Silver Tox Experts (TE) Call

Draft minutes, Call 15 February 2019 (10:00-11:30 CET)

1 Welcome and Introduction

1.1 Reminder on Confidentiality and Competition Law

Participants were reminded on their obligation to comply with Confidentiality and Competition Law.

1.2 Tour de table and apologies

The list of participants is available in Annex 1.

1.3 Approval of the agenda

The agenda is available on slide 3 in Annex 2. No remarks / additions; agenda approved.

1.4 Approval of the minutes of the last meetings (19 Dec) and status of action points

No remarks / additions; minutes of the meetings of 19 December (EPMF TE meeting and EPMF+ESTF TE meeting) were approved. Tables with the status of the action points from these meetings are available on slides 29-30 in Annex 2. All action points have appropriately been addressed / are ongoing where possible, or will be discussed during the call.

2 Ag gut biome study: preliminary results

Cf. slides 5-27 in Annex 2 (with slides 5-15 already discussed during the 19 December TE meeting). Prof. Lison kindly presented the latest available results from the study. Comments/additions:

- Slide 15: results from **Se and Cu measurements** in serum should be available in the next few weeks.
- Slide 16: Ag levels in blood of rats are consistent with earlier results. Comparison of the blood **Ag content** to other oral TK studies shows that oral bioavailability is higher via gavage than dietary administration. There are still data gaps for TK data of some specific Ag compounds. Saturation kinetics were not achieved at 28 days since exposure levels were too low. Ag measurements in 10 week blood samples and tissue samples should be available in the next few weeks.
- **Biome determination** was done by next generation sequencing (NGS) analysis at MR DNA lab. Raw sequences were identified by comparing to a database to generate operational taxonomic units (OTU). OTUs were grouped based on analogy at phylum / family level. Results are available on slides 17-24:
    - Slides 18-19 (phylum): the statistics mentioned at the top of each graph are the classical p-values, and Q-values integrate the false-discovery rate (reasonable threshold is 0.10). Two statistics were applied, ANOVA (is there a difference among the groups? any difference) and trend-tests (is there a dose-dependent trend? most relevant). There are changes in the phylum proportions both in males and females. A **significant increase of cyanobacteria** is observed in both sexes, but these represent only a small proportion of bacteria in the gut. The biological relevance of this change is not yet determined (**AP1**). With rats, there is a possibility of a homogenisation effect in a cage, but this is unlikely here since only 2 rats per cage, meaning the observation is treatment related. Cyanobacteria are unlikely to be pathogenic, and no sign

of infection or inflammation was observed. It was questioned if there is a link between cyanobacteria and neurotox (**AP2**).

- It was noted that the biome effects in the nanoAg mouse study (van den Brule et al. 2016) seemed more pronounced. This might possibly be related to the local bioavailability in the gut being higher for nanoAg than for ionic Ag, as nanoAg causes local delivery of ionic Ag (something similar has been observed in sludge studies, where the effects of nanoAg are higher than for ionic Ag because of local hotspots with high Ag^+ concentrations). It was further noted that ionic Ag can form secondary NPs in the gut.
- Slides 21-22 (family): the main effect seems a **significant reduction of S24-7** in both sexes (this was also observed in the nanoAg study). Again, the biological relevance of this change is not yet determined (**AP2**). Looking at the **Campylobacter** family (for which in humans and cattle, overgrowth of pathogenic forms can lead to adverse reproductive outcomes (e.g. abortions)), it appears that some animals show an increase in proportion of these bacteria after AgAc treatment and some do not. For the animals responding, the data should be checked in more detail (Were they in the same cage? What was their blood Ag content?) (**AP3**).
- Slide 23 (analysis of the **α-diversity**; richness and evenness within a microbial community): no significant effect.
- Slide 24 (analysis of the **β-diversity**; differences in composition among communities): there is a dose-dependent effect of AgAc treatment on the β-diversity or the equilibrium of the biome, in both sexes. The upper graphs are a 2-D representation of an analysis that integrates much more dimensions. The figures at the top of the graphs (ADONIS ~ ANOVA, and trend-test) show a significant difference in β-diversity between the different treatments both in males and females (more pronounced in females). The trend is further illustrated in the bottom graphs. Again, the biological relevance of this change is not yet determined (**AP2**).
- Conclusion: AgAc treatment has a significant effect on the rat gut microbiome, and this shows the possibility of a potential secondary effect on reprotox. The next step is to look at the **biological relevance of the observed biome changes** / look at the functionality of the biome. This is usually done by looking at the short-chain fatty acids (SCFAs like butyric acid, lactic acid…), key faeces metabolites generated by gut bacteria fermentation. These can be analysed in the faeces via GC-MS. Faeces have been stored, but not yet analysed. (**AP2**).
- In terms of potential endocrine interference, in both humans and rats there is evidence that circulating estrogen levels are linked to functional microbiomes, such as the gut microbiome. Dysbiosis involving the GI tract has a potential to depress circulating estrogen. This is due to loss of β-glucuronidase capacity in the gut resulting in decreased deconjugation of estrogen hormones into their active forms (cf. Baker et al. 2017). In the case of significant microbiome shifts, it is worth considering whether estrogen levels have been affected (**AP2**).
- It is noted that the inclusion of the DIT cohort in the EOGRTS is already proposed, so this additional investigation is more for clarification than for decision making about EOGRTS test-setup.
- Prof. Lison anticipates that 2 months will be needed to determine SCFA and estrogen levels. A cost proposal will be provided.

- **Uterus tissue samples** have been reserved and it was agreed at the last TE meeting to proceed to histopath of uterus samples for the control and the high dose. This could be done by Prof. E. Marbaix at Louvain University and we are awaiting an estimation of the costs.

(Post-meeting note: Conventional tissue analysis requires 10% formalin fixation followed by cassette placement with appropriate orientation of the uterus and ovaries and paraffin embedding, followed by histological sections at 5 µm thickness and staining with hematoxylin and eosin. The histological sections will be examined by light microscopy and their analysis will be reported with photomicrographs of the lesions or tissue changes of interest where appropriate. The costs are estimated at 121 € per rat or 4.840 € for 40 rats)

- **Ag localisation in the brain**: we suspect that Ag does not pass the blood-brain-barrier but that it binds to the vascular epithelial cells or blood cells. Ideally, this should be confirmed with ultrastructural localisation of Ag in the brain which can be done with different techniques (autometallography (AMG); EM-EDX / EM-EELS; XRF; laser capture microscopy with ICP-MS). AMG is the quickest and most cost-efficient way to check but there are some doubts about the techniques' sensitivity. Therefore, we would perform AMG first, check what comes out and later on decide whether more advanced imaging techniques would be required. AMG could be done at Louvain University (1.500 €/sample) or Antwerp University (awaiting estimation of costs).

3 Ag read-across

Cf. slide 32 in Annex 2. No questions / feedback on the Ag TK review report. The TE support the proposal for a detailed follow-up discussion on the TK work + read-across (**AP4**).

4 Preparation EOGRTS

- Updated **process and timeline** is available on slide 34 in Annex 2. Comments on the DD were submitted by 8 Feb 2019 and are now under consideration by ECHA, who may amend the DD accordingly. Afterwards, ECHA will notify the MSCA of the (amended) DD and they will have the chance to submit comments as well. The DD will then be referred to MSC, who may seek agreement in written procedure or at its MSC meeting. In any case, based on the decision-making process, our **current estimate for receiving the final decision is June 2019 at the earliest.** It is noted however that the ECHA guidelines on dossier evaluation mention 'ECHA notifies the MSCAs of the (amended) decision, which generally occurs within **3-9 months** from receipt of your comments'. This would mean that the second commenting period for EPMF (on the PfAs) could be anywhere between Jun and Dec 2019.
- **Feedback received from CROs in preparation of comments to DD**: an overview on feedback received from the CROs (Charles River, Citoxlab, LPT and Envigo) on timing/costs of the EOGRTS is available on slide 35 in Annex 2. Although it is not entirely clear which analyses (type + number) is included in each offer, the price for the EOGRTS seems quite comparable (700-850 k€) between the different labs with Citoxlab being the cheapest. It is noted that it has recently been announced that Charles River will acquire Citoxlab, probably in Q2 2019. **AP5**
- A preliminary assessment of available data on Ag reprotox was done by David Esdaile from Citoxlab in order to assess what kind of DRF would be needed before starting the EOGRTS. This assessment contained **further critique of the Sprando et al. study** (cf. slide 36 in Annex 2) and the suggestion to request the raw data and historical control data for the study. The TE agreed to ask for this data (**AP6**).
- Mark Raffray briefly outlined the considerations for **dose level setting for the EOGRTS**: cf. slides 37-38 in Annex 2. This will be further discussed during the face-to-face meeting (**AP4**). In order to decide on the usefulness of the gut biome results for the dose-setting discussion, we should ideally have

further information on the biological relevance of the results. In addition, we should also take into accounts the Cu metabolism MoA.

- **Possible CROs / study monitors**: as possible study monitor, Lindsay Aveyard was mentioned during the Dec TE meeting. She has extensive metal experience and has proved to provide very valuable input on other metal cases. The TE agree to contact her already (and check if she would be willing to attend the face-to-face meeting). **AP7**

5 Feedback ECHA on disconnect REACH / BPR / CLH following submission CLH dossier AgNO3 by KemI

Cf. slide 41 in Annex 2.

Annexes

1. List of participants
2. Slides presented at the call

Actions

Table 1. Actions agreed at the 15 February 2019 Ag Tox Experts call

	What?	Who?	When?
Ag gut biome study			
1.	Investigate link between cyanobacteria and neurotox.	Prof. Lison	Mar 2019?
2.	Send further offer for: • checking the biological relevance of the observed changes in the gut biome / SCFA analysis in feces; • checking estrogen levels.	Prof. Lison	Mar 2019
3.	Compare different data from animals showing an increase in proportion of bacteria from Campylobacter family after AgAc treatment.	Prof. Lison	Mar 2019?
Ag read-across			
4.	Organise detailed follow-up discussion on TK work + read-across.	EPMF Sec	Mar 2019
Ag EOGRTS			
5.	Follow-up offers for EOGRTS with different labs.	EPMF Sec	Mar-Apr 2019
6.	Ask for raw data and historical control data of the Sprando et al. study	EPMF Sec	ASAP
7.	Contact Lindsay Aveyard as possible study monitor for the EOGRTS	EPMF Sec	ASAP

Annex 1: Participants

Katrien ARIJS, consultant for EPMF (ARCHE, Belgium)

Arno BUTHE, Heraeus (Germany)

Marie-Laure LEDRICH, consultant for Traxys (Luxembourg)

Olga LEMKE, BASF (Germany)

Dominique LISON, University of Louvain (Belgium) – *for agenda point 2*

Jelle MERTENS, EPMF (Belgium)

Mark RAFFRAY, Consultant, Raffray Biosciences Ltd (United Kingdom)

Nissanka RAJAPAKSE, Johnson Matthey (United Kingdom)

Steven VERBERCKMOES, Umicore (Belgium)