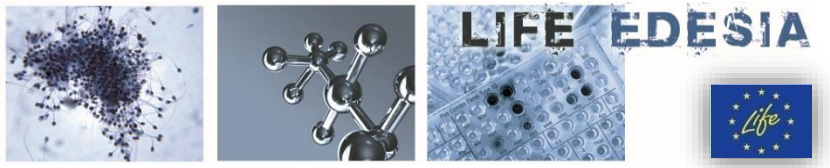
Searching for plasticizer's alternatives by animal free screening in the LIFE-EDESIA project



Stefano Lorenzetti

Istituto Superiore di Sanità (ISS)

Department of Food Safety and Veterinary Public Health

Unit of Food and Veterinary Toxicology

THE VERY BEGINNING…

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.

… WHERE WE STARTED FROM

REACH Regulation
1907/2006/CE

ENDOCRINE DISRUPTORS
as Substances of Very High Concern (SVHC)
Artt. 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

- **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**

- **"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.

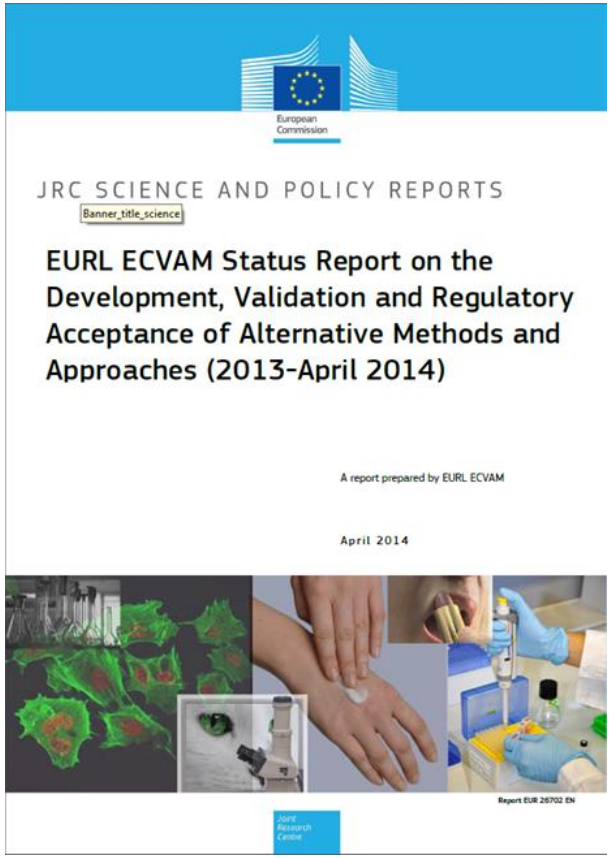
The former is in danger of being insufficiently discriminatory, the latter shifts the problem to defining what adversity should mean in an endocrine context, which could be too restrictive and not inclusive enough.

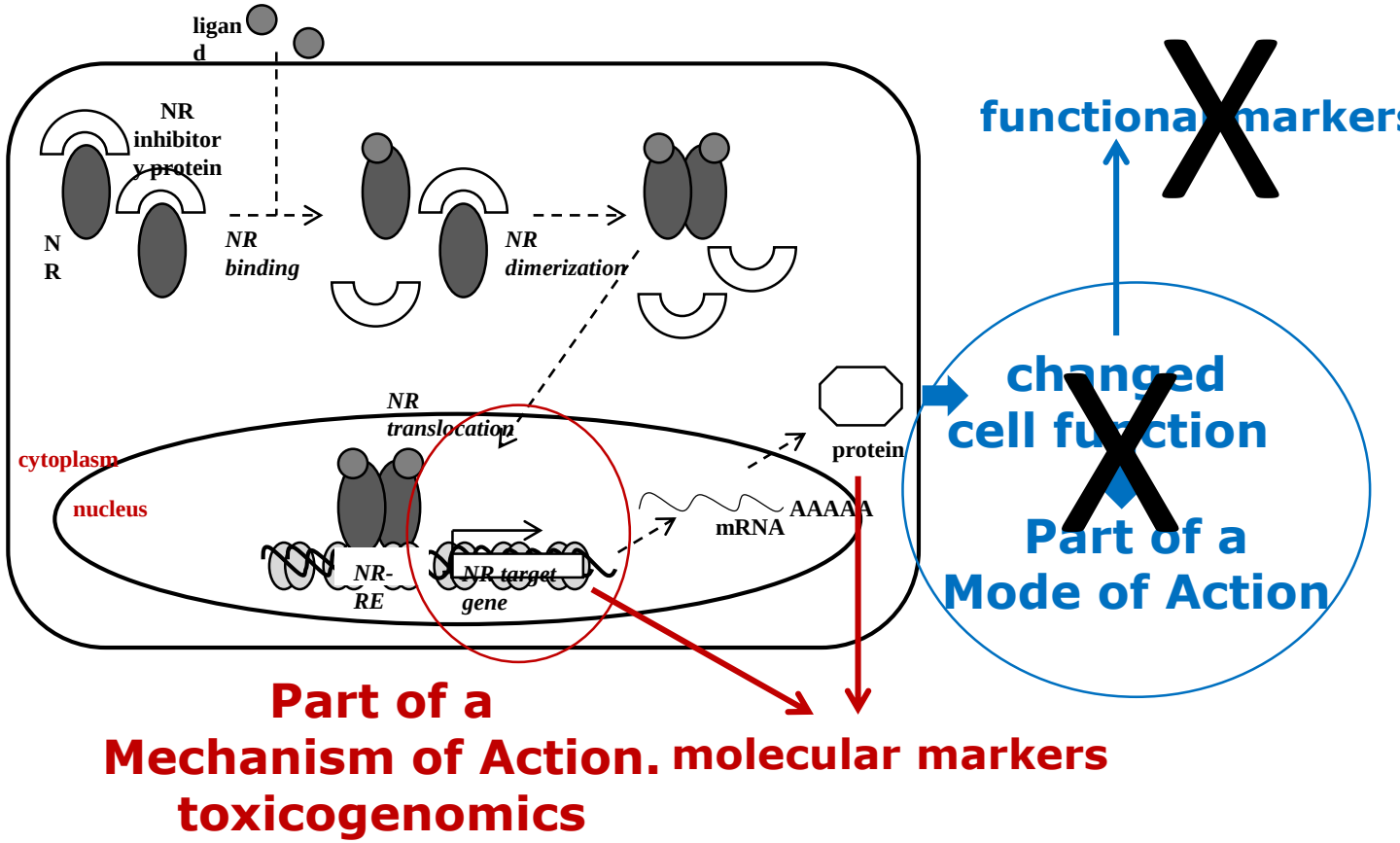
At the core of this dilemma is the fact that "endocrine disruption" cannot presently be anchored to specific assay outcomes in a straightforward way.

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

… ALWAYS LOOKING AT GENE REPORTER ASSAY ?!

HOW THE INTERNATIONAL COMMUNITY IS MOVING ON…

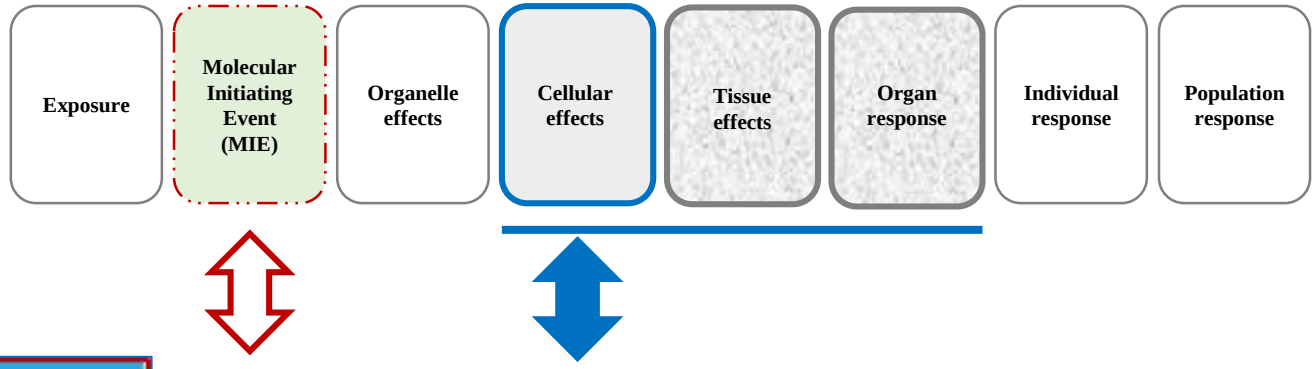




Adapted from Lorenzetti and Narciso, 2012
DOI: 10.1039/9781849735353

SCREENING FOR ENDOCRINE DISRUPTION:
mechanism- *vs* effect based-approach

Adverse Outcome Pathway (AOP)



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

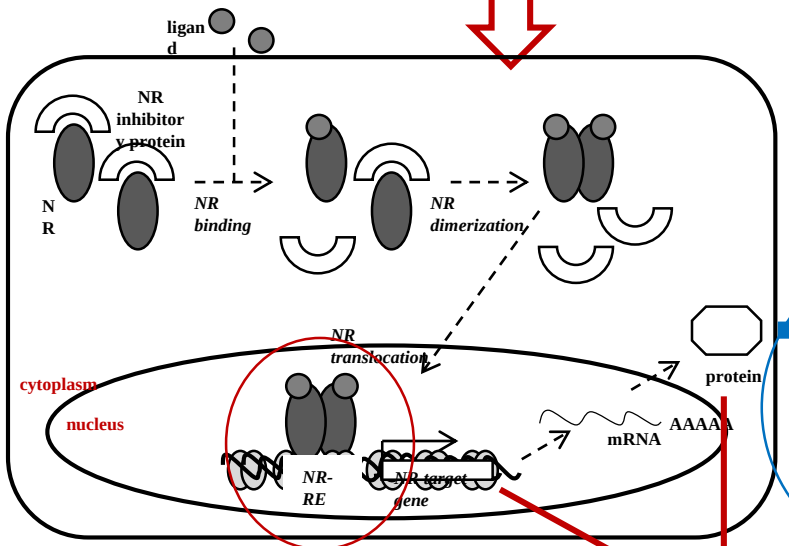
- **AN ENDOCRINE ACTIVITY ?**
- **AN ADVERSE EFFECT ?**

... *in vitro* screening:
so far, mostly applying at
GENE REPORTER ASSAYS
without to know the cell output(s)

SCREENING FOR ENDOCRINE DISRUPTION:
mechanism- *vs* effect based-approach

Adverse Outcome Pathway (AOP)

Exposure	Molecular Initiating Event (MIE)	Organelle effects	Cellular effects	Tissue effects	Organ response	Individual response	Population response



transcriptional activation

Part of a Mechanism of Action (incl. toxicogenomics)

molecular markers:
mRNA, miRNA, proteins, metabolites

changed cell function

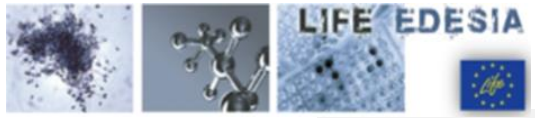
Part of a Mode of Action

Effect-based functional markers

WHICH BIOMARKERS TO SCREEN FOR:

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

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

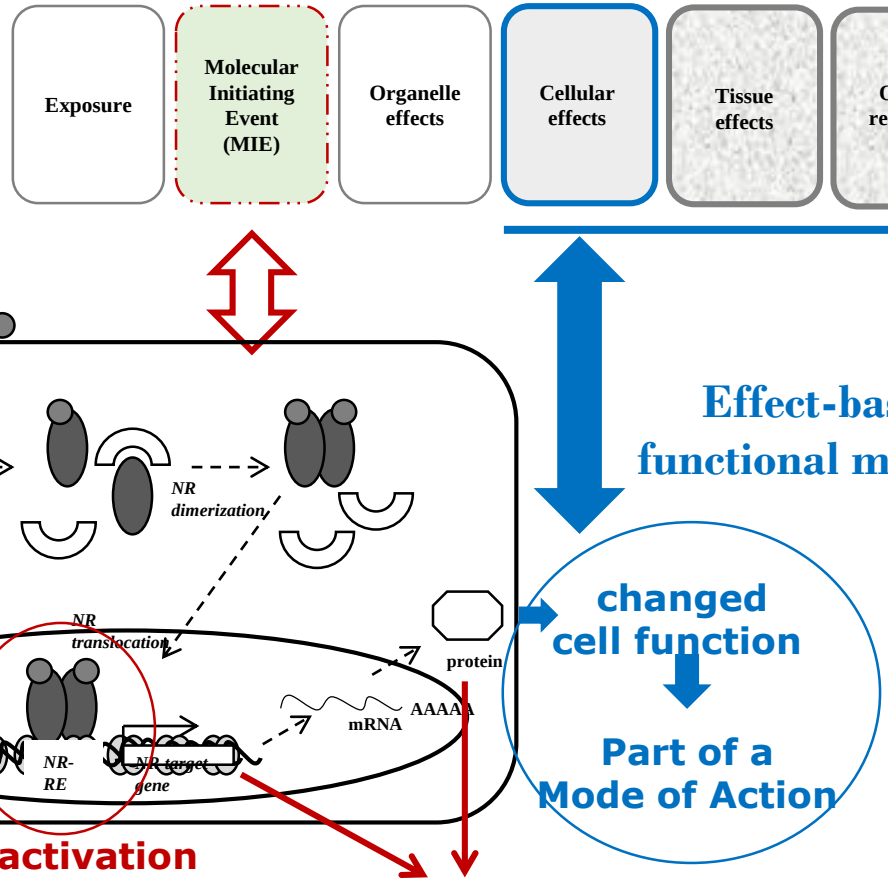


- **PSA secretion – androgen disruption in prostate epithelium**
- **βhCG secretion – estrogen disruption in trophoblast-like cell (placenta)**
- **AFP secretion – metabolic disruption in liver**

SCREENING FOR ENDOCRINE DISRUPTION:
mechanism- *vs* effect based-approach – the case of DEHP /phthalates

In vitro **screening of EDs by any AR-gene reporter assay could never detect its Mode-of Action: anti-androgenicity**

Adverse Outcome Pathway (AOP)



Effect-based functional markers

changed cell function

Part of a Mode of Action

transcriptional activation

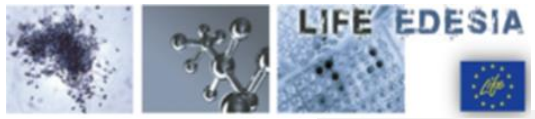
Part of a Mechanism of Action (incl. toxicogenomics)

molecular markers:
mRNA, miRNA, proteins, metabolites

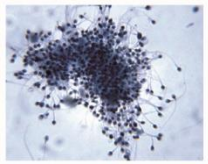
WHICH BIOMARKERS TO SCREEN FOR:

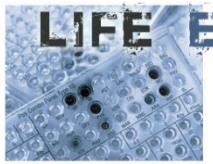
- **AN ENDOCRINE ACTIVITY ?**
- **AN ADVERSE EFFECT ?**

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**





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

LIFE12 ENV/IT/000633

LIFE-EDESIA in ACTION(s)

A. Preparatory actions

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

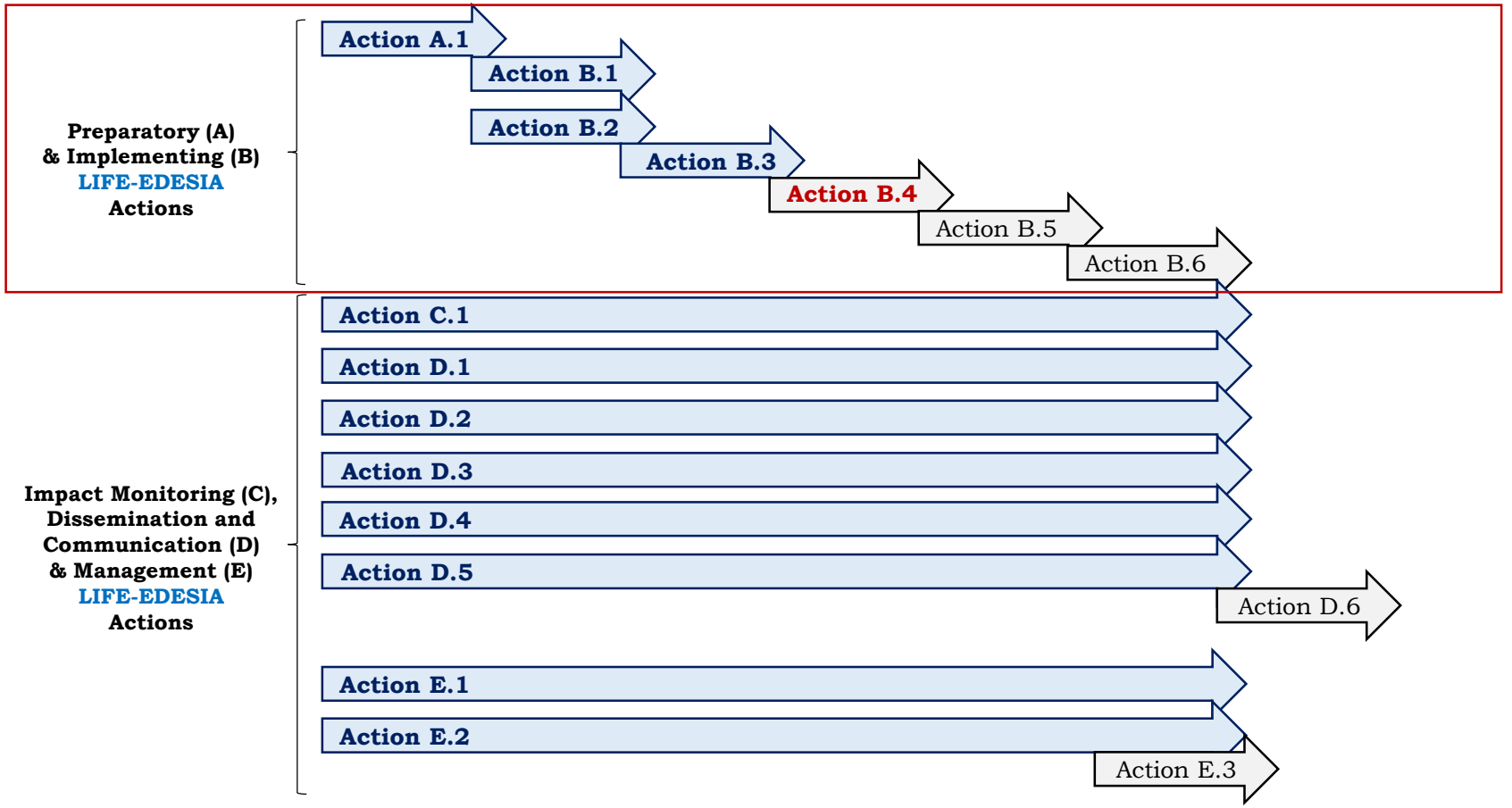
E. Project management and monitoring of the project progress (obligatory)

E1 Project management

E2 Project monitoring

ISTITUTO SUPERIORE DI SANITÀ

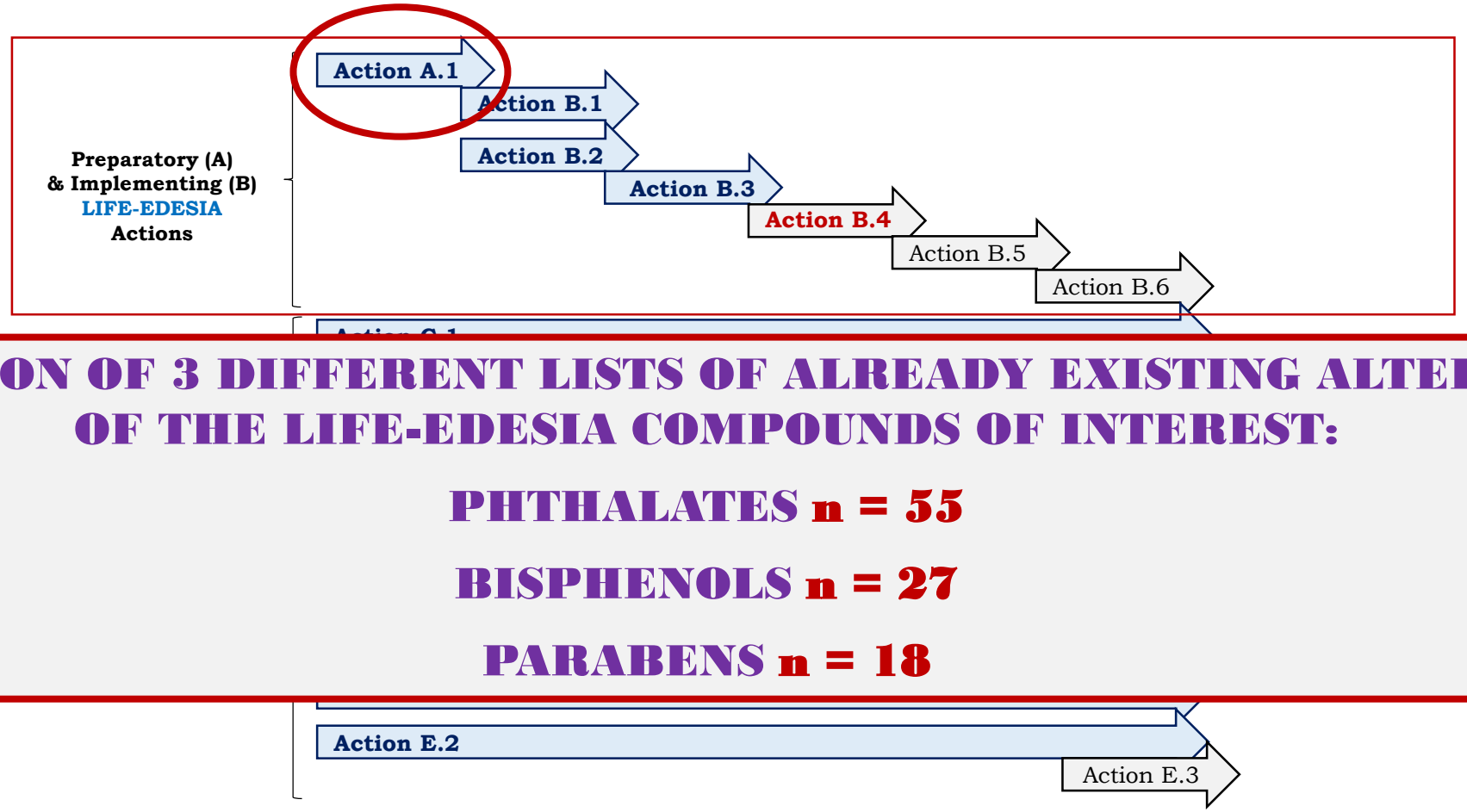
Progress Update – September, 2016



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 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

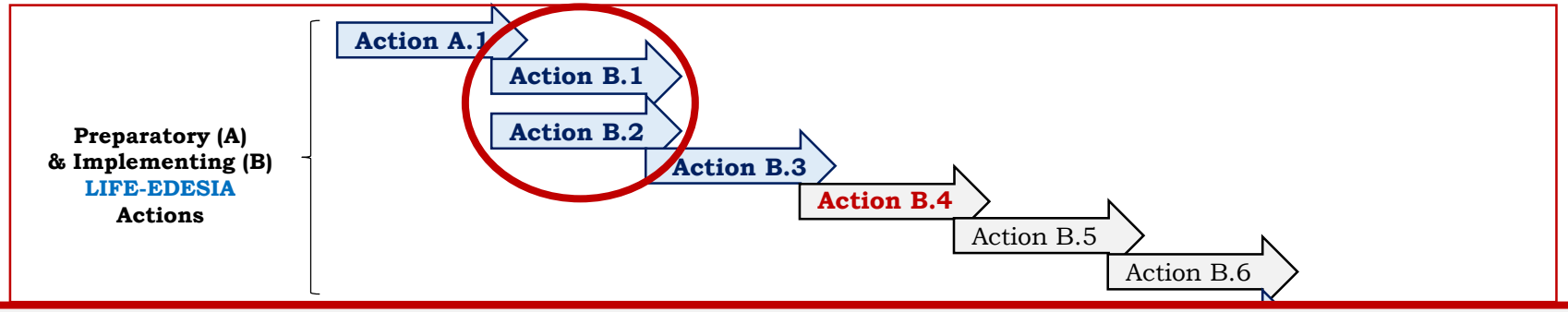
BISPHENOLS n = 27

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

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



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

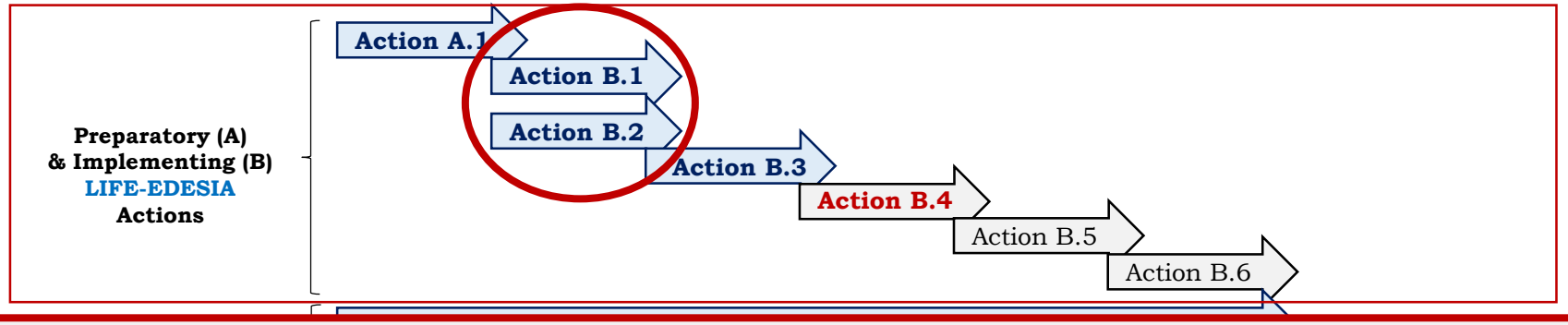
(*e.g., cancerogenic, mutagenic, binding to nuclear receptors*) assessed by tools implemented in the VEGA platform on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life*

(***data available on request***)

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 3

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



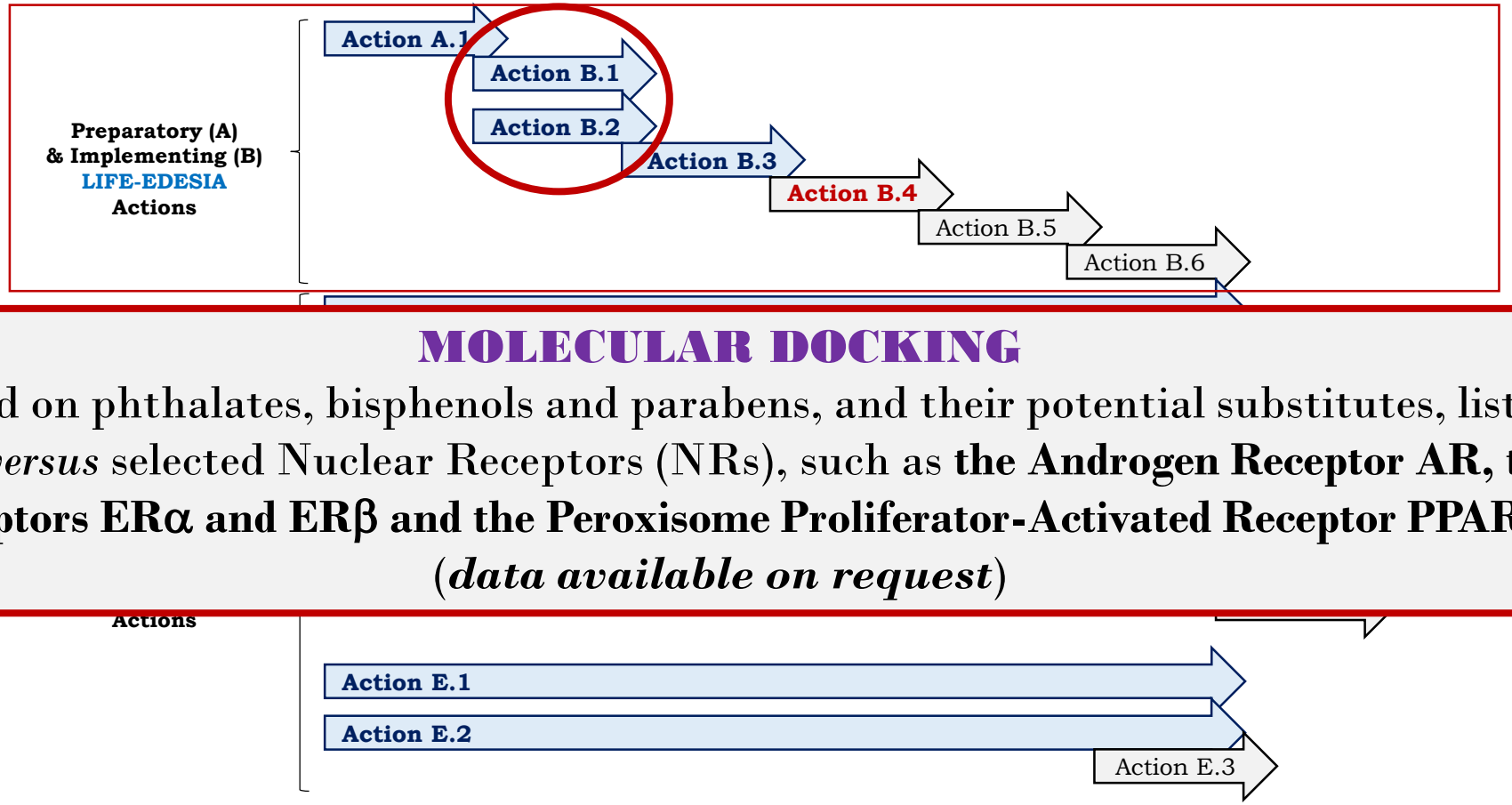
QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIP (QSAR)

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***)

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 4

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



MOLECULAR DOCKING

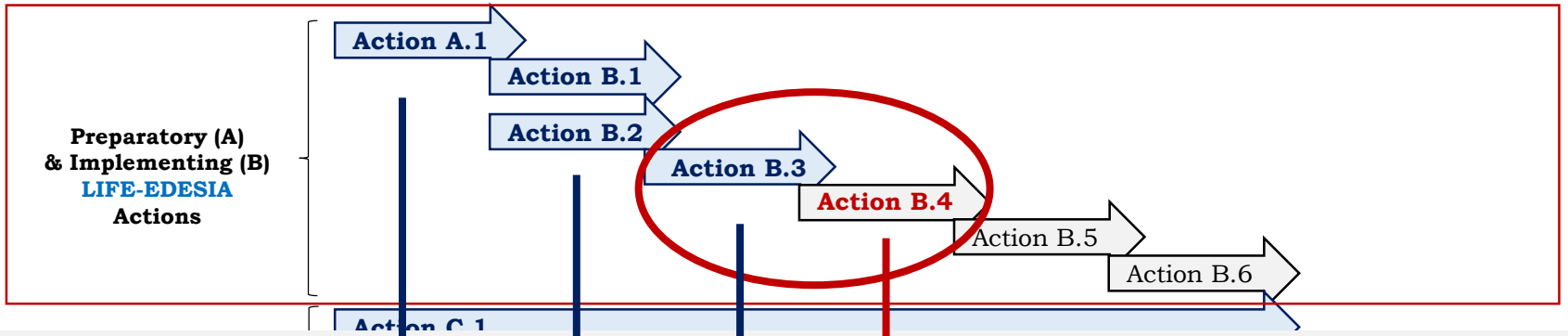
performed on phthalates, bisphenols and parabens, and their potential substitutes, listed on *www.iss.it/life*, *versus* selected Nuclear Receptors (NRs), such as **the Androgen Receptor AR, the Estrogen Receptors ERα and ERβ and the Peroxisome Proliferator-Activated Receptor PPARγ**

(***data available on request***)

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 - CONCLUSION 1

In silico **selection (by Benfenati group in Milan and Cozzini group in Parma) of alternatives to be tested** ***in vitro*** **upon their chemical synthesis (by Perissutti group in Naples)**



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.

in vitro studies using ED-dependent functional biomarkers

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.**

in vitro studies using ED-dependent functional biomarkers

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

in vitro studies using ED-dependent functional biomarkers

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.

ACTION B.4
THE ACTIVITY *IN PROGRESS* - 6

in vitro studies using ED-dependent functional biomarkers

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**) •

• **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.**

ACTION B.4
THE ACTIVITY *IN PROGRESS* - 7

in vitro studies using ED-dependent functional biomarkers

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**) •

• **OECD guidelines for the testing of chemicals** http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

… & CONCLUSION 2

Gene reporter assay(s) DO(ES) NOT IDENTIFY A MODE OF ACTION but only AN ENDOCRINE DISRUPTING PROPERTY - part of a potential mechanism of action of a chemical - whose endocrine disruption property remain to be assessed… ! :

by a functionally-relevant endpoint (as in LIFE-EDESIA)

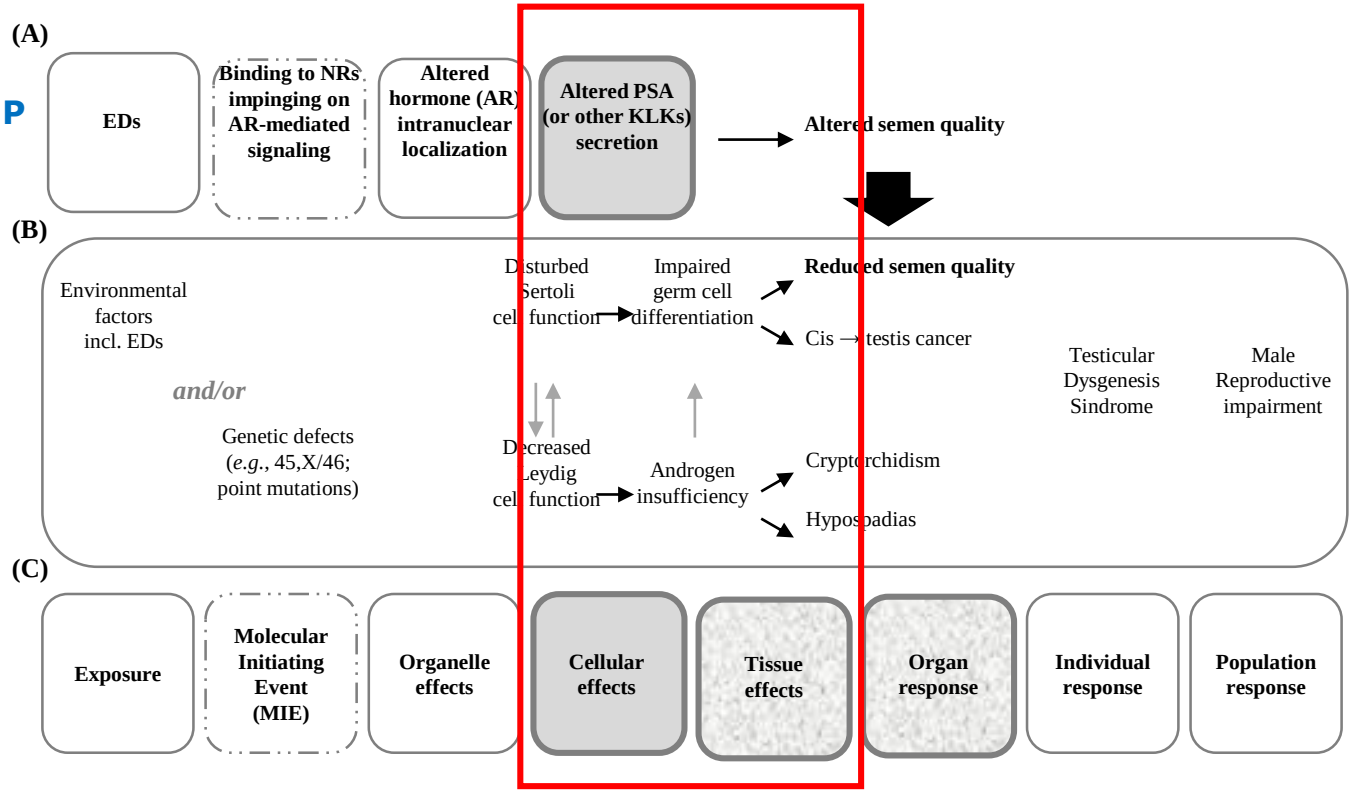
Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 1

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).

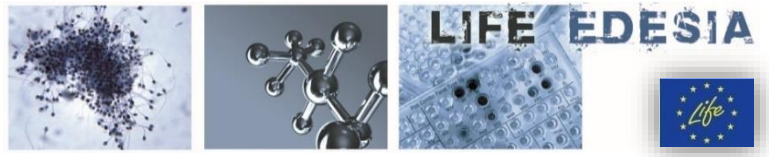
In vitro model (LAPC4 & LNCaP prostate epithelium)

Testicular Dysgenesis Syndrome (TDS)

Adverse Outcome Pathway (AOP)



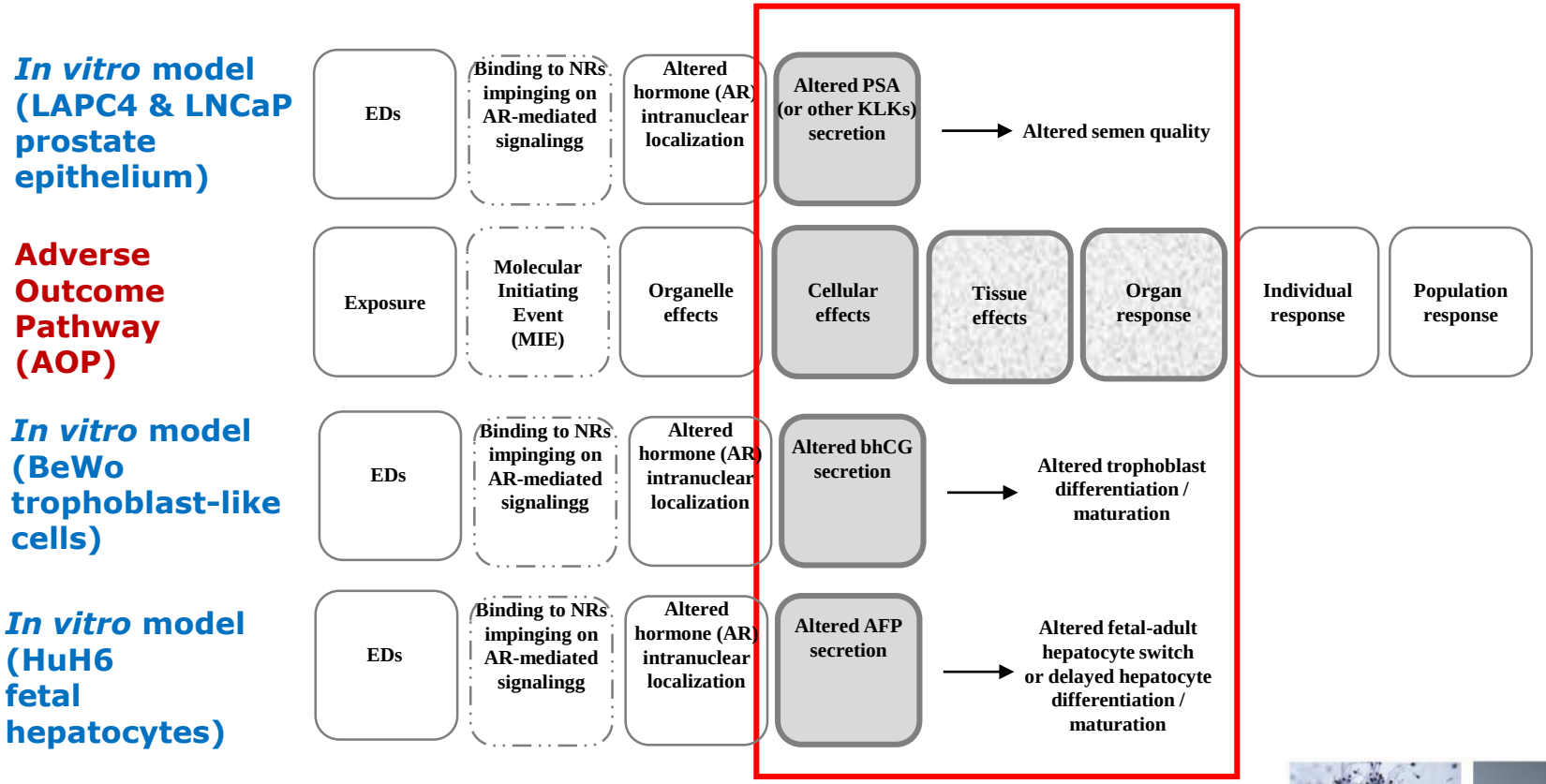
Adapted from Lorenzetti *et al.*, Annals 2015



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

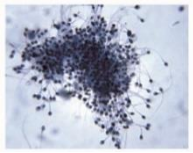
Biomarker-based, cell-specific bioassays as the best screening approach to build an Adverse Outcome Pathway for Endocrine Disruption - 2

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



Adapted from Lorenzetti *et al.*, Annals 2015

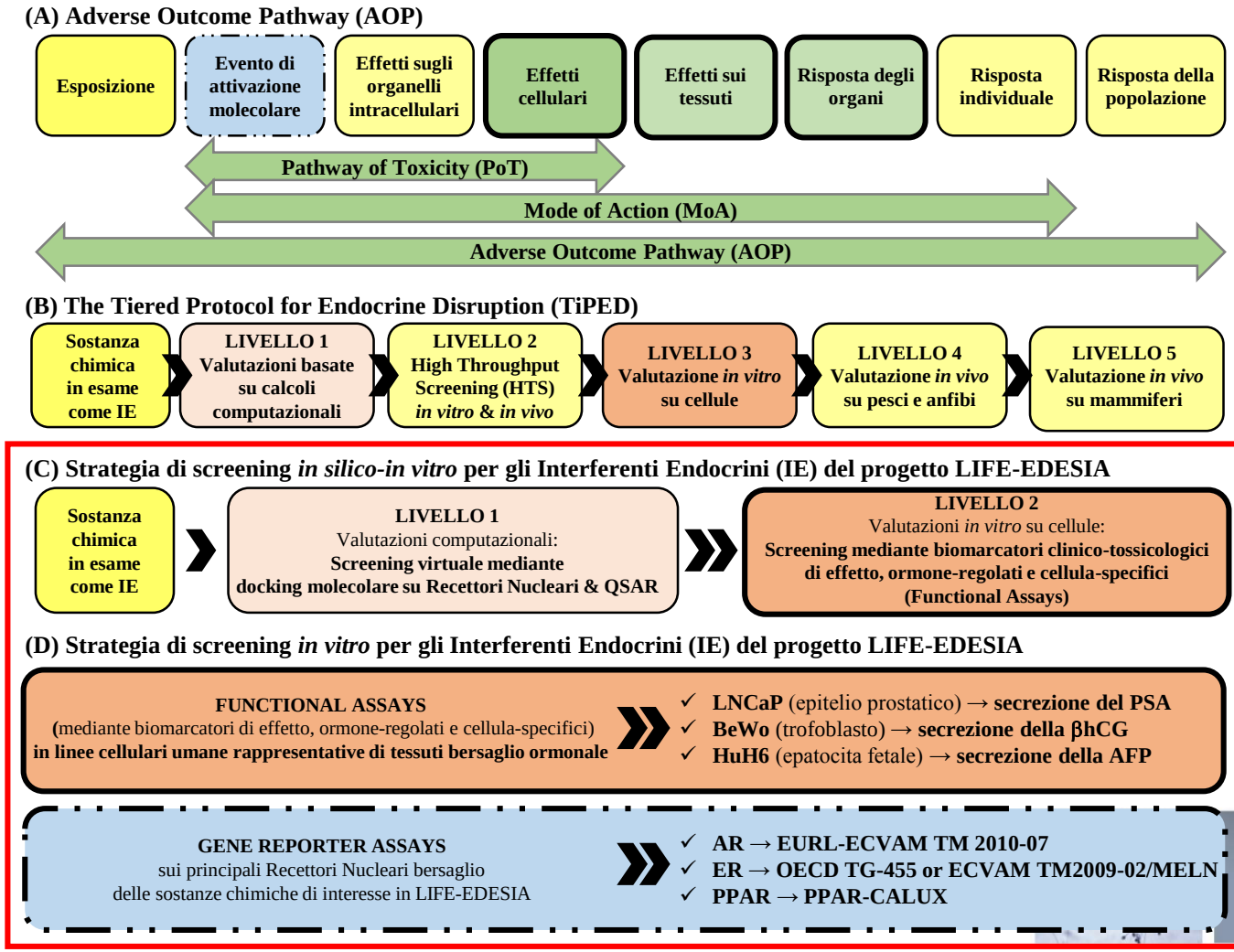
see also Verze *et al.*, Nature Rev. Urology 2016



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

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).

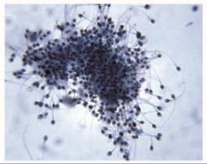


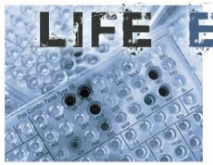
Adapted from
Lorenzetti *et al.*, Annals 2015

ISTITUTO SUPERIORE DI SANITÀ

LIFE EDESIA

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





ACKNOWLEDGMENTS 1

Grazie

ISS

Alberto MANTOVANI
Stefano LORENZETTI
Daniele MARCOCCIA
Monica GIULIVO
Chiara CLEMENTINI

IRFMN

MARIO NEGRI
ISTITUTO DI RICERCHE FARMACOLOGICHE

Emilio BENFENATI
Alessandra RONCAGLIONI
Maria Pina INCISIVO
Pier Paolo LA FAUCI

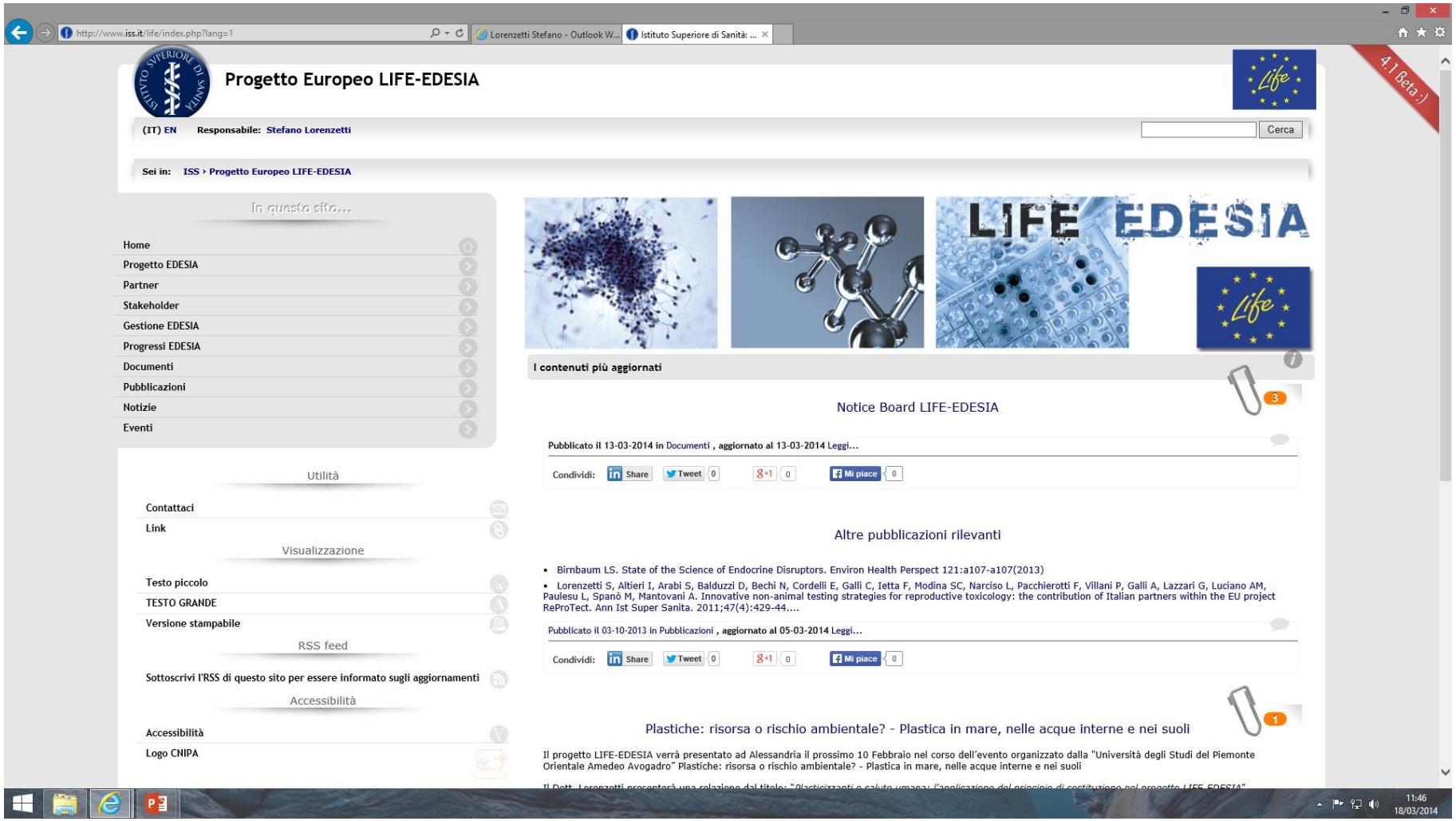
UniNAPOLI

Elisa PERISSUTTI
Ferdinando FIORINO

UniPARMA

Pietro COZZINI
Luca DELLAFIORA
Tiziana GINEX

ACKNOWLEDGMENTS 2

questions are wellcome &…

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