Precious Metals Consortium

Precious Metals & Rhenium Consortium

Silver expert meeting Reprotoxicity

23 November 2017 | Brussels

Precious Metals Consortium

1. Welcome and introduction

Katrien Arijs

1.1 Reminder on confidentiality and competition law

Attendees should refrain from any discussion on pricing, market shares and volumes and other information that could be considered as market sensitive and covered by antitrust legislation

DO	DON'T
Application of competition law	
Art. 101 and 102 TFEU may be applicable to the conclusion of any preliminary agreement and activities of any preliminary phase.	Don't assume that conflicts with competition law are excluded simply by the fact that the Agreement complies with the provisions of the REACH Regulation.
Consultation in Matters of Competition Law	
Consult an in-house legal expert or the compliance officer of your company or an external lawyer whenever there are uncertainties respecting compliance with competition law. Stop all meetings/discussions which are not in compliance with these Compliance Guidelines until a legal expert has been involved.	Don't assume that these Compliance Guidelines deal with all competition law issues exhaustively. Basically, compliance with Art. 101 and 102 TFEU can be determined only on the basis of market impact in each individual case. These Compliance Guidelines may therefore be regarded only as a means of providing general conduct recommendations.
Activities in any preliminary phase and at any other stage of operation of the Consortium	
Restrict cooperation within the scope of the preliminary phase to the initially defined goals and purposes of the cooperation.	Pursuant to Art. 101 and 102 TFEU, activities which have the object of the effect of preventing, restricting and/or distorting competition are prohibited within the scope of this Agreement, including: - Coming to agreement, including arrangements or collusions, about prices, markets and customers (see Art. 101 paragraph 1 a)-e) TFEU); - Joint boycotting of other companies; - The unjustified unequal treatment of trade partners; - The abusive exploitation of a dominating market position.
Exchange of Confidential Information	
Involve a Trustee for the exchange of Confidential Information.	The exchange of Information concerning market behaviour and having the object or the effect of preventing, restricting and/or distorting competition is inadmissible; in particular, this relates to : - Production capacities; - Productions or sales volumes; - Import volumes; - Market shares; - Price policy; - Distribution and marketing terms; - Marketing strategies; - Information regarding the relationship with suppliers.
Documentation on Cooperation	
Keep minutes of all meetings which detail the subject of the meeting. In case of uncertainty, have the contents of the minutes reviewed by an external legal expert prior to sending them to all parties of the Agreement. Stop all meetings which are not in compliance with these Guidelines until a legal expert has been involved.	

1.2 Tour de table and apologies

- Cf. participants list included in agenda
- Please briefly explain your tox specialism

1.3 Approval of the agenda

1. Welcome and Introduction *(K Arijs)*
2. Silver reproductive toxicity – current status *(M Raffray)*
3. Process / timeline EOGRTS Testing proposal (TP) *(K Arijs)*
4. RAC mindset on reprotox classifications
   - Examples *(K Arijs, O Lemke, S Verberckmoes)*
   - Assessment of impact on situation for silver *(M Holsapple)*
5. Stress-testing examples *(All)*
6. Defence of EOGRTS TP *(M Holsapple)*

FOR APPROVAL

LUNCH

7. Adjustments to EOGRTS TP *(M Holsapple)*
8. Mechanism of action (MoA) insights *(M Raffray)*
9. Enabling work *(M Holsapple)*
10. Re-visit stress-test / confirm technical defence plan *(All)*

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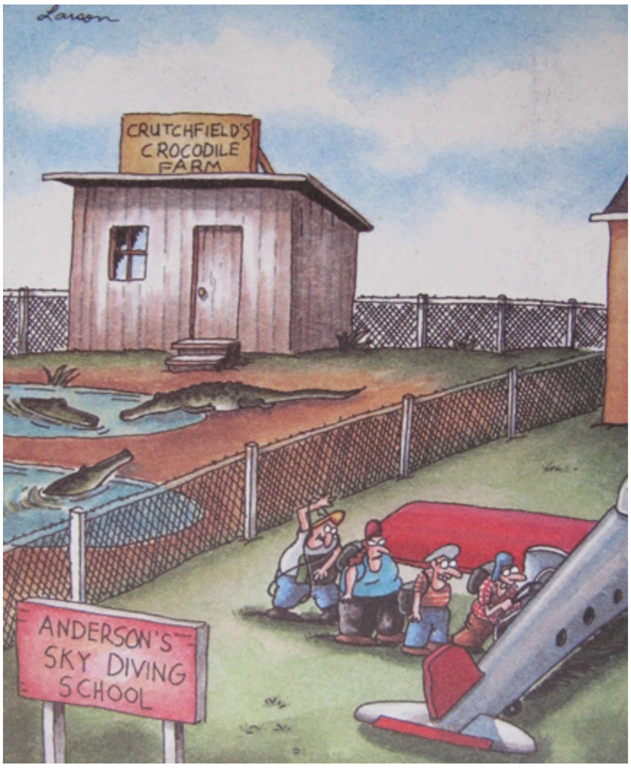
2. Silver reproductive toxicity - current status

Mark Raffray

2 ▸ Current status: Ag^+ Reproductive toxicity



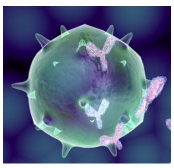
2 ▸ Current status: Ag^+ Reproductive toxicity

Developmental

- Main battleground (*MR view*)
- Pre- & post-natal effects
- Uncertainty: indirect effects, MoA

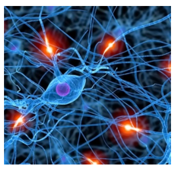
Fertility

- Limited evidence (Sprando study)
- SCAS data (2-gen) no effect



Developmental immunotoxicity

- Babu study
- Indicative not firm evidence
- Weaknesses in study approach



Developmental neurotoxicity

- DNT Ag partial/complex picture
- PMC understood risks to TP
- Ag-Cu axis impact?

2 ▸ Key Ag⁺ developmental studies [I]

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Shavlovski et al. (1995)

Non-TG conform dev. tox study (AgCl) 188 mg Ag/kg bw/d [D] Exposure either GD 1-20 or GD 7-15	Post-implantation loss ↑ Fetal death ↑ / Viability index ↓ Fetal weight ↓ Pup soft tissue abnormalities ↑: kidney (*hydronephrosis*); testis	• Disparity in effects GD 1-20 group vs absence in GD 7-15 group suggests early vulnerability window • Tissue Cu levels ↓ (foetal, placental, maternal serum) • Effects reversed with Cp admin. [IP]

US NTP (2002)

TG conform dev. tox study (AgAc) 6.5, ~20, 65 mg Ag/kg bw/d [G] Exposure either GD 1-20 or GD 7-15	No effects on malformations or variations No effects on other developmental parameters NOAEL (dev.): 65 mg Ag/kg bw/d	• Can be criticised due to exposure only during GD 6-19/period of organogenesis (see above)

D = dietary G = gavage IP = Intraperitoneal
All treatment levels normalised to Ag equiv. values

2 ▸ Key Ag⁺ developmental studies [II]

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Sprando et al. (2017)

OGRTS (AgAc) 0.26, 2.6 or 26 mg Ag/kg bw/d [W] 10-wk exposure including gestation & lactation	Implantation nr ↓ Pup mortality ↑ Viability index ↓ Fetal weight ↓ Runts↑ No malformations/ anomalies/ soft tissue abnormalities Fertility ↓ Nr. litters ↓ (HD only)	• Dev. LOAEL / NOAEL: 2.6 / 0.26 mg Ag/kg bw/day • Embryofetal tox early HD / late MD? • No effect thymus wt. (*cf. below*) • Fertility parameters incomplete • DIT reported in statellite study (Babu et al., 2016)

SCAS Silver Zinc Zeolite; SZZ (2002)

2-gen study (TG 416) conform (SZZ) ~1.8, ~11, ~23 mg Ag/kg bw/d [D] Exposure in maturation, mating, gestation & lactation	Fetal mortality ↑ Stillbirth ↑ Pup bwt ↓ Thymic wt ↓ Pup kidney, UT abnormalities ↑	• Mainly F1 HD/MD; F2 MD (HD lost). Males generally more affected. • Hydronephrosis causation ascribed to zeolite moiety; query Cu axis? • No clear effect on fertility • Limited data: Ag, Zn, Cu tissue levels (F2 pups) • Outcomes read-across to SZ

D = dietary W = drinking water

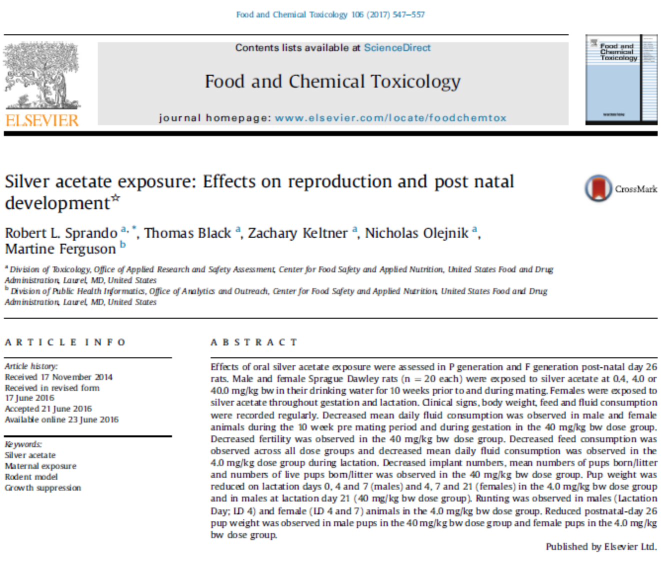
2 ▸ Key Ag⁺ developmental studies [III]

SCAS Silver Sodium Hydrogen Zirconium Phosphate SSZHP (2002)

2-gen study (TG 416) conform	Litter size ↓ (F1/F2 HD)	• Non-zeolite comparator with low Zr release characteristic
(SSZHP) ~2, ~10, ~40 mg Ag/kg bw/d [D]	Group litter wt ↓ (F1/F2 HD)	• All developmental effects marginal
Exposure during maturation, mating, gestation & lactation	Thymic wt ↓ (slight; F1/F2 HD/MD)	• No effect on fertility parameters
		• Other data: no embryotoxicity in 2x dev. tox (TG 414) studies up to 25 mg Ag/kg bw/d [G]; exposure during GD 6-15
		• Comparative bioelution data SZZ : SSZHP limited; however, long-term Ag release observed with SSZHP @ pH 4 & 8 (phosphate buffer)

D = dietary G = Gavage

2 ▸ Impact of Sprando et al. (2017)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Food and Chemical Toxicology

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Silver acetate exposure: Effects on reproduction and post natal development☆

Robert L. Sprando [a,*], Thomas Black [a], Zachary Keltner [a], Nicholas Olejnik [a], Martine Ferguson [b]

[a] Division of Toxicology, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, Laurel, MD, United States
[b] Division of Public Health Informatics, Office of Analytics and Outreach, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, Laurel, MD, United States

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ABSTRACT

Effects of oral silver acetate exposure were assessed in P generation and F generation post-natal day 26 rats. Male and female Sprague Dawley rats (n = 20 each) were exposed to silver acetate at 0.4, 4.0 or 40.0 mg/kg bw in their drinking water for 10 weeks prior to and during mating. Females were exposed to silver acetate throughout gestation and lactation. Clinical signs, body weight, feed and fluid consumption were recorded regularly. Decreased mean daily fluid consumption was observed in male and female animals during the 10 week pre mating period and during gestation in the 40 mg/kg bw dose group. Decreased fertility was observed in the 40 mg/kg bw dose group. Decreased feed consumption was observed across all dose groups and decreased mean daily fluid consumption was observed in the 4.0 mg/kg dose group during lactation. Decreased implant numbers, mean numbers of pups born/litter and numbers of live pups born/litter was observed in the 40 mg/kg bw dose group. Pup weight was reduced on lactation days 0, 4 and 7 (males) and 4, 7 and 21 (females) in the 4.0 mg/kg bw dose group and in males at lactation day 21 (40 mg/kg bw dose group). Runting was observed in males (Lactation Day; LD 4) and female (LD 4 and 7) animals in the 4.0 mg/kg bw dose group. Reduced postnatal-day 26 pup weight was observed in male pups in the 40 mg/kg bw dose group and female pups in the 4.0 mg/kg bw dose group.

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- In latest SCAS dossiers for RAC
- Seen as supporting dev. tox conclusions drawn from SZZ 2-gen study & Shavlovski work
- Cf. SCAS: Ag effect isolated from confounders (e.g. Zn, zeolite, Zr)
- Mitigation: Dev. effects not severe. Fertility segment flawed. Indirect effects not evaluated. MoA open.
- Overall the study can be criticised vs TG 443 (EOGRTS)

2 ▸ Impact of Babu et al. (2016)


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Effects of maternal silver acetate exposure on immune biomarkers in a rodent model

Uma S. Babu [b,*], Kannan V. Balan [b], Elmer Bigley [b], Marion Pereira [b], Thomas Black [a], Nicholas Olejnik [a], Zachary Keltner [a], Robert L. Sprando [a]

[a] Division of Toxicology, Office of Applied Research and Safety Assessment, CFSAN, U.S. FDA, Laurel, MD, United States
[b] Division of Virulence Assessment, Office of Applied Research and Safety Assessment, CFSAN, U.S. FDA, Laurel, MD, United States

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ABSTRACT

Male and female rats (26-day old) were exposed to 0.0, 0.4, 4 or 40 mg/kg body weight silver acetate (AgAc) in drinking water for 10 weeks prior to and during mating. Sperm positive females remained within their dose groups and were exposed to AgAc during gestation and lactation. Splenic and thymic lymphocyte subsets from F1 generation PD (postnatal day) 4 and 26 pups were assessed by flow cytometry for changes in phenotypic markers. Spleens from PD4 pups had lower percentages of $CD8^+$ lymphocytes in 4 and 40 mg/kg AgAc exposed groups and reduced Concanavalin A (Con A) response at all AgAc exposure groups. Splenic maturation increased in PD26 pups compared to PD4 pups. Con A and lipopolysaccharide (LPS) mediated splenic responses were lower in PD26 pups exposed to 40 mg/kg AgAc. Changes in PD 26 pup splenocyte phenotypic markers included lower TCR + cells at 4 and 40 mg/kg AgAc exposure and higher B cell population in the 40 mg/kg AgAc. PD26 pup splenic natural killer cell (NK) activity was higher in the 0.4 AgAc group and unchanged in 4 and 40 mg/kg AgAc groups. In conclusion, maternal exposure to AgAc had a significant impact on rat splenic development during the early lactation period.

- Oddly not yet on radar for RAC ! EFSA have considered
- Refer to Prof. Holsapple's review for full interpretation / impact assessment ▶ **Indicative** of DIT rather than firm evidence.
- Sub-optimal design vs. EOGRTS DIT protocols
- But concern must be how RAC would view it also integrated with Sprando et al. findings
- Is Cat. 1B conclusion for ionic Ag now a bigger risk ?

2 ▸ PMC Updated Strategy

Predicted trajectory adverse ▶ Defence of TP key to sector

Given: TP if maintained, must be amended (▶ complex EOGRTS)

Ideal final position could include any/all of following:

- ▶ **robust evidence that Ag reprotox is secondary effect***
- ▶ **MOA is of no / limited relevance to humans***
- ▶ **Ag reprotox is confined to exposure levels significantly above those relevant to human risk assessment**

Onion defence ▶ ideally no classification, but avoid Cat. 1B

No delays in building defence / Maintain initiative

*** Preferred outcome**

Brief re-cap report on the Assessment of DIT Studies for Silver

Michael P. Holsapple, PhD, ATS

holsappl@msu.edu
michael.holsapple@gmail.com
Mobile: 517-898-4940

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q1: What is significance of Babu et al. (2016) [and linked Sprando et al. (2017)] study on DIT-related considerations for ionic silver?**
- **Q2a: What is impact on DIT-aspects of previously submitted PMC EOGRTS Testing Proposal?**
- **Q2b: How should TP be updated in this respect and what are justifications for doing so?**
- **Q3: If we want to be proactive, what possible enabling ITOX work could we undertake that would further inform on viability of proceeding w/ EOGRTS?**
- **Q4: What is possible impact of latest DIT data on ionic Ag classification?**

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q1: What is significance of Babu et al. (2016) [and linked Sprando et al. (2017)] study on DIT-related considerations for ionic silver?**
- "*suggestive evidence … for silver … cause DIT*"
- Suppressed splenic mitogen responses
- No effect on thymic mitogen responses or splenic NKC response
- Total lack of any kind of a dose-response
- Failure to site Gao et al. (2015) – no effect on developing thymus
- 2015 version of TP – "*perform enabling TK investigations including those covering transplacental distribution studies*"
- I did not comment: NOAEL/LOAEL = 0.4/4.0 mg/kg bw/day

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q1: What is significance of Babu et al. (2016) [and linked Sprando et al. (2017)] study on DIT-related considerations for ionic silver?**
- Possibility that doses used in these studies may have triggered some sort of stressor response
- As mentioned in other review of the Sprando paper "*It is crucial to differentiate between a primary immunosuppressive effect and indirect effects mediated by stress*"
- NTP ITOX studies – doses should not cause obvious stress (e.g., >10% decrease in body weight gain)
- Doses from 2015 TP even higher – 10, 30, 100 mg/kg bw/day – than those used in Babu / Sprando papers

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q2a: What is impact on DIT-aspects of previously submitted PMC EOGRTS Testing Proposal?**
- "*suggestive evidence … for silver … cause DIT*"
- "*reliance on mitogen responses as principle indicator of DIT*" = weak evidence for ITOX
- Cohort 3 of EOGRTS would include TDAR (gold standard)
- Included 3 other papers demonstrating important relationship between nanosized silver & ionic silver
- Re-emphasized importance of MPS on the distribution of ionic silver – van der Zande et al. (2012) showed silver in liver > spleen > **testis** > kidney > **brain** > lungs

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q2b: How should TP be updated in this respect and what are justifications for doing so?**
- Include Cohort 3 in EOGRTS … TDAR = "gold standard"; "*suggestive evidence*" coupled with distribution studies
- Reconsider doses from 2015 TP – NO rationale for doses of 10, 30 and 100 mg/kg bw/day; > Babu & Sprando studies
- EPA RfD (1991) for silver = 5 μg/kg bw/day; EPA (1981) human argyria after retention of 1 g of Ag (200,000x RfD); no significant physiological effects except "coloration"
- Gao & Sprando studies – reported results consistent with silver-induced argyria (doses = 80x, 800x & 8,000x RfD)
- WHO (2004): NOAEL for gen'l population = 5 μg/kg bw/day

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q3: If we want to be proactive, what possible enabling ITOX work could we undertake that would further inform on viability of proceeding w/ EOGRTS?**
- Provided DIT chapter – "*basic design for DIT*" = "*quite consistent with EOGRTS*"; TDAR = 'gold standard'
- Both 2015 TP and review of Sprando et al (2017) paper underscored "*importance of gut microbiota*"
- TP: "*oral exposure … to high doses … significant disturbances in intestinal lumen microbiota*" (Williams et al, 2015)
- My report – "*sponsors consider contacting an expert on the role that the gut microbiome plays in immune system development*"

Brief re-cap of my report on the Assessment of DIT Studies for Silver

- **Q4: What is possible impact of latest DIT data on ionic Ag classification?**
- I am not familiar with CLP regulation / classification criteria
- Focused on Chapter 3.7 – reprotox classification criteria
- Not able to find any language that addressed how a positive finding in Cohort 3 from EOGRTS (DIT) would impact classification criteria … would affect 'decision tree'
- Premature to discuss impact of Babu paper – other than as "*suggestive evidence*" to 'trigger' inclusion of Cohort 3
- EOGRTS is designed to provide the rigor to enable that discussion (e.g., about possible DIT) to take place

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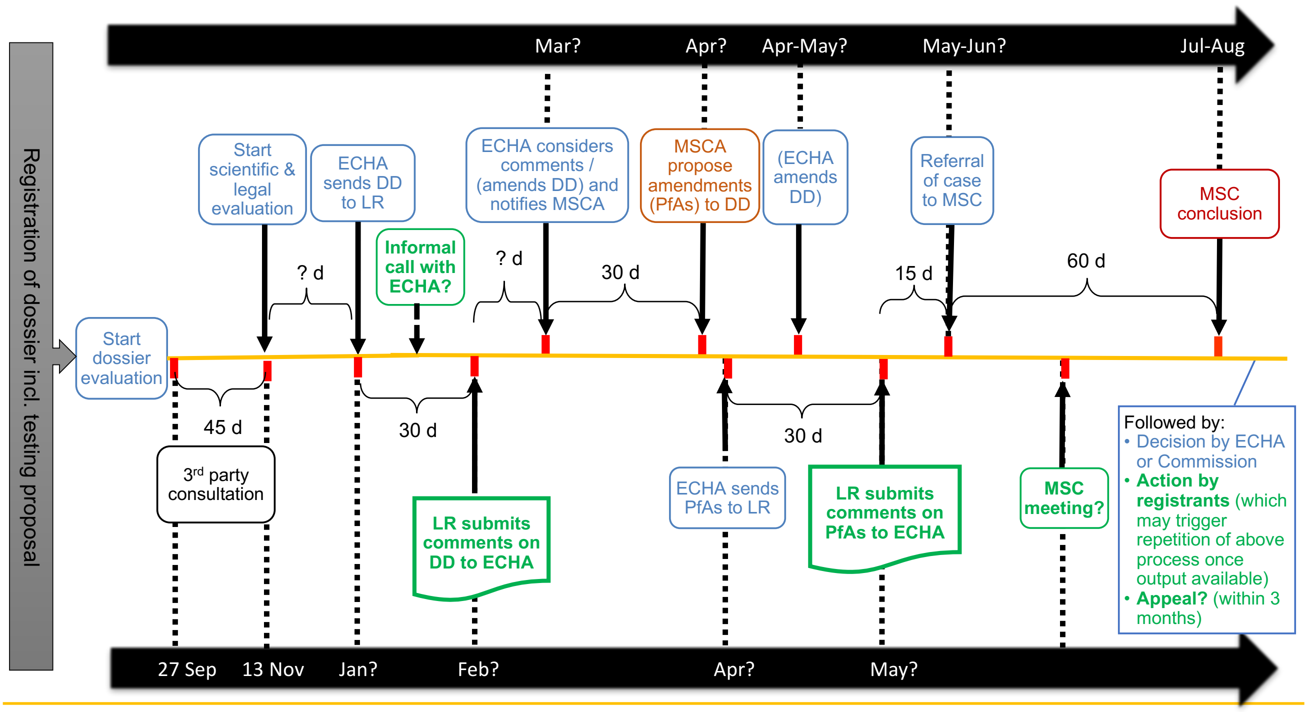
3. Process / timeline EOGRTS Testing Proposal (TP)

Katrien Arijs

Dossier evaluation process

DD = Draft Decision
LR = Lead Registrant
PfAs = Proposal for Amendments



Dossier evaluation process – involvement DUs

- Increasing communication with our DUs seems to become more and more urgent but message needs to be properly tailored
- Proposal:
  - Clarify and agree on our **strategy** for Ag reprotox
  - Review **DU mapping** performed in 2016
  - Finalize the **SEA data gathering**: having a good mass flow analysis could help us identify priority DUs and develop arguments towards authorities on classification impact
  - Draft **key messages** and **contact some key DUs** proactively

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4. RAC mindset on reprotox classifications

4 ▸ MEMO GHS/EU Classification scheme

Reproductive toxicant	Criteria	Remarks
Category 1A	• Known human reproductive toxicant (development and/or fertility)	• Few substances classified to this level. • Metals: Pb/Pb cmpds
Category 1B	• Mainly based on animal studies • Strong presumption relevance to humans • Evidence must be clear (dev./fert.) • Not secondary i.e. non-specific consequence (including 2º due to marked maternal toxicity, stress etc.)	• SVHC implications: regulatory controls & possible end-user deselection • Metals: Cmpds of Co, Cr^{6+}, Ni; metallic Hg
Category 2	• Evidence not sufficiently convincing to assign Cat. 1 • Typically only animal data • Same caveat: should not be a non-specific consequence of other toxicity	• Due to REACH testing increasingly common classification (eventually >1000 substances?)

4 ▸ RAC criteria applied to reprotox assessments

Based on recent CLH precedents

- Standard considerations of biological plausibility, d-r, statistical significance etc.
- Outside historical control range: dose level with maximal effect
- Severity of effect important (judgements can be inconsistent)
- MoA, if known, relevant to humans (but precautionary principle applied)
- Not indirect secondary toxicity / non-specific MoA
  - Severe & confounding parental (maternal) toxicity would exclude
  - But RAC have classified when reprotox co-exists with quite appreciable maternal systemic toxicity

Case study 1: silver zinc zeolite (SZZ)

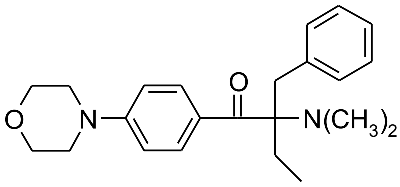
- CLH proposal by Sweden: **Repr Cat.1B**
  - ➢ 2-gen study with SZZ
    - o effects relevant for 1B classification were primarily based on foetal/pup mortality, ↓ pup bwt and ↓ thymus wt, that were not considered secondary non-specific consequences of marked toxicity in the dams
  - ➢ supporting data on silver chloride and silver acetate
- RAC: agreed on a **Repr Cat.2**
  - ➢ 2-gen study with SZZ
    - o provided evidence of adverse effects
    - o ↓ pup bwt and ↓ thymus wt and ↑ frequency of hydronephrosis considered general systemic tox rather than dev tox
    - o tox at highest conc too high to be used for establishing Cat.1B classification
    - o pup cardiac enlargement appeared in presence of maternal toxicity (mainly hydronephrosis and haematological alterations) and cannot be totally disregarded for classification → RAC considers these effects on the heart as relevant for classification of SZZ as toxic to development Cat 2

Case study 2: boric acid

- CLH proposal by Poland: Repr Cat.1B → **Repr Cat.2**
  - ➢ while no dev effects seen in highly exposed populations, the epi studies of dev effects not considered as robust as the fertility studies, and would therefore warrant Repr Cat.2 classification
  - ➢ based on adverse dev effects of B in rats and rabbits, boric acid should be classified Repr Cat.2
- RAC: agreed on a **Repr Cat.1B**
  - ➢ tox data from 4 different species (mice, rats, rabbits and dogs) provide clear evidence of an adverse effect on sexual function, fertility, and development in absence of other toxic effects
    - o Dev tox: malformations in rats and rabbits – no indications that effects were secondary to other toxic effect
  - ➢ no reprotox evidence observed in the epi studies but designed to cover only male fertility effects + methodological limitations → no evidence that the effects observed in animals are not relevant to humans

CASE STUDY 3: 2-BENZYL-2-DIMETHYLAMINO-4'-MORPHOLINOBUTYROPHENONE

CAS: 119313-12-1, EC: 404-360-3



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Dossier submitter: BASF SE
RAC rapporteur: Marja Pronk
Adviser: Marjolijn Woutersen

DEVELOPMENTAL TOXICITY

- One-generation study (OECD 415) in rats
  - 30, 100, and 300 mg/kg bw/day, by oral gavage
- Developmental effects
  - Stillbirth
  - Post-natal pup mortality day 1-4
  - Reduced pup weight
- Maternal effects
  - Reduced bw, bw gain, food consumption
  - Discolorations in liver, kidneys, glandular stomach
  - Increased weight of liver and adrenals
  - Histopathological findings in liver, adrenals and glandular stomach

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DEVELOPMENTAL TOXICITY

- DS proposal: cat. 2, based on ↑ pup mortality and ↓ pup wt at highest dose, in the presence of maternal toxicity (liver tox, stress, ↓bw(g)/fc)

- Reduced bw(g), food consumption → only at high dose
  - ↓ fc: only during lactation (up to 20%)
  - ↓ bwg: only during gestation (-15%)

    Gain lowest in 4 dams with small litters; no clear link between bwg of individual dams and no. of stillborns
  - ↓ bw: at most 8% during gestation/lactation (vs 13-24% ↓pup bw)
  - Feed restriction studies: 30 and 50% feed reduction did not affect pre-or postnatal mortality, but reduced maternal bw (by 10-20 and 17-32%, resp.) and pup bw (by 10-20 and 12-47%, resp.)

  → Effects on bw, bwg and fc not directly causative for dev.tox

DEVELOPMENTAL TOXICITY

- Histopathological lesions → only at high dose
  - Liver: slight to minimal hypertrophy. Adaptive rather than true liver toxicity; no clear link between liver toxicity and pup mortality for individual dams
  - Glandular stomach: minimal to slight mucosal hyperemia (probably due to local irritation). No dose-relation, and only in 3 high dose dams whereas 8 had stillborn pups
  - Adrenal gland: cortical cells with condensed eosinophilic cytoplasm devoid of lipid vacuoles in the zona fasciculata. Considered stress-related by DS

  → Organ effects not directly causative for dev.tox

Conclusion Developmental toxicity

- Maternal toxicity only at high dose, but developmental toxicity (increase in stillbirth and post-natal mortality, decrease in pup bw) at mid and high dose

- Maternal effects not considered directly causative for developmental effects

→ Developmental effects not considered a secondary non-specific consequence of maternal toxicity

→ Classification is warranted

Classification for Developmental toxicity

- Arguments for Cat. 2
  - Maternal toxicity at the top dose
  - Only statistical significant effect at mid dose is increase in stillbirth, but this was still within historical control range

- Arguments for Cat. 1B
  - Study is of good quality
  - Stillbirth and post-natal mortality, in particular, are severe effects, showing statistical significance (as did ↓pup bw at high dose)
  - Effects on stillbirth and pup bw were dose-related

- Rapporteur considers Cat. 1B more appropriate than Cat. 2 → Does RAC agree?

Extra slides

Developmental toxicity

- Dose-dependent increase in stillbirth

	Historical data	0 mg/kg bw/day	30 mg/kg bw/day	100 mg/kg bw/day	300 mg/kg bw/day
Number of litters		18	19	17	17
Total number of pups		194	190	190	162
Pups delivered per dam (mean)	9.3-12.8	10.8	10.1	11.2	9.5
Females with stillborn pups, (N (%))		0 (0)	1 (5.3)	5 (29)*	8 (47)*
Stillborn pups, (N (%))	(0-4.5)	0 (0)	2 (1.0)	6 (3.2)*	**9 (5.6)***
Live birth index (%)	95-100	100	99	97	94

DEVELOPMENTAL TOXICITY

- Increase in post-natal pup mortality

	Historical data	0 mg/kg bw/day	30 mg/kg bw/day	100 mg/kg bw/day	300 mg/kg bw/day
Pups dead day 0 (N (%))		0 (0)	1 (0.5)	0 (0)	4 (2.6)
Pups dead day 1-4 (N (%))		0 (0)	3 (1.6)	3 (1.6)	**18 (12)***
Pups dead day 5-21 (N (%))		0 (0)	0 (0)	1 (0.5)	1 (0.7)
Viability index; N (%)	(94-100)	194 (100)	185 (98)	181 (98)	131* (86)
Lactation index; N (%)	(94-100)	134 (100)	139 (100)	129 (99)	103 (99)

DEVELOPMENTAL TOXICITY

- Pup and maternal weight

	Historical data	0 mg/kg bw/day	30 mg/kg bw/day	100 mg/kg bw/day	300 mg/kg bw/day
Pup bw day 1 (g)	5.8 – 6.9	6.3	6.3	5.9 (-6%)	**5.5*** (-13%)
Pup bw day 21 (g)	41.3 – 53.7	46.6	46.3	44.3 (-5%)	**36.9*** (-21%)
Maternal bw GD 0 (g)		218.10	217.80	224.90	209.20
Maternal bw GD 20 (g)		315.80	311.70	314.50	**292.7*** (-7%)
Maternal bw gain during gestation (g)		97.7	93.9	89.6	83.5 (-15%)
Maternal bw LD 0 (g)		248.8	246.4	252.4 (+1%)	**232.0*** (-7%)
Maternal bw LD 21 (g)	226.7 –307.7	273	274.5	278.6 (+2%)	**260.3*** (-5%)
Maternal bw gain during lactation (g)		24.2	28.1	26.3	28.3

Case study 4: cobalt

- Short update from Steven Verberckmoes

4. RAC mindset on reprotox classifications

(Assessment of impact on situation for silver (*M. Holsapple*)

- ❑ As noted in my report I am not familiar with CLP regulation / classification criteria
- ❑ I have printed and scanned the following documents: 2.1, 2.2, 2.3, 4.1, 4.2, 4.3, and the relevant components of the EFSA report on the re-evaluation of silver (E 174) as a food additive
- ❑ I am still not able to find any language that addressed how a positive finding in Cohort 3 from EOGRTS (DIT) would impact classification criteria … would affect 'decision tree' … What is the relationship between Repr Cat.1B / Repr Cat.2 and a trigger to include Cohort 3 in an EOGRTS?

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5. Stress-testing examples

Round table discussion

5 ▸ Stress-Testing examples

Argument	Rebuttal possibilities
TP unnecessary – new OGRTS (Sprando et al.) negates need for EOGRTS	• Sprando study protocol weaknesses: e.g. gaps in reproductive organ histopath. and org. wt. data; gaps re fertility parameters • Fertility effects evidence weak / not replicated in 2-gen studies with SCAS • Possibility of 2º toxicity being influential • New EOGRTS (adapted) would provide precision
The overall weight-of-evidence for Ag+ reprotox is sufficient	• See also above. • MOA poorly understood / mechanistic relevance to humans not well defined. • Various axes for 2º toxicity not excluded so far, e.g. biocidal impact on microbiome • New EOGRTS (adapted) could provide better precision
Latest findings re developmental immunotoxicity (Babu et al.) represent a classification imperative	• The DIT findings in Babu et al., are indicative only and have multiple associated uncertainties (Holsapple opinion) • Key DIT parameters omitted from the study (e.g. TDAR) • Support for DIT postulate weak (adult Ag^{+} immunotox absent or weak evidence / no unique developmental immunotoxicants) • New EOGRTS with DIT cohort would address

5 ▸ Stress-Testing examples

Argument	Rebuttal possibilities
The mammalian toxicity of Ag is understood, and its reprotox profile is explainable	• Mammalian toxicity of Ag^{+} is comparatively low (versus some other metals) • But Ag^{+} remains a data-poor substance in mechanistic terms • For instance, it is a potent broad-spectrum microbiocide - knowledge still formative stage re linked <u>indirect</u> effects on adult and foetal/neonatal homeostasis (including reproductive/immune systems) • Enabling/further main studies are required on MOA
Argyria is of toxicological significance and has been associated with the adverse reprotox findings	• Weight-of-evidence is that argyria is not toxicologically significant and represents adaptive/protective change (q.v. PMC CLH input) • RAC precedent: RAC 35 decision regarding limited significance of argyria • Causal association/correlation between argyria and reprotox has not been robustly demonstrated

6. Defence of EOGRTS TP

Michael Holsapple

6. Defense of EOGRTS TP (*M. Holsapple*)

- *Arguments for why TP should be maintained in context of recent reprotox / DIT data*
- "*<u>suggestive</u> evidence … for silver … cause DIT*"
- Cohort 3 of EOGRTS would include TDAR (gold standard)
- Distribution of ionic silver – van der Zande study showed silver in liver > **spleen** > testis > kidney > brain > lungs
- Possible affect of silver on gut microbiome = plausible MOA for a unique developmental immunotoxicant
- Testing this hypothesis would require at least a OGRTS – e.g., cannot be tested in adults

6. Defense of EOGRTS TP (*M. Holsapple*)

- *After adaptation, how will TP address key weaknesses in other studies & data gaps*
- Integrating Cohort 3 into the EOGRTS will include the TDAR – definitive endpoint for ITOX … holistic assay measuring multiple components of immunocompetence
- Rationale to include Cohort 3 would be diminished if biodistribution of silver to spleen of F1 was <u>not</u> seen
- Rationale to include Cohort 3 would be diminished if exposure to silver did <u>not</u> affect gut microbiome in F1
- Interpretation of results from Cohort 3 would be compromised if unreasonably high doses of silver were used (e.g., no evidence of argyria?)

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7. Adjustments to EOGRTS TP

Michael Holsapple

7. Adjustments to EOGRTS TP (*M. Holsapple*)

- *Technical adjustments to make TP more viable/defensible (accounting for latest triggers)*
- Integrate a reasonable dose-response into TP (e.g., "*It is crucial to differentiate between a primary immunosuppressive effect and indirect effects mediated by stress*")
- Confirm biodistribution of silver to spleen of F1 – Can be done either as part of ultimate EOGRTS or as part of stand-alone studies
- Address possible MOA by determining if exposure to silver affects gut microbiome in F1 – Can be done either as part of ultimate EOGRTS or as part of stand-alone studies

7. Adjustments to EOGRTS TP (*M. Holsapple*)

- *Key test parameters for inclusion (standard & adapted to silver situation)*
- 'Reasonable' dose-response for EOGRTS
- Assess biodistribution of silver to immunocompetent organs in F1
- Assess possible MOA of silver-induced changes in gut microbiome in F1

7. Adjustments to EOGRTS TP (*M. Holsapple*)

- *Dose setting for EOGRTS*
- Keep in mind that both EPA (RfD) and WHO (NOAEL) used 5 µg/kg bw/day
- Keep in mind that Babu / Sprando studies showed possible argyria at high dose – 40 mg/kg bw/day
- 2015 TP doses: 10, 30 and 100 mg/kg bw/day would be 2,000x, 6,000x and 20,000x EPA RfD / WHO NOAEL
- Don't know much about exposures associated with uses of silver by members of Precious Metals Consortium
- As a starting point, consider … 0.5, 5.0 and 15.0 mg/kg bw/day … 100x, 1,000x and 3,000x EPA RfD / WHO NOAEL

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8. Mechanism of action (MoA) insights

Mark Raffray

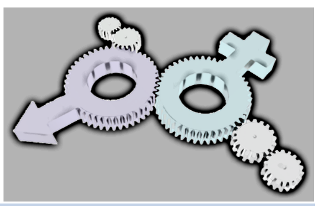
8 ▸ Mechanism of Action (MoA)

Includes:

1) Ag^+ effects on **Cu homeostasis**

2) Indirect effects via **gut microbiome** (maternal / fetal)

3) More (speculative) possibilities
   - Generalised **stress responses** related to indirect reprotox
   - Ag^+ depletion of Se ▶ consequential **Se micronutrient disorder**
   - **Other?** (q.v. Ag toxicity recent publications)



8 ▸ Key MOA theme: Ag-Cu axis effect

Effects on offspring of maternal Cu deficiency during pregnancy

Short term	Long term
Connective tissue abnormalities	Cardiac ultrastructure abnormalities
Pulmonary insufficiency	Immune suppression
Neuronal degeneration	Impaired cognitive and behavioural function
Skeletal defects	
Lower survival rate	

From: Iron, copper and fetal development; Gambling & McArdle (2004)

See also reviews of micronutrients in reproduction / deficiency:-

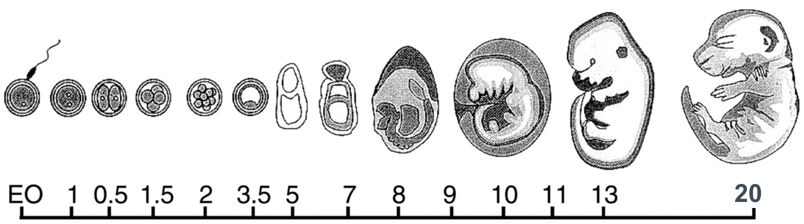
Gambling L, McArdle HJ (2004) Iron, copper and fetal development. Proc Nutr Soc. 63: 553-562. Review.

Gambling L, Kennedy C, McArdle HJ (2011) Iron and copper in fetal development. Semin Cell Dev Biol. 22: 637-644. Review.

8 ▸ Ag-Cu axis effect

Effects of Ag on Cu transport/deficiency

- From prior RAC consideration their key mechanistic focus
- Shavlovski / other rodent work (St. Petersburg teams): Ag effects on fetus caused by Cu displacement by Ag / interference with transporter protein (Ceruloplasmin; Cp)
- Defence issue: several of the favourable developmental studies (on AgAc & SCAS) do not cover full susceptibility window



8 ▸ Data gap: Fetal/neonatal Cu levels after Ag dosing

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Very limited data interrelating Cu levels to reprotox at low-moderate Ag^{+} exposures
Table ex. SZZ 2-gen study: Ag, Zn, Cu tissue levels* (mg/kg bw) **F2** pups culled @ PND 4

Dietary level SZZ ppm	control		1000		6250	
	Males	Females	Males	Females	Males	Females
Silver	<1	<1	1.04	1.06	1.68	2.2
	<1	<1	1.06	<1	1.1	<1
	<1	<1	<1	<1	1.07	1.84
Zinc	7.77	10	8.87	8.05	8.65	10.4
	6.44	6.31	11.8	6.88	7.32	7.56
	8.01	7.62	5.57	5.63	8.85	11.9
Copper	2.24	2.18	1.97	1.67	<1.5	1.86
	2.07	2.49	2.19	1.61	<1.5	<1.5
	2.15	2.72	1.61	1.76	1.96	1.52

$\bar{x}$ 89% of control $\bar{x}$ 68% of control

[LD ~2 mg Ag/kg bw/d]

But Zn treatment also depletes Cu levels ! [so-called '*Zn-Cu antagonism*']

Might this help explain why SZZ 2-gen study outcomes more severe than with SSZHP ?

*Analysis of whole animal homogenates. Values are for Individual animals.

8 ▸ MoA site of effect not an academic point

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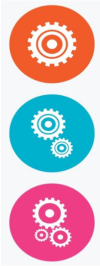
Maternally-mediated, direct embryo-fetal, or both?

- EBRC opinion (2015) / basis PMC comments: Interpretation of Shavlovski study (1995) viz. Ag influence on Cu status / Cp function
  - *"Classification for "effects observed in studies with inorganic silver is not justified, since the observed effects are secondary to non-specific disruption of maternal homeostasis"*
- Some researchers argue induced-Cu-deficiency primarily mediated at embryo-fetal level (i.e. directly embryotoxic)
  - Mieden et al. (1986) ▶ rat embryo culture model (ref. not reviewed by RAC)
- Ag^{+} case unclear: RAC decision leant toward possibility of direct embryo-fetal effects but acknowledged evidence was limited
  - Direct embryotoxicity bears on classification; Cat 2 vs 1B
  - Bearing on human situation / RA mitigation arguments



8 ▸ Relevance of Ag-Cu axis effect

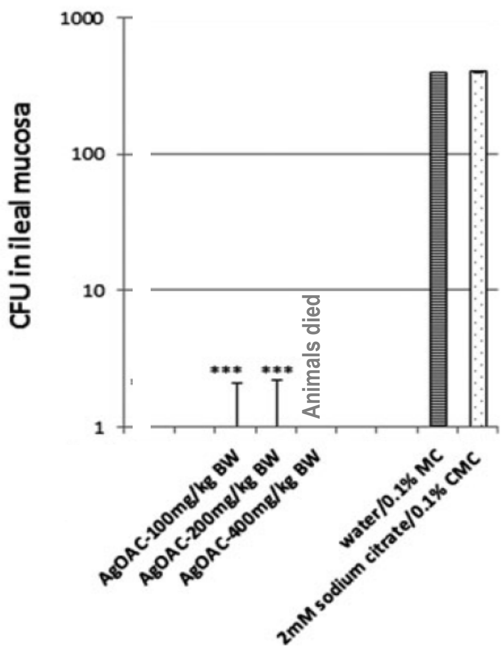
But multiple uncertainties / defence leverage points, for instance:

- As mentioned, correlation of dev. tox endpoints with Cu levels (especially embryofetal) is lacking at Ag^+ exposures below those in the Russian MoA studies on Ag-induced Cu depletion in rodents
- Criticality?
  - Cu transport & homeostasis are normal in ceruloplasmin knock-out mice
  - Humans with aceruloplasminemia have low plasma Cu, but Cu metabolism normal: indicates no essential role for Cp in Cu homeostasis
  - Though criticality Cp:Cu in development could differ from adult situation
- Human pregnancy / risk assessment relevance?
  - Human pregnancy ▶ serum Cp [& Cu] levels ↑ 3–4 fold: would significant Ag^+ exposure be needed in humans to cause reprotox?
  - If MoA relevant, modelled hazard for worst-case human Ag^+ exposure ?

Seek advice from expert on micronutrients in pregnancy:
Prof. Harry McArdle ?
(ex. Rowett Inst., UK)

8 ▸ Key MOA theme: Ag^+ impacts on gut microbiome

Bacteria attached to ileal mucosa of female rats



From Williams et al., 2014:
Adult rats, 90-d exposure by oral route with soluble Ag (Ag acetate)

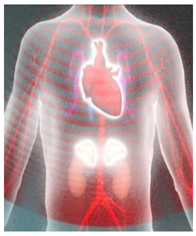
Severe microbiome effects at lowest tested dose level ≡ 64 mg/kg/d Ag^+

Shifts in key microbial populations (incl. depression of 'good' microbes)

But:
- **Data lacking at lower Ag^+ exposure levels …**
- **No knowledge at all of effects on microbiome in new-born animals**

8 ▸ Generalized indirect stress effects

- Could speculate on probability of influence on Ag^{+} reprotox
  - TK: Ag distribution to adrenals significant (% radiolabel / g tissue)
  - Moderate decreases in food and water consumption in RDT/repro studies
  - Thymic weight effects
  - Unexplained mortalities
  - Sprando et al. study flawed re some markers
- But cohesive picture of parental animal stress lacking
  - Organ wt overall picture; histopath etc.
  - Dossiers/RAC decisions have excluded significant maternal tox / stress
- Would need robust evidence
- Notoriously difficult to design studies to prove influence
  - EOGRTS with additional stress markers incorporated?
  - Might aid case if other indirect effects shown (e.g. microbiome)



8 ▸ Selenium ▶ role in reproductive biology

Se deficiency effects (humans / other animals)	
♂	**Impaired fertility** (oligospermia, abnormal spermatozoa, impaired motility)
	Depressed testosterone synthesis
	Increased oxidative DNA damage to spermatozoa
♀	**Impaired fertility**
	Increased oxidative damage to ova(?)
	Fetal death / abortion
	Retained placenta
	Neonatal development disorders, incl. soft tissues

Some important Selenoproteins
Glutathione peroxidases (GPx 1-6)
Thioredoxin reductase
Cytochrome c oxidase
Other selenoproteins

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9. Enabling work

Michael Holsapple

9. Enabling work (*M. Holsapple*)

- *Key uncertainties and data gaps where enabling studies could inform (ideally short-term work and/or desktop efforts). Cost-benefit and timeline prediction*
- Addressing statement from '**Stress-testing examples**', "*no unique developmental immunotoxicants*" – see next few slides

Background – What's so special about the developing immune system?

- It is known that common infectious diseases can **occur more often** and are **usually more severe** in the very young when compared to adolescents and adults.
- Yet, infants can mount a vigorous immune response to tissue and organ allografts ('non-pathogenic' foreign antigens) and to vaccines.
- Infants are easily immunosuppressed; susceptible to immune toxicities and immune manipulations.

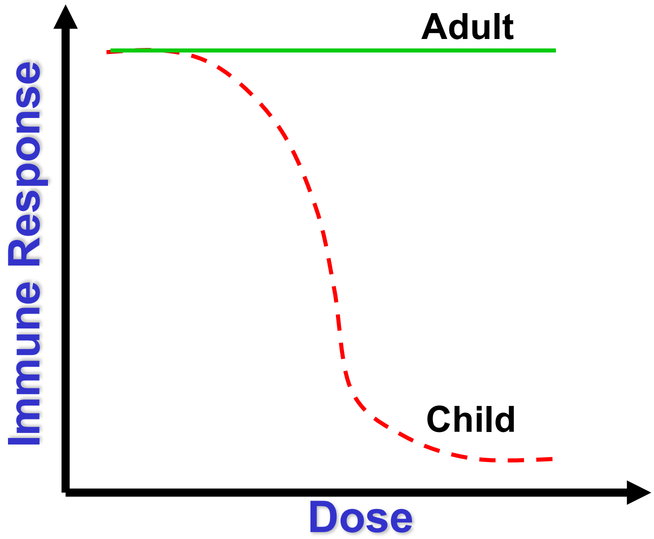
Background – What's so special about the developing immune system?

Basic Tenet: Children differ significantly from adults in their biological and/or physiological responses to environmental exposures.

Hypothesis: The **developing** immune system demonstrates **greater susceptibility** to chemical perturbation than the adult immune system.

How might kids be at greater risk?
A qualitative difference

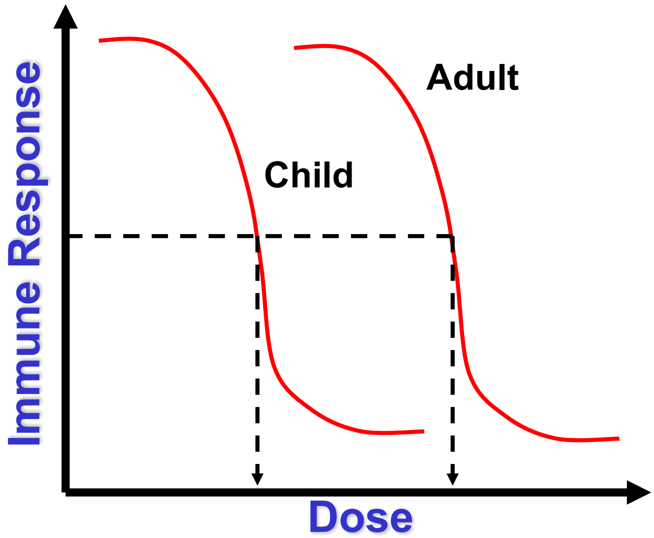
Chemicals alter the developing immune system, but not that of the adult.



Burns-Naas et al, Invited Review – What's So Special About the Developing Immune System? *International Journal of Toxicology* (27:223-254, 2008)

How might kids be at greater risk?
A quantitative difference

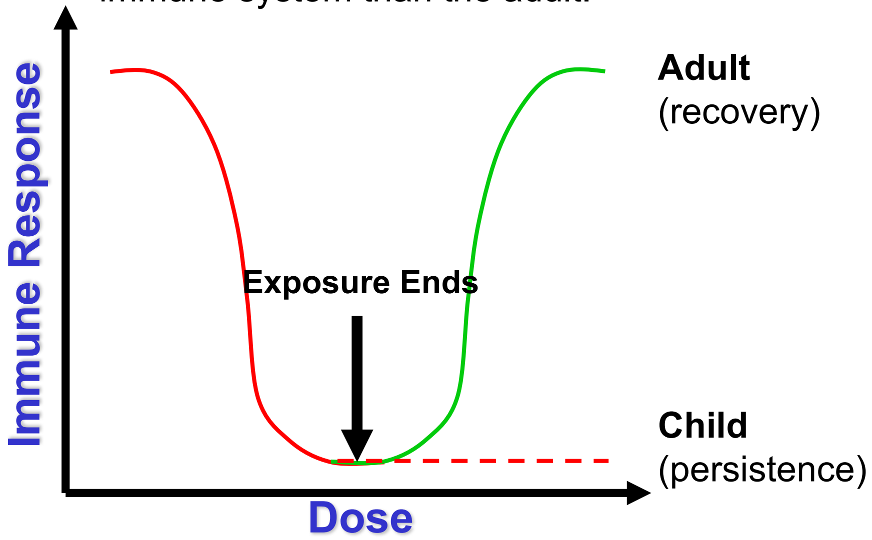
Chemicals alter the developing immune system at lower doses than the adult.



Burns-Naas et al, Invited Review – What's So Special About the Developing Immune System? *International Journal of Toxicology* (27:223-254, 2008)

How might kids be at greater risk?
A temporal difference

Chemicals exhibit more persistent effects on the developing immune system than the adult.



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What's the evidence for these differences?

Luebke R.W., et al, (2006) – The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B Crit. Rev. 9:1-26*

- **Diethylstilbestrol (DES)**: Effects more persistent following developmental exposure.
- **Diazepam (valium)**: Suppression occurs at lower doses and effects are persistent, following developmental exposure.
- **Lead**: Developmental exposures that result in BLL near or below human BLL limit affect cell-mediated immune function, and tend to stimulate IgE production.
- **TCDD**: DIT seen after exposure during immune system maturation in rats at doses <10X dose that affects adult immune function, and effects more persistent.
- **TBTO**: The immature immune system was marginally more sensitive, but the effects were more persistent.

9. Enabling work (*M. Holsapple*)

- *Key uncertainties and data gaps where enabling studies could inform (ideally short-term work and/or desktop efforts). Cost-benefit and timeline prediction*
- Addressing statement from '**Stress-testing examples**', "*no unique developmental immunotoxicants*" – see next few slides
- Addressing question … Could a silver-induced change in gut microbiome function provide an MOA for a "*unique developmental immunotoxicant*"? – see next slide

Supportive Evidence for Feasibility of Impact on Gut Microbiome as a Possible MOA for a Unique Developmental Immunoxicant

- Role of Microbiota in Immunity and Inflammation … *Cell*, 157(1):121-141 (2014) … Y. Belkaid and T. Hand
- *"The microbiota plays a fundamental role on the induction, training, and function of the host immune system"*
- Biomarkers for the 21st Century: Listening to the Microbiome … *Toxicol. Sci*, 144(2): 208-216 (2015) … R. Dietert and E. Silbergeld
- "*Our challenge is to consider these multiple interactions between and within the microbiome, the immune system, and other systems of the host in terms of exposure to exogenous agents, including environmental stressors*"

Supportive Evidence for Feasibility of Impact on Gut Microbiome as a Possible MOA for a Unique Developmental Immunoxicant (cont'd)

- The microbiome-immune-host defense barrier complex (microimmunosome) and developmental programming of noncommunicable diseases … *Repro. Toxicol.*, 68:49-58 (2017) … R. Dietert
- *"Through its role as gatekeeper and filter to the external world, the microbiome affects developmental programming of physiological systems including the immune system*"

9. Enabling work (*M. Holsapple*)

- *Linkage to EOGRTS design – e.g., dose-level setting, MOA, DIT aspects etc.*
- "*dose-level setting"* ... Covered in Slides #50, #51 and #52
- "*MOA"* … Covered in Slides #47, #48, #50, #51, #52, #72 and #73
- "*DIT aspects*" … Covered in Slides #42, #47, #48, #50, #64, #65, #66, #67, #68, #69, #70, #71, #72 and #73

9. Enabling work (*M. Holsapple*)

- *Confirm which studies should be progressed as enabling efforts versus integration of sub-studies/parameters in new EOGRTS*
- Covered in Slides #47, #48 and **#50**
- "*Confirm biodistribution of silver to spleen of F1 – Can be done either as part of ultimate EOGRTS or* ***as part of stand-alone studies***" … "***enabling efforts***"
- "*Address possible MOA by determining if exposure to silver affects gut microbiome in F1 – Can be done as part of ultimate EOGRTS or* ***as part of stand-alone studies***" … "**enabling efforts**"

Mechanisms / Enabling data / Key data gaps [1]

Investigative Work Strawman (for discussion with MPH)

Ref	Theme	Remarks / Rationale
MOA_1	In vivo tissue/circulating levels of Cu; Ag (see TK below), Se; serum Cerulosplasmin and/or Cp oxidase, Cp-associated-metal profiling (parental animals & foetal/pup)	Relates to Cu (& Se) homeostasis MOA: do developmental effects correlate / is dose-response plausible? . Main data gaps exists for foetal/pup data. See also MOA_1A below. Could also add foetal/neonatal ultrastructural studies (pathognomonic markers of Cu-deficiency)
MOA_1A	Ex vivo studies (rat embryo culture) related to Ag-Cu axis. Direct embryonic toxicity vs indirect maternal	Easier/quicker to study thresholds and developmental stage window. Do Ag-induced changes match Cu deficiency? Degree of induced Cu deficiency key?
MOA_2	Augmented TK data dataset, especially addressing current foetal (exposure) TK data gaps	Enabling TK data also key for EOGRTS design, including transplacental data (dose-level setting, distributional correlation with reprotox effects etc.). Beyond TG 443, Pt.17 recommendations. Tissue as well as blood levels for Ag.

Mechanisms / Enabling data / Key data gaps [1]

Investigative Work Strawman (for discussion with MPH)

Ref	Theme	Remarks / Rationale
MOA_4	Neonatal gut microbiome evaluation of Ag+ effects. Including low-end dose-response (a data gap)	Biome critical in proper immune system development. Postulate: significantly biocidal Ag concentrations irrelevant to worst-case human exposures
MOA_5	Parental gut microbiome evaluation of Ag+ effects	Indirect maternal effect potential / key support function of maternal microbiome
MOA_4A/5A	Ag reprotox in germ-free/gnotobiotic model	Isolate relevance of possible indirect effects of Ag+ on microbiome
MOA_4B/5B	Parenteral Ag^+ dosing comparator reprotox study	Avoids direct exposure gut microbiome by oral route (alternative effect isolation strategy)

Note: In attempting to null out the Sprando 1-gen/SCAS 2-gen datasets, if biome MOA is important a key data gap is how dosing at these relatively low Ag^+ levels affects gut microbiome(s). In any case, either enabling studies or EOGRTS dose-level setting efforts will need to address this issue.

Mechanisms / Enabling data / Key data gaps [1]

Investigative Work Strawman (for discussion with MPH)

Ref	Theme	Remarks / Rationale
MOA_6	Evaluate induced stress as an indirect mechanism. Include stress-related parameters: HPA axis/ biochemical markers, adrenal ultrastructure, 2º sex organ wt. etc. (w-o-e approach)	Not high probability of success. Differentiating mild-moderate stress effects from direct toxicities is challenging; definitive and compelling evidence needed. Confounder arguments re generalised organ/tissue/reproductive changes if Cu-mediated mechanism is in play. Might be useful if other indirect mechanisms proven
MOA_7	Argyria (ultrastructural) studies	Evidence related to direct linkage of tissue argyria with adverse histopathological change
RA_1	Risk assessment related: confirm predicted worst case adult and neonatal exposures to Ag	Assess Margin of Exposure. May integrate nanoAg. Achieve via desk-top study

10. Re-visit stress-test / Confirm technical defence plan

Round table discussion

Stress-Testing examples

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TP unnecessary – new OGRTS (Sprando et al.) negates need for EOGRTS	• Sprando study protocol weaknesses: e.g. gaps in reproductive organ histopath. and org. wt. data; gaps re fertility parameters • Fertility effects evidence weak / not replicated in 2-gen studies with SCAS • Possibility of 2º toxicity being influential • New EOGRTS (adapted) would provide precision
The overall weight-of-evidence for Ag+ reprotox is sufficient	• See also above. • MOA poorly understood / mechanistic relevance to humans not well defined. • Various axes for 2º toxicity not excluded so far, e.g. biocidal impact on microbiome • New EOGRTS (adapted) could provide better precision
Latest findings re developmental immunotoxicity (Babu et al.) represent a classification imperative	• The DIT findings in Babu et al., are indicative only and have multiple associated uncertainties (Holsapple opinion) • Key DIT parameters omitted from the study (e.g. TDAR) • Support for DIT postulate weak (adult Ag^+ immunotox absent or weak evidence / no unique developmental immunotoxicants) • New EOGRTS with DIT cohort would address

Stress-Testing examples

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Argyria is of toxicological significance and has been associated with the adverse reprotox findings	• Weight-of-evidence is that argyria is not toxicologically significant and represents adaptive/protective change (q.v. PMC CLH input) • RAC precedent: RAC 35 decision regarding limited significance of argyria • Causal association/correlation between argyria and reprotox has not been robustly demonstrated

THANK YOU

www.epmf.be | info@epmf.be
Avenue de Broqueville 12, B-1150 Brussels
+32 (0)2 761 01 00