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G-TwYST Stakeholder Consultation

Responses by G-TwYST team members to questions and comments concerning the study plans raised by stakeholders

G-TwYST
GMP Two Year Safety Testing
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G-TWYST
GM PLANTS TWO YEAR SAFETY TESTING

Acknowledgment and Disclaimer

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This document expresses the view of the G-TwYST consortium, and does not reflect an official opinion of the European Commission. Responsibility for the information and views expressed therein lies entirely with the authors.

In the whole document, the acronym "**G-TwYST**" has been used to refer to the project.

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List of abbreviations

AMPA	AminoMethylPhosphonic Acid, a metabolite of glyphosate
BMD	Benchmark Dose
CA	Competent Authorities
CADIMA	Central Access Database for Impact Assessment of Crop Genetic Improvement Technologies
CLI	CropLife International
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
GLP	Good Laboratory Practice
GM	Genetically Modified
GMO	Genetically Modified Organism
GRACE	GMO Risk Assessment and Communication of Evidence
GRAS	Generally Recognized as Safe
G-TwYST	GMO Plant Two Year Safety Testing
HC	Historical Control
JKI	Julius Kühn-Institut
MOE	Margin Of Exposure
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
SES	Standard Effect Size
SOP	Standard Operating Procedure
SZU	Slovenská Zdravotnícka Univerzita
TG	Test Guideline
WP	Work Package

1. Introduction to the report

This document presents the comments on the study plan provided by stakeholders in writing after the first G-TwYST Stakeholder Workshop held on 16-17 December 2014 in Vienna and the responses to those comments by the G-TwYST team members. A previous report has described the entire planning stage consultation process and documented the workshop inputs and discussions (available at http://www.g-twyst.eu/reports/workshop-documentys-planning-phase). A general strategy for stakeholder engagement and communication - underlying all activities in the project is available at http://www.g-twyst.eu/communication-and-governance/stakeholder-engagement-and-communication-strategy.

Objective

Openness and inclusiveness are key elements in G-TwYST's stakeholder involvement activities. Since the questions, answers and discussions during the workshop could have given rise to additional comments, stakeholders were also offered the opportunity to send written comments on the draft G-TwYST study plans.

Scope

The consultation was to focus on issues related to methodology, design and analysis of the planned 90-day and 2-year animal feeding trials with GM maize. More general questions such as the value of these studies for GMO risk assessment or possible triggers to conduct such studies will be discussed in another stakeholder consultation on the results and draft interpretation of the studies conducted at a later stage of the G-TwYST project.

Response

Stakeholders were invited to file written comments on the workshop documents and on the workshop discussions. Seven sets of comments were provided by stakeholder organisations or individuals before the deadline of 16th of January, 2015. Overall, a total of 131 written comments were received.

The processing of written comments

The G-TwYST team received written comments from 7 stakeholders: 3 comments from Competent Authorities, 2 from Research Institutes, 1 from a CSO and 1 from Industry. The full written comments were included in the appendix of the G-TwYST Stakeholder Workshop Report available at http://www.g-twyst.eu. In this report we indicated the source of each comment in terms of stakeholder category (CA, industry, CSO, science).

Comments were split in categories:

1. General comments
2. Cultivation
3. Feed production and plant analysis
4. Feed quality
5. Design of the feeding trials
6. Data collection
7. Statistical analysis
8. Other issues

Several comments received during the workshop were also considered by the G-TwYST team and added to this report. Some of these comments required additional information from subcontractors

which was not readily available. This partly explains why the compilation of responses took considerable longer than expected.

2. Responses to the written comments

2.1. General comments

A number of general comments was made on the necessity of the studies or part of the studies.

Comment 1 (CSO): At the stakeholder workshop, it was claimed several times that G-TwYST was initiated for political reasons in answer to the Séralini study rather than due to a concrete scientific concern. We would like to remind the project coordinators and the European Commission (EC) that the rules for project evaluation laid down in Article 38 of Directive 2010/63/EU also apply for research projects initiated by the EC. Thus, we believe that the 2-year feeding trials as they are planned now for G-TwYST do not fulfil any of the requirements or comply with Article 38, nor those laid down in Article 4, 5, 6 or 13 of the before mentioned Directive.
Reply 1: The G-TwYST agrees with the first sentence of the comment. Regarding the rest of the comment, it is the European Commission that issued the call and selected the G-TwYST project.

Comment 2 (CSO): It is not clear to us why the several EU projects that deal with the assessment of the validity of feeding studies and with potential risks posed by GM plants in food and feed were not planned in such a way that all testing data and project outcomes can be compared, i.e. there is now "need" to conduct a new 90-day feeding trial even though a 90-day feeding trial was already conducted in the frame of the GRACE project. The "need" to perform a new 90-day feeding trial is justified by the project coordinators of G-TwYST by stating that they use different GM maize breeds than those in GRACE. This is of very great concern to us because this inconsistency in planning results in more and new animal tests/feeding trials that could have been avoided if the experimental set-up of all respective EU projects would have been designed to build on from each other (i.e. use of the same GM breeds, same animal strains, treatment of plants, etc.).
Reply 2: One cannot use the data on a certain GM plant to predict the potential adverse effects of another GM plant. Therefore, each GM plant needs to be tested individually.

Comment 3 (Industry): The conduct of the 2-year study with MON 810 is unnecessary (several arguments given in the CLI letter of January 2015). Consequently, given that the performance of a long-term test is a requirement of the European Commission call KBBE.2013.3.5-03, we recommend shifting the financial resources planned for the conduct of this study to one combined chronic toxicity/carcinogenicity study that is sufficiently robust and thus has the greatest potential to deliver interpretable results.
Reply 3: The G-TwYST team agrees; financial resources have been shifted.

Comment 3a (Industry): The precise objectives of the study currently remain unclear, particularly regarding specific testable hypotheses. These need to be decided upon and clearly indicated in order to be able to select the most appropriate treatment structure and level of replication. Treatment structure also has the potential to impact on the method of statistical analysis.
Reply 3a: The G-TwYST projects aims at:

- enabling risk assessors (applicants, authorities) and policy makers to evaluate and decide about the necessity of (long-term) feeding studies while at the same time,
- clarifying about uncertainties raised through the outcomes and reports from recent (long-term)rodent feeding studies with whole GM food/feed, and
- elaborating a scientifically sound approach for performing extended feeding studies based on the OECD test guideline 453 and subsequent EFSA recommendations.

In order to achieve this objective, G-TwYST will perform rat feeding trials with GM maize NK603 to test its potential subchronic toxicity, chronic toxicity and carcinogenicity.

Comment 4 (Science): The study plan doesn't highlight the specificities of the chronic toxicity and the carcinogenicity parts of the study, like the OECD Guideline 453 does.
Reply 4: There is no need to do this. The study plan has to describe in detail how the feeding trial will be performed.

2.2. Cultivation

2.2.1. Herbicide use, other chemical treatment, residues and pest infestation

During the workshop in December 2014 several questions were already asked regarding herbicide use, pest infections, seed treatment and storage of the harvest. The following information on the application of herbicides and other chemical treatment was supplied:

Treatment: According to Good Agricultural Practices, Roundup treatment was applied to the fields in the USA and Canada only once. Further management measures included:

- Canada: Broadcast pre-plant incorporated 220 kg/ha of 20-0-30 + 2.4% Sulphur (granular), In-furrow at planting 47 L/ha of 7-27-3 + 5% Zinc liquid starter fertilizer, injected 116 kg/ha actual Nitrogen as 28% liquid nitrogen fertilizer; May 2,2014: Primextra II Magnum 3,5 L/ha – all blocks (S-Metolachlor + Atrazine), on June 19June 20, 2014 : Roundup Transorb HC (540 g/L) 2,5 L/ha = 1.35 kg/ha Glyphosat (Potassium Salt);
- USA: Five gallons per acre of 10-34-0 (N-P-K) fertilizer were applied at planting with another 150 lbs of N and 50 lbs of K at about 4-leaf stage; pre-emergence application of Keystone herbicide (acetochlor and atrazine) was applied at a rate of 1.5 quarts/Acre; June 14, 2014
- Seed treatment is currently not reported. This question will be addressed to the partners at the production site

One of the participants commented that there is the risk that the feeding material you are receiving is not representative, and you do not have the possibility to control this anymore. The G-TwYST team replied that:
a) The varieties to be used for the feed production are commercially cultivated varieties in North America. The management is regional practice.
b) What is a representative "feeding material" for testing transgenic events in whole food/feed? In principle the test should be robust towards alterations in origin, site, varieties etc. The projects GRACE and G-TwYST will provide data to consider the robustness and general applicability.

Pest infestation: In Canada, infestations of pests or diseases has not been reported and, hence, additional management measures were not taken. In the USA, minor infestations of corn earworm, leaf rust and *Fusarium* were observed, but they were below the thresholds for any measures

Residue analysis: Analyses will comprise a broad pesticide screen.

2.2.2. Plot design

Comment 5 (CA): It would be justified to provide more details on the actual test design and the underlying concept used at the production sites; in particular, whether or not a fully randomised plot design, as outlined in current EFSA guidance (EFSA 2011), has been used. If not, the reasons behind the presented concept should be explained and the approach should be set forth conclusively.
Reply 5: Cultivation did not follow a "randomized plot design". The reference (EFSA 2011) specifically refers to field trials conducted for a comparative assessment and does not refer to the production of maize used for toxicological studies. Regarding the amount of maize needed in our studies, a randomised block design for cultivation would have led with a greater probability to edge effects (such as outcrossing of events, pesticide drift etc.) and an additional mixing of the harvest for each variety. In such a case, management would have been necessary to ensure sufficiently constant

quality of the material throughout the studies. Hence, such an approach would have needed more efforts to control the performance of the feeding studies. Moreover, if the results of the feeding trials were time- and site-specific, the method cannot be considered a valid standard approach. The robustness of the feeding studies regarding variation of site, time and variety is essential for a valid safety testing of transgenic events following the proposed concept of feeding studies. Within GRACE, a set of 90-day feeding studies with different varieties was undertaken to address this issue. The studies conducted in GRACE, G-TwYST and GMO90+ will all in all elucidate this critical issue.

Comment 6 (Industry, workshop comment): There is no randomization in the maize fields. If you grow identical varieties in adjacent fields, you'll see differences in the omics results. There is a risk of attributing differences to GM crop, which may be actually caused by other factors.
Reply 6: We agree. The use of ~omics to characterize "unintended effects" is not recognized (up to now) as an established approach for impact or risk assessment and is not recommended by the project at the present time. In the frame of G-TwYST, transgenic and conventional varieties as well as herbicide-treated MON810 and NK603 were cultured at two independent agricultural sites. The omics analyses will be carried out on the two replicates. The data generated throughout G-TwYST will complement an already existing database on omics-data of GM crops established by WUR/RIKILT and CRAG within the project GRACE, which shall help to broadly explore the approach.

2.2.3. The use of non-identical varieties

Comment 7 (Industry): According to the stakeholder meetings, four varieties (2 transgenic and 2 conventional) were grown for the study. Each transgenic variety (P8906R or Prairie Brand 882RR2) had a near isogenic, conventional variety (P8906 or Prairie Brand 882) grown in a similar geographic region under similar environmental conditions. Consequently, they would be a suitable concurrent control item for their respective transgenic variety. However, neither the transgenic varieties are identical to each other, nor are the conventional varieties the near isogenic control of the other transgenic variety (i.e., P8906 is not the near isogen of Prairie Brand 882RR2). Thus, in accordance with national (EFSA, 2011) and international (Codex, 2009) guidances on GM feeding studies, they should not be used interchangeably. CLI recommends revision to clearly indicate the variety chosen for testing and provide GLP-compliant identification as well as descriptive and characterization information. Lastly, the variety chosen for testing should also replace "NK603" throughout the text of the document.
Reply 7: The transgenic variety grown in Canada is P8906R and the corresponding isogenic variety is P8906. The transgenic variety grown in the USA is Prarie Brand 882RR2 and its isogenic variety is Prarie Brand 882. Diets will be prepared from P8906R and P8906 or Prarie Brand 882RR2 and Prarie Brand 882.

Comment 8 (Industry): Please clearly indicate the variety chosen as the control and provide GLP-compliant identification as well as descriptive and characterization information.
Reply 8: Once the test material has been chosen, the variety will be accordingly identified.

2.3. Feed production and plant analysis

During the stakeholder workshop in December 2014 a number of issues concerning storage conditions, logistics, and analyses of plant material were raised.

2.3.1. Storage conditions:

Comment 9a (Industry): What are the storage conditions? Will temperature be controlled?
Comment 9b (Industry): To ensure proper storage and Study Plan compliance, please indicate the controlled temperature and humidity range (e.g., 22 ± 3°C and relative humidity 50 ± 20%).

Reply 9: Storage of maize: >7°C, the upper limit was not known for the original site in spring 2015. Another storage site of the maize was chosen in autumn 2015 due to insufficient pest control at the previous site. Temperature and humidity are controlled but not regulated. The site is regularly inspected by official veterinary and feed inspection services for HACCP compliance

Comment 10 (CA, workshop comment): Diets will be irradiated. Is there any information that irradiation may change the composition, especially the proteins? Will this be checked?
Reply 10: If you apply heat, there will be a change. Irradiation has been performed in the frame of GRACE. Analyses have been conducted, and we will check whether there is a change. There shouldn't be changes with the amount of irradiation that was applied.

Comment 11 (CA): With respect to the irradiation of the feed material it should be properly documented that no chemical changes of important nutrients (e.g. protein isomerisation) occur.
Reply 11: This will be the case.

Comment 12 (CA, workshop comment)(: Proteins could change from the L to the D isomers (research in Vienna hospital, reference to literature?), which could be a cause of mutagenicity.
Reply 12: The G-TwYST team will check this.

Comment 13 (CA): Are you using a closed storage room protected against rodents, insects?
Reply 13: All our storage rooms (for feed, bedding and other materials) are closed, protected against rodents and insects and monitored regarding temperature and humidity.

Comment 14 (Industry, workshop comment): What is the shelf life of the diets?
Reply 14: This will be requested from the producer or tested.

2.3.2. Logistics

Comment 15 (Industry): Specifics regarding dietary sample size, number, identification, and storage conditions, shipping contact information, shipping conditions and the analyses to be conducted on the samples should be included. A physical description of each diet should be included in the study record (recorded prior to treatment) to minimize the likelihood that diets with grossly different appearances would be administered to different treatment groups in the same study. Gross differences in appearance may be an indicator of substantial differences in content or composition (two factors which could confound study results), and thus should be avoided.
Comment 16 (Industry): Please indicate whether or not the listed activities will be GLP-compliant and the individuals responsible for the conduct of each activity.
Reply 15 & 16: Early 2015 the call for tender was still pending and the information was not considered necessary in a study plan. The information that was requested could only be provided at a later stage. In this case, the additional information is now provided (see below).
Part of the issues raised have been included in the contract with the feed provider and could only be provided after the contract was granted. Further issues are monitored.
Detailed analyses of the diets will be performed but cannot be realised in parallel to the feeding studies as the cashflow of funding does not balance all the costs incurred at any time. Therefore, some analyses have had to be postponed to ensure the supply with feed and the services for performing the trial itself. A limited pretesting of the basal compounds used for feed production has been performed to allow some basic monitoring as a control.
The production site of the diets has been visited. The shape of the diets will always be the same as the same machine and equipment (pasta press) is used. The modification may affect the length or hardness of the diet pellets. These parameters are tested by the producer, and diet samples are kept at JKI.

2.3.3. Analyses of plant material

Comment 17 (Science, workshop comment): Are you doing the chemical and microbial analysis on the plants and the grains?
Reply 17: The harvest/kernels will be analyzed, and these analyses are/will be subcontracted.

Comment 18 (Science, workshop comment): Will you make a comparison with available commercial varieties?
Reply 18: This will be done in the frame of the GMO90+ project and the result of the analyses will be forwarded to the G-TwYST project; additional varieties will not be analysed within the project.

Comment 19 (Industry): While growing the GM and comparator crops in separate (albeit adjacent) fields may be an acceptable compromise as far as generating the feed material is concerned, it does not provide an appropriate basis for comparative –omics analysis. The absence of randomization means that there is no guarantee that any differences observed would actually be due to the genetic modification and not to the different growing conditions. Moreover, the fact that –omics responses are known to be sensitive to small differences in growing conditions means that the risk of such bias is high. Indeed, many researchers including Ricroch et al. (2013) have discussed that –omics comparisons have revealed that genetic modifications have a lesser impact on gene expression and composition than does conventional plant breeding. Likewise, at this point in time, the proposed –omics technologies are not useful for understanding potential health effects in humans or other animals, primarily, because –omics technologies have not been validated for diagnostic purposes. Relying on relatively new and not validated –omics technologies to determine the absence or presence of unintended adverse effects will not substantially improve hazard identification or risk assessment. Indeed, we must first understand the normal natural variation of the –omics endpoints. Once this is understood, they might be applied to the routine safety evaluation of biotech crops if they add meaningfully to the hazard identification process. At the present time –omics profiling studies are highly heterogeneous (Ricroch, 2013) and should be standardized and independently validated to reach sound conclusions regarding their ability to detect relevant effects (Blankenburg et al., 2009). Lastly, a fundamental flaw when using –omics technologies for diagnostic purposes is that there is no specific hypothesis to test, which ultimately leads to a bias towards false positive results (Chassy 2010).
Reply 19: Agreed. The use of ~omics to characterize "unintended effects" is not recognized (up to now) as an established approach for impact or risk assessment and is not recommended by the project at the present time. In the frame of G-TwYST, transgenic and conventional varieties as well as herbicide-treated MON810 and for NK603 were cultured in two independent agricultural sites. The omics analyses will be carried out on the two replicates. The data generated throughout G-TwYST will complement an already existing database on omics-data of GM crops established by WUR/RIKILT and CRAG within the project GRACE, which shall help to broadly explore the approach.

Comment 20 (CA): How did you select parameters for analyses of plant material?
Reply 20: Omics analyses are non-targeted approaches; thus, we do not select any specific transcript, protein, etc., but jointly analyze a very large number of mRNA, protein or metabolite targets. Once (putative) targets with an altered expression level have been identified in GM and conventional varieties, targeted approaches can be used to elucidate the biological significance of these alterations.

2.4. Feed quality

Stakeholders made several comments concerning environmental contaminants, nutritional components, GMO content of the control group, diet formulation and the availability of historical data.

2.4.1. Contaminants and nutritional components

Comment 21 (Science): It must be noted that "Analyses for nutritive components and relevant possible contaminants are performed regularly. Certificates of analysis are retained". The contamination of the feed should be extensively measured. Generally, rat feeding studies only measure a few contaminants, such as banned organochlorine pesticides. This limited assessment of the tested substances can question the reliability of the conclusions (Mesnage et al., 2014).
Reply 21: The G-TwYST team agrees, but analyses can only be made to the extent that can be covered by the given budget. Efforts will have to be balanced with other activities in the project. Detailed results of the performed analyses will be made available. The spectrum of analytes will be equivalent to the spectrum analysed within GRACE.

Comment 22 (Science): Recent analytical methods can measure hundreds of pesticides (including the most recently introduced ones) at a reasonable cost with a good sensitivity. For instance, the QuEChERS method (Eitzer et al., 2014) measuring more than 300 pesticides is available in accredited laboratories (ISO 17025 and ISO 9001:2008) (European and French Standard NF EN 15662 from January 2009 for foods of plant origin).
Reply 22: See previous reply. Accredited labs will be employed.

Comment 23 (Science): If the corn analysis does not reveal Roundup residues in the diet incorporating the NK603 sprayed with Roundup, an additional group of rats fed the control diet supplemented with Roundup residues should be added to ensure the capacity of the experimental design to cover all the possible health risk sources.
Reply 23: An additional group cannot be included because of limited animal housing capacity.

Comment 24 (Industry): CLI recommends adding specific information regarding dietary analysis for environmental contaminants such as aflatoxins, pesticide residues, and heavy metals; moreover, nutritional components should be included (please refer to the detailed comments submitted on 9 January, 2015 for reference). This information and the formulation records will demonstrate that the diets are nutritionally balanced and free of contaminants that could confound interpretation of the study results.
Reply 24: The analyses of diets will follow the analyses conducted for GRACE by taking into account financial constraints (i.e. a prioritisation will be undertaken). Mycotoxins and other feed deteriorating substances are tested prior to the selection of the maize varieties to be used for diet preparation.

2.4.2. GMO content of control group (isogenic non-GMO)

Comment 25a (CA): It should be verified by analysis that all diet components except for the GM test material are free from GMOs, i.e. the control maize as well as all other diet ingredients (soy meal etc.). GMO contamination in the diet may mask effects of the test item and therefore has to be excluded. The test item itself has also to be free from contamination with any other GM material.
Comment 25b (CA): Has a contamination threshold been established? (0.9% would be appropriate from our perspective; this is the threshold for adventitious or technically unavoidable traces of GM in food and feed.) Will the charges be checked with respect to GMO content?
Reply 25: The call for tender is pending. The issue has been taken care of. Nevertheless, the seed sown has the usual regional quality, and the GMO content will be tested.

Comment 26 (CA): At which times contamination tests will be performed? (after harvest, before processing, before feeding?)
Reply 26: Contaminations are tested before processing and for selected batches of feed (due to financial constraints). Samples of the batches will be stored.

Comment 27 (CA): Would certain levels of contamination lead to elimination of the charge?
Reply 27: The trial(s) will be stopped in case that contaminations could seriously interfere with the interpretation of the results to be obtained. Documented recommendations/thresholds for contaminants in laboratory animal feed will be considered.

2.4.3. Diet formulation and availability of historical data

Comment 28 (Industry): Please clearly indicate the dietary formulation to be administered to the animals and whether or not it is known to be nutritionally balanced and equivalent between test and control groups. Per comments on diet production provided separately to G-TwYST on 9 January 2015, a nutritionally balanced diet similar to the diet fed to animals in Harlan's Historical Control (HC) database should be fed to the animals. This will facilitate use of the Harlan HC data to help determine whether or not differences observed are outside the normal range for the test species. This is an important way of determining the toxicological relevance of differences in all toxicology studies, including GM crop feeding studies. If the diets being fed on this study are not similar to the HC database, then comparisons to HC may not be valid. It is anticipated that Harlan and the company nutritionist will be able to provide diet formulation information regarding the diets prepared for this study. Furthermore, comparisons between treatment groups in the study should only be made if the diets fed to the groups are nutritionally balanced and equivalent.
Reply 28: In spring 2015, the call for tender was pending. Data will be available together with the study plan or addendum or in separate files. Comments were considered while preparing the call. We will take into account what has been said regarding the concentration of total protein in the feed as well as further recommendations. The diets of the different treatment groups shall be nutritionally balanced and equivalent. Moreover, G-TwYST tries to use diets, which are qualitatively similar diets to those in the GRACE project to enable a comparison with "historical" data generated by the lab at the animal housing facility of SZU itself. The relevance of using own historical data, as stated in various OECD documents, is acknowledged.

Comment 29 (Science): What is the composition of the bottles? Why are the bottles autoclaved and not cleaned with detergent and disinfectant in the same way as cages?
Reply 29: We use polycarbonate bottles from Tecniplast Italy. In a first step, the bottles are cleaned in the same way as cages. Thereafter, bottles are autoclaved and filled with drinking water.

Comment 30 (Science, workshop comment): As you don't have a lot of historical data, you have a risk with the protein levels in your diet.
Reply 30: The G-TwYST team agrees. Therefore, we will control that the protein level in the diets is appropriate, but not too high to induce renal toxicity.

The lack of historical data and its consequences for the discussion of the findings was also addressed during the workshop in December 2014. The G-TwYST team replied that regarding the 90-day feeding trial we now have the results obtained in two 90-day feeding trials in the frame of the GRACE project. The lack of historical data from the own facility regarding 1- and 2-year feeding trials cannot be circumvented. One of the participants also commented that having only one control group and no historical data is very critical because you'll lose a lot of animals and you are testing a new diet. The G-TwYST team replied that it is not possible to include further groups because of animal housing limitations.

2.5. Design of the feeding trials

Comments on the design of the feeding trials focused on dose levels, the number of animals in the different groups (90-day/2-year studies and NK603/MON810 studies), the characteristics of the test animals (strain, gender, age), animal housing, feed dosage and route of administration as well as the method of euthanasia.

2.5.1. Dose levels

Dose levels were already extensively discussed at the stakeholder meeting in December 2014. Several stakeholders followed up on the discussion with written comments on this issue.

Comment 31 (CA): We would recommend a 40% and a 20% dose group, because – based on the experience with shorter-term studies that did not show very substantial GMO caused effects in the past – it would be advisable to go up with the upper dose as high as possible without reaching an imbalance and therefore potential toxicity levels (except for perhaps less weight gain or other minimal effects derived from shorter-term studies as lege artis requested for the highest dose in long term/ carcinogenicity studies). In this context, 50% dose groups could already pose a risk, and, thus, we would not recommend to reach that level in rat studies. The 20% dose group would be a usual gradation (high dose divided by two – geometric series) in toxicity tests.
Reply 31: We will use 11 and 33% doses for the long-term study, and will test the 50% dose in an additional 90-day feeding trial.

*Comment 34a (CA):*As it is not expected that GM maize NK603 and MON810 will be highly toxic, the GM maize content in the highest dose group should be as high as possible. Hence, we support EFSAs proposal to use a higher incorporation than 33% maize in the highest dose group.
Comment 34b (Science): The 90 days study with the 50 % group should be continued with a 1 year and a 2 years studies.
Comment 34c (Science): To avoid imbalances in important nutrients and protein levels that can occur with large concentrations of the tested substances, we suggest the use of 11, 22 and 33% inclusion rates of NK603 in a standard diet.
Comment 34d (CA): The high dose (33% inclusion rate) used/proposed in the subchronic study on MON810 (conducted in GRACE) or on NK603 (to be conducted in G-TwYST) is not supported by any sound justification. Actually, 33% was the incorporation rate previously tested by the applicant (again with no clear justification). Considering the issues/criticisms to the recently published paper on the results of the GRACE 90-day study, it would be advisable to select the high dose level to ensure a more robust toxicological study. This comment concerns both the subchronic study (NK603), as well as the combined chronic/carcinogenicity study on NK603, and the carinogenicity study on MON810.
Reply 34: We will not go up higher than 33% in the long-term study, and will test the 50% dose in an additional 90-day feeding trial. We do not have the animal housing capacity and financial resources to test such a high dose in a 1- or 2-year feeding trial. Most probably, a 50% dose will lead to a nutritional imbalance after 1 year.

Comment 35 (CA): The rodent feeding studies performed in the context of G-TwYST will be interlinked with other research projects (GRACE, GMO90+). This interlinkage is claimed to increase comparability of results, expand data basis for political decisions, join forced and share costs. Against this background, it is recommended to select doses allowing such interlinkage. This particularly refers to subchronic studies on NK603 (G-TwYST and GMO 90+).
Reply 35: We will select doses that allow the mentioned interlinkage.

Comment 36 (CA): The low dose (currently 22%) could be modified accordingly. This comment concerns both the subchronic study (NK603) as well as the combined chronic/carcinogenicity study on NK603 and the carcinogenicity study on MON810.
Reply 36: We will test the 11 and 33% doses in the 90-day and in the combined chronic toxicity/carcinogenicity feeding trials, and will test the 50% dose in an additional 90-day feeding trial.

Comment 37 (CA): Discussion with the diet provider on the experience/ possibility of a high dose >33%maize incorporation rate is recommended. This comment concerns both the subchronic study (NK603), as well as the combined chronic/carcinogenicity study on NK603, and the carinogenicity study on MON810.
Reply 37: In spring 2015 a discussion with the diet provider regarding an incorporation rate higher than 33% was foreseen.
In the meantime, the nutritionist of the diet provider suggested a maximal incorporation rate of maize of 40%. The 50% incorporation rate was chosen because of the EFSA statement based on a single study reporting that feeding a diet with 50% maize did not lead to any negative effects.

Comment 38 (CA): A literature search on the incorporation rate of maize in a carcinogenicity study is recommended.
Reply 38: A literature search will be performed.

Comment 39 (CA): The dose levels in feeding studies with rodents should be selected in accordance with the appropriate guidance document (OECD Technical Guidances, EFSA documents - e.g. EFSA Scientific Committee Guidance on 90-day studies, 2011; EFSA Scientific Report on Considerations on the applicability of OECD TG 453 to whole food/feed testing, 2013). In the case of whole food/feed, in particular, the well-known limitations regarding the high dose selection should be considered.
In any case, a clear justification of the dose levels selected should be provided for all protocols of the G-TwYST feeding studies in rodents.
Dose levels might differ in different types of studies. In particular, it is highlighted that in a comprehensive project aimed to support the safety assessment of a test substance, making use of several animal studies, the studies schedule is pivotal to achieve sound results and must comply with the 3Rs principles. Typically, subchronic studies provide information relevant to set the dose for chronic and, in particular, for carcinogenicity studies. Unfortunately, during the Stakeholder meeting it was explained that, due to time/budget constraints, this is not possible in the context of G-TwYST (in particular for NK 603), with obvious limitations regarding a sound rationale for dose selection.
Reply 39: The G-TwYST team agrees with all comments. The rationale when selecting the adequate doses will be to adhere to the internationally recognized test guidelines and recommendations published by OECD and EFSA.

2.5.2. The number of test animals

Comment 40 (CSO): We welcome the fact that the project coordinators have made an effort to revise the experimental set-up and provided an alternative set-up that refrains from conducting the 2-year carcinogenicity feeding trial with MON810 GM Maize. We would like to encourage the project coordinators to stick to this commitment in order to reduce the number of animals used in the G-TwYST programme.
Reply 40: The number of animals in the G-TwYST project will be reduced wherever it is feasible.

Comment 41a (Industry): Justification for the selection and number of animals: Justification should be given on the high number of animals used for the chronic phase of the study. It is true that 20 animals per sex and gender are typically utilized for chronic studies (OECD TG 452) for statistical reasons. However, OECD TG 453 allows for a reduced animal number (10 per sex/group): “Each dose group (as outlined in paragraph 22) and concurrent control group intended for the chronic toxicity

phase of the study should contain at least 10 animals of each sex, in the case of rodents. It should be noted that this number is lower than in the chronic toxicity study TG 452. The interpretation of the data from the reduced number of animals per group in the chronic toxicity phase of this combined study will however be supported by the data from the larger number of animals in the carcinogenicity phase of the study." Thus, the current design indicates that 100 animals more than is necessary are being used. Additional justification for increased animal usage should be provided. Also, the fate of the sentinel animals is not described in sufficient detail. Any procedures or endpoints to be investigated for these animals should be clearly defined in the document.
Comment 41b (CSO): There were numerous attempts during the stakeholder workshop to reason that the new 90-day feeding trial was "needed" because in the frame of the GRACE project the GM maize MON810 was used, while in the frame of G-TwYST it is planned to only assess GM maize NK603. It was also explained by the project coordinators that, therefore, data and results could not be compared due to the different characteristics of the two GM breeds. In our opinion this is a very alarming issue. At least the feeding trials conducted in the frame of GRACE were supposed to deliver results to help in the assessment of the validity of feeding trials with GM plants for risk assessment (e.g. for human health) in general, not for one particular type of breed of GM maize. We see the danger that, there will be a further need identified to create projects where each and every new or existing breed of GM plant is tested in feeding trials. From an animal welfare point of view, this cannot be justified.
Reply 41: The number of animals in our study is selected following OECD Test Guidelines. Moreover, the results obtained with a certain genetic event cannot be generalized, i.e. each type of GM crop will have to be tested separately.

Comment 42 (Industry): A GLP-compliant 90-day study with NK603 has been conducted, and the results have been published in the peer-reviewed scientific literature (Hammond et al., 2004) and reviewed by regulatory authorities around the world. Reviewers have deduced that the data supports the conclusion that NK603 is as safe as conventional maize when fed to rats for 90 days. Consequently, the scientific rationale, and Animal Care and Use justification, for repeating this study is not apparent. Furthermore, the current 90-day study is to be run concurrently with the longer term study; this defeats an important part of its purpose. For chemicals, a 90-day toxicity study is normally run as a precursor to longer term studies (e.g., a combined chronic toxicity/carcinogenicity study) to ensure that the high dose selected for the chronic study does not exceed the Maximum Tolerated Dose – a dose anticipated to produce limited toxicity when administered for the longer period of the chronic study. When the 90-day and chronic/carcinogenicity studies are run concurrently, at similar inclusion levels, the utility of the 90-day data is diminished. Especially, due to the availability of the 90-day NK603 study results from Hammond et al. (2004).
For these reasons CLI believes the repeated conduct of the 90-day study with NK603 is unnecessary. The financial resources planned for the conduct of this study could be shifted to the combined chronic toxicity/carcinogenicity study planned with herbicide tolerant maize to enhance its robustness. A more robust chronic/carcinogenicity study will maximize its potential for producing interpretable results.
Reply 42: The resources to be used in the G-TwYST feeding trials (diets, animals, methods to determine the different endpoints, etc.) are not the same as in the 90-day NK603 study by Hammond et al. (2004). Therefore, we cannot take over the Hammond data and have to perform our own 90-day study.

Comment 43 (Industry): The robustness of the study can be improved with slight modifications of the indicated study design. The current Groups 2 and 3 (unsprayed, low and high GM maize incorporation rates, respectively) could be omitted and the animals utilized to include two reference groups fed conventional maize varieties in the study. In the absence of HC data for 2-year feeding studies at the testing facility, these reference groups would provide a better understanding of the

normal range of variability under the conditions of testing. While HC data from the animal supplier may partially address this need, a more robust study would lead to a better understanding of normal variability at the lab's site and with their equipment. Moreover, the utility of including groups fed unsprayed grain is called into question by recent EFSA guidance on the conduct of rodent feeding studies with GM crops, which indicates that the test items should be grain treated with the intended herbicide when the test item is an herbicide-tolerant variety (EFSA, 2014). This makes inherent sense because a farmer is highly unlikely to pay more for seed containing an herbicide tolerance trait, and then not utilize that technology to improve agronomic performance. To maximize the potential of the study to reach definitive conclusions, more than 2 reference groups should be added to the design; perhaps as many as 6 reference groups. CLI understands that a finite amount of funding has been granted. However, the 90-day NK603 study and 2-year MON 810 study could be halted based on the existing safety data. Likewise, other cost-saving measures have been identified in the comments on this Study Plan (see Comments 49, 56, 63 and 70a). These revisions would allow G-TwYST to redeploy the financial resources from these unnecessary studies and endpoints to the current chronic toxicity/carcinogenicity study to improve its design, robustness, and ultimately the interpretability of its results.
Reply 43: We need the groups 2 and 3 in order to assess whether the effect is or not related to the use of the herbicide (this was specifically asked for by the EC). Furthermore, we cannot leave out the 90-day NK603 feeding trial (see Reply 43).

2.5.3. Age, gender and strain of the test animals

Comment 44 (Science): The protocol does not include the major windows of sensitivity to toxic effects, which are the prenatal period, puberty, and late life (Diamanti-Kandarakis et al., 2009).
Reply 44: G-TwYST follows the internationally recognized test guidelines and recommendations from EFSA and OECD for a 2-year study, i.e. in these documents exposure does not include the periods mentioned.

Comment 45 (Science): The early treatment is different according to gender. Consequently, haematological and biochemical analyzes will be shifted, with a possible effect time analysis between gender.
Reply 45: Due to working load limitation, this issue cannot be avoided.

Comment 46 (Science): The choice of the Wistar rat from Harlan is not inappropriate, but it makes the results difficult to compare with those of previous studies with the same GMO performed with Sprague-Dawleys.
Reply 46: We agree that the comparison is difficult, but there are a number of arguments in favour of using Wistar rats such as a low incidence of spontaneous tumors.

2.5.4. Animal housing and randomization

Comment 47 (Industry): An acclimation period of 4-6 days is unusually short for a longer term feeding study; ≥2 weeks is more common. Morover, OECD TG 453 requires healthy animals be acclimated for at least 7 days. Either way, these acclimation timelines are inconsistent with the study schedule which indicated animal delivery in February 2015 and the beginning of treatment in March-April 2015. Please revise for consistency between text of the document and study schedule.
Reply 47: In a first step, animals are acclimated for a period of 6 days. On day 6, animals are assigned to the different experimental groups by randomisation according to the scheme presented on page 20-22 of the study plan.

Comment 48 (Industry): Five animal rooms are indicated for 14 racks of animals, thus an even number of racks cannot be distributed among the rooms. In either this section or the Randomization

section please indicate how the racks will be distributed among the rooms to control for the impact of slightly different environmental conditions in each room. For example, the racks could be distributed as indicated below if two larger (arbitrarily designated 308 and 309) and three smaller (arbitrarily designated 310, 311, and 312) rooms were available: (see separate document 'table proposed by CLI'
Reply 48: In the study plan, a detailed scheme of the randomised distribution of animals in each rack is described. All animals will be housed in 5 rooms next to each other. In all rooms, climatic conditions are exactly the same.

Comment 49 (Industry): Lines 264-298: The level of detail provided in this section seems inconsistent with that provided in the other sections (i.e., it is far more detailed). For the sake of continuity, and utility of the document to the technical staff conducting the study, please consider moving some of the details (e.g., tables indicating randomization schemes and ANOVA details) to an appendix. Given that the animals are reported to fall within a reasonably wide initial weight range (+/- 20% of the mean), blocking by initial weight should be seriously considered (a) to avoid the possibility of having two rats of very different sizes in the same cage, and (b) to help reduce the residual error.
Reply 49: The technical staff will follow a detailed standard operating procedure for randomisation in order to circumvent the problems mentioned in the comment.

Comment 50 (Science): All animals will be housed in 4 separate rooms. Is there a possible room housed effect?
Reply 50: All animals will be housed in 5 rooms next to each other. In all rooms, climatic conditions are exactly the same. All 5 rooms have one common handling area.

Comment 51 (Science): What is the light period (which hours in the day)?
Reply 51: 12 hours light (6 a.m. to 6 p.m.)/12 hours darkness.

Comment 52 (Industry): CLI recommends utilizing filters that will minimize the potential for all environmental contaminants (microorganisms, metals, chemicals, etc.) in the drinking water.
Reply 52: Drinking water is controlled by the Waterwork Bratislava. Water is passed through a microbiological filter and autoclaved in the bottle.

2.5.5. Feed dosage and route of administration

Comment 53 (Industry): To avoid the potential for cross-contamination of diets, CLI recommends filling feeders outside of the animal room. Color coding diets and cage cards for treatment groups should also minimize the potential for errors in diet administration.
Reply 53: All studies use color coding. All procedures during the study (filling of cages, weighing of feed and animals, clinical control etc.) are performed in the handling area.

2.5.6. Method of euthanasia

Comment 54 (Industry): The method of euthanasia is never distinctly mentioned in the protocol. To ensure uniform termination between treatment groups and genders a distinct method (i.e., CO2 inhalation, cervical dislocation, etc.) should be included. This will minimize the potential for introducing variability into the study design.
Reply 54: Euthanasia will be performed by using ketamine/xylazine according to SOP ŠPP/TOX/V005 of the Slovak Medical University.

2.6. Data collection

Comments on methods for data collection included procedures for sample collection, urinalysis, clinical chemistry, hematology, physical examination and functional assessment, gross necropsy, histopathology, ophthalmologic examination as well as - in this context - the availability of historical data.

2.6.1. Procedures for sample collection

Comment 56 (Industry): Under the heading "OBSERVATIONS (CHRONIC TOXICITY PHASE)" paragraph 38 of OECD TG 453 indicates these exams, "…should be carried out on all animals prior to the first administration of the test substance", and, "At the termination of the study…" (OECD, 2009). Please clearly indicate in the Study Plan when the initial and final exams will be conducted for the chronic toxicity phase animals.
Reply 56: Detailed clinical observations - paragraph 37, OECD TG 453, 2009
The detailed physical examination and functional assessment of animals are described on page 24, last paragraph of the Study Plan. Rats will be examined inside the cage **every day twice**, outside the cage **once weekly**. Any deviations from normal status will be recorded in terms of nature and severity, date and time of onset, duration and progress of the observed response. An initial examination will be conducted during the acclimatization period (day 4-5) and 2) immediately prior the first administration of the test substance. A final examination will be conducted during the last week before euthanasia and repeated on the day of euthanasia (for the chronic toxicity phase as well as the carcinogenicity phase of the study).

Ophthalmological examination, using an ophthalmoscope - paragraph 38, OECD TG453, 2009
Ophthalmological examination is described on the page 25, second paragraph, of the Study Plan. The eyes of all animals will be examined prior to the administration of the test feeds (day one) and at the end of the study (one week before the termination of the study) for both chronic toxicity phase as well as the carcinogenicity phase of the study.

Comment 57 (Industry): A single blood sample will be divided for hematology and clinical chemistry analyses (Lines 367-368). This is highly unusual as optimal samples for hematological and clinical chemistry analyses are conducted on fundamentally different biological matrices (whole blood and serum, typically). However, it could be a misstatement because the document later indicates hematology samples will be collected in the presence of EDTA (Lines 401-402) and clinical chemistry samples will be collected in the absence of an anticoagulant (Lines 420-426). The collection methods indicated in Lines 401-402 and 420-426 are consistent with typical sample collections for hematology and clinical chemistry of serum samples on other rodent feeding studies. CLI recommends clarifying Lines 367-368 to state that separate blood samples will be collected for hematological and clinical chemistry analysis.
Reply 57: We apologize for the imprecise wording in lines 367-368. Haematology samples will be collected in the presence of EDTA and clinical chemistry samples will be collected in the absence of an anticoagulant.

Comment 58 (Science): The study plan says: "At the end of the first and second year 500 µl plasma/rat and 2-3 ml urine/rat from 16 rats per experimental group and at the end of the second year liver and kidney samples from 16 rats per experimental group will be sent by SZU to the French consortium GMO90+, which will analyse the expression of a number of biomarkers of effects in the above-mentioned samples". How will the 16 rats (compared to 20 or 50 by group) be chosen?
Reply 58: Rats will be chosen using random sampling.

Comment 59 (Industry): Recommend adding a body weight collection interval "immediately prior to randomization" to have a better understanding of body weight at the study start, and refining the "at the end of the study" interval to indicate unfasted body weights the day prior to necropsy and fasted body weights the day of necropsy. Unfasted body weights are a better comparator for other body weight intervals in the study and fasted body weights are necessary for the proper calculation of organ-to-body-weight ratios if desired (or applicable, Page 17 of OECD TG 453).
Reply 59: Recommended body weight collection intervals are already included in the study plan. Randomization is performed using the body weight collected immediately prior to randomization (early morning on the day of randomization). This day is day one / starting day of the study. Unfasted body weights are measured and recorded at defined intervals. The fasted body weights are measured on the day of the necropsy - immediately after removing the animals from the metabolic cages.

Comment 60 (CA): The animal sample processing steps are separated between several laboratories dispersed over Europe, and thus require an immense load of coordination, documentation and management. The execution of the experiments at a single renowned institution with a long and honoured record of expertise would have been preferable.
Reply 60: It has already been proven in the frame of GRACE that the decentralized animal sample processing steps can successfully be performed.

Comment 61 (CA): The quality control at the tissue sample analysis institution/pathology should be executed by an external expert (not by a related party of the owner of the company).
Reply 61: The quality control will be performed by an external expert.

Comment 62 (Science): In a recent study on dairy cows performed with MON 810, gene expression pattern of markers for apoptosis, inflammation and cell cycle were assessed from liver and gastrointestinal tract. It would be interesting to collect and store gastrointestinal tissue for possible additional studies.
Reply 62: This will be done.

2.6.2. Urinalysis, hematology and clinical chemistry

Comment 63a/b/c (Industry): (Three seperate but similar comments on 1) clinical biochemistry, 2) hematology, and 3) urinalysis have been merged to one comment) Per OECD TG 453, "Measurements at 3 months, either in rodents or nonrodents, need not be conducted if no effect was seen on urinalysis, clinical biochemistry or hematological parameters in a previous 90 day study carried out at comparable dose levels." As Hammond et al., (2004) do not report any adverse effects on clinical chemistry, hematological or urinalysis endpoints from the feeding of NK603 it is not necessary to conduct these evaluations at this interval. Likewise, urinalysis, clinical chemistry and hematology analysis or at the end of the carcinogenicity phase is likely to be confounded by geriatric changes and tumor formation. Consequently, these endpoints are frequently considered optional. Furthermore, the latest endpoint in the HC data available from Harlan is >70 weeks. As the carcinogenicity phase of this study is scheduled to run 104 weeks, and this precise interval is not given in the HC database, it is uncertain that suitable hematology, urinalysis and clinical chemistry HC data will be available to evaluate differences in these endpoints. Also, as mentioned in the hematology section, most regulatory agencies and subject matter experts recommend that clinical pathology endpoints not be included at the end of a 2-year study due to the confounding affects of geriatric changes and tumors. For these reasons G-TwYST should examine whether the financial resources committed to the conduct of clinical chemistry at the end of the carcinogenicity phase could be better utilized to improve the robustness of other study parameters or design elements. For the reasons indicated above G-TwYST should carefully consider whether conducting clinical chemistry analyses at two unnecessary intervals (3 month and the end of the carcinogenicity phase)

is appropriate. Eliminating 1200 unnecessary clinical chemistry evaluations (700 evaluations at 3 months and 500 evaluations at 2 years) could provide substantial savings and those funds could be used to enhance the robustness of other aspects of the study design.
Comment 63d: Generally, hematology, clinical chemistry and urinalysis should not be performed at the very end of the 2-year study. These parameters are as critical as the organ weights which will not be determined "...since geriatric changes and the development of tumours will confound the usefulness of organ weight data..." (stated in line 487-489). Geriatric changes and tumors certainly confound not only organ weights, but clinical pathology endpoints as well. Therefore, applicable OECD TGs do not mandatorily require the examination of these parameters, EPA- and EU-specific guidelines as well as Japanese guidelines do generally not require these examinations after 2 years either. Several publications dealing with this matter have also come to the same conclusion regarding the usefulness of organ weight and clinical pathology data at the end of a 2-year study (Weingand et al., 1992; Long and Symanowski, 1998; Young et al., 2011).
Reply 63: The rationale for performing clinical chemistry and hematological analyses is the following: Firstly, the feeding study on NK603 published by Hammond et al. (2004) was conducted in a different strain of rats (in Sprague-Dawley rats). Our study will be conducted in Wistar Han RCC rats and as such will be hardly comparable with one performed with a different strain of rats. In addition, Sprague Dawley rats are known to have a higher incidence of spontaneous tumours. Secondly, at the beginning of the 2-year study, no results from the 90-day study on NK 603 will be available at the testing facility. In the present study, clinical chemistry and hematological analyses as well as urinalysis will be performed in 20 male and 20 female rats from each group, i.e. 200 animals at a time after 3 and 6 months will be examined. The number of animals is sufficient for an appropriate statistical analyses. Geriatric changes that very likely will be observed at the end of the carcinogenicity phase of the study do not necessarily constitute a problem (confounding factor), since all animals in the study and control groups will undergo the same ageing processes.

Comment 64 (Science): The study plan says: "Blood samples will be stored at room temperature (17-25°C), maximally up to 4 hours, until measurement". Were the storage conditions and temperature until measurement validated?
Reply 64: Storage of blood samples at room temperature (17-25°C) and up to 4 hours before measurement are conditions recommended for measurement of hematologic and clinical chemistry parameters according to the Operation Manual of the haematological analyser (Sysmex XS-1000i, Instructions for use Chapter 6) and the Operation Manual of the clinical chemistry analyser (Vitros Test Methodology Manual, Cat.No. 8321622).

Comment 65 (Science): There is no shift between first and last blood sample collection?
Reply 65: The time shift between the first and the last blood sample collection is up to 1.5 h. Three to five trained persons will be taking blood from rats in parallel.

Comment 66a(Industry): The diagnostic utility of gamma-glutamyl transpeptidase (GGT) in rat studies has been characterized as "limited" (Ennulat et al., 2010), and as a result CLI recommends that another biomarker suitable for hepatobiliary evaluation be used instead. OECD TG 453 indicates other biomarkers such as 5' nucleotidase, total bilirubin, total bile acids are suitable for this purpose.
Comment66b: Is bilirubin (total) measured?
Reply 66: The activity of two transaminases as well as that of alkaline phospatase in blood will be measured. Moreover, the albumin and total protein levels in blood will be determined. Total bilirubin is not included in the panel of clinical chemistry assays. In case that liver pathology becomes evident, a further biomarker such as e.g. 5'-nucleotidase could be included, but is not *a priori* foreseen.

Comment 67 (Industry): The analysis of 17β-estradiol, testosterone, T3, and T4 are also indicated as endpoints. These are not requirements of OECD TG 453, and as such are unlikely to be evaluated in other toxicology studies conducted according to this guideline. Accordingly, it is unlikely that there is robust HC data available for these endpoints in studies of this length, particularly at the later time points. Additionally, because hormone levels are known to fluctuate with the female cycle, evaluating potential differences in hormone levels between groups is further complicated and may be prone to misinterpretation without sufficient HC data. Accurately interpreting the relevance of any differences detected will be difficult at best. Presuming the availability of suitable HC data for these endpoints at earlier time points (i.e., perhaps the 6 month interval) CLI recommends limiting the analysis of these endpoints to those intervals with suitable HC data.
Reply 67: The serum levels of 17β-estradiol, testosterone, T3, and T4 will be measured at the end of the 90-day subchronic toxicity study 1 with GM maize NK603.
As is stated in the study plan, vaginal smears will be taken daily between 08:00-09:00 a.m. during 10 days prior to necropsy and on day of necropsy. Ten female rats in each group will be sacrificed on day 91. According to vaginal smears, the blood samples from the female rats in the estrous stage of the estrous cycle will be collected, centrifuged and serum will be stored at -20°C until it is used for the hormonal assays. The other 6 female rats in each group will be sacrificed on the day of the first estrous after day 91 and the collected serum will be stored at -20°C until it is used for the hormonal analyses. In this way, at least 6, but most probably more than 6 serum samples will be obtained at the same stage of the estrous cycle, thereby making sure that the hormonal background is the same, and potential differences between the experimental groups might become visible.

Comment 68 (Science): Will calculated globulin and albumin/globulin ratio be tabulated?
Reply 68: Yes, the albumin/globulin ratio will be calculated and tabulated.

Comment 69 (CA): Parameters especially indicating immunotoxicity should be included. For this aim, immunoglobulin concentrations (for instance IgE, IgM, IgG in blood or other endogenous fluids of the test animals could be measured). Appropriate omics analysis could help to evaluate potential endocrine disrupting effects (see for example "Genomic approaches for cross-species extrapolation in toxicology" [Benson and Di Giulio 2007]).
Reply 69: Parameters indicating immunotoxicity will be included. No omics analysis will be performed to assess endocrine effects, since this analysis form has not been standardized and validated up to now.

2.6.3. Clinical signs, detailed physical examinations and functional assessment

Comment 70a (Industry): The outcome of e.g. gait changes will be recorded according to a specific SOP. In addition, "… animals will also be assessed for gait disturbances using Accuplacer treadmill equipment." Is this examination procedure often performed in the test facility? Were positive control studies ever been done to assure sensitivity and reliability of these examinations? How often during the course of the study will this be done? Is it part of the DCO? Please specify.
Comment 70b (Science): The study plan says: "The outcome of this examination will be recorded for each animal in accordance with the SOP ŠPP/TOX/V003 (Origin of score system: Országh A. et al. [1985] Veterinárna ortopédia a rontgenológia, Bratislava: Príroda, 243 p.". Has the score system been validated compared to Irwin or FOB test?
Reply 70: The outcome of e.g. gait changes will be recorded according to a specific SOP. A 4-channel training track for rats (AccuScan Instruments, Inc.; AccuPacer Treadmill – MNL032306Rev.- USA) will be used.
This examination procedure has often been performed at the test facility in the past, e.g. in the projects APVV-21 a ITMS č. 26240120033). In the frame of the G-TwYST project, we will use the model for each animal twice during the study:

- At week 46-50: Time: 25 min /speed: 15 m/min
- At week 96-100: Time: 25 min /speed: 15 m/min

2.6.4. Gross necropsy

Comment 71 (Science): The draft study plan says: "The dose groups will be unblinded at the time of necropsy." What is the interest to perform the study blinded until this time of the experiment ?
Reply 71: It is important to avoid any subjective influence by the experimentators when determining the different endpoints.

Comment 72 (Industry): CLI recommends that a supervising pathologist be at both scheduled necropsy intervals (i.e., chronic toxicity and Carcinogenicity phases). Perhaps this was the aim, but as currently written this could be interpreted to mean that only the last necropsy interval will include a pathologist.
Reply 72: A well qualified expert at SZU is in charge of the necropsy protocol, and the SOPs will include forms that have been tested before the start of the feeding trial.

Comment 73 (Industry): Weights of the sternum with bone marrow and the thymus (likely to be involuted and hardly traceable at the indicated time points) are not indicated in OECD TG 453, and as such are unlikely to be evaluated in other toxicology studies conducted according to this guideline. Accordingly, it is unlikely that there is robust HC data available for these endpoints in studies of this length. Thus, interpreting the relevance of any differences detected in these endpoints will be difficult. For these reasons CLI recommends omitting these endpoints.
Reply 73: We agree - there is no point in weighing the sternum or the thymus.

Comment 74 (Science): During the chronic toxicity phase all animals in the study shall be normally subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. However, provision may also be made (in the interim kill or satellite groups) for measurements to be restricted to specific, key measures such as neurotoxicity or immunotoxicity (see paragraph 21). These animals need not be subjected to necropsy and the subsequent procedures described in the following paragraphs. Sentinel animals may require necropsy on a case-by-case basis, at the discretion of the study director."
Reply 74: We will exclude sentinel animals from the histopathological examination of the main study. Including them would possibly compromise the statistical analysis of neoplasms, which is the main point of the study.

2.6.5. Histopathology

Comment 75 (CA): A fully blinded evaluation of the tissue samples should be expected- not a mixture of blinded and non-blinded steps during the evaluation process.
Comment 75a (Industry): The question of non-blinding in histopathology was also raised during the workshop in December 2014: What about a peer review?
Reply 75: The only analysis that will be performed unblinded is the histophatological evaluation (as recommended e.g. by the European Society of Toxicologic Pathologists).
Referring to a 2004 publication on best practices, a blind study is much slower (takes 3 times as long) and you have a higher chance of missing effects. The problem of non-blinding can be solved by a 2nd pathologist who would typically look at each neoplasm and about 10% of the animals completely. We will be doing this.

Comment 76 (Industry): Certain tissues are not optimally preserved by immediate preservation in 10% neutral buffered formalin. For example, testes, epididymides and eyes are commonly preserved in Davidson's solution first and subsequently transferred to 10% neutral buffered formalin after the

initial fixation is achieved. This enables the production of optimal sections for evaluation of these tissues and CLI recommends following these procedures as well.
Reply 76: We agree. Testes, epididymides and eyes should be preserved in Davidson's solution first and subsequently transferred to 10% neutral buffered formalin after the initial fixation is achieved.

Comment 77 (Industry): Line 88, Additional responsibilities: Please indicate that peer review means histopathological examination and assessment.
Reply 77: Peer Review of histopathology will be conducted according to recently issued OECD Guidelines. Details of the procedures and the Peer Reviewing Pathologist will be added in an amendment.

Prior to the written comments mentioned above, stakeholders made the following comments on histopathology during the workshop in December 2014:
Comment 78 (CA): Does the histopathologist give advice to Bratislava how to prepare the necropsies/organs?
Reply 78: Yes, very detailed, completely standardized protocols will be followed. There will be some practice sessions before the study starts.

Comment 79 (CA): Consistency is needed in the description of what you see. Will the histopathologist will be present in Bratislava to check during necropsy?
Reply 79: Yes, the histopathologist will be present during the final necropsy. The system we use is PathData, which allows to enter necropsy data in English, French, German (and Slovak, predefined). In Bratislava, all data will be entered in English as well as in Slovak.

Comment 80 (Industry): Can you share your thoughts about the use of tissue from animals that have suffered?
Reply 80: A carcinogenicity study is a "lifetime" study; it is inevitable that some control and treated animals will die before the end of the study. Well established criteria to sacrifice animals before the end of a particular study exist and will be followed to avoid unneccesary suffering of the animals.

2.6.6. Ophthalmologic examination

Comment 81 (Industry): OECD TG 453 does not indicate that ophthalmologic examination is a requirement for the carcinogenicity phase of the study. Therefore, such evaluations are unnecessary. As currently written, this section of the Study Plan is unclear and could be misinterpreted by readers to indicate that all animals will receive an ophthalmologic exam at the end of the study. However, if it is G-TwYST's intention to evaluate all animals at all endpoints, this is unnecessary. By eliminating the ophthalmic exams in the carcinogenicity phase 1000 unnecessary ophthalmic exams (500 animals evaluated twice) could be omitted to provide substantial savings, and those funds could be used to enhance the robustness of other aspects of the study design.
Reply 81: Ophthalmological examination, using an ophthalmoscope, will be conducted prior to the administration of the test feeds and at the end of the study.
In the chronic toxicity phase, OECD TG 453 recommended to examine at least the high dose and control groups of animals. However, our study is blinded and, therefore, we should conduct the examination in all animals.
Nevertheless, the ophthalmological evaluation is not required in the carcinogenicity phase of the study but we still consider this examination an important part of the clinical observations at the end of the carcinogenicity phase of the study.

Comment 82 (Industry): The current Study Plan contains assessment intervals and endpoints not indicated in OECD Test Guideline (TG) 453. Reverting to the recommended assessment paradigm in OECD TG 453 would enable a substantial reduction in the number of ophthalmic exams (1000

exams; see Specific Comment 11 for details) and clinical pathology assessments (1200 hematology, clinical chemistry, and urinalysis evaluations; See Specific Comments 56, 63 and 70a for details). The funding currently intended to support these unnecessary assessments could be used to design a more robust study with multiple Reference Groups to expand the Historical Control (HC) data and analyses to confirm that each batch of diet prepared is suitable for administration prior to feeding. These are important considerations particularly if a 2 year study is conducted with higher doses of the grain than what is available in the normal routine rodent chow, and considering that (i) the testing facility does not have HC data for chronic toxicity/carcinogenicity studies, and (ii) that contaminants or nutritional imbalances in the diet could hopelessly confound the results of the feeding study.
Reply 82: We agree with the comment that sampling all animals is costly and time-consuming. Therefore, we have developed the Appendix to the Study Plan in accordance with recommendations in the OECD TG 453: In the chronic phase of the study, blood will be sampled from all animals, i.e. 20 males and 20 females/group. In the carcinogenicity phase, using random sampling, blood will be taken from 20 males and 20 females/group.
The OECD TG 453 indicates: "In order to maximise the information obtained from the study, especially for mode of action considerations, blood samples may be taken for haematology and clinical biochemistry, although this is at the discretion of the study director. Urinalysis may also be appropriate."

2.6.7. Use of historical data

Comment 84 (Science): Concerning the chronic toxicity phase it is generally considered that baseline haematological and clinical biochemistry variables need be determined before treatment for dog studies, but need not be determined in rodent studies (38). However, if historical baseline data (see paragraph 58) are inadequate, consideration should be given to generating such data."
Reply 84 : There is no possibility of generating such data in the frame of G-TwYST.

Comment 85 (Science): For the carcinogenicity and chronic toxicity study, historical control data may be valuable in the interpretation of the results of the study, e.g., in the case when there are indications that the data provided by the concurrent controls are substantially out of line when compared to recent data from control animals from the same test facility/colony.
Reply 85: The consortium agrees with the statement.

Comment 86 (Science): Historical control data, if evaluated, should be submitted from the same laboratory, relate to animals of the same age and strain, generated during the five years preceding the study in question.
Reply 86: The consortium agrees with the statement.

2.7. Statistical analysis

Comments on the statistical analyses part of the proposal focused on sex differences, the use of equivalence limits, effect size and confidence intervals, the use of historical data, the multiple comparisson procedure, the statistical analysis of histopathological data and the statistical power.

Comment 87 (Industry): In Lines 161-163 it is stated that the studies will provide a "comparative assessment" of the results of shorter term subchronic toxicity studies versus extended chronic toxicity and carcogenicity studies. The use of the term "comparative assessment" is perhaps misleading because the term is usually reserved for cases in which formal statistically-based comparisons can be made (as in a traditional regulatory safety study, for example). Here, any comparison between studies will be more qualitative in nature and not a true comparative assessment in the statistical sense.

Reply 87: The consortium agrees with this statement.

2.7.1. Sex differences

Comment 88 (Science): Females and males should not be statistically analysed together, but separately. Even liver and kidneys are sex-differentiated organs (Chang et al., 2013). General principles in endocrinology have taught us that we cannot expect similar responses in males and females after lifetime exposures (Diamanti-Kandarakis, 2009).
Reply 88: Not all response variables are expected to be sex-differentiated. And if they are, it may concern only main effects, i.e. differences between males and females that are the same for all treatment groups in the study. For any variable without sex by treatment interaction, a joint analysis has two advantages: 1) a more concise presentation of results, and 2) an increased statistical power. A test for interaction between sex and effect can still be performed in a combined analysis, allowing go back to separate analyses if a statistically significant interaction is found.
In spite of the theoretical advantages sketched above, the analyses in G-TwYST will follow the traditional approach because this is supposed to be better received by stakeholders, i.e. endpoints will be analysed separately for males and females.

Comment 89 (Industry): While the rationale for staggering the start date between males and females is understood, this effectively invalidates any test of significance of the sex effect, as does housing males and females in separate rooms. This is of no real consequence as there is no interest in the comparison of sexes per se, but this limitation should be made clear in the protocol.
Reply 89: The GTwYST team fully agrees with this comment. Investigation of sex differences is not an aim of this study.

2.7.2. The use of equivalence limits

Comment 90 (CA): According to the presentations at the workshop, the evaluation and statistical analysis of the animal feeding studies will make use of equivalence limits instead of NOAEL or benchmark dose (BMD) approaches, which are mentioned by OECD test guidelines. Even though we understand the problems with background variability and normal biological variation in the current studies, however, the usual approach of establishing safety criteria for human exposure would be to calculate NOAEL and reference dose. For exposure assessment, a worst case scenario can be used. This is still regularly done by GMO applicants and is based on the assumption that any conventional food product will be replaced by a food derived from the GMO. By doing this, even a "margin of exposure" (MOE) could be calculated.
Reply 90: The approach proposed in this comment (NOAEL or BMD + exposure --> Margin of Exposure) is very useful for well-characterized and quantifiable hazards (e.g. pesticide concentrations). In the case of GMO safety assessment, there is no such identified hazard variable. Difference and equivalence testing in the context of a comparative assessment belong to Hazard Identification rather than to Hazard Characterization or Risk Assessment. We agree that for identified hazards a BMD+exposure+MOE approach would be useful as a follow-up.

The issue of applying equivalence was also raised during the December 2014 workshop. One of the participants commented that we have to prove that there is no difference. When applying equivalence (as regulation requires), you may be missing differences/effects. The response from the G-TwYST team was that it is impossible to prove the null hypothesis by a statistical analysis. It can only be rejected (at a pre-specified significance level) or accepted (means: not rejected). Exactly for this reason, we focus on equivalence tests, where a null hypothesis of non-equivalence has to be rejected in order to prove equivalence. In addition, difference tests will still be done to avoid missing differences/effects.

2.7.3. Effect size and confidence intervals

Comment 91 (Industry): The design as currently proposed does not provide a basis for estimating the level of natural variation across the population of crop varieties. This being the case, it is difficult to see the value of including some form of formal equivalence test based on pre-specified effect sizes as this would not tell us anything more than can be deduced from the proposed difference test for which results are expressed as point estimates and confidence intervals. That is, if the effect size of interest is x, and the upper confidence interval for the difference is less than x, then equivalence can be concluded. (While there is arguably an issue of "sidedness" here between difference tests and equivalence tests, this should not give cause for concern provided results are interpreted pragmatically, especially bearing in mind that, in feeding studies, toxicologists routinely consider the potential relevance of all results regardless of their statistical significance). If equivalence testing is to be included then the pre-specified effect sizes should be stated in the protocol.
Reply 91: Pre-specified values or effect sizes for equivalence testing (equivalence limits), or procedures by which such values or effect sizes will be derived, will be specified in the Statistical Analysis Plan prior to analyzing the data. It can be noted that it is very difficult to obtain equivalence limits, yet they are absolutely essential for the project. If uncertainty about equivalence limits can be quantified, then this uncertainty can be integrated into the equivalence testing approach.

Comment 92 (Industry): Industry statisticians and toxicologists remain skeptical of the value of performing statistical analysis based on standardized effect sizes in addition to the analyses performed on the natural scale. There are several reasons for this. Firstly, there is no relationship between SES and toxicological relevance. Secondly, SES does not provide a basis for comparison with historical ranges. Thirdly, all endpoints arising from the same experimental design will appear equally sensitive regardless of the differences in underlying levels of variability.
Reply 92: This is agreed. SES was used in an EFSA report only as an example based on the rather artifical assumption that 1 SD (residual between cages within the experiment) would be a limit of no toxicological relevance. In the frame of G-TwYST, research is directed to finding a better model, or at least explaining the problems better. Attempts are made to base a useful standardization on either external data or on expert opinions. Given interpretable standardization factors related to natural variation or as a limit of toxicological relevance, the use of such standardized effect sizes is considered useful in an overview that has to look simultaneously at many variables. For interpreting each variable in detail, the results can (and will) also be presented on the natural scale.

2.7.4. Use of historical data

The use of external reference data was already extensively discussed at the stakeholder workshop in December 2014. One of the stakeholders pointed out that the use of Harlan data as a reference would raise questions concerning the comparability as these have been collected in the context of pharmaceutical testing. It is therefore important to make sure that it is transparent what data go in and where the data come from. The G-TwYST team replied that to translate biological relevance into equivalence limits you want a wide range of variation where you are sure there is no concern. Further comments followed in the written contributions.

Comment 93 (Science): For the statistical analysis, it is planned to use the historical data of the Harlan rat strain as a secondary control. These are not relevant because the rat feed may have been contaminated with different pesticides residues, heavy metals, and dioxins, and the rats may have been rised under different conditions. The bibliography on this topic is extensive.
Reply 93: G-TwYST has investigated the possibilities to use the Harlan data, but has come to the same conclusion that these data cannot be used as external reference data. Other data such as those from the GRACE project will be used instead whenever possible.

Comment 94 (Science): While a verbal assurance was made by the panel at the GM-TwYST workshop in Vienna (Dec 2014) that historical control data would only be used as an additional guide and not in the primary comparison, it should be written in the protocol that the researchers recognize that historical control data must not be used to dismiss statistically significant differences in the GM-fed group on the grounds that they are within the range of the historical control data
Reply 94: Historical control data from the own animal housing facility will be used as an additional guide.

2.7.5. Multiple comparison procedure

Comment 95 (Industry): Although the treatment list has yet to be finalized, it is very likely that there will be more than two treatments. This being the case, there is a need to consider the issue of multiplicity and whether, for example, some form of multiple comparison procedure should be considered in place of standard t-tests. There is also a need to consider whether testing the significance of specific treatment differences should depend on first obtaining a statistically significant F-test for the overall treatment effect. Provided that the comparisons of interest are specified a priori, then arguably the most appropriate course of action would be to disregard the overall F-test and focus directly on the comparisons of interest.
Reply 95: Multiplicity problems arise for two reasons: 1) multiple variables, and 2) multiple treatment groups. Whereas there are more or less standard procedures for the second type of multiplicity, the first type is much more important in this study. There are no generally accepted procedures to handle the multiplicity due to analyzing many variables at the same time (unless one would move to a multivariate statistical model, which is uncommon and also leads to various problems). We therefore choose to accept the multiplicity for what it is, and focus directly on the comparisons of interest, in line with the suggestion in this comment.

2.7.6. Statistical analysis of the histopathology results

Comment 96 (Industry): In the event that the Study Pathologist requests statistical analyses of the histopathology results, CLI recommends a formal amendment to the Study Plan as detailed in Lines 102-108.
Reply 96: The study pathologist will carry out an age-adjusted Peto statistical analysis of neoplastic data using the PathData pathology software. This has been the standard procedure for carcinogenicity studies for over 30 years; without this, the data is meaningless. Selected non-neoplastic findings may also be analysed at the pathologist's discretion. No protocol amendment is necessary.

2.7.7. Statistical power analysis

Comment 97 (Science): What is the rational to test only 16 animals?
Reply 97: A power analysis will be performed a priori to establish the adequate number of animals per group to be used.

Comment 98 (Industry): Given the overall aim of this study is to inform on the design and value of future studies rather than to assess the safety of the chosen test materials, replication can be set pragmatically rather than strictly on the basis of statistical power. This is especially relevant given that the accuracy of power calculations is dependent on having a reliable estimate of the expected experimental error, which we do not have due to the lack of relevant data.
Reply 98: We agree. However, the power analysis is expected to be still very useful to inform on the design and value of all separate endpoints in future studies.

During the stakeholder workshop one of the participants stated that the first goal in carcinogenicity studies is to detect tumours: more or earlier. For this purpose, we have the Peto test. You have to

focus on a number of parameters. The G-TwYST team responded that less groups with more animals will increase the statistical power. It would be safer to have 6 groups of 60 animals. It was also made clear that we have no trigger, and G-TwYST simply fulfills the requirements of the EC call, i.e. we will perform feeding trials with different extents of time. G-TwYST will also vary the number of animals by taking into account the result of the power analysis.

2.8. Other issues

Additional comments concerned a lack of information in the study plan on GLP-compliance, logistics and archiving.

2.8.1. GLP compliance

Comment 99 (Science): As to the Multisite study, attention must be underlined regarding the communication between test sites. According to an Consensus Document of the Working Group on Good Laboratory Practice: "The Application of the OECD Principles of GLP to the Organisation and Management of Multi-Site Study":
"For a multi-site study to be conducted successfully, it is imperative that all parties involved are aware of their responsibilities. In order to discharge these responsibilities, and to deal with any events that may need to be addressed during the conduct of the study, the flow of information and effective communication among the sponsor, management at sites, the Study Director, Principal Investigator(s), Quality Assurance and study personnel is of paramount importance.
The mechanism for communication of study-related information among these parties should be agreed in advance and documented.
The Study Director should be kept informed of the progress of the study at all sites".
Reply 99: The G-TwYST team fully agrees. It has to be noted that some constraints arise because G-TwYST is a EU-funded project. There is already an intense flow of information between partners in preparing the trials (including visits, common language and wording).

Comment 100 (Industry): All activities conducted outside of Slovak Medical University (SMU) should be clearly identified as GLP or non-GLP and Principal Investigators assigned as appropriate. While this has been done for certain study components (i.e., histology, histopathology, and biostatistics), other elements of the study design are less certain (-omics analyses as part of the French consortium GMO90+).
Comment 100a (Industry): For the purposes of transparency, please clearly identify the components of the study which are not GLP-compliant.
Reply 100: The Center of Testing Laboratories (Laboratory of Toxicology, Laboratory of Immunotoxicology - including the Laboratory of Haematology - and the experimental animal housing rooms) of the Slovak Medical University has received a statement of GLP compliance from the Slovak National Accreditation Service (certificate No. G-036). The Laboratory of Clinical and Experimental Biochemistry of the Slovak Medical University is not GLP compliant, but holds an accreditation certificate (ISO 17025 certificate No. M-013) and is subject to the National Quality Control Programme for Clinical Biology. All procedures performed by the above-mentioned laboratories are described in standard operating procedures approved by the QAU. Each Principal Investigator at the histology processing test site and the histopathology examination test site is responsible for compliance with their national GLP regulations.

Comment 101 (Industry): In some sections the Study Plan lacks sufficient information and direction to the study staff. Per OECD's GLP guidelines, the Study Plan or Protocol should contain, "Detailed information on the experimental design, including a description of the chronological procedure of the study, all methods, materials and conditions, type and frequency of analysis, measurements, observations and examinations to be performed, and statistical methods to be used (if any)." Some

of the Specific Comments below attempt to address sections where insufficient detail may be observed.
Reply 101: We consider this a general comment that was split in more specific comments. The G-TwYST has replied to the specific comments.

2.8.2. SOPs and archiving

Comment 102 (Science): SOPs for necropsy, histopathology and ophthalmologic examination are not indicated
Reply 102: SOPs are not necessary.

Comment 103 (Industry): SOPs are referenced throughout the document. To increase transparency and facilitate understanding among stakeholders, CLI requests that these SOP documents be included on the website and be accessible to stakeholders.
Reply 103: In our test facility, all referenced SOPs are available and can be published on the G-TwYST website. However, all SOPs are designed for our staff (Slovak study personnel) and, therefore, are available in the Slovak version only. We are willing to provide the documents in the Slovak version on the website. Since we have no financial budget and no personal capacity for translation of all SOPs, people that are interested may use the automatic translator.

Comment 104 (Science): What is planned after the 10-years archiving?
Reply 104: In compliance with the Slovak legislation, after 10 years paper documents will be destroyed. Moreover, the biological material will then be destroyed by a special company.