



Precious Metals
Consortium

Precious Metals & Rhenium Consortium

Silver TE Call: Study Ag effects on the biome

23 February 2018

Outcome TE call 16 Jan: Design Ag microbiome study I

Basic design aspects

- Rat; sexually mature adult (~6 wk)
- Strain: Wistar (SD also possibility)
- Test article: Ag acetate (AgAc)
- Dietary administration (diet formulation to be performed at external lab)
- 10 animals per sex/group
- Group sizes augmented by satellite animals for ancillary investigations, as required

Target treatment levels [mg/kg bw/d] if based on Sprando et al.:

0 0.4 4 40 AgAc

0 0.26 2.6 26 Ag \equiv

Sprando devtox LOAEL /
NOAEL: 2.6 / 0.26
mg/kg/d

Devtox LOAEL for main
SCAS (SZZ) was ~11
mg/kg/d in 2-gen design

- Duration **28 days**
- Sprando et al. (2017): females dosed for **2 wk prior to mating**, then **throughout gestation** (~22 d) & lactation [males 10-wk dosing covering spermatogenesis]
- Williams et al. (2014) existing most comprehensive rat study of Ag⁺ (**AgAc**) impact on microbiome after 90-d dosing (LD = 65 mg Ag⁺/kg bw/d)
- Van den Brule (2016) study in mouse with **AgNP** was 28-d ► clear effects evident after this duration

Design input on microbiome aspects of study is awaited from Univ. Louvain



Outcome TE call 16 Jan: Design Ag microbiome study II

Ancillary investigations

TK

Ag levels in:
Blood
Faeces
Intestinal contents

Ask to freeze tissue sets / possibly analyse later

- Blood/faeces to be sampled twice: mid-point (7 or 14-d, TBD) + 28-d terminal (proof of exposure)
- Intestinal contents to be sampled at 28-d (gut content Ag analysis may aid understanding biome d-r)
- Other tissues of potential interest: reproductive system (female & male); spleen/thymus (immunotox); brain (neurotox); liver & kidney (main distribution sites/comparator to other studies)
- Benchmark good quality TK study exists for Ag⁺ ► van Zande et al. 2012: 28-d; rat; gavage; 5.7 mg Ag⁺/kg bw (as AgNO₃)
 - Broad range of tissues (28-d terminal), blood (weekly), faeces (weekly) & intestinal contents (terminal)

Cu/Cp MoA

- Serum Cu levels
- Serum Ceruloplasmin oxidase activity

- To be sampled twice: mid-point (7 or 14-d, TBD) + 28-d terminal
- Rationale: to obtain data on Cp oxidase activity depression / serum Cu depletion at exposures comparable to Sprando et al. + SZZ 2-gen study (where only fetal homogenate data exists)

Se MoA

Serum Se levels

- To be sampled twice: mid-point (7 or 14-d, TBD) + 28-d terminal
- Rationale: Se sequestration of Ag (also by S) is protective mechanism in producing inert Ag depots in tissues (argyria)
- Repeated oral Ag exposure might conceivably also interfere with Se uptake in gut
- Se is a key micronutrient in fertility & normal fetal development
- Data gap exists as to how Ag treatment of adult rats impacts on Se homeostasis (at low-end Ag doses shown to provoke apparent devtox & possible antifertility effects)
- Any work outside the context of an associated reprotox protocol could only be indicative of an area meriting follow-up

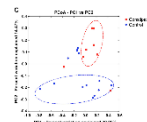
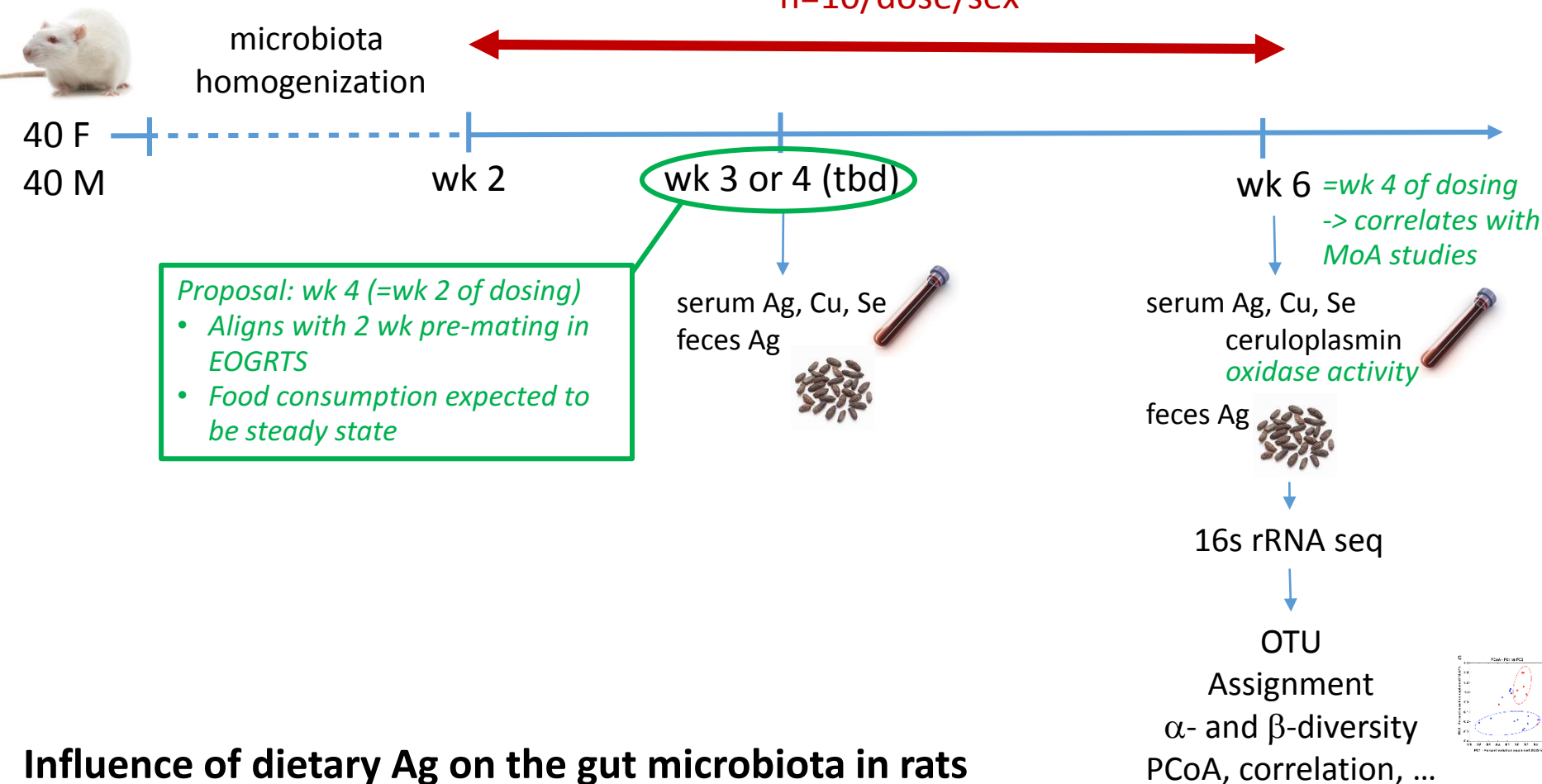
Proposal van den Brule & Lison Feb 2018



Dietary formulation:

- Carfil: 1000 €
- Analysis included?

0, 0.4, 4 and 40 mg AgAc/kg/d
n=10/dose/sex



Influence of dietary Ag on the gut microbiota in rats
(S. van den Brule, D. Lison; Feb 2018)

Quote for the study on the effects of soluble silver on the gut bacterial microbiota

Product or task	Description	Provider	unit price	quantity	total price
Wistar rats	females, 4 weeks, 100-124 g	Janvier	€ 13,61	40	€ 544,40
	males, 4 weeks, 100-124 g	Janvier	€ 13,61	40	€ 544,40
	delivery boxes	Janvier	€ 27,00	14	€ 378,00
	transport	Janvier	€ 125,00	1	€ 125,00
mixing Ag Ac with diet		PMC Carfil?	€ 1.000,00	1	€ 1.000,00
animal facility	room (price/month)	Animalerie Centrale UCL	€ 249,00	2	€ 498,00
	entry (price/person/month)	2 persons	€ 52,00	4	€ 208,00
	litter	Animalerie Centrale UCL	€ 34,30	3	€ 102,90
	feed	Animalerie Centrale UCL	€ 42,00	2	€ 84,00
Consumables	tubes, tips, ...				€ 2.000,00
QIAamp DNA stool mini kit	50 DNA extractions	Qiagen	€ 272,00	2	€ 544,00
NGS (16S bacterial sequencing)	80 samples (5600 \$)	MR DNA lab	€ 56,80	80	€ 4.544,00
	shipping		€ 200,00	1	€ 200,00
ICP-MS (Ag, Cu and Se levels)	in blood (7 or 14 and 28d), feces (7 or 14 and 28d) and intestinal contents (28d)	St Luc Any experience?	€ 30,00	640	€ 19.200,00
ceruloplasmin activity test	100 tests, in blood (7 or 14 and 28d)	Sigma	€ 469,00	2	€ 938,00
experimental design and data analysis	Sybille van den Brule (month)	UCL	€ 8.000,00	1	€ 8.000,00
NGS data analysis	Jérôme Ambroise (month)	UCL	€ 8.000,00	1	€ 8.000,00
sample collection, DNA extractions and other assays	Saloua Iboursadaten (month)	UCL	€ 4.000,00	1	€ 4.000,00
Animal care and exposure	Mihaly Palmai-Pallag (month)	UCL	€ 4.000,00	1	€ 4.000,00
<p>Blood: 3 analytes (Ag, Cu, Se) x 2 times x 80 rats = 480 samples</p> <p>Feces: 1 analyte (Ag) x 1 time x 80 rats = 80 samples</p> <p>Gut content: 1 analyte (Ag) x 1 time x 80 rats = 80 samples</p>				total	€ 54.910,70
				overhead	€ 10.982,14
				VAT	€ 11.531,25
				TOTAL	€ 77.424,09



Time schedule		
	weeks	
Test Ag, Cu and Se measurement by ICP-MS	2	
rat acclimation	2	
exposure duration	4	
DNA extraction	1	
Ceruloplasmin activity	1	
Ag, Cu and Se measurement by ICP-MS	2	
mrDNA sequencing	4	
sequencing analysis	4	
overall analysis	2	
total	22	5 months

- **+ lead time of min. 2 months** (for ordering rats, food, development Ag analysis...)
- **Most critical aspect: ICP-MS measurement** of Ag in biological tissues which might require significant development
- **No formal guarantee that all Ag measurements will be available at the end of the 5 months** (but would not affect main study objective, i.e. impact on microbiota)
- **Alternative: outsource ICP-MS measurements** (suggestions?)

Additional measurements in the Ag rat biome study?

- 1) Measurement of **testicular Ag levels** (Ag concentrations per g tissue + organ weight + histopath): this is something that was not looked at in the Sprando study while other testicular studies were performed at lower exposure levels.
- 2) Measurement of **luminal Ag concentrations in the gut** at the end of the study: this was measured in a study on effects of nanoAg in mice (Bergin et al. 2016, Ref ID#6). However, this measurement may require satellite animals.



Timeline?

- Study would help in EOGRTS design and could provide argumentation to
 - 1) further defend our TP and
 - 2) avoid classification -> point stands even if TP is eventually denied! (i.e. study has fallback value even if not directly useful for EOGRTS design)
- Even after DD on TP is issued, we still have chances to defend it (through comments on the decision / informal call with ECHA) -> proposal / timeline from Prof. Lison useful in discussions with ECHA on timeline needed for EOGRTS
- **16 Jan TE call:** TE agreed to wait for proposal / timeline from Prof. Lison before making decision on biome study (might already have DD by then)
- **20 Feb Mgmt Cttee meeting:**
 - Ag strategy discussed
 - Very unlikely to have results biome study before DD -> no rush to start study
- Mail sent to ECHA to ask clarity on timing

Gao et al. 2017

'Toxicity of nano- and ionic silver to embryonic stem cells: a comparative toxicogenomic study'

- US FDA study, done in parallel with Sprando and Babu studies; **Klimisch 2**
- Study represents enhanced version of the EST coupled with toxicogenomics and is the first of its kind on nanoAg/Ag+
- *In vitro* cytotoxicity after 24h exposure of pluripotent mouse embryonic stem cells (ESC) to nanoAg and Ag+ (AgAc) -> both caused concentration-dependent cytotoxicity (Ag+ > nanoAg) (difference between mono-layer culture and embryoid body drop culture with reduced cytotoxicity in the latter)
- Differential gene expression analysis -> both nanoAg and Ag+ impacted multiple groups of genes involved in general development, morphogenesis, embryonic development, cell differentiation, and metabolic pathways – suggesting potential for embryotoxicity
- Note: limitations of *in vitro* model for developmental tox endpoint + interpretation of toxicogenomic outcomes challenging.
- **External reviewers may conclude that this *in vitro* study adds support to the *in vivo* developmental findings on AgAc reported by Sprando et al. (2017)**





THANK YOU

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