GRACE
GMO Risk Assessment and Communication of Evidence

G-TWYST
GM PLANTS TWO YEAR SAFETY TESTING

Mandatory 90-day feeding trials in rats

- A “must” when assessing the safety of genetically modified plants? -

SEVENTH FRAMEWORK PROGRAMME

Key objectives of the GRACE project

To test GM maize MON810 varieties in subchronic and chronic animal feeding trials and alternative *in vitro* methods in order to determine how suitable the above-mentioned test systems are and whether they provide useful scientific information for the health risk assessment of GM food and feed.

	Monsanto MON810/ near isogenic control	Pioneer MON810/ near isogenic control	Conv. 1	Conv. 2	Conv. 3	Conv. 4
Study A (90 days)	X		X	X		
Study B (90 days)		X			X	X
Study C (1 year)	X[1]			X[1]		
Study D (longitudinal, metabolomics, 90 days)	X					
Study E (longitudinal metabolomics, 90 days)		X				

[1] Different planting season

- **Two 90-day feeding trials with two different GM maize MON810 varieties were performed: Their study design was based on the OECD Test Guideline 408 for Testing of Chemicals - Repeated dose 90-day oral toxicity study in rodents, and recommendations of the European Food Safety Authority (EFSA) were taken into account.**

- **The selected MON810 varieties were the two most widely used by local farmers in Catalonia (Spain).**

- **In each feeding trial the corresponding near-isogenic non-GM maize variety as well as two additional conventional maize varieties were tested (collection of historical data).**

Study design of the 90-day feeding trials A and B

Group	Dose (% w/w feed)				No. of animals	
	non-GM isogenic	MON810	conv. 1	conv. 2	male	female
1	33	0	0	0	16	16
2	22	11	0	0	16	16
3	0	33	0	0	16	16
4	0	0	33	0	16	16
5	0	0	0	33	16	16
Total					80	80

Feeding trial A	
Diet	**Maize variety content (%)**
33% near-isogenic non-GM maize	33% DKC6666[a]
11% MON810	11% DKC6667-YG[b] + 22% DKC6666
33% MON810	33% DKC6667-YG
33% conventional 1	33% PR33W82[c]
33% conventional 2	33% SY NEPAL[d]

[a] Near-isogenic maize variety of DKC6667 YG, from Monsanto
[b] Transgenic maize variety (MON 810), from Monsanto
[c] Conventional maize variety, from Pioneer Hi-Bred
[d] Conventional maize variety, from Koipesol Semillas

Feeding trial B	
Diet	**Maize variety content (%)**
33% near-isogenic non-GM maize	33% PR32T16[a]
11% MON810	11% PR33D48[b] + 22% PR32T16
33% MON810	33% PR33D48
33% conventional 1	33% PR32T83[c]
33% conventional 2	33% DKC6815[d]

[a] Near-isogenic maize variety of PR33D48, from Pioneer Hi-Bred
[b] Transgenic maize variety (MON 810), from Pioneer Hi-Bred
[c] Conventional maize variety, from Pioneer Hi-Bred
[d] Conventional maize variety, from Monsanto.

- **The following experimental steps were undertaken:**
  - **the composition of the feed was analysed**
  - **the body weight and the feed consumption were monitored**
  - **clinical and ophthalmological observations were recorded**
  - **haematology and clinical biochemistry parameters were quantified**
  - **a gross necropsy including the determination of the absolute and relative organ weights as well as a histopathological analysis were performed**

Histological findings in male and female Wistar Han RCC rats in the 90-day feeding trials A and B

A

Organ	Histological finding	33% isogenic non-GM maize	33% GM maize
Males			
prostate	focal fibrosis	1/16	0/16
	interstitial mononuclear infiltration	2/16	2/16
seminal vesicles	interstitial mononuclear infiltration	0/16	2/16
Females			
kidney	cysts	1/16	0/16
ovary	cystic follicles	0/16	1/16
small intestine	lymphoepithelioid granuloma	2/16	1/16
	calcified lymph nodes	0/16	1/16
uterus	mucification of the endometrial epithelium	1/16	0/16

B

Organ	Histological finding	33% isogenic non-GM maize	33% GM maize
Males			
adrenal gland	cortex vacuolization	1/16	0/16
epididymis	focal epididymitis	0/16	1/16
heart	mononuclear cell nodule	0/16	2/16
prostate	interstitial mononuclear infiltration	2/16	1/16
	focal fibrosis, prostatitis	0/16	1/16
Females			
uterus	mucification of the endometrial epithelium	0/16	1/16
mesentery	lipoma	0/16	1/16

Conclusions: *90-Day feeding trials with the GM maize MON810*

- **The results obtained in the 90-day feeding trials performed in the frame of GRACE show that the MON810 maize at a level of up to 33% in the diet did not induce adverse effects in male and female Wistar Han RCC rats after subchronic exposure, independently of the different genetic backgrounds of the event.**
- **It is obvious that statistically significant differences regarding several parameters between the control groups and the groups being fed the GMO diets for 90 days were observed, but the differences were interpreted as being unrelated to the MON810 event.**

Publications

Zeljenková *et al*. (2014) 90-day oral toxicity studies on two genetically modified maize MON810 varieties in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). Arch. Toxicol. 88: 2289-2314.

Schmidt *et al*. (2016) Enhancing the interpretation of statistical P values in toxicology studies: implementation of linear mixed models (LMMs) and standardized effect sizes (SESs). Arch. Toxicol. 90: 731-751.

Schmidt *et al*. (2016) Proposed criteria for the evaluation of the scientific quality of mandatory rat and mouse feeding trials with whole food/feed derived from genetically modified plants. Arch. Toxicol. 90: 2287-2291.

Zeljenková *et al*. (2016) One-year oral toxicity study on a genetically modified maize MON810 variety in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). Arch. Toxicol. 90: 2531-2562.

Schmidt *et al*. (2017) Variability of control data and relevance of observed group differences in five oral toxicity studies with genetically modified maize MON810 in rats. Arch Toxicol. 91: 1977-2006.

Sharbati *et al* (2017) Transcriptomic analysis of intestinal tissues from two 90-day feeding studies in rats using genetically modified MON810 maize varieties. Frontiers in Genetics 8: 222.

Tulinská *et al*. (2018) Humoral and cellular immune response in Wistar Han RCC rats fed two genetically modified maize MON810 varieties for 90 days (EU 7th Framework Programme project GRACE). Arch. Toxicol. 92: 2385-2399.

Key objectives of the G-TwYST project

In 2012 a controversially discussed study described the long-term toxicity of a Roundup herbicide and the Roundup-tolerant genetically modified maize NK603. Moreover, EFSA was requested to assist the EC by providing supplementary guidance on key elements to consider for a 2-year carcinogenicity trial in rats with whole food/feed if requested in the course of a GMO risk assessment. In this context, the G-TwYST consortium performed two 90-day feeding trials, one with maize inclusion rates of 11 and 33% and one with inclusion rates of up to 50%, as well as a combined chronic toxicity/carcinogenicity study with inclusion rates of 11 and 33%.

90-day feeding trial 1 with GM maize NK603

Group	Content in the diet (%)			No. of animals	
	Isogenic non-GM	NK603	NK603 + Roundup	Males	Females
1	33	0	0	16	16
2	22	11	0	16	16
3	0	33	0	16	16
4	22	0	11	16	16
5	0	0	33	16	16
Sentinels				6	6
Total				86	86

90-day feeding trial 2 with GM maize NK603

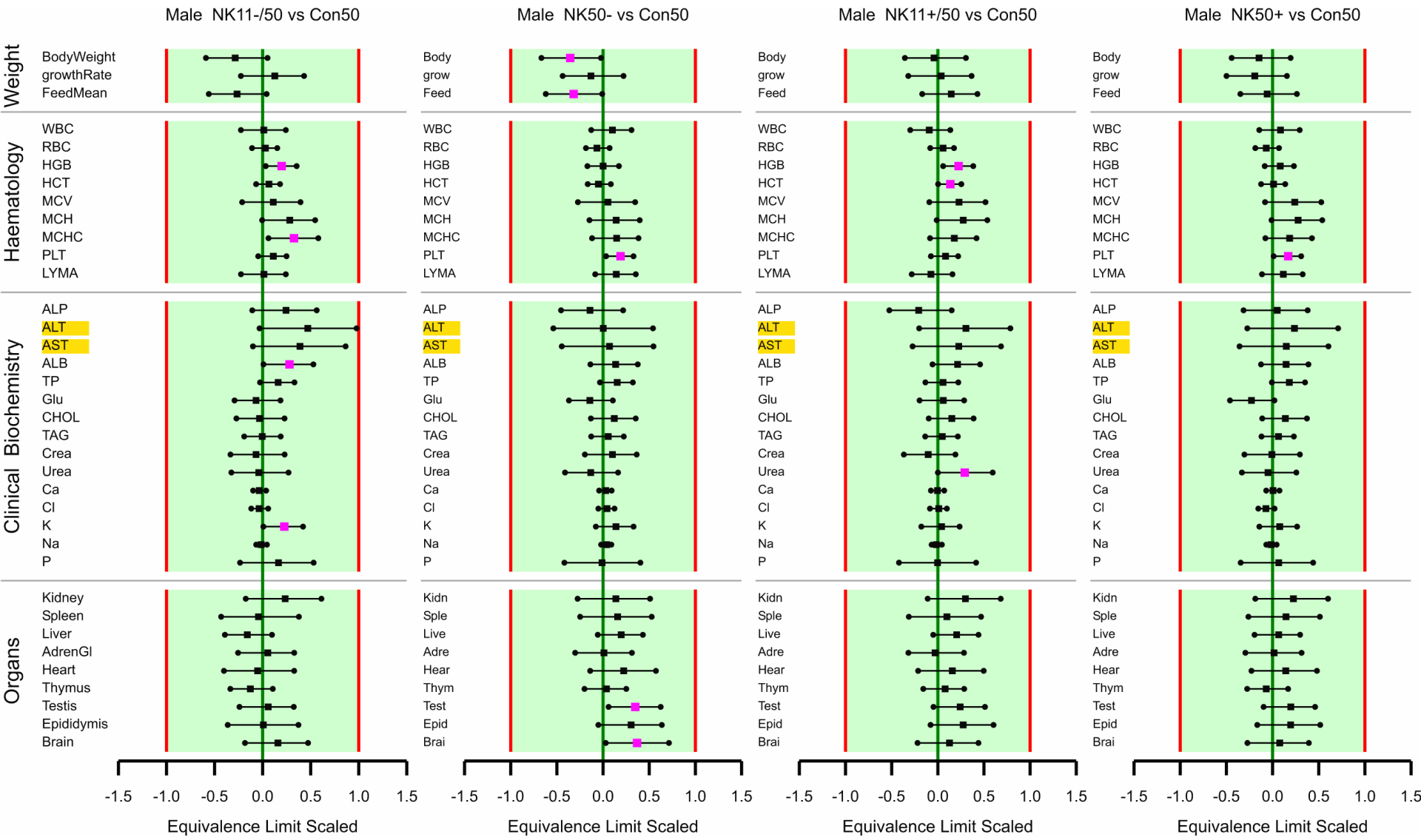
Group	Content in the diet (%)			No. of animals	
	Isogenic non-GM	NK603	NK603 + Roundup	Males	Females
1	33	0	0	16	16
2	0	33	0	16	16
3	0	0	33	16	16
4	50	0	0	16	16
5	39	11	0	16	16
6	0	50	0	16	16
7	39	0	11	16	16
8	0	0	50	16	16
Sentinels				6	6
Total				134	134

Equivalence and difference tests

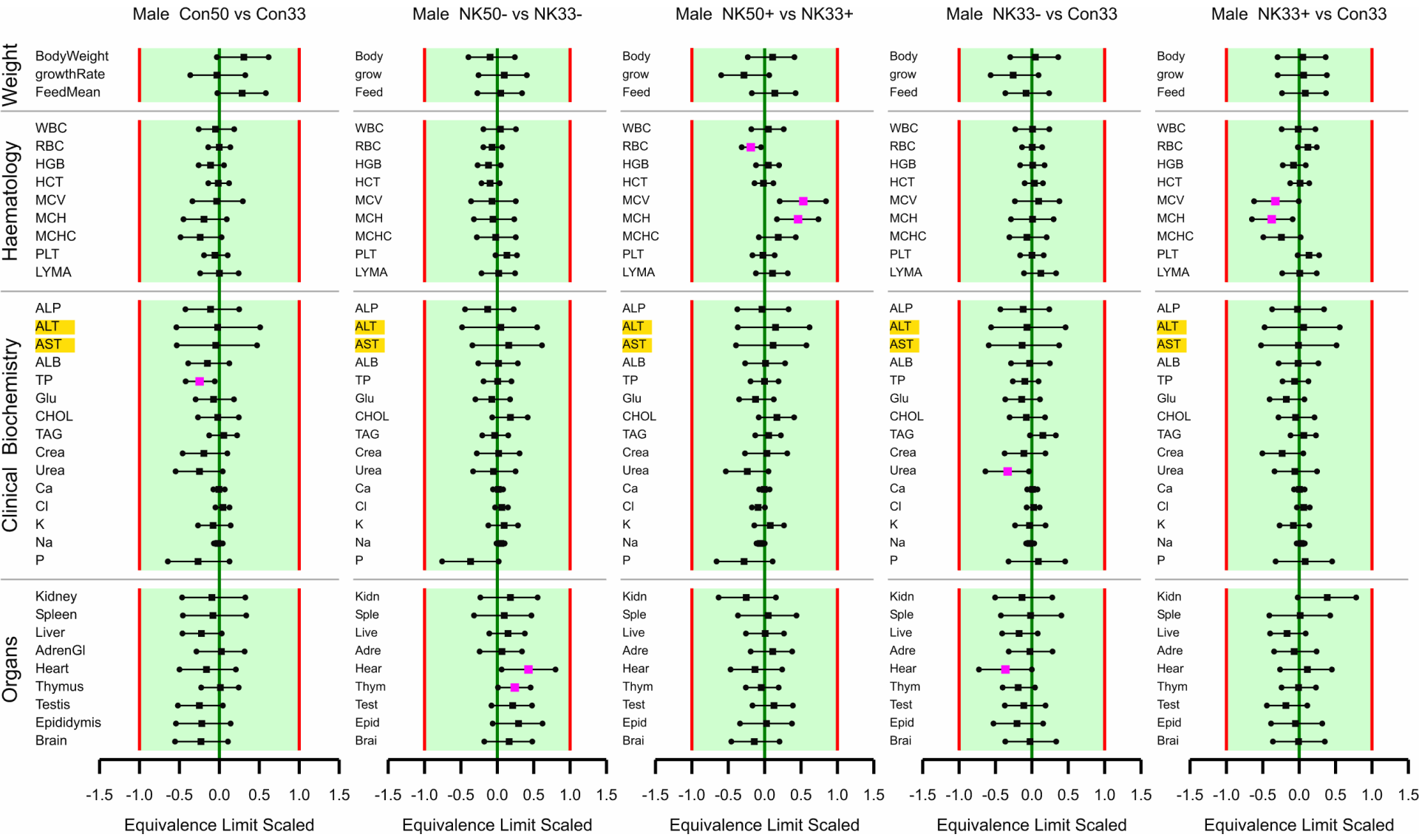
(Visual overview of results: next 4 slides)

- **Graphs of confidence intervals for differences between the treated groups and the control group**
- **Equivalence-limit scaled, based on the observed variation in historical (GRACE) non-GM data**
- **When the interval does not contain 0 → proof of difference**
- **When the full interval lies within [-1,+1] → proof of equivalence**
- **Some endpoints have a high residual variation**

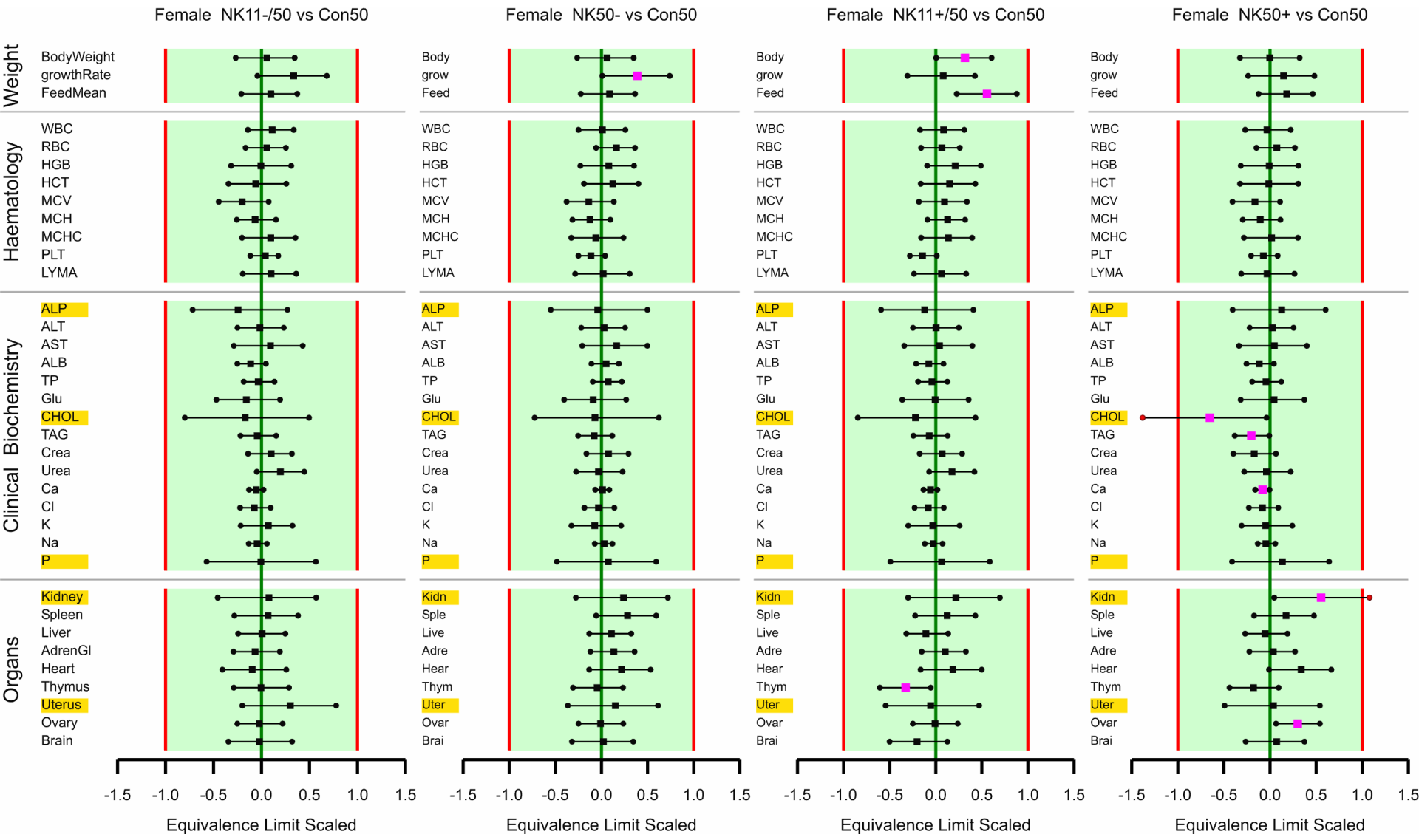
Equivalence tests, 90-day feeding trial 2, male rats



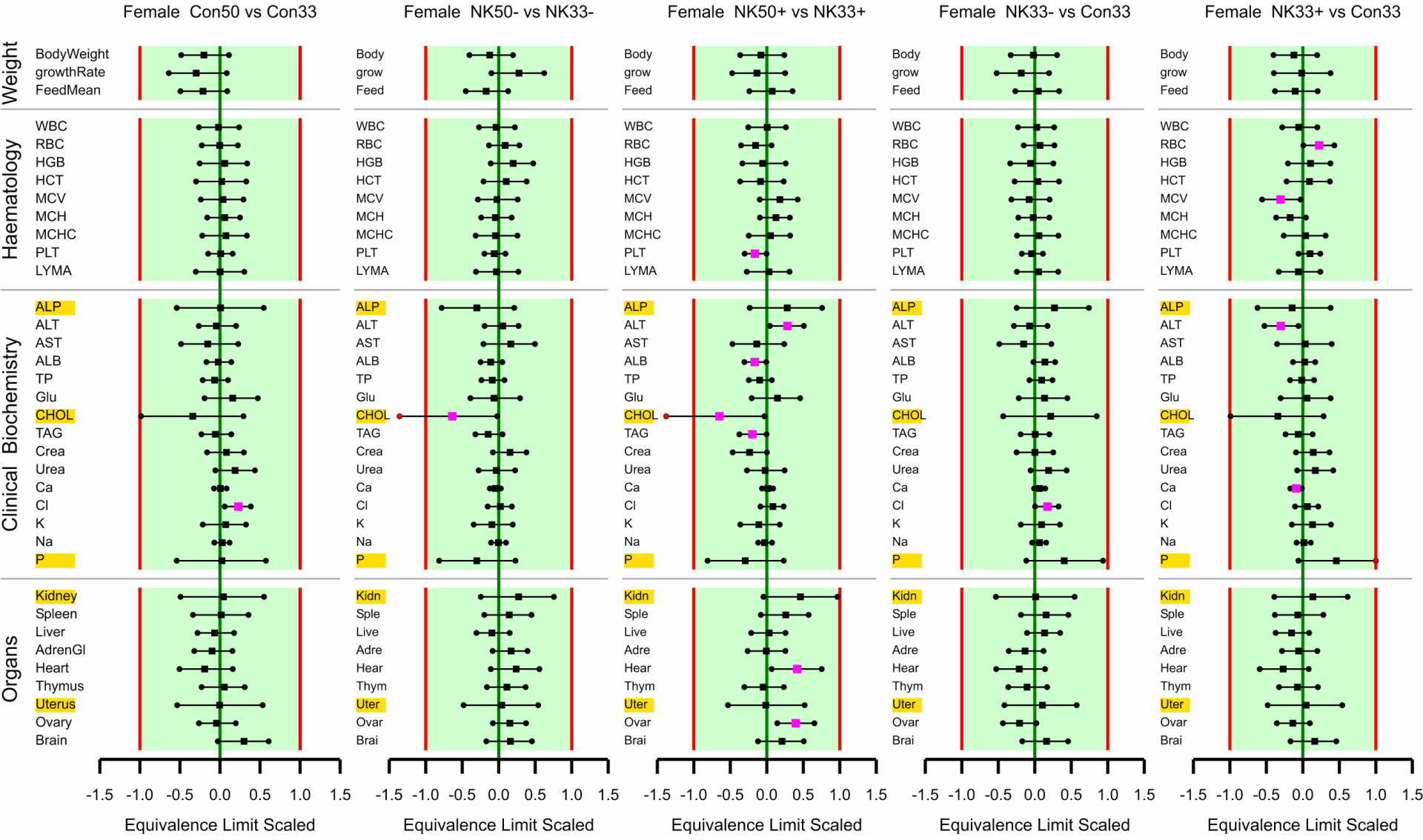
Equivalence tests, 90-day feeding trial 2, male rats (cont'd.)



Equivalence tests, 90-day feeding trial 2, female rats



Equivalence tests, 90-day feeding trial 2, female rats (cont'd.)



Summary of the difference and equivalence tests in the 90-day feeding trial 2

- **46 significant differences (7% of 648 tests)**
- **643 significant equivalences (99% of 648 tests)**
  - **Intervals outside [-1,+1] for blood cholesterol (CHOL) and phosphorus (P) levels as well as for the kidney weight (females only)**
  - **In all these cases, the within-group variation in the 90-day feeding trial 2 was higher than in the GRACE project data**

Histopathology data (I)

Liver								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of male rats	16	16	-	-	16	1	-	16
Infl. cell foci	15	13	-	-	15	-	-	13
Grade 1	14	12	-	-	14	-	-	12
Grade 2	1	1	-	-	1	-	-	1
Vacuolation; PP	2	-	-	-	1	-	-	3
Grade 1	2	-	-	-	1	-	-	2
Grade 2	-	-	-	-	-	-	-	1
Vacuolation; CL	-	1	-	-	-	-	-	-
Grade 1	-	1	-	-	-	-	-	-
Necrosis; focal	-	1	-	-	-	-	-	-
Grade 2	-	1	-	-	-	-	-	-
Hyperpl; bile duct	-	1	-	-	-	-	-	-
Grade 1	-	1	-	-	-	-	-	-
Necrosis; single cell	1	-	-	-	-	-	-	-
Grade 1	1	-	-	-	-	-	-	-
Pigment; hepatocytes	-	-	-	-	-	-	-	1
Grade 1	-	-	-	-	-	-	-	1
Pigment; macrophages	-	-	-	-	-	-	-	1
Grade 1	-	-	-	-	-	-	-	1

PP: Periportal; CL: Centrilobular

Histopathology data (II)

Kidneys								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of male rats	16	16	1	4	16	3	3	16
Cyst; cortex	-	-	1	-	-	-	-	-
Grade 1	-	-	1	-	-	-	-	-
Hemorrhage; agonal	1	3	1	2	-	2	1	4
Dilation; pelvis	-	-	1	1	2	-	2	-
Grade 1	-	-	1	1	-	-	1	-
Grade 2	-	-	-	-	2	-	-	-
Grade 3	-	-	-	-	-	-	1	-
Tubular basophilia	2	3	-	-	1	-	-	2
Grade 1	2	3	-	-	1	-	-	2
Tubular casts	-	-	-	-	1	-	-	-
Grade 1	-	-	-	-	1	-	-	-

Histopathology data (III)

Liver								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of female rats	16	16	-	-	16	-	-	16
Infl. cell foci	11	10	-	-	6	-	-	7
Grade 1	11	10	-	-	6	-	-	7
Grade 2	-	-	-	-	-	-	-	-
Vacuolation; PP	5	1	-	-	3	-	-	4
Grade 1	5	1	--	--	3	--	--	4
Pigment; hepatocytes	1	-	-	-	-	-	-	1
Grade 1	-	-	-	-	-	-	-	1
Grade 2	1	-	-	-	-		-	-

Histopathology data (IV)

Kidneys								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of female rats	16	15	1	-	16	2	-	16
Dilation; pelvis	-	-	-	-	1	1	-	1
Grade 1	-	-	-	-	1	1	-	-
Grade 2	-	-	-	-	-		-	-
Grade 3	-	-	-	-	-		-	1
Infiltrate; lymphoid	-	-	-	-	1	-	-	-
Grade 1	-	-	-	-	1	-	-	-
Tubular basophilia	-	-	-	-	1	-	-	-
Grade 1	-	-	-	-	1	-	-	-
Tubular casts	1	-	-	-	-	-	-	-
Grade 1	1	-	-	-	-	-	-	-
Scar; cortex	-	-	1	-	1	-	-	-
Grade 2	-	-	-	-	1	-	-	-
Grade 3	-	-	1	-	-	-	-	-

Histopathology data (V)

Uterus								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of female rats	16	16	1	-	16	-	-	16
Dilated lumen	6	6	1	-	9	-	-	7
Grade 1	3	1	-	-	6	-	-	6
Grade 2	3	5	1	-	3	-	-	1

Ovaries								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of female rats	16	16	-	1	16	-	-	16
Corpora lutea	15	16	-	-	16	-	-	16
Cyst; luteal	1	-	-	-	-	-	-	-
Cyst; follicular	1	-	-	-	-	-	-	-
Yolk sack carcinoma	-	-	-	1	-	-	-	-

Histopathology data (VI)

Vagina								
	Control33	Control50	NK11-	NK33-	NK50-	NK11+	NK33+	NK50+
No. of female rats	16	16	-	-	15	-	-	15
Diestrus	6	8	1	-	7	-	-	4
Proestrus	6	4	-	-	5	-	-	5
Estrus	3	3	1	-	2	-	-	3
Metestrus	-	1			1	-	-	3

→ **No histopathological alterations in the mammary glands**

Conclusion of the histopathology analysis

There were no treatment-related necropsy or histopathological findings following the administration of genetically modified NK603 maize or genetically modified NK603 maize plus Roundup (up to a 50% maize inclusion rate) to rats for 90 days.

Publications

van der Voet *et al*. (2017) Equivalence testing using existing reference data: An example with genetically modified and conventional crops in animal feeding studies. Food Chem. Toxicol. 109: 472-485

van der Voet (2018) Safety assessments and multiplicity adjustment: Comments on a recent paper. J. Agric. Food Chem. 66: 2194-2195

Chereau *et al*. (2018) Rat feeding trials: A comprehensive assessment of contaminants in both genetically modified maize and resulting pellets. Food Chem. Toxicol.121: 573-582

Steinberg *et al*. (2019) Lack of adverse effects in subchronic and chronic toxicity/carcinogenicity studies on the glyphosate-resistant genetically modified maize NK603 in Wistar Han RCC rats. Arch. Toxicol., https://doi.org/10.1007/s00204-019-02400-1

van der Voet et al. (2019) Equivalence limit scaled differences for untargeted safety assessments: Comparative analyses to guard against unintended effects on the environment or human health of genetically modified maize. Food Chem. Toxicol. 125: 540-548

Overall conclusions and recommendations

- **The GRACE and G-TwYST projects provided a broad set of data indicating that the performance of rat feeding trials with whole food/feed for the risk assessment of the GM maize varieties MON810 and NK603 would not result in additional information pointing at possible health risks of the two GM plants when compared to the earlier risk assessments published by EFSA.**
- **No potential risk has been identified in the course of the initial molecular characterization or in the compositional, phenotypic and/or agronomic analyses of the GM maize varieties MON810 and NK603. The GRACE and G-TwYST data from 90-day and long-term animal studies did not identify potential risks as well, and therefore support the result from the initial analyses.**

- **The necessity to perform a feeding trial with whole food/feed should be carefully evaluated given the high number of animals needed.**
- **The protocols outlined in the OECD Test Guidelines 408 for subchronic toxicity testing and 453 for a combined chronic toxicity/carcinogenicity testing in rodents have been designed for the testing of single chemicals.**
- **Chemicals can be administered individually to rodents at doses several multiples higher than the amount of the chemicals to which humans are exposed in order to test whether they may lead to toxicity, while whole food/feed contains a mixture of constituents and can only be administered to rodents at rather limited levels in order to avoid a nutritional imbalance.**

- **Therefore, it is unlikely that substances present in small amounts and with a low toxic potential in whole food/feed will cause any observable effects in animal feeding trials (EFSA GMO Panel Working Group on Animal Feeding Trials 2008).**

- **Consequently, the studies aim at differences that do not show up in the targeted analyses and nevertheless have an effect that might be observed in by their nature highly insensitive animal feeding trials.**

- **Criteria to evaluate the scientific quality of 90-day and extended feeding trials on whole food/feed derived from genetically modified plants have been proposed by the G-TwYST Consortium (Schmidt et al. 2016). These should be taken into account when evaluating a rodent feeding trial in the course of a pre-market approval procedure.**

- **If 90-day, 1-year and/or 2-year feeding studies on whole food/feed derived from GM plants are planned to be performed in the course of research projects not related to a pre-market approval procedure, these should be based on the corresponding OECD Guidelines for the testing of single chemicals and take into account EFSA recommendations as well as the quality criteria proposed by G-TwYST.**

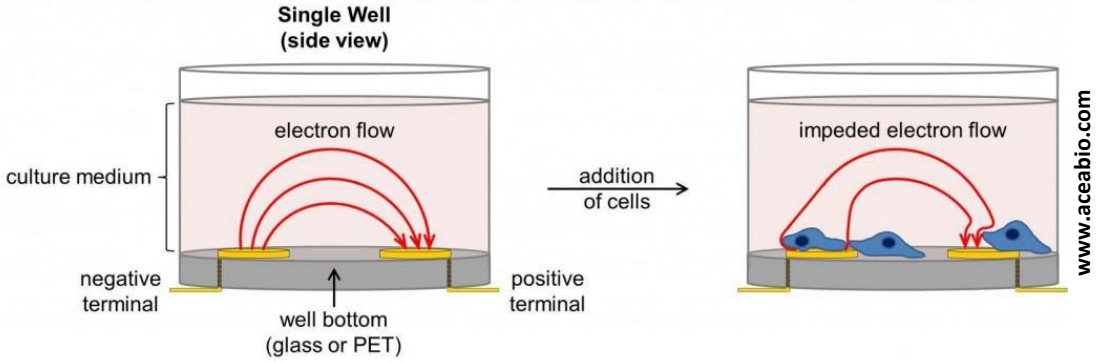
In vitro test systems

An alternative to rat feeding trials for the safety testing of genetically modified plants?

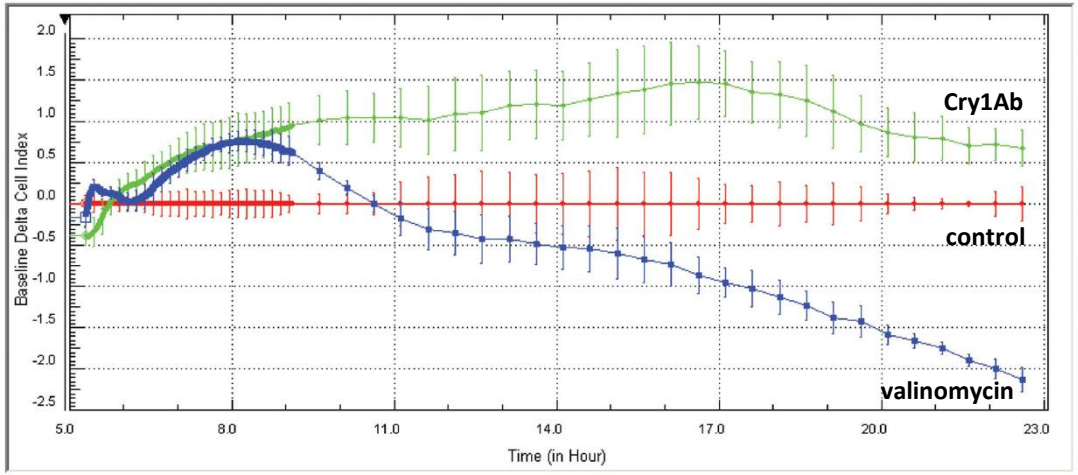
PLoS ONE 8: e67079 (2013)

Cry1Ab Treatment Has No Effects on Viability of Cultured Porcine Intestinal Cells, but Triggers Hsp70 Expression

Angelika Bondzio[1]*, Ulrike Lodemann[2], Christoph Weise[3], Ralf Einspanier[1]



xCELLigence system (Roche Applied Science and ACEA Biosciences)



Cell line: IPEC-J2 cells
Test item: Purified Cry1Ab protein

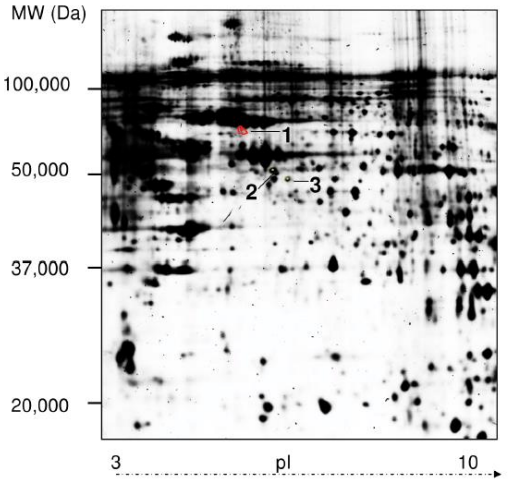


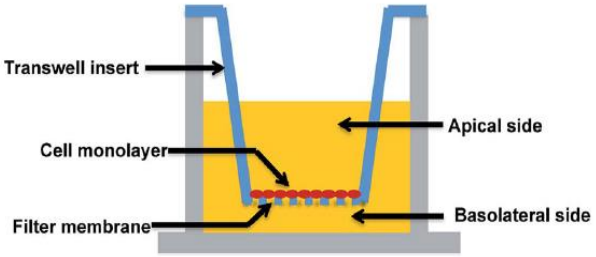
Figure 4. Proteomic profiling of IPEC-J2 cells in response to Cry1Ab treatment as revealed by 2D DIGE analysis. Image shows one representative spot map of Cry1Ab treated IPEC-J2 cell extracts (n = 5) indicating spot boundaries of proteins, whose expression level is increased (red) or decreased (green) in comparison with the corresponding untreated controls (only medium) ($P<0.05$) as revealed by Decyder V.7.0 software. Spots marked with a number, correlating to the identified proteins in Table1.

Food Chem. Toxicol. 115: 451-459 (2018)

Extended exposure duration of cultured intestinal epithelial cell monolayers in characterizing hazardous and non-hazardous proteins

C. Zimmermann[a], A.D. Eaton[b], B.B. Lanter[b], J. Roper[c], B.P. Hurley[b,*,1], B. Delaney[a,1]

www.corning.com



www.pubs.rsc.org

T84 — 24 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	-	-	+	+	+	Hazardous
SLO	-	-	-	-	-	
PHA-E	-	-	-	-	-	
WGA	+	-	-	-	+	
Fbn	-	-	-	-	-	Innocuous
BSA	-	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

Caco-2 — 24 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	+	+	+	+	+	Hazardous
SLO	-	-	-	-	-	
PHA-E	-	-	+	-	-	
WGA	+	-	+	+	-	
Fbn	-	-	-	-	-	Innocuous
BSA	-	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

T84 — 48 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	+	+	+	+	+	Hazardous
SLO	-	-	-	-	-	
PHA-E	-	-	-	-	+	
WGA	+	-	-	-	+	
Fbn	-	-	-	-	-	Innocuous
BSA	-	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

Caco-2 — 48 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	+	+	+	+	+	Hazardous
SLO	-	-	-	-	-	
PHA-E	-	-	+	+	+	
WGA	+	-	+	+	+	
Fbn	-	-	-	-	-	Innocuous
BSA	-	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

T84 — 72 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	+	+	+	+	+	Hazardous
SLO	+	-	-	-	-	
PHA-E	-	-	+	-	+	
WGA	+	+	-	-	+	
Fbn	-	-	-	-	-	Innocuous
BSA	+	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

Caco-2 — 72 Hour

Protein	Cytotoxicity LDH	Cytotoxicity MTT	Barrier Integrity FitC-inulin	Barrier Integrity HRP	Barrier Integrity TEER	
ToxA	+	+	+	+	+	Hazardous
SLO	-	-	-	-	-	
PHA-E	-	+	+	+	+	
WGA	+	+	+	+	+	
Fbn	-	-	-	-	-	Innocuous
BSA	-	-	-	-	-	
PSA	-	-	-	-	-	
Tx-100	+	+	+	+	+	(+) control

Overall Hazard analysis: Individual proteins were considered (+) with a red box if they produced a significant ($p > 0.05$) and reproducible (≥ 3 indepent experiments) effect of any magnitude tested in a specific cell monolayer-based assay. If no reproducible effect was observed for a specific assay, the response was considered (-) and assigned a blue box.

Future Sci. OA 3: FSO0185 (2017)

The ascendance of microphysiological systems to solve the drug testing dilemma

Eva-Maria Dehne*[,1], Tobias Hasenberg[1] & Uwe Marx[1]

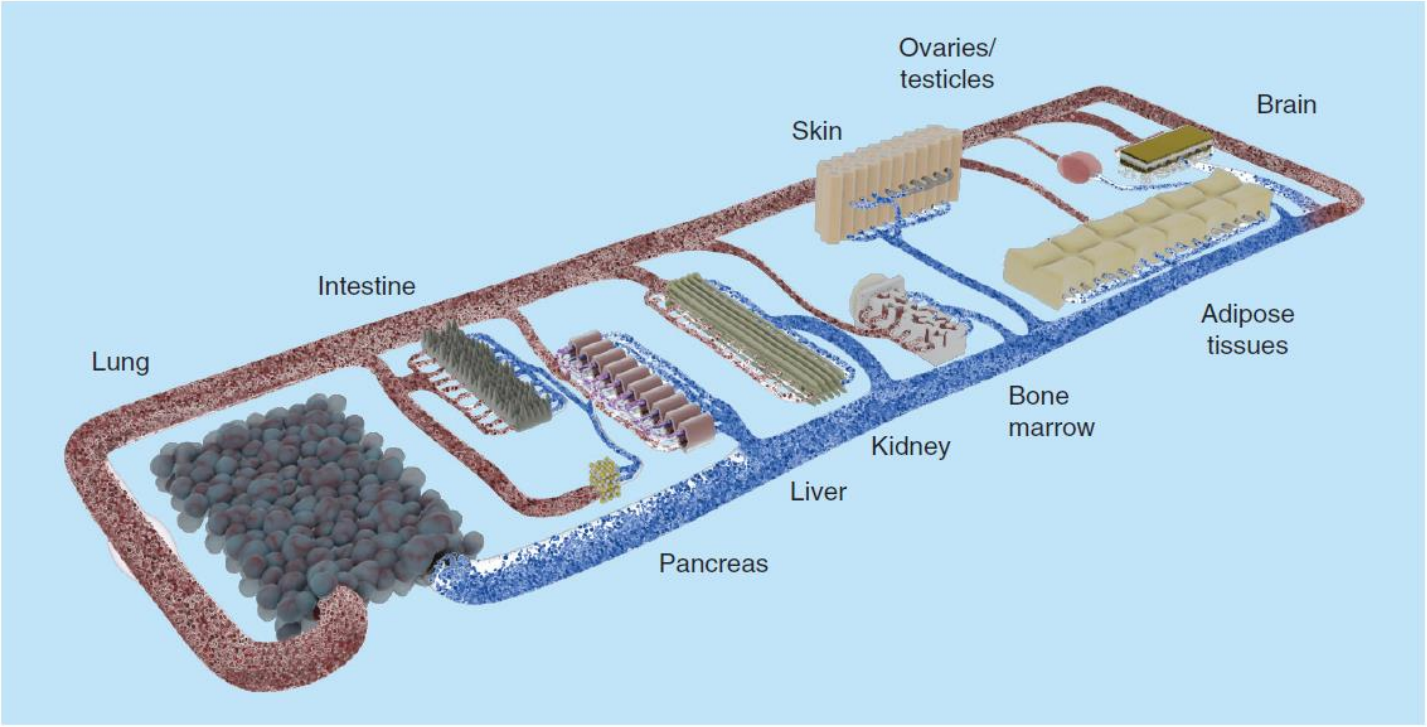
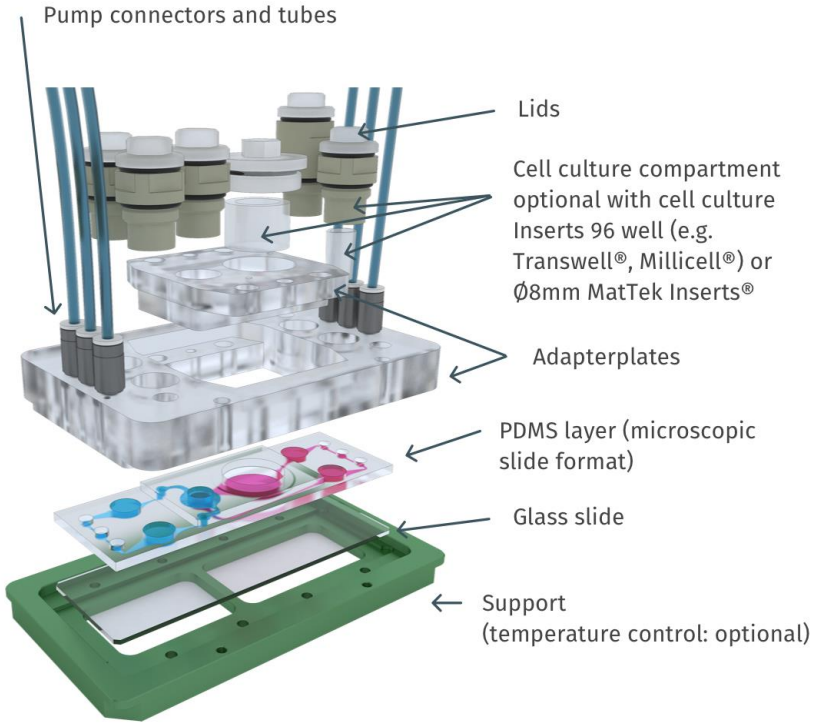
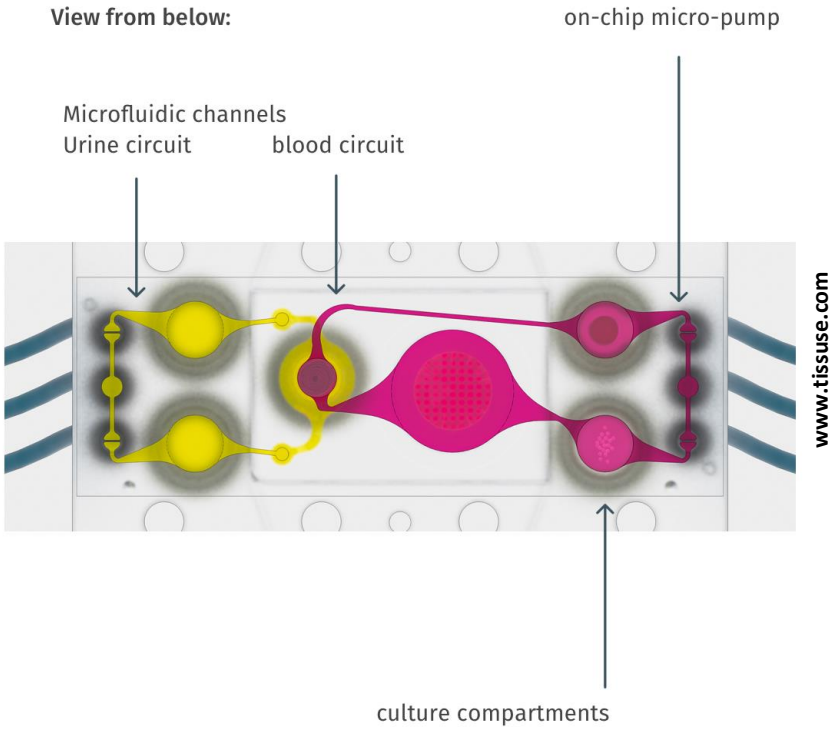


Figure 3. Concept of a human-on-a-chip. The pursuit of the most important bodily functions will lead to a miniature organism on a chip. Common tests conducted in rodents should be realizable with this device. Therefore, the products of the organoids (e.g., urine) need to be discharged in separate compartments. Oral, dermal and intravenous uptake routes, and through inhalation, need to be possible. The transparent device enables optical analysis. Incorporated electrodes will assess barrier resistance, electrophysiological data and key parameters in the supernatants.

Example for a microphysiological system: The 4-Organ- Chip (4-OC) from TissUse GmbH





Cell culture systems in the frame of the overall risk assessment of GM plants:
More questions than answers (open to discussion!)

1) Currently, there are no good *in vitro* methods for complex endpoints/chronic pathological processes (e.g. cancer).

2) The development of such assays will require major scientific advances.

3) The validation of novel *in vitro* tests requires the comparison to available *in vivo* data.

4) Positive controls (GM plant extracts) for *in vitro* test systems are not available.

5) The concentration of plant proteins/extracts tested in *in vitro* test systems is much higher than the human exposure levels to the plant proteins/extracts.

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Thank you very much for your attention!

SEVENTH FRAMEWORK PROGRAMME