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Workshop W13

Non-Monotonic Dose-Response Curve in Hormonally Active Substances

Dose-response relationship: monotone vs non-monotone curve

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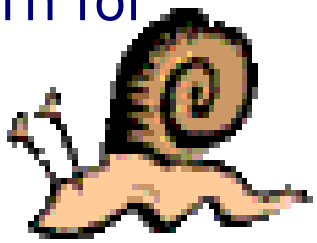
ISTITVTO SVPERIORE DI SANITÀ

The evidence that exposure to some environmental contaminants **interferes with the endocrine system** in wildlife animal populations (e.g.: **imposex in bivalves and crustaceans, effects on fish**) leads to some concern for similar effects in humans.



Some chemicals can indeed interfere with one or more steps regulating the functions of the endocrine network.

The interaction between the chemical and the endocrine system can lead to an **'homeostatic response'** which may progress to an adverse effects when the so called **"threshold of adversity"** (Piersma et al, 2011) is exceeded (that is the homeostatic control is overcome).

Chemicals inducing non adverse interferences can be defined as **EAS: Endocrine active substances** (EFSA, 2010; 2013).

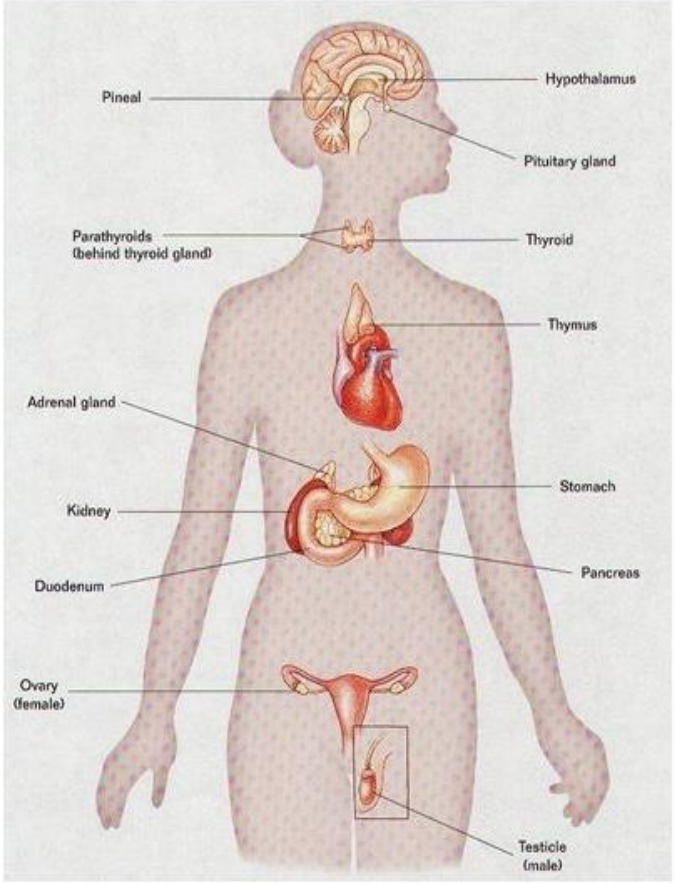
Man-made chemicals and by-products released in the environment with EAS properties

- industrial chemicals (e.g. PCB, dioxins).
- pharmaceuticals (e.g. contraceptive pills, drugs to treat hormone-responsive tumors)
- pesticides (e.g. DDT e chlorinated compounds)
- ingredients in consumer products (e.g. phthalates)

Natural chemicals produced by plants and mushroom with known EAS properties (phytoestrogens):

- genistein, cumestrol and isoflavones in soya beans (exposure via diet: food or food supplements)

When an EAS can be considered an Endocrine Disruptor ?

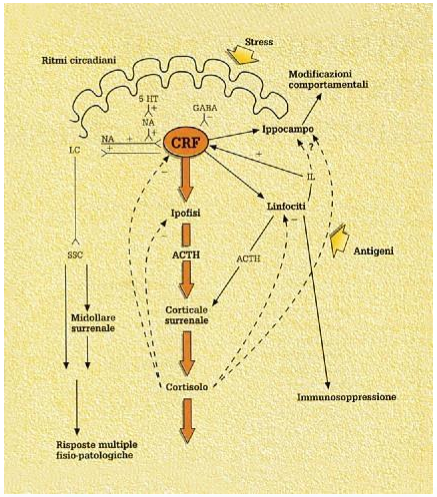


The International Programme for Chemical Safety (2002) defined *endocrine disrupters* as follows:

Endocrine disrupters are exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations.

The same definition has been endorsed by EFSA in its opinion on ED (2013) and by WHO-UNEP (2013).

***"An ED is an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action"* (Zoeller et al., 2012), adopted by the Endocrine Society**



This and other definitions are ambiguous since they do not allow to distinguish between:

- alterations falling within the range of physiological reactions as response to a stimulus thank to the plasticity of the endocrine system (*endocrine modulation* or **adaptive responses**)

and

- Alterations leading to the onset of an **adverse effect** (i.e. an exaggerated undesired response to a stimulus obtained at the wrong time/level causing the loss of hormonal homeostasis)

The endocrine system is specifically designed to respond to external stimuli. Such adaptive responses are characterised by discrete changes of our hormonal status (measurable endocrine changes) without consequent adversity in a living organism, when transient and within the normal homeostatic range (Goodman et al., 2010; Rhomberg et al., 2012) :

- ✓ **reaction to food** (e.g.: insulin secretion following sugar-reach food ingestion)

- ✓ **emergency reactions (**e.g.: adrenalin secretion in case of stress or danger),
- ✓ **reactions related to sexual behaviour**

Adverse effect definition (WHO/IPCS, 2004) :
"*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*".

Further US EPA "...does **not consider endocrine disruption to be an adverse effect *per se*, but rather to be a mode or mechanism of action potentially leading to other outcomes,** e.g. cancer, reproductive/developmental toxicity. A similar position has been endorsed by EFSA (2013).

There are at least two minimal requisites needed for defining a chemical as an ED : induction of an **adverse effect** through a MoA compatible with **'endocrine disruption'** (biological plausibility).

Mechanisms through which a chemical can interfere with the functioning of the endocrine system are various:

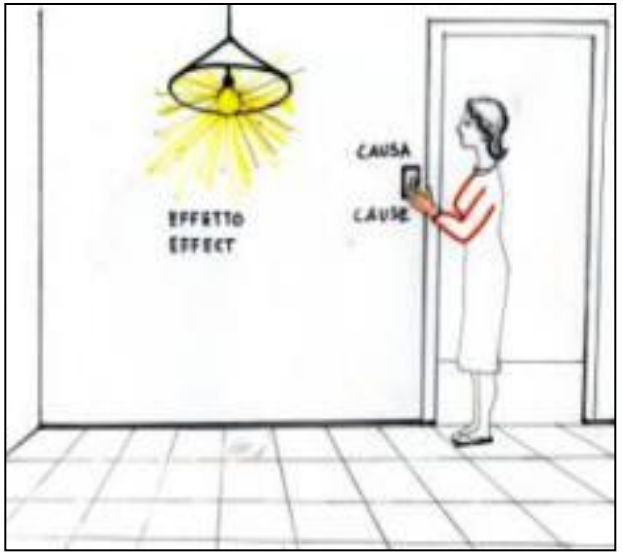
- ✓ **mimicking** a natural hormone action activating similar reaction in the organism (e.g.: receptor binding followed by a cascade of events);
- ✓ **blocking** hormone receptors in target cells, preventing the physiological action of natural hormones);
- ✓ **altering synthesis, transport, biotransformation or excretion (i.e. kinetics) of hormones**, affecting their circulating concentration (e.g.: PCBs are potent sulfotransferase inhibitors; the enzymes conjugate estradiol before excretion. Their inhibition could in principle prolong the action of endogenous estrogens, an alteration potentially relevant for some hormonally-dependent tumors).

Indipendently on the mechanism, a chemical can be defined as an **ED** when its biological activity results in an **adverse effect**

Beside the biological plausibility of the MoA, the term '***consequently***' in the WHO definition indicate the need of a **cause-effect relationship between the endocrine activity and the adverse effect.**

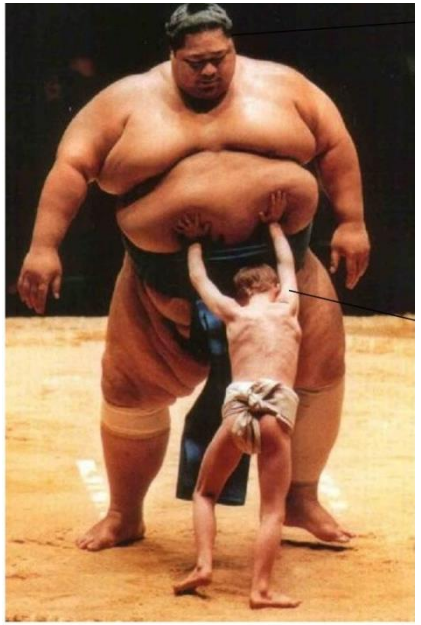
At present general/standardised **criteria** to check this kind of causality are **not available** but a simple co-occurrence of the two events are not *per se* a proof of causality.

Opinion on this topic are different, often contrasting: the toxicological relevance of data available is left to the **expert judgement** on a **case-by-case** basis in a very **transparent** way, using a **WoE approach**, without any prejudice.



The **potency issue** has to be taken into account in order to understand the relevance for humans.

Exogenous chemicals in order to 'efficiently' affect the endocrine system must act against the background of **circulating levels of endogenous hormones**, which are usually much more potent than any exogenous ED.



Estrogenic potency: known environmental estrogens are generally 3-7 orders of magnitude weaker than endogenous hormones (e.g. nonilphenol has an estrogenic activity as low as $1/10^5$ when compared to 17β-estradiol.

Therefore human exposure to the most potent environmental estrogens would need to be at least 1000-fold higher than this level, for adverse effects relevant to the human male to be induced, and such levels of exposure are remote (Sharpe 2003; Borgert et al, 2012; Nohynek et al., 2013)

Chemical	Use/origin	Effective Dose* (mg/kg/day)
Diethylstilbestrol (DES)	Drug	0.0001
Ethynilestradiol	Contraceptive	0.0003
Estrone	Estrogen	0.0012
Cumestrol	Legumes	0.03
Genisteine	Soya	8
4-MBC	Sun filter	300
Buthyl-paraben	Preservative	600
Benzyl-paraben	Preservative	2500

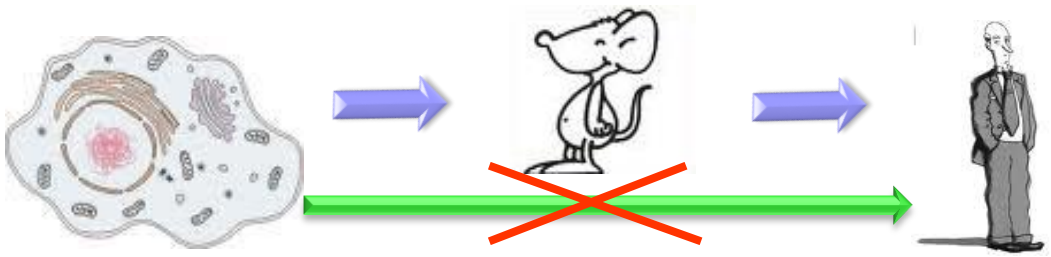
$>10^7$

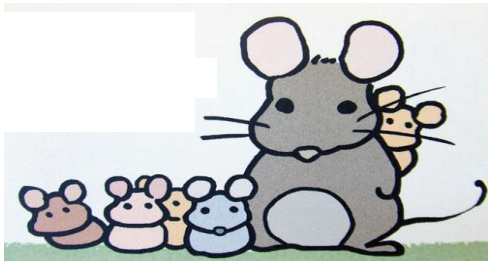
* uterotrophic test (screening test on rats – oral treatment)

The term '**intact organism**' in the WHO definition indicates not only that at present ***in vivo* studies** are needed to identify an ED, but also the limited relevance of *in vivo* models in which animals have been **'manipulated'** (e.g. castrated males or ovariectomised females).

These animals have **lost integrity** of their hypothalamic–pituitary–gonad (HPG) endocrine axis, the normal **dynamic physiological capacity to compensate** for the actions of external hormonal activity has been compromised; whilst useful for identifying compounds with relevant hormonal/anti-hormonal activity, such artificial systems have limited utility in evaluating risk of the compound in question.

Evidences exclusively coming from ***in vitro* studies** are not sufficient to define an ED (e.g. due to lack of kinetics). They can be used to identify a potential EAS, possible MoA, to prioritize for further testing (e.g. in the tiered approach described by the OECD framework or in the EPA strategy for ED).





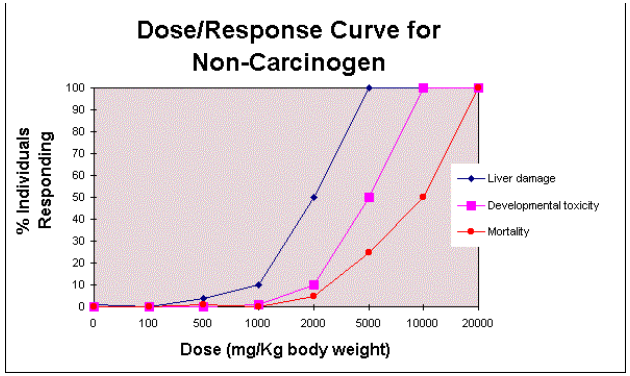
Another relevant factor is the existence of **critical exposure windows**, during which the susceptibility of the organism is increased (e.g.: in utero exposure during the organogenesis period).

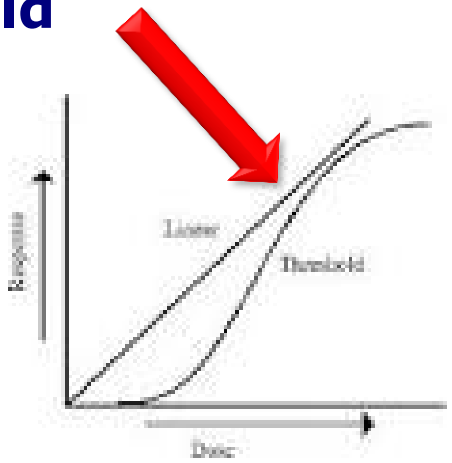
Some critical windows are covered by the internationally accepted methodology (e.g.: multi-generation reproductive toxicity) but this is not always the case. By knowing the MoA it is possible to understand whether long latency effects are covered. However in the animal-to-human extrapolation it is crucial to consider **species differences in the endocrine system**

Pregnancy:
The estrogen circulating concentration are 100 fold lower in mice vs. humans ⟹ **pregnant mice can be more susceptible than pregnant women to estrogenic compounds.** In women estrogen are mainly of placental origin, while in female mice are of maternal origin (*corpus luteus*).

A debate exists on the applicability of the actual Risk Assessment paradigm, mainly related to difference positions on the possibility that ED can:

- **Act without a threshold**





- **Result in a non monotonic dose-response (NMDR) curve** in any point in the range of concentrations tested, from the true "low dose" range up to the high pharmacological range (Vandenberg, 2014).

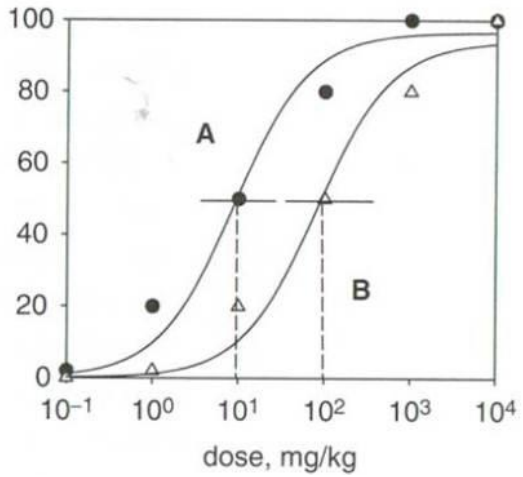
Starting from Paracelsus statement

Omnia venenum sunt: nec sine veneno quicquam existit. Dosis sola facit ut venenum non sit

(All substances are poisons. The dose makes the poison)

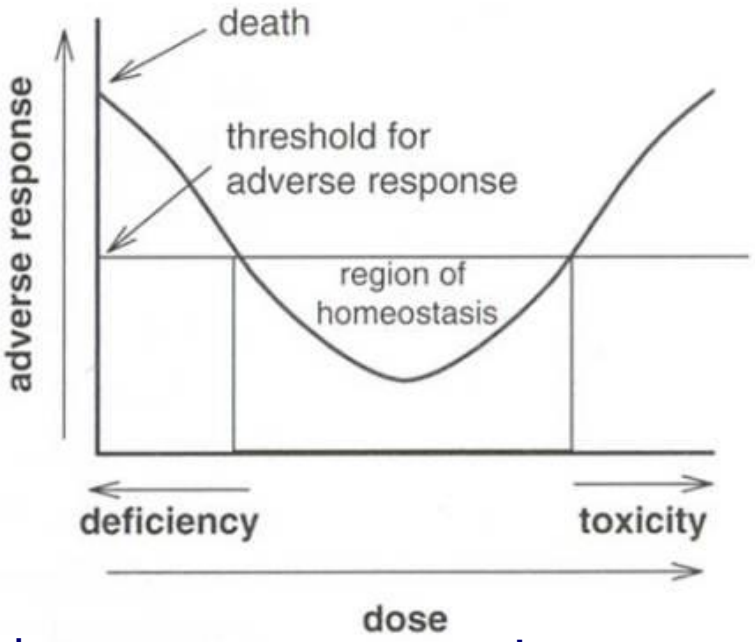
the paradigm in toxicology and risk assessment is that the individual response of an organism to a chemical increases/decreases proportionally to the exposure (dose).

This gives rise to a monotonic dose-response relationship
In monotonic responses the effect either increases or decreases over the full dose range tested.



It is generally accepted that for most chemicals (with no genotoxic potential) there is a **threshold dose** below which there is no adverse effect.

The possibility exists that two competing monotonic curves overlap to produce effects that manifests as a **non monotonic dose-response relationship occurs (NMDR)** with U-shaped or inverted U-shaped profile



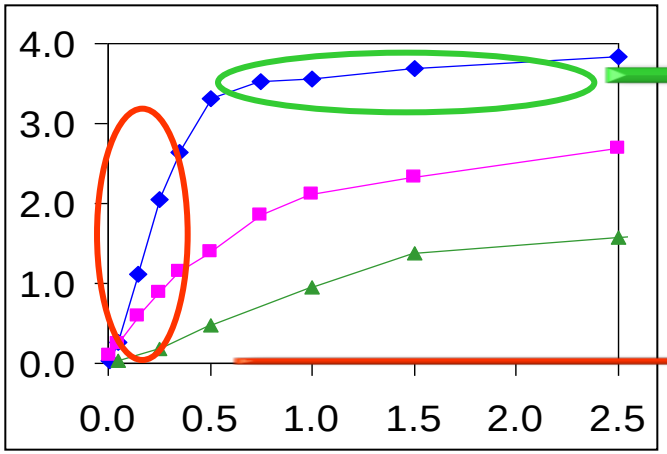
The case of **ETE** is well known. As an example the effects due to copper deficiency are much more severe than the ones due to the its excess.

A dose-response curve is non-monotonic when **the slope of the curve changes sign** somewhere within the range of doses examined. **Non monotonicity is not synonymous with low dose.**

Low dose effects are defined as any biological changes occurring in the range of typical human exposures, or biological changes that occur at doses below those used in traditional toxicology studies (Melnick et al., 2002).

Non linear relationships are not uncommon in toxicology, especially when the **kinetic behaviour** is concerned and the MoA is dependent on the concentration at the target site:

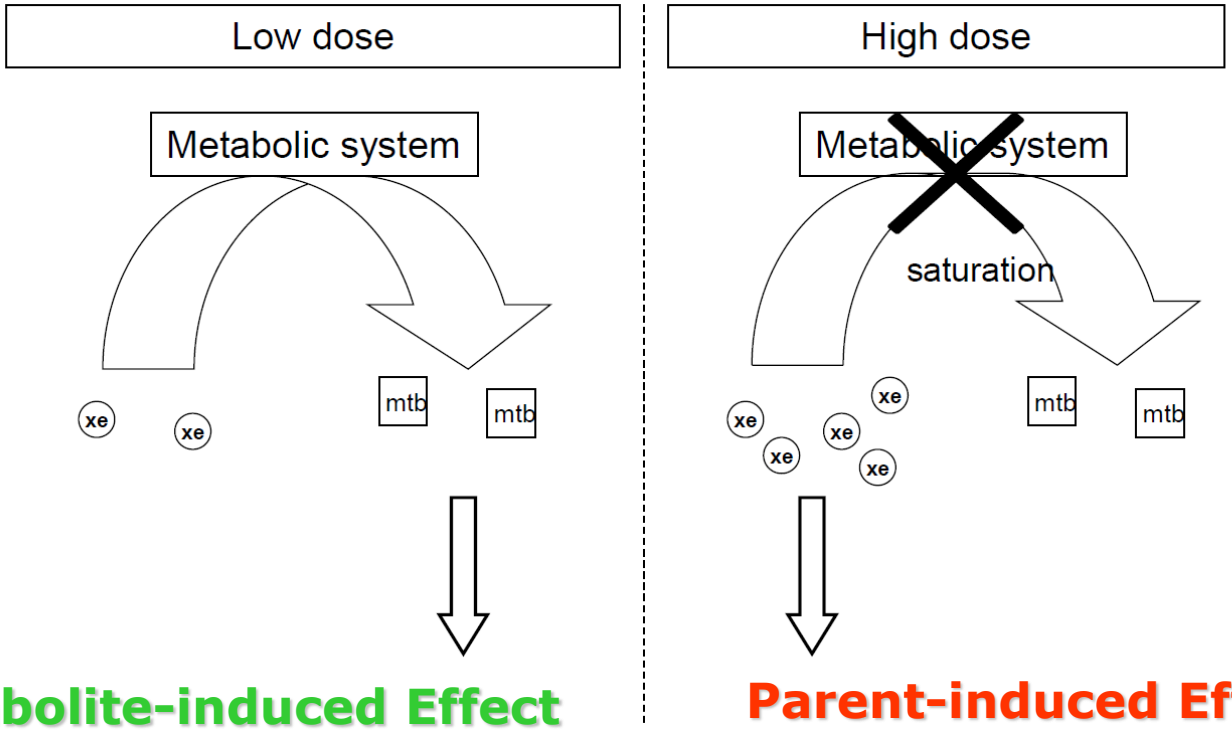
- ✓ 2 antagonist receptors with different affinities and actions
- ✓ 2 enzymes involved in the biotransformation with different affinities and producing different metabolites, with those produced at higher doses masking/counteracting the effects induced by metabolites produced at lower doses
- ✓ saturation, induction/inhibition of biotransformation enzymes of a single metabolic pathway



High doses: saturation of metabolites formation-accumulation of the parent – possible different effect or counteracting effects due to metabolites

Low doses: the effect due to metabolites increases with the dose

- at **low doses** the effects due to the metabolite are evident
- at **high doses** the effects due to the parent are evident possibly masking the ones induced by metabolites because of enzyme inhibition (e.g.: sustrate inhibition or 'suicide' irreversible inactivation)



Metabolite-induced Effect

Parent-induced Effect

(Modified from Lagarde et al, 2015)

NMDR can be observed in studies in which high-doses tested alters the experimental model (cell, organ or animal), thus decreasing the observed response.

This could occur when **cytotoxic doses** are tested in in vitro studies or in vivo, when using doses that are excessively toxic to animals (doses **exceeding the maximum tolerable dose**): the reduced viability can reduce the onset of an effect.

The same could happen when the formation of aggregates, colloids or micelles at high concentrations can **reduce bioavailability** thus apparently decreasing the degree of toxicity that appeared at lower concentrations

It is likely that these phenomena could contribute to generate only apparent NMDR

The low doses hypothesis impacts on RA challenging the regulation of chemicals: this would require careful evaluation of the shape of the dose-response curve, scientifically-based decisions regarding the adverse nature of the effects seen, and consideration of study designs incorporating if needed also endpoints beyond current OECD methods.

- ✓ The presence of a response at one dose level only is not sufficient to demonstrate a causal relationship or a NMDR by comparison with other studies.
- ✓ A wide dose range and reasonably closely spaced dose intervals (<10-fold within the same study) is necessary to demonstrate U-shaped dose-responses with statistical plausibility
- ✓ Poorly described experiments in non-validated models should not be used.
- ✓ Data should be reproducible by different groups

Up to now no scientific consensus has been reached

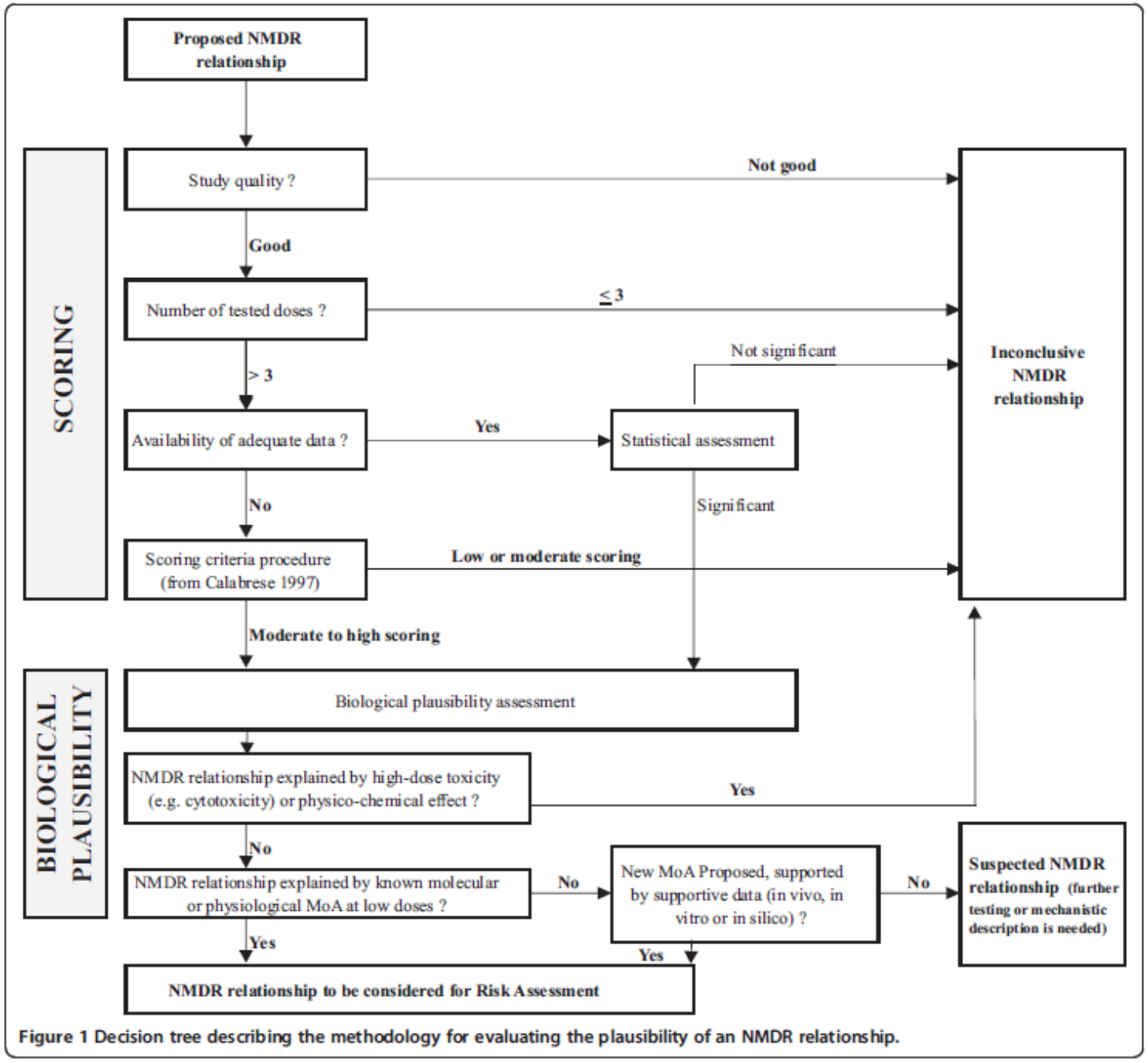


Figure 1 Decision tree describing the methodology for evaluating the plausibility of an NMDR relationship.

Decision tree for NMDR analysis as proposed by Lagarde et al. 2015

Zoeller and Vandenberg (2015) published a comment to the Lagarde et al (2015) paper specifically on three elements :

1) the use of Klimisch scores to evaluate study quality,
2) evaluating study quality without topical experts' knowledge,
3) the requirement of establishing the biological plausibility of an NMDR before consideration for use in risk assessment.

In favor of

1) dismissing the Klimisch score for assessing study quality;
2) evaluation of study quality requiring experts in the specific field
3) understanding of mechanisms not required to accept observable, statistically valid phenomena.

Vandenberg et al. (2012) reported a high number of **NMDR** to show how **common** they are in the area of ED.

More recently (2014), Vandenberg published a pilot study of the **BPA *in vitro* literature** illustrating that **NMDR** occur in more than 1/5 of experiments and in more than 1/3 of studies that were appropriately designed to assess dose responses (i.e. studies that examined more than one dose and identified at least one endpoint affected by BPA)

The **Danish Centre on Endocrine Disrupters** examined the evidence presented by Vandenberg et al. (2012) for NMDRs (DTU Food, 2013) :

1) the **majority** of data were from **in vitro studies** and inappropriately included findings for which the U-shaped or inverted U-shaped curves were the product of general toxicity
2) 45% of the in vitro examples cited were the result of **cytotoxicity** and thus were not examples of true NMDRs
3) one-third of the remaining examples were judged to be false; another one-third were considered questionable
4) only **5 out of the 34 in vivo examples** cited were considered to show "clear evidence" of NMDRs

Examples of NMDRs do exist, but they are not as common as Vandenberg et al. (2012) suggests.

USEPA also conducted an expert review of the experimental evidence for NMDRs (USEPA,2013):
they are not uncommon in *in vitro* studies and often relate to 'lower-order biological endpoints' rather than apical endpoints.

Assays that provide data at a lower level of biological organization (such as proteomics or transcriptomics) are more likely to identify NMDRs than studies that provide data on apical adverse events further downstream from the molecular initiating event.

***"There is currently no reproducible evidence that the early key events involved in the expression of NMDRs that are identified at low doses are predictive of adverse outcomes that may be seen in humans or wildlife populations for estrogen, androgen or thyroid endpoints"*(USEPA,2013).**

***Reproducibility* of NMDRs is important in establishing *plausibility* of a response and its potential applicability as part of the hazard characterization. It is influenced by i) study design, ii) robustness of physiologic compensation producing changes in slope and iii) competing processes– induction of metabolism, repair, or independent mechanisms.**

NMDRs for adverse effects have been occasionally seen in intact organisms for effects involving estrogen, androgen or thyroid hormones.
When observed, there is not sufficient evidence of NMDRs for adverse effects below the NOAELS or BMD. For some MoA the scientific database remains too limited to conclude.

NMDRs are relatively uncommon and when reproducible occur above NOAEL/BMD

Thus, the current testing approaches do not fail to identify or establish appropriate NOAELS in the low rose range of exposure, even if not all effects for every chemical are identified. The goal of chemical testing is to identify the potential for hazard after exposure to the xenobiotic of concern, not to identify and describe 100% of all the possible biological effects.

NRC revised the draft USEPA evaluation and recommend to improve aspects related to systematic literature searches, data extraction, and evaluation of the evidence on NMDR relationships (NRC-NAS,2014) .

Risk vs hazard

A **hazard-based approach is not appropriate** as the starting point to take decisions on the identification and related regulation for EDs (as for any other chemical)

What about some **extreme cases**?

Hydrogen Oxide H_2O at high doses can alter aldosterone levels, renine and angiotensine, causing strong effects up to death.

Is water an ED? based on hazard…. It seems so!

GRAZIE!

Obrigada!

Thank you!