- **IATA definition**

INTEGRATING APPROACHES TO TESTING AND ASSESSEMENT
"a structured approach that strategically integrates and weights all relevant data to inform regulatory decisions regarding potential hazard and/or risk and/or the need for further targeted testing and therefore optimizing and potentially reducing the number of tests that need to be conducted."

Integrated Approaches to Testing and Assessment (IATA) are flexible approaches for chemical safety assessment based on the integration and translation of the data derived from multiple methods and sources.

In addition to traditional in vitro and in vivo tests, IATA can incorporate new approach methodologies such as high throughput screening and high content imaging methods, along with computational methods that are used not only for data generation, but also interpretation and integration.

IATA components may also include defined approaches, which are rule-based approaches that make predictions based on multiple sources of data. The design and application of IATA involves a degree of expert judgement.

Comprende in vivo, in vitro, in silico, in chemico, oics tecnologies

- **What is meant for validation?**

Means to identify the Reliability, Relevance, Reproducibility and Predictivity of a method.

Validation process follow some steps:

1. Development of Tests Scientific Basis (Field of Application, Test Protocol, Prediction Model)
2. Prevalidation (Improvement of Tests Protocols, Inter-laboratory Comparison, Improvement of Prediction Model)
3. Validation (Interlaboratory Test under Blind Conditions, Relevance & Reliability for the Field of Applications Designated)
4. Indipendent Evaluation (peer review)
5. Official Approval

- **Skin irritation and alternative methods**

Skin irritation is a local inflammation, of non-immunological origin, characterized by erythema, edema or necrosis due to single or repeated exposure to a substance. An irritating potential can cause effects on the stratum corneum, epidermis and dermis.
Skin irritation Is define as a reversible damage of the skin (test substance for 4 h)

Some factors such as age, sex, genetic factors, application site, Membrane permeability, Frequency and method of use (short term, long term ecc) can influence the toxicity of the substances.
Skin irritation can be caused by Chemicals, Cosmetics Drugs and biologics, Food and Feed Additives

REGULATIONS

- Cosmetics Directive (76/768/EEC)
- Classification, Labelling and Packaging (CLP) Regulation
- REACH Regulation

TEST:

IN VIVO TEST – DRAIZE TEST for irritation (rabbit test for skin corrosion/irritation testing)

ENDPOINT: Severe erythema, Erosions, Ulcers, Abscesses Necrosis

We assign scores for the test about Formation of erythema and eschar (0-4) and Edema formation (0-4)

IN VITRO

We can use:

- Monolayer of keratiocytes
- 3D culture models: barriers
- 3D models air-lift technique
- Stem cells

Validated EU models OECDTG 439 (EU B-46) :

are based of Reconstructed Human Epidermis (RHE) Model Test

- EPISKIN (MTT reduction and IL-1a release)
- SkinEthic RHE (with MTT reduction)
- EPIDERM MODIFIED (with MTT reduction)

All of these are test of **REPLACEMENT** with endpoint cell viability (misured by MTT test). Episkin have as endpoint also interleukine -1a release

These are test for chemicals and they make a differentiation between substances classifiable by irritation (No Cat.) and irritants (Cat 2) not for weak irritants (Cat 1)

They are not suitable for Not for aerosols and gaseous substances

- **OECD definition**

Organization for Economic Cooperation and Development

is an international organisation in which governments work together to find solutions to common challenges, develop global standards, share experiences and identify best practices to promote better policies for better lives.

- **TG definition**

TG stands for “Test guidelines”. Is an harmonising Guidelines for the Testing of Chemicals. Each guideline is specific for an aim and is identify by a number (es OECDTG346 for skin irritation)

- **BCOP LINK DA LEGGERE**

Bovine Corneal Opacity and Permeability

Is an in vitro assay that directly assesses the effects of potential irritant substances on the opacity and permeability of freshly obtained bovine corneas

Following exposure to a test substance/chemical, corneal opacity is directly measured with an opacitometer.

Corneal permeability is determined using sodium fluorescein (a dye that usually cannot pass through epithelial cells of the cornea) and measured spectrophotometrically through changes in optical density.

An endpoint irritation score is calculated from these data, which can then be used to compare irritancy potential of different test substances/chemicals.

BCOP assay was developed as an alternative to the Draize rabbit eye irritation test. With certain limitations, it can be used to screen for potential ocular corrosives and severe irritants.

It can also be used as part of wider sequential testing strategies and as part of weight-of-evidence approaches.
In a non-regulatory context it can also be used to elucidate biological mechanisms in appropriate experimental designs. According to regulatory guidelines, a positive result is accepted as indicative of a substance being a severe irritant or ocular corrosive. In this case no further (animal-based) testing is required. In the case of a negative result, substances should be assessed with other in vitro methods for eye irritancy or finally in rabbits.

SLIDE:
is an ex vivo models where eyes are a by-product of slaughter. Animals are killed for other pourpose
eyes must arrive within 4 – 5 hours since excision
The cornea is cut from the globe around outside and is lifted from the globe and placed on a holder (anterior and posterior). Test substances are applied to the anterior chamber of the corneal holder
Toxic effects of a test substance to the cornea are measured by its ability to induce opacity (opacitometer) and increased permeability in the isolated bovine cornea (visible light spectrophotometer)

- **Internal Dose concept**

Internal dose concept is considered the free concentration reaching the cellular target.
Lesson 7 ultime slide

- **Mest**

MeST mouse embryonic stem cell test – in vitro test used to detect the potential teratogenicity of the substances. Is used as a model to assess embryotoxicity in vitro.
The test can result in 3 different classics:

- Strong embryotoxic
- Weak embryotoxic
- Not embryotoxic

The ability of MeST to shift from a pluripotent to differentiated state makes them a optimal candidate as a screening tool for hazard identification
The test use 2 permanent cell lines:

- ESC D3 (representing embryonic cells)
- BALB/C 3T3 fibroblast (representing adult cells)

The test use the capacity of embryonic stem cells to differentiate spontaneously into contracting cardiomyocytes. The potential embryotoxicity is showing when there is an interference with the cell differentiation. The disturbance of the embryonic stem cells differentiation after the exposure at the substance indicate a potential embryotoxicity.
To predict the embryotoxic potential of a test substance, three endpoints are assessed:

1. inhibition of differentiation into beating cardiomyocytes (we evaluate the contractility)
2. cytotoxic effects on stem cells
3. cytotoxic effects on 3T3 fibroblasts.

EVALUATION OF CARDIOMYOCYTES CONTRATILITY

1. Drops of embryonic stem cells suspension are placed in the lid of petri dish and incubate.
2. After 3 days and aggregation of stem cells are transfer to a new petri dish.
3. 2 days later the embryoid body are transfer in a 24 well plates with the testing solution and incubate for 5 days
4. At the 10^{th} day it is possible to evaluate the cells

CYTOTOXICITY effects on D3 (murine embryonic stem cells) and BALBS 3T3 (fibroblast) are evaluated with MTT test

- **NAM definition**

With the term "new approach methodologies" (NAMs) we include any non-animal-based approaches that can be used to provide information in the context of chemical hazard and risk assessment. These new approaches include integrated approaches to testing and assessment (IATAs), defined approaches for data interpretation, and performance-based evaluation of test methods.

- **ITS definition**

Integrated testing strategies (ITS), as opposed to a single definitive test or fixed batteries of tests, are **expected to efficiently combine different information sources in a quantifiable fashion to satisfy an information need**, in this case for regulatory safety assessments.
The principle of ITS, as they are used in this context, is to incorporate multiple data from various information streams, derived from different test methods, test method batteries, tiered test schemes, modeling techniques

integrated testing strategies involve the use of multiple approaches for obtaining the information necessary for a regulatory assessment that circumvents the laundry-list of animal toxicity tests, where possible

Potential benefits of integrated testing strategy approaches include:

- Better protection of human and animal health and the environment by the rapid prioritization of more toxic materials for additional testing
- Reductions in animal testing requirements
- Time and cost savings

- **Skin corrosion and alternative methods**

Skin corrosion is an irreversible damage after an application of a test substance for 4 h.

IN VIVO TEST - Draize Test skin corrosion: TG 404 (Eu B4) Acute toxicity: dermal irritation/corrosion
REGULATIONS:

- Cosmetics Directive
- Classification, Labelling and Packaging (CLP) Regulation (1272/2008)
- REACH Regulation (1907/2006

Related cosmetics and chemical substances in category 1

EX VIVO TEST
Validated EU models TG 430 (EU B-40) are represented by:

- TER: Trans Epithelial Electrical Resistance ex vivo (TG430) 2004

Make a discrimination between CORROSIVE and NON CORROSIVE.
It is not for aerosols and gaseous substances
Characteristics:

➢ EXVIVO METHOD (in some references it is also reported IN VITRO)
➢ RAT SKIN (humanely killed rats 28-30 DAYS)
➢ DISCRIMINATION BETWEEN CORROSIVE AND NON CORROSIVE SUBSTANCES
➢ ENDPOINT: ALTERATION OF THE SKIN BARRIER

IN VITRO
There are other Validated methods based on RHE for in vitro skin corrosion

Validated EU models TG 431 (EU B-40bis) - In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Model Test
They are:

- EPISKIN
- EPIDERM SCT (skin corrosion test)
- SKINETHIC RhE
- epiCS (EST 1000)

they make a discrimination between CORROSIVE and NON CORROSIVE for chemical substances and mixtures, but it is limited the evaluation for formulation
ENDPOINT: CELL VIABILITY (with MTT test for all the models)

There is another test but it is not validated
TG 435 (NOT Validated EU)
It is not included in EU legislation because it is based on a synthetic membrane
CORROSITEX – in vitro test for acids/basis/certain derivates
➢Very limited application
➢Synthetic macromolecular biobarrier
➢ ENDPOINT: Colorimetric reaction

- **Skin sensitization alternative methods**

Skin sensitization is an immunoreaction to an external substance in genetically predisposed subjects
It manifests itself as itching, urticaria, eczema. The exposure to chemical sensitizer gives allergic contact dermatitis (ACD)

REGULATION is related to:
➢Plant Protection Products Regulation
➢Biocides regulation
➢Classification, Labelling, Packaging (CLP Regulation 1272/2008) regulation
➢REACH regulation (EC, 2006)

Skin sensitisation is **an immunological process that is described in two phases: the induction of sensitisation and the subsequent elicitation of the immune reaction**. The first phase includes a sequential set of events which are described in this Adverse Outcome Pathway (AOP).
Skin sensitization is based on complex immunological mechanisms, which include two phases, a first phase of induction (memory induction), and a second phase of elicitation (cell mediated responce)
These 1 phases are complex, but we can individuate few key points:

1. Percutaneous absorption (bioavailability)
2. Protein binding (Hapten)
3. Cytokine release
4. Activation of DCs (Dendritic cells /Langherans'cells) (up-regulation CD54 – CD86)
5. Maturation and migration of DCs
6. Antigen presentation to T lymphocytes helper

HO LA SOSTANZA, VIENE ASSORBITA DALLA PELLE, POI C'è IL BIDING DELLE PROTEINE, POI EFFETTI PROINFIAMAMATORI (CITOCHINE). SI ATTIVANO LE CELLULE DENDTRITICHE E LA REGOLAZIONE DI CD 54 E CD86. LE CELLULE DENDRITICHE MATURANO ESPONENDO L'ANTIGENE E MIGRANO AL SIST LINFATICO DOVE PRESENTANO L'ANTIGENE AGLI T HELPE

TEST:

IN VIVO TEST- LLNA (Local Lymph Node Assay)

► Method for assessing the skin sensitization potential of chemicals

► In vivo method

► "Reduction-refinement method"

► it is based on the careful evaluation of the immunological outcomes stimulated by chemicals during the induction phase of sensitization.

►OECD TG 429 (revised TG 429 adopted July 2010

If we compare LLNA with the previous used test GPMT (guinea pig maximization), LLNA:

► provides quantitative data

► reduces the number of animals used

► reduces animal suffering (does not require stimulation of skin hypersensitivity reactions; does not require the use of adjuvant)

HOW IT WORKS:

The sensitizers induce a proliferation of lymphocytes in the lymph node responsible for the drainage of the area of application of the chemical that is proportional to the applied dose and the potency of the allergen.

The LLNA evaluates this proliferation through the use of the Stimulation Index (SI)

SI is THE ratio between the proliferation of lymphocytes in the treated groups and that of the negative controls treated with the vehicle alone

Elected Species: Mouse Females

Number of animals: (REDUCED IF WE COMPARE TO GPMT):

4 animals for treat group and 3 concentrations of chemicals.

➢ 4 animals per treatment group 3 concentrations of the test chemical

➢ 4 animals for the negative control group (treated with the vehicle only)

➢ 4 animals for the positive control group (when required)

➔ TOT 12 animals

+ 4 animals for PC

+ 4 animals for NC

➔ TOT 20 ANIMALS

In the day 1-2-3 we apply the substance - day 6 injection of 3Hmethyl thymidine - 5h later: suppression and removal of ear lymph node – then Cell suspension preparation – then Measurement of incorporated 3H-methyl thymidine by β-scintillation counting

The results are expressed through the Stimulation Index (SI). It is calculated by dividing the radioactive incorporation (DPM) by each treatment group by the incorporation of the negative control group

- SI ≥ 2,5 → the substance is considered a skin sensitiser
- SI ≤ 1,7 → the substance is not considered a sensitiser

Is it possible to find an alternative to an in vivo test?

In order to find an alternative we have to consider the key points we told above. It is a bito complex to find an alternative methods because che key point are more. For this reason we use an integrated strategi:

ALTERNATIVE APPROACH

1. Percutaneous absoption
   - RHE model test (vedi sopra per skin irritation)
2. Protein binding
   - QSAR OR DPRA Peptide Binding Assay (cysteine or lysine)

   Direct Peptide Reactivity Assay – DPRA- (OECD TG 442C) IS A Chemico test that aims to evaluate the MIE (Molecular Initiating Event) by quantifying the reaction the reactivity of the chemical with a

synthetic peptide model containing cysteine and lysine (OECD TG 442C, Feb 2015) The percentage of cysteine and lysine depletion is used to classify the substances

3. Inflammatory response and gene expression
   ARE-Nrf2 Luciferase Test Method (OECD TG 442D) - IN vitro method
   KeratinoSens (HaCaT cells are the immortalized human keratinocytes): based on the activation of luciferase and cytotoxicity (measured with MTT)
4. Activation of Dendritic cells
   In vitro Skin Sensitization (OECD TG 442 E) - This TG provides three in vitro test methods addressing the same Key Event on the AOP (Adverse outcome pathway):
   1- the human cell Line Activation Test or h-CLAT method - activation of dendritic cell which formally evaluated in a EURL ECVAM-coordinated validation study in collaboration with JaCVAM is currently undergoing peer review (finalized)
   2- the U937 Cell Line Activation Test or U-SENS
   3- the Interleukin-8 Reporter Gene Assay or IL-8 Luc assay
5. Activation and proliferation of T lymphocytes – IN VIVO Local Lymph Node Assay
   - LLNA (OECD 429)
   - rLLNA-DA (OECD 442A)
   - rLLNA-BrDU ELISA (OECD 442B)

Reassuming as alternativa approach we use an integrated system in chemico + in vitro + in vivo

KEY POINT	TEST		VIA
Percutaneous absoption	RHE model test		
Protein binding	Direct Peptide Reactivity Assay – DPRA- (OECD TG 442C)	Aims to evaluate the MIE (Molecular Initiating Event) by quantifying the reaction the reactivity of the chemical with a synthetic peptide model containing cysteine and lysine (OECD TG 442C, Feb 2015) The percentage of cysteine and lysine depletion is used to classify the substances	In chemico
Inflammatory response and gene expression	ARE-Nrf2 Luciferase Test Method (OECD TG 442D)	KeratinoSens (HaCaT cells are the immortalized human keratinocytes): based on the activation of luciferase and cytotoxicity (measured with MTT)	IN VITRO
Activation of Dendritic cells	In vitro Skin Sensitization (OECD TG 442 E)	1- the human cell Line Activation Test or h-CLAT method - activation of dendritic cell which formally evaluated in a EURL ECVAM-coordinated validation study in collaboration with JaCVAM is currently undergoing peer review (finalized) 2- the U937 Cell Line Activation Test or U-SENS 3- the Interleukin-8 Reporter Gene Assay or IL-8 Luc assay	IN VITRO
Activation and proliferation of T lymphocytes	Local Lymph Node Assay - LLNA (OECD 429) - rLLNA-DA (OECD 442A) - rLLNA-BrDU ELISA (OECD 442B)	The sensitizers induce a proliferation of lymphocytes in the lymph node responsible for the drainage of the area of application of the chemical that is proportional to the applied dose and the potency of the allergen. The LLNA evaluates this proliferation through the use of the Stimulation Index (SI)	IN VIVO

- **Endocrine Disruptor Chemicals: testing approach**

https://www.efsa.europa.eu/en/topics/topic/endocrine-active-substances

- **EDC definition**

An endocrine disruptor 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
EDCs can be Natural hormones, natural chemicals (es toxin produces by plants), synthetically produced pharmaceuticals, man made chemicals (by products released into the environment)
Mainly they have reproductive, behavioral and immunosistemichal effects.

- **Topical toxicity and alternative methods**

Skin toxicity is an alteration of cellular homeostasis able to influence the ability of skin cells:

➢ Fit
➢ Riproduce
➢ Survive
➢ Alterations with specific functions

SKIN TOXICITY EFFECTS:

- Irritative reaction: (irritant contact dermatitis) - local inflammation, of non-immunological origin, characterized by erythema, edema or necrosis due to single or repeated exposure to a substance (HCl, HF). An irritating potential can cause effects on the stratum corneum, epidermis and dermis.
- Direct cytotoxicity (corrosion): severe skin ulceration and subsequent desquamation (NH3, HCl)
- Allergic sensitization - immune reaction to external substances (e.g. neomycin, sulphonamides, metals such as Ni, Hg, Cr). Effects: itching, urticaria, eczema.
- Phototoxicity– Toxicity after exposure to solar irradiation
- Tumor promotion - mutagenic action due to solar radiation

Toxicity is influenced by some factors such as age, sex, genetic factors, application site, membrane permeaility and frequency of use

TESTS FOR ACUTE DERMAL TOXICITY – OECD TG 402

- **AOP definition**

ADVERSE OUTCOME PATHWAY

An AOP is a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (for human health) or population level (for ecotoxicological endpoints). The key events in an AOP should be definable and make sense from a physiological and biochemical perspective. AOPs incorporate the toxicity pathway and mode of action for an adverse effect. AOPs may be related to other mechanisms and pathways as well as to detoxification routes (OECD, 2013) AOP include theTP and the MoA

- **ITS: some examples**

SKIN SENSITIZATION → in chemico + in vitro + in vivo

Genotoxicity → AMES + MNT

CARCINOGENESIS:

1. In silico studies (PBPK or QSAR)

2. Mutagenesis and genotoxicity studies (we cannot discriminated between genotoxic and not genotoxic carginogens but its necessary)
3. CTA
4. Gene expression (cDNA microarrays) studies
5. Human clinical trials
6. Epidemiological study

- **Eye irritation alternative methods**

Eye irritation is an ocular changes following the application of test substance on the front surface of the eye, TOTALLY REVERSIBLE within 21 days from application (OECD TG 405)

EU CLASSIFICATION :
- NI: NON IRRITANT
- R36: OCULAR IRRITANT
- R41:RISK OF SERIOUS EYE DAMAGE

IN VIVO TEST – DRAIZE EYE TEST ON ALBINO RABBIT
Effects of substance are evaluated on iris, cornea, conjiunctiva
CURRENTLY: DO NOT EXIST AN IN VITRO "STAND ALONE" TEST FOR EYE IRRITATION ABLE TO REPLACE THE DRAIZE EYE TEST. For this reason it is necessary to apply a tiered testing strategy

Alternative to IN VIVO TEST: Draize test OECD TG-405: → vedi anche pdf expert

EX VIVO:

1. Bovine Cornea Opacity and Permeability Test (BCOP) in vitro EX VIVO (OECD 437)) (NC e CAT1)
   For identify:
   - Chemicals inducing severe damage (CAT 1-CLP)
   - test method for identifying chemicals not requiring classification for eye irritation or serious eye damage
   BCOP uses isolated corneas from eyes of cattles died for commercial pourposes (etically value because avoid the use of lab animals)
   Toxic effects on cornea are measured (endpoint) by opacity and permeability. When combined gives an in vitro irritancy score (IVIS) – non irritant, weak irritant, moderate irritant, severe irritant

2. Isolated Chicken Eye Test (ICE) in vitro EX VIVO (OECD 438)) (NC e CAT1)
   Can be used to classify substance cat 1 or as not requiring classification
   Uses eye collected from chickens killed for human consumption (no lab animal)
   Endpoint : corneal opacity, corneal thickness, fluorescein retention, surface morphological damage.
   The endpoints are evaluated separately assigned a score and the combined
   The histopathology of the eye can be evaluated as well

IN VITRO:

3. Fluorescein Leakage Test (FL) in vitro (OECD 460)) (CAT 1)
   In vitro test for substances in cat 1 (ocular corrosion and severe irritants)
   Used for identify water soluble substances and severe irritants
   Is performed in a well where cells are used as a separation between 2 chambers.
   This test shold be part of a tiered test strategy

4. Short Time Exposure In vitro Test Methods (OECD 491)) (NC e CAT1)

In vitro test to identify chemicals Inducing Serious Eye Damage and for identifying chemicals not requiring classification for eye irritation or serious eye damagel
The test is performed by a monolayer of silk cells. The decrease of cell viability is used to predict the potential adverse effect leading to a ocular damage

5. Reconstructed Tissues (RhCE) : Skinethic TM HCE and Epiocular TM LabCyte Cornea Model 24-EIT and others (OECD 492)) (NC)
   HCE Epiocular EIT OECD 492 - Reconstructed human corneal like epithelium test methods
   For identify chemicals not requiring classification
   ENDPOINT: cytotoxicity (MTT)
   Reconstructed human corneal like epithelium that mimics the histological, morphological, biochemical and physiological properties of human corneal epithelium.
   This test evaluate the ability of the chemical to induce cytotoxicity on the tissue measured with MTT assay
6. Vitrigel-eye irritancy test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage (OECD 494)
7. In The in vitro macromolecular test method Ocular Irritection (OECD 496) in vitro macromolecular test method for identifying chemicals inducing serious eye damage and chemicals not requiring classification for eye irritation or serious eye damage (OECD 496) EYE IRRITATION/CORROSION

- **In silico methods: definition**

In silico methods are computer-based estimations or simulations. Examples include structure–activity relationships (SAR), quantitative structure–activity relationships (QSARs), molecular modelling techniques, and expert systems (OECD, 2005)
The in silico methods are currently used to obtain preliminary information on toxicity substance

- **QSARs definition (vedi file word lezione 15 marzo**

A QSAR is a theoretical model for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). QSARs relate quantitative measures of chemical structure to continuous or categorical variables describing the property to be predicted (OECD, 2005

- *modelli **quantitativi di relazione struttura-attività** (modelli **QSAR**) sono modelli di regressione o classificazione utilizzati nelle scienze chimiche e biologiche e nell'ingegneria. Come altri modelli di regressione, i modelli di regressione QSAR mettono in relazione un insieme di variabili "predittrici" (X) alla potenza della variabile di risposta (Y), mentre i modelli di classificazione QSAR mettono in relazione le variabili predittive con un valore categorico della variabile di risposta.*
- *Nella modellazione QSAR, i predittori consistono in proprietà fisico-chimiche o descrittori molecolari teorici di sostanze chimiche; la variabile di risposta QSAR potrebbe essere un'attività biologica delle sostanze chimiche. I modelli QSAR riassumono innanzitutto una presunta relazione tra strutture chimiche e attività biologica in un set di dati di sostanze chimiche. In secondo luogo, i modelli QSAR prevedono le attività di nuove sostanze chimiche.*
-

- **Ames testC**

The Ames test uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis. These strains require histidine for growth, but cannot produce it. The method tests the capability of the tested substance (xenobiotic) in creating mutations that result in a return to a "prototrophic" state, so that the cells can grow on a histidine-free medium. This is called REVERSE MUTATION ON.
The bacteria is exposed to the xenobiotic and let it grow in a low histidine substrate, and incubated for 2 days at 37°. If the xenobiotic is mutagen the bacteria will be able to grow.

Limitation: S*almonella typhimurium* is a prokaryote, therefore it is not a perfect model for humans. Rat liver S9 fraction is used to mimic the mammalian metabolic conditions. Before the exposure the chemical is metabolically activated.
However, there are differences in metabolism between humans and rats that can affect the mutagenicity of the chemicals being tested

- **Reproductive toxicology and alternative methods**

"Reproductive toxicity" will refer to any manifestations of xenobiotic exposure reflecting adverse effects on the physiological processes and associated behaviors and/or anatomical structures involved in reproduction or development
A Teratogen is a xenobiotic which, if taken by the mother during pregnancy, is able to act on the somatic cells of the product of conception by determining changes in the correct development of organs and tissues
In humans the critical periods is 21-56 days pregnancy

TEST
CELL BASED MODEL → EST TEST (EMBRIONIC STEM CELL TEST)
TISSUE BASED MODEL → MICROMASS TEST
WHOLE EMBRYO CULTURE APPROACHES

MeST mouse embryonic stem cell test – in vitro test used to detect the potential teratogenicity of the substances. Is used as a model to assess embryotoxicity in vitro.
The test can result in 3 different classics:

- Strong embryotoxic
- Weak embryotoxic
- Not embryotoxic

The ability of MeST to shift from a pluripotent to differentiated state makes them a optimal candidate as a screening tool for hazard identification
The test use 2 permanent cell lines:

- ESC D3 (representing embryonic cells)
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The test use the capacity of embryonic stem cells to differentiate spontaneously into contracting cardiomyocytes. The potential embryotoxicity is showing when there is an interference with the cell differentiation. The disturbance of the embryonic stem cells differentiation after the exposure at the substance indicate a potential embryotoxicity.
To predict the embryotoxic potential of a test substance, three endpoints are assessed:

4. inhibition of differentiation into beating cardiomyocytes (we evaluate the contractility)
5. cytotoxic effects on stem cells
6. cytotoxic effects on 3T3 fibroblasts.

EVALUATION OF CARDIOMYOCYTES CONTRATILITY

5. Drops of embryonic stem cells suspension are placed in the lid of petri dish and incubate.
6. After 3 days and aggregation of stem cells are transfer to a new petri dish.
7. 2 days later the embryoid body are transfer in a 24 well plates with the testing solution and incubate for 5 days
8. At the 10th day it is possible to evaluate the cells

CYTOTOXICITY effects on D3 (murine embryonic stem cells) and BALBS 3T3 (fibroblast) are evaluated with MTT test

MICROMASS TEST (MM)

Is based on embryonic tissues (from limb / cephalic tissue) at the stage of embryo organogenesis

ENDPOINTS:

- Cell differentiation (alcian blue)
- Cell viability (NEUTRAL RED U)
- Cell proliferation

It is a predictive model to evaluate non embryotoxic, weak embryotoxic, strong embryotoxic substances

Rodent post implantation Whole Embryo Culture

Predictive model for Strong Embryotoxic and Malformations during organogenesis.

Endpoint:

- Morphology of the Embryo
- Functionality of the Embryo
- Embryo growth
- Cell viability

- **Read across definizione**

Prediction from data for reference substance(s) within the group or "category" of substances by interpolation to other substances in the group

Read-across is a technique for predicting endpoint information for one substance (target substance), by using data from the same endpoint from (an)other substance(s), (source substance(s))

- **Cell transformation assay**

CELL TRANSFORMATION ASSAY (CTA) is based on the evaluation of phenotypic changes induced by chemical compounds (or physical agents) in cultured cells. Used for both, genotoxic and non genotoxic carcinogen, but cannot discriminate them. If we want to discriminate we need to use in parallele a genotoxicity test.

Statement: the transformation process in vitro is able to mimic some stages in vivo

Cellular models used are:

- Syrian hamster embryo cells (SHE): to evaluate the early stages of the carcinogenesis
- BALB/c 3T3 and C3H10T1/2 (mouse embryonic fibroblasts): to evaluate later carcinogenic effects
- Bhas 42 iniziatiion and promotion

2 steps:

1. Cytotoxicity assay -> based on a Dose-response curve with calculation of NOEL (no observe adverse effect level), IC 50 (half maximal inhibitor concentration), IC 90 (90% inhibitor concentration)
2. Transformation assay -> depends on the cell used: SHE (8-10 days), BALB and C3H10T (4-6 week)

We evaluate the morphological results: formation of colony for SHE, formation of foci for the others

LIMITS: it is not a stand alone test. Cannot substitute in vivo test, but it can be used in an integrated strategy

INTEGRATED STRATEGY FOR CARCINOGENESIS:

7. In silico studies (PBPK or QSAR)
8. Mutagenesis and genotoxicity studies (we cannot discriminated between genotoxic and not genotoxic carginogens but its necessary)
9. CTA
10. Gene expression (cDNA microarrays) studies
11. Human clinical trials
12. Epidemiological study

FOCUS ON SHE CTA:
She cells are divided in 2 categories and used as a feeder cells (x-ray irradiated to inactivate tehir capability to replicate and add as a nutrient base and support for metabolic activity), and target cells (used to assess morphological transformation of colonies)

-day 0 →the feeder SHE cells (2 ml) are seeded in x number of dishes and the cultures are incubated for 24 h
- day 1 → adding target cells (2 ml) to each dish and incubate for 24 h
- day 2 → are prepared 5 concentration (200 µg/mL, 100 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL) + Positive and Negative control (medium). 4 ml of test substance is add in each well and incubate for 7 days
- day 9 → discharge the medium, wash with buffer saline, cover with fixing solution, then remove it an colored with Geimsa

Now we can evaluate morphological transformation under stereomicroscope
We calculate:
- MTF (morphological transformation frequency) for morphological transformation
- PE (plating efficiency) and RPE (relative plating efficiency) to evaluate cytotoxicity

- **Phototoxicity in vitro validation test**

Phototoxicity or photoirritation is an acute reaction / irritation of the skin related to a nonimmunological xenobiotic exposure, acute chemically induced in the presence of light.
Light that causes irritation (usually within the UVA spectrum, that is, 320 - 400 nm).
It is caused by simultaneous exposure to xenobiotic and light
Exposure to the photoactive xenobiotic can occur for
➢ Topically
➢ Orally
➢ Inhalation route
➢ Parenterally
The reaction can be evoked in all subjects as long as the concentration of the chemical and the dose of light are sufficient

TEST:
OECD TG 432- In vitro 3T3 NRU phototoxicity test
In vitro test, considered a stand alone test
➢ Neutral red uptake assay with Balb / c3T3 cells (European Directive B 41 and OECD guideline 432)
Neutral red assay with Balb / c3T3 cells Immortalized mouse fibroblasts (Balb / c3T3 clone 31)

PROTOCOL:
1. Exposure to the test substance
2. Irradiation

3. Evaluation of the viability of cells through Neutral red uptake assay/ viability test
   This is a predictive test for photosensitizing and non-photosensitizing substances
   Limit: water solubility of the test substance

3D models (MTT test): based on RHE with endpoint viability measured by MTT

➢ Target Tissue (RHE is a target tissue)

➢ Valid also for complex mixtures and pharmacological patch

➢ we ca do an histological evaluation in order to see the effects of the phototoxic substances

➢ Barrier

➢ Advantages related to the solubility of the substances (this was the weak point of the NRU)

• Reconstructed Human Epidermis Phototoxicity Test (RhE PT) - Method EpiDerm™ (EPI200) (validated OECD 498)

In conclusion 3t3 NRU PT is a stand alone method in OECD guidelines and unther EU legislation
We che use an tiered strategy where we first apply 3T3 NTU PT (phototoxicity test), followed by Epiderm H3D-PT RHD

- **Botulinum toxin and 3Rs**

Botulism is a severe intoxication from a gram + produced a neurotoxin. C. Botulinum has 7 serotypes (A to G). Each species have a specie-specific serotype that have toxic effect. Es. Human (type A,B,E), equine (C,D) bovine, (B,C,D), swine (C).
Botulinum toxin A is the most powerful in nature. The toxin is thermolabile. The spore are present in the soil and can be destroyed at 115-120° exposure.
MECHANISM OF ACTION→ inhibition of the release of Ach, it result in a flaccid paralysis
In vitro studies demonstrate the inhibition of Ach
BoNT → Botulinum neurotoxin is used either for therapeutic application or for aesthetic reason. This is why the use of the toxin has increased in the recent years.
Injection of the toxin into the muscle leads to a temporary paralysis (2 to 6 months).
BoNT can be used:

- Therapeutic uses (treatment of diseases like cervical dystonia, blepharospasm, hyperhidrosis, strabismus)
- Aesthetic use (licensed for temporary treatment)

In 2008 the FDA reported systemic advance reaction in relation to the aesthetic use of the toxin
BoNT is regulated by European Pharmacopoeia but not in the Cosmetic Regulation

IN VIVO TEST → MOUSE LD50 ASSAY
Require a lot of animal: 100 mice divided in group
Administration of different doses of botulinum toxin, with a lot of sufferance for animals cause the endpoint is death by choking after 3-4 days suffering
That's why it's very important use the 3R

IN VIVO TEST → Mouse flaccid paralysis assay → Compare to LD 50 is apply REDUCTION and REFINEMENT, not REPLACE
→ The endpoint is not death but local paralyses and less mice are use (32 for each preparation), and the duration in less (24-48 h)

EX VIVO TEST (Tissue based assay) → Mouse Phrenic Nerve (MPN) Assay (REDUCTION)

Diaphragm removal including the phrenic nerve and administration of different concentrations of botulinum toxin.
Endpoint: evaluation of the loss of muscle contractility) after electrical nerve stimulation

EX VIVO TEST (Tissue based assay) → Rat Intercostal Neuromuscular Junction (NMJ) Assay (REDUCTION)
Isolated preparations of rat intercostal muscle
Endpoint: evaluation of the loss of muscle contractility) after electrical nerve stimulation

IN VITRO TEST → Endopeptidase assay
Molecular biological test that measures the cleavage of the SNAP-25 protein complex that carries acetylcholine by the botulinum toxin.
Faster and less expensive method than currently validated tests, very sensitive. Use of mouse-derived antibodies

IN VITRO TEST → CBPA CELL BASED POTENCY ASSAY
Are used Differentiated cells of Human Neuroblastoma (SiMa)

Lots of in vitro test are waiting for validation but the process seems to be long

- **CBPA → BOTULINUM SLIDE**

Cell-Based Potency Assay