Food Packaging Forum

Workshop

Food packaging and chemical safety:
What does the future hold?

8 October 2015

Animal free (toxicological) screening of plasticizer alternatives

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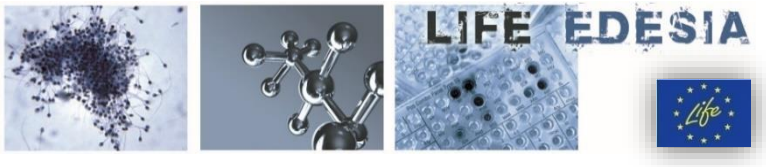

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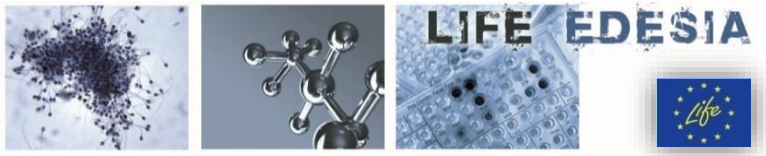
OUTLINE

- ✓ **Why to search for plasticizer alternatives?**
- ✓ **Endocrine Disruption and adverse effects**
- ✓ **Endocrine Disruptor (ED)-screening: mechanism-based *versus* effect-based**
- ✓ **The *in vitro* LIFE-EDESIA approach:**
  Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption

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



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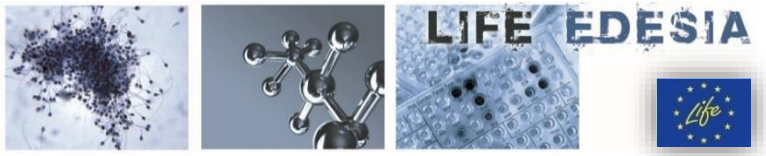

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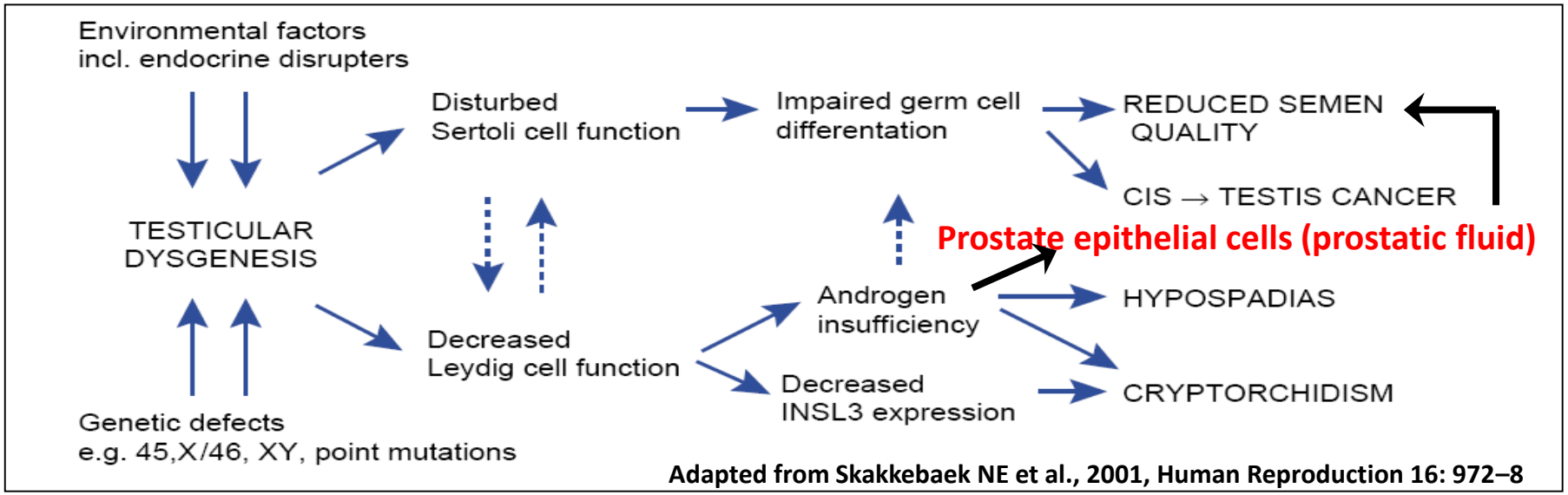
Why to search for plasticizer alternatives ? - A HEALTHY ISSUE 1

➢ Testicular Dysgenesis Syndrome (TDS) in humans

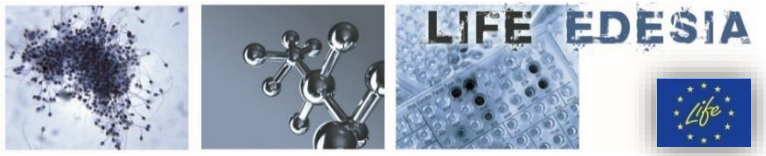
exposure *in utero* to environmental factors (anti-androgenic compounds) in Western Europe and USA are responsible of male infertility and associated-diseases/malformations.



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



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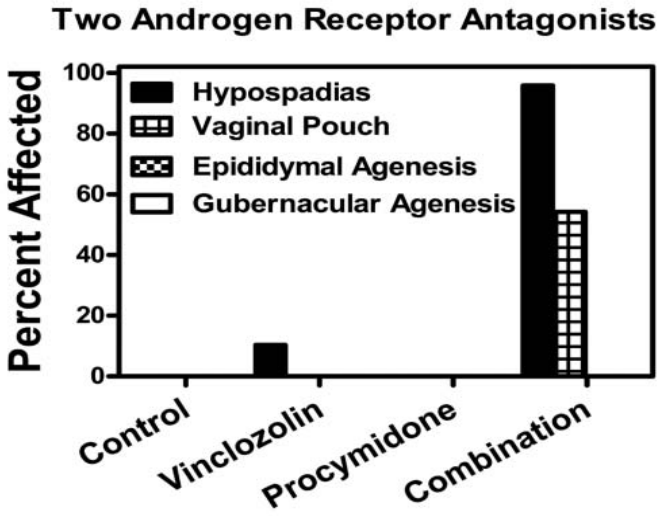
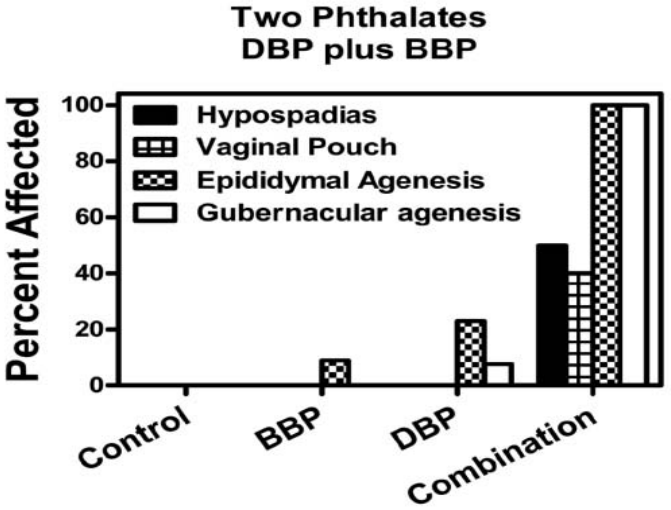
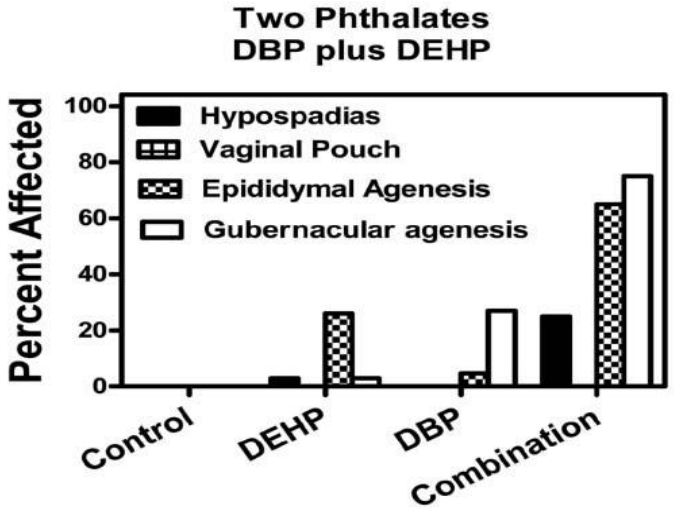
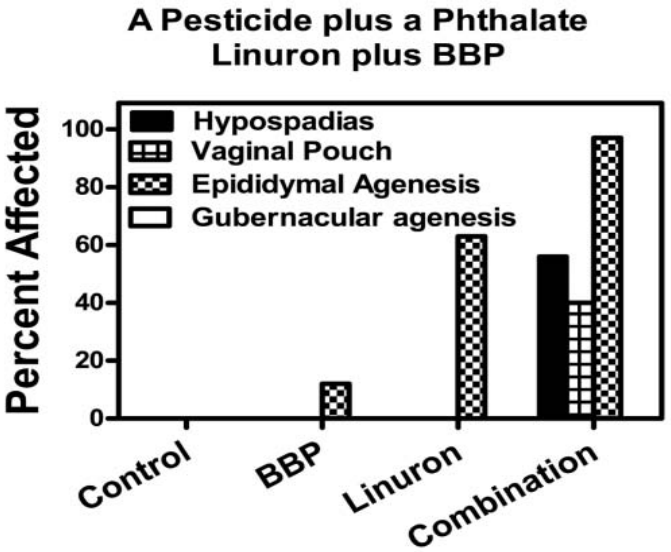
Why to search for plasticizer alternatives ? - A HEALTHY ISSUE 2





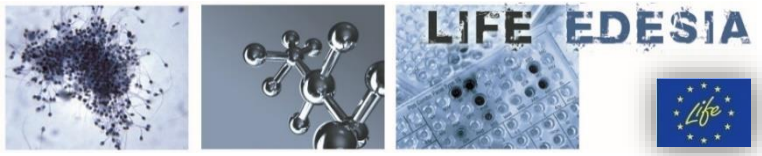




«Phthalate syndrome» (rodent TDS) and pesticides: the role of EDC mixtures

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

Rider *et al*. Toxicol. Pathol. 2009



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Why to search for plasticizer alternatives ? - A HEALTHY ISSUE 3

➢ **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.

Why to search for plasticizer alternatives ? - A LEGISLATIVE ISSUE 1

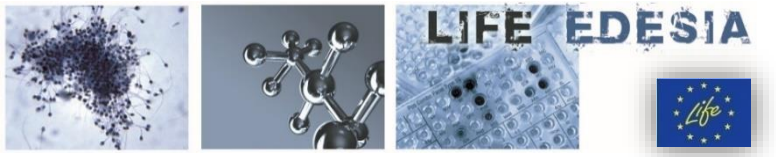
➢ EU regulatory framework

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 to search for plasticizer alternatives ? - A LEGISLATIVE ISSUE 2

Candidate List of Substances of Very High Concern (SVHCs) for Authorisation:

151 entries (updated: 16 December 2013)

Substance name	EC number	CAS number	Date of inclusion	Reason for inclusion	Decision number	IUCLID 5 substance dataset	*details*
Di-*n*-hexyl phthalate (**DNHP**)	201-559-5	84-75-3	2013/12/16	Toxic for reproduction (Article 57 c)	ED/121/2013		X
Di-*n*-pentyl phthalate (**DNPP**)	205-017-9	131-18-0	2013/06/20	Toxic for reproduction (Article 57 c)	ED/69/2013		X
N-pentyl-isopentyl phthalate	-	776297-69-9	2012/12/19	Toxic for reproduction (Article 57 c)	ED/169/2012		X
Diisopentylphthalate (**DIPP**)	210-088-4	605-50-5	2012/12/19	Toxic for reproduction (Article 57 c)	ED/169/2012		X
Bis (2-methoxyethyl) phthalate (**DMEP**)	204-212-6	117-82-8	2011/12/19	Toxic for reproduction (Article 57 c)	ED/77/2011		X
Diisobutyl phthalate (**DIBP**)	201-553-2	84-69-5	2010/01/13	Toxic for reproduction (Article 57 c)	ED/68/2009		X
Benzylbutyl phthalate (**BBP**)	201-622-7	85-68-7	2008/10/28	Toxic for reproduction (Article 57 c)	ED/67/2008		X
Bis (2-ethylhexyl) phthalate (**DEHP**)	204-211-0	117-81-7	2008/10/28	Toxic for reproduction (Article 57 c)	ED/67/2008		X
Dibutyl phthalate (**DBP**)	201-557-4	84-74-2	2008/10/28	Toxic for reproduction (Article 57 c)	ED/67/2008		X

Why to search for plasticizer alternatives ? - A LEGISLATIVE ISSUE 3

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03 PVC

Why to search for plasticizer alternatives ? - A LEGISLATIVE ISSUE 4

Annex XIV - Authorization list

22 entries (updated 17 April 2013)

Substance name	EC number	CAS number	Sunset date	Latest application	Exempted (categories of) uses	*details*
Diisobutyl phthalate (**DIBP**)	201-553-2	84-69-5	21/02/2015	21/08/2013		
Dibutyl phthalate (**DBP**)	201-557-4	84-74-2	21/02/2015	21/08/2013	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC.	X
Bis(2-ethylhexyl) phthalate (**DEHP**)	204-211-0	117-81-7	21/02/2015	21/08/2013	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC.	X
Benzyl butyl phthalate (**BBP**)	201-622-7	85-68-7	21/02/2015	21/08/2013	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC.	X

WARNING: **so far, DEHP is the most effective plasticizer to be used in a key medical device such as BLOOD BAGS**

Why to search for plasticizer alternatives ? - A LEGISLATIVE ISSUE 5

Annex XVII - Restriction list

(adopted 22 june 2009)

Substance name	EC number	CAS number	Sunset date	Latest application	Exempted (categories of) uses	*details*
Diisobutyl phthalate (**DIBP**)	201-553-2	84-69-5	21/02/2015	21/08/2013		
Dibutyl phthalate (**DBP**)	201-557-4	84-74-2	21/02/2015	21/08/2013	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC.	X
Bis(2-ethylhexyl) phthalate (**DEHP**)	204-211-0	117-81-7	21/02/2015	21/08/2013	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC.	X
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WARNING: so far, DEHP is the most effective plasticizer to be used in a key medical device such as BLOOD BAGS BUT WILL NOT BE BANNED IN 2015 AS ORIGINALLY EXPECTED

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Endocrine Disruptors *in silico / in vitro*
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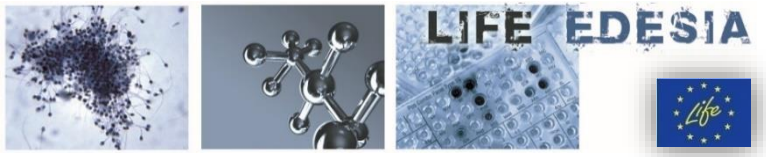


http://www.iss.it/life/

Endocrine Disruption and adverse effects - 1

➢ 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 Journal 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

➢ "EDs are EASs causing adverse effects mediated by endocrine mechanisms"

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

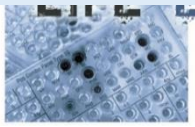
In summary, **currently available definitions of "endocrine disrupter"** are **either** neutral in terms of specifying the toxicological relevance of the effects to be described, **or** they introduce the idea of adversity.

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





Endocrine Disruption and adverse effects - 2

➢ Endocrine Activity…

"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

➢ … as a sum of different Mode of Actions

"A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences".

Federal Institute for Risk Assessement (BfR), Berlin workshop 2009"

OECD 2002

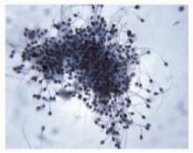
OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

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)

***In vitro* Nuclear Receptor binding & regulation of gene transcription IS SUFFICIENT TO DEFINE:**

- ✓ **AN ENDOCRINE ACTIVITY ?**
- ✓ **AN ADVERSE EFFECT ?**



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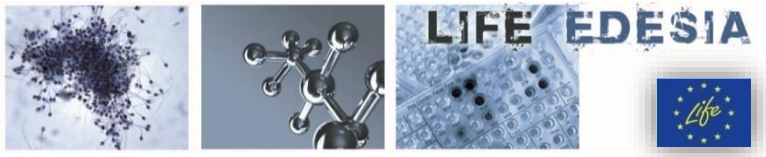

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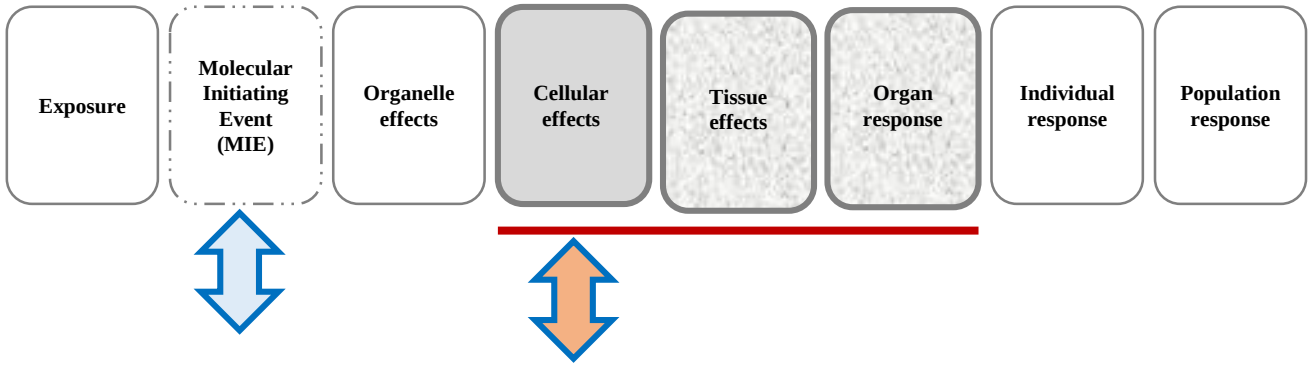
ED-screening: mechanism-based *versus* effect-based - 1

Adverse Outcome Pathway (AOP)



In vitro **Nuclear Receptor binding & regulation of gene transcription IS SUFFICIENT TO DEFINE:**

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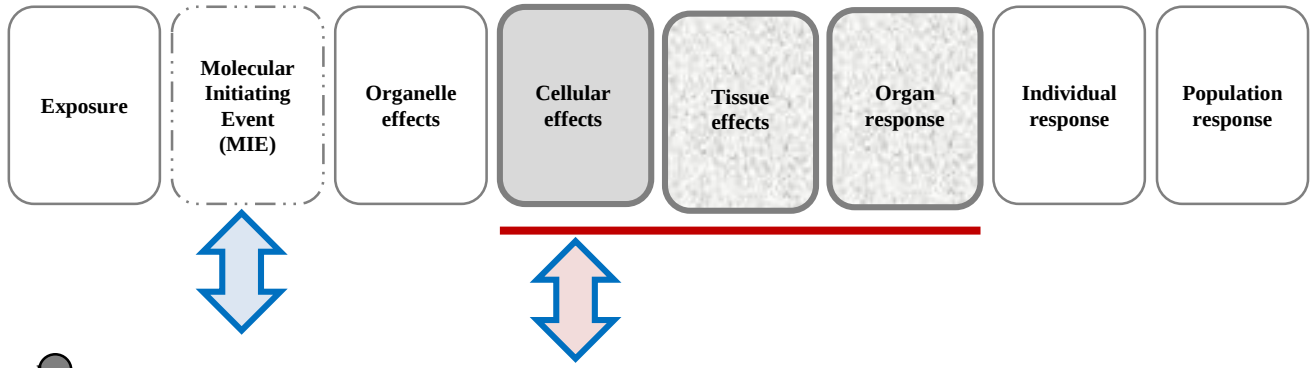
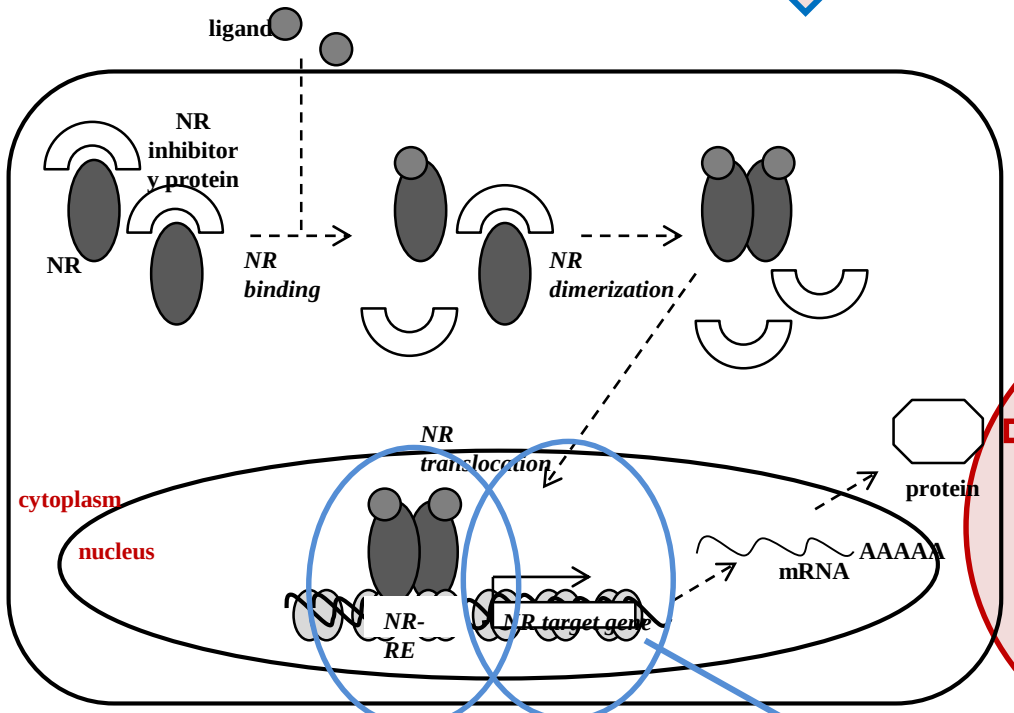
ED-screening: mechanism-based *versus* effect-based - 2

Adverse Outcome Pathway (AOP)





effect-based (functional) markers

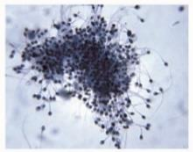
changed cell function

Part of a Mode of Action

Part of a Mechanism of Action

mechanism-based markers

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



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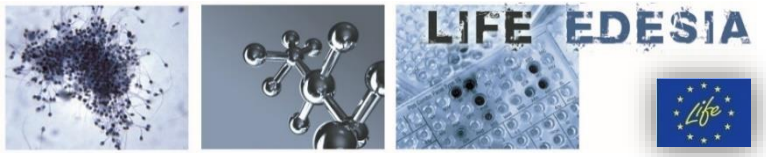


http://www.iss.it/life/

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.

The APPROACH

- ✓ **Multiple clinical-, physiologically-relevant endpoints will be used to translate the *in vitro* toxicological profile to a suitable prediction for human health.**
- ✓ **Endocrine disrupting properties** of the selected alternative substances to phthalates, bisphenols and parabens - in comparison with their reference EDCs - **will be assessed by an integrated set of *in vitro* battery test of alternative methods that**, although not yet validated, **have been so far accepted by the academic community as directly focusing on specific and reliable endocrine endpoints.**
- ✓ **Well characterized human-derived cell lines representing recognized tissue target of the EDCs to be substituted have been selected.**

The overall LIFE-EDESIA approach

Action A1

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***)

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***)

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

The EXPERIMENTAL MODELS

Endocrine disrupting properties will be tested only in cell lines of human sources, whose employment is well proven, integrating tests representative of the endocrine activities of the following human tissues:

- **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

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.

THE CORE OF ACTION B4 AT A GLANCE

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)

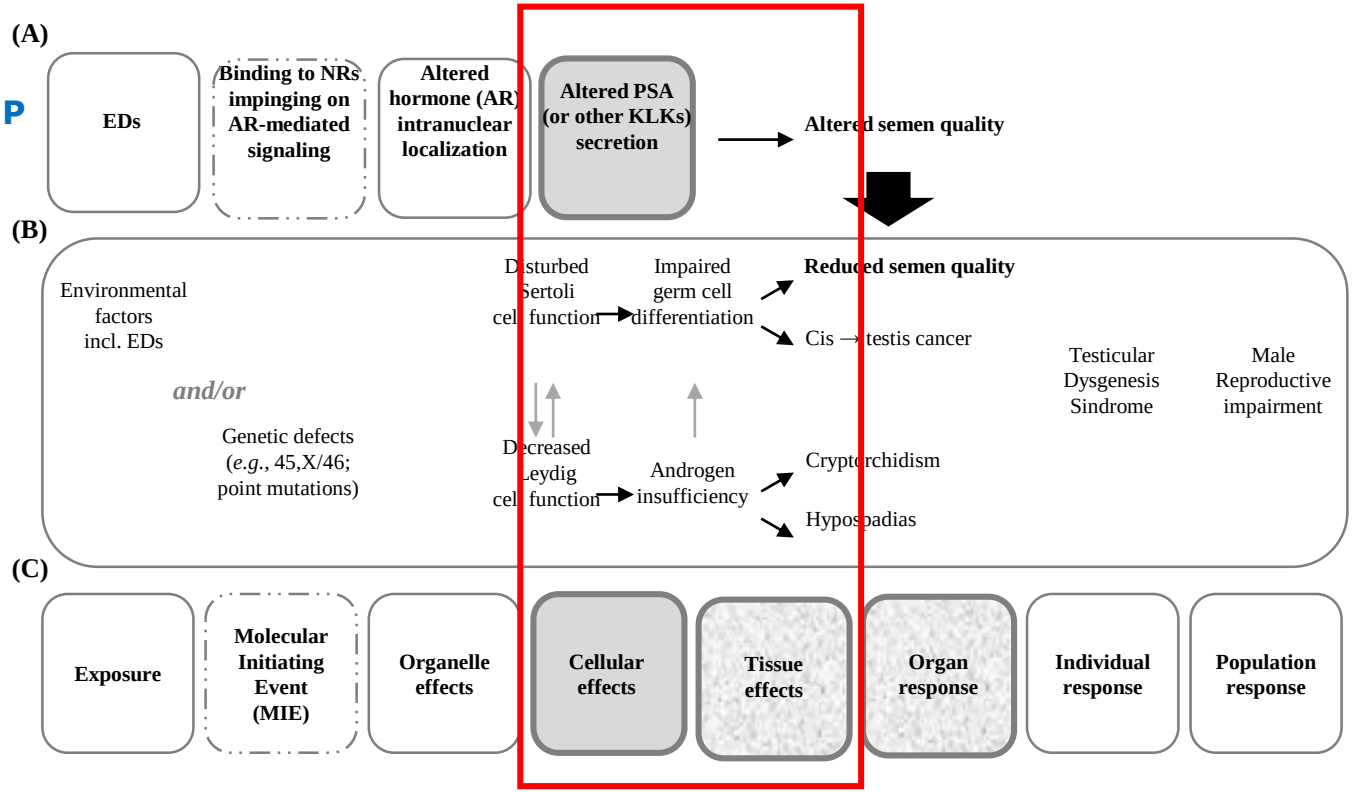
Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 1

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

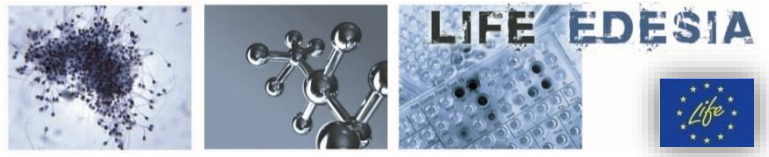
In vitro model (LAPC4 & LNCaP prostate epithelium)

Testicular Dysgenesis Syndrome (TDS)

Adverse Outcome Pathway (AOP)



Adapted from Lorenzetti *et al.*, Annals 2015



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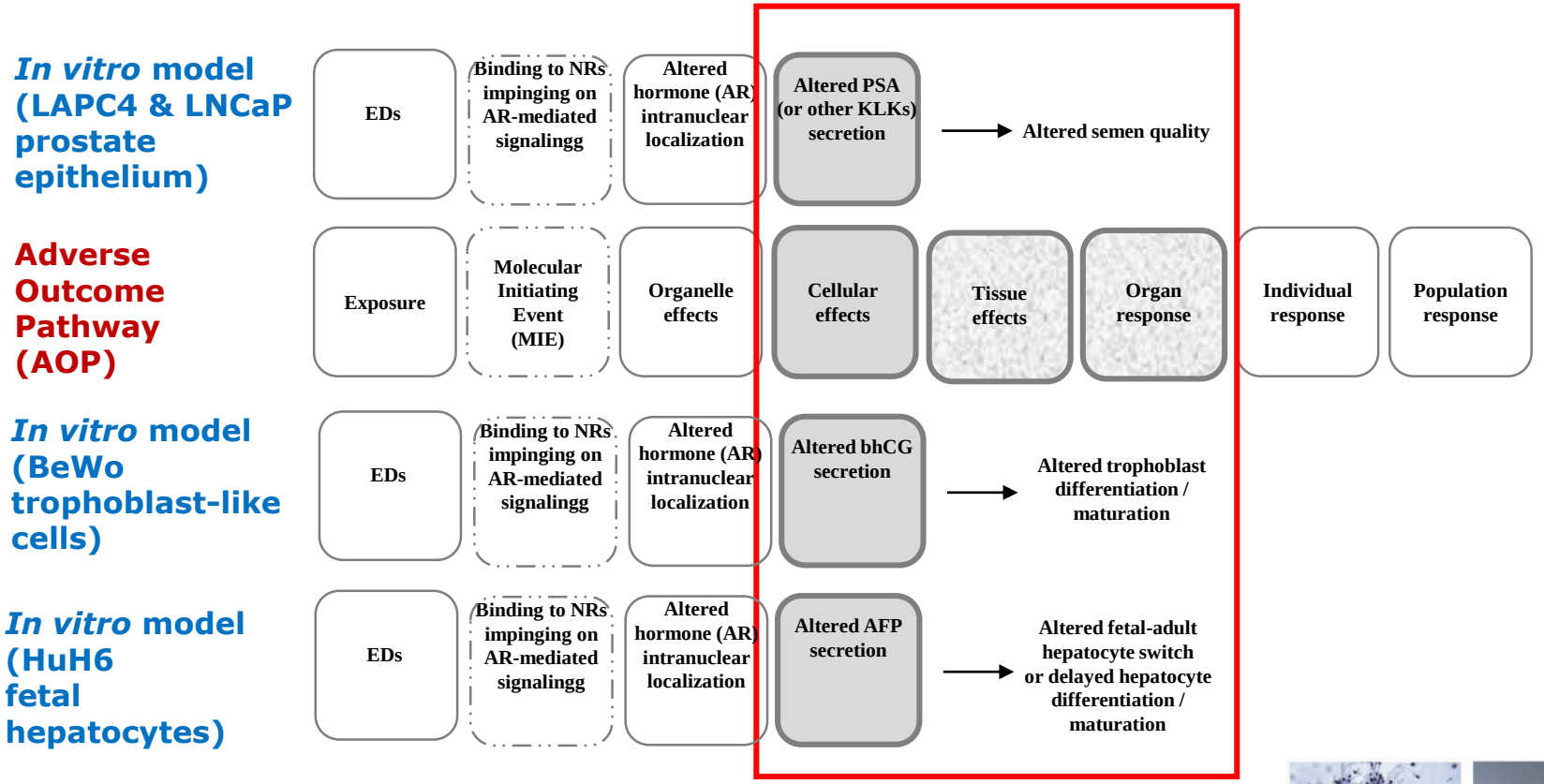
Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption . 2

Integrating the LIFE-EDESIA Endocrine-based Screening using Cell-specific, ED-targeted Functional Biomarkers as an Adverse Outcome Pathway.



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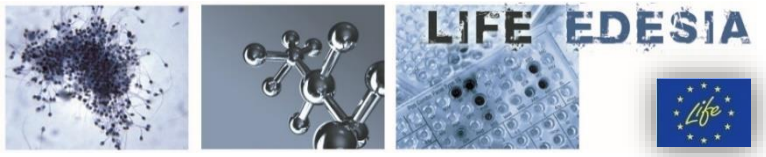
Lorenzetti *et al.*, Annals 2015



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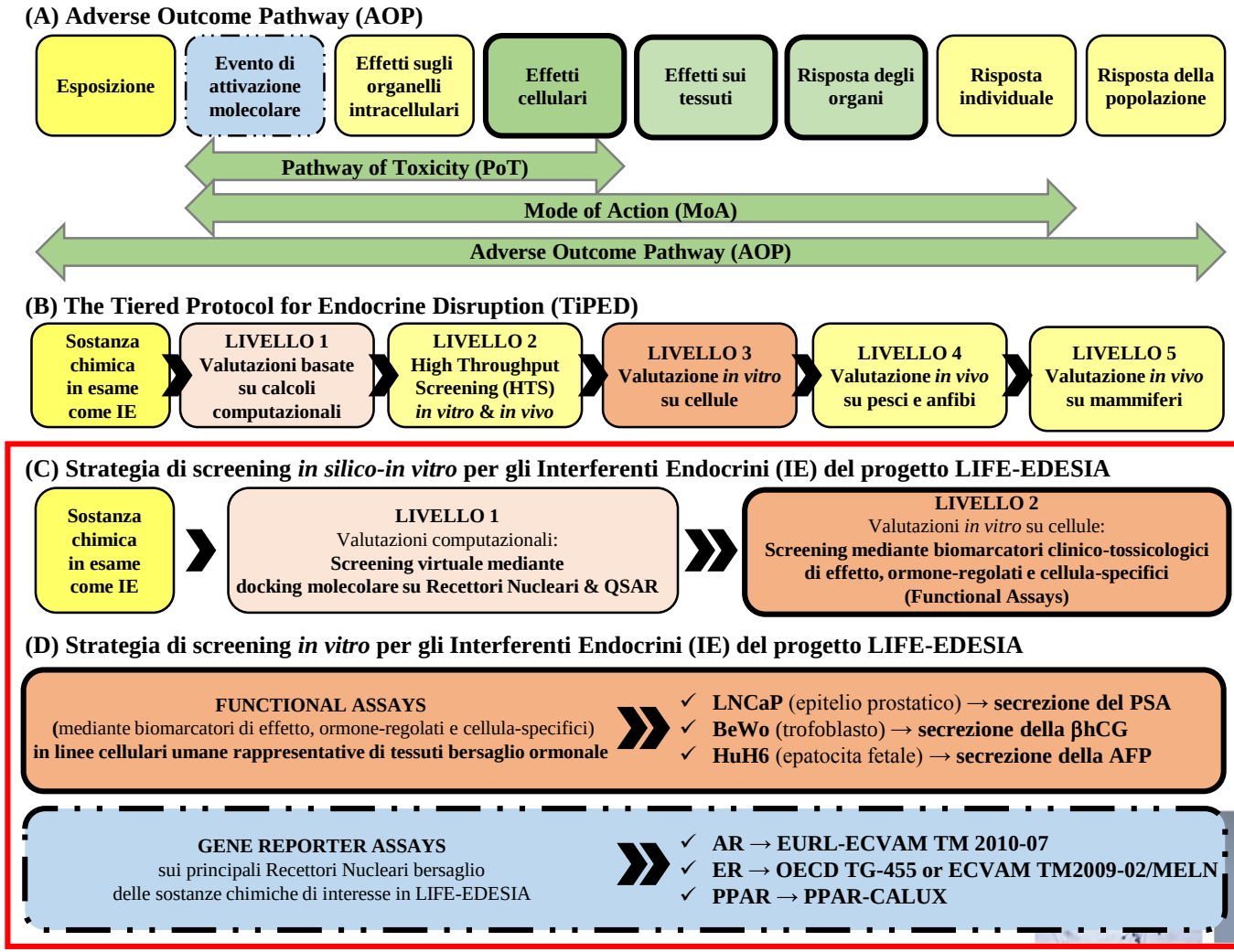
Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 3

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

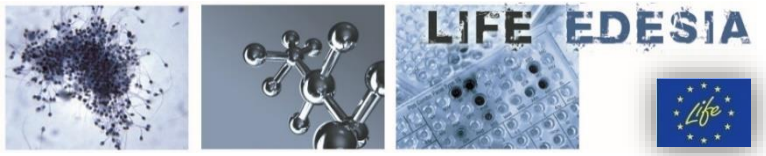
CONCLUSIONS

- ✓ **So far, only the *in silico* part (Actions A1 and B1-B3) of the LIFE-EDESIA project has been completed: Actions A1 data are available online, whereas Actions B1-B3 data will be soon available online.**

- ✓ **The *in vitro* part (Action B4) of the LIFE-EDESIA approach has been already set up for the 3 *in vitro* experimental models selected to perform our biomarker-based, cell-specific bioassays to screen for ED-like alternatives.**

- ✓ **The last but most important part (Action B5) that deals with demonstration of of the industrial applicability of the selected LIFE-EDESIA alternatives will start at the end of Action B4.**

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



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ACKNOWLEDGEMENTS

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

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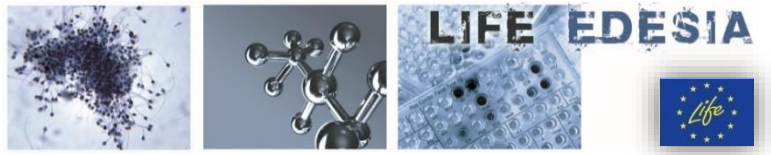

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