the possible role of metabolic disruptors in these epidemics and have identified research needs in order to provide more relevant data on the role of environmental chemicals in these diseases. The objective is both to indicate the strength of the current data and to provide a roadmap for further studies. A coherent, enhanced research agenda will help identify strategies to prevent metabolic diseases through actions that can be taken by individuals as well as public health agencies. History shows that prevention is always the best strategy. Increased understanding of the importance of the metabolic disruptor hypothesis to the epidemics of obesity and metabolic syndrome offers the potential for these diseases to be mitigated by modifying exposures, thereby creating a healthier environment for future generations.

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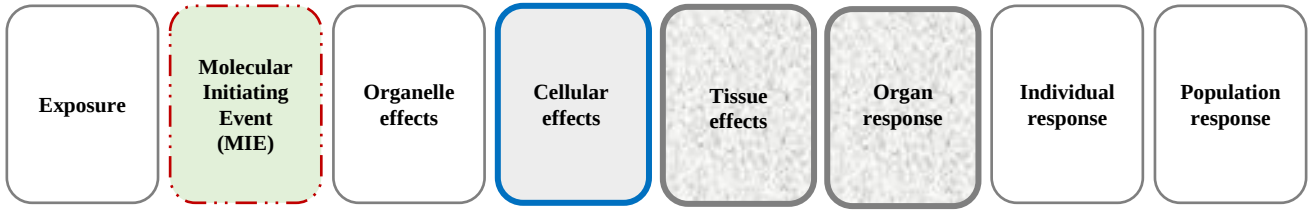
CURRENT «ALTERNATIVES» FOR ENDOCRINE DISRUPTION : mechanism-based approaches

Adverse Outcome Pathway (AOP)



… *in vitro* screening:
so far, mostly applying at GENE REPORTER ASSAYS

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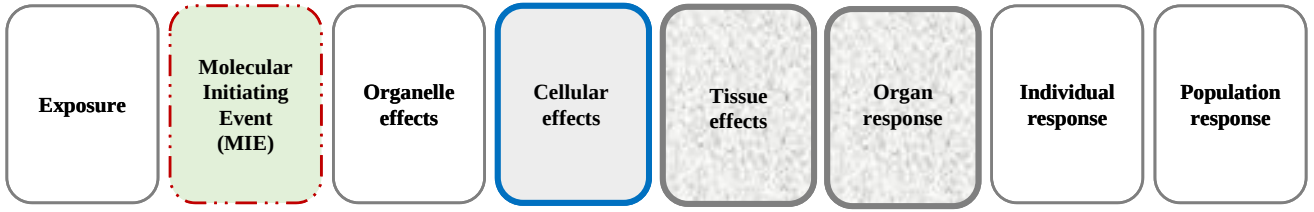

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In vitro Nuclear Receptor binding & regulation of gene transcription (*gene reporter assays*)
IS SUFFICIENT TO DEFINE…

- AN ENDOCRINE ACTIVITY ?

NO, if an endocrine activity is a Mode-of-Action
WHO/IPCS 2002 – Weybridge definition
STATE OF THE ART ASSESSMENT OF ENDOCRINE DISRUPTERS (EC)

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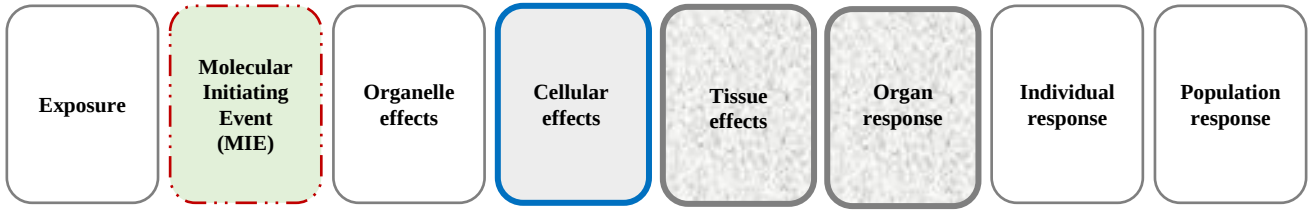

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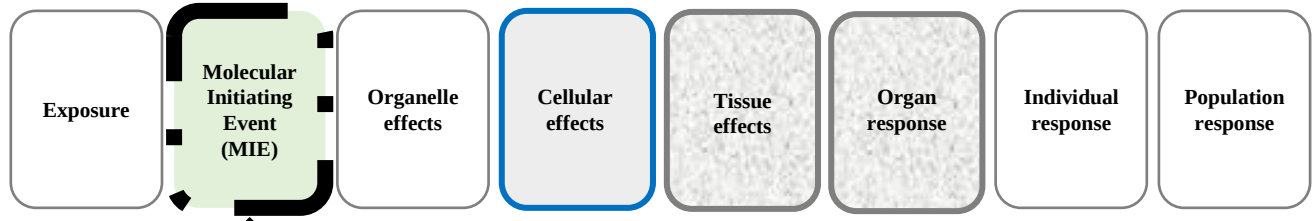
- AN ADVERSE EFFECT ?

NO, because a binding to a Nuclear Receptor (or its transcriptional regulation) does not define any cellular output(s) in terms of ADVERSITY

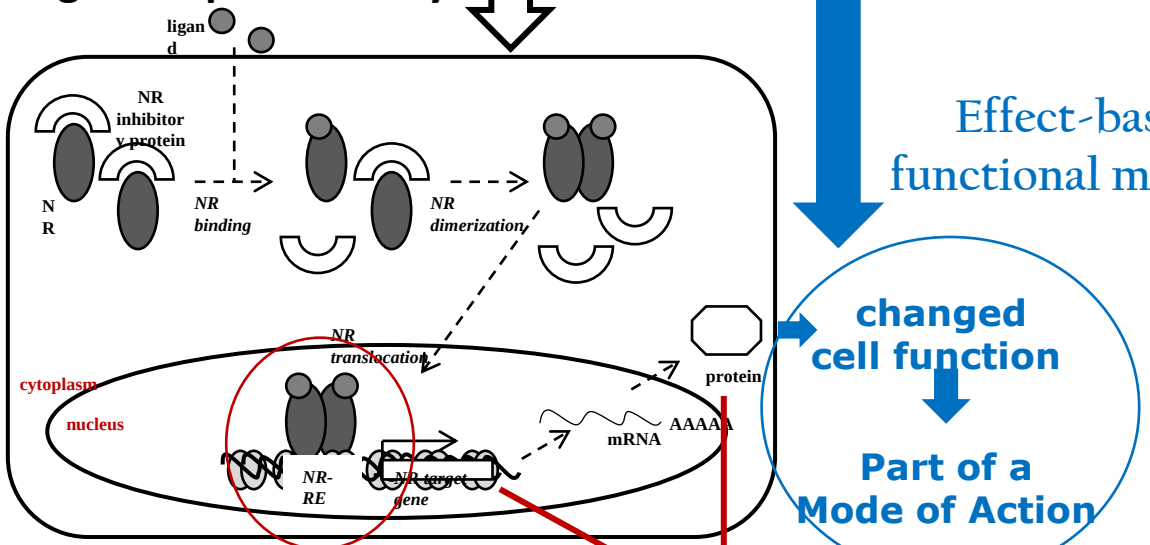
CURRENT «ALTERNATIVES» FOR ENDOCRINE DISRUPTION : an example of a mechanism-based mistake: the case of the anti-androgenic phthalate DEHP

In vitro screening of EDs by any Androgen Receptor (AR)-gene reporter...

Adverse Outcome Pathway (AOP)



AR-gene reporter assay



Effect-based functional markers

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

... it will detect a lack of binding to AR (no activation of AR-mediated gene transcription)
BUT it will never detect its already known Mode-of Action: anti-androgenicity !

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

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The overall LIFE-EDESIA approach - 1. computational prioritization

Compilation of 3 different lists of already existing alternatives of the LIFE-EDESIA compounds of interest

(*phthalates/DEHP, bispheols/BPA, parabens/methyl paraben*)

CHEMICO-PHYSICAL PROPERTIES

(*e.g.,* solubility by the ***ACD/Solubility DB*** and lipophilicity by the octanol-water partition coefficient ***LogP*** and by the apparent partition coefficient D for dissociative systems ***Log D***) assessed on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life* (***data available on request***)

TOX PROPERTIES

(*e.g., cancerogenic, mutagenic, binding to nuclear receptors*) assessed by tools implemented in the VEGA platform on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life* (***data available on request***)

QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIP (QSAR)

performed on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life*, *versus* selected NRs, namely AR and ERa, using i) a CART model also implemented in the VEGA platform, ii) SARpy model developed on the basis of the CERAPP (Collaborative Estrogen Receptor Activity Prediction Project) dataset, iii) the German Federal Environment Agency (UBA) ED-scan for ER and AR binders, and iv) the Estrogen Receptor Binding and the rtER Expert System ver.1 – USEPA profilers available to investigate Eds in the OECD QSAR application Toolbox (***data available on request***)

MOLECULAR DOCKING

performed on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life*, *versus* selected Nuclear Receptors (NRs), such as the Androgen Receptor AR, the Estrogen Receptors ERa and ERb (***data available on request***), and on the Peroxisome Proliferator-Activated Receptor PPARg (***in progress***)

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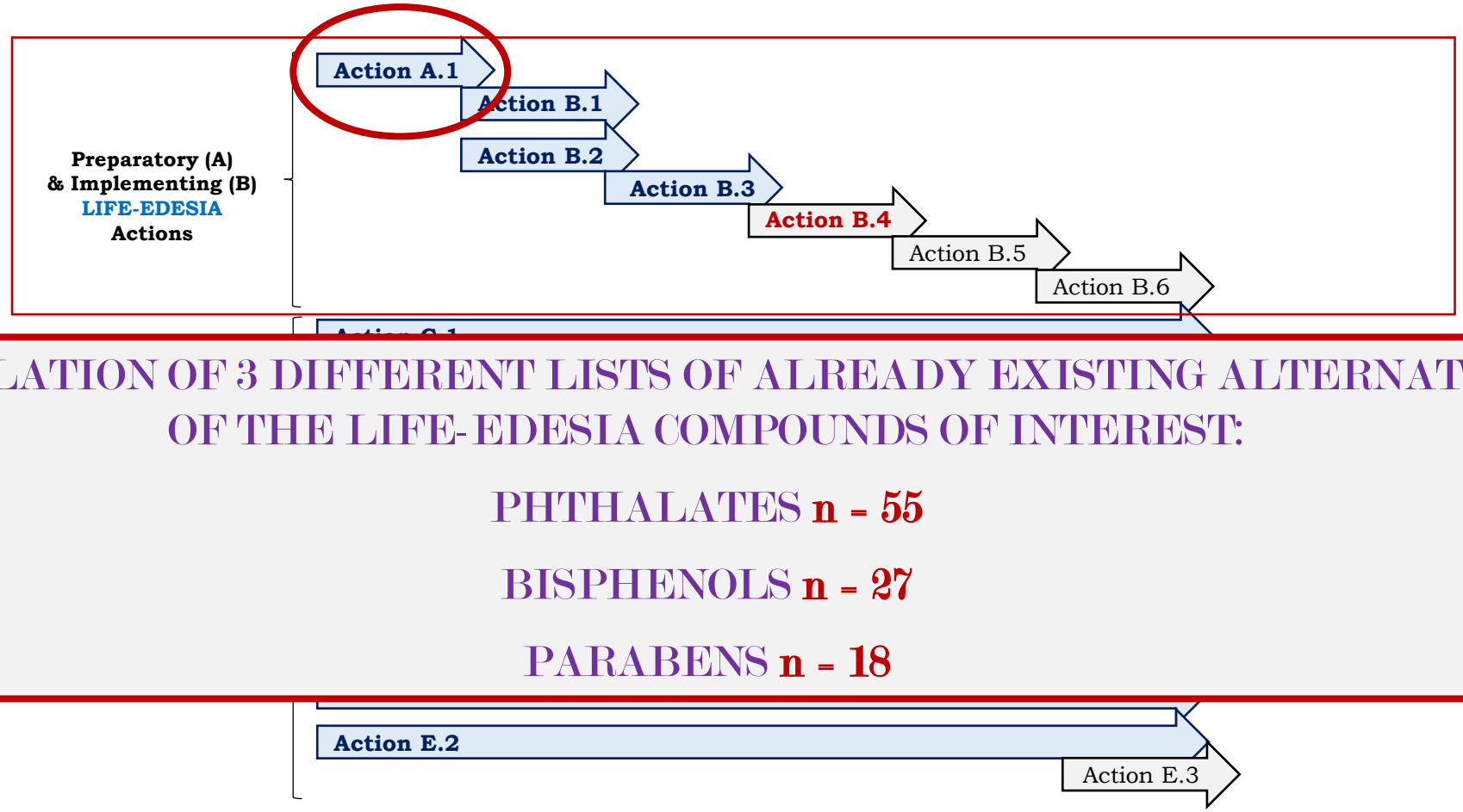
SUMMARY OF *IN SILICO* PROJECT RESULTS 1

✓ ***In silico*** **selection (by Benfenati group in Milan and Cozzini group in Parma) of alternatives to be tested *in vitro*...**



COMPILATION OF 3 DIFFERENT LISTS OF ALREADY EXISTING ALTERNATIVES OF THE LIFE-EDESIA COMPOUNDS OF INTEREST:

PHTHALATES **n = 55**

BISPHENOLS **n = 27**

PARABENS **n = 18**

Completed Actions and whole project-running Actions are depicted in pale blu arrows. Actions highlighted in red are *in progress*, whereas those ones not yet started are in pale grey arrows.

SUMMARY OF *IN SILICO* PROJECT RESULTS 2

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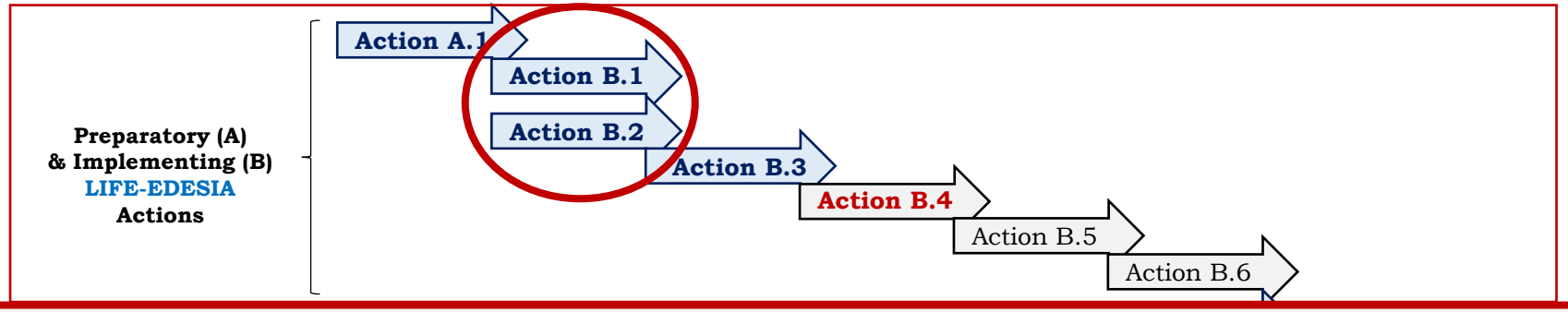

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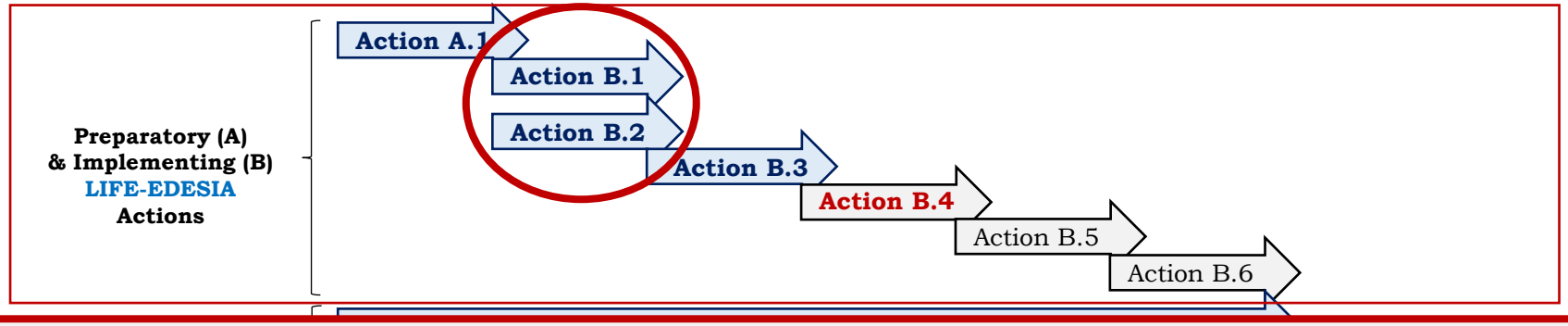

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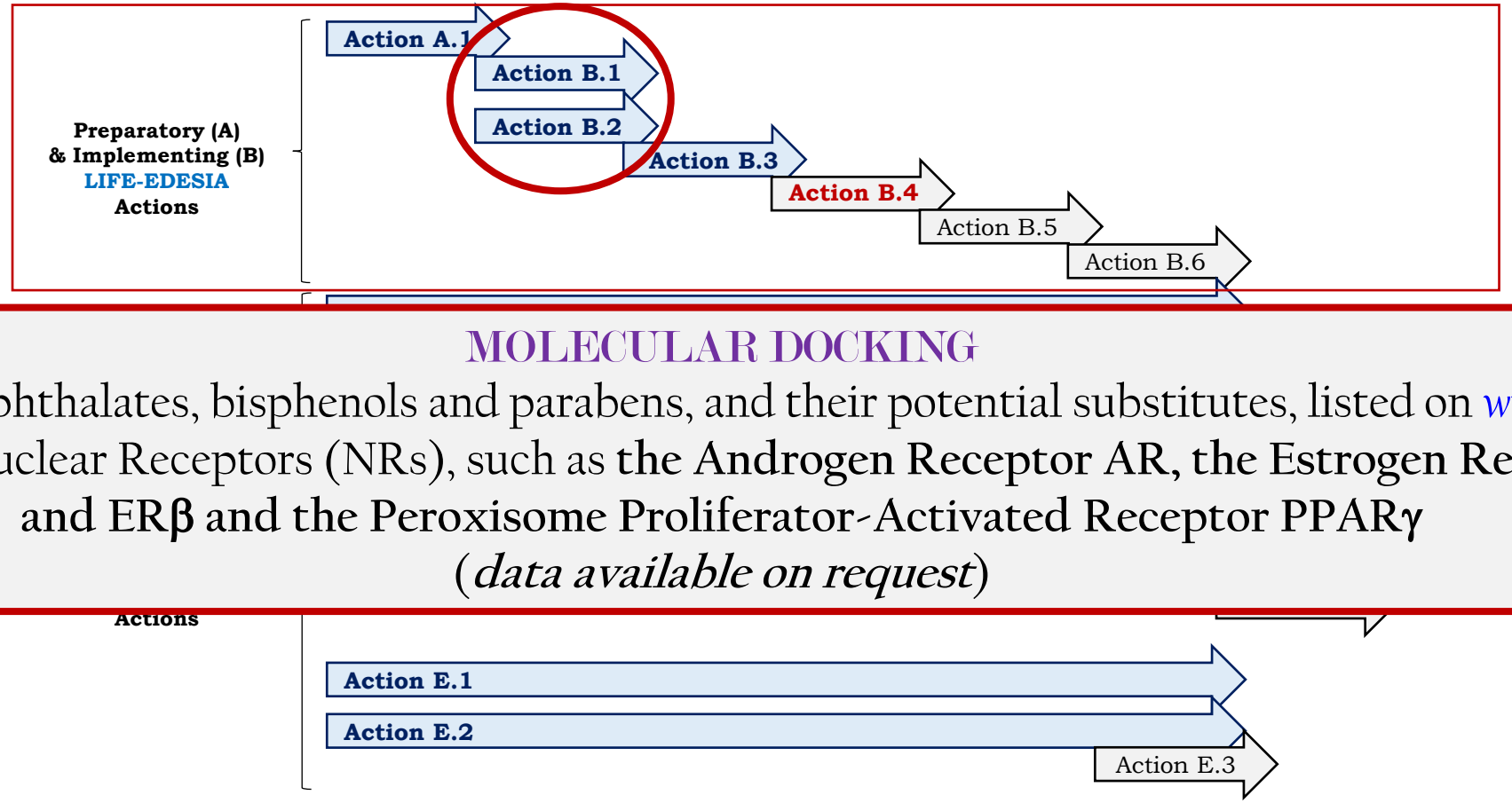

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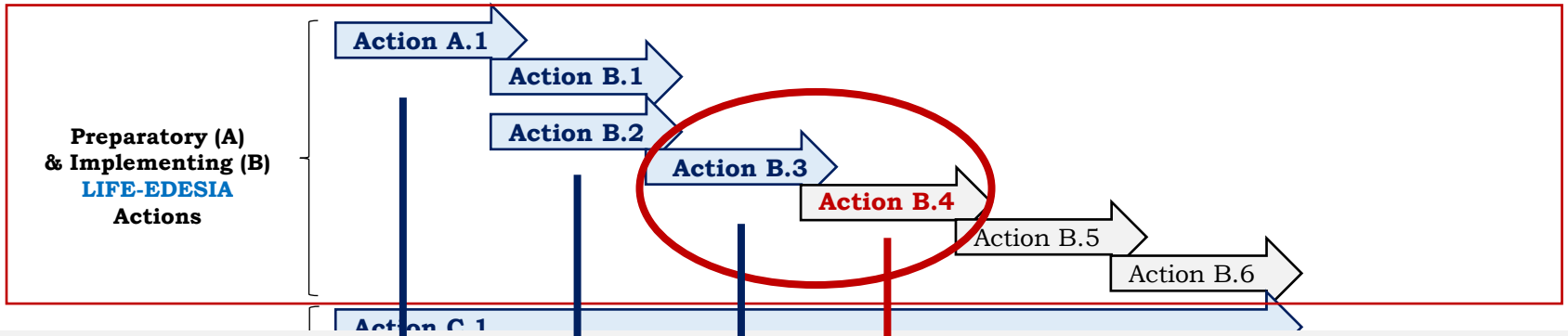
The overall LIFE-EDESIA approach: computational prioritization

in silico toxicological selection of potential substitutes of the LIFE-EDESIA chemicals of concern (*phthalates/DEHP, bispheols/BPA, parabens/methyl paraben*)



Phthalates (*e.g.* DEHP) alternatives	n=55	...	5	*in progress*, at ISS, in comparison to reference molecules (*e.g.* DEHP)
Bisphenols (*e.g.* BPA) alternatives	n=27	...	3	*in progress*, at ISS, in comparison to ref. molecules (*e.g.* BPA)
Parabens alternatives	n=18	...	4	*in progress*, at ISS, in comparison to ref. molecules (*e.g.* methyl paraben)

pale grey arrows.

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The overall LIFE-EDESIA approach - 2. testing by ED-dependent, biomarker-based, cell-specific bioassays

The AIM To characterize *in vitro*, in multiple ED-targeted human cells, if the alternatives identified in previous actions are "less toxic" considering their endocrine disrupting properties.

Cell aspecific endpoint:
Cell Viability
(MTS assay)

Cell specific endpoint:
Functional Assay – Phenotypic anchoring
- prostate: PSA secretion
- trophoblast: βhCG secretion
- liver: intracellular lipid accumulation and AFP secretion

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

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

● OECD guidelines for the testing of chemicals http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm
JRC-IHCP website http://ec.europa.eu/dgs/jrc/index.cfm : *e.g.*, the "Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists" (OECD TG 455); the "BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists" (OECD TG 457).

✓ Lorenzetti S, Mantovani A. 2014. Reproductive and Developmental Toxicity Testing: issues for 3Rs implementation. In: Reducing, Refining, and Replacing the Use of Animals in Toxicity Testing (Chapter 12, pp. 330-347), edited by Dave G Allen and Michael D Waters, RSC Publishing, Cambridge (UK); DOI:10.1039/9781849737920-00330.

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The METHODS

Within the three model systems will be used in parallel an approach based on the use of three cell-based assays:

a) cytotoxicity/cell proliferation test (by MTS assay, a metabolic-based assay relying on mitochondrial functionality) that will assist to distinguish if the changes observed in the other tested endpoints (b. and c.) are cell specific or merely due to cell damages;

b) assessment of gene expression (by real time RT-PCR) of a set of nuclear receptors (NRs) known molecular mediators of the actions of parabens, bisphenols and phthalates;

c) "phenotypic anchoring" by measurements of clinical-, physiologically-relevant endpoints: to allow the assessment of the physiological relevance of detected change in NR gene expression by the measurement of cell specific cellular biomarkers already employed in clinical practice and well recognized as endocrine endpoints modulated by both endogenous and exogenous hormone-like stimuli.

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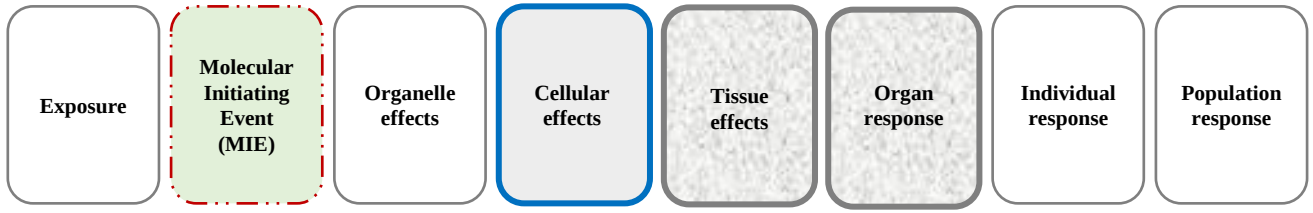
gene expression of Nuclear Receptors of interest
(qPCR)

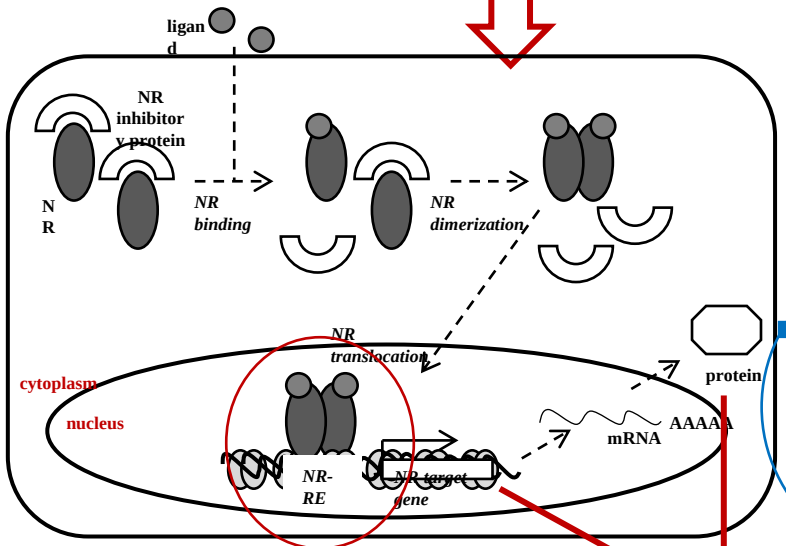
The EXPERIMENTAL MODELS

- **prostate**, to investigate ED androgen receptor (AR)-mediated effects on the male reproductive system
  - Lorenzetti *et al.*, 2010, Reprod.Toxicol. 30:25-30; Lorenzetti *et al.*, 2011, Ann Ist Super Sanita. 47(4):429-44
- **trophoblast**, to investigate ED estrogen receptor (ER)-mediated effect on the placenta and hence the transgenerational effects on nutrient exchange between mother-child
  - Morck *et al.*, 2010, Reprod.Toxicol. 30:131; Lorenzetti *et al.*, 2011, Ann Ist Super Sanita. 47(4):429-44
- **liver**, to investigate multiple ED nuclear receptor (NR)-mediated effects on the programming of the metabolic syndrome.
  - Grasselli *et al.*, 2013, Chemosphere. 91(8):1123-9

BEING MORE «ALTERNATIVES» FOR ENDOCRINE DISRUPTION : effect-based approaches

Adverse Outcome Pathway (AOP)





transcriptional activation

Part of a Mechanism of Action (incl. toxicogenomics)

molecular markers:
mRNA, miRNA, proteins, metabolites

Effect-based functional markers

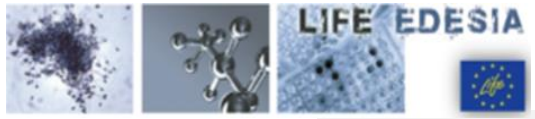
changed cell function

Part of a Mode of Action

WHICH BIOMARKERS TO SCREEN FOR:

- AN ENDOCRINE ACTIVITY ?
- AN ADVERSE EFFECT ?

cell-specific, clinically relevant, hormone-dependent biomarkers of effects

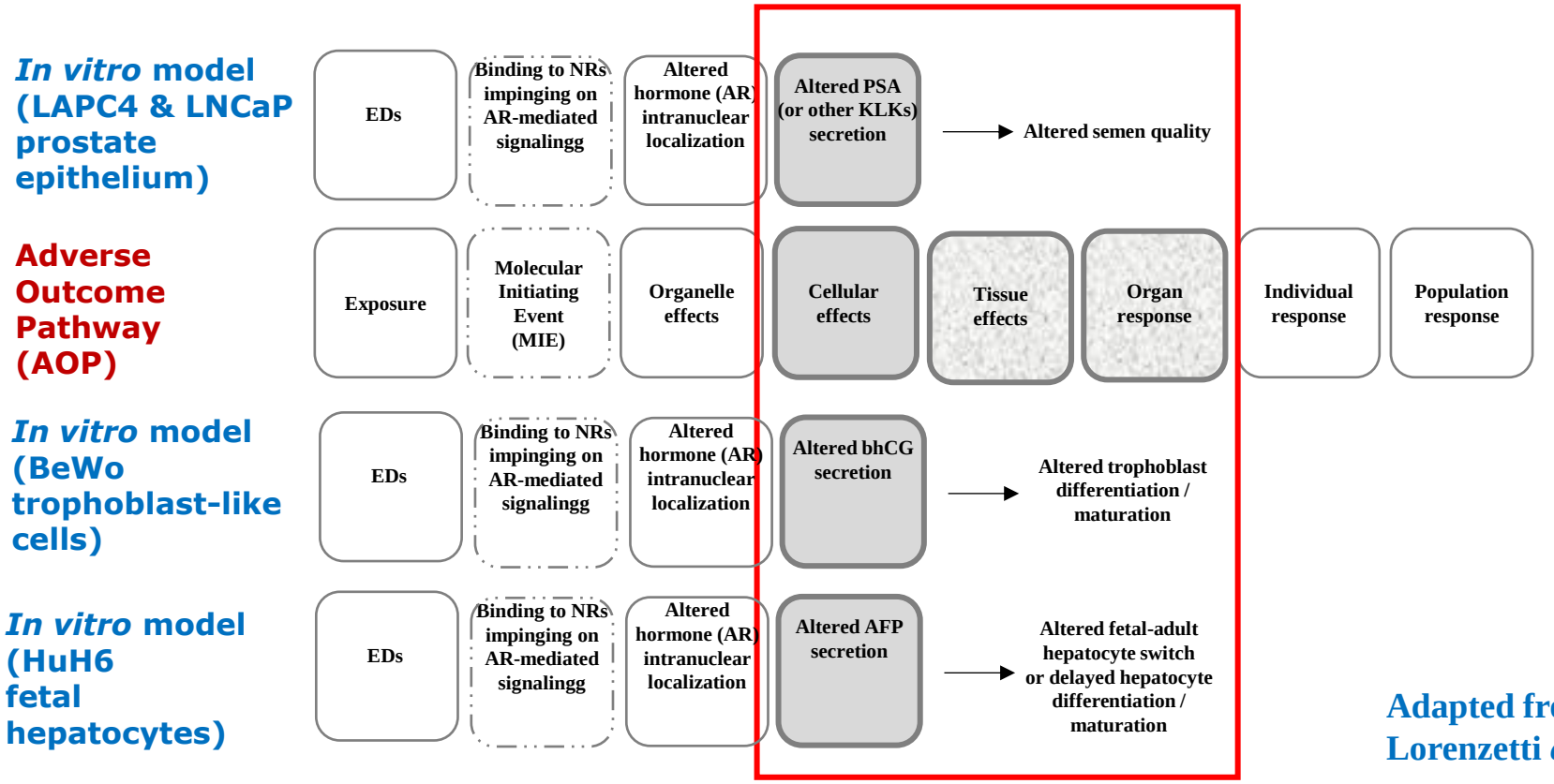


- PSA secretion – androgen disruption in prostate epithelium
- βhCG secretion – estrogen disruption in trophoblast-like cell (placenta)
- AFP secretion – metabolic disruption in liver

OUTLINE

- ✓ **Why alternatives to animal testing**
- ✓ **Endocrine Disruption and adverse effects**
- ✓ **Endocrine Disruptor (ED)-screening: mechanism-based *versus* effect-based**
- ✓ **The *in vitro* LIFE-EDESIA approach:**
  computational prioritization
  plus
  ***in vitro* testing by ED-dependent, biomarker-based, cell-specific bioassays**
- ✓ **Let's go to the end**

Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 1



Adapted from Lorenzetti *et al.*, Annals 2015

Monolayers and 3D-cultured cells STOP here

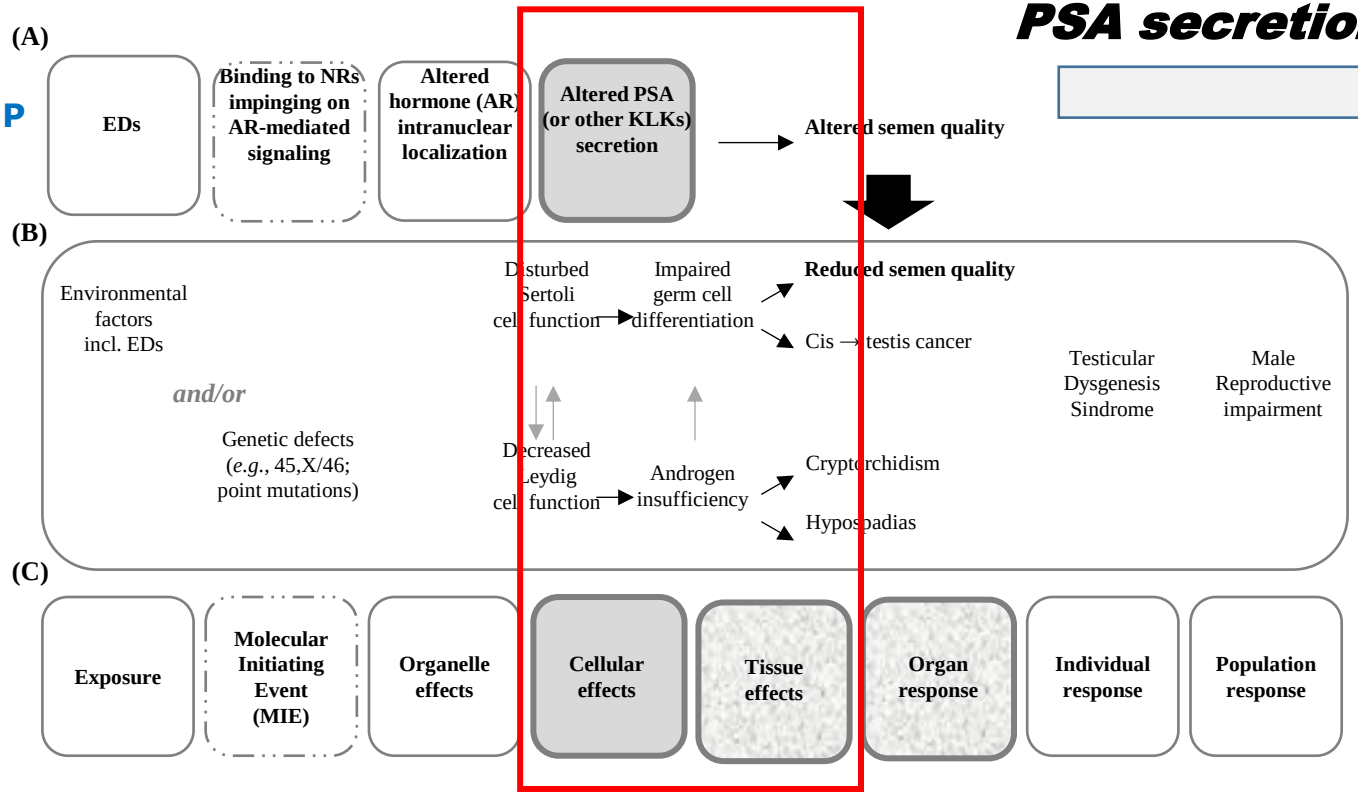
Co-cultured cells & organoids can eventually STOP here

Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 2

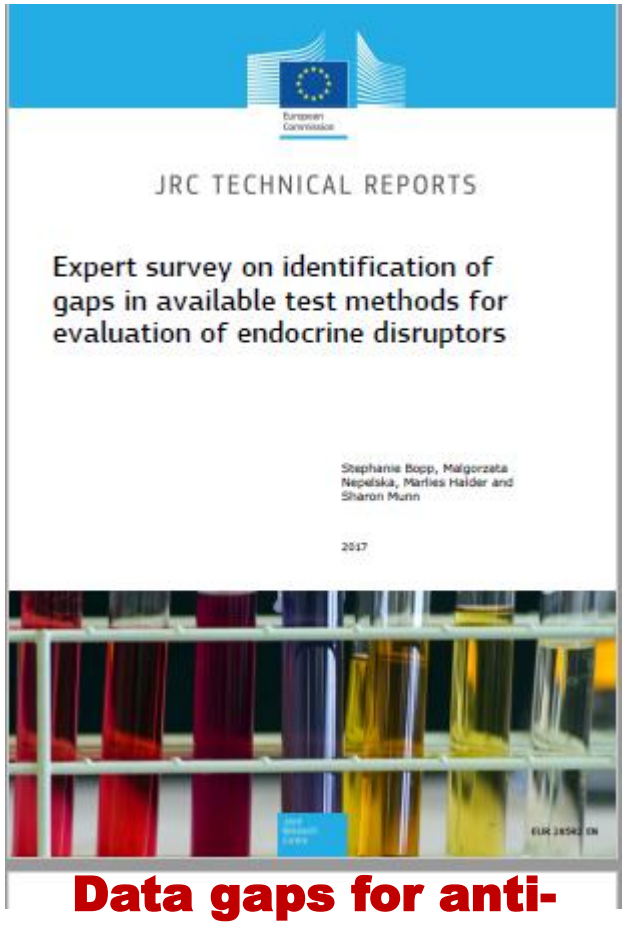
***In vitro* model (LAPC4 & LNCaP prostate epithelium)**

Testicular Dysgenesis Syndrome (TDS)

Adverse Outcome Pathway (AOP)



PSA secretion test

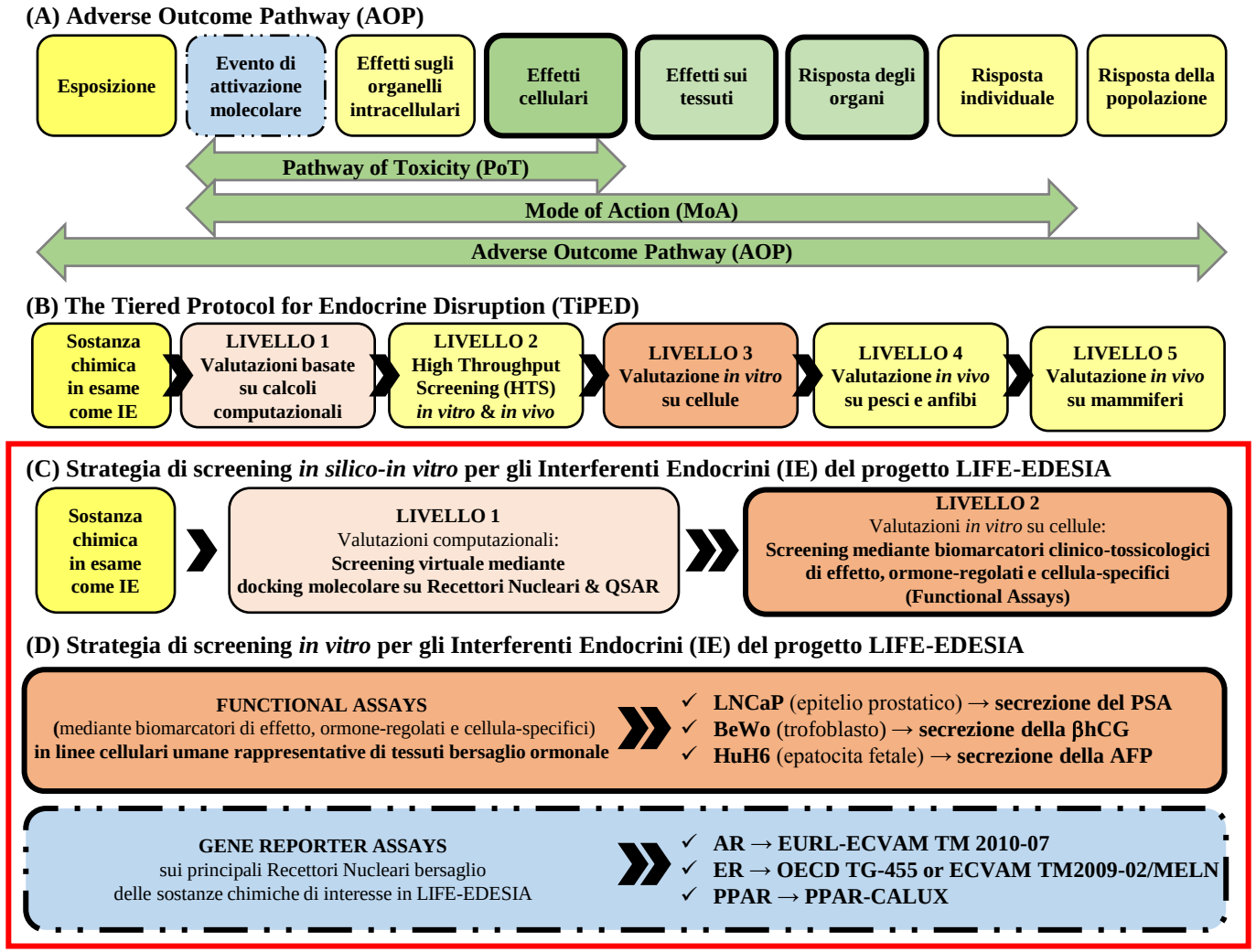


Data gaps for anti-androgenicity in male accessory glands

Adapted from
Lorenzetti *et al.*, Annals 2015

Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 3

Integrating the LIFE-EDESIA Endocrine-based Screening using Cell-specific, ED-targeted Functional Biomarkers (C, D) within the Testicular Dysgenesis Syndrome (B) as an Adverse Outcome Pathway (A).



Adapted from Lorenzetti *et al.*, Annals 2015

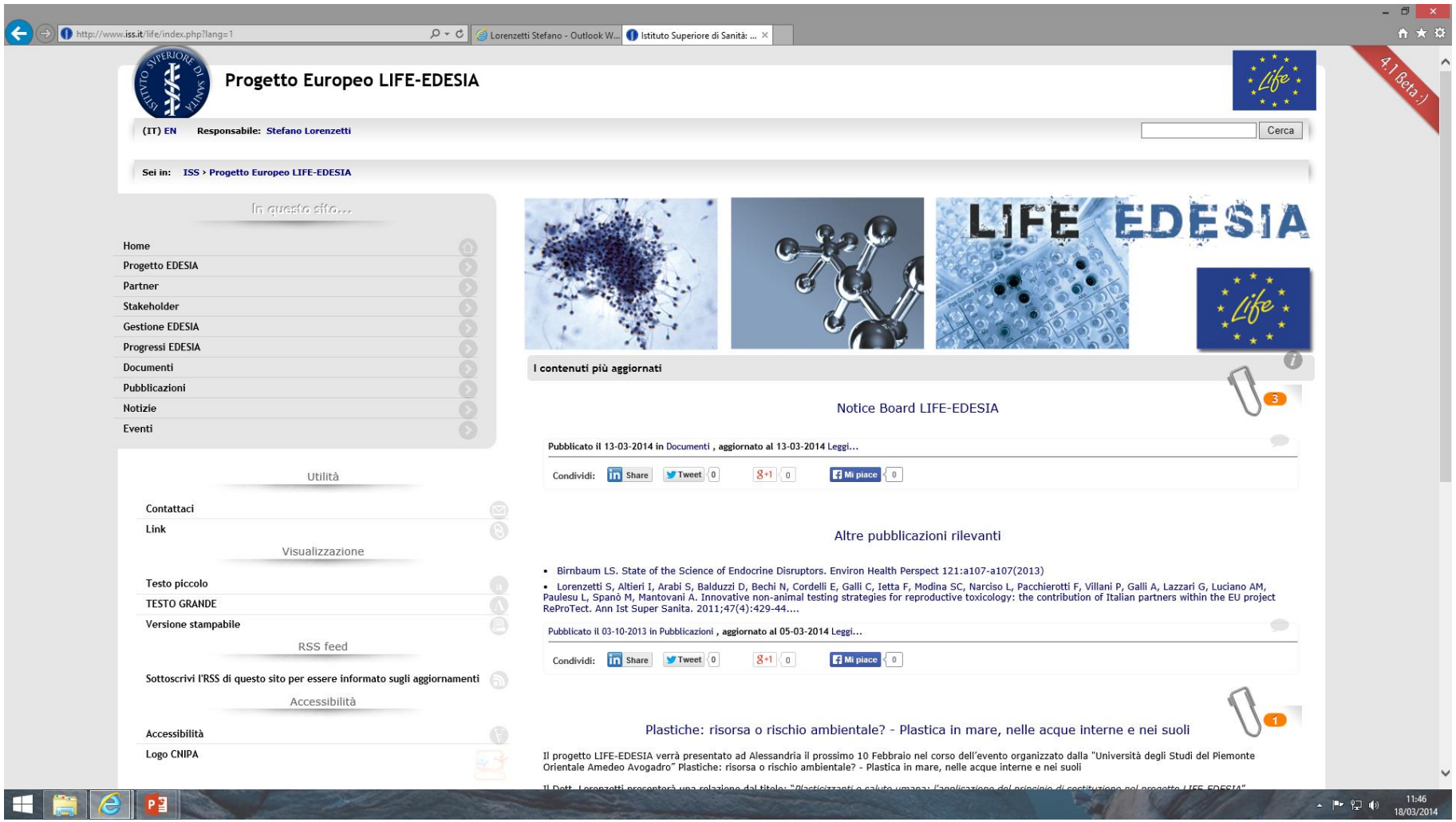
TAKE HOME MESSAGEs

- **About prioritisation and testing:**

- **Always remind the limitations of your favourite alternative assay to animal testing**
- **Always remind the concept «fit-for-purpose» not only to test your hypothesis but also to dismiss a wrong application of a test method**
- **Try to develop new screening methods as closed as possible to the (human) physiological reality**

MORE INFO ON LIFE-EDESIA

come to visit our website www.iss.it/life



MORE INFO ON ALTERNATIVE TEST METHODS

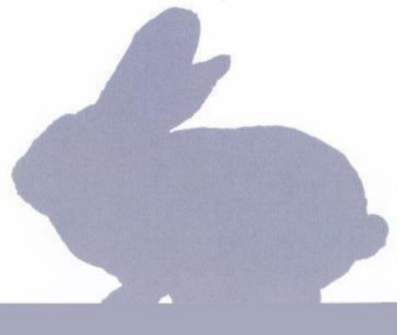
come to visit www.ipamitalia.org

IPAM

Associazione Nazionale no-profit

Fondata nel 2003

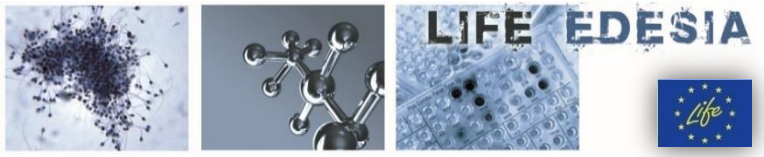
Italian Platform on Alternative Methods



ACKNOWLEDGEMENTS

Endocrine Disruptors *in silico / in vitro*
Evaluation and Substitution for Industrial Applications

LIFE12 ENV/IT/000633



http://www.iss.it/life/

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