ANNEX 1

Bioveiligheidsraad
Conseil de Biosécurité

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Secretariaat
Secrétariat

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Title: Advice of the Belgian Biosafety Advisory Council on the article by Séralini *et al.*, 2012 on toxicity of GM maize NK603

Issue

On 19 September 2012, the Journal Food and Chemical Toxicology published online a research paper, written by G-E. Séralini *et al.*, entitled: "Long term toxicity of a Roundup herbicide and Roundup-tolerant genetically modified maize"[1].
The authors presented this study as the first detailed documentation of long-term adverse effects arising from the consumption by rodents of a genetically modified (GM) glyphosate-tolerant maize and of the Roundup herbicide, a commercial glyphosate-containing formula. The paper states that *the study clearly demonstrates that low levels of complete agricultural glyphosate herbicide formulations induce severe hormone-dependent mammary, hepatic and kidney disturbances*. Further, it is stated that *disruption of biosynthetic pathways that may result from overexpression of the epsps transgene in the GM maize NK603 can give rise to comparable pathologies that may be linked to abnormal or unbalanced phenolic acids metabolites, or related compounds, without excluding other mutagenic and metabolic effects of the edible GMO*.

Mandate

As a result of the publication of the abovementioned research paper, the Federal Minister of Public Health asked the Biosafety Advisory Council (BAC) on 21 September 2012 to evaluate the paper. The BAC was asked to inform the Minister whether this paper (i) contains new scientific information with regard to risks for human health of GM maize NK603 and (ii) whether this information triggers a revision of the current authorisation for commercialisation for food and feed use of this GM maize in the European Union (EU).

Procedure

Within the framework of this mandate, the BAC, under the supervision of a coordinator and with the assistance of its Secretariat, contacted scientists with expertise in statistics, toxicology, oncology, haematology, anatomopathology and clinical biology to review the research paper. They were invited to consider in particular the robustness of the conducted research, the applied methods and the interpretation of the results. To avoid any conflicting interest, experts of the common list drawn up by the BAC and the Biosafety and

[1] Séralini GE., Clair E., Mesnage R., Gress S., Defarge N., Malatesta M., Hennequin D., de Vendômois JS. 2012. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem. Toxicol. doi: 10.1016/j.fct.2012.08.005

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Biotechnology Unit (SBB) who were involved in previous safety assessment of GM plants were explicitly not invited to participate in this consultation.
The following experts answered positively to this request and provided their feedback:
Prof. Adelin Albert (Université de Liège), Prof. Dominique Cassart (Université de Liège), Prof. Corinne Charlier (Université de Liège), Prof. Dr. Dirk De Bacquer (Universiteit Gent), Dr. Bart De Ketelaere (Katholieke Universiteit Leuven), Prof. Joris Delanghe (Universiteit Gent), Prof. Philippe Delvenne (Université de Liège), Prof. Frédéric Farnir (Université de Liège), Prof. Pascal Gustin (Université de Liège), Dr. Dominique Lison (Université catholique de Louvain), Dr. Ir. Viviane Planchon (Centre wallon de Recherches agronomiques, Gembloux).

This document provides a summary of the main elements and conclusions addressed by the experts in their analysis reports as well as the conclusions drawn by the BAC on this basis.

Background information

The GM maize NK603 (Unique Identifier MON-ØØ6Ø3-6) has been developed for tolerance to glyphosate by the introduction, via particle gun acceleration, of a gene coding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (CP4 EPSPS).
This GM maize is currently authorised for commercialisation in the EU for food and feed use (for further details, see the EU Register of authorised GMOs at http://ec.europa.eu/food/dyna/gm_register/index_en.cfm).

In October 2005, Monsanto has submitted to EFSA under Regulation (EC) No 1829/2003 an application (Reference EFSA-GMO-NL-2005-22) for authorisation of NK603 for cultivation, food and feed uses and import and processing, as well as an application for renewal of the authorisation of existing feed materials and food and feed additives produced from maize NK603 (Reference EFSA/GMO/RX/NK603).

In the frame of the evaluation of these two applications, the BAC has issued a comprehensive advice on 2 October 2009 (Reference WIV-ISP/BAC/2009_01367). In this advice, the BAC concludes that it agrees with the GMO Panel of EFSA that no major risks for human and animal health associated with the use of GM maize NK603 in food and feed were identified.
When drafting this advice, the BAC took into consideration all relevant available information, including a 90-day study in rats fed GM maize NK603 either as 11% or 33% of the total diet, or a control diet containing 11% or 33% non-GM maize having a comparable genetic background to GM maize NK603 (data published in Hammond et al., 2004)[2].

General information about the design of the Séralini'study and toxicity/carcinogenicity studies

The paper of Séralini et al. presents a long-term (2 years) experiment on feeding trials using 200 Virgin albino Sprague-Dawley rats (100 males and 100 females). The 100 animals of each sex have been randomised into 10 distinct groups of 10 rats each. For each sex, one control group had access to plain water and standard diet containing 33% of the closest isogenic non-GM maize control; six groups were fed with diet containing 11, 22 and 33% of GM NK603 maize either treated or not with RoundUp. The final three groups were fed with the control diet and had access to water supplemented with three different concentrations of Roundup.

[2] Hammond B., Dudek R., Lemen J., Nemeth M. 2004. Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. Food Chem. Toxicol. 42, 1003–1014.

The aim of the study, as far as can be judged from the publication, was to carry out a prolonged oral chronic toxicity study in rodents to investigate the effects of GM maize NK603 (treated or not treated with Roundup) consumed over the long term. The starting point to design the study was the usual parameters for a 90-day toxicity study (OECD Guideline No 408[3]) to which the authors added some additional parameters and prolonged biochemical and haematological measurements or disease status as recommended for combined chronic toxicity/carcinogenicity studies (they refer to OECD Guideline No 453[4]).

The OECD Guideline No 453 recommends that each dose group and concurrent control group intended for the chronic toxicity phase of a study should contain at least 10 animals of each sex, while for the carcinogenicity phase of a study each group should contain at least 50 animals of each sex. The recommended period of dosing and duration of the study is 12 months for the chronic phase, and 24 months for the carcinogenicity phase (representing the majority of the normal life span of the animals to be used). The Guideline also states that "*interpretation of the data from the reduced number of animals per group in the chronic toxicity phase of a combined study will however be supported by the data from the larger number of animals in the carcinogenicity phase of the study.*"

Information for the design of long-term chronic toxicity studies is also available in the OECD Guideline No 452[5]. For rodents, it is recommended that at least 20 animals per sex per group should be used at each dose level so that at the end of the study enough animals in every group are available for thorough biological and statistical evaluation. The Guideline is designed as a 12 month chronic toxicity study, although longer or shorter durations may also be chosen depending on specific requirements.

Analysis of the research paper published by Séralini et al. (2012) Summary of the main elements addressed by the experts in their analysis reports

Design of the study

- Three experts were of the opinion that the long duration of this study is a positive aspect since most of the toxicity studies on GMOs are performed on shorter periods.

- Seven experts considered the number of animals used by Séralini et al. (10 rats/sex/group) as being too low and not fully complying with the recommended standards for a long-term toxicity study and/or for a carcinogenic study (see general information above).
However, one expert referred to biocides toxicological evaluation in the frame of REACH where sub-chronic or long-term studies are performed on 10 or 15 animals, which is quite the same as in the Séralini paper. Although the OECD recommends the use of at least 20 animals, 10 animals in each group is better than what is made in many other studies.

- The study was performed using Sprague-Dawley (SD) rats. While this strain is commonly used in studies for drug discovery and in short-term toxicity studies, its relevancy in a two-year study was questioned by five experts in particular in the context of the analysis of tumour incidence. There are indeed numerous references in the scientific literature showing that SD rats have a high background incidence for certain types of tumours, especially mammary and pituitary tumours, with probabilities that rapidly increase during the last quarter of their life, and suggesting a clear effect of feeding strategy. These studies also show that there is a non-

[3] OECD (1998). Test No. 408: *Repeated Dose 90-Day Oral Toxicity Study in Rodents*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.
[4] OECD (2009). Test No. 453: *Combined Chronic Toxicity/Carcinogenicity Studies*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.
[5] OECD (2009). Test No. 452: *Chronic Toxicity Studies*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.

negligible variability in this probability, probably related to specific "settings" of the studies (feed type and amount, ...).
In such a situation confidence intervals for small sample sizes (such as n = 10) are broad. Moreover, this can create a problem of interpretation of the lesions with a possible confusion between lesions possibly related to age and lesions caused by the products tested.

- Three experts noted that it is somewhat unusual that only 10 control animals per sex were used for a total of 90 animals per sex in the experimental groups. The interpretation of the reported results will depend largely on the expected survival rate / tumour incidence rate of control animals. Therefore, in order to determine a control group probability of developing pathologies, a substantial amount of control animals is compulsory, 10 being considered too low. Through this power imbalance in favour of the exposure groups, the importance of the observations in the control group as reference is not sufficiently emphasized. To partly accommodate this, thorough balanced statistical analysis would therefore be necessary, but is made difficult in this case due to the low number of animals per group.

- One expert indicated that the number of animals per cage was unclear. Section 2.3 refers to "two animals of the same sex per cage" while Table 1 refers to "one or two animals of the same sex by cage". Although this might be seen as a detail, it should be noted that rats are rather gregarious and isolation could generate endocrine deregulation potentially leading to the apparition of tumours (reference to a scientific paper was cited).

- One expert indicated that the experimental design was not "complete", in the sense that not all possibilities generated by diet and water were utilised. Actually, the experimental design consists of two distinct "dose-response" studies using the same control group, respectively (control + 3 groups fed with 11, 22 and 33% of GM NK603 maize either treated or not with RoundUp) and (control + three groups fed with the control diet and water supplemented with three different concentrations of Roundup). Interestingly, the authors do not explicitly look at the groups as originating from dose-response experiments. The 10 groups are viewed as qualitatively distinct, i.e. without any ordering (see the OPLS analysis on biochemical parameters). Indeed, nowhere in the paper have the groups been considered or treated as "ordered".

Endpoints

According to one expert, the endpoints of this study have not been clearly defined. What is the primary endpoint, what are the secondary endpoints? Several possible endpoints (or outcome measures) can be identified:

- Survival or death over the study period (binary variable)
- Time to death (time-to-event variable with censoring)
- Tumour development (binary variable Yes/No)
- Time to tumour development (time-to-event variable with censoring)
- Number of tumours developed (count variable: 0, 1, 2 ...)
- Number of pathological findings (count variable: 0, 1, 2 ...)
- Biochemical tests (quantitative continuous variables repeated over time)

These endpoints can be binary, time-to-event or lifetimes, counts or quantitative variables. Appropriate statistical methods should be used to analyse the corresponding data. This has not been done.

Anatomopathological observations

- Three experts commented this part of the study. Their general feeling was that the paper does not clearly specify the type of tumours that have been observed. Table 2 is unclear and mixes different categories of tumours. The legend of Figure 3 refers to adenocarcinomas and fibroadenomas. These should be clearly distinguished as they correspond respectively to

benign and malignant tumours. Galactoceles and hyperplasias are also mentioned in Figure 3 although it is unusual to consider them as tumours.

- One expert noted that while, from the data presented, the diagnosis of fibroadenomas can be considered likely, histological analysis does not allow to formally conclude the presence of adenocarcinomas. Research of myoepithelial cells by immunohistochemistry may be useful to confirm the histological diagnosis (adenocarcinoma *vs* fibroadenoma). Accordingly, it is surprising, given the size of tumours, that only two animals had developed metastases (a characteristic of malignant tumours - results not shown). Another expert concluded that it was impossible to know, from the data available, whether the reported lesions were regressive, inflammatory or neoplastic (i.e. with the potential to evolve in cancer).
It should also be noted that in human pathology, fibroadenoma (a dual - epithelial (glandular) and fibre - component tumour) is not considered the precursor of the classical mammary adenocarcinoma. Indeed, its degeneration into cancer is considered exceptional.

- One expert noted that, even though the authors state several times in the paper that tumours were studied by electron microscopy, the results are not reported nor discussed.

Biochemical analyses

- One expert noted that the results were presented in a way that makes their interpretation very difficult. Table 3 reports only about the percentages of variation of tested parameters. The main limitation in this approach is that biochemical parameters often reveal large variations between animals, so presenting percentages of variation is not sufficient. Crude data should be provided as well as other information such as description of the analytical methods used for all determination (section 2.4 only states that parameters were assessed "according to standard methods"), total error or standard deviation of the methods...

- One expert was of the view that the authors' opinion that biochemical parameters indicate kidney and liver failures is questionable. In particular he noted that:
- The hepatic biochemical parameters (ALT, total protein, cholesterol synthesis, coagulation) are very similar between control and treated groups and do not indicate serious liver disease.
- The biochemical parameters for kidneys are also satisfactory. The reported reduced levels of creatinine in urine for all treatment groups in comparison to female controls (Table 3) should be interpreted very carefully, in particular given the absence of any information about the methodology used. Creatinine levels in rats are much more lower than in humans, which makes their measurement very unreliable (see new standard SRM 967 for use in establishing calibrations for routine creatinine measurement procedures in human serum). Moreover the reported nephropathies in the treated groups seem unlikely due to the very limited changes in urea and creatinine levels.
- The significance of the observed changes in Na and Cl excretion is very unclear. Contrary to what should be expected, no hyponatraemia is observed. In case of salt-losing nephritis an "Urémie par manque de sel" should normally be detected. The study of the possible renal tubular damage (typically associated with kidney injury) is very superficial in the paper.

Statistical analyses

- Four experts noted that the differences in the incidence of the primary health effects that were observed, namely mortality and tumour development (Figures 1 and 2), were not subjected to statistical testing.

- One expert suggested that the following statistical design should have been performed: Proportions (e.g. death rates) in the various groups should be compared by a chi-square test

or by Fisher exact test. To account for the ordering of the groups (dose effect), it would even be better to apply a logistic regression analysis. To compare counts, a Poisson regression model should be used, to compare lifetimes in different groups or according to dose, a Cox PH regression model would be required. For continuous variables, the classical multiple regression analysis should be used.

- One expert noted that the SEM (standard error of mean) used by the authors in Figure 1 and 2 is known to reduce standard deviation.

- Under the heading "Mortality" of the results section, the authors state that "*30% control males (three in total) and 20% females (only two) died spontaneously, while up to 50% males and 70% females died in some groups on diets containing the GM maize*". Some experts indicated that such a statement needs to be considered with great caution. By applying other hypothesis tests to this scenario (e.g. Fisher's exact test), it appears that for both male and female animals, these differences in mortality are all but statistically significant. In other terms, results could have well been observed by chance alone. Therefore, there is no sufficient statistical evidence to demonstrate differences between the groups.

- One expert noted that the fact that the experiment includes a large number of experimental groups necessitates a multiple comparison correction to control the number of false positive discoveries. However, this important aspect was ignored by the authors. It could simply be done e.g. by a Bonferroni adjustment of the significance level.
Comment: This expert provided a detailed and extensive statistical simulation study showing that the (interpretation of the) results depend(s) heavily on the control probability for developing pathologies, a quantity that is not estimable with high precision based on only 10 control animals for each sex.

- Given the limited sample size of animals per exposure group, one expert wonders whether a statistical analysis is even feasible. In consequence it is unclear to what extent the observed differences can be explained as being coincidence or not.

- To illustrate this, one expert noted that due to the small samples size, it would be critical to have larger differences between the control and treated groups (for example, the maximum difference of tumours incidence between control groups (30%) and "worst" treated groups (80%) is only significant with $p = 0.03$).
However, one expert was of the opinion that even if the control animals developed some tumours, the frequency in tested rats was significantly higher than in the control group.

- A statistical analysis was performed for the biochemical parameters, based on the OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis) regression technique. Although there are some arguments for the choice of this technique[6] it is important to note that it seemingly aims to find differences than rather testing whether there can be differences demonstrated in the measured biochemical parameters between tested and control groups. A discriminant analysis starts therefore from *a priory* belief that two groups are different.

- One expert was of the view that biochemical parameters should be analysed by the more advanced "General Linear Mixed Model (GLMM)" which accounts for repeated measurements of the laboratory tests in the comparison of the groups.

- One expert was of the opinion that the discriminant analysis performed in this case on the biochemical data seems to present some shortcomings because of (i) the small sample size for each analysis made, and (ii) the validation strategy chosen, excluding a testing phase on an independent test set.

[6] OPLS-DA is often used in cases where the number of variables is large when compared to the number of samples taken, and where a large correlation amongst the variables is present.

- One expert noted that given the multitude of comparisons that are made (because of the many biochemical data as well as the number of groups being compared), a correction of the significance threshold is required to reduce the risk of "false-positive" findings ("multiple testing"). This was not considered in the paper. Here again, the statistical analysis provided in the paper should be interpreted with great caution and does not support the conclusion of any treatment-related toxicity or carcinogenicity.

- One expert was of the view that more detail would be needed in order to better understand some seemingly paradoxical results: although most (76%) discriminant variables are kidney related, kidney related pathologies do not seem significantly more frequent in diets than in controls. Probably, this only indicates disturbances in the kidney parameters, but with no clear marked effect on the occurrence of a disease.

- Some experts noted that OPLS-DA for biochemical data (Figure 5) are only presented for one group (females that had received feed with 33% NK603 maize compared to the control group), and in a way that does not provide a clear basis to perform a statistical evaluation with sufficient accuracy. Moreover data were only analysed at 15 months ignoring time evolution and group ordering.

Other issues

Some experts noted that the paper lacks information on some aspects and basic parameters that are important for a proper assessment of the reported effects and should be reported for this type of study. Although the authors state (page 4) that "*All data cannot be shown in one report, and the most relevant are described here*", it is unclear on which basis specific pieces of data were included or not into the paper. If only those data "showing the largest differences" were selected, the authors introduced a selection bias into their results. Complementary data should have been provided in an appendix or online supplement, as it is usual for peer-reviewed articles. Missing information mentioned by the experts includes:

- Details on diet composition. The authors state that "*All feed formulations consisted in balanced diets, chemically measured as substantially equivalent except for the transgene, with no contaminating pesticides over standard limits*". However, detailed and clear information on rodents diet is important since differences observed amongst animals may be due to dietary compositional differences. For instance, no information is available regarding the levels of herbicide residues in treated corn, the presence of plant metabolites, or the potential presence of confounders such as mycotoxins.

- Figures for feed and water consumption. The amount of feed and water animals consumed can also have an important influence on many aspects of animal responses, including tumour development and kidney function. In particular in SD rats, it has been reported in the literature that diet restriction increases 2-year survival when compared to *ad libitum* feeding. Although the feeding regime is not described in full detail in the paper, it can be assumed that rats were fed *ad libitum* (in section 2.3 it is stated that animals had "free access to feed and water").

- Information about whether or not the study was blinded. In such studies where anatomopathological data (tumour size measurements, etc.) are used, the investigator should not know the exposure group from which a tested animal is coming from. This is crucial to minimise the risk of biased interpretation of the observations.

Interpretation of results

- One expert noted that Figure 1 shows that the various diets have similar mortality rates in males, although few individuals seem to be dying earlier in the GMO+Roundup diet. The

situation in females seems more in favour of an hypothesis of increased morbidity for the GMO and Roundup diets, with higher mortality and higher euthanasia rates.

- On page 8 the authors state that "*Our data show that, as is often the case for hormonal diseases, most observed effects in this study were not proportional to the dose of the treatment, non-monotonic and with a threshold effect*".
Three experts were of the opinion that the apparent absence of dose-response relationship rather argues against a genuine treatment-related effect. The hypothesis offered by Séralini et al. to explain the absence of dose-response relationship (non-monotonic responses) is not supported by the data of the study or by the general toxicological literature. At least, this indicates that the findings of this study should be interpreted with great caution.

- Another expert commented on the variability within the groups: assuming, as the study suggests and as the authors discuss, that potential deleterious effects of the GMM and/or Roundup are threshold dependent, various doses somehow correspond to replicates of similar situations. Using that argument, it can be observed from the results (for example, from Table 2), that the variation across samples is quite high (for example, for pathologies of the pituitary gland, some diets show less problems than the controls, while for others, the incidence is doubled with respect to controls). This observation again underlines the need for larger samples sizes.

- On page 9, the authors suggest that adverse effects associated with consumption of GM maize NK603 could be explained by reduced levels of caffeic and ferulic acids (secondary metabolites of the plant shikimate pathway) in the GM diets. The authors add that such reduced levels may result from overexpression of the *epsps* transgene in maize NK603. According to the authors, "*this may lower their protective effects against carcinogenesis and even mammalian tumors. Moreover, these phenolic acids and in particular ferulic acid may modulate estrogen receptors or the estrogenic pathway in mammalian cells.*"
One expert was of the opinion that the hypothesis of possible protective effects of caffeic and ferulic acids on tumour development is a matter of discussion and can not be fully substantiated by relevant scientific information. He provided references to scientific papers where adverse effects associated with these components are reported.

Conclusions of the experts

- The experimental design used in this study allows estimation of the effect of water contamination and of the effect of GMO diet, but not the cumulative effect of both combined, in male and female rats. The endpoints are not clearly defined and so are the statistical methods used to test the null hypotheses at hand. The study lacks expert data modelling which would lead to scientifically sound conclusions.

- The study provides some indications that GMO and Roundup based diets potentially might have deleterious effects on health, at least in rats. A major result of the paper is that the (potential) occurrence of problems takes time well above the usual duration used for this type of experiences, which strongly indicates that future experimentations should consider longer terms effects than what is usually done.
No definitive conclusion can be drawn before the experience is repeated with a similar design but with larger cohorts, and maybe with other rat lines. The dose-effect relationship would deserve more attention. And, assuming a threshold effects as done by the authors, a better characterisation of the allowable threshold should be made in order to eventually come with recommendations.

- The results of the study have to be considered with many caution, and of course further experimentations are needed to confirm or not the present findings.

- One can say that the way the study was designed, the data were analysed and the results selectively proposed, is not sufficiently convincing to reach the conclusions mentioned in the paper. Despite the many methodological shortcomings, it can nevertheless be stated that the results of Séralini et al could give rise to further, larger and independent research on the health long term effects of genetically modified food.

- It seems reasonable to assume that the publication of Prof. Séralini, without providing definitive conclusion as to carcinogenicity in rats and even less about the underlying mechanisms, provides a reasonable and sufficient doubt to promote research on the impact of GMOs and pesticides associated with this type of culture, on the fauna and flora as well as mammals exposed. Rather than rejecting these results, should we not, according to the scientific approach, encourage new experiments to verify the reproducibility of the results by correcting any shortcomings of the current publication. All this calls for extreme caution and to discuss these issues with great care.

- Results of the Séralini study can not be regarded as results to take decisions. They must be accompanied by other studies that confirm (or not) the results of this exploratory study.

- This study is not really convincing and a lot of question marks remain.

- The results are rather suggestive than scientifically well-backed and additional/new experiments are needed in order to invalidate former tests performed on GMO and that did not reveal an increased toxicity / risk.

- Challenging existing knowledge and paradigms is of course the basis of scientific progress, and revisiting those current views could be appropriate and welcome. It would need, however, to apply solid scientific standards; the paper by Séralini et al. fails largely in this respect. The work is scientifically very weak, with flaws in the experimental design, in the interpretation of the results as well as their (over)interpretation and reporting. It should never have been accepted for publication in a scientific journal. The process of peer review which is usual before acceptance for publication in scientific journals has clearly failed here.

Conclusions of the Biosafety Advisory Council

1. Given the shortcomings identified by the experts regarding the experimental design, the statistical analysis, the interpretation of the results, the redaction of the article and the presentation of the results, the Biosafety Advisory Council concludes that this study does not contain new scientifically relevant elements that may lead to reconsider immediately the current authorisation for food and feed use of GM maize NK603.

2. Considering the issues raised by the study (i.e. long term assessment), the Biosafety Advisory Council proposes EFSA urgently to study in depth the relevance of the actual guidelines and procedures. It can find inspiration in the GRACE project to find useful information and new concerted ideas.

p.o Bl. Ph Herman
Prof. B. Reheul
President of the Belgian Biosafety Advisory Council

Annex I: Minority opinion

Annex 1: Minority opinion

"Considering the uncertainties on long term effects of GM maize NK603 on health, we ask for a reassesment of the advice of the BAC on the initial dossiers of the maize NK603, regarding effects on human and animal health, using the same critical analysis that was applied by the BAC's experts to the Seralini *et al.* study."

Jean-Claude Grégoire, Damien Winandy, Lucette Flandroy and Philippe Baret

Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Stellungnahme des Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit (BVL) zu der Veröffentlichung *"Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize"* von Séralini et al. 2012

1. Fazit des BVL:

Das BVL ist zu dem Ergebnis gelangt, dass die Schlussfolgerungen der Autoren aufgrund von Unzulänglichkeiten des Studiendesigns sowie der Art der Datenauswertung und der Datenpräsentation wissenschaftlich nicht gerechtfertigt sind.

Aufgrund dieser Mängel können die Inhalte der Veröffentlichung für eine Risikobewertung der gentechnisch veränderten Maislinie NK603 nicht berücksichtigt werden.

Die Notwendigkeit einer erneuten Risikobewertung von NK603 Mais lässt sich auf Basis der genannten Veröffentlichung nicht begründen.

Die beiden zentralen Schlussfolgerungen der Autoren, dass Ratten, welche mit NK603 Mais gefüttert werden, früher sterben sowie früher und häufiger an Krebs erkranken würden, lassen sich aufgrund der präsentierten Daten nicht begründen und sind daher abzulehnen.

Das BVL hat eine eigene statistische Analyse zur Überlebensdauer der Versuchstiere durchgeführt (Anlage 1) und ist zu dem Ergebnisse gelangt, dass **keine Unterschiede zwischen den Behandlungsgruppen in Bezug auf die Überlebensdauer nachweisbar sind**.

Fundierte Aussagen zur Krebsanfälligkeit sind allein aufgrund des ungeeigneten Studiendesigns nicht möglich. Zweijährige Studien mit Ratten müssen gemäß internationaler Richtlinien (z. B. OECD-Richtlinie 451 oder 453) 50 Tiere je Gruppe und Geschlecht enthalten. In der vorliegenden Studie wurden jedoch nur jeweils 10 Tiere eingesetzt. Eine statistisch aussagekräftige Analyse der Daten wurde von den Autoren nicht vorgelegt. Aufgrund unzureichender Präsentation der Daten ist hier eine sinnvolle statistische Auswertung durch das BVL nicht möglich. **Eine durch die Verfütterung von NK603 Mais erhöhte Krebshäufigkeit ist daher nicht nachweisbar.** Ein Vergleich der präsentierten Daten zu historischen Kontrolldaten des genutzten Rattenstammes deutet nicht auf mögliche Auswirkungen der Fütterung auf die Krebshäufigkeit hin.

Eine dritte Schlussfolgerung der Autoren, dass Ratten, welche mit NK603 Mais gefüttert werden, toxikologische Störungen an Nieren und Hormonsystem erleiden würden, lässt sich aufgrund einer unzureichenden Auswertung und Präsentation der Daten nicht rechtfertigen.

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Unterschiede zwischen den Behandlungsgruppen bezüglich der Blut- und Urinwerte sowie histologischer und pathologischer Parameter wurden von den Autoren statistisch nicht abgesichert und die Ergebnisse wurden nur rudimentär präsentiert.

Aus den Ergebnissen der Versuchsreihe mit “WeatherMAX“-behandeltem Mais der Linie NK603 können keine eindeutigen Schlüsse auf erhöhte Todesraten und Tumorbildungen in Abhängigkeit von Glyphosatrückständen in den verfütterten Maiskörnern gezogen werden, da die Ausgangsmengen an Glyphosat in diesem Futter nicht berichtet werden. Die Anzahl von Todesfällen und Tumorbildungen nach Gabe von angeblich rückstandsbelastetem Futter unterscheiden sich wegen der zu geringen Zahl der eingesetzten Versuchstiere nur unwesentlich von denen der Kontrollgruppen.

Ebenso uneindeutig bleiben die Befunde nach den Dosierungen über das Trinkwasser, zumal auch in den Kontrollgruppen zum Teil beide Effekte (Todesfälle und Tumorbildungen) in ähnlicher Größenordnung auftraten als bei den in der Höhe unterschiedlichen Dosierungen. Auch hier kann nur gefolgert werden, dass die zu den Ausgangsbedingungen gehörenden Angaben der tatsächlich aufgenommenen Glyphosatmengen fehlen und dass die Zahl der Versuchstiere zu gering war, um mögliche Trends der Ergebnisse zuverlässig ableiten zu können. Darüber hinaus wurde als weiterer Einflussparameter die mögliche Wirkung des im verwendetem “Roundup GT Plus“ enthaltene tallowaminhaltige Netzmittel auf die Gesundheit der Versuchstiere nicht weiter diskutiert.

Eine wissenschaftlich fundierte Bewertung toxikologischer Ergebnisse ist daher auf Basis der vorliegenden Daten nicht möglich.

Nach Angaben der Autoren wurde jedoch eine ausreichende Datenmenge erhoben, um aussagekräftige toxikologische Analysen durchzuführen. Das BVL hat daher die Autoren gebeten, weitere Daten zu übermitteln, damit entsprechende Analysen inklusive angemessener Statistik durchgeführt werden können (Anlage 3). Bisher haben die Autoren auf entsprechende Anfragen von BfR, EFSA und BVL nicht reagiert, jedoch bei einer Anhörung in Frankreich eine weitere Veröffentlichung zu diesem Thema in einer Fachzeitschrift angekündigt.

In der Diskussion der Ergebnisse werden von den Autoren verschiedene mögliche Wirkmechanismen angeführt, welche die Beobachtungen erklären sollen. Nach Ansicht des BVL ist eine solche Diskussion derzeit abzulehnen, da eine geeignete Grundlage fehlt. Vor einer Diskussion möglicher Wirkmechanismen müssten Auswirkungen der Behandlung auf die Versuchstiere wissenschaftlich fundiert gezeigt und statistisch abgesichert werden. Dies gelingt den Autoren in der vorliegenden Studie jedoch nicht.

2. Bewertung anderer Institutionen

BfR

Am 28.09.2012 hat das BfR dem BVL eine Stellungnahme zur Publikation von Seralini *et al.* (2012) übermittelt. Die vollständige Stellungnahme ist diesem Bericht als Anlage 2 beigefügt. Im Ergebnis kommt das BfR zu folgender Bewertung „*Auf der Grundlage der Publikation ist das BfR zu der Einschätzung gelangt, dass die Hauptaussagen der Veröffentlichung experimentell nicht ausreichend belegt sind und zudem aufgrund der Unzulänglichkeiten des Studiendesigns sowie der Art der Präsentation und Interpretation der Daten wesentliche Schlussfolgerungen der Autoren nicht nachvollziehbar sind“* Diese Einschätzung wird vom BVL geteilt. Von BfR und BVL werden die gleichen Mängel der Studie erkannt und als Begründung für die Bewertung genannt.

ZKBS

Das BVL wird die Stellungnahme der ZKBS an das BMELV nachreichen, sobald diese nach der nächsten Sitzung der ZKBS am 06.11.2012 verfügbar ist.

3. Weitere eingeleitete Schritte

A) Das BVL hat zwei Mitglieder der Zentralen Kommission für die Biologische Sicherheit (ZKBS), Prof. Dr. Steinberg und Prof. Dr. Maser, an der vorläufigen Einschätzung der Studie beteiligt. Auf ihrer nächsten planmäßigen Sitzung am 6. November wird die ZKBS zu der Studie Stellung nehmen.

B) _Das BVL hat die Arbeitsgruppe um Prof. Seralini am 27. 09. 2012 per Email sowie am 11.10.2012 schriftlich (Anlage 3) gebeten, weitere Daten für eine detaillierte Bewertung zur Verfügung zu stellen.

4. Gegenstand der Bewertung

Gegenstand der folgenden Bewertung ist die Publikation *“Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize”* von Séralini *et al.*, die am 19. September 2012 von der Fachzeitschrift *“Food and Chemical Toxicology”* veröffentlicht wurde.

Ziel der Studie war es nach Angaben der Autoren, mögliche toxische Effekte durch die Verfütterung von gentechnisch verändertem Glyphosat-toleranten Mais NK603 sowie der

Verabreichung einer kommerziellen glyphosathaltigen Formulierung (Roundup GT Plus) in einer Langzeit-Rattenfütterungsstudie über zwei Jahre zu untersuchen.

Zusammenfassend sind die Autoren zu dem Ergebnis gelangt, dass Tiere der Testgruppen früher und häufiger als die Kontrolltiere sterben und mehr sowie früher Tumore entwickeln. Die Autoren der Studie vermuten, dass dies auf hormonelle Störungen durch das Herbizid Roundup bzw. auf Stoffwechselprozesse, die durch die gentechnische Veränderung ausgelöst werden, zurückzuführen sei.

Nähere Information zur Studie (Material und Methoden) finden sich in Anhang 1. Eine Sachstandszusammenfassung zum gentechnisch veränderten Mais NK603 (Art der gentechnischen Veränderung, Zulassungsstatus in der EU, bisherige Stellungnahmen des BVL) in Anhang 2.

5. Hauptkritikpunkte

5.1. Studiendesign

Die Gruppengröße ist zu gering und entspricht nicht international anerkannten Standards

Die Autoren verweisen im Hinblick auf die von ihnen durchgeführte Langzeitrattenfütterungsstudie auf die OECD-Richtlinie 408 („*Repeated Dose 90-day Oral Toxicity Study in Rodents*“), auf deren Grundlage die Herstellerfirmen üblicherweise Fütterungsversuche mit gentechnisch veränderten Organismen (GVO) durchführen. Hierauf Bezug nehmend weisen die Autoren darauf hin, dass sie in ihrer Langzeitstudie Daten zu mehr Parametern und häufiger als in den standardmäßig durchgeführten Tests gemäß OECD-Richtlinie 408 erhoben haben.

Das BVL weist darauf hin, dass das Design der Studie nicht den international anerkannten Standards für Langzeitstudien entspricht, welche vor allem dazu konzipiert wurden, Aussagen über die Kanzerogenität des Testmaterials - wie von den Autoren im vorliegenden Fall gemacht - zuzulassen (z. B. OECD-Richtlinie 451 oder 453). Während in der vorliegenden Studie pro Behandlungsgruppe nur je 10 männliche und 10 weibliche Tiere untersucht wurden, fordert die OECD in den entsprechenden Langzeitstudienprotokollen eine Mindestanzahl von 50 weiblichen und 50 männlichen Tieren pro Gruppe, da aufgrund altersbedingter pathologischer Veränderungen große Unterschiede zwischen den einzelnen Versuchstieren einer Gruppe auftreten können. Entsprechend erlaubt das vorliegende Studiendesign mit nur 10 Tieren pro Geschlecht pro Gruppe keine Differenzierung der zwischen den Gruppen auftretenden Unterschiede.

Historische Kontrolldaten werden nicht berücksichtigt

Überdies berücksichtigen die Autoren in keiner Weise die historischen Kontrolldaten, die für den verwendeten Rattenstamm vorliegen. Generell bewegt sich die zweijährige Versuchsdauer (Tiere sind zu Versuchsbeginn acht Wochen alt) im Bereich der durchschnittlichen Lebenserwartung von Laborratten wie dem Sprague-Dawley-Stamm (siehe beispielsweise Nakazawa *et al.*, 2001), was auch die vorliegende Studie bestätigt. Sprague-Dawley-Ratten weisen zudem eine relativ hohe Spontantumorrate auf, was in der verfügbaren Literatur gut dokumentiert ist (Suziki *et al.*, 1979; Nakazawa *et al.*, 2001; Tennekes *et al.*, 2004; Brix *et al.*, 2005; Dinse *et al.*, 2010). So fanden sich in Langzeitstudien mit unbehandelten Sprague-Dawley-Ratten Tumorinzidenzraten von 70-76,7 % für Männchen und 87-95,8 % für Weibchen (Nakazawa *et al.*, 2001). Die Tumorinzidenz steigt mit zunehmendem Alter der Tiere an, allerdings ist auch für junge Tiere das spontane Auftreten von Tumoren dokumentiert. So beschreiben Kuzutani *et al.* (2012) das spontane Auftreten eines Mamma-Adenokarzinoms bei einem 12 Wochen alten Sprague-Dawley-Weibchen. Unbehandelte weibliche Sprague-Dawley-Ratten weisen (insbesondere bei *ad-libitum*-Fütterung) vor allem eine hohe spontane Tumorrate für Mammakarzinome auf (Nakazawa *et al.*, 2001; Tennekes *et al.*, 2004; Brix *et al.*, 2005; Dinse *et al.*, 2010). Unbehandelte Sprague-Dawley-Ratten beiderlei Geschlechts weisen zudem hohe Spontantumorraten für Tumore der Hypophyse und der Nebennieren auf. Überdies sind Sprague-Dawley-Ratten suszeptibel für das Auftreten spontaner Tumore in einer Reihe weiterer Organe, darunter Leber und Pankreas. Vor diesem Hintergrund ist es aus der Sicht des BVL nicht überraschend, dass auch in der Langzeitstudie von Seralini *et al.* eine beträchtliche Anzahl von Tieren aus unterschiedlichen Gründen spontan oder altersbedingt erkranken und sterben, da dies bereits für unbehandelte Sprague-Dawley-Ratten beschrieben und demzufolge zu erwarten ist. Auch dass Seralini *et al.* in ihrer Studie vor allem Mamma- und Hypophysentumore finden, entspricht der hohen Inzidenz für diese beiden Tumorarten, die für unbehandelte Sprague-Dawley-Ratten charakteristisch ist. Die Verteilung der Todesfälle und Tumorraten auf die unterschiedlichen Behandlungsgruppen kann im Falle der Seralini-Studie zufällig sein, da - wie bereits zuvor ausgeführt - eine Gruppengröße von 10 Tieren pro Geschlecht zu gering ist, um einen Trend oder einen Effekt abzusichern (Vgl. mit international anerkannten Standards, die für derartige Studien eine Gruppengröße von 50 Tieren pro Geschlecht fordern).

5.2. Statistische Analyse und Darstellung der Ergebnisse

Fehlende statistische Analyse der Mortalitäts- und Tumordaten

In Bezug auf die Mortalität und die Tumorhäufigkeit zeigen Seralini *et al.* lediglich deskriptive Daten, die zudem in schwer verständlicher Form präsentiert werden. Eine hypothesenbasierte Auswertung, die untersucht, ob die bestehenden Unterschiede zwischen

den Gruppen zufallsbedingt oder statistisch signifikant sind, fehlt. Die Anzahl der Tumore wird kumuliert über alle Tiere einer Gruppe dargestellt, eine Bewertung des Anteils krebserkrankter Tiere in der Gruppe ist damit unmöglich,und die Daten sind einer sinnvollen statistischen Auswertung nicht zugänglich.

Für eine statistische Auswertung der Mortalität der Versuchstiere hat das BVL Werte aus Abbildung 1 der Publikation entnommen. Diese sind zugleich die einzigen zugänglichen Rohdaten, die für eine weitere Analyse genutzt werden können. Die aus der genannten Abbildung gewonnen Daten sind nach Ansicht des BVL geeignet, um eine statistische Auswertung von ausreichender Aussagekraft zu erhalten. Diese Daten für die Mortalität der Versuchstiere hat das BVL einer Kaplan-Meier Analyse zur Schätzung der Überlebensfunktion unterzogen (Anlage 1).
Diese Analyse der Mortalität in den einzelnen Behandlungsgruppen zeigte im Ergebnis, dass keine Unterschiede zwischen den Gruppen nachweisbar sind. Ursache für die fehlende Signifikanz sind zu geringe Unterschiede zwischen den Gruppen bezogen auf die geringe Gruppengröße.
Mögliche Auswirkungen der Behandlung auf die Mortalität können damit nicht postuliert werden.

Selektive Darstellung biochemischer Parameter mit einer wenig bekannten statistischen Methode, die hochgradig abgeleitete Ergebnisse präsentiert

Die Darstellung der Daten zur Analyse der biochemischen Parameter ist schwer nachvollziehbar und die Ergebnisse lassen sich nicht bewerten. Nach Aussage der Autoren wurden jeweils 47 Blut- und Urinwerte zu 11 Zeitpunkten für die Tiere beider Geschlechter der 10 Gruppen erhoben. In der Studie präsentiert wurden jedoch nur die Blut und Urinwerte einer Behandlungsgruppe (33% NK603 Mais) zu einem Zeitpunkt (15 Monate) für ein Geschlecht (weiblich) im Vergleich zur zugehörigen Kontrollgruppe. Dies entspricht der Auswahl von etwa 1% der Daten für die Darstellung und Auswertung der Ergebnisse biochemischer Parameter. Selbst für diese ausgewählten Vergleichsgruppen wurden weder Rohdaten noch beschreibende Statistiken (Mittelwert, Extreme, Standardabweichung) präsentiert. Einen Teil der Rohdaten wären möglicherweise aus Abbildung 5B zu entnehmen, wenn eine geeignete Achsenbeschriftung verfügbar wäre. Im Übrigen ist zu erwähnen, dass die Verbindung der Werte einzelner Tiere zu einer Kurve in Abbildung 5B keinen Sinn ergibt und damit irreführend ist.
Stattdessen werden Ergebnisse der relativ neuartigen Methode der Diskriminanzanalyse präsentiert, ohne deren Anwendung ausreichend zu rechtfertigen. Diese Methode wird üblicherweise dazu eingesetzt, Strukturen und relevante Parameter in sehr umfangreichen Datensätzen mit vielen gemessenen Parametern zu identifizieren. Sie setzt jedoch voraus,

dass zu jedem gemessenen Parameter viele Messpunkte verfügbar sind. Im vorliegenden Fall stehen aber 47 Parametern nur je 10 Messwerte gegenüber, was die Anwendung der Methode problematisch macht und zumindest eine äußerst vorsichtige Interpretation der Ergebnisse erfordert. Die Ergebnisse einer Diskriminanzanalyse zeigen lediglich, welche der gemessen Parameter am besten geeignet sind, zwischen den Gruppen zu differenzieren, d.h. die welche Parameter am besten geeignet wären, die Zugehörigkeit zu einer bestimmten Gruppe zu prognostizieren. Eine Nachweis, ob für den jeweiligen Parameter tatsächlich ein signifikanter Unterschied zwischen den Gruppen vorliegt, ist damit nicht verbunden.
Die Autoren kommen auf Basis der Diskriminanzanalyse zu dem Schluss, dass verschiedene Parameter, die im Zusammenhang mit der NaCl Ausscheidung stehen, geeignet sind, zwischen der Kontroll- und der Behandlungsgruppe zu unterscheiden. Der naheliegende Test, ob Unterschiede zwischen den Gruppen in diesen Parametern statistisch nachweisbar sind, erfolgt jedoch nicht. Abbildung 5B deutet dagegen eher darauf hin, dass für die NaCl bezogenen Parameter keine signifikanten Unterschiede zu erwarten sind, da nahezu alle Werte innerhalb eines Intervalls von +/- 2 Standardabweichungen (entsprich etwa 95% Vertrauensintervall) liegen. Die Autoren diskutieren die biochemischen Daten auch nicht vor dem Hintergrund der natürlichen Variabilität der untersuchten Parameter. So könnte etwa ein Vergleich der absoluten Werte der Parameter, die im Zusammenhang mit der NaCl Ausscheidung stehen, mit historischen Kontrolldaten auf einfache Weise darüber Klarheit verschaffen, ob gefundene Unterschiede klinisch bedeutsam sind oder sich innerhalb der natürlichen Schwankungsbreite für eine gesunde Ratte befinden.
Aus den präsentierten Daten Hinweise auf mögliche Nierenschädigungen in der untersuchten Behandlungsgruppe zum untersuchten Zeitpunkt abzuleiten, ist nach Ansicht des BVL wissenschaftlich nicht haltbar. Noch weniger begründet ist damit die Postulierung möglicher Nierenschädigungen in anderen Behandlungsgruppen oder zu anderen Messzeitpunkten und durch NK603 Mais oder glyphosathaltige Pflanzenschutzmitteln im Allgemeinen.

Die Autoren präsentieren weiterhin Daten zu Testosteron- und Östradiolspiegeln von weiblichen Ratten der 33 % Mais NK603-Gruppe, die zum Zeitpunkt 15 Monate bestimmt wurden, im Vergleich zur Kontrollgruppe (dargestellt sind die Hormonspiegel von Einzeltieren). In diesem Zusammenhang berücksichtigen oder diskutieren die Autoren in keiner Weise die natürliche Schwankungsbreite der Hormonspiegel, die charakteristisch für den weiblichen Sexualzyklus ist. Da nicht davon auszugehen ist, dass die untersuchten Tiere alle denselben Zyklusstatus hatten und eine Östrusbestimmung unterblieben ist, sind starke Schwankungen für die beiden gemessenen Hormonspiegel zwischen Einzeltieren nicht unerwartet, was gerade in Anbetracht der geringen Gruppengröße von Bedeutung ist. Im Übrigen gilt wiederum, dass die Tatsache, dass nahezu alle Messwerte innerhalb eines

Intervalls von +/- 2 Standardabweichungen (entsprich etwa 95% Vertrauensintervall) liegen, gegen die Annahme signifikanter Unterschiede zwischen den Gruppen spricht.

Das BVL weist darauf hin, dass der laut Veröffentlichung erhobene Datensatz sehr umfangreich ist und daher grundsätzlich nach einer geeigneten statistischen Analyse für eine toxikologische Bewertung geeignet wäre. Für eine entsprechende Analyse zu Zeitpunkten bis zu 12 Monaten ist auch die Gruppengröße von 10 Tieren je Geschlecht ausreichend und den OECD Richtlinien entsprechend.
Da eine entsprechend fundierte Auswertung der Daten in der Veröffentlichung nicht durchgeführt wurde, hat das BVL die Autoren gebeten, weitere Daten für eine entsprechende Analyse zur Verfügung zu stellen.

<u>Bewertung der Fütterungsstudie hinsichtlich der Dosierungen der Versuchstiere mit Glyphosat-Formulierungen über das Trinkwasser</u>
Eingesetzt wurde das Pflanzenschutzmittel „Roundup GT Plus“, welches u. a. in Belgien und Frankreich zugelassen war/ist. Gemäß eigener Recherche war dieses Herbizid zur Zeit der Versuchsdurchführung mit POE-Tallowaminen als Netzmittel formuliert.
Es wurden drei Konzentrationen verabreicht, die, so die Autoren, Folgendem entsprechen würden:

1. **50 ng/L:** den Glyphosatmengen in einigen Leitungswässern
2. **400 mg/L:** dem in den U.S.A. zulässigen Rückstandshöchstgehalt an Glyphosat in Futter aus glyphosatresistenen Pflanzenmaterial,
3. **2,25 g/L:** einer halben minimalen praxisgerechten Spritzbrühenkonzentration

Diese Konzentrationen im Trinkwasser der Ratten werden in Prozent angegeben. Die tatsächlich verwendeten Mengen wurden zwar massenspektroskopisch bestimmt, jedoch nicht berichtet. Die Verhältnismäßigkeit dieser gewählten Basiswerte für die durchgeführten Untersuchungen wird wie folgt kommentiert:

Zu 1.
Die Angabe von Befunden von 50 ng/L Glyphosat in Leitungswasser ist pauschal. Die Herkunft dieses Wertes bleibt in der Studie unbekannt.
Mit einem Auftreten von Glyphosat im Trinkwasser/Leitungswasser ist in Deutschland nicht zu rechnen. Somit ist die Bevölkerung über diesen Aufnahmeweg nicht exponiert. In Untersuchungen der Überwachungsbehörden wird Glyphosat im Grundwasser nur in Ausnahmefällen gefunden. Ein Auftreten ist auch sehr unwahrscheinlich, da der Wirkstoff gut sorbiert, also an Bodenpartikel gebunden wird. Damit ist aus Grundwasser gewonnenes Trinkwasser gut vor Glyphosateinträgen geschützt. In Oberflächengewässern wird der

Wirkstoff häufiger nachgewiesen. Diese Einträge stammen z. B. aus der Abschwemmung von befestigten Flächen in Oberflächengewässer. Bei der Gewinnung von Trinkwasser aus Oberflächenwasser sind jedoch verschiedene Verfahren wie die Uferfiltration, die Grundwasseranreicherung über die Versickerung von Oberflächenwasser oder die Aufbereitung des Rohwassers in den Wasserwerken (z. B. Aktivkohlefiltration) zwischengeschaltet. Der Wasserversorger muss gemäß Trinkwasserverordnung gewährleisten, dass Pflanzenschutzmittelwirkstoffe den Trinkwassergrenzwert von 0,1 µg/l nicht überschreiten.

Zu 2.
Zur überhöhten Exposition auf Basis des zulässigen Rückstandshöchstgehaltes (RHG) für Glyphosat im verfütterten gentechnisch veränderten Mais NK603 ist folgendes zu bemerken:

Der CODEX – MRL für Glyphosat wurde für Mais auf 500 mg/kg festgelegt. Dieser Wert gilt insgesamt für Maiskorn und Grünmais (Frischfutter und Silagemais) und zwar für konventionelle und gentechnisch veränderte, Glyphosat resistente Maislinien. Abgeleitet wurde der verhältnismäßig hohe RHG offensichtlich aus Rückstandsdaten, die in den U.S.A. an kompletten Grünmaispflanzen erarbeitet wurden. Die vorliegenden Daten zu trockenem Körnermais aus den U.S.A. und aus EU-Staaten erreichen aber nach mehrfacher Anwendung überhöhter Aufwandmengen im Vergleich zu zugelassenen Wirkstoffaufwandmengen im Lauf der Vegetationsdauer der Maispflanzen nur Rückstandswerte, die dem aus den EU-Daten abgeleiteten zulässigen Rückstandshöchstgehalt von 1 mg/kg für Maiskorn entsprechen. Für Grünmais als Futtermittel ist gemäß Verordnung (EU) Nr. 396/2005 kein RHG festgelegt.
Gefüttert wurden die Ratten jedoch mit einem maiskornhaltigen Mischfutter, das eine nicht berichtete Rückstandsmenge an Glyphosat nach Anwendung des in den U.S.A. zugelassenen Mittels „WeatherMAX“ enthielt.

Zu 3.
Die gewählte Spritzbrühenkonzentration kann sich exemplarisch auf die praxisgerechte Anwendung von „Durano“ beziehen. Dieses Mittel wird in Getreide zwecks Sikkation 14 Tage vor der Ernte in der Menge von 5 L/ha in 100 – 400 L Wasser, entsprechend 1800 g/ha Glyphosat angewendet. Daraus ergibt sich die halbe Minimalkonzentration der Spritzbrühe von 2,25 g/L Glyphosat.
Anwender, Arbeiter und Nebenstehende sind ggf. während bzw. nach der Ausbringung der betreffenden Pflanzenschutzmittel ausschließlich dermal und inhalativ gegenüber der Spritzbrühe, dem Spritznebel sowie dem Spritzbelag auf den behandelten Pflanzen exponiert. Hierbei handelt es sich um kurzzeitige Ereignisse und nicht um lebenslange

Dauerbelastungen. Der Anwender als ungünstigster Fall, ist nach Abschätzung mit dem deutschen Modell nur zu einem tausensten Teil beim Ansetzen der Spritzbrühe und während der Ausbringung exponiert. Die Exposition ist damit niemals so hoch, wie im beschriebenen Rattenversuch, in dem die Tiere der angegebenen unverhältnismäßig hohen Konzentration im Trinkwasser, also der oralen Mittelaufnahme ausgesetzt waren.

Die tatsächlich von den Ratten aufgenommenen Glyphosatmengen mit dem Futter und mit dem Trinkwasser werden nicht berichtet. Dies ist wegen der Art der Versuchsanstellung auch nicht möglich, da einerseits zwei Ratten pro Käfig gehalten wurden und die tatsächlich aufgenommenen Wirkstoffmengen nicht berichtet werden. Weiterhin gibt es keine Angaben über die Technik der Verabreichung (über Trinkflaschen?) und über Wasserverluste beim Trinken der Tiere.

Somit ist es dem Leser nicht möglich, eine klare Kausalitätsbeziehung zu den beobachteten Effekten zu erkennen. Aus den tabellarischen und graphischen Darstellungen der Ergebnisse sind dosisabhängige Todesfälle und Tumorbildungen daher nicht ableitbar. Tumorbildungen einschließlich Karzinome sind auf Grund der Erkenntnisse aus Langzeitstudien mit reinem Glyphosat nicht zu erwarten. Entsprechende Langzeiteffekte durch das enthaltene Tallowamin-haltige Netzmittel liegen den Behörden bisher nicht vor. Dieser Aspekt findet jedoch keine Beachtung in der aktuellen Studie.

Undifferenzierter Vergleich der Kontrolldaten gegen die Testgruppen

Vor allem hinsichtlich der Mortalitätsrate und der Tumorinzidenz bevorzugen die Autoren der Studie eine Angabe in Prozent, eines „Vielfachen“ (z. B. *x times more*) sowie der Nennung eines Maximalwertes (*up to*). Dabei wird die Kontrollgruppe oftmals undifferenziert gegen die Testgruppen verglichen, wobei für die Testgruppen der höchste gefundene Wert genannt wird. So heißt es beispielsweise, dass in der Kontrollgruppe 30 % der männlichen Ratten sterben, wohingegen in einigen Testgruppen bis zu 50 % der Männchen sterben. Allerdings wäre es nach dieser Darstellungsmethode anhand der vorliegenden Daten genauso richtig zu sagen, dass in der Kontrollgruppe 30 % der männlichen Ratten sterben, wohingegen in anderen Testgruppen nur 10 % der Männchen sterben. Nach Ansicht des BVL ist ein direkter und unabhängiger Vergleich der Kontrollgruppe gegen jede Behandlungsgruppe einzeln unentbehrlich. In diesem Zusammenhang ist auch zu bedenken, dass in der vorliegenden Studie völlig unterschiedliche Testmaterialien (gentechnisch veränderter Mais NK603 und Roundup-Formulierung) und somit unterschiedliche Fragen untersucht werden, die jeweils zunächst einer Einzelbetrachtung bedürfen.

Selektive und willkürliche Auswahl/Darstellung von Einzelbefunden

Die Autoren der Studie erheben im Verlauf der von ihnen durchgeführten zweijährigen Studie eine Vielzahl von Daten (Vgl. Anhang 1), von denen im Ergebnisteil aber nur eine sehr begrenzte und nach subjektiven Kriterien der Autoren bestimmte Auswahl an Ergebnissen gezeigt werden (*„All data cannot be shown in one report, and the most relevant are described here.“*). So werden willkürlich Messzeitpunkte und Behandlungsgruppen herausgegriffen (s. o.), zu denen die Daten präsentiert werden, ohne darauf einzugehen, welche Daten in den anderen Gruppen und oder zu den weiteren Messzeitpunkten erhoben wurden. Eine Interpretation solcher selektiv herausgezogenen Daten ohne Berücksichtigung der Datengesamtheit hält das BVL für nicht zulässig. Teilweise werden Einzelbefunde, die für nur eine oder wenige Behandlungsgruppen gefunden wurden, unzulässigerweise auf alle Behandlungsgruppen übertragen. Auch Befunde, die bei einzelnen Tieren einer Behandlungsgruppe detektiert wurden, werden in Form von Bildern/Fotos so dargestellt, dass der Eindruck erweckt wird, der Befund sei charakteristisch für alle Tiere der Gruppe. Die Präsentation der Daten in „%“ oder unter Nennung der Angabe „x-fach“ lässt die Ergebnisse in Anbetracht der geringen Tierzahlen beeindruckender erscheinen als bei einer Darstellung unter Nennung der Zahl der Fälle pro Gruppe.

Undifferenzierte Darstellung von Daten

Oftmals werden Daten unvollständig und/oder undifferenziert präsentiert. So fehlt bei den erwähnten pathologischen Befunden, die beispielsweise in Tabelle 2 nur aufgelistet werden, eine differenzierte Darstellung nach Organ, Effekt bzw. differentialdiagnostische Charakterisierung und Schweregrad. Auch zur Histopathologie werden nur exemplarische Bilder gezeigt, ohne eine komplette Histologie zu präsentieren und genau zu quantifizieren, wie oft man welchen Befund genau gefunden hat.

6. Diskussion

In der Diskussion der Ergebnisse werden von den Autoren verschiedene mögliche Wirkmechanismen angeführt, welche die Beobachtungen erklären sollen. Nach Ansicht des BVL ist eine solche Diskussion derzeit abzulehnen, da eine geeignete Grundlage fehlt. Vor einer Diskussion möglicher Wirkmechanismen müssten Auswirkungen der Behandlung auf die Versuchstiere wissenschaftlich fundiert gezeigt und statistisch abgesichert werden. Dies gelingt den Autoren in der vorliegenden Studie jedoch nicht.

Weder für die Mortalität noch für die Tumorhäufigkeit wurden von den Autoren signifikante Unterschiede zwischen den Gruppen gezeigt. Für die Mortalität konnte das BVL zeigen, dass dies auch nicht möglich ist. Für die Tumorhäufigkeit sind aufgrund der geringen Gruppengröße ähnliche Ergebnisse zu erwarten.
Hiervon ausgehend, verbietet sich eine weitere Diskussion möglicher Auswirkungen der Verfütterung von NK603 Mais oder Roundup auf Mortalität und Tumorhäufigkeit. Ebenso lässt sich nicht von einer fehlenden Dosis-Wirkungs-Beziehung sprechen, solange keine Wirkung nachgewiesen wurde. Damit wird auch der Schluss auf eine endokrin disruptive Wirkung sowie ein beträchtlicher Teil der vorgelegten Diskussion, welcher auf diesem Schluss basiert, gegenstandslos.
Ebenso fehlt die Grundlage für eine fundierte Diskussion möglicher toxischer Wirkungen auf Basis von biochemischen Daten, da entsprechende Daten nur zu einem sehr geringen Teil und zudem ungeeignet präsentiert wurden.

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Anhang 1

Material und Methoden

A) Test- und Kontrollmaterial

Als Testmaterial wurde der infolge gentechnischer Veränderung Glyphosat-tolerante Mais NK603 verwendet. Dabei wurde sowohl mit als auch ohne Roundup-behandeltes NK603-Material eingesetzt. Die Roundup-Behandlung des Feldes entsprach drei Litern der Formulierung Weather-MAX (540 g Glyphsat pro Liter) pro Hektar. Als Kontrollmaterial diente nah-isogener, nicht gentechnisch veränderter Mais, der nicht mit Roundup behandelt wurde. Die Produktion des Test- und Kontrollmaterials erfolgte am gleichen Ort. Die Identität und Reinheit der gentechnisch veränderten Saatkörner wurde mittels qPCR bestätigt. Eine Grenzwert überschreitende Kontaminierung mit Pestiziden lag nicht vor.

Die kommerzielle Roundup-Formulierung GT Plus (450 g Glyphosat pro Liter, Genehmigung 2020448, Monsanto, Belgien) wurde verwendet, um sie dem Trinkwasser beizumischen. Die Herbizidgehalte wurden durch Bestimmung des Glyphosatgehaltes in den unterschiedlichen Verdünnungen mittels Massenspektrometrie bestimmt.

B) Diäten

Insgesamt umfasste der Versuch neun Testbehandlungen und eine Kontrolle:

1) Diät mit 33 % nah-isogenem Mais (Kontrolle)
2) Diät mit 11 % Mais NK603 ohne Glyphosatbehandlung
3) Diät mit 22 % Mais NK603 ohne Glyphosatbehandlung
4) Diät mit 33 % Mais NK603 ohne Glyphosatbehandlung
5) Diät mit 11 % Mais NK603 mit Glyphosatbehandlung
6) Diät mit 22 % Mais NK603 mit Glyphosatbehandlung
7) Diät mit 33 % Mais NK603 mit Glyphosatbehandlung
8) Diät mit 33 % nah-isogenem Mais + Trinkwasser mit 50 ng/l „Roundup GT Plus“
9) Diät mit 33 % nah-isogenem Mais + Trinkwasser mit 400 mg/l „Roundup GT Plus“
10) Diät mit 33 % nah-isogenem Mais + Trinkwasser mit 2,25 g/l „Roundup GT Plus“

Die Diäten wurden auf der Grundlage des Standardfutters A04 (Safe, France) hergestellt. Laut Angaben der Autoren bestanden alle Futterformulierungen aus balancierten Diäten, deren substantielle Äquivalenz (mit Ausnahme des Transgens) chemisch gemessen wurde. Überdies lag keine Grenzwert überschreitende Kontaminierung mit Pestiziden vor. Die Konzentration des Transgens wurde für die drei Dosierungen jeder Diät mittels qPCR bestätigt.

C) Rattenstamm, Haltung und Studiendesign

Durchgeführt wurde eine 2 Jahre-Rattenfütterungsstudie. Die Autoren verweisen in diesem Zusammenhang auf die OECD-Richtlinie 408 (*„Repeated Dose 90-day Oral Toxicity Study in Rodents“*), auf deren Grundlage die Herstellerfirmen üblicherweise Fütterungsversuche mit gentechnisch veränderten Organismen (GVO) durchführen. Hierauf Bezug nehmend verweisen die Autoren darauf, dass sie in ihrer Langzeitstudie Daten zu mehr Parametern und häufiger als in den standardmäßig durchgeführten Tests gemäß OECD-Richtlinie 408 erhoben haben.

Verwendet wurden männliche und weibliche Sprague-Dawley Albinoratten, die im Alter von fünf Wochen von Harlan (Gannat, France) bezogen wurden. Nach einer 20-tägigen Akklimationsphase wurden die Tiere zufällig auf die zehn Behandlungsgruppen verteilt (gewichtsbasiert). Pro Behandlungsgruppe waren 20 Tiere (je zehn männliche und zehn weibliche) enthalten, was einer Gesamtzahl von 200 Tieren entspricht. Die Verfütterung der Kontroll- und Testdiäten erfolgte für 730 Tage (= Versuchsende).

Die Haltung der Tiere fand bei 22 ± 3 °C unter Einhaltung eines 12 h Hell/Dunkel-Zyklus statt. Pro Käfig wurden jeweils zwei Tiere eines Geschlechts gehalten. Futter und Wasser wurden *ad libitum* verabreicht. Das mit „Roundup GT Plus“ versetzte Trinkwasser der Behandlungsgruppen 8-10 wurde wöchentlich gewechselt.

Die Tiere wurden zweimal wöchentlich eingehend beobachtet und abgetastet, um klinische Zeichen, eventuell vorhandene Tumore, Futter- und Wasseraufnahme sowie das individuelle Körpergewicht zu bestimmen.

D) Biochemische Analysen

Zu den Zeitpunkten 1, 2, 3, 6, 9, 12, 15, 18 21 und 24 Monaten wurden Blutproben aus der Schwanzvene unter Isofluranbetäubung entnommen. Nach Standardmethoden wurden 31 Parameter untersucht, darunter: Hämatologie- und Koagulationsparameter, Albumin, Globulin, Gesamtproteinkonzentration, Kreatinin, Harnstoff, Calcium, Natrium, Kalium, Chlorid, anorganischer Phosphor, Triglyceride, Glukose, Gesamtcholesterin, Alaninaminotransferase, Aspartataminotransferase, gamma-Glutamyltransferase, Östradiol und Testosteron. Überdies wurde nach 12 und nach 24 Monaten das C-reaktive Protein bestimmt. Zu elf Zeitpunkten (ähnlich denen der Blutentnahme) wurden Urinproben über 24 h in metabolischen Käfigen gesammelt. Auf deren Grundlage wurden 16 Parameter quantifiziert, darunter: Kreatinin, Phosphor, Kalium, Chlorid, Natrium, Calcium, ph-Wert und *Clearance*. Leberproben, die zum Versuchsende genommen wurden, ermöglichten überdies die Durchführung von *Assays* der CYP1A1-, 1A2-, 3A4 und 2C9-Aktivitäten mit Glutathion-S-Transferase und gamma-Glutamyltransferase.

E) Pathologie

Bei Versuchsende, bzw. wenn Tiere aus ethischen Gründen vorzeitig euthanisiert werden mussten, wurden folgende Organe entnommen: Gehirn, Kolon, Herz, Nieren, Leber, Lunge, Ovarien, Milz, Hoden, Nebennieren, Nebenhoden, Prostata, Thymus, Uterus, Aorta, Blase, Knochen, Duodenum, Ösophagus, Augen, Ileum, Jejunum, Lymphknoten, Lymphoretikuläres System, Brustdrüsen, Pankreas, Nebenschilddrüse, Peyer-Plaques, Hypophyse., Speicheldrüsen, Ischiasnerv, Haut, Rückenmark, Magen, Schilddrüse und Trachea. Von einem Großteil der genommenen Organe wurden Paraffinschnitte gefertigt, die einer HES (Hämatoxylin-Eosin-Safran) Färbung unterzogen wurden. Von Leber, Nieren und Tumoren wurden Schnitte für die Transmissionselektronenmikroskopie gefertigt.

F) Statistik

Neuere statistische Methoden zur multiplen Regression wurden genutzt, um Beziehungen zwischen den Ergebnissen biochemischer Parameter und den einzelnen Behandlungsstufen zu identifizieren. Es handelt sich hierbei um Methoden der explorativen Statistik, sie werden genutzt, um Strukturen und mögliche Korrelationen innerhalb komplexer Datensätze zu identifizieren. Ein Nachweis ursächlicher Zusammenhänge ist mit diesen Methoden grundsätzlich nicht möglich und die Ergebnisse sollten demzufolge auch nicht so interpretiert werden.

In Bezug auf die Mortalität und Tumorhäufigkeit wurde lediglich eine beschreibende Statistik (%-Anteile) zum Ende der Lebensdauer präsentiert bzw. kumulierte Häufigkeiten graphisch dargestellt (schwer lesbar). Statistische Tests, die signifikante Unterschiede zwischen Behandlungsgruppen zeigen könnten, wurden nicht präsentiert.

Anhang 2

Sachstand/Mais NK603

NK603 ist ein herbizidtoleranter Mais. Die Herbizidtoleranz wird durch das aus dem Bodenbakterium *Agrobacterium tumefaciens* stammende *epsps*-Gen vermittelt, dessen Genprodukt eine Rolle in der Synthese aromatischer Metabolite spielt. Pflanzen besitzen ein eigenes EPSPS-Protein, das jedoch durch den herbiziden Wirkstoff Glyphosat gehemmt wird. Das vom bakteriellen Gen kodierte EPSPS-Protein ist jedoch unempfindlich gegen Glyphosat. Mais NK603 kann daher Herbizidapplikationen mit Glyphosat (Markenname Roundup) schadlos überstehen, während Beikräuter absterben.

Mais NK603 besitzt gemäß Richtlinie 2001/18/EG eine Genehmigung zum Inverkehrbringen für Import und Verarbeitung in der EU (C/ES/00/01; Kommissionsentscheidung 2004/643/EC v. 19.07.2004). Ferner besitzen aus Mais NK603 gewonnene Lebensmittel und Lebensmittelzutaten eine Genehmigung als neuartige Lebensmittel gemäß der Verordnung (EG) 258/97 (Kommissionsentscheidung 2005/448/EG v. 03.03.2005).

Für Hybriden, welche Mais NK603 als Elter enthalten, existieren folgende Zulassungen:

- MON863xMON810xNK603, KOM-Entscheidung v. 02.03.2010 (2010/139/EU)
- MON863xNK603, KOM-Entscheidung v. 02.03.2010 (2010/141/EU)
- NK603xMON810, KOM-Entscheidung v. 14.10.2007 (2007/701/EG)
- 1507xNK603, KOM-Entscheidung v. 24.10.2007 (2007/703/EG)

Zu folgenden zum Inverkehrbringen nach Verordnung (EG)1829/2003 beantragten Hybriden sowie zum Antrag auf Anbau von NK603 liegen befürwortende Stellungnahmen der EFSA vor:

- 1507xMON89034xNK603 (EFSA-GMO-NL-2009-65), EFSA-Opinion v. 08.09.2010
- MON89034xNK603 (EFSA-GMO-NL-2007-38), EFSA-Opinion v. 09.09.2009
- 59122x1507xNK603 (EFSA-GMO-UK-2005-21), EFSA-Opinion v. 03.04.2009
- 59122xNK603 (EFSA-GMO-UK-2005-20), EFSA-Opinion v. 19.11.2008
- NK603 (EFSA-GMO-NL-2005-22, Anbau), EFSA-Opinion v. 27.05.2009

Stellungnahmen des BVL:

Das damals zuständige Robert-Koch-Institut und die ZKBS gab mit Datum 21.03.2003 eine befürwortende Stellungnahme zum ersten Antrag auf Inverkehrbringen von C/ES/00/01 als Lebens- und Futtermittel ab. In der toxikologischen Prüfung wurden damals folgende Untersuchungen berücksichtigt: Stabilität von EPSPS-Protein in simulierter Säuger Gastrointestinalflüssigkeit, eine Akut-Toxizität Prüfung an Mäusen (Limit-Test mit einer Dosierung), ein Datenbankabgleich mit bekannten Toxinen und pharmakologisch aktiven Substanzen, eine subchronische Fütterungsstudie mit den auch bei Seralini et al (2012) verwendeten Sprague-Dawley Ratten (90-Tage Rattenfütterungsstudie) mit einem Anteil von 33% Mais im Futter sowie eine Hühner-Fütterungsstudie für 42 Tage mit 65% Maisanteil im Futter. Schädliche Auswirkungen konnten nicht festgestellt werden. Zu einem entsprechenden Schluss kam auch die ZKBS mit Beschluss v. 27.03.2003.

Auch in den weiteren oben genannten Anträgen zum Inverkehrbringen von Hybriden sowie dem Antrag auf Anbau nach Verordnung (EG) 1829/2003 ergaben sich keine zusätzlichen Daten, die die bisherige toxikologische Bewertung von NK603 in Frage stellten. So liegen aus dem Antrag auf Anbau (EFSA-GMO-NL-2005-22) auch weitere Fütterungsversuche mit Ochsen, Milchkühen und Mastschweinen ohne negative Befunde vor.

Anlage 1

Statistische Analyse der Mortalität von Ratten in Seralini *et al.* 2012 nach der Kaplan-Meier Methode

Aus Abbildung 1 (Seralini *et al.* 2012) wurden Werte für die Überlebenszeit der Versuchstiere mit Hilfe grafischer Methoden entnommen. Wegen der Qualität der Grafik konnten diese Werte nur näherungsweise ermittelt werden. Aufgrund übereinstimmender Mittelwerte und Standardfehler für die Kontroll- und Behandlungsgruppen in Seralini *et al.* bzw. den vom BVL ermittelten Daten (701 +/- 20 Tage in Seralini *et al.* 2012 vs. 700 +/- 20 Tage bei BVL für weibliche Tiere bzw. 624 +/- 21 Tage in Seralini *et al.* 2012 vs. 623 +/-20 Tage bei BVL für männliche Tiere), kann jedoch von einer ausreichenden Übereinstimmung der entnommenen Werte mit den tatsächlichen Werten ausgegangen werden.
Diese Werte wurden einer Kaplan-Meier Analyse zur Schätzung der Überlebensfunktion unterzogen. Es handelt sich um einen nichtparametrischen Test der Überlebensfunktion, welche häufig im Rahmen von klinischen Studien genutzt wird, um Überlebenszeiten in verschiedenen Patientengruppen zu vergleichen. Dabei können auch rechts-zensierte Daten, d.h. Überlebenszeiten, welche bis über das Ende des Beobachtungszeitraums hinaus gehen, verarbeitet werden. Die Methode scheint daher geeignet, die vorliegenden Daten zu analysieren.

Männliche Ratten

Der Mittelwert der Überlebensdauer liegt für männliche Ratten der Kontrollgruppe mit 623 Tagen knapp über dem Gesamtmittel von 620 Tagen. Die höchste durchschnittliche Überlebensdauer wurde bei der Gruppe mit dem höchsten GVO Anteil (33%) im Futter gefunden, knapp gefolgt von der Gruppe mit dem höchsten Roundup Gehalt (2,25g/L) im Trinkwasser.
Die durchschnittliche Überlebensdauer liegt für die beste Gruppe ca. 10 % über, für die schlechteste ca. 14 % unter der der Kontrollgruppe.

Mittelwerte für die Überlebenszeit

Gruppe	Mittelwert(a)				
			95%-Konfidenzintervall		
			Untere	Obere	
	Schätzer	Standardfehler	Grenze	Grenze	%
GMO 33	682,50	16,48	650,20	714,80	109,64
C	681,00	16,86	647,95	714,05	109,40
GMO 22	654,00	23,30	608,33	699,67	105,06
B	642,00	26,78	589,51	694,49	103,13

A	639,50	19,41	601,46	677,54	102,73
Kontrolle	**622,50**	**20,12**	**583,07**	**661,93**	**100,00**
GMO 33 +R	617,00	21,89	574,10	659,90	99,12
GMO 11 +R	586,00	44,83	498,14	673,86	94,14
GMO11	540,00	58,80	424,75	655,25	86,75
GMO 22 +R	537,50	60,42	419,08	655,92	86,35
Gesamt	620,20	12,06	596,56	643,84	99,63

Tabelle 1

Ein Gesamtvergleich über alle Gruppen mit verschiedenen Teststatistiken deutet darauf hin, dass kein Unterschied zwischen den Gruppen nachweisbar ist.

Gesamtvergleiche **männlich**

	Chi-Quadrat	Freiheitsgrade	Sig.
Log Rank (Mantel-Cox)	8,88903	9	0,448
Breslow (Generalized Wilcoxon)	10,58382	9	0,305
Tarone-Ware	9,47365	9	0,395

Test auf Gleichheit der Überlebensverteilungen für die verschiedenen Stufen von Gruppe.

Tabelle 2

Dennoch wurde jeweils ein paarweiser Vergleich zwischen der Kontrollgruppe und den Behandlungsgruppen durchgeführt, um mögliche signifikante Unterschiede auf diesem Niveau zu erfassen. Der paarweise Log-Rank Test auf Unterschiede in den Überlebensfunktionen zeigt zunächst, dass das Signifikanzniveau von 5 % bei zwei Gruppen knapp unterschritten wird. Daraus ergäbe sich eine signifikant **höhere** Lebenserwartung der beiden Gruppen mit maximalem GVO (33%) bzw. Roundup (2,25g/L) Anteil im Futter/Trinkwasser gegenüber der Kontrollgruppe. Um die Gesamt-Irrtumswahrscheinlichkeit unter Kontrolle zu halten, wurde eine Bonferroni bzw. Holm-Bonferroni Korrektur der Signifikanzniveaus durchgeführt. Diese Korrekturen werden eingesetzt, um bei einer großen Anzahl von Vergleichen die Häufigkeit falsch positive Befunde zu begrenzen.

Paarweise Vergleiche zur Kontrolle **männlich**		Log Rank (Mantel-Cox)		
	Chi-Quadrat	Sig.	Bonferroni korr. Sig.	Holm-Bonferroni korr. Sig.
GMO 33	3,971	**0,046***	0,417	0,417
C	3,885	**0,049***	0,439	0,390
GMO 22	1,620	0,203	1,828	1,422
B	1,283	0,257	2,315	1,544
GMO 11 +R	0,383	0,536	4,825	2,681
A	0,252	0,616	5,540	2,462
GMO 33 +R	0,087	0,767	6,907	2,302
GMO 22 +R	0,006	0,936	8,427	1,873
GMO 11	0,002	0,964	8,676	0,964

Tabelle 3

Die korrigierten Signifikanzniveaus zeigen, dass sich in keinem Fall Unterschiede zwischen den Gruppen nachweisen lassen.

Weibliche Ratten

Der Mittelwert der Überlebensdauer liegt für weibliche Ratten der Kontrollgruppe mit 700 Tagen ca. 50 Tage über dem Gesamtmittel von 651 Tagen. Die geringste durchschnittliche Überlebensdauer wurde bei der Gruppe mit dem GVO Anteil von 22% im Futter gefunden. Die durchschnittliche Überlebensdauer liegt für die schlechteste Behandlungsgruppe ca. 17% unter der der Kontrollgruppe

Mittelwerte für die Überlebenszeit					
Gruppe	Mittelwert(a)				
			95%-Konfidenzintervall		
			Untere	Obere	
	Schätzer	Standardfehler	Grenze	Grenze	%
Kontrolle	**700,00**	**19,80**	**661,19**	**738,81**	**100,0**
GMO 11	697,50	11,30	675,36	719,64	99,6
GMO 33 +R	676,50	21,40	634,55	718,45	96,6
GMO 11 +R	668,50	27,00	615,58	721,42	95,5
C	646,50	33,96	579,93	713,07	92,4
B	644,00	39,12	567,32	720,68	92,0
GMO 22 +R	638,00	23,61	591,72	684,28	91,1
A	635,00	38,73	559,09	710,91	90,7
GMO 33	623,50	42,29	540,61	706,39	89,1
GMO 22	583,00	45,39	494,04	671,96	83,3
Gesamt	651,25	10,69	630,29	672,21	93,0

Tabelle 4.

Ein Gesamtvergleich über alle Gruppen mit verschiedenen Teststatistiken deutet darauf hin, dass kein Unterschied zwischen den Gruppen nachweisbar ist.

Gesamtvergleiche **weiblich**

	Chi-Quadrat	Freiheitsgrade	Sig.
Log Rank (Mantel-Cox)	7,62170	9	0,573
Breslow (Generalized Wilcoxon)	8,34624	9	0,500
Tarone-Ware	7,93615	9	0,541

Test auf Gleichheit der Überlebensverteilungen für die verschiedenen Stufen von Gruppe.

Tabelle 5.

Dennoch wurde jeweils ein paarweiser Vergleich zwischen der Kontrollgruppe und den Behandlungsgruppen durchgeführt, um mögliche signifikante Unterschiede auf diesem Niveau zu erfassen. Der paarweise Log-Rank Test auf signifikante Unterschiede in den Überlebensfunktionen zeigt zunächst, dass das Signifikanzniveau von 5 % bei drei Gruppen knapp unterschritten wird. Daraus ergäbe sich eine signifikant **geringere** Lebenserwartung der Gruppen mit der geringsten Roundup-Konzentration im Trinkwasser (50ng/L) sowie mit 22% GVO bzw. 22% Roundup-behandeltem GVO im Futter gegenüber der Kontrollgruppe. Um die Gesamt-Irrtumswahrscheinlichkeit unter Kontrolle zu halten, wurde eine Bonferroni bzw. Holm-Bonferroni Korrektur der Signifikanzniveaus durchgeführt.

Paarweise Vergleiche

zur Kontrollgruppe

weiblich		Log Rank (Mantel-Cox)		
Gruppe	Chi-Quadrat	Sig.	Bonferroni korr. Sig.	Holm-Bonferroni korr. Sig.
GMO22R	4,472	**0,034***	0,310	0,310
A	4,224	**0,040***	0,359	0,319
GMO22	3,871	**0,049***	0,442	0,344
GMO33	3,062	0,080	0,721	0,481
C	1,823	0,177	1,593	0,885
GMO11R	1,693	0,193	1,739	0,773
B	1,548	0,213	1,920	0,640
GMO11	1,240	0,265	2,388	0,531
GMO33R	0,781	0,377	3,392	0,377

Tabelle 4

Die korrigierten Signifikanzniveaus zeigen, dass sich in keinem Fall Unterschiede zwischen den Gruppen nachweisen lassen.

Zusammenfassung

Insgesamt ist davon auszugehen, dass die Gruppengröße bzw. die beobachteten Unterschiede in den Überlebensfunktionen der einzelnen Gruppen zu gering sind, um mögliche Effekte der Behandlungen auf die Überlebensdauer statistisch absichern zu können.

Daher geht das BVL davon aus, dass die Aussage, Ratten, welche NK603 Mais im Futter bzw. Roundup im Trinkwasser erhalten haben, würden früher sterben, durch die Ergebnisse von Seralini *et al.* 2012 nicht gestützt wird.

Literatur

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Grafisch ermittelte Überlebenszeiten

Männliche Tiere

Tier	1	2	3	4	5	6	7	8	9	10
Cont	490	575	590	600	610	620	650	680	680	730
GMO33	580	625	640	650	690	720	730	730	730	730
GMO22	490	600	610	610	655	680	705	730	730	730
GMO11	100	425	430	500	505	600	650	730	730	730
GMO33R	440	590	600	625	630	630	640	655	665	695
GMO22R	125	345	345	545	590	630	640	695	730	730
GMO11R	295	445	480	495	630	640	685	730	730	730
A	545	590	590	600	610	630	665	705	730	730
B	505	540	545	580	680	690	690	730	730	730
C	580	620	630	650	695	715	730	730	730	730

Weibliche Tiere

Tier	1	2	3	4	5	6	7	8	9	10
Cont	540	620	730	730	730	730	730	730	730	730
GMO11	640	650	655	690	690	730	730	730	730	730
GMO22	290	470	480	510	530	630	730	730	730	730
GMO33	390	420	465	640	685	715	730	730	730	730
GMO11R	500	510	665	670	690	730	730	730	730	730
GMO22R	500	550	600	610	620	640	670	730	730	730
GMO33R	555	600	600	630	730	730	730	730	730	730
A	345	505	545	675	675	700	715	730	730	730
B	310	580	630	630	640	730	730	730	730	730
C	455	470	545	650	695	730	730	730	730	730

Anlage 2

Opinion of the Federal Institute for Risk Assessment (BfR)

Feeding study in rats with genetically modified NK603 maize and with a glyphosate containing formulation (Roundup) published by Séralini et al. (2012)

BfR-Opinion 037/2012 of 1 October 2012

In mid-September 2012, a scientific team headed by Gilles-Eric Séralini at the University of Caen in France published the results of a long-term study with rats which had been fed genetically modified glyphosate-tolerant NK603 maize. One part of the maize had been treated with a glyphosate-containing plant protection product (*Roundup*) during cultivation, whereas another part was untreated. In each case the maize was administered in three doses. In addition, other animals fed with conventional feed received *Roundup* via the drinking water, also in three doses. The only control group was fed a non-genetically modified maize. The authors reported that the animals in some of the test groups developed increased incidences of several tumours and other non-neoplastic lesions and died earlier than animals in the control group. The effects could have been caused by hormonal effects of *Roundup* and specific constituents of the genetically modified maize, respectively. The Federal Institute for Risk Assessment (BfR) has evaluated the study in terms of its relevance for the evaluation of the health risk of genetically modified glyphosate-tolerant maize NK603 and also with regard to the evaluation of the health risk of the glyphosate-containing formulation. On the basis of the publication, the BfR has come to the conclusion that the authors' main statements are not sufficiently corroborated by experimental evidence, due to deficiencies in the study design and in the presentation and interpretation of the study results. Therefore, the main conclusions of the authors are not supported by the presented incomplete data. The study does not comply with internationally recognised standards for long-term carcinogenicity studies. The rat strain used shows a relatively high spontaneous tumour rate, especially for mammary and pituitary tumours, and the number of animals used was too small and insufficient for assessing the claimed differences between the test groups and the control group. Also the authors' hypothesis that the observed effects could result from adverse effects on the endocrine system is not sufficiently supported by the data presented. Furthermore, the BfR criticises that the glyphosate dose administered was not determined in the studies with the glyphosate-containing plant protection product *Roundup*. Due to these deficiencies the BfR has asked the authors to provide the complete study report including the individual animal data. Moreover, it has asked specific questions in order to allow for a further evaluation of the reported effects.

1 Subject

This opinion refers to the study "*Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize*" published by Séralini and co-authors in the journal *Food and Chemical Toxicology* on 19 September 2012 (Séralini et al., 2012).

2 Result

After having reviewed the publication, the German Federal Institute for Risk Assessment (BfR) is of the opinion that the experimental data do not support the main statements in the publication. Further, due to shortcomings in the study design as well as in the presentation and interpretation of the data, relevant conclusions drawn by the authors are not comprehensible.

For further assessment, the BfR has asked the authors to provide the complete study report including the individual animal data and has also put forward specific questions. This request has not yet been answered.

3 Justification

Based on scientific opinions of the European Food Safety Authority (EFSA) (EFSA, 2003a; EFSA, 2003b), the NK603 maize that was used in the study was authorised for feed use in accordance with Directive 2001/18/EC on 19 July 2004 and for food use in accordance with Regulation (EC) No 258/97 on 10 October 2004. An application for renewal of these authorisations in accordance with Regulation (EC) No 1829/2003 was already assessed by EFSA (EFSA, 2009).

The active ingredient in the *Roundup* formulation used in the study was glyphosate which was included in Annex I of Directive 91/414/EEC in 2002 for a ten-year period based on Directive 2001/99/EG from 20.11.2001 (European Commission, 2002). The submitted data were evaluated in the monograph (Draft Assessment Report, DAR) by Germany as Rapporteur Member State (RMS) with the involvement of the BgVV, the predecessor of the BfR, in 1998. This comprehensive assessment in the DAR has been supplemented several times. Following a decision of the European Commission, the inclusion of glyphosate in Annex I was prolonged until 31 December 2015 with Directive 2010/77/EC from 10 November 2010. Currently, a renewal of the assessment of glyphosate is ongoing within the AIR2 programme based on Regulation (EC) No 1141/2010. Germany acts again as RMS and will, with involvement of the BfR (responsible for drafting the chapters on toxicology, residues and analytical methods), establish a new DAR that will be discussed in the framework of the Community centralised procedure led by EFSA. Numerous glyphosate containing plant protection products have been authorised in EU countries (currently 75 herbicides in Germany including 13 different *Roundup* formulations).

Beside studies on potential health effects from genetically modified NK603 maize the researchers led by Gilles-Eric Séralini had published a series of papers on effects of glyphosate containing plant protection products. Some of them (Richard et al., 2005; Benachour et al., 2007; Bellé et al., 2007; Gasnier et al., 2009; Benachour and Séralini, 2009) had been already commented by the BfR.

The aim of the now published study by Séralini and co-authors was to examine potential effects of the genetically modified glyphosate-tolerant NK603 maize and of one glyphosate containing formulation (*Roundup*) administered to rats over two years. Groups of 10 male and 10 female Sprague Dawley rats (Harlan) were fed diets containing 11, 22 or 33 per cent of NK603 maize (Monsanto Corporation, USA), which had been treated or not treated with *Roundup* during cultivation. The diet for the control group contained 33 per cent of a non-genetically modified maize line. The animals of another test series received drinking water containing 1.1×10^{-8}, 0,09 or 0,5 per cent *Roundup* .

The authors concluded from the results of the study that the mortality of female animals in all treated groups as well as male animals in three of the groups that had received NK603 maize was higher than in the control group and deaths occurred earlier. According to the authors, all results were hormone and sex dependent, and the pathological profiles were comparable. It was postulated that females developed more frequently large tumours of the mammary gland, and the pituitary was the second most affected organ. In treated males, pathologic lesions in the liver (congestions and necrosis) and kidney (severe nephropathies) were more frequent, the latter was confirmed by biochemical data. The authors further indicated that these results could be explained by non-linear endocrine-disrupting effects of *Roundup*, but also by overexpression of the transgene in the NK603 maize and its metabolic consequences.

BfR noticed with interest that for the first time a long-term feeding study was performed with a glyphosate containing formulation. Long-term studies were not yet available because for regulatory purposes such studies are worldwide requested only with the active substances. Glyphosate itself has been comprehensively tested. Numerous long-term studies in rats and mice showed no indications of either a carcinogenic potential or increased mortality or any effects on the endocrine system, as reported by Séralini and co-workers in their publication.

However, the BfR is aware of certain co-formulants, in particular surfactants from the group of polyethoxylated alkyl amines (POEA, often designated as tallow amines), that might affect the toxicity of glyphosate containing herbicides. The toxic effects are in some cases more severe compared to studies with the active ingredient. Therefore, the results of the study performed by Séralini's group could provide an experimental contribution to the elucidation of the possible influence of formulants on long-term effects of plant protection products.

While the performance of a long-term study in the case of the glyphosate containing formulation is in principle appreciated, it needs to be mentioned that the published study shows significant shortcomings in the study design and further shortcomings due to incomplete and unclear presentation of the collected data. Furthermore, the main statements were not supported by the experimental data. As outlined in detail below, it is therefore impossible to comprehend the main conclusions of the authors.

3.1 Comments on the study design

Long-term studies are highly complex and elaborate as rats spontaneously develop tumours and other age-related alterations. The published study was not conducted in accordance with internationally accepted standards, such as OECD Test Guidelines No. 451 or Nr. 453 (OECD, 2009a; OECD, 2009b). Instead, the authors have chosen a study design (OECD Test Guideline Nr. 408) that was developed for 90 day (subchronic toxicity) studies (OECD, 1998). Therefore, only 10 animals per sex instead of 50 have been assigned to each group.

However, subchronic studies show a substantially lower variation of age-related pathological changes between animals within a group while those changes are inevitable in long-term studies. As the published study has confirmed, the two-year duration of the study is of the order of the expected life span in rats including the Sprague Dawley strain that was used in the study. This strain, provided by the breeder Harlan, is known to develop spontaneous tumours, particularly mammary and pituitary tumours, at relatively high rates compared to other strains (Brix et al., 2005; Dinse et al., 2010). Therefore, it can be expected that a significant number of animals develop age-related illnesses or die for diverse reasons already during conduct of the study. The distribution of the cases of death between groups can be random, and a number of 10 animals per sex and group is too low to confirm a trend or an effect. Furthermore, no statements on statistically significant dose-response-relationships can be made. Larger sample sizes, as recommended for carcinogenicity studies in OECD Test Guidelines No. 451 or No. 453, would be required in order to allow precise statements with respect to the findings.

Regarding the design of the study, another point of criticism is that the mean levels of the daily applied doses of *Roundup* have not been determined. It should also be noted that the glyphosate containing formulation (Weather-MAX) used for the treatment of NK603 maize during cultivation was different from the formulation (GT Plus) used in the test series with *Roundup*. Further details on the composition of the applied formulations are lacking.

The publication does not inform whether the diets of all groups contained a total of 33 per cent maize, i.e. whether the diets with 11 and 22 per cent have been supplemented with non-genetically modified maize. The only information given by the authors is that balanced diets were fed and that these diets were considered "substantially equivalent" except for the newly introduced gene. However, detailed information on the composition of the diets is lacking. Moreover, data on feed and water consumption as well as body weight development are missing. The question therefore is, whether balanced diets really had been administered. There are also no further details on the identity of the control maize line that is referred to as "nearest isogenic non-transgenic control". Furthermore, it has to be critically stated that the maize varieties used in the study were not analysed for the presence of mycotoxins.

3.2 Comments on the presentation of results

The first part of the study considers mortality, tumour incidences and other pathological changes and contains descriptive data while statistical analyses are lacking. The presentation of the data in percentage terms or as “x times more”, suggest more impressive results compared to absolute figures.

The BfR is of the opinion that the treatment-related increase in mortality as reported by the authors is not confirmed by the published data. The two cases of death caused by Wilms’ tumours (nephroblastoma) in male animals of two not clearly indicated test groups fed with *Roundup* treated NK603 maize are not chemically induced and are correctly not claimed to be treatment-induced. Therefore, they should not be used as evidence for a higher mortality compared to the non treated control group. Likewise, no effects of *Roundup* on the mortality of male rats can be detected.

In female rats mammary tumours are indicated as the main cause of mortality. However, this type of tumour occurs rather frequently particularly in Sprague Dawley rats and if feed is offered *ad libitum*. In the current study this type of tumour also occurred in approximately 50 per cent of the animals in the control group. As outlined above, the number of animals is not sufficient for an assessment of the difference to the treated animals (60 to 100 per cent without a clear dose dependence). The reported comparison with historical control data published in 1992 is not acceptable.

The incomplete and undifferentiated presentation of the data makes evaluation very difficult. For example, it is absolutely insufficient to mention only findings in liver and the digestive tract, as done in table 2, without characterising them from a differential diagnostic standpoint and assessing the grade of severity. Further, the graphs demonstrating mortality and tumours, respectively, are not always in agreement with the statements in the text or can not be followed, as in the case of the observed deaths caused by Wilms' tumours.

A statistical analysis was only performed for the biochemical parameters. This was done with a special kind of principal component analysis (OPLS-DA = Orthogonal Partial Least Squares Discriminant Analysis), but results were only presented for one group (females that had received feed with 33 per cent NK603 maize compared to the control group). In addition, figure 5, presenting biochemical parameters, is difficult to understand. Their assessment would require data of all measurement time points.

3.3 Comments on the mechanisms suspected by the authors

One hypothesis of the authors was that specific compound(s) present in the genetically modified NK603 maize and in the applied glyphosate containing formulation, respectively, could

account for the observed increased tumour incidences, particularly in female test animals, by affecting the endocrine system. However, the BfR is of the opinion that no convincing arguments have been provided to support this. The following points are discussed.

- The authors indicate that most of the observed effects show a non-monotonic dose-response-relationship and show a threshold. They consider this as a clear indication that the endocrine system is adversely affected. The authors refer to a recent review published by Vandenberg et al. (2012). However, a detailed look into this paper reveals that its content is not correctly reflected by Séralini et al.. Vandenberg et al. explicitly question the existence of a threshold for adverse effects induced by endocrine disruptors. Thus the cited literature is not suitable to support the authors' claims. Furthermore, the presence of a non-monotonic dose-response- relationship does not mean that the effects are caused by an impairment of the endocrine system. Non-monotonic dose- response-relationships have also been described for other substances. For example some essential minerals show a non-monotonic dose-response-relationship (Stern, 2010; Calabrese, 2008) yet without affecting the endocrine system. Considering a non-monotonic dose-response-relationship, a quantitative relationship between the dose and the effect is observed which, however does not proceed in a monotonic manner over the examined dose range. Instead of a non-monotonic dose-response-relationship, the data presented does not allow the identification of any obvious relationship between the observed adverse effects and the applied dose. Rather, the datasets consisting of 3 dose levels and the control group with animal numbers <10 per group and sex show no statistically significant relationship between the observed effects and the applied dose.

- To further support their thesis the authors refer to their results obtained by measurement of testosterone and estradiol levels (figure 5B). The figure presents the data for hormone levels of the single female animals of the control group and the group, which had received a diet with 33 per cent NK603 maize, 15 months after the commencement of the study. A balanced scientific discussion should include a critical discussion of specific points by the authors. For example, statistically significant differences in hormone levels might easily be assessed on the basis of mean values plus standard deviation. However, figure 5B does not provide a clear basis to perform a statistical evaluation with sufficient accuracy. In addition, the respective data for male animals were not shown. Furthermore, the natural variation in hormone levels caused by the circadian rhythmic and during the estrous cycle was not acknowledged by the authors as a possible cause for the results given in figure 5. It is also known that Sprague Dawley rats develop estrous cycle abnormalities relatively early (from 4-6 months of age; OECD, 2009). The differences observed between treated and control animals 15 months after study begin could thus also be due to variations in hormone levels independent of the applied substances. If the authors were right in stating that the particularly higher incidence of mammary tumours could be related to the estradiol level, one would expect a statistically significant difference in the estradiol level of the female animals in the group, which had received a diet with 33 per cent NK603 maize, when compared to the control animals. However, this is not identifiable on the basis of the data presented.

- The authors also hypothesise that NK603 maize and *Roundup* could cause hormonal disturbances via an impact on the estrogen system. In this respect, the authors regard lower contents of specific organic acids (caffeic and ferulic acid) present in NK603 maize as being responsible for the observed effects. These acids are claimed to exert protective effects in the experimental animals and to impact on the estrogen

metabolism. However, significant differences in the estrogen levels of female animals in the group fed with 33 per cent NK603 maize can not be identified on the basis of the data presented. Additional factors, for example a possible modulation of the ER-receptor expression have not been addressed experimentally. Furthermore, the discussion of possible protective effects by plant constituents on tumour development does not reflect the current state of scientific knowledge. With regard to effects induced by the glyphosate containing formulation, the authors discuss the possibility of aromatase inhibition as well as an interaction with cellular estrogen or androgen receptors. However, these anticipated mechanisms have not been tested experimentally in this work. They are based on results from *in vitro* studies, which have been questioned by the BfR in previous opinions. The thesis of the authors that the observed effects could result from adverse effects on the endocrine system, exerted by the genetically modified NK603 maize and *Roundup*, respectively, are therefore not sufficiently supported by the experimental data presented in the publication.

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Anlage 3

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IHR ZEICHEN
IHRE NACHRICHT VOM

AKTENZEICHEN
(bitte bei Antwort angeben)

DATUM 11. October 2012

Publication in Food and Chemical Toxicology

Follow up to my email from September 27, 2012.

Dear Prof. Dr. Seralini,

The Federal Office of Consumer Protection and Food Safety (BVL) is the German national management authority for health-related consumer protection. Therefore, the paper "*Long term toxicity of Roundup herbicide and a Roundup-tolerant genetically modified maize*" published by you and co-authors in "*Food and Chemical Toxicology*" was perceived with high attention.

In order to conclude on any necessary management measures that might result form new findings in your study, we would kindly ask you to provide further information.

Our special interest focuses on the biochemical analysis, in which you collected 31 blood and 16 urine parameters at 11 times for 9 treatment groups and the respective controls. Unfortunately, only very little of this extensive dataset is accessible from your publication. We therefore wish to ask you to kindly provide us with at least descriptive statistics (mean, SD, min., max.) for all measured parameters at each point of time and each group. Additionally, appropriate test statistics would allow for the identification of any significant difference between treatment groups and conclusions on potential signs of toxicity.

Depending on the outcome of this analysis an investigation into individual animal and group histories might be necessary. We would therefore appreciate very much if raw data and anatomopathological data on an individual animal basis can be provided.

The BVL will use the provided raw data exclusively for the assessment of potential risks and decisions on appropriate management measures within the regulatory framework and ensures confidentiality.

Since the Federal Office of Consumer Protection and Food Safety strives for providing a maximum of health-related consumer protection, we highly appreciate your collaboration in this issue in order to rule out any potential hazard due to the use of the substances investigated in your publication.

With kind regards

Prof. Dr. Detlef Bartsch

DTU Fødevareinstituttets vurdering af nyt langtidsstudie med gensplejset majs NK603 og med sprøjtemidlet Roundup

DTU Fødevareinstituttet finder, at det nye studie ikke er designet korrekt, at der ikke gøres brug af den korrekte statistik, og til sidst at forfatterne ikke diskuterer deres data, som videnskabelig praksis foreskriver inden for toksikologi. DTU Fødevareinstituttet konkluderer, at artiklen er af ringe faglig kvalitet, og at den ikke burde have været publiceret i et peer-review tidsskrift.

Indledning

Det nye studie, der henvises til, fremgår af artiklen Séralini G, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, Spiroux de Vendômois J 2012: Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize som er accepteret til publikation i tidsskriftet Food and Chemical Toxicology.

Artiklen beskriver resultater fra et toårs fodringsforsøg med rotter af typen Sprague-Dawley (SD). Formålet er at vise effekter på rotter efter fodring med forskellige koncentrationer af den gensplejsede NK603 majs samt effekter på rotter efter tilsætning af Roundup i drikkevand. NK603 majs er gensplejset til at kunne tåle glyfosat, der er det aktive stof i ukrudtsmidlet Roundup. I forsøget indgår både NK603 majs, der har været sprøjtet med Roundup og NK603 majs, der ikke er sprøjtet med Roundup. Majsen NK603 er godkendt til konsum i EU samt en lang række andre lande.

I alt er anvendt 200 SD rotter til studiet, 100 af hvert køn, med opdeling i hold hver med 10 rotter. For hvert køn blev 3 hold fodret med NK603 majs i koncentrationer på hhv. 11, 22 og 33 % i foderet. En anden gruppe med 3 hold fik tre forskellige koncentrationer af Roundup i drikkevandet. Den tredje gruppe fik 3 forskellige koncentrationer (11, 22 og 33 %) af den gensplejsede NK603 majs der var sprøjtet med Roundup. Det sidste hold var kontrollen, hvor hverken GMO majs eller Roundup indgik i foderet.

Artiklen forsøger at vise, at flere rotter døde i de hold, der fik NK603 majs eller Roundup i drikkevand end i kontrolhold. Artiklens forsøger også at vise, at der var en højere forekomst af kræftknuder, og at rotterne døde tidligere i de hold, der var fodret med NK603 majs eller fodret med Roundup i drikkevandet, end kontrolholdet der blev fodret med traditionel majs, og som ikke fik Roundup i drikkevand.

DTU Fødevareinstituttet har som en af sine opgaver at foretage risikovurderinger og kommunikere resultater, også på området gensplejsede fødevarer. Det er vores vurdering, at artiklen ikke lever op til sædvanlig videnskabelig standard på dette område og således har givet anledning til at skabe unødig bekymring omkring effekter af gensplejset majs og sprøjtemidlet Roundup. Vi har derfor ment, at det vigtigt at fjerne misforståelser ved at påpege, at artiklen har metodiske fejl, der giver fejlagtige konklusioner.

DTU Fødevareinstituttets vurdering

DTU Fødevareinstituttet har grundigt gennemlæst artiklen og fundet en lang række problemstillinger, der gør det umuligt at drage konklusioner om effekter af hverken den gensplejsede majs eller Roundup. De data der præsenteres i artiklen giver således ikke grundlag for at ændre på tidligere vurderinger af hverken den gensplejsede majs NK603 eller det aktive stof glyfosat, der indgår i sprøjtemidlet Roundup.

De vigtigste indvendinger imod artiklen baserer sig på, at der anvendes for få dyr i hvert hold til at vise effekter, og at forsøget ikke lever op til de internationale accepterede retningslinjer for udførelse af langtidsforsøg. Der mangler basal statistik på dyreforsøget, og de statistiske metoder, som er blevet anvendt til dokumentation af de observerede biokemiske effekter, er ikke udført i overenstemmelse med de gældende OECD retningslinjer for statistisk afrapportering.

Forfatterne konkluderer, at der er en højere dødelighed og flere af tumorer i de behandlede dyr, men dødeligheden samt antal og typer af tumorer ligger i alle testhold indenfor den variation man ser i kontrolhold fra andre undersøgelser med Sprague-Dawley rotter.

De vurderinger om effekter af NK603 majs og Roundup, som forfatterne fremkommer med, er ikke i overensstemmelse med andre studier, hvor NK603 majs eller Roundup er undersøgt.

DTU Fødevareinstituttet finder det etisk uforsvarligt at lade dyrene gå så længe med tumorer uden at det bidrager til opnåelse af vigtige data.

DTU Fødevareinstituttet finder, at artiklen er af ringe faglig kvalitet, og at den ikke burde kunne publiceres i et peer-review tidsskrift.

Begrundelsen for denne vurdering er baseret på en række forhold i forsøgsdesign og i fortolkningen af resultater, som fremgår af artiklen og er nærmere beskrevet nedenfor.

Forsøgsdesign

- Der anvendes ikke en forsøgsprotokol, der opfylder OECD's standard for denne type dyreforsøg. Blandt andet er antallet af dyr på 10 i hver gruppe langt under det anbefalede. Det betyder, at resultaterne ikke kan tillægges større vægt da mindre forskelle vil blive skjult i de store tilfældige udsving.

- Normalt anvendes som udgangspunkt i sådanne forsøg, det samme antal dyr i kontrol- og testhold. Samme kontrolhold kan i visse tilfælde anvendes til flere testhold. I dette forsøg anvendes 10 dyr pr. hold, og kun et af holdene for hvert køn er kontrolhold, der bruges til sammenligning med alle ni andre testhold. Som udgangspunkt er dette alt for få dyr i hvert hold i et langtidsstudie som dette. Dels forventes det, at der med 10 dyr pr. hold i slutningen af forsøget er for få dyr tilbage til at lave statistik på, og dels kan kontrolgruppen let afvige meget af tilfældige årsager, uden at dette kan be- eller afkræftes. I det sidste tilfælde kan et unormalt kontrolhold fremkalde falske indikationer på, at alle testhold afviger fra det normale. Flere kontroldyr, sammenholdt med en fornuftig statistisk analyse, kunne være anvendt til at mindske usikkerheden omkring vurderingen af om kontrolholdet repræsenterer det "normale", og om de fund som blev rapporteret for testholdene kan

relateres til behandling. Udvælgelsen af rottestammen Sprague-Dawley (SD) er ikke velvalgt, fordi stammen er kendt for hyppigt at få spontane tumorer (prevalence ca 45 %) og har en relativ høj dødelighed (i 2 års forsøg vil mindre end 50 % i gennemsnit være i live[1]). Valget af rottestamme mangler begrundelse i artiklen.

- Ud fra en etisk synsvinkel er studiet problematisk på flere områder. Antallet af dyr er så lavt, at eventuelle effekter ikke kan afsløres ved statistisk analyse af data. Forfatterne refererer selv til en artikel[1], der beskriver overlevelsesrater for SD rotter under langtidsforsøg, og er derfor vidende om problemet allerede inden de starter forsøget. Et andet aspekt er, at de lader tumorer vokse sig meget store, før dyrene aflives. Der mangler en faglig begrundelse for ikke at aflive dyrene med tumorer på et tidligere tidspunkt. Vi finder det etisk uforsvarligt at lade dyrene gå så længe med tumorer, hvis det ikke bidrager til, at der opnås vigtige data. De retningslinier DTU Fødevareinstituttet anvender ved dyreforsøg sikrer, at tumorerne ikke påvirker dyrets tilstand. Vores kriterier er, at den enkelte tumors vægt ikke overstiger 4 g svarende til en diameter på 20 mm, og at den samlede tumormasse ikke overstiger 10 % af rottens normalvægt. Disse kriterier er i overensstemmelse med Dyreforsøgstilsynets retningslinier for tilladelse til at udføre sådanne dyreforsøg.

  Det fremgår ikke klart af artiklen hvor tæt, genetisk, kontrol-majsen er beslægtet med den gensplejsede majs NK603. Der er heller ikke data om svampetoksin (mykotoksin) indhold eller andre dyrkningsmæssige forhold der kan have betydning for forsøget. Mykotoksiner kan være leverskadende, nyreskadende eller kræftfremkaldende og derfor burde forfatterne have foretaget disse målinger. Det er ikke oplyst, om alle dyrehold fodres med samme koncentration af majs (om der kompenseres med traditionel majs i foderblandinger hvor der f.eks. gives 11 % GM majs).

- Der er ingen data for dyrenes indtagelse af foder eller væske. Det er væsentlige parametre for at vurdere dyrenes tilstand, fx kan det tænkes at rotter undlader at drikke eller drikker mindre alene, fordi drikkevand med Roundup smagsmæssigt afholder dem fra at drikke efter behov.

Resultater og konklusioner

- I studiet er der en forsøgsserie, hvor SD rotter får forskellige koncentrationer af Roundup i drikkevandet. Den aktive ingrediens i Roundup er glyfosat. Glyfosat er undersøgt i mange langtidsforsøg i både rotter og mus. Ingen af disse studier viser tegn på, at glyfosat har potentiale til at være carcinogent, eller at dyrene har øget dødelighed eller har givet anledning til hormonale effekter. Ud over glyfosat indeholder sprøjtemidlet Roundup forskellige co-formulanter, fx overfladeaktive stoffer, men det fremgår ikke af artiklen hvilke stoffer der er tale om.
  Designet af studiet er så mangelfuldt, at det ikke kan udledes om indgivelse af Roundup i drikkevand har effekt på rotterne i langtidsforsøg.
  Derfor er de konklusioner som forfatterne drager på dette materiale ikke valide.

[1] Brix, A.E., Nyska, A., Haseman, J.K., Sells, D.M., Jokinen, M.P., Walker, N.J., 2005. Incidences of selected lesions in control female Harlan Sprague-Dawley rats from two-year studies performed by the National Toxicology Program. Toxicol. Pathol. 33, 477–483.

- De eneste resultater som forfatterne fremlægger af deres sammenlignende studier af indholdsstoffer i NK603 majs og kontrol majs er, at ferulinsyre og kaffesyre koncentationerne er væsentlig lavere i NK603 majs – det gælder både Roundup behandlet og ikke-Roundup behandlet majs. Forfatterne argumenterer for, at disse syrer har en beskyttende effekt på dyrene, og at NK603 majs derfor mangler den beskyttende effekt, hvilket kan forklare visse negative effekter på nyrerne i dyr fodret med NK603. Disse forskelle er ikke set i tidligere undersøgelser, hvor den gensplejsede majs NK603 er undersøgt, hvilket forfatterne undlader at nævne.
  Det kan derfor udledes, at det ikke er det nye protein, CP4-EPSPS, som findes i NK603, eller andet relateret til gensplejsningen i NK603, der er årsag til de lavere målinger af ferulinsyre og kaffesyre i forsøget. Eventuelle negative effekter på nyrerne som følge af det lave indhold af ferulinsyre og kaffesyre er derfor ikke relateret til den gensplejsede majs, men til alle sorter af majs med lavt indhold af disse syrer.

- Der er ingen dosis-respons relationer for parametre rapporteret i studiet. Der vil normalt forventes en vis dosis-respons relation, så de dyr der er fodret med de største mængder af et teststof udviser de største effekter. I dette studie ses denne relation ikke. Forfatterne henviser til, at dette ikke er tilfældet for alle stoffer, men er afhængigt af stoffets effekt på dyrene.
  DTU Fødevareinstituttet er bekendt med disse forhold, men finder ikke, at fortolkningen af de opnåede resultater, herunder at det kan skyldes hormonale effekter, er underbyggede.

- Der drages konklusioner ud fra forskelle baseret på få dyr som forfatterne mener, kan tillægges betydning. Her mangler en neutral statistisk analyse af data bl.a. for dødelighed og antal tumorer til sammenligning mellem de forskellige hold.

- De data om dødelighed og forekomst af tumorer som forsøget leverer, falder indenfor de historiske data for SD rottestammen[2], fx er overlevelsesraten: 17-62,9 % i hanner og 20-62 % i hunner. Forfattere oplyser ikke hyppigheden af dyr med maligne og godartede tumorer i mammavævet separat. De tumorer de finder i vævet i mælkekirtler hos hunrotter (fibroadenoma og adenocarcinoma) er kendt at forekomme hyppigt hos aldrende hunrotter og at udvise stærkt varierende forekomst mellem undersøgelserne[3].

- Der mangler en afbalanceret videnskabelig diskussion. Generelt undlader forfatterne at henvise til relevant og vigtig litteratur på området og sammenholde deres resultater med tilsvarende publicerede undersøgelser. I artiklen argumenteres for, at hanrotter hovedsagligt døde pga. en væsentlig nedsat funktion af lever og nyrer. Men disse ændringer er en del af patologien af den aldrende rotte dvs. dette er ganske normalt. Derfor skal deres relation til behandling evalueres med stor forsigtighed og en potentiel relation til behandling bør kun fremsættes, hvis forekomsten i de behandlede grupper er statistisk signifikant højere end i kontrollerne, og der er en tydelig relation mellem dosis og respons. Artiklen indeholder ingen data der kan dokumentere udsagnet, herunder data fra mikroskopiske undersøgelser af lever og nyrer, leverfunktions målinger, urinanalyser og cytochrom aktivitet. Til støtte for deres argumenter henviser forfatterne til deres tidligere

[2] Compilation of Spontaneous Neoplastic Lesions and Survival in Crl:CD® (SD) Rats from Control Groups. Charles River Laboratories March 2004.

[3] Greaves P, 2000: Mammary Gland and Haematopoietic and Lymphatic Systems In: Histopathology of Preclinical Toxicity Studies. Interpretation and Relevance in Drug Safety Evaluation. 2nd edition, pp 55-125, Elsevier Science.

publikation [4]. Denne tidligere publikation, der er en statistisk reanalyse af eksisterende data, er imidlertid tidligere blevet alvorligt kritiseret af både eksperter og myndigheder for at være uden plausible videnskabelige forklaringer.

[4] Spiroux et al. Int. J. Biol. Sci. 5:706-726, 2009

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RÉPUBLIQUE FRANÇAISE

Ministère des Affaires sociales et de la Santé

Ministère délégué auprès du Ministère de l'économie et des finances, chargé de l'économie sociale et solidaire et de la consommation

Ministère de l'Écologie, du Développement durable et de l'Énergie

Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt

Monsieur Marc MORTUREUX

Directeur général de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

Monsieur Jean-François DHAINAUT

Président du Haut conseil des biotechnologies

Paris, le 24 septembre 2012

Monsieur le Directeur général,

Monsieur le Président,

Un article intitulé « *Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize* » vient d'être publié dans la revue scientifique Food and Chemical Toxicology par l'équipe du professeur Gilles-Eric Séralini. Cet article porte sur une étude conduite sur des rats ayant consommé pendant 2 ans du maïs génétiquement modifié NK603 traité ou non avec l'herbicide Roundup, ou de l'herbicide Roundup seul.

Nous vous demandons, par la présente saisine, de bien vouloir vous rapprocher afin de procéder à une analyse de l'étude rapportée par cet article afin de déterminer si elle est de nature à remettre en cause ou non les conclusions des évaluations précédentes sur cet OGM et notamment si elle peut être considérée comme conclusive quant au risque sanitaire que pourraient présenter les aliments issus de plantes OGM comportant l'événement NK603.

Il est d'autre part demandé à l'ANSES de déterminer si cette étude est de nature à remettre en cause ou non les conclusions des évaluations précédentes de l'ANSES sur l'herbicide Roundup.

Sur la base de cette analyse, nous vous demandons d'évaluer si le protocole mis en œuvre et les conclusions de cette étude remettent en cause les lignes directrices actuelles ou à venir en matière d'évaluation des risques sanitaires.

Nous vous saurions gré de bien vouloir rendre un avis sur cet article avant le 20 octobre 2012 et sur la pertinence des modalités d'évaluation des risques sanitaires et des propositions d'aménagements des lignes directrices, si nécessaire, avant le 20 novembre 2012.

Nous vous prions de croire, Monsieur le Président, Monsieur le Directeur général, à l'assurance de notre considération distinguée.

La Ministre des Affaires sociales et de la Santé	Le Ministre délégué auprès du Ministre de l'économie et des finances, chargé de l'économie sociale et solidaire et de la consommation	La Ministre de l'Écologie, du Développement durable et de l'Énergie	Le Ministre de l'Agriculture, de l'Agroalimentaire et de la Forêt
Marisol TOURAINE	Benoît HAMON	Delphine BATHO	Stéphane LE FOLL

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RÉPUBLIQUE FRANÇAISE

Ministry of Social Affairs and Health	Ministry with responsibility for the Social Economy and Consumer Affairs in the Ministry for the Economy and Finance	Ministry for Ecology, Sustainable Development and Energy	Ministry of Agriculture, Food and Forestry

Mr Marc Mortureux

Director-General,
National Agency for Food,
Environmental and
Occupational Health Safety

Mr Jean-François Dhainaut

President,
High Council for Biotechnology

Paris, 24 September 2012

Dear Mr Mortureux,

Dear Mr Dhainaut,

A paper entitled '*Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize*' has recently been published in the scientific journal *Food and Chemical Toxicology* by the team of Professor Gilles-Eric Séralini. This paper concerns a study conducted for 2 years on rats fed genetically modified maize NK603, treated or untreated with Roundup, or the Roundup herbicide alone.

We request you, by this referral, to confer together to undertake an analysis of the study reported by this paper in order to determine whether or not it is likely to cast doubt on the findings of previous assessments of this GMO and in particular whether it may be considered conclusive regarding the possible health risk of food derived from GM plants containing event NK603.

ANSES is further requested to determine whether or not this study is likely to cast doubt on the findings of ANSES previous assessments of the Roundup herbicide.

On the basis of this analysis, you are requested to assess whether the study's protocol and findings call into question current or future guidelines for health risk assessment.

Would you please deliver an opinion on this paper by 20 October 2012 and on the suitability of health risk assessment procedures and proposed adjustments to guidelines, if necessary, by 20 November 2012.

Yours sincerely,

Marisol Touraine	Benoît Hamon	Delphine Batho	Stéphane Le Foll
Minister of Social Affairs and Health	Minister with responsibility for the Social Economy and Consumer Affairs in the Ministry for the Economy and Finance	Minister for Ecology, Sustainable Development and Energy	Minister of Agriculture, Food and Forestry

Maisons-Alfort, 19 October 2012

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

concerning an analysis of the study by Séralini *et al.* (2012) "Long term toxicity of a ROUNDUP herbicide and a ROUNDUP-tolerant genetically modified maize"

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are made public.

On 24 September 2012, ANSES received a formal request from the Minister for Social Affairs and Health, the Minister of Ecology, Sustainable Development and Energy, the Minister of Agriculture, Food and Forestry, and the Minister for the Social Economy, Solidarity and Consumer Affairs attached to the Ministry of Economics and Finance, to issue an Opinion based on an analysis of the recently published study "Long term toxicity of a ROUNDUP herbicide and a ROUNDUP-tolerant genetically modified maize" (Séralini *et al.* 2012).

1. BACKGROUND AND PURPOSE OF THE REQUEST

On 19 September 2012, the journal Food and Chemical Toxicology published a study by Séralini *et al.* on the long-term toxicity of ROUNDUP and NK603 maize, which is glyphosate-tolerant. In particular, the authors noted an increase in mortality and the incidence of tumours in several groups of rats fed for two years on NK603 glyphosate-tolerant maize, whether or not the maize had been treated with ROUNDUP WEATHER MAX or given drinking water containing various doses of the herbicide ROUNDUP GT PLUS.

ANSES was immediately informed of a future request with the aim of:

- establishing whether or not this publication casts doubt on the conclusions of previous assessments of this genetically-modified organism (GMO) or of the herbicide ROUNDUP, and in particular whether it can be considered conclusive with regard to the possible health risk of food from plants containing the NK603 event,
- assessing from this whether the experimental protocol and the conclusions of this study cast doubt on current or future guidelines for the assessment of health risks.

At the same time, the French Government also asked the French High Council for Biotechnologies (HCB) to investigate the aspects specifically related to NK603 maize.

On the same day this paper was published in the journal Food and Chemical Toxicology, it was the subject of comment in an article in the French weekly *Nouvel Observateur*. The study has attracted considerable attention from the media, reviving the intense public debate on the issue of GMOs and plant protection products.

A number of scientists or scientific groups rapidly expressed opinions in the written press or *via* the Internet and, in many cases, cast doubt on the scientific value of the study, while others highlighted the relevance of the questions raised, the innovative nature of the study and its intrinsic qualities.

Moreover, several Agencies in Member States of the European Union and the European Food Safety Authority (EFSA) also received requests and have issued opinions restricted to a scientific analysis of the study, especially EFSA, Germany's BfR[1], the Dutch RIVM[2] and the Danish DTU[3]. These bodies found that the author's conclusions are insufficiently supported by experimental evidence as a result of the study's inadequate protocol, presentation and interpretation. They also call for the authors to publish the full data on which the study was based. The BfR does underline, however, that the results of this study can be seen as a contribution to the experimental study of the possible influence of co-formulants on the long-term effects of plant protection products.

The documents sent to ANSES by Monsanto express similar criticisms.

It is therefore in a context of intense debate, with a short timeframe and after the issuing of opinions by a considerable number of scientists and collective expert assessment agencies, that ANSES now offers its own analysis of the work described in the paper by Séralini *et al*. (2012) and also gives an opinion on the relevance of reconsidering the way in which the health risks attached to GMOs and plant protection products are assessed.

2. ORGANISATION OF THE EXPERT ASSESSMENT

This expert appraisal was carried out in accordance with the French standard NF X 50-110 "Quality in Expertise - General Requirements of Competence for Expert Appraisals (May 2003)".

ANSES set up an emergency collective expert assessment group (the "NK603 R" ECEAG), made up of experts from a range of disciplines. Its composition (Annex 1) and the experts' public declarations of interest will be made public at the same time as this Opinion. The ECEAG held meetings on 28 September and 3 and 15 October and validated its assessment report electronically on 19 October 2012. ANSES also had several discussions with the HCB and a meeting with representatives of the ECEAG took place on 17 October.

ANSES participated in several meetings for the exchange of information organised by EFSA, on 28 September and 11 and 18 October.

ANSES held two hearings on 10 October 2012, the minutes of which have been validated by the interviewees and are being published at the same time as this Opinion. These concern:

- Several of the co-authors of the study (Messrs Séralini, Spiroux de Vendômois, Defarge, Gress and Mesnage), who were asked to present the results, answer certain questions from ANSES, and give their opinions on current methods for assessing the risks to health of GMOs and plant protection products.
- François Veillerette, President of the "Générations Futures" association, who was asked to express his association's view on current methods for assessing the risks to health of GMOs and plant protection products.

Monsanto was also invited, but in view of the short timeframe preferred to send information in written form on 17 October, which is also being published simultaneously with this opinion.

After the hearing, Mr Séralini sent ANSES, on 15 October, raw data on the mortality of the experimental animals (the data corresponding to Figure 1 in the article by Séralini *et al.*, 2012) and

[1] Bundesinstitut für Risikobewertung
[2] National Institute for Public Health and the Environment
[3] Danmarks Tekniske Universitet

the onset of non-regressive tumours (data corresponding to Figure 2 in the same article), without distinguishing the nature of the tumours.

3. ANALYSIS AND CONCLUSIONS OF THE EMERGENCY COLLECTIVE EXPERT ASSESSMENT GROUP (ECEAG)

In order to understand the context in which the paper by Séralini et *al.* (2012) was published, it is necessary to:

- recall the regulatory context and the general scientific principles underpinning the authorisation process for GMOs and plant protection products,
- list the scientific publications relevant to this Opinion so as to be able to undertake a critical review of the main publications directly related to the issues raised.

Contents of Chapter 3

3.1 Regulatory context for the authorisation of genetically-modified organisms and plant protection substances

3.1.1. Expert assessment process and requirements for the authorisation of GMOs

3.1.1.1 Expert assessment process and authorisation of GMOs

Genetically-modified organisms (GMOs) are defined as organisms or microorganisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (Article 2 of Directive 2001/18/EC).

Using genetic engineering techniques, it is possible to transfer selected genes from one organism to another, sometimes between different species. This technique enables the introduction into the genetic make-up of the modified organism of one or more genes coding for proteins conferring new properties. Any sequence of DNA inserted into another organism is called a "transgene". Transferred genes can come from a wide range of living organisms as a result of the universal nature of the genetic code. This technique can be applied to microorganisms, plants or animals. To date, applications for which marketing authorisations (MAs) have been requested for use in food or feed mainly concern genetically-modified plants (GMPs).

Since 2003[4], there has been a specific European regulatory framework governing the use of GMOs (Regulation EC 1829/2003[5]). This Regulation defines, for GMOs (animals, plants, microorganisms, etc.), methods for assessing the risk to food and feed and to the environment, and entrusts this assessment to the European Food Safety Authority (EFSA).
However, as concerns GMOs, EFSA enables the competent authorities of each of the Member States to assess the dossiers and to provide their comments. In France, the competent authorities are the Ministry for the Social Economy, Solidarity and Consumer Affairs attached to the Ministry of Economics and Finance and the Ministry of Agriculture, Food and Forestry. These authorities requested that ANSES investigate the risk to humans and animals of food and feed containing GMOs and also that the HCB (French High Council for Biotechnologies) investigate more specifically the environmental risks.
The procedure followed by ANSES involves an examination of the data provided, verification of their scientific validity and their compliance with regulatory requirements, and an assessment of the health risks in view of the information submitted. When the procedure is complete, conducted according to the collective expert assessment principles applied by ANSES, the Agency's conclusions are presented in an Opinion. This Opinion guides the French vote during the authorisation procedure at the European Commission.

In 55% of the cases examined concerning genetically-modified plants, the Agency deemed that the applicant had submitted insufficient data to enable a conclusion to be drawn on the health issues related to consumption of the GMO in question, and in these cases additional comments were submitted to EFSA.

3.1.1.2 Guidelines for the assessment of GMOs

In 2002, AFSSA drew up guidelines for assessing the safety of GMOs in respect of their use in food and feed (AFSSA 2002). When Regulation EC 1829/2003 was adopted, EFSA developed guidelines detailing the requirements of this Regulation. According to these guidelines (EFSA 2006; EFSA 2009b; EFSA 2011b), the application dossier must include a series of studies designed to identify and characterise any harmful effects related to the consumption of genetically-modified plants or products derived from them, by humans or animals.
The guidelines lay out the information to be provided by the company that must be included in the application for a GMO marketing authorisation in Europe.

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[4] Before 2003, applications for use in "human foodstuffs" were governed by Regulation EC No.258/97, known as the "Novel Foods Regulation". This Regulation required that foodstuffs not traditionally consumed in Europe before 1997 (including GMOs), undergo risk assessment prior to authorisation. The use of GMOs in animal feed was regulated by Directive 2001/18.

[5] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically-modified food and feed.
http://ec.europa.eu/food/food/animalnutrition/labelling/Reg_1829_2003_en.pdf

3.1.1.2.1: Analytical data

Molecular characterisation
An initial set of data are used for the molecular characterisation of the GMP. The application dossier must therefore include:

- The DNA sequence of the transgene introduced into the plant to verify that it corresponds fully with the sequences described in the vector used for transgenesis,
- The results of analysis for describing the newly expressed proteins in the GMP,
- Characterisation of the genomic regions flanking the transgene () to verify that the insertion has not occurred in a coding sequence, and to assess the possible production of chimeric proteins or peptides,
- Bioinformatics analyses to search for homologies between the proteins produced by the transgene, the peptides that can be produced in the regions flanking the insert and the proteins or peptides known to be toxic or allergenic,
- The results concerning the genetic stability of the insert in the plant over successive generations.

Comparative analysis of the GM plant and the comparator
The application dossier should also include agronomic and chemical composition data to enable comparison of the GM plant with its closest control (near-isogenic line) (EFSA 2011a) on the basis of its agronomic and phenotypic properties and its chemical composition. The chemical composition analysis concerns several dozen principal compounds (amino acids, fatty acids, etc.), compounds with important nutritional properties such as vitamins, and certain metabolites and antinutritional factors. The nature of the latter depends on the plant species and is laid down in specific OECD[6] guidance documents[7] for each species.
The samples studied come from plants cultivated in fields at several different sites and sometimes over several seasons to take different environmental conditions into account. The experimental design must rigorously follow EFSA's recommendations in terms of repetitions and numbers of sites and must include non-transgenic commercial varieties of the same species. The analysis of the data must follow precise statistical methods (EFSA 2009a). In conclusion, the analysis of these results shows whether or not the GM plant and the non-GM control have the same composition regarding the analysed compounds.

3.1.1.2.2: Toxicity, food-grade and allergenicity studies

Toxicity studies
The assessment of potential toxicity of the GMP and the absence of harmful effects on human and animal health is an essential step in assessing the application. It depends on toxicological studies using laboratory animals (mostly rodents). These are internationally-recognised standard toxicity tests (OECD, Annex 2) conducted under conditions that comply with good laboratory practice (GLP) (Annex 2) for which the applicant must also provide the full study report.
The following animal tests must be conducted:

- An acute toxicity study by single administration of the protein produced by the transgene to several groups of mice which are then examined for 14 days. In the case of new proteins, these are re-administered to the mice for 28 days (EFSA 2011b)[8],
- A repeated–dose 90-day oral toxicity study in rodents on whole food/feed by administration of a part of the plant. The results in terms of growth, consumption and haematological, biochemical and urinary parameters are compared between the groups of animals having consumed the GMP and the groups of animals having received the control plant.

[6] OECD: Organisation for Economic Cooperation and Development
[7] Consensus Documents for the Work on the Safety of Novel Foods and Feeds
http://www.oecd.org/science/biosafety-biotrack/consensusdocumentsfortheworkonthesafetyofnovelfoodsandfeeds.htm
[8] It should be pointed out that these tests, which require a large quantity of proteins, are often carried out with a protein coded for by the same gene but produced in a bacterium. A series of studies demonstrating the equivalence between the protein tested and the protein expressed in the GMP is provided in the application dossier.

This 90-day oral toxicity study on whole food/feed in rodents was not systematically mandatory in the EFSA guidelines, but was decided on a case-by-case basis. In France, ANSES will not rule on applications concerning primary genetic transformation events without this test. The European Commission is currently in the process of making these studies mandatory as part of the process to consolidate the guidelines that appeared as an annex to Regulation EC 1829/2003.

At present, toxicological studies targeting reproduction and development functions (reproduction, development, teratogenicity, etc.) are not mandatory. They can however be requested depending on the potential exposure, the nature and quantitative significance of the differences in chemical composition observed between the GMO and its non-GMO control, or the results of the nutritional assessment and 90-day oral toxicity study on whole food/feed.

Nutritional studies of genetically modified feed

In order to demonstrate that products intended for animal feed have equivalent nutritional qualities, the chemical composition analysis is often supplemented with a nutritional study. The purpose is to verify that groups of animals given feed from a genetically-modified plant show the same zootechnical characteristics (in terms of growth, weight, state of health, etc.) as groups receiving feed from isogenic plants and commercial varieties of the same plant. These tests are often carried out on chickens, over a period covering the usual economic lifetime of broilers (42 days).

This part of the application dossier can also include other elements from *in vitro* or bioinformatic analysis:

- *In vitro* digestibility tests simulating intestinal or gastric digestion in humans or animals and verifying that the resulting proteins and peptides are broken down during the digestion process,
- Physico-chemical properties of the protein(s) produced by the transgene,
- Bioinformatic analysis comparing sequences of the protein(s) produced by the transgene with sequences of the proteins and peptides catalogued as toxic in public databases.

Assessment of potential allergenicity

EFSA recently published a detailed Opinion following its assessment of the allergenicity of GMOs intended for food or feed (EFSA 2010). The new issues raised in this Opinion were incorporated in the revised guidelines (EFSA 2011b). Therefore, the revised guidelines include:

- an assessment of the allergenicity of the newly-expressed protein(s), including the origin of the gene, the structural, biological and physio-chemical characteristics, a comparison of the homology of the amino acid sequence between the newly expressed proteins and known allergens, and *in vitro* tests for resistance to pepsin, and digestibility;
- an assessment of the food or feed's allergenicity involving the whole GMP with, if necessary, an analysis of any possible over-expression of natural endogenous allergens.

3.1.1.3 The Agency's contribution to changes in the guidelines

Like other EU Member State Agencies and jointly with EFSA, ANSES contributes in drawing up and modifying the guideline documents for use by industrial applicants.

From 2002, AFSSA contributed significantly to reinforcing the requirements that industrial applicants had to satisfy (in terms of data and tests), by identifying the sensitive aspects of health risk assessments related to the consumption of GMOs (AFSSA 2002). The Agency was the first in Europe to consider adapting the protocol for 90-day subchronic oral toxicity studies to the assessment of GMPs.

In addition, in 2011 ANSES issued an Opinion on methods for the statistical analysis of data for this study, which resulted in recommendations for the implementation of the protocols and analytical methods to be used to guarantee the reliability of results. In particular, this Opinion recommended increasing the number of animals to increase the statistical power of the tests (ANSES 2011).

3.1.2: Collective expert assessment process and requirements concerning the authorisation of plant protection substances

3.1.2.1: Process of collective expert assessment and authorisation of plant protection substances and preparations

The assessment of active substances in plant protection formulations, and of the formulations themselves with regard to marketing, is strictly regulated and harmonised at European level by Regulation EC 1107/2009[9], replacing Directive 91/414/EEC[10], which was in force until June 2011.

The process requires two phases:

- the **first phase**, carried out jointly by EU Member States, involves identifying the hazards of **active substances** and assessing the risks related to a reference product, with a view to ruling on whether or not these substances should be approved in Europe.
- the **second phase**, for approved active substances, consists in assessing the agricultural benefits and the risks related to commercial **formulations**; for this purpose, Europe is divided up into three geographical zones (North, Centre and South): France is in the South zone.

In France, applicants submit an MA[11] application dossier to ANSES's Regulated Products Department.
To investigate the application, ANSES:

- examines the data supplied and verifies their scientific validity as well as their compliance with regulatory requirements,
- assesses the agricultural risks and benefits related to the use of the formulation.

The investigation is carried out in accordance with the collective expert assessment principles applied by ANSES. When it is complete, the conclusions of the assessment, in some cases together with recommendations for management measures, are laid out in an Opinion. Conclusions relative to the acceptability of risk refer to the criteria indicated in Regulation (EU) 546/2011[12]. They are expressed as either "acceptable" or "unacceptable", with reference to these criteria.
The Directorate General for Food then uses the ANSES Opinion to decide whether or not to grant an MA or any modification of a current MA. This MA is issued when, under normal conditions of use associated with good agricultural practice, the formulation is deemed effective and free of unacceptable affects on human or animal health or the environment. The decision concerning MA details:

- the crop(s) targeted by this treatment,
- the pest(s), disease(s) or weed(s) targeted,
- the dose, period and frequency of application for the formulation, with any other agricultural practices associated with the treatment,
- restrictions concerning the conditions of use and management measures.

It should be noted that active substances and their associated formulations must be reassessed systematically according to a schedule laid down in Regulation (EC) 1107/2009.

[9] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC.
[10] Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market, transposed into French Law by the Order of 6 September 1994 implementing Decree 94/359 of 5 May 1994 on the control of plant protection products.
[11] MA: Marketing Authorisation
[12] Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products.

3.1.2.2 Guidelines for the assessment of plant protection products

The implementing regulations for Regulation (EC) 1107/2009 (i.e. Regulation (EU) 544/2011[13] for active substances and Regulation (EU) 545/2011[14] for formulations) specify the information to be included in application dossiers and the methods to be followed to obtain it. These Regulations refer explicitly to methodology guidelines adopted by European or international organisations such as OECD, FAO[15] and EPPO[16] and are supplemented by almost 200 technical guidance documents, adopted at European level, detailing the models to be used, the parameters to be taken into account and the default values to be included in the models in the absence of valid information in the application dossier. These guidance documents are available from the European Commission website. The absence of required data or the presentation of non-compliant information result in a conclusion of unacceptable risk or in default values being used (if available), which is always disadvantageous for the applicant.

Regarding more specifically the data used for the assessment of risk to human health, the requirements are summarised below.

Applications concerning **active substances** must enable the intrinsic properties of these substances to be characterised and therefore the hazards they pose for humans and the environment. To assess the effects on human health, they must include full reports of the following toxicity and metabolism studies in mammals, carried out according to the guidelines defined by the regulations and good laboratory practice:

- metabolism studies in animals,
- acute toxicity studies for exposure by the oral or dermal routes or by inhalation,
- dermal or ocular irritation studies,
- skin sensitisation study,
- studies of toxicity by repeated oral administration in the short, medium and long term, and carcinogenic studies,
- mutagenicity studies,
- toxicity study for reproduction over two generations and studies on the effects on development,
- neurotoxicity studies depending on the properties of the substances,
- other studies depending on the results obtained from the preceding studies, especially for better identification of the effects and mechanisms of action.

The OECD guidelines for long-term toxicity studies and carcinogenic studies are summarised in Annex 2.
More specifically, to characterise the long-term effects of the active substance, such as those studied in the article by Séralini *et al.* (2012), the guidelines insist on two long-term and carcinogenesis studies performed on different species (rats and mice: 50 animals per group) to assess the general effects and potential carcinogenic effects of the substance when administered daily over the rodent's entire lifetime.

In these studies, numerous physiological, biochemical and histological parameters are monitored and measured in the animals. They enable a study of the dose-effect relationship, the toxicological mechanism of action, the reversibility of effects, whether or not there is a threshold for the undesirable effects, species specificity and the potential for extrapolating effects to humans.
On the basis of the most sensitive effects observed in these studies, toxicity reference values (ADI[17], ARfD[18], AOEL[19]) are calculated at the end of the European collective assessment for each

[13] Commission Regulation (EU) No 544/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for active substances
[14] Commission Regulation (EU) No 545/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for plant protection products
[15] FAO: United Nations Food and Agriculture Organization.
[16] EPPO: European and Mediterranean Plant Protection Organization
[17] ADI: the Acceptable Daily Intake of a chemical is an estimate of the quantity of the active substance in food or drinking water that can be ingested every day over the entire lifetime, with no appreciable risk to the health of the consumer, bearing in mind all known factors at the time of the assessment. It is expressed in milligrams of chemical substance per kilogram of body weight (WHO, 1997).

active substance. They will be used later to determine the risks associated with the use of plant protection formulations containing the substance.

Application dossiers must also contain study reports on metabolism and residues in the plants (and in foodstuffs of animal origin where concerned). For each foodstuff intended for human consumption, whether of plant or animal origin, the nature of the residue is defined (active substances and any relevant metabolite(s)). An MRL[20] is then determined for each active substance and each foodstuff, in order to ensure that consumer exposure remains below the values considered to be without risk to health in the short and long term.

A **plant protection formulation** consists of one or more active substances, most often associated with one or more co-formulants, which play a role in preparing or stabilising the formulation (water-based, powder, granules, suspension, etc.) or in modifying the availability of the active substance to the target pest (parasites or weeds). The MA application is submitted for one or more specific uses, a use being defined for the crop treated, the target pest, the quantity of product used per hectare, the period and frequency of use. Regarding human health, formulations are assessed for the risk to workers applying the treatment, agricultural workers handling the treated plant and bystanders[21], as well as the risks to consumers (chronic and acute risks to adults, toddlers[22] and infants, for different diets and for drinking water).
Marketing authorisation application dossiers must contain information enabling the characterisation of the formulations, of the concentration of active substances and co-formulants they contain and their associated hazards and in particular they must include full reports of the following toxicity and metabolism studies in mammals, carried out according to the guidelines defined by the regulations and good laboratory practice:

- Acute toxicity studies for the formulation, particularly to determine the toxicity of the product relative to the active substance and, if possible, the toxic mode of action, by the oral route, the dermal route and, if exposure by this route is possible, by inhalation,
- Dermal and ocular irritation studies,
- Skin sensitisation study.

The application dossier also contains toxicology data relative to the non-active substances.
Thanks to a process harmonised at European level[23], classifications based on a hazard assessment coordinated by the European Chemicals Agency have been published for a large number of substances and co-formulants.
The range of information available makes it possible to characterise the formulation and, in particular, to propose, on the basis of its composition in active substance(s) and co-formulant(s) and their properties, a classification corresponding to the hazards presented by this formulation.

The risk assessment takes into account the hazard determined for the formulation and the level of exposure, measured during tests or calculated using models.
The following tests must be carried out to estimate exposure for each formulation, and their full reports must be included with each application dossier:

- Dermal absorption study
- Tests for residues in the products treated and the derived food and feed.

In the particular case of plant protection formulations for treating GM crops that are tolerant to the active substance, residual tests on the GM crop concerned are required (Annex 3).

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[18] ARfD: The Acute Reference Dose of a chemical is the estimated quantity of a substance found in food or drinking water, expressed as a proportion of body weight, that can be ingested over a short period, usually in the course of a meal or a day, with no appreciable risk to the health of the consumer, bearing in mind all known factors at the time of the assessment. It is expressed in milligrams of chemical substance per kilogram of body weight (WHO, 1997).

[19] AOEL: The Acceptable Operator Exposure Level is the maximum quantity of active substance to which an operator can be exposed on a daily basis, with no hazardous effect on his/her health.

[20] MRL: The Maximum Residue Level.

[21] Persons who are located within or directly adjacent to the area where the pesticide application is in process

[22] Children from 13 to 18 months.

[23] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures.

At present, in accordance with the opinion of the European experts, the regulations do not require any long-term toxicity study on the formulated preparation (active substance + co-formulants). Such a requirement would necessitate a far greater number of tests on vertebrates, whereas it is currently considered that these should be limited to what is strictly necessary.
The long-term effects of the active substance are characterised and, as regards co-formulants, toxicological data are available. Regulation (EC) 1107/2009 includes a list (in its Annex III) of co-formulants that may not be included in the composition of plant protection formulations. This list is currently being compiled and will be updated to take into account any new knowledge about hazards. Any new information revealing a hazard is nonetheless already taken into account. For example, the use of polyethoxylated derivatives of nonylphenol, as a co-formulant in plant protection formulations and biocides, is prohibited[24], because of their endocrine-disrupting properties. In the case of insufficient data or of doubt about the long-term toxicity of a co-formulant, the provisions of the REACh[25] Regulation for the assessment of chemical substances authorise assessment agencies to ask applicants to carry out further studies.
Furthermore, the results of the acute toxicity studies conducted with the formulation are compared with the results expected in view of the known properties of the active substance(s) in order to identify any deviations raising questions about the toxicity of the co-formulants.

The issue of accumulated risk is the subject of much discussion at European level. ANSES has chosen an assessment methodology that it employs for plant protection formulations whenever several constituent substances have been classified as having properties that are carcinogenic, mutagenic or toxic to reproduction. This methodology, based on an approach involving target organs and mechanisms of action, is described in a memorandum available on the ANSES website.

Certain co-formulants can influence the exposure of humans to active substance(s) in the formulation, either during application, by acting on the way these substances are absorbed, or, for those consuming the foodstuff, by modifying the level of residues found in the treated crop. This is why applicants must systematically provide a dermal absorption study, together with tests designed to measure residue levels in the treated plants after treatment, conducted with the plant protection formulation.

3.2 An inventory of toxicology studies available on GMOs and glyphosate-based formulations

The toxicology studies needed for examining regulatory dossiers and applications for the authorisation of GMOs and plant presentation products, described in the previous section, are examined by the bodies responsible for ruling on these dossiers. The studies available during the regulatory investigation of NK603 maize and ROUNDUP are described in greater detail in the annexes concerning the assessment history of these dossiers (Annexes 3 and 4).
These regulatory studies are not always published in the scientific literature. On the other hand, other research teams use experimental protocols that are not directly linked to the initial authorisation of these products. These studies can be found in peer-reviewed scientific journals. An inventory of scientific publications useful in clarifying the questions raised by the study by Séralini *et al.* (2012) has been drawn up.

3.2.1 A literature search of relevant studies in the context of this Request and concerning genetically-modified plants

Several summary reviews have been compiled from the literature on assessment of the risks related to genetically-modified plants. A recent review was published in the journal "*Environment International*" in February 2011 by J.L. Domingo and Bordonaba (Domingo and Giné Bordonaba 2011). This summary describes the wide range of protocols used that are not systematically applied according to the recommendations in the international scientific literature (Domingo 2007). The

[24] Directive 2003/53/EC of the European Parliament and of the Council of 18 June 2003 amending for the 26th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (nonylphenol, nonylphenol ethoxylate and cement).

[25] Regulation (EC) No.1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals.

listed assessment protocols may have widely differing durations (from 28 days to 104 weeks), use various animal models (rats, mice, chickens, macaques, etc.) and investigate very different parameters (growth, pathological effects, biochemical parameters, etc.). This summary concludes that the number of publications specifically devoted to the assessment of the risks related to GMOs remains limited. However, it emphasises the fact that for the first time, a balance seems to be emerging between the number of authors who state that the genetically-modified plants studied are as safe and have the same nutritional properties as their respective conventional non-GM plants, and the number of authors raising concerns. This summary does not take a critical position regarding the validity of the interpretations of the authors cited, though certain among these have been questioned by the scientific world. It states, furthermore, that the studies concluding that GMPs are safe have often been carried out by companies responsible for marketing these GM plants.

A considerable number of studies described in this publication are tests referred to as 90-day subchronic toxicity studies that administer the product through feed, using part of the genetically-modified plant. The purpose of these tests is to assess the toxic potential of GMPs and the safety of subchronic consumption. These studies are based on the OECD's protocol 408, which is used to test the subchronic toxicity of chemicals in rodents. There are about 20 such publications (Annex 5) in the scientific literature. Most of these studies are carried out by major companies or by Contract Research Organisations (CROs) at the request of major companies and are frequently found in MA application dossiers, such as the one by Hammond *et al.* (Hammond *et al.* 2004) for the application concerning NK603 maize.
Three quarters of the GMPs tested are first-generation GMPs, i.e. resistant to insects and/or tolerant to herbicides. The animal groups contain between 10 and 20 subjects, three quarters of these being Sprague Dawley rats. The percentage of inclusion in the animals' diets varies according to the plant species tested. Maximum percentages are often 30% for maize and may reach 60% for rice. According to the authors, none of these studies has revealed any harmful effects on health.

Another review of the literature, published in 2011 in the journal "*Food and Chemical Toxicology*" (Snell *et al.* 2012) made a particular assessment of "the health impact of GMP diets in long-term and multigenerational animal feeding trials". It examined 12 long-term and 12 multigenerational studies. The description of these long-term studies confirms the variation in the protocols, species and results observed by (Domingo and Giné Bordonaba 2011) for all the studies. They involve a wide range of species (rats, mice, dairy cows, salmon, macaques) and the observation period varies from 26 to 104 weeks for species with varying lifetimes. The vast majority of these studies concerned glyphosate-tolerant soybeans, and none were performed on NK603 maize.
The authors of this summary conclude that:

- these 24 studies suggest that there is no particular hazard and that, although differences are sometimes observed between the control animals and the animals fed with the GMPs studied, these can be explained by the range of biological variation naturally observed between individuals of the species. According to the authors, they do not show any evidence of a toxic effect of GMOs,
- none of these studies describe observations that would require further information to be provided in addition to the 90-day subchronic toxicity studies on rodents laid down by OECD guideline 408 (OECD 1998). The 90-day subchronic toxicity studies would seem to be sufficient as a basis for regulatory assessment of GMOs. However, one cannot rule out the possibility that long-term studies should be carried out on a case-by-case basis for regulatory assessments if reasonable doubts persist after an examination of the 90-day subchronic toxicity study.

Protocol for a literature search of relevant studies in the context of this Request and concerning NK603 maize
In view of the questions asked, the literature search focused on identifying all studies concerning NK603 maize. It was broadened to include all genetically-modified plants carrying the CP4*epsps* gene and long-term studies concerning all genetically-modified plants.
The investigation particularly sought any new publications not referenced in previous reviews and likely to provide input to this investigation. Two databases were searched (Scopus© and Medline) with the use of keywords.

The results were compared with the references cited in the summary literature reviews (Domingo and Giné Bordonaba 2011; Snell *et al.* 2012) and ANSES's own literature on the subject. No new publications were identified that had not been previously considered, with the exception of the article by Séralini *et al.* that is the subject of this Request.

Only two studies, carried out over a period close to the average lifetime of the animals concerned, using a glyphosate-resistant GMP (soybeans) and which could therefore be compared to the study by Séralini *et al.* (2012), were identified. These are studies by Sakamoto et al. (Sakamoto *et al.* 2008) and Malatesta et al. (Malatesta *et al.* 2008). ANSES commissioned an English translation of the paper by Sakamoto *et al.* which had been published in Japanese.

3.2.2 A literature search of relevant studies in the context of this Request for glyphosate-based formulations

Several studies were examined during the investigation of the active substance glyphosate in view of its inclusion in Annex I of Directive 91/414/EEC, and the MA application dossiers for the plant protection formulations containing this substance. Annex 3 summarises the data taken into account, some of which, found in the literature, were produced by the American National Toxicology Program (NTP).
In view of the questions asked, the literature search focused on identifying all studies concerning glyphosate-based formulations. It was also broadened to include glyphosate, co-formulants associated with it in ROUNDUP formulations, and long-term studies. The investigation particularly sought any new publications not referenced in previous review and likely to provide input to this investigation. Two databases were searched (Scopus© and Medline) with the use of keywords (ROUNDUP, glyphosate, long term studies, 104-week study, toxicity).

Regarding more specifically the long-term studies (lasting for one year or more), there are no experiments reported in the scientific literature carried out on ROUNDUP GT PLUS, nor on other glyphosate-based formulations, nor with the co-formulant found in the formulation ROUNDUP GT PLUS.

3.3 Critical analysis of the most relevant long-term studies

3.3.1 Description of the studies

3.3.1.1 The study by Malatesta *et al.*

3.3.1.1.1 Objective and study protocol

The study by Malatesta *et al.* (Malatesta *et al.* 2008) examines the effects on the liver of a diet containing 14% genetically-modified soybeans (CP4 EPSPS event) treated with ROUNDUP, administered for two years to female Swiss mice (10 mice per group). It is not a regulatory toxicity study but a study undertaken for research purposes that therefore does not fall within the scope of the OECD guidelines. This study combines a proteomic approach to determine whether a GM diet leads to protein level changes in the liver with the investigation of morphological and cellular parameters. In this framework, the number of mice (ten female mice per group) appears comparable to that used in other publications having the same objective.

3.3.1.1.2 Results

According to the authors, the GM diet did not result in any significant differences in mortality or animal and liver weights. Moreover, no macroscopic alterations, pathologic lesions or onset of tumours were observed in the mice's organs.
As far as proteins were concerned, no differences were observed in the total protein content of the liver and the number of identified proteins, which remained stable (approximately 1400). However, the proteomic analysis found 49 proteins that were expressed differently in the GM-fed mice, including 39 that were up-expressed and 10 down-expressed. Twenty of these proteins were identified by mass spectrometry and were proteins involved in hepatocyte metabolism, stress response, calcium pathways and mitochondrial function. At the morphological level, the authors

report nuclear and mitochondrial changes that appeared only after the first year. The authors conclude that GM soybeans affect the metabolic capacity of hepatocytes and the ageing process. They underline the importance of undertaking research into the long-term effects of a GM diet.

3.3.1.2 The study by Sakamoto *et al.*

3.3.1.2.1 Objective and study protocol

Sakamoto *et al.* (Sakamoto *et al.* 2008) assessed the long-term safety of genetically-modified soybeans (CP4 EPSPS event) in F344 rats fed a diet containing these soybeans at a concentration of 30% for two years. Three groups of rats were used. They were respectively fed a diet containing 30% genetically modified soybeans (50 rats/sex), a diet containing 30% near-isogenic soybeans (50 rats/sex) and a standard soybean-free diet (35 rats/sex). Of the long-term toxicity studies found in the literature, the one by Sakamoto *et al.* is the closest to a regulatory study. Most of the recommendations in OECD protocol 453 were followed, particularly in terms of the large number of animals (50/group).

3.3.1.2.2 Results

At the end of the study, the authors did not note any differences in survival rates, body weights or food intake related to GM soybeans. Some haematological parameters (haemoglobins, haematocrit levels, mean corpuscular haemoglobin concentration) were significantly decreased in the GM group compared to the non-GM group, but these variations were all below 4% and not biologically relevant. The only biochemical (transaminases, creatinine) and organ weight changes involved soybean treatments *versus* a standard diet. The only significant differences in the onset of neoplastic tumours were also observed in animals fed a soybean diet *versus* a standard diet. In conclusion, this study shows that GM soybeans administered to F344 rats for two years did not lead to significant changes compared to rats fed non-GM soybeans. However, a diet made of 30% soybeans may have potentially harmful effects.

3.3.1.3 The study by Séralini *et al.*

3.3.1.3.1 Objective and study protocol

The study by Séralini *et al.* (2012) examined for rats the long-term dietary toxicity of ROUNDUP GT PLUS and a glyphosate-tolerant maize administered via their feed. The glyphosate-tolerant maize used in the study contained the NK603 transformation event.
Over a two-year period, ten groups of ten rats of each sex were fed a diet containing either:

- 33% non-GM maize (control group),
- 11, 22 or 33% NK603 maize not treated with ROUNDUP WEATHER MAX,
- 11, 22 or 33% NK603 maize treated with ROUNDUP WEATHER MAX,
- 33% control maize and drinking water containing three different doses of ROUNDUP GT PLUS.

The authors monitored rat mortality and tumour incidence in each group. They performed histological staining and electron microscopy on organs presenting a pathology during the experiment. The publication presents a discriminant statistical analysis (OPLS-DA[26]) of biochemical blood and urine parameters (measured at 15 months) in the group of females fed 22% NK603 maize not treated with ROUNDUP WEATHER MAX compared to the control group.

3.3.1.3.2 Results

The authors describe earlier and higher mortality and tumour incidence in all of the treated groups compared to the control group for females and in the GM-fed groups for males. The observed pathologies were sex-dependent mainly with mammary tumours and pituitary abnormalities in

[26] Orthogonal Partial Least Squares-Discriminant Analysis

females and pathologies related to the liver, hepatodigestive tract and kidneys in males. Images of histopathological and electron microscopy sections illustrate these results. Ferulic and caffeic acid levels in the rats' diets were lower in the chow (pellet feed) containing NK603 maize. Biochemical data for the female group fed 22% untreated NK603 maize and the control group were analysed using a statistical method that shows that the most discriminant variables for the two groups are kidney related. Blood oestrogen levels were modified. According to the authors, the study's results are due to hormone-dependent food toxicity, non-linear in relation to the dose, having different effects for each sex.

These three study protocols are summarised in the following table:

	Séralini *et al.*	Sakamoto *et al.*	Malatesta *et al.*
PROTOCOL			
Species and Strain	Sprague Dawley rats	F344 rats	Swiss mice
Study period	2 years	2 years	2 years
Number of animals/group/sex	10	4 x 50 and 2 x 35	10
Number of groups	20	6	2
Total number of animals	200	270	20
Sex	Male and female	Male and female	Female
Plant material	NK603 maize	ROUNDUP Ready soybeans	40-3-2 soybeans
Transgene	two CP4-epsps genes	one CP4-epsps gene	one CP4-epsps gene
Protein	two CP4-EPSPS proteins	CP4-EPSPS	CP4-EPSPS
ROUNDUP plant treatment	Depending on the group	Not specified but presence of glyphosate residues	Yes
Tested doses for the various groups (**abbreviations used in the text**)	11%, 22%, 33% NK 603 maize seeds not treated with ROUNDUP **(% GMO)** 11%, 22%, 33% NK 603 maize seeds treated with ROUNDUP WEATHER MAX (3 L/ha) **(% GMO + R)** 50ng/L **(RA)**, 400mg/kg **(RB)**, 2.25 g/L **(RC)** of ROUNDUP GT PLUS in drinking water	30% soybeans	14% ROUNDUP-treated soybeans
Doses in the control groups	33% near-isogenic	30% near-isogenic Soybean-free diets	14% commercial soybeans
Feed composition analysis/balance between the groups	Yes but not shown Hearing: Absence of mycotoxins (< LOQ)	Yes	No
Age at start of experiment	5 weeks	4 weeks	Age at weaning
COLLECTED DATA			
Presence of the transgene in the plant	Yes	Yes	No
Consumption data	Not provided	Yes	No
Growth data	Not provided	Yes	No
Mortality	Yes	Yes	No
Organ weight	No	Yes	The liver only

	Séralini *et al.*	Sakamoto *et al.*	Malatesta *et al.*
Anatomical pathology tests	Yes Unclear number and description of histopathological observations	Yes Clear number and description of histopathological observations (non-neoplastic lesions, number of hepatocellular foci, nephropathies, neoplastic lesions) Histopathologies	No
Other tests	Electron microscopy		Liver examinations: Proteomic analysis Electron microscopy Cell morphometry Immunohistochemistry
Tumour frequency	Yes	Yes	No observed tumours
Onset time	Yes	No	
Biochemical parameters	Yes (haematology, blood and urine biochemistry)	Yes (haematology, blood biochemistry)	No
Organs weight	Not provided	Yes (8 in males and 9 in females)	Yes (liver)
Steroid hormones	Yes in the blood	No	No
Behavioural analysis/clinical follow-up	Twice a week: observation and palpation, recording of clinical signs	Daily observation	No
Ophthalmological tests	Yes	No	No
Statistical data processing	No statistical tests on treatment differences for mortality and pathology incidence. Discriminant analysis based on the OPLS method for biochemical data. No estimation of dose/sex/ROUNDUP GMO effects or calculation of confidence intervals for these effects	Statistical analysis consistent with the OECD recommendations (mean and frequency comparisons, calculation of confidence intervals) For growth, intake, organ weight, biochemical and haematological data Student's t-test for comparing GM and non-GM groups	GMO/Control difference tests for proteomics

3.3.2 Critical analysis of the literature

3.3.2.1 The study by Malatesta *et al.*

This an original study that assesses the effects of GMOs on nonstandard parameters using sophisticated techniques (proteomics and electron microscopy). In this study, the soybeans administered to the control group are poorly defined and are not near-isogenic non-GM soybeans. Moreover, none of the groups received non-herbicide-treated GM soybeans, which could have distinguished effects related to the latter. The analysis focused solely on the detection of liver changes. This study shows the effects of administration for two years of a diet containing GM soybeans on the morpho-functional characteristics of hepatocytes. However, the biochemical blood and urine parameters traditionally assessed to express the harmful effects on this organ for toxicological purposes are not presented.
In the end, in spite of the observed molecular and cellular differences, the Swiss mice that were exposed for 24 months to a diet containing 14% GM soybeans treated with ROUNDUP did not

show signs indicating any carcinogenesis process. That said, this study was undertaken in a line of mice (Swiss) that is known in the literature for developing very few tumours (Annex 6).

3.3.2.2 The study by Sakamoto *et al.*

The study is the closest to a standard carcinogenicity study protocol in rats and uses the recommended number of animals. It can be criticised for not measuring renal function parameters and a lack of information about tumour-onset times.
Regarding the study's experimental protocol, it is not specified whether or not the GM soybeans were treated with a glyphosate formulation. However, the trace amounts (0.1 ppm) of glyphosate detected in these soybeans suggest that this was the case (Sakamoto *et al.* 2007). If so, like in the above study, it is unfortunate that there are no GM groups not treated with a glyphosate formulation in addition to the other groups. In terms of statistical analysis, the methods used are consistent with the OECD's recommendations, with mean and frequency comparisons and a calculation of confidence intervals to compare the group fed the GM soybeans with the control groups (non-GM soybeans and soy-free diet). In the end, no biologically significant differences were observed between the various groups of rats that may reflect harmful effects related to the genetic modification of soybeans.

3.3.2.3 The study by Séralini *et al.*

Considering the subject matter of the Request, this study has been analysed in detail.

3.3.2.3.1 Protocol

A broad study
The study by Séralini *et al.* (2012) was undertaken in an experimental research framework and was not intended to be strictly compared to studies undertaken for the authorisation of products and substances (regulatory studies). Domingo *et al*. (Domingo 2007) confirm that a number of GMO publications are based on studies that do not follow the guidelines recommended in the context of these regulatory studies.

With regard to such research protocols, the study by Séralini *et al.* (2012) is an ambitious study that was undertaken with considerable resources. It is worth highlighting on account of its originality; indeed, very few publications describe work examining both the long-term effects of GMOs and the herbicide to which they are tolerant.
This study is unique in that over this long period and using several doses, it tests both a GMP cultivated with and without treatment by a plant protection product and the complete plant protection formulation by itself. In this respect, no equivalents have been found in the literature. It is also distinctive in that it monitors a large number of blood and urine parameters and the authors indicate that it was undertaken in a GLP environment[27].

The main criticisms that the authorities have made thus far involve a lack of information in the publication about the composition of the feed and the types of tested diets, the choice of doses, the strain of rat, the number of rats per group and the statistical analysis of data.

Missing information
During the hearing, Mr. Séralini's team answered a number of questions regarding data that do not formally appear in the publication. They provided the missing information or offered guarantees in relation to the following:

- The periods and number of crop treatments with ROUNDUP WEATHER MAX and other plant protection products for the maize grown in this study,
- The chemical composition of the seeds and their levels of mycotoxins, glyphosate and its residues,

[27] The claim for GLP status made for the study published by Séralini *et al*. (2012) implies application of the principles listed in the OECD ENV/JM/MONO (2002) document addressing the specific case of studies using multiple sites.

- The composition of the diets and the fact that the feed of the rats in the 11% and 22% groups was supplemented with near-isogenic maize to reach 33% maize in the feed,
- The feed storage method.

The choice of doses

Regarding the doses of GMOs in the feed, which were 11%, 22% and 33%[28], the study protocol is comprehensive and standard. These doses (11% and 33%) correspond to those generally used in regulatory 90-day subchronic toxicity studies in rats.

However, the levels of ROUNDUP GT PLUS administered in the drinking water of the rats raise two points. The first involves the scale of variation between the three tested doses, which range from 50 ng/L to 2.5 g/L, i.e. a multiplicative factor of around fifty million. The gap between the high doses and the low dose is therefore too large to be able to determine a dose-response relationship. The second point involves the relevance of these three doses in terms of exposure in consumers and users of glyphosate formulations.

The three concentrations tested in this study were compared to the available exposure data:

- The first tested dose corresponds to a level that the author describes as the glyphosate contamination level in tap water: 50 ng/L. The regulatory standard in France stipulates no more than 100 ng/L in drinking water. For the water supply, of 43,741 tests that screened for glyphosate (2007-2009 period)[29], only 95 (0.2%) detected quantifiable levels of glyphosate. These quantifiable results were found in a limited number of distribution stations (0.2 to 0.4% of the 21,864 tested stations). 50 ng/L is therefore a realistic value that could potentially be observed but only in a very limited number of French stations. Moreover, these analytical data apply only to glyphosate and not glyphosate combined with co-formulants. The ROUNDUP GT PLUS co-formulant is not mobile in the soil (Koc = 2500 to 9600, DT50 soil = 1-2 days)[30]. The likelihood of finding the tested quantities in groundwater appears negligible.

- The second tested dose corresponds to a contamination level that the author describes as ‘equivalent’ to the US MRL for glyphosate in GM feed (400 mg/kg). In Europe (source: the DG Sanco website), the MRL for glyphosate is set at 0.1 mg/kg for sweetcorn (as a vegetable-fruit) and 1 mg/kg for maize as a cereal. Therefore, the level to which European consumers are exposed is far lower than the level tested in this protocol. Furthermore, as in drinking water, since co-formulants are not systemic, consumers will primarily be exposed to glyphosate and not glyphosate combined with a co-formulant.

- The third tested dose corresponds to a level that the author describes as "half of the minimal agricultural working dilution" (2.25 g/L). Taking into account the concentration of glyphosate in the formulation (ROUNDUP GT PLUS), the quantity to be applied per hectare (data from the French Ministry of Agriculture's E-phy database) and the dilution recommended by the manufacturer, the level of glyphosate in spray mixtures would be approximately 7 g/L, which is the same order of magnitude as the dose tested in the publication. However, users will mainly be exposed to the diluted ROUNDUP GT PLUS formulation through dermal contact and potentially inhalation. The route of administration described in the study protocol (oral exposure) is therefore not the most appropriate for assessing the risks related to the product's application.

[28] Extrapolated to humans, the dose of 33% corresponds to a daily dose that is approximately 40 times higher than average intakes of maize. Monsanto indicates a proportion 84 times higher in its submitted document.

[29] Source: the French Ministry of Health's SISE-Eaux database

[30] JP Giesy, S Dobson and KR Solomon (2000) Ecotoxicological Risk Assessment for Roundup Herbicide, Rev. Environ. Contam. Toxicol., 167:35-120

Number of rats and rat strain
During their hearing, the authors of this study reiterated that one of the study's initial objectives had been to assess the ability of 90-day subchronic toxicity studies to predict the onset of long term effects.
In this framework, it seemed appropriate to choose Sprague Dawley rats, which are the most frequently used rats for this protocol[31] and the choice of ten rats per group was justified because it is commonly used in the context of subchronic toxicity studies.
It should be noted that the number of rats per group is a decisive factor when attempting to prove the safety of a product (regulatory studies) because it determines statistical test power and the probability of detecting an effect. By using a small number of animals per group, the authors ran the risk of not being able to find statistically significant differences between the groups and therefore of having conducted an inconclusive study, considering the study's duration, which was 2 years[32] instead of 90 days, and the susceptibility of the rat strain used.

The data in the literature on Sprague Dawley rats (Annex 6) show high mortality rates and high incidence rates for mammary tumours in control groups, which were the main abnormalities observed by Séralini *et al.* (2012). These phenotypic characteristics should have been taken into account when calculating the required number of animals.

Size and number of control groups
A significant criticism is the use of only one control group and the small number of male and female control animals which considerably limit interpretations of this study. During the hearing, the study's author agreed that this point was unfortunate.

3.3.2.3.2 Results and discussion

For information, the authors describe more and earlier deaths in the female population in all treated groups and 'generally'[33] earlier and larger tumours. They describe liver damage for all of the GM-treated males.
The main criticism of the study's results concerns the lack of statistical data analysis supporting these findings.

The authors simply note that the treated groups were generally more affected than the control group without testing the possibility that these results may have been due to chance. The authors, when asked about this point, indicated that they had simply wanted to report their results, which they had found disturbing, not in the form of a statistical analysis but rather in the form of a description as practised in human clinical research.

> *"Gilles-Éric Séralini's team added that this study corresponded to a research protocol and was not at all intended to be a regulatory test protocol. The results are presented factually for both tumours and mortality. The team did not wish to conduct statistical analyses for these points, as it was keenly aware that with 10 rats per group, tests would not be sufficiently powerful. The team had criticised the Monsanto study for just this. Gilles-Eric Séralini stated that thorough statistical analyses had however been undertaken for biochemical parameters, confirming disturbances that can lead to the observed pathologies" (Extracted from the verbatim report of the hearing with the study's authors)*

Following the hearing, ANSES asked Mr Séralini to submit all of the study's raw data. The authors did not grant this request (see hearing) but submitted quantitative data on mortality and the onset of non-regressive tumours. The ECEAG was able to use some of these data (on mortality) in addition

[31] OECD protocols 452 and 453 are not prescriptive with regard to the rat strain to be used and while the Fischer 354 strain is often used in carcinogenicity studies, mainly because it is the best known, the Sprague Dawley strain is currently being evaluated by the National Toxicology Program (NTP).
[32] As two years is a rat's average lifespan, a high percentage of animals risk dying before the end of long-term studies.
[33] almost always more often

to those available in the publication (Figure 1 and Table 2) to determine the significance of certain results.
In order to increase the probability of detecting effects, the ECEAG first undertook one-tailed tests[34] considering Type I error risks[35] of 5% without taking the effect of multiple testing into account. This approach increases statistical power and yet also increases the risk of false positives (false discovery). It is considered the most favourable statistical test for highlighting a maximum number of effects that need to be interpreted biologically. The statistical tests were then corrected so as to limit risks of false discoveries (FDR (False Discovery Rate) control or correction)[36] (Benjamini and Hochberg 1995). Indeed, undertaking multiple statistical tests on a given dataset rapidly increases the rate of Type I errors, i.e. the probability of observing falsely significant differences.

Three series of statistical tests were undertaken by the ECEAG:
The first series of statistical tests aimed to determine whether there were significant differences in mortality rates at the end of the study between the control group of rats and the GMO and/or ROUNDUP groups of rats[37]. These tests compared the null hypothesis H0 'Mortality rate in the control group = Mortality rate in the GMO and/or ROUNDUP groups' and the alternative hypothesis A 'Mortality rate in the control group < Mortality rate in the GMO and/or ROUNDUP groups'. This series of tests was undertaken using the data extracted from Figure 1 of the study by *Séralini et al. (2012).* The risk of a Type 1 error (probability of wrongly rejecting H0) was calculated by conducting Fisher's exact test on 2 dead rats out of the 10 rats of the female control group and 3 dead rats out of the 10 rats of the male control group. The results (Table 1) show that the differences in mortality rates are significant at the 5% level before correction (FDR) for two in 18 groups of rats:

- for the female group, GMO at the 22% dose,
- and for the female group, GMO + R at the 22% dose.

When taking multiple testing into account (FDR), no significant differences are found.

Table 1. Results of statistical tests (P=probability of a Type I error) on mortality rates. *The P values have not been corrected to take multiple testing into account. When these corrections are applied, no differences are significant.*

	Males		Females	
Group	Mortality rate	P-value	Mortality rate	P-value
Control	3/10	NA	2/10	NA
GMO 11%	5/10	0.3250	3/10	0.5
GMO 22%	1/10	0.9567	7/10	***0.0349***
GMO 33%	1/10	0.9567	4/10	0.3142
GMO 11%, R	4/10	0.5000	4/10	0.3142
GMO 22%, R	5/10	0.3250	7/10	***0.0349***
GMO 33%, R	3/10	0.686	4/10	0.3142
RA	3/10	0.686	5/10	0.1749
RB	4/10	0.5000	5/10	0.1749
RC	1/10	0.9567	4/10	0.3142

The second series of tests aimed to determine whether the rats in either of the GMO and/or ROUNDUP groups died earlier than the rats in the control group. The ECEAG used the Log-Rank test for this purpose. This test compared survival probabilities for the various groups. Three series of comparisons were conducted successively: Control *vs.* GMO alone, Control *vs.* GMO treated with ROUNDUP WEATHER MAX, Control *vs.* ROUNDUP GT PLUS. The tests were undertaken with

[34] Tests that assume that a difference will be in a particular direction. For this analysis, the assumption is that the groups treated with GMOs or ROUNDUP are likely to have adverse effects but not positive effects.
[35] Probability of wrongly rejecting hypothesis H0, which is the hypothesis that there are no differences between the groups.
[36] A procedure that controls the risk of false positives related to a high number of tests undertaken with the same data.
[37] Tested doses in the groups:
11%, 22%, 33% NK 603 maize seeds not treated with ROUNDUP **(% GMO**)
11%, 22%, 33% NK 603 maize seeds treated with ROUNDUP WEATHER MAX (3 L/ha) **(% GMO + R**)
50ng/L **(RA)**, 400mg/kg **(RB)**, 2.25 g/L **(RC)** of ROUNDUP GT PLUS in drinking water

and without correction (Sidak correction) for the number of comparisons made per series using the raw mortality data submitted by the author after his hearing. The results (Table 2) show that there are two significant differences out of 18 with the uncorrected tests:

- for the female group, GMO at the 22% dose,
- and for the female group, GMO + R at the 22% dose.

After correction, no differences are significant.

Table 2. Results of Log-Rank tests on reduced life expectancies with and without correction for multiple testing.

Comparison		Males		Females	
		Uncorrected	Corrected (Sidak)	Uncorrected	Corrected (Sidak)
GMO 11%	Control	0.661	0.9999	0.4522	0.9956
GMO 22%	Control	0.2357	0.911	**0.0159**	0.1341
GMO 33%	Control	0.0907	0.5751	0.122	0.6899
GMO 11%, R	Control	0.4797	0.9972	0.3233	0.9702
GMO 22%, R	Control	0.8953	1	**0.0448**	0.3378
GMO 33%, R	Control	0.7233	1	0.4666	0.9965
RA	Control	0.5778	0.9996	0.0841	0.5464
RB	Control	0.3179	0.968	0.239	0.9144
RC	Control	0.084	0	0.2501	0.925

The third series of tests aimed to determine whether the frequency of pathologies was higher in the GMO and/or ROUNDUP groups than in the control group. These tests compared the null hypothesis H0 ‘Frequency of pathologies in the control group = Frequency of pathologies in the GMO and/or ROUNDUP groups' with the alternative hypothesis A ‘Frequency of pathologies in the control group < Frequency of pathologies in the GMO and/or ROUNDUP groups’. These tests were undertaken using Table 2 of the study by Séralini *et al.* (2012) for the six listed pathologies with the six GMO treatments (three doses of GMO without ROUNDUP + three doses of GMO with ROUNDUP WEATHER MAX) and the three ROUNDUP GT PLUS treatments. The Type I error risk was calculated separately for each pathology and each diet with Fisher’s exact test. The results are shown in Table 3 below.

Of the 54 comparisons, five are significant at a level of 5% before FDR correction.

- ‘hepatic pathologies’ described by the author as liver congestions, macroscopic spots and microscopic necrotic foci
  - for the males in the group fed 22% GMO,
  - for the males in the RB group.
- mammary tumours
  - for the females in the RB group.
- pathological signs in the mammary glands (other than tumours described by the authors as galactoceles and mammary hyperplasias)
  - for the females in the RA group,
  - for the females in the RB group.

After FDR correction for multiple testing, there are no significant differences at the 5% level.

Table 3. Results of statistical tests (P = probability of a Type I error) on the incidence of pathologies (percentage of animals with at least one tumour or pathological lesion). The probabilities have not been corrected to take multiple testing into account. When these corrections are applied, no differences are significant at the 5% level.

Organs and associated pathologies	GMO 11%	GMO 22%	GMO 33%	GMO 11% + R	GMO 22% + R	GMO 33% + R	RA	RB	RC
Males, in liver	0.31	**0.035**	0.085	0.31	0.31	0.18	0.18	**0.035**	0.18
In hepatodigestive tract	0.5	0.33	0.5	0.5	0.5	0.33	0.07	0.18	0.67
Kidneys, CPN	0.5	0.33	0.09	0.33	0.5	0.5	0.19	0.33	0.67
Females, mammary tumours	0.33	0.33	0.18	0.5	0.33	0.07	0.07	**0.02**	0.07
In mammary glands	0.18	0.33	0.18	0.18	0.18	0.07	**0.02**	**0.02**	0.07
Pituitary	0.15	0.31	0.82	0.15	0.91	0.5	0.31	0.5	0.5

In general, more specific information about the observed pathologies is required to determine the biological significance of the statistical results before correcting for multiple testing as recommended, particularly in the ANSES report (ANSES 2011). The ECEAG considers it unfortunate that the definitions of the groups of pathologies described in the publication are unclear and that there are so few useable biochemical data. Nonetheless, the statistical analysis results as a whole show that:

- The increased mortality and reduced life expectancy (Tables 1 and 2) observed for the females in the GMO 22% and GMO 22% + R groups (before correction) are not confirmed by any underlying pathologies (Table 3). This finding is striking and additional information on the cause of death for each animal in these groups would be necessary to interpret it.
- The increase in pathologies highlighted in the publication is significant at a level of 5% (before correction) for only a small number of treatments and is difficult to interpret from a biological standpoint due to the unclear definitions of the pathologies.
- The increase in the incidence of hepatic pathologies in the ‘GMO 22% male' group (before correction) is not found at the 11% and 33% doses nor for the ‘GMO 22% + R male’ group. This result does not appear coherent since it occurs at a single intermediate dose and is not found in a group fed the same percentage of GM maize.
- The increase in hepatic pathologies observed in males for the RB dose of ROUNDUP GT PLUS (before correction) may be consistent with the LOAEL for glyphosate. However, the other pathologies (mammary tumours, galactoceles and mammary hyperplasia) observed in females at the RB dose do not appear consistent with the toxicological data on glyphosate (long-term studies in rodents). Furthermore, none of these effects are found at the highest dose (RC). This finding does not support biological coherence even though it could be expected that this high dose would interfere with the eating behaviour of rats. It would be useful to have data on the water and feed consumption of the treated animals.
- The significant increase in the frequency of mammary gland pathologies (excluding tumours) (Table 3) observed at the lowest dose of ROUNDUP GT PLUS (RA) (before correction) caught the attention of the ECEAG and may suggest an unexpected effect at a very low dose. However, in order to determine a biologically significant effect, it is necessary to have individual data, comprehensive biochemical data and historical data on the SD strain provided by the CRO.

Conclusions on study results

The significant results obtained before correction are not biologically coherent overall. However, biological data on the results would be needed to draw a definitive conclusion. At this point in time, in light of the information provided in the publication, the ECEAG's experts consider that the authors' interpretations are not sufficiently corroborated by the study data.

Moreover, during the hearing, the study's authors admitted that this study was not conclusive by itself and that, though subject to improvement, it had the merit of opening up an interesting line of research.

> *"The team's members firmly believe that, having used all techniques available, what they observed was not random. The study could certainly be improved but the team simply opened up a path and we must now collectively do better. "For Gilles-Éric Séralini's team, there is endocrine disruption because there is disruption of testosterone/oestradiol ratios and female pituitary glands in particular. These experiments need to be repeated since this was the first time that tests were undertaken with a pesticide as a whole at a low dose" (Extracted from the verbatim report of the hearing with the study's authors)".*

3.3.2.3.3 Assumptions

The mechanistic assumptions put forth by the authors are not corroborated by results and are therefore speculative. The ECEAG's members nonetheless considered it would be worthwhile to further discuss the merits of these assumptions.

Plausibility of an endocrine disrupting effect and low-dose effects

The assumption put forth by Séralini *et al.* (2012) to explain the development of mammary tumours in females is a mechanism of action related to endocrine disruption. According to the authors, this assumption is based on:

- variations in circulating levels of oestradiol and testosterone in the females in the treated groups,
- the onset of tumours in hormone-sensitive tissues (mammary and pituitary glands) in the treated groups,
- the results of prior studies published by the same team reporting *in vitro* effects on aromatase (an enzyme that converts testosterone to oestradiol) activity with ROUNDUP and those published by other authors (Romano *et al.* 2012; Romano *et al.* 2010; Walsh *et al.* 2000) reporting the effects of ROUNDUP on steroidogenesis, reproduction and development,
- relatively low levels of caffeic and ferulic acids in foods made with genetically-modified maize, which could lead to endocrine disruption,
- non-monotonic dose-response curves considered a characteristic of endocrine disruption

A close examination of the publication indicates that this assumption is not sufficiently corroborated by the study results. Indeed, judging by Figure 5 and Table 3, which show circulating levels in female rats at 15 months, the reported values do not indicate any significant effects for the treatments and there is no link between the observations made for the pituitary and mammary glands. Moreover, it should be noted that hormone levels in female rats vary considerably over the oestrous cycle and depending on the time of sampling during the day, which makes it difficult to interpret the data without having precise experimental details on the sampling conditions. Other hormones (*e.g.* prolactin, LH, FSH), hormone-sensitive tissues (testicles, ovaries, adrenal glands) and enzymatic activities involved in steroidogenesis would need to have been examined to draw any conclusions. Although the assumption of endocrine disruption with ROUNDUP has already been described in the literature (Romano *et al.* 2012; Romano *et al.* 2010), this article offers no evidence of these effects. Furthermore, on the basis of current knowledge, it is difficult to agree with the arguments put forth by Séralini *et al.* regarding an endocrine disrupting mechanism to explain (unproven) effects on mammary tumours related to the consumption of NK603 maize without exposure to a glyphosate formulation. Although most endocrine disruptors have effects that do not correspond to a monotonic curve, the lack of a dose-response relationship for the 'GMO' and 'GMO + R' groups can in no case be regarded as evidence of endocrine disruption.

Plausibility of effects related to secondary metabolites

In addition to its 'own' EPSP synthase, NK 603 maize contains a bacterial EPSP synthase (encoded by two copies of the CP4 EPSPS gene from *Agrobacterium tumefaciens*). This bacterial enzyme is glyphosate-tolerant and involved in a very early stage of the so-called 'shikimic acid' pathway (Annex 8). The authors suggest disruptions to the secondary metabolism of plants caused by genetic modification. Changes in the chemical composition of GMPs do indeed have to be documented as part of authorisation applications. In this context, each application must include a comparative analysis of the chemical composition of the GMP and that of its non-GM control.

Thus, differences in levels of certain secondary metabolites, and particularly phenolic acid metabolites, are noted and highlighted by the authors. These metabolites can have protective or endocrine disrupting effects.

The data presented in the article involve two types of compounds measured in rat diets (chow - pellet feed): isoflavone phyto-oestrogens and two phenylpropanoids: caffeic acid and ferulic acid. The authors indicated during the hearing that they had other data for other compounds (e.g. tocopherols) that had not yet been made available to the scientific community.

Regarding isoflavones: the authors did not observe any differences between the diets used in the experiments for these compounds. Isoflavone levels in maize are extremely low (< or << 100 µg/kg) (Kuhnle *et al.* 2009). Isoflavones are compounds that are known for being *selective oestrogen receptor modulators* (which largely explains their so-called 'phyto-oestrogen' properties). They are characteristic of food plants such as soybeans, yet their levels in maize are not in principle high enough to explain any protective or endocrine disrupting effects (AFSSA/AFSSAPS 2005)[38].

The phenylpropanoids measured in the study are caffeic acid and its *O-methyl* counterpart, ferulic acid. The latter has higher levels in maize by far (~ 90% of the total phenol content, ~ 50 times more than caffeic acid). Ferulic acid is a secondary metabolite that has traditionally been measured to compare the chemical composition of GM maize and its controls, together with other compounds (phytic acid, trypsin inhibitors, vitamin E, coumaric acid and raffinose). Maize can be described as a plant that is rich in ferulic acid (total ferulic acid content of around 1-3 g ferulic acid/kg of maize, dry-weight) (Ridley *et al.* 2002); (De La Parra *et al.* 2007) (OECD 2002). The levels reported by the authors (and measured in the diets containing 33% maize), once extrapolated to the levels in the initial maize, are consistent with the data in the literature.

In the specific case of NK603 maize, Ridley *et al*. (Ridley *et al.* 2002) analysed the two types of maize (NK603 *vs* non-GM control). With an average level of 2 g/kg, concentrations ranged from 1.5 to 2.5 g/kg (NK603) and from 1.7 to 2.3 g/kg (non-GM control). Ferulic acid levels in this maize therefore vary naturally by approximately 40%. In conclusion, the 16-30% difference described in the publication between the groups of feed corresponds to the 'natural' variability of this compound in maize.

The possible protective role (particularly in the liver) of ferulic acid (and numerous similar compounds) shown in a series of studies can be noted. For example, a protective effect has been found against mammary tumours induced by 7,12-dimethylbenz[*a*]anthrecene in Sprague Dawley rats (Baskaran *et al.* 2010). However, none of the scientific data currently available in the study by Séralini *et al.* support a protective role of dietary ferulic acid in rats or a supposedly harmful effect related to a 16-30% decrease in ferulic acid as observed in the study. More generally, it is difficult to assess the role of such a substance due to the multiple pleiotropic biological properties for which chemopreventive potential is often claimed with no solid epidemiological data.
To further study such assumptions on the effects of these natural substances, it would have been wise to have a comprehensive study on the composition of maize and diets (chow - pellet feed) containing the secondary metabolites that are commonly evaluated for maize.

[38] Refer to the 2005 joint AFSSA/AFSSAPS report "*Safety and benefits of dietary phyto-oestrogens – recommendations*", which can be viewed at the following address:
http://www.afssa.fr/Documents/NUT-Ra-Phytoestrogenes.pdf.
The recommendations are a maximum daily intake of 1 mg/kg body weight of isoflavone equivalents in humans.

Conclusion to section 3.3

In conclusion, after critically examining the relevant publications in the framework of this Request, the ECEAG notes the lack of publications involving long-term toxicological studies on formulated plant protection products and the limited number of publications on the long-term effects of GMPs. The publication by Séralini *et al.* (2012) combines these two approaches. Its major weakness is that in order to do so, it reduced the number of control groups and animals in each group. The results on mortality and tumour incidence are presented descriptively and are not statistically analysed. The authors thus make interpretations that are not supported by the study's data. The assumed mechanisms proposed by the team of Séralini *et al.* (2012) to explain the results were not confirmed by the ECEAG's analyses.

The two long-term studies on GMPs identified by the ECEAG (Malatesta *et al.* 2008; Sakamoto *et al.* 2008) do not offer evidence of GMP-related effects comparable to those described by Séralini *et al.* (2012) (onset of tumours and increased animal mortality). However, it should be noted that these study results cannot be fully applied to the Séralini study, since they were not conducted with maize but with glyphosate-tolerant soybeans, even though this tolerance was obtained through the synthesis of a CP4 EPSPS protein, like for NK603 maize. Furthermore, for Malatesta *et al.* (Malatesta *et al.* 2008), the study was conducted on a limited number of animals, a different species and only on female mice.

3.4 Conclusions drawn by the ECEAG

Séralini *et al.* (2012) conducted an ambitious study, employing considerable research resources, that was published in an internationally recognised food toxicology journal. This study is commendable for having addressed novel issues.

However, upon examination, the ECEAG experts consider that the authors' conclusions are not sufficiently supported by the data presented in the paper. Furthermore, the analysis provided by the ECEAG does not confirm the hypotheses on the mechanisms of action that the authors formulated to explain their results.

As a result, the ECEAG experts conclude that the results of the study as they have been published do not challenge the conclusions from previous risk assessments of NK603 maize and the use of ROUNDUP herbicide. This study cannot therefore be regarded as conclusive as to the potential health risk of food products derived from NK603 GM maize or of ROUNDUP.

Nevertheless, the ECEAG experts note the lack of studies on the potential effects of long-term exposure to various glyphosate-based formulations and the limited number of studies that have addressed the long-term effects of consuming GMOs.

Regarding the issue concerning revisions of GMO and plant protection product assessment principles , the ECEAG considers that it is too early to issue recommendations, which cannot in any case be based on a single study.

Regarding GMOs, the ECEAG experts note that there has been a gradual improvement in safety assessment criteria and standards; in particular, the strengthening of the substantial equivalence approach by implementing subchronic toxicity feeding studies on animals. However, whether current assessment methods can detect potential long-term effects and the plausibility of these effects are subjects of controversy in the scientific community. Given that there are so few studies documenting these effects, it is difficult to overcome this controversy. The ECEAG deems that these issues should be debated, especially in regard to the growing and foreseeable complexity (GM stacked events) of genetically-modified plants. The ECEAG experts therefore feel that it is necessary to deliberate further on whether the scientific principles for evaluating safety should be revised and this deliberation should be based on all the studies that have been conducted on a national level, particularly by ANSES, but also on an international level[39].

[39] In particular, current research projects (GRACE FP7-KBBE project, Project reference: 311957).

Regarding plant protection products, the regulations for placing these products on the market do not require long-term studies for commercial formulations. In particular, cumulative effects can be addressed using methods that are currently being developed in Europe. These methods are intended for application in studies on the cumulative effects of active substances present in the same formulation, particularly on exposed workers. The ECEAG experts consider that it would be appropriate to apply these methodologies to co-formulants, especially those, given their properties, for which toxicity reference values have been set. The experts also consider that more methodological research on the ‘cocktail effect' of formulations is needed.

4. AGENCY'S CONCLUSIONS AND RECOMMENDATIONS

ANSES endorses the conclusions and recommendations made by the ECEAG.

ANSES recalls its recent work on issues underlying risk assessment of GMOs and its methodological approaches for these assessments. Accordingly, as part of an innovative approach on the European level, ANSES issued an Opinion in 2011 recommending more rigorous conditions under which 90-day subchronic toxicity studies should be carried out, and proposed a very strict data analysis methodology. A draft European regulation is being finalised and was submitted to Member States in spring 2012; it requires that 90-day feeding studies be carried out using the conditions advocated by ANSES.

Regarding plant protection products, ANSES has actively participated in methodological developments at the European level to more effectively address the cumulative effects of active substances. These methods are currently being included in European safety assessment standards. They are intended for the study of cumulative effects of active substances and co-formulants.

Moreover, ANSES has put considerable effort into addressing the questions underlying the *Séralini et al.* study. Over the past few years, ANSES has carried out wide-reaching studies on endocrine disruptors and more generally on the issue of low doses. In addition, as part of the Périclés programme, ANSES research has also addressed the mixture effect of xenobiotics and the identification of their potential synergistic effects ("cocktail effects").

In general, the fact that publication of a study on the potential long-term effects of a GMO associated with a common plant protection product has sparked such an active public debate shows that more scientific knowledge is required in this area.
This debate is part of a wider scientific context that includes other diverse studies. On the one hand, there are studies funded by industry to meet regulations, and on the other hand, there is publicly-funded research, with more limited resources, that seeks to investigate potential health effects that have been little documented thus far. Although this situation is not specific to GMOs, GMOs attract considerable public attention and there is a particularly acute public desire for independent, objective research.

Thus, more generally speaking, ANSES calls for more public funding on the national and European levels for broad-scope studies to consolidate scientific knowledge on insufficiently documented health risks.

In light of the needs for studies and research highlighted by the ECEAG, ANSES recommends:

- more research on the potential health effects associated with the long-term consumption of GMOs or long-term exposure to plant protection products. This research should focus in particular on the issue of exposure to GMOs and to residues of associated plant protection preparations. These studies should be conducted using public funds and based on precise research protocols that address specific questions (investigated effects, monitored parameters, research methodology, number and nature of animals studied, complexity of the GMO, type of exposure, etc.). ANSES is prepared, along with other partners, and particularly other European health agencies, to work toward defining the general principles for these study protocols;
- and more broadly speaking, fostering research on the health issues associated with chronic exposure to xenobiotics (active substances, co-formulants), their mixtures and their potential interactions, especially regarding their effects when combined with GMOs.

The Director General

Marc Mortureux

KEY WORDS

GMOs, plant protection products, NK603, ROUNDUP, long-term study

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ANNEXES

Annex 1

Members of the emergency collective expert assessment group (NK603-R ECEAG)

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Ms Marie-Anne ROBIN – Research director, INSERM, Rennes

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Annex 2

OECD standards for long-term studies and Good Laboratory Practice

It is important to distinguish between non-carcinogenic and carcinogenic chronic effects. Three OECD guidelines correspond to these effects:

- OECD 451 – Carcinogenicity studies

The purpose of long-term carcinogenicity studies is to observe test animals over most of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration. This guideline is intended primarily for use with rats and mice, and for oral administration. Both sexes should be used. Each dose group and concurrent control group should contain at least 50 animals of each sex. At least three dose levels and a concurrent control should be used. The test substance should be administered daily to animals via the oral route (or via dermal or inhalation administration) and the mode of exposure should be adjusted according to the toxicokinetic profile of the test substance. The duration of the study will normally be 24 months for rodents. For specific strains of mice, a duration of 18 months may be more appropriate. Termination of the study should be considered when the number of survivors in the lower dose groups or the control group falls below 25 percent. The results of these studies include measurements (weighing, food consumption), and, at least, daily and detailed observations, as well as gross necropsy and histopathology.

- OECD 452 – Chronic toxicity studies

The purpose of chronic toxicity studies is to characterise the profile of a substance in mammalian species (primarily rodents) following prolonged and repeated exposure. The guideline focuses on rodents and oral administration. Both sexes should be used. For rodents, at least 20 animals per sex per group should normally be used at each dose level, while for non-rodents a minimum of 4 animals per sex per group is recommended. At least three dose levels should be used in addition to the concurrent control group. Frequency of exposure is normally daily, but may vary according to the route chosen (oral, dermal or inhalation) and should be adjusted according to the toxicokinetic profile of the test substance. The duration of the exposure period should be 12 months. The study report should include measurements (weighing) and regular detailed observations (haematological examination, urinalysis, clinical chemistry), as well as necropsy procedures and histopathology.

- OECD 453 – Combined chronic toxicity/carcinogenicity studies

The objective of a combined chronic toxicity/carcinogenicity study is to identify carcinogenic and chronic effects in mammalian species, and to determine dose-response relationships following prolonged and repeated exposure. The rat is typically used for this study. For rodents, each dose group and concurrent control group intended for the carcinogenicity phase of the study should contain at least 50 animals of each sex, while for the chronic toxicity phase of the study they should contain at least 10 animals of each sex. At least three dose levels should be used, in addition to the concurrent control group for both the chronic toxicity phase and the carcinogenicity phase of the study. The three main routes of administration are oral, dermal, and inhalation. The guideline focuses on the oral route of administration. The duration of the study is normally 12 months for the chronic toxicity phase, and 24 months for the carcinogenicity phase. The study report should include measurements (weighing) and regular detailed observations (haematological examination, urinalysis, clinical chemistry), as well as necropsy procedures and histopathology. All these observations enable the detection of neoplastic effects and the determination of carcinogenic potential as well as general toxicity.

For active plant protection substances, the OECD 453 guideline is used in order to assess chronic and carcinogenic effects in a single study. A study in rats and a study in mice are required.

- Good Laboratory Practice (GLP)

Initially developed by the US Food and Drug Administration (FDA) in 1976 and then adopted by the OECD in 1978, the principles of Good Laboratory Practice (GLP) make up an organisational process that covers all organisational and operational aspects of the non-clinical safety testing of chemical products. Their objectives are to guarantee the quality, reproducibility and integrity of data generated for regulatory purposes so they may be accepted on an international level without duplicative testing.

For the European Union, GLP principles are defined in Directive 2004/10/EC. Study compliance with GLP principles is ensured through national programmes to verify studies and the inspection of testing laboratories. In Europe, the inspection and verification of Good Laboratory Practice are addressed in Directive 2004/9/EC.

The specific case of studies involving multiple sites is covered by special provisions (OECD ENV/JM/MONO(2002)9).

Annex 3

Prior assessments of ROUNDUP™ GT plus

Roundup GT Plus is the formulation that was used in the Séralini *et al.* (2012) study for administration in drinking water. The active ingredient in this formulation is glyphosate (in isopropylamine salt form).

Glyphosate is an active ingredient that was approved for use in Europe in 2001 and reference values were set at that time. The EU is currently reassessing the safety of glyphosate. Germany is the Rapporteur Member State and is responsible for reviewing all the regulatory toxicological data and the data found in the literature. This reassessment started in May 2012 and will be made available to EFSA[40] and the other Member States in June 2013.

In the assessment report on glyphosate and its addendum, the following studies, submitted by the many notifiers employing this active ingredient, were reviewed by the German authorities. This assessment report was also reviewed by experts in other Member States.

Glyphosate as the active ingredient:

- kinetics (absorption, distribution, metabolism, elimination): 12 studies, including 3 from the literature on glyphosate or ROUNDUP.
- acute oral toxicity in rats, mice: more than 20 studies.
- acute dermal toxicity in rats and rabbits: 15 studies.
- acute toxicity by inhalation in rats: 9 studies.
- skin irritation in rabbits: 12 studies.
- eye irritation in rabbits: 11 studies.
- skin sensitisation in guinea pigs: 9 studies.
- subacute oral toxicity: in rats (3 studies over 28 days), mice (1 study over 30 days) and dogs (2 'range-finding' studies).
- subchronic oral toxicity: 9 studies in rats (90 days) including one from the literature conducted by the NTP[41], 3 studies in mice (90 days) including one from the literature conducted by the NTP, 6 studies in dogs (durations of 3 months to 1 year).
- subacute dermal toxicity: 3 studies in rabbits (21 or 28 days), 1 study in rats (21 days).
- subacute toxicity by inhalation: 1 14-day study in rats and 1 literature review on 28-day studies in rats with ROUNDUP.
- *in vitro* genotoxicity studies: 9 Ames tests, 2 chromosomal aberrations assays, 1 genetic mutation test on mammal cells, 6 DNA repair tests.
- *in vivo* genotoxicity studies: 3 micronucleus or chromosomal aberration assays in rats or mice, 3 germ cell tests (dominant lethal tests).

40 EFSA: European Food Safety Authority
41 NTP: National Toxicology Program
http://ntpsearch.niehs.nih.gov/query.html?qt=glyphosate&col=015abst&col=020rpt&charset=iso-8859-1

The studies show that glyphosate does not have any genotoxic properties *in vivo*.

- Chronic and carcinogenesis studies: 4 studies in rats and 4 studies in mice, summarised below:

Species/Duration	Doses	NOEL[42]/NOAEL[43]	Target organ	Reference
Wistar Rat/2 years 50 rats of each sex in each group.	0 – 100 – 1000 – 10,000 ppm	NOAEL: 1000 ppm (60 mg/kg/day) NOEL: 100 ppm (6.3 mg/kg/day)	Liver damage (biochemical indications). Cataracts (weak evidence) These effects are observed at 10,000 ppm (LOAEL). At 1000 ppm (LOEL), the observed effects on alkaline phosphatase were not consistent throughout the study.	Suresh, 1996
Sprague Dawley rats/2 years 85 rats of each sex in each group.	0 – 10 – 100 – 300 mg/kg/day	NOEL: 10 mg/kg/day	Salivary glands (histological effects). Mild hepatic toxicity These effects are observed from 100 mg/kg/day.	Atkinson *et al.*, 1993
Sprague Dawley rats/2 years 60 rats of each sex in each group.	0 – 2000 – 8000 – 20,000 ppm	NOEL: 2000 ppm (89 mg/kg/day)	Cataracts, mild liver damage at 20,000 ppm. Gastric inflammation at 8000 ppm (LOEL).	Stout and Ruecker, 1990
Sprague Dawley rats/26 months 50 rats of each sex in each group.	0 – 3 – 10 – 31 mg/kg/day in male rats. 0 – 3.4 – 11 – 34 mg/kg/day in female rats.	NOEL: 31 mg/kg/day	No treatment effects observed.	Lankas, 1981
CD-1 mice/2 years 50 mice of each sex in each group.	0 – 100 – 300 – 1000 mg/kg/day	NOAEL: 1000 mg/kg/day	No treatment effects observed.	Atkinson *et al.*, 1993
Balb/c mice/18 months 25 mice of each sex in each group.	0 – 75 – 150 – 300 ppm	NOAEL: 150 ppm (15 mg/kg/day)	Decrease in weight gain and in food consumption at 300 ppm.	Bhide, 1988 *
CD-1 mice/2 years 50 mice of each sex in each group.	0 – 1000 – 5000 – 30,000 ppm	NOEL: 1000 ppm (157 mg/kg/day)	Liver damage (30,000 ppm) and bladder damage (5000 ppm) observed only in males.	Knezevich and Hogan, 1983
CFLP/LATI mice/18 months 50 mice of each sex in each group.	0 – 100 – 300 ppm	NOAEL: 300 ppm (30 mg/kg/day)	No treatment effects observed.	Vereczkey and Csanyi, 1982 (rev. 1992)

*Study not appropriate for evaluating carcinogenic effects.

None of these studies show any significant increase in the incidence of tumours in animals treated with glyphosate.

[42] NOEL: No observed effect level
[43] NOAEL: No observed adverse effect level

- Studies on reproductive functions: 2 studies on one generation in rats, 3 studies on two generations in rats, 3 studies on three generations in rats, 1 literature study of the specific effects on fertility.
- Developmental toxicity studies: 5 studies in rats, 5 studies in rabbits and 1 study in mice.

The results of these studies show that glyphosate does not lead to any alteration in reproductive functions.

Studies have also been carried out on AMPA[44], which is the main metabolite of glyphosate:

- Acute toxicity by three exposure routes, dermal irritation and eye irritation, dermal sensitisation, subacute and subchronic studies (4 studies in rats for 14 to 90 days, 2 studies in dogs for 1 month and 90 days). There are also 3 studies on developmental toxicity.

Based on these studies, the following reference values (expressed in dose of active ingredient) were derived:

- ADI: 0.3 mg/kg/day
- ARfD: not applicable
- AOEL: 0.2 mg/kg/day

Based on these figures, the hazard classifications of glyphosate and its salts were determined by a European expert group for classification and labelling.
The harmonised European classifications according to Regulation (EC) no. 1272/2008 are the following:

For glyphosate acid:
H318 Eye Irritant Cat. 1 (R41 in the former classification system)
H411 Aquatic Chronic 2 (R51/53 in the former classification system)

For glyphosate salts:
H411 Aquatic Chronic 2 (R51/53 in the former classification system)

Glyphosate-based formulations
Fourteen different preparations were studied in the EU assessment report. For most of these formulations, the required data were provided: acute toxicity by three administration routes, dermal irritation and eye irritation, dermal sensitisation, dermal absorption (*in vitro* on human and monkey skin, *in vivo* in monkeys).
*In vitro g*enotoxicity studies have also been carried out with formulations containing glyphosate and a surfactant (Williams 2000[45]): 3 Ames tests, 2 tests on *Drosophila*, 1 chromosomal aberration assay, 2 sister chromatid exchange tests.
Some *in vivo* genotoxicity studies are also available: 6 micronucleus tests. There are also 3 tests on DNA effects.
The results do not indicate that Roundup formulations have genotoxic properties.

Co-formulants used in ROUNDUP formulations
A summary document from the US EPA (2009)[46] reviews the main toxicological studies available on ROUNDUP co-formulants. These co-formulants have also been assessed by ANSES as adjuvants in herbicide sprays.
The toxicological dossiers of these substances include acute toxicity studies, studies on irritation, sensitisation, *in vitro* genotoxicity studies (Ames tests, mutagenicity and chromosomal aberrations),

[44] AMPA: aminomethylphosphonic acid
[45] Williams GM, *et al.* (2000) Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology* **31**(2 I), 117-165
[46] US-EPA proposed the following TRVs (source: http://www.epa.gov/fedrgstr/EPA-PEST/2009/June/Day-17/p14113.pdf)

subchronic toxicity studies for 90 days in rats (2 studies) and in dogs (1 study), 2 screening studies for reproductive toxicity properties in rats.

By cross-examining the data available for other adjuvants considered to be equivalent, the reference toxicological values were determined for these adjuvants:

- ADI: 0.15 mg/kg/day
- ARfD: 0.72 mg/kg
- AOEL: 0.15 mg/kg/day

Chemical formulation of ROUNDUP GT Plus

The formulation of ROUNDUP GT Plus contains isopropylamine salt of glyphosate, a co-formulant and water. AFSSA assessed this formulation in 2006 for use in home gardens as a weed-killer before planting, the use intended by the manufacturer.

The results relating to human health risks presented in the AFSSA opinion of 16 April 2007 are as follows:

Regarding toxicological properties

The acceptable daily intake (ADI) of glyphosate acid is 0.3 mg/kg/day, a value that was set when glyphosate was included in Annex I of Directive 91/414/EEC. This ADI was derived by applying a safety factor of 100 to the no-effect dose obtained in a two-year study of oral administration in rats.

Other studies carried out with comparable formulations, containing the same co-formulant and 490 g/L of glyphosate instead of 450 g/L in the ROUNDUP GT Plus formulation gave the following results:

- an LD_{50}[47] by the oral route and dermal route in rats of more than 5000 mg/kg,
- mild eye irritation in rabbits,
- no skin irritation in rabbits,
- no skin sensitisation in guinea pigs.

Given these results and the data available on co-formulants, this formulation does not require any hazard classification with regard to its acute toxicity or its irritant or sensitisation potential.

Regarding data with regard to operator, bystander and worker exposure

The acceptable operator exposure level for glyphosate acid, set when it was included in Annex I of Directive 91/414/EEC, is 0.2 mg/kg/day. This AOEL was derived by applying a safety factor of 100 to the no-effect dose obtained from an oral teratogenicity study in rabbits. The level of dermal absorption used for the operator exposure assessment is 3% (determined from an *in vitro* study on human skin and an *in vivo* study in Rhesus monkeys).

In consideration of the conditions in which ROUNDUP GT Plus is applied in home gardens, without gloves, the systemic operator exposure was estimated from specific studies available using the following parameters:

- application dose: 5.6 mL/10m², with 460 g/L glyphosate and 898 g/L co-formulant;
- application method: spray application with a pre-pressurised aerosol can.

[47] LD_{50}: The median lethal dose (lethal dose, 50%) is a statistical value of the dose of a substance or formulation at which a single administration by the oral route causes death in 50% of the treated animals.

The estimated exposure, expressed as a percentage of the AOEL is as follows:

% AOEL glyphosate	% AOEL co-formulant
35	31

In light of these results, the health risk to home garden users without protective gloves is considered to be acceptable[48] during preparation and application of the formulation.

An additional risk assessment to account for potential long-term cumulative effects with regard to the presence of several substances in a mixture can be performed.

Various approaches for assessing cumulative exposure risks are described in the literature. The approach described below is based on that advocated by the Chemical Regulation Directorate (CRD UK) and on that presented in ANSES's report of June 2010[49].

The methodology[50] used is based on calculating risk quotients (RQ) defined for each active ingredient as the ratio of estimated exposure levels to the reference value (AOEL). The sum of the risk quotients (Σ RQ) for each substance is then calculated to determine the risk index (RI).
If the RI is <1 then the risks for the operator, bystanders and workers are considered acceptable.
If the RI is >1 then the risks for the operator, bystanders and workers are considered unacceptable.

The % of AOEL, the RQs for each active ingredient as well as the RIs are as follows:

% AOEL [Risk quotients (RQ)]		Sum of risk quotients or risk indices
Glyphosate 0.2 mg/kg/day	Co-formulant 0.15 mg/kg/day	
35% (0.35)	31% (0.31)	0.66

The exposure of home gardeners (without gloves) is less than 100% of the AOEL for glyphosate and the co-formulant.

The RI estimating the cumulative risk of active ingredients in the formulation is <1 (0.66). The risk due to the simultaneous exposure to glyphosate and the co-formulant can therefore be considered as acceptable.

Regarding the data on residues and consumer exposure
The intended use does not lead to any direct exposure of crops when the formulation is sprayed. However, given the systemic properties of glyphosate (translocation within the plant) a risk assessment is necessary. This assessment considers the level of absorption of the substance by subsequent crops planted in the treated area.

Available data on the active ingredient
There have been studies on metabolism in the main categories of plants (23 types of crop) and in animals (goats and layer hens) as well as studies on the processing of plant products and residues in subsequent crops. These studies show that glyphosate can be included as a potential residue found in plant- and animal-derived products. These results were used for the risk assessment.

[48] Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products

[49] Proposal of a methodology for assessing the aggregated and cumulative health risks associated with exposure to a mixture of benzylbutylphthalate and dibutyl phthalate. Expert Committee on Assessment of Risks associated with Chemical Substances, June 2010, final version no. 1, www.afsset.fr

[50] An information note is available on the ANSES website.

Consumer risk assessment for glyphosate

Based on the ADI of 0.3 mg/kg bw/day, the assessment of consumer exposure[51] shows that for an adult, toddlers (13-18 months) and infants (7-12 months), the theoretical maximum daily intake (TMDI) estimated from the maximum residue levels (MRLs) determined for products of plant and animal origin represents less than 18% of the ADI. The chronic risk for all consumers together is considered as acceptable.

For the various intended uses, the intervals before harvesting have been set to ensure that the level of residues in food is lower than the MRLs.

Consumer risk assessment for the co-formulant

The intended use does not lead to any direct crop exposure when the formulation is sprayed. Given the data reported in the literature, it appears that the co-formulant does not have systemic properties and it stays on the plant where it was applied. Its metabolites, in particular C14 fatty acid (myristic acid) can migrate in plants but it is considered to be of low toxicity and, as part of a European assessment of fatty acids (C7-C20), it has not been deemed necessary to set toxicological reference values for them. In addition, studies found in the scientific literature on the degradation of this type of co-formulant in the environment[52,53,54] show that these substances are rapidly broken down by microorganisms. Moreover, the high Koc[55] values indicate that the co-formulant binds strongly to soil when coming into contact with it. Based on these data, it is estimated that the absorption by roots of the co-formulant in the ROUNDUP GT Plus formulation in plants that are growing after treatment is negligible. It is therefore highly unlikely that this co-formulant would leach into groundwater or that it could be found there.

In conclusion, under conditions of intended use, consumer exposure to the co-formulant is considered negligible. Therefore, the cumulative risk assessment of glyphosate and the co-formulant, which could be conducted using the methodology indicated above or using the one currently being developed by the EU (European ACROPOLIS programme), does not appear to be warranted.

The risk to consumers for the intended use can be considered as acceptable.

Glyphosate (or ROUNDUP) with NK603

The French Food Safety Agency issued an opinion on 9 March 2010 regarding a marketing authorisation application from Monsanto Agriculture France SAS for the glyphosate-based ROUNDUP READY formulation for use as a weedkiller on maize crops (only on glyphosate-tolerant maize with the NK603 transformation event and expressing the CP4 EPSPS protein).

Residue trials carried out using the ROUNDUP READY formulation on glyphosate-tolerant maize crops were submitted and examined as part of the application process.

The operator, consumer and environmental risks assessed were all deemed acceptable. The Opinion[56] is available for consultation on the ANSES website.

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[51] PRIMo revision 2 (EFSA, 2007). Reasoned opinion on the potential chronic and acute risk to consumers' health arising from proposed temporary EU MRLs according to Regulation (EC) No 396/2005 on Maximum Residue Levels of Pesticides in Food and Feed of Plant and Animal Origin. 15 March 2007.

[52] Behaviour of three nonionic surfactants following foliar application - Peter J. Holloway, Dawn Silcox - Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol, BS18 9AF, UK.- British Crop Protection Conferences – weeds(1985).

[53] Behavior of Polyoxyethylene sorbitan ^{14}C-monooleate in Tobacco and kidney bean leaves.-Yukio Sugimura and Tsuneyyuki Takeno -J. Pesticide Sci 10, 323-239(1985).

[54] Behavior and fate of ethoxylated alkyl phenol nonionic surfactant in barley plants - Gary E Stolzenberg, Prudence A Olson, Richard G Zaylstie, Eugene Mansager - J Agri Food Chem (1982) 637-644.

[55] Koc: soil organic carbon-water partition coefficient: ratio of the mass of a chemical that is adsorbed in the soil per unit mass of organic carbon in the soil.

[56] AFSSA Opinion No 2007-3111 of 9 March 2010 on a marketing authorisation application for the glyphosate-based formulation ROUNDUP READY, submitted by Monsanto Agriculture France SAS.

Annex 4

Prior assessments of NK 603 maize

The NK603 event confers glyphosate tolerance to maize. The genes introduced into this maize come from a common soil bacterium, *Agrobacterium sp.* strain *CP4.* The gene construct used contains two genes that are inserted in tandem into a single insertion site, allowing for the expression of two 5-enolpyruvylshikimate-3-phosphate synthase enzymes: CP4 EPSPS and CP4 EPSPS L214P. One gene is regulated by the rice actin promoter and the other by the cauliflower mosaic virus 35S promoter. The two proteins differ only by one amino acid substitution of proline for leucine at position 214 (CP4 EPSPS L214P).
EPSPS proteins are enzymes involved in the shikimic acid metabolic pathway (Annex 7), a route used by plants, fungi and micro-organisms for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan). These proteins are ubiquitous in these organisms but not in animals, which do not produce their own aromatic amino acids and have to obtain them from food.

Glyphosate is an herbicide that acts in the step catalysed by the EPSPS protein by blocking the synthesis of the three aromatic amino acids. In susceptible plants, this blockage subsequently prevents the synthesis of proteins, auxin and lignin, deregulates the chorismate pathway and ultimately leads to plant death.
Two transgenes derived from *Agrobacterium sp.* CP4 are used in NK603 genetically-modified maize, resulting in the synthesis of the bacterial EPSPS protein, which is less susceptible to glyphosate and allows the maize to synthesise aromatic amino acids (Padgette *et al.*, 1996). Maize containing the NK603 event is therefore tolerant to glyphosate at doses used for the control of susceptible weeds.

NK603 maize has been the subject of two AFSSA Opinions for feed (AFSSA, 2003 and 2004) and two for food (AFSSA, 2003 and 2004); having requested additional documents from the applicant, AFSSA did not make a decision until January 2004. EFSA issued a favourable Opinion on 25 November 2003 (EFSA, 2003). NK603 maize authorisations have been issued based on two European regulations: Directive 2001/18/EC for animal feed and Regulation 258/97/EC for human food.
The European Commission has published two decisions authorising the marketing of foods and food ingredients derived from the genetically modified maize line NK603 as novel foods or novel food ingredients[57] and for animal feed[58].

In 2006, a new marketing authorisation application of the genetically modified glyphosate tolerant maize NK603 for cultivation, food and feed uses and import and processing was submitted under Regulation (EC) no. 1829/2003. It did not contain any information that had not already been included in those applications previously examined by AFSSA when assessing the health risks related to consumption of this maize in humans and animals, apart from presenting studies assessing the environmental risks related to its cultivation. These studies, which did not fall within AFSSA's scope of expertise, were assessed by the French Biomolecular Engineering Commission (CGB). EFSA issued an Opinion on 27 May 2009 on both the framework of Regulation (EC) no. 1829/2003 and the renewal of the authorisation for NK603 maize for products authorised in the former regulation (EFSA, 2009). AFSSA's opinion was not sought for the renewal of the marketing authorisation for this maize.

The applications assessed by AFSSA for NK603 maize (Requests 2003-SA-0027, 2003-SA-0047, 2003-SA-0401 and 2003-SA-0242) contained information related to:

[57] Commission Decision of 3 March 2005 (2005/448/EC) authorising the placing on the market of foods and food ingredients derived from genetically modified maize line NK 603 as novel foods or novel food ingredients under Regulation 258/97/EC (OJEU 21/06/05).
[58] Commission Decision of 19 July 2004 (2004/643/EC) concerning the placing on the market, in accordance with Directive 2001/18/EC, of a maize product (*Zea mays* L. line NK603) genetically modified for glyphosate tolerance (OJEU 18.09.04).

- the genetic modification and molecular characterization of NK 603 genetically modified maize,
- the chemical composition of grain maize and the whole plant and its nutritional qualities,
- CP4 EPSPS and CP4 EPSPS L214P protein levels in maize tissues,
- an assessment of the toxic potential of the CP4 EPSPS and CP4 EPSPS L214P proteins through acute toxicity studies[59], *in vitro* degradation experiments and searches for sequence homology with toxic and allergenic proteins,
- a nutritional value study in growing chickens,
- a 90-day feeding toxicity study undertaken in rats (see following paragraph) and calculations of margins of exposure for maize.

The allergenic potential of NK603 maize was assessed in light of a number of points as recommended in the guidelines:

- the lack of known allergenic potential for the source organism (*Agrobacterium*),
- the lack of protein sequence identity (including for eight consecutive amino acids) between the primary structures of the CP4 EPSPS and CP4 EPSPS L214P proteins and those of known allergenic and toxic proteins,
- rapid *in vitro* hydrolysis of the CP4 EPSPS and CP4 EPSPS L214P proteins.

With regard to these points, these two proteins and NK603 maize had no suspected allergenic potential.

Subchronic toxicity study

A 90-day subchronic toxicity study was undertaken in 2001 in rats of both sexes (20 rats of each sex/treatment) to examine the effects of a diet containing two incorporation rates (11 and 33%) for NK603 grain maize compared to a diet containing maize with the same genetic base and six other maize varieties. The maize was treated with glyphosate. This study did not show any differences regarded by experts as relevant for any of the observed biological parameters, between the control rats and those fed the diets containing GM maize (AFSSA, 2003).

In December 2009, AFSSA issued an internal Request to analyse the results of a publication by Spiroux de Vendômois *et al*. (Spiroux de Vendômois *et al.,* 2009) which re-examined the data of this subchronic toxicity study. This publication showed significant differences in certain groups and treatments for liver and kidney function. In its Opinion 2009-SA-0322, AFSSA assessed modified parameters in treated and control groups and considered that these heterogeneous variations, which were unrelated, were a perfect example of the lack of correlation between statistically significant variations and their biological relevance.

Bibliography for Annex 4

AFSSA 2003-SA-0047, Avis de l'Agence française de sécurité sanitaire des aliments relatif à un dossier d'autorisation de la mise sur le marché d'un maïs génétiquement modifié tolérant au Roundup Ready lignée NK 603 en vue de son utilisation comme tout autre maïs, à l'exclusion de la culture, sur le territoire de l'Union européenne, au titre de la directive 2001/18/CE, le 7 mars 2003 http://www.anses.fr/Documents/BIOT2003sa0047.pdf

AFSSA 2003-SA-0242, Examen des compléments d'information en réponse aux objections des Etats membres relatifs à un dossier d'autorisation de la mise sur le marché d'un maïs génétiquement modifié tolérant au Roundup Ready lignée NK 603 en vue de son utilisation comme tout autre maïs, à l'exclusion de la culture, sur le territoire de l'Union européenne, au titre de la directive 2001/18/CE2003-SA-0242, le 5 janvier 2004.
http://www.anses.fr/Documents/BIOT2003sa0242.pdf

AFSSA 2003-SA-0027, Avis de l'Agence française de sécurité sanitaire des aliments relatif au rapport d'évaluation initiale établi par les autorités néerlandaises concernant la mise sur le marché

[59] No toxic effects were found at doses above 572 mg/kg bw for CP4EPSPS and 817 mg/kg bw for CP4EPSPS L214P (single-dose acute toxicity in mice)

de grains et de produits dérivés de maïs de la lignée NK 603 résistant au glyphosate (Roundup Ready) au titre du règlement 258/97, le 21 février 2003.
http://www.anses.fr/Documents/BIOT2003sa0027.pdf

AFSSA 2003-SA-0401 Examen des compléments d'information en réponse aux objections des Etats membres relatifs à un dossier d'autorisation de la mise sur le marché de grains et de produits dérivés de grains de maïs génétiquement modifié tolérant au Roundup Ready lignée NK 603 au titre du règlement (CE) n°258/97, le 13 janvier 2004
http://www.anses.fr/Documents/BIOT2003sa0401.pdf

AFSSA 2009-SA-0322 Avis de l'Agence française de sécurité sanitaire des aliments relatif à son auto-saisine sur l'article publié dans 'International Journal of Biological Sciences' et intitulé 'A comparison of the effects of three GM corn varieties on mammalian health, le 5 février 2010.
http://www.anses.fr/Documents/BIOT2009sa0322.pdf

EFSA, 2003 Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the safety of foods and food ingredients derived from herbicide-tolerant genetically modified maize NK603, for which a request for placing on the market was submitted under Article 4 of the Novel Food Regulation (EC) No 258/97 by Monsanto1 Opinion adopted on 25 November 2003 The EFSA Journal (2003) 9, 1-14

EFSA, 2009 Scientific Opinion of the Panel on Genetically Modified Organisms on applications (EFSA-GMONL-2005-22 and EFSA-GMO-RX-NK603) for the placing on the market of the genetically modified glyphosate tolerant maize NK603 for cultivation, food and feed uses and import and processing, and for renewal of the authorisation of maize NK603 as existing product. The EFSA Journal (2009) 1137, 1-50.

Padgette et al, 1996 The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *J Nutr.* 126(3):702-16.

Spiroux de Vendômois J. S., Roullier F., Cellier D., Séralini, G. E. 2009 A comparison of the effects of three GM corn varieties on mammalian health. *International Journal of Biological Sciences* **5**(7), 706-726.

Annex 5

List of publications relative to 90-day subchronic oral toxicity studies, conducted in the context of risk assessments for GMPs

References and title	GMP	Newly expressed proteins	Tested material and incorporation rates	Treatments	Rats strain Number of rats/treatment group/sex	Results
Appenzeller *et al.*, 2008 Subchronic feeding study of herbicide-tolerant soybean DP-356Ø43-5 in Sprague-Dawley rats. Food and Chem. Tox. 46 2201-2213.	HT genetically modified Soybean DP-356043-5	GAT GM-HRA	Toasted meal (20% w/w) and ground hulls (1.5% w/w).	6 groups: -GM soybean 356043 -GM soybean 356043 treated intended herbicides -near-isoline control, -3 non transgenic commercial varieties	Sprague Dawley 12	No adverse effects
Appenzeller et al., 2009a Subchronic feeding study with GM stacked trait lepidopteran and coleopteran resistant (DAS-1507-1xDAS-59122-7) maize grain. Food and Chem. Tox. 47 1512-1520.	HT and IR genetically modified Maize 1507x59122	Cry1F PAT Cry1Ab34 Cry1Ab35 PAT	Maize grain (34%w/w)	6 groups: -GM maize 1507x59122 - near-isoline control - 3 non transgenic commercial varieties	Sprague Dawley 12	Grain from 1507x59122 maize is as safe and nutritious as that obtained from non-GM maize
Appenzeller et al., 2009b Subchronic feeding study of grain from herbicide tolerant maize DP-98140-6 in Sprague Dawley rats. Food and Chem. Tox. 47 2269-2280.	HT genetically modified Maize 98140	GAT	Maize grain (35 and 38% w/w)	6 groups: -GM maize 98140 -GM maize 98140 treated intended herbicide - near-isoline control - 3 non transgenic commercial varieties	Sprague Dawley 12	No adverse health effects
Dryzga et al., 2007 Evaluation of the safety and nutritional equivalence of a GM cottonseed meal in a 90 day dietary toxicity study in rats. Food and Chem. Tox. 45 1994-2004.	IR and HT genetically modified cotton	Cry1F Cry1Ac PAT	Meals (10% concentration)	5 groups -GM Widestrike -near-isoline control -3 non transgenic commercial varieties	Sprague Dawley 12	Lack of any toxicity of GM Widestrike cottonseed

References and title	GMP	Newly expressed proteins	Tested material and incorporation rates	Treatments	Rats strain Number of rats/treatment group/sex	Results
Hammond et al., 2004 Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. Food and Chem. Tox. 42 1003-1014.	HT genetically modified maize NK603	CP4 EPSPS	Maize grain (11 and 33%)	10 groups -11 or 33% GM maize -11 or 33% near isoline control -6 non transgenic commercial varieties 33%	Sprague Dawley 20	This study confirms ROUNDUP ready corn grains is as safe and nutritious as existing commercial corn hybrids.
(Hammond, Dudek et al. 2006) Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. Food and Chem. Tox. 44 1092-1099	IR genetically modified maize MON810	Cry1Ab	Maize grain (11 and 33%)	10 groups -11 or 33% GM maize -11 or 33% near isoline control -6 non transgenic commercial varieties 33%	Sprague Dawley 20	MON810 is considered to be substantially equivalent to, and as safe and nutritious as, conventional corn varieties.
(Hammond, Dudek et al. 2006) Results of a 90-day safety assurance study with rats fed grain from rootworm-protected corn. Food and Chem. Tox. 44 147-160	IR genetically modified maize MON863	Cry3Bb1 NptII	Maize grain (11 and 33%)	10 groups -11 or 33% GM maize -11 or 33% near isoline control -6 non transgenic commercial varieties 33%	Sprague Dawley 20	MON863 is considered to be substantially equivalent to, and as safe and nutritious as, conventional corn varieties.
(Hammond, Lemen et al. 2008) Safety assessment of SDA soybean oil: Results of a 28-day study and a 90-day/one generation reproduction feeding study in rats. Regul. Tox. and Pharmacol. 52, 311-323.	SDA genetically modified soybean (rich in stearidonic-acid)	Δ6 and Δ15 desaturases	Soybean oil 4 g/kg body weight	4 groups -4g or 1.5g SDA soybean oil (GM) -4g near isogenic control soybean oil -4 g menhaden oil	Sprague Dawley 25	The result of the 90 day/one generation reproduction feeding study found no evidence of treatment related adverse effects up to the highest dosages of SDA soybean oil tested.
(He, Huang et al. 2008) Comparison of grain from corn rootworm resistant T DAS -59122-7 maize with non-T maize grain in a 90 day feeding study in Sprague Dawley rats. Food and Chem. Toxicol. 46 1994-2002.	IR and HT genetically modified maize DAS-59122	Cry34Ab1 Cry35Ab1 PAT	Maize flour (50% and 70%)	5 groups -50 or 70% GM maize 59122 -50 or 70% near isoline control maize -43.3% maize flour (=control diet)	Sprague Dawley 10	The results demonstrated that it was as safe and nutritious as non-transgenic maize grain
(He, Tang et al. 2009) A 90-day toxicology study of transgenic lysine-rich maize grain (Y642) in Sprague Dawley rats, Food and Chem. Toxicol. 47 425-432.	Lysine-rich genetically modified maize (Y642)	sb401 (a gene from potatoes)	Maize grain (30 and 76%)	5 groups: -30% and 76% GM Y642 -30% and 76% near isoline control maize -43.3% maize flour (=control diet)	Sprague Dawley 10	The results demonstrated that Y642 is as safe and nutritious as conventional non-GM maize grain

References and title	GMP	Newly expressed proteins	Tested material and incorporation rates	Treatments	Rats strain Number of rats/treatment group/sex	Results
(Healy, Hammond et al. 2008) Results of a 13-week safety assurance study with rats fed grain from corn rootworm-protected, glyphosate-tolerant MON87017 corn. Food and chem. Toxicol. 46 2517-2524.	IR and HT genetically modified Optimum GAT maize MON87017	Cry3Bb1 CP4EPSPS	Grain (11 and 33%)	9 groups: -11% and 33% GM MON87017 -11% and 33% near isogenic control -6 non transgenic commercial varieties 33%	Sprague Dawley 20	No adverse health effects were detected in rats following 13 weeks of dietary exposure to grain from genetically modified Optimum GAT maize.
(Malley, Everds et al. 2007) Subchronic feeding study of DAS-59122-7 maize grain in Spague-Dawley rats, Food and Chem. Tox. 45 1277-1292.	IR and HT genetically modified maize DAS-59122	Cry34Ab1 Cry35Ab1 PAT	Grain (35%)	5 groups -35% GM maize DAS-59122 - 35% non transgenic near isogenic control 1 variété commerciale - - 2 non transgenic commercial varieties 35%	Sprague Dawley 12	Results from the current study demonstrated that 59122 maize grain is as nutritious and wholesome as conventional maize grain when evaluated in a subchronic feeding study in rats.
(Poulsen, Kroghsbo et al. 2007) A 90-day safety study in Wistar rats fed genetically modified rice expressing snowdrop lectin Galanthus nivalis (GNA) Food and Chem. Tox. 45 350-363.	Genetically modified Rice GNA (expressing lectin galanthus nivalis)	GNA lectin galanthus nivalis	Rice flour	2 groups: 60% GM rice GNA 60% control parental rice	Wistar 16	In the present study several differences were observed between rats fed diets with GM and parental rice. Most of these differences appeared to be related to the increased water intake of the rats fed GM rice, which probably relates to the GNA lectin content, but none of the effects were considered to be adverse.
Liu et al. 2012 A 90-day subchronic feeding study of genetically modified maize expressing Cry1Ac-M protein in Sprague-Dawley rats. Food and Chem. Tox. 50 3215-3221.	IR genetically modified maize	Cry1Ac_M	Maize	7 groups 12.5, 25, 50% GM maize 12.5, 25, 50% non GM maize Maize commercial line	Sprague Dawley 10	Safe as conventional
(Schroder, Poulsen et al. 2007) A 90-day safety study of genetically modified rice expression Cry1Ab protein (Bacillus thuringiensis toxin) in Wistar rats. Food and Chem. Tox. 45 339-349.	IR genetically resistant Rice (KMD1)	Cry1Ab	Rice flour	2 groups: 60% GM rice KDM1 60% non-transgenic parental wild type rice	Wistar 16	The results show no adverse or toxic effects of KDM1 rice when tested in the design used in this 90-day study.

References and title	GMP	Newly expressed proteins	Tested material and incorporation rates	Treatments	Rats strain Number of rats/treatment group/sex	Results
(Wang, Wang et al. 2002) Toxicological evaluation of transgenic rice flour with a synthetic *cry1Ab* gene from bacillus thuringiensis. J. Sci. Food Agric. 82 738-744.	IR genetically resistant Rice (KMD1)	Cry1Ab	Rice flour	4 groups: 64% GM rice KDM1 32% GM rice KDM1 16% GM rice KDM1 64% non-transgenic parental wild type rice	Sprague Dawley 10	KDM1 rice flour was safe to rats in general.
Zhu et al. 2012 A 90-day feeding study of glyphosate-tolerant maize with the G2-aroA gene in Sprague-Dawley rats. Food and Chem Tox 18. S0278-6915	HT genetically modified maize	G2-AroA gene	Maize	7 groups 12.5, 25, 50% GM maize 12.5, 25, 50% non GM maize Maize commercial line	Sprague Dawley 10	Safe and nutritious
Zhou et al 2011 A 90-day toxicology study of high-amylose transgenic rice grain in Sprague-Dawley rats. Food Chem Tox, 2011 49(12):3112-3118.	High amylose	RNAi	Rice	3 groups 70% GM 70% isogenic Control diet	Sprague Dawley	as safe as the conventional non-transgenic rice for rat consumption

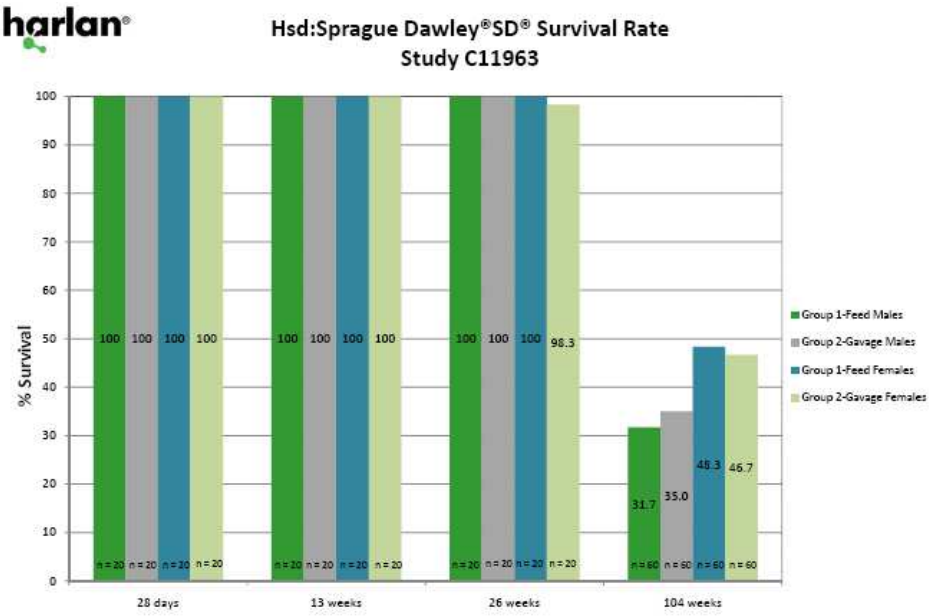
IR: Insect resistant
HT: Herbicide tolerant

Annex 6

Comparison between data in the literature on mortality rates and incidences of tumours, and the study by Séralini *et al.* (2012)

Mortality (Figure 1 and Table 1)
Table 1 and Figure 1 show the mortality rates described in the literature for Sprague Dawley rats, the strain used in the experiment by Séralini *et al.* (2012) (Chandra *et al.* 1992; Dinse *et al.* 2010; Nakazawa *et al.* 2001; Prejean *et al.* 1973). Séralini *et al.* (2012) observed two early deaths (males: 100 days 11% GMO and 120 days 22% GMO + R) for which the exact causes are not given. The literature reports a few rare cases of the onset of mammary tumours before 140 days (Kuzutani, 2012).

Figure 1: Mortality in Sprague Dawley rats from Harlan Laboratories, which supplied the rats for the study by Séralini *et al*. (2012).



Incidence of tumours (Tables 2, 3, 4 and 5)
In females, the literature describes a high incidence of mammary and pituitary tumours. This corresponds to the observations reported by Séralini *et al*. (2012).
However, in males, several studies show a high incidence of testicular, dermal and adrenal tumours (Chandra, Riley 1992, Nakazawa 2001). The article by Séralini *et al*. (2012) does not mention tumours in these organs. The incidence of liver and kidney tumours is very low in Sprague Dawley rats (Chandra, Riley 1992, Nakazawa 2001). It is difficult to assess these results and compare them to the observations made by Séralini *et al*. (2012). Indeed, the article by Séralini *et al*. (2012) mentions pathologies of the liver and kidneys without specifying whether or not they are tumour-related.

Table 1: Percentage of mortalities in Sprague Dawley rats

	Prejean (1973) (77 weeks or 540 days) 180 rats/sex	Nakazawa (2001) Groups A and B 120/sex	Chandra (1992) 2 years 1340 males 1329 females	Iffa Crédo (105 weeks or 2 years) 100 rats/sex	Dinse (2010) 2 years 330 females
Males	33%	A 11% B 24%	51%	50%	
Females	58%	A 58% B 65%	54%	35%	28-51%

Table 2: Frequency in % of tumours observed in Sprague Dawley rats in publications by Nakazawa (2001) and Chandra (1992)

	Sprague-Dawley Shizuoka Japan Nakazawa *et al.* 2001			Sprague-Dawley Charles River Chandra *et al.* 1992		
Organs	**Male (240)**	**Female (240)**	**Total (480)**	**Male (1340)**	**Female (1329)**	**Total (2669)**
Liver	3.33 (8)	2.08 (5)	5.41 (13)	2.6(35)	0.83 (11)	1.7 (46)
Pituitary gland	36.67 (88)	69.59 (167)	53 (255)	27.8 (373)	49.4 (659)	38.6 (1032)
Mammary gland	–	32.92 (79)	32.92 (79)	1.5 (20)	31.6 (420)	
Kidneys	1.25 (3)	0.83 (2)	1 (5)	0.97 (13)	0.45 (6)	0.7 (19)
Adrenal gland	12.92 (31)	10 (24)	11.4 (55)	6.1 (82)	3 (40)	4.6 (122)

Table 3: Frequency in % of tumours observed in Sprague Dawley rats and Swiss mice from the publication by Prejean *et al.* 1973.
(Size of groups) Analysis at 540 days

	Sprague-Dawley Charles River Prejean *et al.* (1973)			Swiss mice Charles River Prejean *et al.* (1973)		
Organs	**Male (179)**	**Female (181)**	**Total (360)**	**Male (101)**	**Female (153)**	**Total (254)**
Liver	0	0	0	2 (2)	0	0.8 (2)
Pituitary gland	16.2 (29)	29.3 (53)	22.8 (82)	0	1.9 (3)	1.2 (3)
Mammary gland	2.2 (4)	32 (58)	17.2 (62)	1 (1)	1.9 (3)	1.6 (4)
Kidneys	0	0	0	2 (2)	0	0.8 (2)
Adrenal gland	7.8 (14)	10 (18)	8.8 (32)	1 (1)	0	0.4 (1)

Table 4: Frequency in % of tumours observed in Sprague Dawley rats and Fisher 344/N rats in publications by Dinse *et al.* (2010) and Brix *et al.* (2005)

(Size of groups) Analysis at 2 years

	Sprague-Dawley Harlan Dinse *et al.* (2010)	Sprague-Dawley Harlan Brix *et al.* (2005)	Fischer 344/N Harlan Dinse *et al.* (2010)
Organs	**Females (473)**	**Females (371)**	**Females (450)**
Liver	1.27 (4)	1.3 (5)	0.89 (4)
Pituitary gland	40.55 (191)	42.3 (157)	45.77 (206)
Mammary gland	80.1 (379)	85.2 (316)	52.88 (238)
Kidneys	1.47 (7)	0.6 (2)	0.22 (1)
Adrenal gland	9.35 (44)	1.1 (4)	3.78 (17)

Table 5: Incidence in % of the onset of tumours in the mammary gland in Sprague Dawley rats. % (number of animals concerned/total number of animals)

Mammary gland			
	Male	**Female**	**Origin**
Okada *et al.* (1981)		20.3 (13/64)* 75.8 (72/95)**	JCL Japan
Chandra *et al.* (1992)	1.5 (20/1340)	31.6 (420/1329)	Charles River
Nakazawa *et al.* (2001)		32.9 (79 /240)	Japan SLC
Brix *et al.* (2005)		85.2 (316/371)	Harlan
Dinse *et al.* (2010)		80.1 (379/473)	Harlan
Séralini *et al.* (2012)		50 (5/10)	Harlan
Harlan (2012)	3.4 (4/~120)	91.6 (109/~120)	Harlan

*420 days; ** - 756 days

Bibliography for Annex 6

Chandra M, *et al.* (1992) Spontaneous neoplasms in aged Sprague-Dawley rats. *Archives of Toxicology* **66**(7), 496-502.

Dinse GE, *et al.* (2010) Comparison of NTP historical control tumor incidence rates in female Harlan Sprague Dawley and Fischer 344/N Rats. *Toxicologic Pathology* **38**(5), 765-75.

Kuzutani K, *et al.* (2012) Spontaneous Mammary Adenocarcinoma in a Twelve-week-old Female Sprague-Dawley Rat. *J Toxicol Pathol* **25**(3), 221-4.

Nakazawa M, *et al.* (2001) Spontaneous neoplastic lesions in aged Sprague-Dawley rats. *Experimental Animals* **50**(2), 99-103.

Okada M, *et al.* (1981) Characteristics of 106 spontaneous mammary tumours appearing in Sprague-Dawley female rats. *British Journal of Cancer* **43**(5), 689-695.

Prejean JD, *et al.* (1973) Spontaneous tumors in Sprague Dawley rats and Swiss mice. *Cancer Research* **33**(11), 2768-2773.

Annex 7

Metabolic pathway of shikimic acid

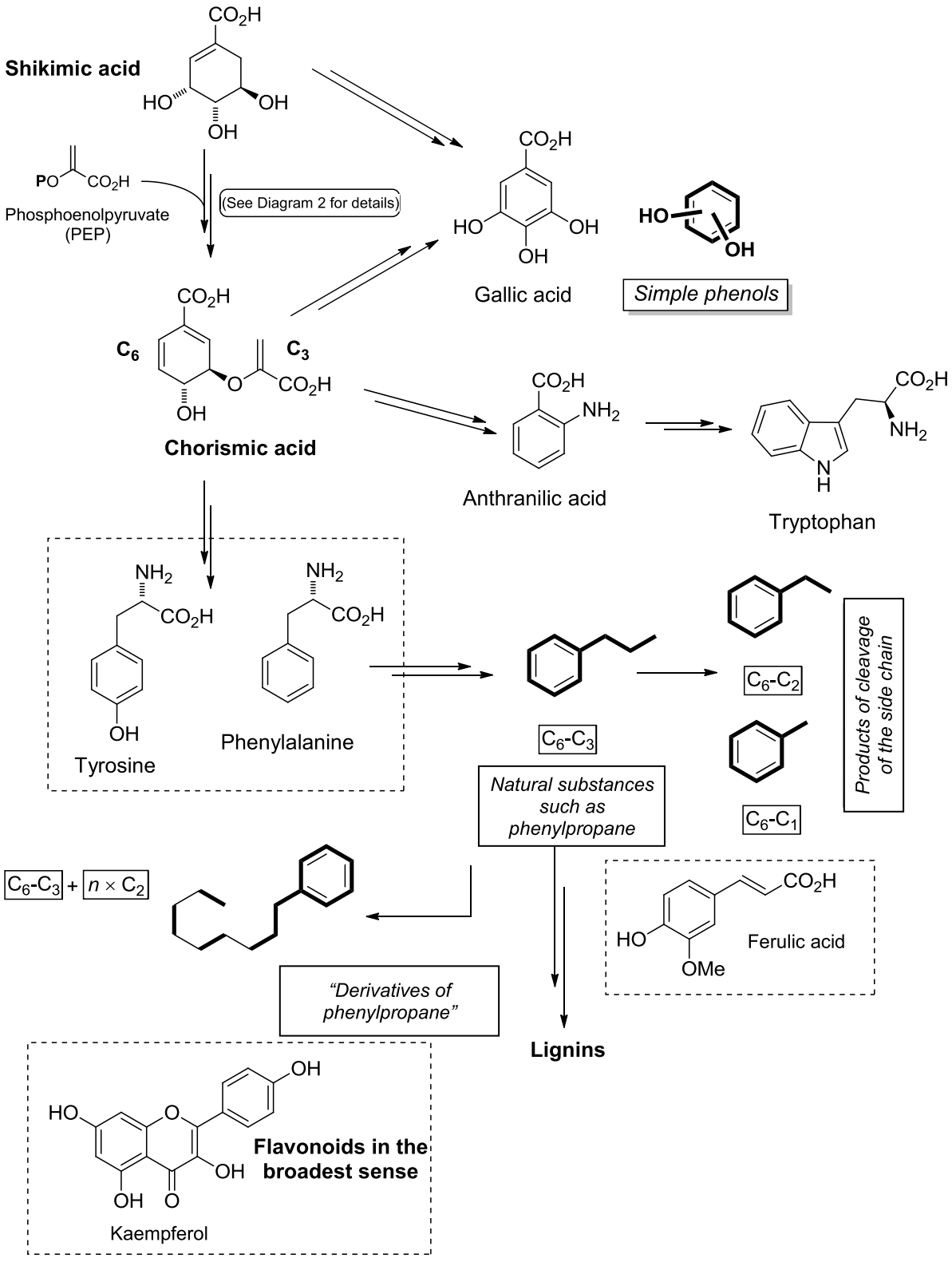


Diagram 1 – Shikimic acid pathway

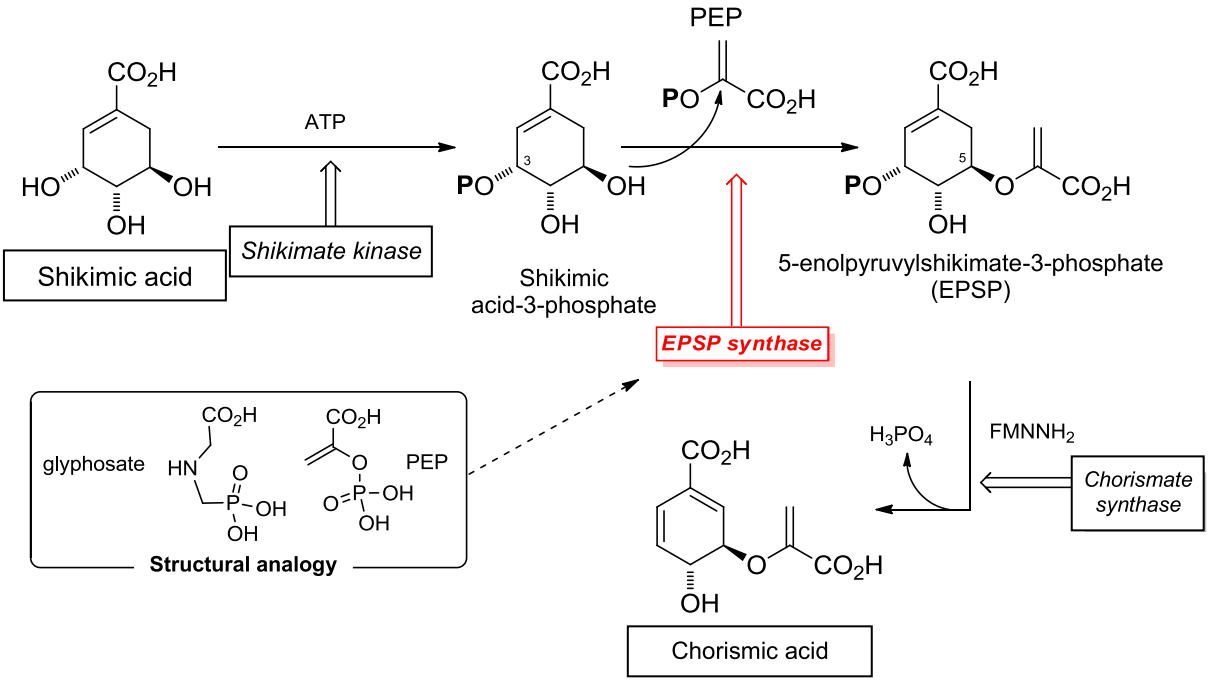


Diagram 2 – Position of EPSP synthase in the metabolism of shikimic acid

HIGH COUNCIL FOR BIOTECHNOLOGY

SCIENTIFIC COMMITTEE

Paris, 19 October 2012

OPINION

on the paper by Séralini *et al.* (*Food and Chemical Toxicology*, 2012)
in response to the referral of 24 September 2012[1].

On 24 September 2012 the High Council for Biotechnology (HCB) was asked by the French Competent Authorities (the Ministry of Social Affairs and Health, the Ministry with responsibility for the Social Economy and Consumer Affairs in the Ministry for the Economy and Finance, the Ministry for Ecology, Sustainable Development and Energy, and the Ministry of Agriculture, Food and Forestry) to provide an opinion on the paper by Professor Séralini's team published in the journal *Food and Chemical Toxicology* reporting harmful effects on rats of long-term consumption of genetically modified maize NK603 and of Roundup®, a glyphosate-based herbicide.

Following preliminary work by a group of experts specially set up in response to this referral, the HCB Scientific Committee[2] examined the paper on 2 October 2012 with Jean-Christophe Pagès in the chair.

[1] This referral is reproduced in Appendix 1.

[2] The Scientific Committee's composition and the outside experts in the *ad hoc* working group are given in Appendix 2.

EXECUTIVE SUMMARY[3]

In response to the referral of 24 September 2012, the Scientific Committee of the High Council for Biotechnology (HCB) has analysed the publication by Professor Séralini's team entitled 'Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize' forthcoming in the journal *Food and Chemical Toxicology* (Séralini *et al.*, 2012). The authors of this article lay claim to the first documented experimental demonstration of the long-term toxicity of consumption of a genetically modified (GM) plant, maize NK603, and a glyphosate-based herbicide, Roundup®.

The object of this first opinion from the HCB Scientific Committee is to determine whether the publication presents conclusive results regarding possible toxicity of maize NK603. The second part of the referral requests HCB to consider suitability of the procedures for GM plant health risk assessment and to propose any adaptation if necessary. This subject will be examined in the next few months, taking into account scientific and contextual aspects.

Following a multidisciplinary expert assessment, the HCB Scientific Committee finds that the publication, which is mainly descriptive, fails to establish any causal relationship between events observed during the study and the consumption of maize NK603, whether or not treated with Roundup®. More specifically, the HCB Scientific Committee notes that:

- The experimental design is not appropriate to the study objectives: the number of rats per group is too low, and the number of control groups is not sufficient to infer statistically significant effects of maize NK603 consumption over two years in terms of chronic toxicity and tumour development in rats;
- The reporting of the results is fragmentary and imprecise. Only some results are selected, reported or commented on; the reporting of these selected results lacks precision and biological relevance and uses non-conventional 'nomenclature'. This imprecise and fragmentary description forms the basis for unproven conclusions, which are then used to construct unjustifiable pathophysiological hypotheses;
- The findings of harmful effects of maize NK603 consumption are not supported by analysis of the results reported in the publication. The data have not been subjected to any appropriate statistical analysis. The HCB Scientific Committee has used standard statistical methodologies to analyse the mortality and tumour observations reported by the authors in the publication. It emerges that no statistically significant differences in mortality or tumour incidence in rats are shown between the groups fed maize NK603 and the control groups. Moreover, use of reference data from the supplier of the animals for the study shows that survival rates and tumour incidence for rats fed maize NK603 generally come within the prediction intervals calculated for rats of this stock. It is worth noting that the survival rate of the female control group used in this study falls outside the prediction interval for rats of this stock. This confirms the weakness in the authors' interpretation of the results on the basis of such small numbers. Lastly, the HCB Scientific Committee shows that the statistical methodology used by the authors to analyse the biochemical parameters is inadequate and cannot be used to infer the existence of statistically significant differences between the groups fed maize NK603 and the control groups.
- The authors of the article offer a speculative interpretation of their results. Since the HCB Scientific Committee has found that these results do not show any statistically significant differences in mortality, tumour incidence or biochemical parameters between experimental groups and control groups, it has not thought it worth commenting on every aspect of the discussion. The HCB Scientific Committee has nevertheless pointed out the unacceptable shortcomings in the authors' argument concerning the hormone dependency of tumours, the flaws in the reasoning behind the hypothesis of endocrine-disrupting effects caused by a change in the phenolic acid content of maize NK603, and the shortcomings and inconsistencies that make it impossible to validate the authors' hypothesis of possible kidney damage in rats fed maize NK603.

In response to the referral, the HCB Scientific Committee therefore finds that the publication by Séralini *et al.* (2012) does not present conclusive results regarding possible toxicity of maize NK603, whether or not treated with Roundup®.

[3] This summary is not a substitute for the full analysis of the article contained in this opinion.

CONTENTS

1. Ministerial referral further to claims by a scientific paper concerning the health impacts of a GMO and a herbicide

1.1. *Publication by Séralini* et al. *(2012)*

Following a two-year rat feeding study, Professor Séralini's team has laid claim to the first documented experimental demonstration of the long-term toxicity of consumption of a genetically modified (GM) plant and a Roundup® herbicide in a paper published online on 19 September 2012 and in press in the scientific journal *Food and Chemical Toxicology* (Séralini *et al*., 2012).

1.2. *Joint referral to ANSES and HCB by four ministries*

Considering the scope of the claims in this paper, the Ministers of Health, Agriculture, Ecology and the Minister with responsibility for Consumer Affairs in the Ministry for the Economy made a joint request for an opinion from both ANSES (French Agency for Food, Environmental and Occupational Health Safety) and HCB (High Council for Biotechnology).

More precisely, it requested ANSES and HCB to *'undertake an analysis of the study reported by this paper in order to determine whether or not it is likely to cast doubt on the findings of previous assessments of this GMO and in particular whether it may be considered conclusive regarding the possible health risk of food derived from GM plants containing event NK603'.*

Because of its responsibility for assessing plant protection formulations with a view to authorisation for placing on the market, ANSES was further requested to *'determine whether or not this study is likely to cast doubt on the findings of ANSES previous assessments of the Roundup herbicide'*.

Lastly, both organisations were asked to '*assess whether the study's protocol and findings call into question current or future guidelines for health risk assessment'*.

The public authorities are careful to distinguish between analysis of the article itself and consideration of guidelines for health risk assessment by specifying a two-stage timetable: '*Would you please deliver an opinion on this paper by 20 October 2012 and on the suitability of health risk assessment procedures and proposed adjustments to guidelines, if necessary, by 20 November 2012.'*

The referral is reproduced in Appendix 1.

1.3. *Organisation of HCB's response to the referral*

In response to this joint referral, ANSES and HCB conferred together and drew up parallel work programmes with separate working groups to facilitate the organisation of expertise whilst maintaining lines of communication for the joint part of the referral. One expert shared between both working groups facilitated exchange between the two bodies. A final meeting for each group to report on its work was held on 17 October 2012.

As provided for in the referral, HCB drew up a two-stage work programme, with an initial opinion covering analysis of the Séralini *et al.* (2012) paper and its implications to be submitted for 20 October 2012 and a second opinion on health assessment guidelines for GMOs to be submitted at a later date.

More specifically, this HCB Scientific Committee opinion aims to determine whether the paper reports conclusive findings with regard to the possible toxicity of maize NK603. The findings specific to the toxicity of Roundup® herbicides, which do not come within HCB's remit or competence, have been examined by ANSES in accordance with the referral.

The HCB Scientific Committee set up a multidisciplinary working group consisting of four outside experts, three in-house experts and the Scientific Committee Chair and Vice-chair (see Appendix 2). The experts were chosen for their expertise in subjects relevant to analysis of the paper (toxicology, cancer research, human and animal health, statistics, plant

physiology), their public-sector affiliation and their independence, ascertained by a declaration of interests. An additional private-sector expert (from the Centre International de Toxicologie) was consulted on technical issues by the working group. The selected outside experts had never worked on GMOs or assessed GMOs in the past and had not commented on this study publicly.

The working group met on 27 September and on 2 and 5 October 2012 and continued its discussion electronically. The paper by Séralini *et al.* (2012) and the working group's analysis were presented on 2 October 2012 to the HCB Scientific Committee.

Professor Séralini and three of his co-authors (doctoral students Robin Mesnage, Steeve Gress and Nicolas Defarge) were questioned by HCB on 10 October 2012.

An opinion was prepared on the basis of the reports by the working group experts and additional comments by Scientific Committee members. The opinion was reviewed by the working group and adopted electronically by Scientific Committee members on 19 October 2012.

1.4. *Background*

There are few long-term toxicity studies on GM plants. In particular, literature reviews by Domingo and Bordonaba (2011) and Snell *et al.* (2012) identify only two two-year studies for rodents (Domingo and Bordonaba, 2011; Snell *et al*., 2012). The authors of one study found no detectable effects in rats (study using 50 rats per group) from a two-year 30% GM soybean diet (Sakamoto *et al*., 2008); the authors of the second study found that a two-year 14% GM soybean diet might have an effect on liver ageing in mice (study using 10 mice per group) (Malatesta *et al*., 2008).

Toxicity studies to assess the health impact of GM plants prior to placing them on the market have limitations. This has been noted on several occasions by the HCB Scientific Committee[4]. The Committee stresses that absence of risk cannot be formulated without an associated probability of error, which necessitates a power analysis (HCB, 2012).

For herbicide-tolerant GM plants, toxicity studies often cover plants that have not been treated with the relevant herbicide during cultivation. In 2011 EFSA[5] published new guidelines recommending that toxicity assessment of herbicide-tolerant GM plants should include assessment of these plants treated with the herbicide to which they were tolerant (EFSA, 2011). The European Commission is currently converting this guidance into a binding text.

Under Directive 2001/18/EC[6] (EC, 2001) and Regulation (EC) No 1829/2003[7] (EC, 2003), which do not provide for any specific rules on independence, it is the applicants who at present conduct, or contract out, regulatory toxicity studies.

Sundry other aspects in assessment of potential toxicity are often not considered and therefore limit our interpretation of these assessments. For example, sensitivity to toxic effects varies according to species and within the same species. This sensitivity is also affected by animals' stress conditions. There are no standard regulatory criteria for such aspects.

Lastly, as emphasised in a 2009 European Commission position paper[8] on OECD Guideline 408 on 90-day toxicity studies in rodents (OECD, 1998), there is no consensus on toxicity tests for novel foods/ingredients, especially whole foods/feeds.

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[4] For example, in its opinion of 31 July 2012, the HCB Scientific Committee pointed out that the applicant had found no major toxic effects of maize GA21 on health on the basis of a 90-day rat feeding study for which no statistical power analysis had been carried out. That finding went beyond what could actually be interpreted from the applicant's results (HCB, 2012).

[5] EFSA: European Food Safety Authority.

[6] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 lays down EU rules on deliberate release into the environment of GMOs. It repeals Council Directive 90/220/EEC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001L0018:EN:HTML.

[7] Regulation (EC) No 1829/2003 is a regulation of the European Parliament and Council of 22 September 2003 on food and feed containing, consisting of or produced from genetically modified organisms: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32003R1829:EN:HTML.

2. Analysis of study reported by Séralini *et al.* (2012)

2.1. *The paper's claims*

The paper published in the scientific journal *Food and Chemical Toxicology* (Séralini *et al*., 2012) reports findings of long-term health effects in rats fed a diet containing genetically modified (GM) maize – maize NK603[9], genetically modified to be glyphosate-tolerant – and drinking water containing a glyphosate-based herbicide formulation marketed under the name of Roundup®.

For two years, the health effects of three types of diet were monitored concurrently for groups of 10 Sprague-Dawley (SD) rats of each sex:

1. Diets containing GM maize in three different proportions: 11, 22 and 33%;
2. Diets containing GM maize treated with Roundup® (WeatherMax)[10] during cultivation, in the same three proportions of 11, 22 and 33%;
3. Diets containing 33% of a non-GM maize variety, described by the authors as '*the nearest isogenic*' to maize NK603, and drinking water to which Roundup® was added (GT Plus)[11], in three different proportions: 1.1 x 10^{-8}, 0.09 and 0.5%.

For each sex, these 9 groups of rats (referred to as 'experimental groups' in this opinion) were monitored in comparison with one control group fed a diet containing 33% of the non-GM maize '*nearest isogenic*' to maize NK603 and water to which Roundup® had not been added.

According to the authors, the results show:

- Higher and earlier mortality for all the female experimental groups and three of the six male groups fed GM maize;
- In female experimental groups: mammary tumours developing '*almost always more often and before controls'* and pituitary gland damage; '*the sex hormonal balance [...] modified by GMO and Roundup® treatments'*; in male experimental groups: '*four times more large palpable tumours than controls [...] up to 600 days earlier*';
- More liver lesions (congestion, necrosis), observed by optical and electron microscopy, and more frequent kidney disease in the male experimental groups, confirmed by biochemical data.

In their conclusions, the authors state, *'These results can be explained by the non-linear endocrine-disrupting effects of Roundup®, but also by the overexpression of the transgene in the GMO and its metabolic consequences.'*

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[8] EC position paper on the document (ENV/JM(2009)4): Proposal to adapt OECD test guideline No. 408 'Repeated dose 90-day toxicity study in rodents' for whole-food testing. 44th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology for its meeting on 10-11 June 2009.

[9] Genetically modified maize NK603 expresses the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme of the CP4 strain of *Agrobacterium tumefaciens* (CP4 EPSPS), which confers tolerance to glyphosate, the active ingredient of non-selective herbicides such as Roundup®. Glyphosate's broad-spectrum toxicity derives from its inhibition of the EPSPS function in the majority of plants. EPSPS is an essential enzyme for production of amino acids and other aromatic compounds in plants, bacteria and fungi. It is not present in animals, which do not synthesise their own aromatic compounds. Various strategies have been employed to develop glyphosate-tolerant plants, the commonest nowadays being use of the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4. This gene has a mutation that renders the enzyme produced, CP4 EPSPS, insensitive to glyphosate inhibition. One or more copies of the gene are thus added to plants to maintain the metabolic pathway of the aromatic compounds while the endogenous plant EPSPS enzyme is inhibited by glyphosate (Duke and Powles, 2008; Funke *et al*., 2006).

[10] The Roundup WeatherMax® formulation contains 540 g/l of glyphosate. This herbicide was used at a dose of 3 litres per hectare.

[11] The Roundup GT Plus® formulation contains 450g/l of glyphosate.

2.2. *Data analysed*

As published, the paper contains a series of flaws, some of which – lack of precision and information in the description of the experimental design, partial reporting of the results, unjustified use of non-conventional classifications – could have been remedied if the paper's authors had supplied supplementary data.

Contrary to scientific practice and despite undertaking to comply with the journal's guidelines[12], the paper's authors have refused to forward the additional information requested by HCB to this end[13].

The HCB Scientific Committee notes however that although these supplementary data would have been useful to clarify certain results, they were not needed to answer the questions set out in the referral.

To support its analysis of the data reported in the paper, the HCB Scientific Committee was able to obtain reference data from Harlan Laboratories, the company that supplied the SD rats for the study[14].

2.3. *Experimental design not fit for purpose*

Confused objectives

A study's experimental design and protocol are usually developed to answer specific questions. The goals of this study are confused. The study seems to have been initially designed to explore long-term biological disruptions resulting from consumption of GM maize and Roundup® herbicide with no specific targets, as evidenced by the multiplicity of parameters measured: 31 blood parameters and 16 urine parameters analysed on 11 dates spread between the beginning and the end of the trial, as well as 6 biological parameters relating specifically to liver function, measured once at the end of the trial. However, a large part of the paper concerns the development of tumours in the rats, which seems to have attracted the authors' attention in the course of the study.

Not enough rats per group to answer the questions raised

The size of the rat groups should be estimated according to the scale of the biologically significant effect to be detected, taking account of the characteristics of the rat strain used for the parameters measured throughout the course of the study and under specific experimental conditions. The paper makes no mention of any initial calculation of the number of subjects required to detect a biologically significant effect in a two-year study.

In the light of these considerations, the numbers recommended by OECD guidelines are 20 rats per group for a 12-month chronic toxicity study[15] (Test Guideline 452 (OECD, 2009b)) and 50 rats per group for a 24-month carcinogenicity study (Test Guideline 451 (OECD, 2009a)) or a combined chronic toxicity/carcinogenicity study (Test Guideline 453 (OECD, 2009c)). With 10 rats per group, this study falls short of the recommended number considered necessary to infer statistically significant effects of long-term treatment for the two types of analysis undertaken (chronic toxicity, carcinogenesis).

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[12] Ethical guidelines for publication in Elsevier journals, which include *Food and Chemical Toxicology*: '*Data access and retention: Authors may be asked to provide raw data in connection with a paper for editorial review, and should be prepared to provide public access to such data (consistent with the ALPSP-STM Statement on Data and Databases), if practicable, and should in any event be prepared to retain such data for a reasonable time after publication*.'

Specific guidelines for *Food and Chemical Toxicology* authors: '*Furthermore, it is understood that with submission of this article, the authors […] are willing to share the original data and materials if so requested.*'

[13] Letter to Professor Séralini sent on 2 October 2012.

[14] Letter to Harlan Laboratories sent on 20 September and e-mail sent on 12 October 2012; data from the company received on 27 September and 16 October 2012.

[15] OECD Test Guideline 452 for chronic toxicity studies states that a 12-month period is *'sufficiently long to allow any effects of cumulative toxicity to become manifest, without the confounding effects of geriatric changes'* (OECD, 2009a).

Moreover, the SD rat strain, commonly used for 90-day subchronic toxicity studies, is known for spontaneously developing mammary and pituitary tumours in the long term. The incidence of natural mammary gland fibroadenoma in Harlan SD rats can be as high as 70% (Brix *et al*., 2005). Data obtained from Harlan Laboratories on SD rats from the same stock as the animals used in the Séralini *et al.* study (2012) show a 60% incidence of mammary tumours in a two-year in-house study begun in 2009 (reference data communicated by Harlan Laboratories). Such information has to be taken into account when developing a long-term experimental design, since the higher the natural incidence of tumour development, the larger the experimental groups must be in order to detect a significant increase in the number of diet-linked tumours (see Appendix 3). These characteristics of the SD rat strain have been considered neither in the study's experimental design nor in the interpretation and discussion of the results.

Control groups too few in number and lacking biological relevance

For each sex, only one control group of 10 rats was used. This one group of 10 rats was systematically compared to the 9 experimental groups of the same sex. This has resulted in such a lack of statistical power that it is very difficult to establish whether differences observed when comparing the control with each of the 9 experimental groups are due to diet or merely to natural variation. Thus if a certain parameter is particularly high in the control group owing to the randomised nature of the groups, all the differences between the experimental groups and the control group will tend to reveal the same trend and show a decrease in the parameter, without its being possible to infer that the effect is due to diet.

Finally, the groups are not clearly defined: the paper does not indicate whether the diets were formulated so that the total maize content was brought up to 33% with non-GM maize. If this is not the case, each experimental group fed a diet with a given proportion of GM maize ought to be compared to a control group fed a diet with the same proportion of non-GM maize. Lastly, there ought to be a control group fed a standard rat diet (without added maize).

This experimental design therefore cannot be used to infer, from observations made during the study, a causal relationship between treatments and reported effects, particularly for tumour development.

2.4. *Imprecise and fragmentary reporting of results*

Although the shortcomings of the experimental design limit the significance of the study's findings, the HCB Scientific Committee has undertaken a full analysis of the results reported in the paper in order to establish the conclusions that might nevertheless be drawn from them.

The study results are reported in a mainly descriptive style. Apart from the biochemical data, which are given special statistical treatment, the reported data have not been subjected to statistical analysis. Such an approach might be acceptable if the authors confined themselves to description and if this description covered all the data obtained. However, only some results have been selected, reported or commented on, and this fragmentary description forms the basis for unproven conclusions, which are then used to construct unjustifiable pathophysiological hypotheses.

More specifically, the description of the results suffers from:

1. Failure to report data required in toxicology

Data required to interpret the toxicity study, such as data on composition and contaminants in diets, dietary intakes and weight gain of animals, are not reported in the paper. Consumption data and the energy balance of the various diets are all the more important as tumour incidence can vary according to dietary intake (Keenan *et al*., 1997).

2. Unjustified selection of results reported

In a context where results are unsupported by statistical analysis, choosing to single out certain results when reporting on multifactorial experimental research deprives the analysis of any scientific value. Without any justification, the authors have chosen to report the results of the four biochemical parameters and two hormonal parameters that they consider to exhibit

the greatest variation from the control group (Fig. 5B). This choice was made after the results were obtained. It is obviously to be expected that there will be differences between the 864 comparisons[16] made by the authors for the 48 biochemical parameters in the 15th month of the study. Reporting of selected results can consequently be misleading for somebody not familiar with multiple comparisons, who will wrongly conclude that the differences observed are due to differences between the experimental groups and the control groups.

Likewise, presentation of photographs is merely illustrative unless the selection of items is justified, balanced and representative for each group in the study. The photographs in Figure 3 illustrate various objective cytological differences. In the absence of numerical data for occurrence of abnormalities, potential differences between groups cannot be assessed: no information can be derived from mere display of a normal parenchyma and a diseased parenchyma. Similarly, for electron microscopy, the choice and representativeness of the compared items are questionable (Fig. 4). Lastly, beyond empathy for the animals, no information can be derived from photographs of tumours in experimental rats without photographs of tumours in rats from the control groups.

3. Selection of data reported in text

The mortality results are commented on selectively: '*Before this period, 30% control males (three in total) and 20% females (only two) died spontaneously, while up to 50% males and 70% females died in some groups on diets containing the GM maize (Fig. 1)*.' The top left-hand panel of Figure 1 of the paper, concerning males, is reproduced in Figure A below. Just by looking at the histogram, we can see that while the group of males fed 11% GM maize had a mortality rate of '50%' (5 dead rats) at 600 days into the study, the groups of males fed larger doses of GM maize had mortality rates of only '10%' (1 dead rat), i.e. a lower mortality rate than that of the control group. These results are not mentioned. Furthermore, choosing to take the mortality rate at 600 days is arbitrary (see section 2.5. *Survival analysis*, below): the mortality rate in the male control group rose from 30% at 600 days to 50% some days later (dotted line). This simple example demonstrates that it is easy to show different effects, or even opposing tendencies, in the results obtained and that statistical analysis is essential to validate any conclusions that the observations might suggest.

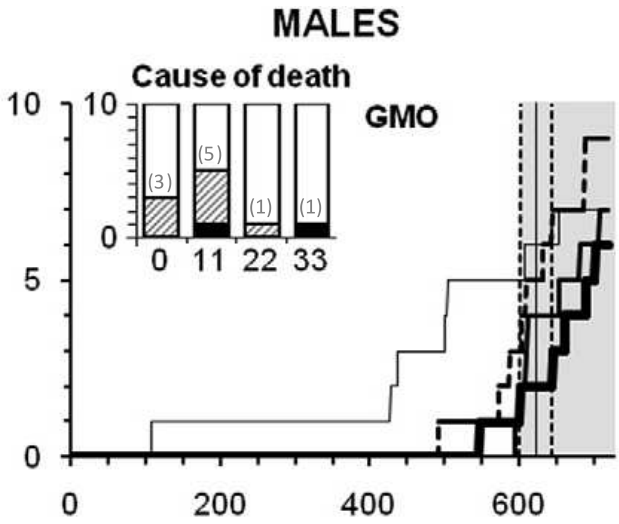

Fig. A. Panel taken from Figure 1 of the paper (Séralini *et al.*, 2012), showing mortality of male rats fed 11, 22 and 33% GM maize diets (thin, medium and bold lines respectively) compared to the control group (dotted line). The histogram shows rat mortality at ~600 days into the study. Cases of euthanasia are shown in black and spontaneous deaths are hatched. The figures in brackets, which have been added to the histogram, indicate the number of dead animals per group, which can be deduced from the stepped line on the y-axis.

[16] This is the total number of comparisons between data on treated groups obtained in the 15th month of the study (18 experimental groups x 48 biochemical parameters = 864) and the corresponding data for the control groups.

The results for tumours are also commented on anecdotally, for example by emphasising two Wilms' tumours (nephroblastomas) in males without its being possible to draw any conclusions: *'It is noteworthy that the first two male rats that died in both GM treated groups had to be euthanized due to kidney Wilm's tumors that were over 25% of body weight. This was at approximately a year before the first control animal died.'* No statistical tests were carried out to compare frequency of tumours between rat groups.

4. Imprecise reporting lacking biological relevance and using non-conventional 'nomenclature'

The graph presentation of mortality and tumour results is imprecise and confusing: the mortality graphs (Fig. 1) cannot be used to compare life expectancy in relation to diet; the graphs showing tumour development (Fig. 2) indicate numbers of 'palpable tumours', which is not a nosological category and cannot be used to draw any statistical, mechanistic or aetiological conclusions.

There is no biological rationale to the description of the biochemical parameters; a single table (Table 3) classifies results according to 'increase' or 'decrease' of parameters while mixing positive and negative values.

The analysis of neoplastic and non-neoplastic pathologies suffers from inadequacies in the description. Histological and macroscopic abnormalities are not clearly identified; no distinction is made between benign tumours, malignant tumours and other organ and tissue lesions (Table 2). It would be impossible to infer a significant difference between experimental groups and control groups solely on the basis of the composite data reported in this paper. Moreover, the authors do not describe their methods for identifying abnormalities (lethal tumours, tumours diagnosed in animals dead of other causes, tumours detected during systematic examination prior to sacrifice at the end of the study) (Gart *et al*., 1986).

The table summarising the most frequent pathologies observed in the different groups (Table 2) uses a 'nomenclature' specific to the authors, which, because it disregards the conventional classifications of anatomical pathology with no explanation, offers no information for specialists in this field (cf. Table 2, Column 1, Line 1, 'Males, in liver'; Line 2, 'In hepatodigestive tract': the latter entity is unknown to specialists; does it include the liver and, if so, are liver abnormalities counted twice?).

2.5. *Unproven conclusions*

Analysis of the results should make it possible to draw, from the data, conclusions relating to differences of effect between treatments and therefore, where appropriate, possible causal relationships between a given treatment and an observed effect. Such conclusions are difficult to justify in principle from this paper not only because the experimental design is not appropriate to the questions raised – particularly tumour development – but also because there is no proper statistical analysis of the data.

The HCB Scientific Committee has used standard statistical methodologies to analyse the results reported in the paper and thus better inform the conclusions that can legitimately be drawn from them. The detail of this analysis is set out in Appendix 4, while its key points are indicated below.

1. Survival analysis

The animals' lifespan has been wrongly estimated by the paper's authors: §3.1. *'Control male animals survived on average 624 ± 21 days, whilst females lived for 701 ± 20'.* These values are inaccurate and are the result of an incorrect calculation, since the authors have not taken into account the phenomenon of censored data (arising, amongst other things, from sacrifice of the animals at the end of the study). In particular, the suggested figures of 20 and 21 days fall considerably short of the accuracy with which the rats' life expectancy can be estimated using these data. It follows that the position of the vertical lines and shaded areas in Figure 1 of the paper, reproduced in Figure A of this opinion, is incorrect.

Inspection of the mortality curves (Fig. 1) provides no evidence of any differences between groups of rats. This would require a statistical procedure that would take into account variation in individuals and therefore survival curves. It is nevertheless possible to test whether the rats from a given experimental group have a tendency to die earlier than the control group. For the study in question, 18 tests would thus be necessary (9 male conditions and 9 female conditions to be compared to their single control groups). For example: H_0 'the 11% GM maize diet has no effect on the lifespan of female rats' vs H_1 'the 11% GM maize diet entails a shorter lifespan for female rats'.

The test statistic, for a rank test, is defined as the sum of the ranks of the control group. Figure B below shows test statistics calculated using the paper's data and ranked in decreasing order, together with the prediction intervals obtained under the null hypothesis and taking proper account of the multiple comparison testing involved. The 18 test statistics are all within the corresponding 90% prediction intervals: consequently, a statistically significant effect of different diets on the rats' lifespan cannot be inferred.

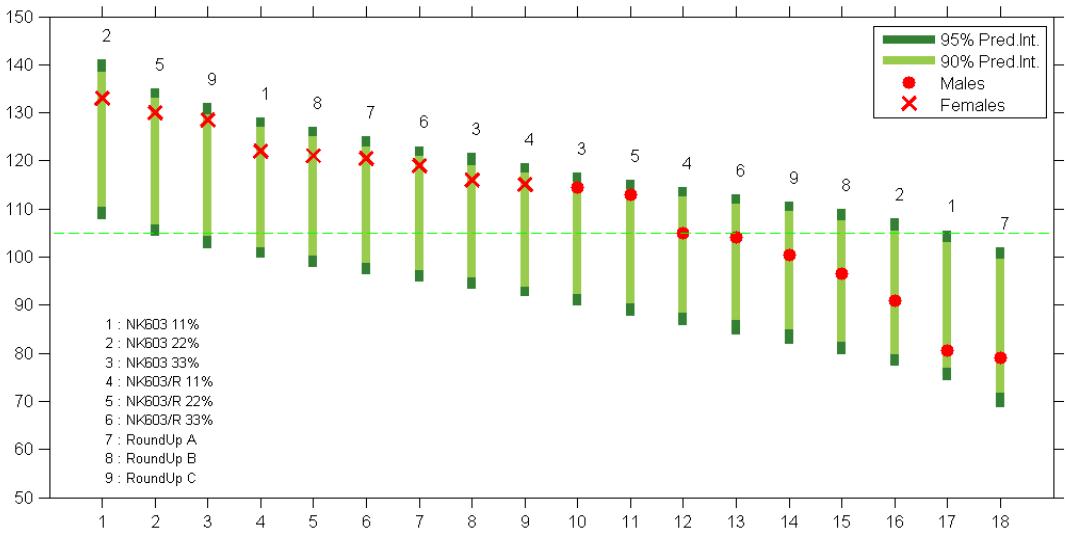

Fig. B. 90% and 95% prediction intervals for 18 test statistics in decreasing order, and observed values of the test statistics for rats' lifespan.

The lack of statistical power, owing to the small number of control rats, prevents us from definitively concluding that the diet does or does not have an effect on mortality, in particular for the female rats. This lack of power can be compensated by introducing *a priori* information on the expected behaviour of control groups. Survival data for the SD rat strain used in the study have been obtained from Harlan Laboratories[17] and offer a useful complement to the information provided by the study reported in this paper.

Use of the reference data provided by the rat supplier confirms that the differences observed in the survival curves of the experimental and control groups cannot be explained by an effect of diet: Figure C shows that the experimental groups are for the most part distributed within the prediction intervals. One experimental group is on the edge of the interval: with 18 groups, it is entirely normal for one observation to be outside the 95% prediction interval. The two-year survival rates observed for the experimental groups are therefore perfectly consistent with the reference data on SD rats provided by Harlan Laboratories. On the other hand, it may be noted that the survival rates observed in the control groups are relatively remote from what the reference values would suggest for this group (the observed proportion of female rats still alive after two years is outside the 95% prediction interval). This further emphasises the statistical weakness of the results obtained from such a small number of cases; it is therefore impossible

[17] Data from a 24-month study by Harlan Laboratories, begun in 2009, on SD rats of the same stock as the rats used in the study by Séralini *et al.* (2012).

to draw any definitive conclusions as to the effect of the different diets on the rats' survival from the data provided in the paper.

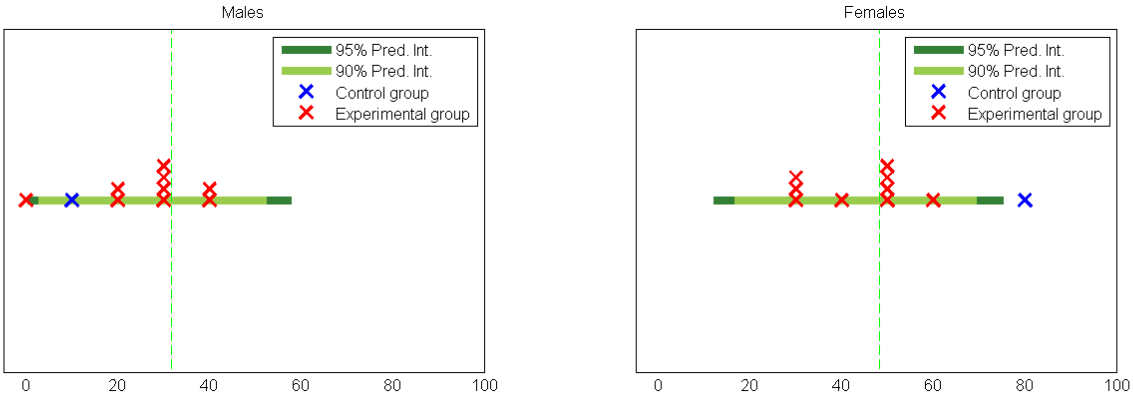

Fig. C. 90% and 95% prediction intervals for 2-year survival rate obtained from data from Harlan Laboratories, and the survival rate observed in the experimental and control groups.

Lastly, a prediction interval for the survival curves of two experimental groups bringing together all the male rats and all the female rats in the study's experimental groups[18] can be constructed by simulation. The survival curves of the control groups are inside the prediction bounds (see Appendix 4). It is therefore impossible to conclude that there is a statistically significant difference between the survival of control rats and that of experimental rats. Here again, the reference data provided by Harlan Laboratories underpin this conclusion since the survival rates observed in the experimental groups are within the prediction intervals for the 2-year survival rate (obtained with a sample of size n = 60) (see Appendix 4).

It cannot therefore be concluded that any experimental diet tested in this study (GM maize, GM maize treated with Roundup®, Roundup®) had a statistically significant effect on survival of the rats.

2. Analysis of tumours

As in the case of the mortality curves, a visual inspection of the curves shown in the paper (Fig. 2) cannot be used to infer any differences in tumour incidence between the groups of rats. This would necessitate a statistical test taking into account variability over time. As in the previous case, the study protocol is not suited to making the 18 proposed comparisons. As shown in Appendix 4, the curves for number of tumours in experimental and control groups are inside the prediction intervals. We therefore cannot infer a statistically significant effect of diet on tumour incidence.

Moreover, as mentioned above, the concept of 'palpable tumours' is open to interpretation; it takes no account whatsoever of the histology of the tumours, and therefore cannot justify any explanation of the effects of the different diets by pathophysiological mechanisms.

3. Analysis of biochemical parameters

In the study, forty-seven biochemical parameters were measured and one biochemical parameter was calculated. For each sex and for each experimental condition, OPLS-DA[19] was used by the authors to discriminate between the control group and the experimental groups. OPLS-DA is frequently used in chemometrics and genomics to identify the subset of variables that can best differentiate different groups. It is pertinent when the number of predictor variables is large in relation to the number of observations. Furthermore, OPLS-DA allows

[18] This strategy of putting the groups together allows construction of more robust and powerful statistical tests; it is used and discussed in Appendix 4.

[19] OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis.

construction of a prediction model, which, for a given set of predictor variables, can give the probability of belonging to each of the groups in question.

The choice of this method and its use by the authors call for some comment (see appendix for full comments):

1. Whatever the method used, it is important to validate the model obtained (i.e. ensure that it possesses good predictive properties) by:

   i) an independent test set, which helps to ensure that the model fitted to the training set retains good predictive properties for new data not previously used to fit the model;

   ii) cross-validation methods, where different subsets of the data are used alternately as training set and test set.

   The study's authors have not validated the models obtained, which cannot therefore be used for predictive purposes.

2. Use of this method assumes a symmetric distribution of predictor variables. Biochemical parameters may have an asymmetric distribution; pre-transformation is therefore necessary. There is nothing to suggest that this was done.

3. Calculating confidence intervals for each parameter is not relevant when many parameters are used, since potential correlations between parameters are totally ignored.

The authors have also selected the data reported. Of the 18 comparisons between experimental groups and control groups, only that for the group of females fed a 33% maize NK603 diet, which supposedly shows the greatest differences, is reported. Moreover, of the 48 biochemical parameters considered, the 6 parameters that, according to the authors, show the biggest differences between the female NK603 (33%) group and the control group are the ones chosen. It is only to be expected that by selecting both the group and the six parameters that exhibit the largest differences, differences between the experimental group and the control group will be conspicuous.

This method does not allow us to explain the observed differences by difference in diet. It is equally impossible to reject the hypothesis that it is natural variability (due to fluctuation of sampling) and/or the parameter selection criteria (choosing the parameters with the largest differences) that explain the observed differences.

As pointed out above, there are no pathophysiological mechanisms to explain the observations made. Taking the example of kidney damage, the term 'severe' applied to lesions observed in dead animals, with no information on the cause or date of death, is not corroborated by the data in Table 3. Variations in blood sodium (1 to 7%) and blood potassium are physiological. The 10% blood potassium variation observed for one group may seem large but is not unusual, since the normal level of blood potassium varies between 4.1 and 4.9 mmol/L. In addition, the absence of proteinuria is uncommon for kidney damage. Lastly, the urine parameters must be treated with caution, owing to the considerable variation in urine collection from rats.

Having analysed the result in the Séralini *et al.* (2012) paper, the HCB Scientific Committee has concluded that the experimental design and statistical tools used suffer considerably from missing data and information and methodological flaws that offer no support for the authors' proposed findings. The HCB Scientific Committee has demonstrated that a rigorous statistical analysis of the paper's data fails to show:

- any statistically significant differences in mortality in rats between the experimental groups and the control groups,
- any statistically significant differences in the number of tumours between the experimental groups and the control groups.

It has further demonstrated that the statistical methodology employed by the authors to analyse the biochemical parameters is inadequate and cannot be used to infer the existence of statistically significant differences between the experimental groups and the control groups.

2.6. *Speculative interpretation*

The discussion section of the paper presents the authors' interpretation of the results obtained in this study. Since the HCB Scientific Committee has found that these results do not show any statistically significant differences in mortality, tumour incidence or biochemical parameters between the experimental groups and the control groups, it has not thought it worth commenting on their speculative interpretation. A few of the points open to criticism may nevertheless be highlighted among the subjects that have not yet been discussed in this opinion:

The argument concerning the hormone dependency of tumours has some unacceptable shortcomings. On the one hand, there is a lack of rigour in discussing tumours' hormone dependency without giving their histological type. On the other, there is no justification for arguing the occurrence, in all experimental groups, of a non-linear endocrine-disrupting effect varying according to the degree of exposure to treatment, on the basis of hormonal parameters (testosterone, oestradiol) reported for a single group (females fed a 33% GM maize diet), using a single measurement interval (sampling at 15 months) and in the form of individual values expressed as percentages of control values. In the absence of (1) numerical data showing means and standard deviations, (2) statistical analysis of variations, and (3) consideration of variation in parameter values as a function of physiological state (oestrous cycle), it is hard to comment on the significance of these observations. The lessons drawn by the authors are basically speculation. The potential effect of hormone imbalance offers a good example of this speculative tendency: the argument is based, on the one hand, on *in vitro* experiments by the authors themselves, whose results have previously been challenged by the scientific community, and, on the other, on references (Vandenberg *et al*., 2012) whose findings are incorrectly reported.

In their discussion, the authors put forward the hypothesis that expression of the transgene in maize NK603 not treated with Roundup® leads to modification of the secondary metabolism of the biosynthesis pathway of phenolic compounds in the GM plant, thus causing endocrine disruption in rats fed this GMO. Two types of secondary metabolite are considered by the authors: isoflavones, and phenolic acids such as caffeic acid and ferulic acid. The authors first note that there is no difference in oestrogenic isoflavone content between the different diets in the study. This is hardly surprising, since this type of compound is not found in maize (Dixon, 2004; Yu *et al*., 2000). Subsequently, the authors state that the GM maize diets have lower levels of caffeic and ferulic acids than the control diets. The authors ascribe this difference to GM maize without examining the other components of the diet. In their opinion, these acids protect against tumour development in mammals and affect the oestrogen metabolism. Phenolic acids rarely exist in their soluble free forms but are incorporated into the plant wall (linked to arabinogalactans and polysaccharides), thus substantially reducing their bioavailability (Buanafina, 2009; Manach *et al*., 2004; Manach *et al*., 2005). Their anticarcinogenic effect is documented mainly by *in vitro* studies on free forms of these secondary metabolites (Gani *et al*., 2012). For this reason, the argument that a reduction in the level of these phenolic acids in GM maize could modulate oestrogen receptors in mammalian cells and cause endocrine disruption remains highly speculative.

As for the biological parameters, the variations described cannot be interpreted as linked to severe liver or kidney damage. Liver damage is described only morphologically, and variations in the blood parameters are either not biologically significant (for example, two parameters that ought to vary along the same lines (ALAT and ASAT) vary diametrically, Fig. 5A) or are not reported by the authors. The variations in the kidney parameters are modest and not always correlated with each other (Fig. 5 and Table 3). Kidney failure is defined by a reduction in the glomerular filtration rate (GFR). Creatinine is the parameter most widely used to estimate GFR, which is calculated by measuring the urinary and serum concentration of the creatinine. Kidney damage results in a rise in serum creatinine and serum urea, a drop in urinary creatinine, a drop in creatinine clearance, and abnormal quantities of protein in urine. In this study, consistency between biological parameters is lacking: in Figure 5A (females fed the 33% GM maize diet) serum creatinine is reduced and urea is normal, urinary creatinine is

reduced and creatinine clearance (termed 'U.Clearance'[20]) is reduced. If reduced clearance is a sign of kidney failure, a reduction in the creatinine present in the blood is abnormal here. With such biological inconsistency, the hypothesis of kidney failure or kidney damage cannot be verified.

3. HCB conclusions and response to first part of referral

Having analysed the results in the Séralini *et al.* (2012) paper, the HCB Scientific Committee has concluded that the experimental design and statistical tools used suffer from missing data and information and unacceptable methodological flaws that offer no support for the authors' proposed findings.

Using rigorous statistical analysis, the HCB Scientific Committee has demonstrated that the paper fails to show:

- any statistically significant differences in mortality in rats between the experimental groups and the control groups,
- any statistically significant differences in the number of tumours between the experimental groups and the control groups.

Moreover, the statistical methodology employed by the authors to analyse the biochemical parameters is inadequate and cannot be used to infer the existence of statistically significant differences between the experimental groups and the control groups.

The hormone disorders claimed by the authors in rats from experimental groups are not specific to a recognised pathology and cannot be distinguished from recognised disorders associated with the Sprague-Dawley strain.

Lastly, despite the authors' highly selective reporting of the results, the pathophysiological relevance of the biological values commented on has not been demonstrated. Kidney and/or liver failure, propounded on the basis of anatomical observations of the animals at autopsy, with no statistical occurrence comparison with the control groups, is not validated by the biological data obtained in the fifteenth month. Moreover, the inadequate and incomplete description of morbid anatomical lesions is not sufficient to corroborate the results. These observations are therefore not conclusive regarding the effect of GM maize on rats.

Consequently, the HCB Scientific Committee considers that the paper offers no information supporting the existence of a health risk associated with consumption of maize NK603, whether or not treated with a Roundup® herbicide formulation.

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Appendix 1: Referral

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RÉPUBLIQUE FRANÇAISE

Ministry of Social Affairs and Health	Ministry with responsibility for the Social Economy and Consumer Affairs in the Ministry for the Economy and Finance	Ministry for Ecology, Sustainable Development and Energy	Ministry of Agriculture, Food and Forestry

Mr Marc Mortureux

Director-General,
National Agency for Food, Environmental and Occupational Health Safety

Mr Jean-François Dhainaut

President,
High Council for Biotechnology

Paris, 24 September 2012

Dear Mr Mortureux,

Dear Mr Dhainaut,

A paper entitled '*Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize*' has recently been published in the scientific journal *Food and Chemical Toxicology* by the team of Professor Gilles-Eric Séralini. This paper concerns a study conducted for 2 years on rats fed genetically modified maize NK603, treated or untreated with Roundup, or the Roundup herbicide alone.

We request you, by this referral, to confer together to undertake an analysis of the study reported by this paper in order to determine whether or not it is likely to cast doubt on the findings of previous assessments of this GMO and in particular whether it may be considered conclusive regarding the possible health risk of food derived from GM plants containing event NK603.

ANSES is further requested to determine whether or not this study is likely to cast doubt on the findings of ANSES previous assessments of the Roundup herbicide.

On the basis of this analysis, you are requested to assess whether the study's protocol and findings call into question current or future guidelines for health risk assessment.

Would you please deliver an opinion on this paper by 20 October 2012 and on the suitability of health risk assessment procedures and proposed adjustments to guidelines, if necessary, by 20 November 2012.

Yours sincerely,

Marisol Touraine	Benoît Hamon	Delphine Batho	Stéphane Le Foll
Minister of Social Affairs and Health	Minister with responsibility for the Social Economy and Consumer Affairs in the Ministry for the Economy and Finance	Minister for Ecology, Sustainable Development and Energy	Minister of Agriculture, Food and Forestry

Appendix 2: Preparation of the opinion

An *ad hoc* working group was set up in response to the referral of 24 September 2012, consisting of expert rapporteurs chosen for their expertise in subjects relevant to analysis of the paper by Séralini *et al.* (2012), their public-sector affiliation and their independence:

- Four expert rapporteurs from outside HCB: Dr Avner Bar-Hen, Professor, Université Paris-Descartes, statistician; Dr Martine Kolf-Clauw, Professor, École Nationale Vétérinaire de Toulouse, veterinary toxicologist; Dr Francelyne Marano, Emeritus Professor, Université Paris-Diderot, specialist in cell biology, toxicology and genotoxicity; and Dr Daniel Marzin, Emeritus Professor, Université de Lille II, toxicologist;
- Three expert rapporteurs from the HCB Scientific Committee: Dr Joël Guillemain, expert pharmaco-toxicologist, lecturer, Université de Tours; Dr Marc Lavielle, Research Director, INRIA, statistician; and Dr Rémy Maximilien, Research Director, CEA, toxicologist;
- Two expert rapporteurs from the HCB Scientific Committee, namely the Chair and Vice-Chair: Dr Jean-Christophe Pagès, Professor of Biochemistry and Molecular Biology, medical practitioner, Tours Faculty of Medicine; and Dr Jean-Jacques Leguay, CNRS Director of Research in biology and plant physiology.

An additional expert from the private sector (Dr Roy Forster, toxicologist, from the Centre International de Toxicologie) was consulted on technical issues by the working group.

The working group analysed the paper by Séralini *et al.* (2012) under the guidance of Dr Rémy Maximilien and submitted a report on 2 October 2012 for consideration by the HCB Scientific Committee. All outside experts signed a non-disclosure commitment and certified that they had no conflicts of interest in this matter. The working group's expert rapporteurs analysed the paper in their own fields of expertise and were questioned by the Scientific Committee. However, they have not contributed directly to preparation of this opinion, for which the HCB Scientific Committee bears responsibility.

The final opinion was prepared by the HCB Scientific Committee chaired by Dr Jean-Christophe Pagès, with Dr Jean-Jacques Leguay as vice-chair, and under the scientific coordination of Dr Catherine Golstein, the HCB Senior Scientific and European Affairs Officer.

The HCB Scientific Committee is a multidisciplinary committee consisting of scientific figures appointed by decree for their specialities in relation to HCB missions. In alphabetical order of surname, the HCB Scientific Committee comprises:

Claude Bagnis, Yves Bertheau, Pascal Boireau, Denis Bourguet, François-Christophe Coléno, Denis Couvet, Jean-Luc Darlix, Elie Dassa, Maryse Deguergue, Marion Desquilbet, Hubert de Verneuil, Robert Drillien, Nathalie Eychenne, Anne Dubart-Kupperschmitt, Claudine Franche, Philippe Guerche, Joël Guillemain, Mireille Jacquemond, André Jestin, Bernard Klonjkowski, Marc Lavielle, Jane Lecomte, Olivier Le Gall, Jean-Jacques Leguay, Didier Lereclus, Rémy Maximilien, Antoine Messéan, Nicolas Munier-Jolain, Jacques Pagès, Jean-Christophe Pagès, Daniel Parzy, Catherine Regnault-Roger, Pierre Rougé, Patrick Saindrenan, Annie Sasco, Pascal Simonet, Virginie Tournay, Bernard Vaissière and Jean-Luc Vilotte.

Antoine Messéan did not contribute to either the preparation or the drafting of this opinion because he is a member of the EFSA GMO panel, and EFSA has also been asked for an opinion on this subject. None of the other Scientific Committee members declared a conflict of interest that might affect preparation of this opinion.

Participation in preparation of this opinion does not imply that the adopted opinion was fully approved by all participants but indicates that there was majority in its favour, within the experts' fields of competence and after presentation of all points of view.

Appendix 3: Statistical aspects of the experimental design

A sample of 200 rats including 100 males and 100 females was randomized into 20 groups of 10 rats of the same sex. Within each group, rats received the same diet.

For each sex, only one control group of 10 rats was used. It is therefore uniquely this same group of 10 rats that is systematically compared to the 9 experimental groups of the same sex.

This results in such a lack of statistical power that it is difficult to establish whether differences observed when comparing the control with the 9 other groups are due to diet, or merely to natural variation within the control group. Thus, if a certain parameter is particularly high for the control group, all of the differences between the experimental and control groups will tend to have the same trend, showing a decrease in the parameter while not necessarily implying that the effect is due to diet. For example, the authors conclude without advising precaution in §3.3 that "*Creatinine or clairance decreased in urine for all treatment groups in comparison to female controls (Table 3)*", though it is likely that the particularly high values of these parameters in the control groups are the reason for these differences.

No initial calculation is provided to suggest the number of subjects required to detect a biologically significant effect. Such a calculation would have been particularly useful for evaluating the amount of information, for example concerning survival and number of tumors, which we might expect to obtain with the chosen protocol. It would have therefore been possible to quantitatively consider the potentially problematic choice of using a strain of rat that naturally develops tumors with a high probability. In effect, the higher the risk of naturally developing tumors, the more animals per group required to exhibit a significant increase in the number of tumors due to diet. Consider for example two strains of rat, A and B, for which the risk of naturally developing a tumor within a given period of time is 10% for A and 60% for B. The tables below show the number of rats required to suggest an increase in the number of tumors of 10%, 20% or 30% when the type I error α and type II error β are both equal to either 5% or 10%. We see that using strain B requires more rats than strain A, independent of the given error risks, when the increase to be revealed is less than 30%.

		Souche de rats	
		A p=10%	**B** p=60%
Effet à mettre en évidence	+ 10%	135	248
	+ 20%	41	60
	+ 30%	24	24

α=β=5%

		Souche de rats	
		A p=10%	**B** p=60%
Effet à mettre en évidence	+ 10%	86	156
	+ 20%	25	36
	+ 30%	15	15

α=β=10%

Table 1. Number of rats required in an experimental group in order to suggest a given increase in the number of tumors for two strains of rat A and B (risk of naturally developing tumors in a given time period is respectively 10% and 60%) for two given type I and II error risks[21].

More generally, the article does not mention the statistical protocol. The authors appear to have undertaken their statistical analysis as a function of the results obtained, entirely contradictory to elementary rules of good statistical practice. In effect, the statistical significance of an observed difference in a given parameter is not the same when the parameter has been selected *a priori* (before obtaining results) or *a posteriori* (from among the parameters exhibiting the largest differences). The authors give in Figure 5.B the 4

[21] The type I error α is the probability of concluding that there is an increase even though there is not. The type II error β is the probability of not detecting an increase even though there is one.

biochemical parameters and 2 hormones, which exhibit the largest differences, within the group that shows the most differences. This choice has been made *a posteriori*, i.e. after the results were obtained. It is statistically expected that some of the 18x48=864 comparisons will provide differences that appear significant. Presenting these partial results in this way may mislead a non-specialist in multiple comparison (here 864) statistics to conclude that the observed differences are due to the difference between experimental and control conditions.

Appendix 4: Statistical aspects of the analysis of the results

1. Statistical inference

Inferential statistics allow us to evaluate the uncertainty and probability of making a mistake when making conclusions about the presence or absence of effects; that is, if the observed differences can be explained by a change in diet or are simply due to random fluctuations in the sampling. Said another way, the natural question to ask here concerns reproducibility of the results: if we repeat the same experiment under the same conditions, what are the chances of obtaining similar results?

As for the survival analysis and the counting of the number of tumors, the authors have entirely ignored this statistical aspect, while proposing unsubstantiated interpretations of their experimental results. On pages 8-9 of the paper, we read:

> *"**All treatments in both sexes enhanced large tumor incidence** by 2–3-fold in comparison to our controls […]"*
>
> *"Suffering inducing euthanasia and deaths corresponded mostly in females to the development of large mammary tumors. **These appeared to be clearly related to the various treatments when compared to the control groups**."*

Not a single statistical argument can be found in this article to suggest a cause-effect relationship of this type. There is not a hint of statistical analysis that would suggest a statistically significant difference in survival and number of tumors between the experimental and control groups.

As for the biochemical parameters, we may read in the article's conclusion p. 10:

> *"The results of the study presented here **clearly demonstrate** that lower levels of complete agricultural glyphosate herbicide formulations, at concentrations well below officially set safety limits, **induce severe hormone-dependent mammary, hepatic and kidney disturbances**."*
>
> *"Altogether, the **significant** biochemical disturbances and physiological failures documented in this work **confirm the pathological effects** of these GMO and Roundup® treatments in both sexes, with different amplitudes".*

Such statements merit rigorous justification and validation. Here, it is absolutely impossible to conclude with certainty about the toxicity of NK603 on such a restricted set of data.

2. Survival analysis

2.1. Lifespan

The authors explain in §3.1 that *"Control male animals survived on average 624 ± 21 days, whilst females lived for 701 ± 20".* These values are the result of an incorrect calculation because the data is censored (we do not know when the animals still alive at end of study would have died naturally since they were euthanized). Instead, what has been calculated is the empirical mean and standard deviation of the uncensored observed values joined with the censored values for still-alive rats (as if a rat still alive at T=720 days is considered dead at T=720 days). The results given are therefore inexact because this procedure introduces a bias by underestimating the average date of death and clearly also the standard error of the estimator. To have chosen not to consider everything that occurs after 624-21=603 days is therefore not justified because this value comes from an incorrect calculation.

Correct calculation of the survival distribution for different groups requires the introduction of a parametric model, but the use of such an approach is constrained, given the limited amount of data per group. For example, if we fit a Gaussian model for the survival time of the males, the

estimated mean and standard deviation are respectively 626 and 68 days. For the females, it is 892 and 206 days. It is not correct to proceed as the authors have done and calculate the standard error for the mean merely by dividing the standard deviation by $\sqrt{10}$, as would be done for uncensored Gaussian variables. Due to censoring, the distribution of the estimator of the mean is much more spread out and asymmetric, meaning that the use of the standard error for calculating confidence intervals is not meaningful.

2.2. Comparisons between experimental and control groups

Survival analysis in this study was limited to graphical representation of mortality curves in each group (number of deceased rats as a function of time), shown in the following figure.

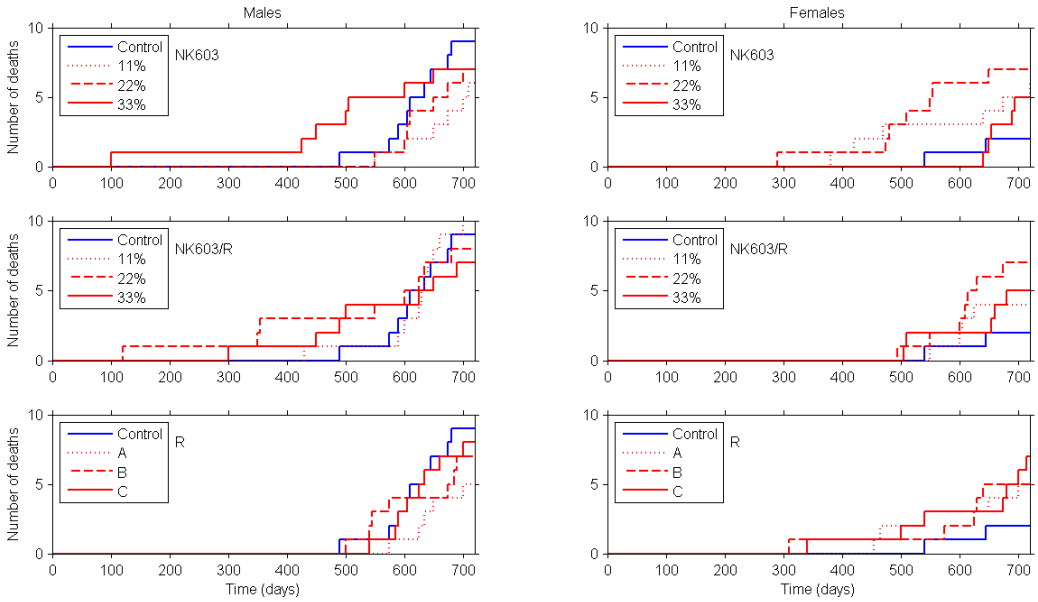

Fig. 1. Mortality curves for the 18 experimental and 2 control groups.

A visual inspection of these observed mortality curves does not provide the slightest evidence of differences between experimental and control groups.

Numerous statistical techniques exist for comparing survival/mortality curves. To begin with, we might consider the 18 possible comparisons between the experimental and control groups. The Wilcoxon rank-sum test is a nonparametric test that allows comparison of the rank statistics of two samples.

In this way we can test whether the rats from a given experimental group have a tendency to die earlier than those of the control group. For example:

H0 "the NK603 11% diet has no effect on the lifespan of female rats"

vs

H1 "the NK603 11% diet leads to decreased lifespan for female rats."

The test statistic is defined as the sum of the ranks of the control group. For each of the 18 comparisons, this test statistic can be calculated and compared with a prediction interval obtained under the null hypothesis. The statistical significance can then be calculated for each of the 18 tests:

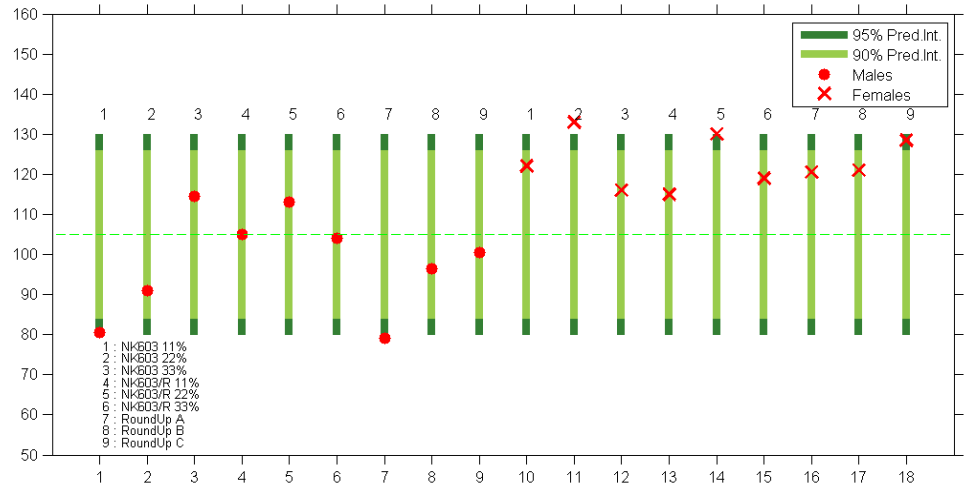

Fig. 2a. 90% and 95% prediction intervals of 18 test statistics along with the observed values of these test statistics.

However, these prediction intervals do not take into account the multiple comparison testing involved. Rather than putting into practice an overly conservative test (which has a tendency to systematically not reject the null hypothesis), we can estimate by either simulation or permutation the probability distribution of the 18 statistics used for this test. The following figure shows the prediction intervals of the 18 test statistics ranked in decreasing order (intervals estimated by simulation here). The 18 test statistics are all within the corresponding 90% prediction intervals:

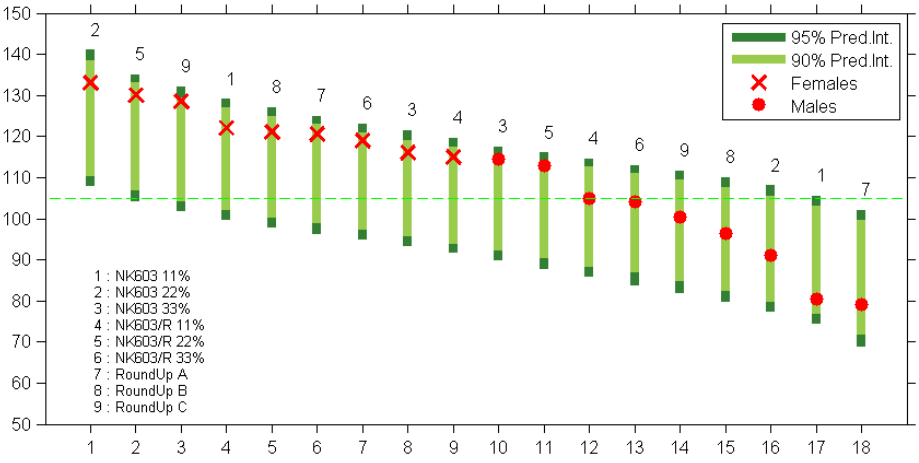

Fig. 2b. 90% and 95% prediction intervals of 18 test statistics put in decreasing order along with the observed values of the test statistics.

We can also estimate the statistical significance of each comparison like an empirical quantile. The table shows for each of the 18 tests the test statistic, *i.e.,* the sum of ranks of the control group (under the null hypothesis, the expected value of this statistic is (1+2+…+19+20)/2=105), the statistical significance of the corresponding rank-sum test, and the adjusted statistical significance that takes into account the fact that multiple tests were performed (by simulation or permutation). Groups that have a tendency to die before the control group (test statistic of over 105) are in red; the other ones are in blue:

Experimental group	Test statistic	Statistical significance	Corrected statistical significance (simulation)	Corrected statistical significance (permutation)
M - NK603 11%	80.5	0.972	0.879	0.895
M - NK603 22%	91	0.865	0.665	0.624
M - NK603 33%	114.5	0.247	0.151	0.073
M - NK603/R 11%	105	0.515	0.385	0.291
M - NK603/R 22%	113	0.285	0.167	0.072
M - NK603/R 33%	104	0.545	0.368	0.263
M - RoundUp A	79	0.978	0.819	0.808
M - RoundUp B	96.5	0.753	0.514	0.452
M - RoundUp C	100.5	0.648	0.422	0.343
F - NK603 11%	122	0.072	0.199	0.174
F - NK603 22%	133	**0.011**	0.158	0.166
F - NK603 33%	116	0.176	0.195	0.124
F - NK603/R 11%	115	0.188	0.185	0.104
F - NK603/R 22%	130	**0.021**	0.122	0.104
F - NK603/R 33%	119	0.116	0.152	0.092
F - RoundUp A	120.5	0.092	0.140	0.098
F - RoundUp B	121	0.085	0.178	0.139
F - RoundUp C	128.5	**0.029**	0.087	0.067

Table 1. The 18 test statistics (Wilcoxon rank-sum test) and the associated statistical significances.

The group that exhibits the largest difference with respect to survival is the female group with the diet of 22% untreated NK603 corn. The statistical significance is 1.1% (i.e., the probability of obtaining a test statistic greater than or equal to 133 under the null hypothesis is 1.1%). Taking into account the multiple tests undertaken, this probability is 15.8% when estimated by simulation and 16.6% by permutation. Thus, the probability that the largest test statistic among 18 is greater than or equal to 133 under the null hypothesis is around 16%. The table therefore allows us to conclude that:

No observed difference between the survival curves of the experimental and control groups is statistically significant.

Lastly, we can show by simulation that, for example, if the death of 5 rats in an experimental group of 10 occurs before the death of a rat in the control group, the statistical significance of the test is 8%. This drops to 2% (resp. 0.8%) if there are 6 (resp. 7) experimental rats that die before a control rat does.

2.3. Use of reference data

The lack of power, due to the small number of control rats, clearly stops us from being able to formally make conclusions as to the presence or absence of an effect of diet on survival, in particular for the female rats. This lack of power can be compensated by introducing *a priori* information on the expected scenario for the control groups. Indeed, survival data for the SD rat strain are available from the Harlan Company and can be used to add pertinent information to the experimental set-up. Obviously, this data has not been obtained under exactly the same conditions as the present study and bias may therefore be introduced by including this *a priori* information. On the other hand, information coming from the control groups is not biased if all groups were treated under the same conditions, but as we have seen, this information is

affected by a large level of variability. A combination of the *a priori* information and the experimental data leads to a good bias-variance compromise.

Here, the data provided by the Harlan Company indicates a 2-year survival rate of 32% for males and 48% for females. For each sex, the number of rats alive after 2 years is a binomial random variable for which we can calculate 90% and 95% prediction intervals[22].

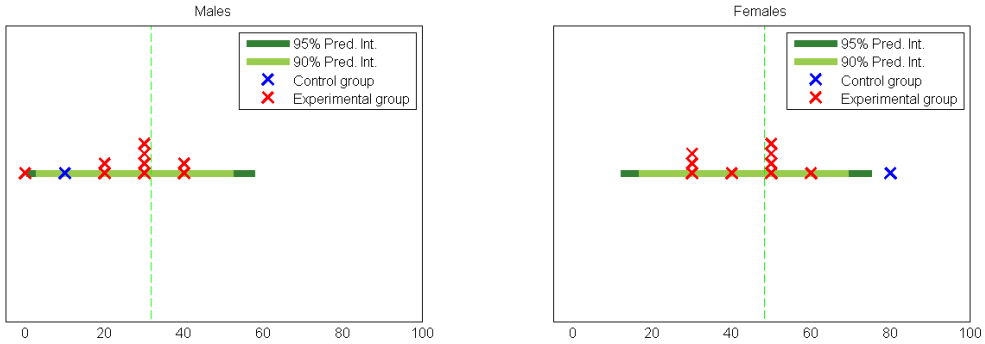

Fig. 3. 90% and 95% prediction intervals for 2-year survival rate obtained from data from the Harlan Company, and the survival rate observed in the experimental and control groups.

The experimental groups are for the most part distributed within these prediction intervals. With 18 groups, it is entirely normal that one observation be found on the edge of the 95% prediction interval. The 2-year survival rates are entirely in agreement with the reference data provided by the company when the rats are raised in normal conditions.

The use of reference data provided by the Harlan Company confirms that we cannot explain the differences observed in the survival curves of different experimental and control groups by invoking the effect of diet.

It can also be noted that the survival rates observed in the control groups are nevertheless relatively far from what the reference values would suggest (the observed proportion of female rats still alive after 2 years is outside the 95% prediction interval). This further emphasizes the fragility of the resulting statistics obtained from such a small number of cases; it is impossible to provide definitive conclusions here.

2.4. Less groups, more power

These results show that it is impossible to consider with sufficient power the 18 possible comparisons between experimental and control groups. The experimental protocol is not adapted for such an ambitious goal.

Limiting the comparisons by grouping together certain groups allows the construction of more powerful and robust tests. For example, we might limit ourselves to test whether mortality in the control group is lower than in the experimental groups in general. We therefore group together the experimental groups for each sex and construct a single survival curve (probability to be alive with respect to time). The two experimental groups (male and female) are now made up of 90 animals: we can therefore reasonably approximate the true survival functions by the empirical ones obtained from the two samples of 90 rats. These can then be compared to the survival functions of the two control groups.

[22] For binomial variables, only the quantiles of order (*i/n, i*=0, 2,…n) can be directly calculated from the probability distribution. We can obtain any other quantile using linear interpolation.

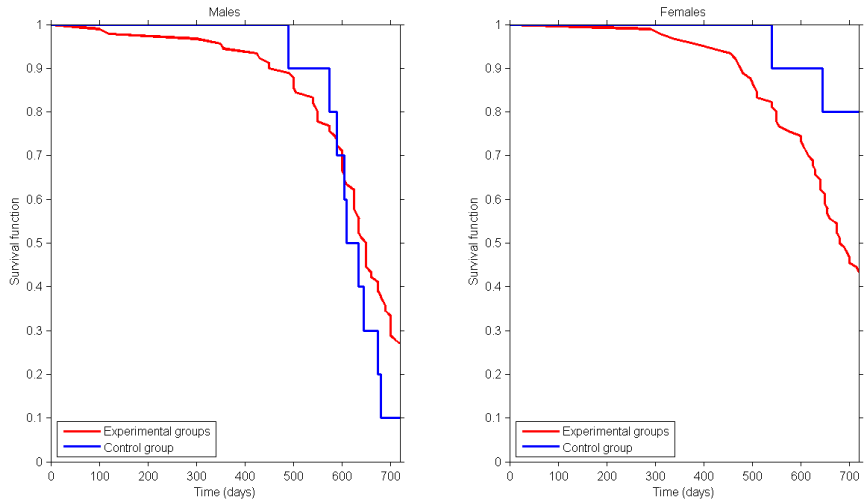

Fig. 4. Survival curves for the experimental and control groups. For each sex, the experimental group is made up of the 9 initial experimental groups.

The question is: could the blue survival curve (observed control group) have been obtained from 10 rats whose survival probability is characterized by the red curve?

A prediction interval for the survival curves of the two experimental groups (male and female) can be constructed easily via simulation. For each sex, we use the survival curve of the experimental group (red) to simulate a very large number (10,000 here) of groups of 10 rats and their dates of death. We can thus construct 10,000 empirical survival curves from the 10,000 groups. From this, we can construct a prediction interval (level 1-α) by calculating at each instant of time the empirical $\alpha/2$ and 1- $\alpha/2$ quantiles of the 10,000 survival curves. We thus obtain a 90% (resp. 95%) prediction interval by calculating the empirical 5% (resp. 2.5%) and 95% (resp. 97.5%) empirical quantiles.

The following figure presents for each sex the survival curves of the 9 experimental groups and the 90% and 95% prediction intervals.

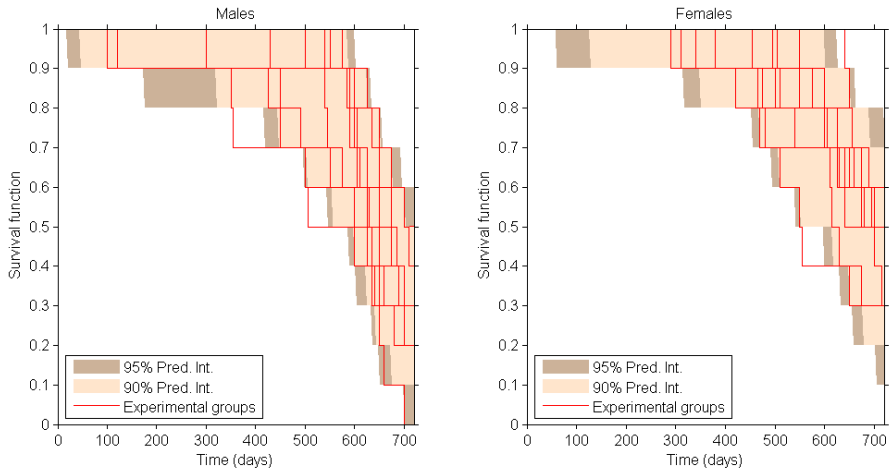

Fig. 5. 90% and 95% prediction intervals for the survival of the combined experimental groups, and the survival curves of the 9 experimental groups seen individually.

The next figure shows for each sex the same prediction intervals, but now with the survival curve of the control groups:

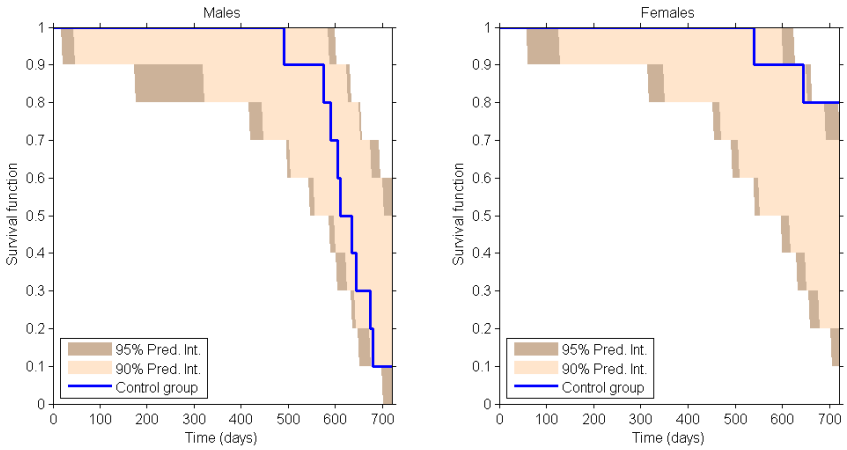

Fig. 6. 90% and 95% prediction intervals for the survival of the combined experimental groups, and the observed survival curves of the control groups.

The survival curves of the control groups are essentially inside the prediction bounds:

We cannot conclude that there is a statistically significant difference between the survival of the control and experimental rats.

Again, the reference data provided by the Harlan Company underpin this conclusion because the prediction intervals of the mortality rate at 2 years (for a sample of size n=90) contain the observed rates in the experimental groups:

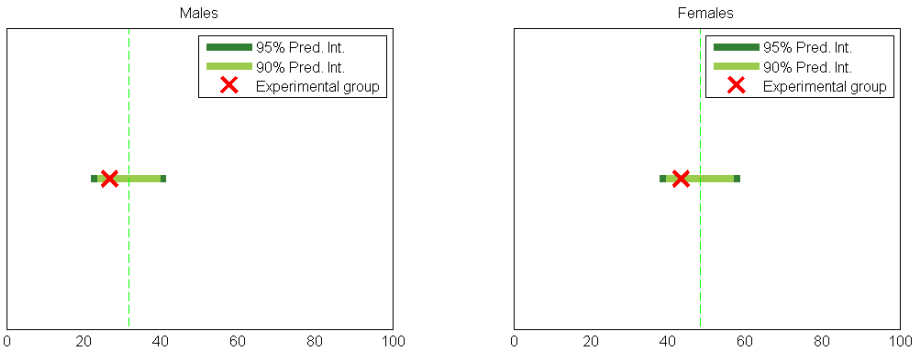

Fig. 7. 90% and 95% prediction intervals for the 2-year mortality rate obtained from the Harlan data, and 2-year mortality rates seen in the experimental groups (each of size 90).

The choice to group together the 9 experimental groups and test whether their distribution is different from the control group is obviously debatable. We might want to implement other tests to test for example whether consumption of a GM corn has an impact on survival, independent of the dose of GM corn and the associated treatment (Roundup® or not). The combined experimental group is then made up of the first 6 experimental groups NK603 and NK603/ Roundup®, 11%, 22% and 33%), while the control group is just the initial control group (no GMO, no GMO/Roundup®, no Roundup®) together with the 3 groups of rats exposed to Roundup®. The figure below shows that no significant differences exist between these groups for either the males or females. Therefore, we are unable to conclude that there is a significant effect of maize NK603 on rat mortality.

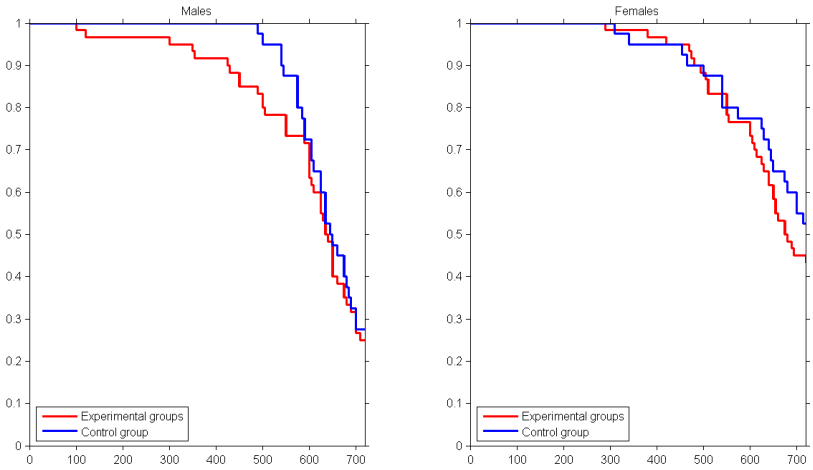

Fig. 8. Survival curves for the experimental and control groups. For each sex, the experimental group contains the 6 initial experimental groups that consumed maize NK603 (with or without RoundUp®). The control group contains the initial control group along with the 3 experimental groups that had not consumed maize NK603.

We can also test whether the absorption of any quantity of Roundup® (in liquid form) has an impact on survival. The experimental group is thus formed of the 3 groups that absorbed Roundup® but not NK603, and the control group is formed by combining all remaining experimental groups with the initial control group. The figure below shows that there is no significant difference in survival between control and experimental groups, both for males and females. We are thus unable to conclude that Roundup® has a significant effect on rat mortality.

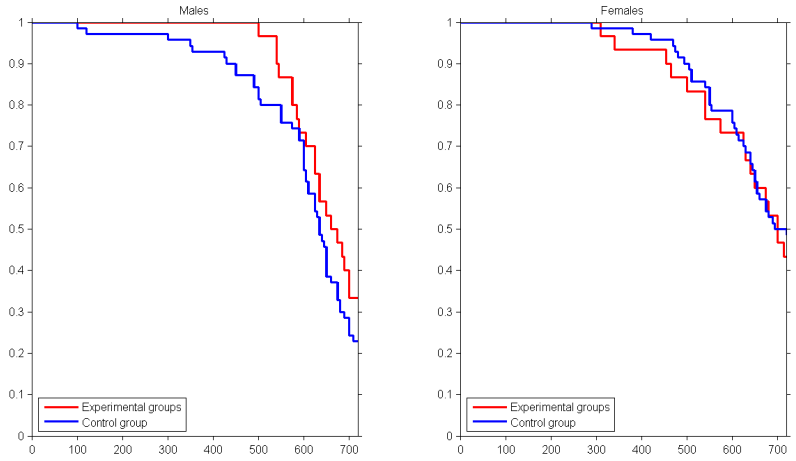

Fig. 9. Survival curves for experimental and control groups. For each sex, the experimental group is made up of the 3 groups that had absorbed Roundup® (but not NK603). The control group is made up of the 6 remaining experimental groups and the initial control group.

In conclusion, we cannot conclude that there is a statistically significant effect of any treatment (NK603, NK603 treated with Roundup®, Roundup®) on the survival of rats.

3. Number of tumors

Analysis in the study of the number of tumors was limited to a graphical representation of the number of palpable tumors observed in each group as a function of time:

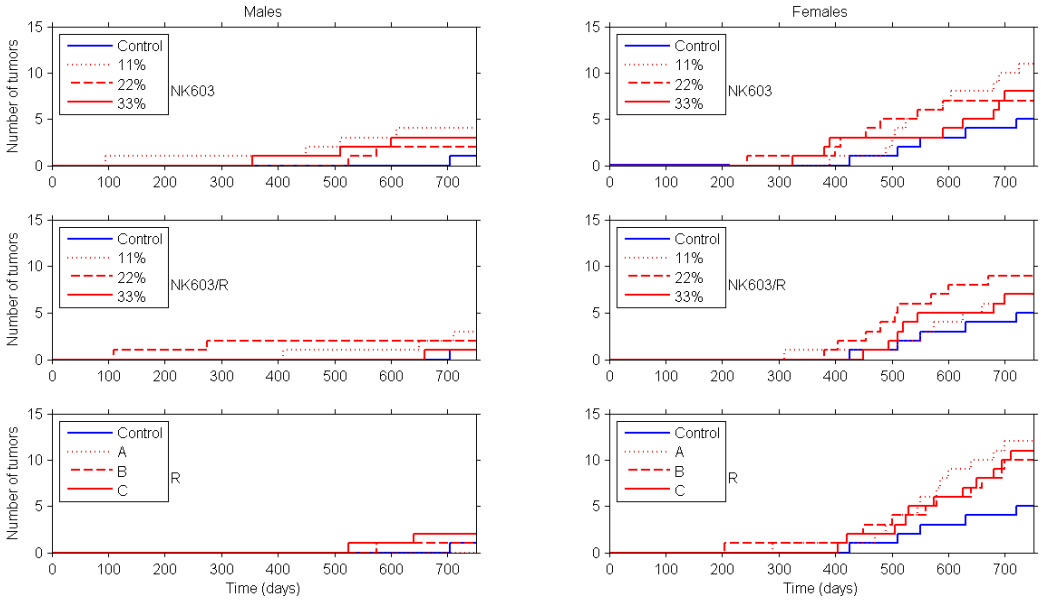

Fig. 10. Evolution of the number of palpable tumors in the 20 groups.

As was the case for the mortality curves, simply looking at the curves here does not allow us to make any confident conclusion as to differences between populations. For that, a rigorous statistical test, which takes into account statistical variability and thus variability in the tumor count curves, would need to be implemented.

As before, the experimental protocol is not adapted for making the 18 proposed comparisons, and as before, we can for each sex group together the experimental groups and construct a single curve for the number of tumors, which can then be compared with that of the control group:

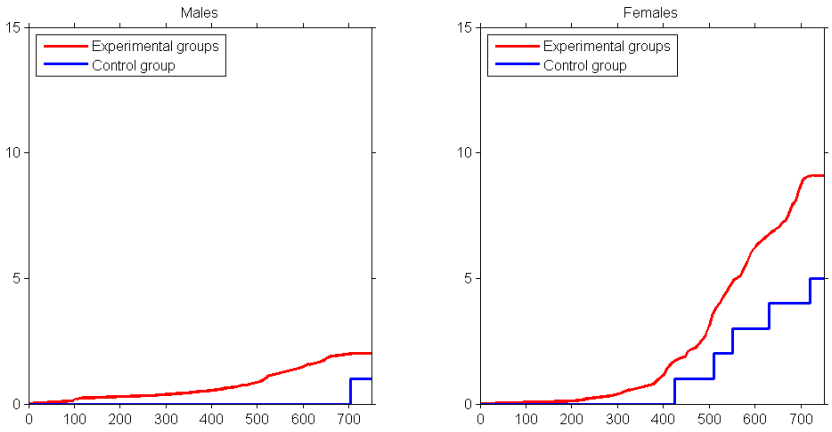

Fig. 11. Number of palpable tumors in the experimental and control groups. For each sex, the experimental group is made up of the combined 9 initial experimental groups.

The question is whether the blue curve for the number of tumors (observed control group) could have been obtained from 10 rats that had been used to create the red curve.

Prediction intervals for the number of tumors in the 2 experimental groups (male and female) can be easily constructed by supposing that for each sex the number of tumors is a non homogeneous Poisson process whose intensity is given at each instant of time by the red curve. In this way, 90% (resp. 95%) prediction intervals can be obtained by calculating at each instant of time the 5% (resp. 2.5%) and 95% (resp. 97.5%) quantiles of a Poisson variable.

The following figure shows for each sex, the number of tumors of the 9 experimental groups and the 90% and 95% prediction intervals:

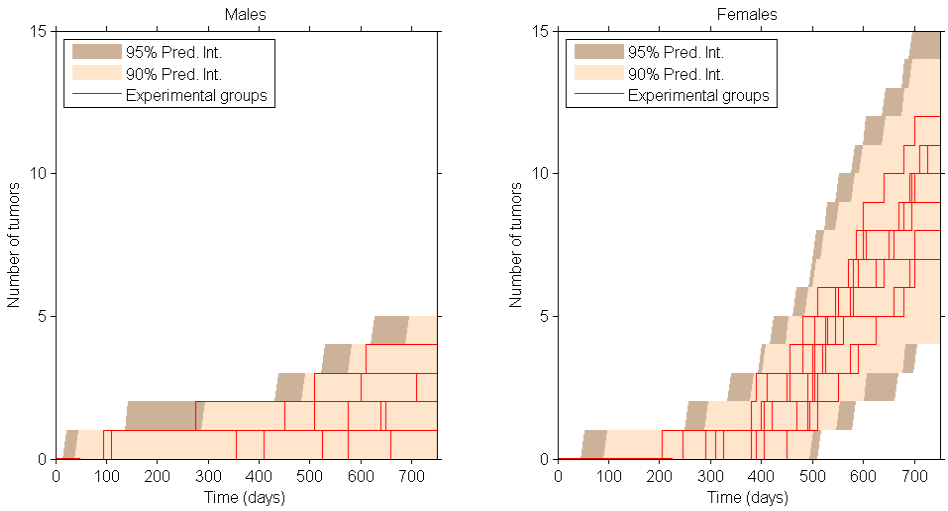

Fig. 12. Intervalles de prévision de niveau 90 % et 95 % des nombres de tumeurs du groupe expérimental et nombres de tumeurs observées des groupes expérimentaux.

The following figure then shows for each sex the same prediction intervals, this time along with the evolution of the number of tumors in the respective control group:

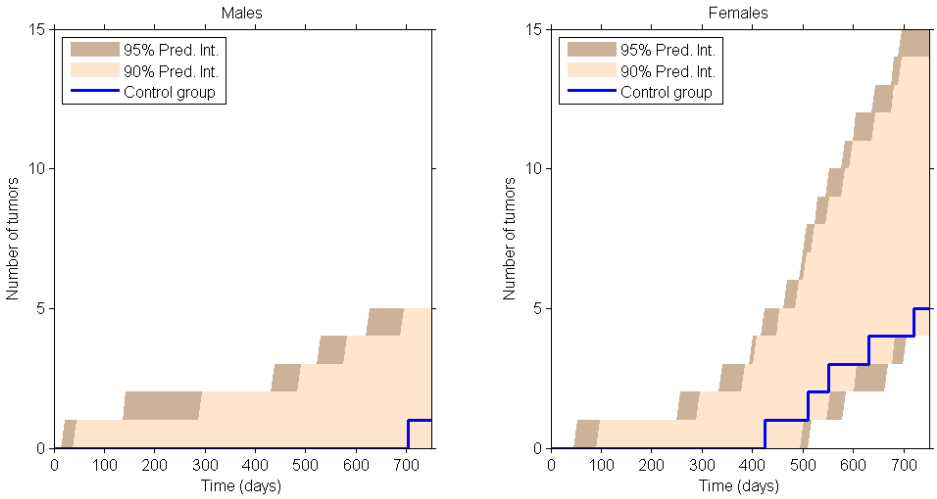

Fig. 13. 90% and 95% prediction intervals for the number of tumors in the experimental groups, and the evolution of the number of observed tumors in the control groups.

The curves for the number of tumors in the control groups are inside both prediction intervals:

We cannot conclude that there is a statistically significant effect of diet on the number of tumors.

4. Biochemical parameters

In the study, 47 biochemical parameters were measured and one additional parameter was calculated for each of the 20 groups. For each sex and for each experimental condition, an "Orthogonal Partial Least Squares Discriminant Analysis" (OPLS-DA) method was implemented to discriminate between the control and experimental group.

The OPLS-DA method is frequently used in chemometrics and genomics for identifying a subset of variables that can best separate different groups. It is particularly pertinent when the number of predictor variables is large with respect to the number of observations. Furthermore, the method allows construction of a predictive model that, for a given set of predictor variables, can output the probability of belonging to each of the groups under consideration.

The choice of this method and its use by the authors in the present context deserves several comments:

1. This type of method is well-known for "over adjusting" the observed data when the number of predictor variables is large with respect to the number of observations (which is the case here). In effect, it is always possible to find a model defined by 48 parameters that perfectly separates 2 groups of 10 subjects, no matter the groups! To validate the obtained model (i.e., to assure oneself that it possesses good predictive properties), one can use:

   - an independent test set, which helps to ensure that the model fitted to the training set retains good predictive properties on new data which has not been previously used to fit the model,
   - cross-validation methods (essentially, different subsets of the data are successively used to play the role of training and test set).

   The authors of the study provide no criteria for validation of the models constructed using the experimental groups of 10 rats. These models cannot therefore be used in a prediction framework.

2. A given model is defined by 48 parameters. Eighteen models are thus each defined by 48 parameters. These many models constructed from so little data is far from ideal: such models will be unstable and have extremely limited predictive power. It would have been more useful to construct a single model that integrated the effects of diet, sex, and perhaps their interaction (or even potential nonlinear dose-effect relationships). The advantage of working with a single model is that it would be built using all of the data, thus helping to reduce the over-parameterization of the model, leading to better stability and predictive power.

3. The use of the method implicitly supposes a symmetrical distribution (as close as possible to a Gaussian distribution) of the predictor variables. It is known that parameters such as biochemical ones have asymmetric distributions and that a pre-transformation is necessary to render them as "Gaussian" as possible. For example, it is standard practice to use certain log-parameters rather than the original parameters. A report by ANSES[23] suggests using the Box-Cox transformation for each parameter, the power parameter being the same for each group meaning that different position parameters characterize each group.

4. Calculating confidence intervals for each parameter is not pertinent when many parameters have been used. In effect, potential correlations between parameters and the multidimensional point-of-view are totally ignored. What is needed therefore is to:

   - be able to calculate confidence ellipses in order to take into account possible correlation between parameters,

[23] Recommandations pour la mise en œuvre de l'analyse statistique des données issues des études de toxicité sub-chronique de 90 jours chez le rat dans le cadre des demandes d'autorisation de mise sur le marché d'OGM

http://www.afssa.fr/Documents/BIOT2009sa0285Ra.pdf

- correct the confidence intervals in order to correctly control the type I error (multiple intervals).

5. These types of classification methods remain quite empirical and are not particularly suited to a prediction context, for which it is necessary to calculate statistical significance (p-values) and/or construct confidence intervals. In effect, the laws of statistics used are poorly understood and methods such as bootstrap and jack-knife, used for calculating confidence intervals, cannot be rigorously justified.

Beyond the questionable choice of using the OPLS-DA method in this study, a methodological error calls into question the results presented. In fact:

i) 18 comparisons are proposed between experimental and control groups. The group of females on the diet containing 33% of maize NK603 is the one that shows the largest differences: this is the group that the authors choose to present;

ii) 48 parameters are compared. The biochemical parameters exhibiting the biggest differences (between the female group on the 33% NK603 diet and the control group) are *Na, Cl, U.Cl, U.N* and the 2 hormones that exhibit the biggest differences are *Testosterone* and *Estradiol*. These are the 6 parameters the authors choose to present.

It is therefore expected that upon selecting both the group and the 6 parameters that exhibit the largest differences, differences between the experimental and control groups will be apparent. **Such an approach does not allow us to:**

- **propose that the observed differences are caused by diet;**
- **reject the hypothesis that it is the data natural variability (due to sampling fluctuation) and the criteria used to select parameters (those with the largest differences) that explains the observed differences.**

5. Conclusion

The experimental protocol and the statistical methods used in the article suffer from serious gaps and methodological shortcomings and do under no instance support the conclusions proposed by the authors.

1. **A rigorous statistical analysis of the results obtained in this study does not show:**
   - **any statistically significant difference in mortality in rats between the control and experimental groups,**
   - **any statistically significant difference in the number of tumors between the control and experimental groups.**
2. **The statistical methodology used to analyse the biochemical parameters is inadequate and does not lead to the conclusion that there are statistically significant differences between the control and experimental groups.**

Ministero della Salute

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Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana
Laboratorio Nazionale di Riferimento per la ricerca di OGM
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OGGETTO: Studio condotto da Gilles-Eric Séralini et al. "*Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize*".

Come noto di recente è stato pubblicato sulla rivista scientifica *Food and Chemical Toxicology* lo studio, riportato in oggetto (http://dx.doi.org/10.1016/j.fct.2012.08.005), sulla potenziale tossicità del mais GM NK603 e di un erbicida che contiene glifosato, i cui risultati concluderebbero che gli Ogm hanno un effetto tossico sugli animali e forse anche sull'uomo.

La Commissione europea ha subito incaricato l'EFSA di effettuare una valutazione su tale studio visto anche l'impatto che una tale conclusione comporterebbe su tutto il settore degli OGM.

In data 4 ottobre u.s. l'Autorità ha pubblicato la prima revisione di detto studio, secondo cui emerge chiaramente la scarsa qualità scientifica della pubblicazione che non rende di conseguenza possibile una valutazione del rischio seria. L'EFSA pertanto ha chiesto agli autori maggiori informazioni considerate fondamentali in quanto al momento ritiene la progettazione della ricerca, il rapporto e l'analisi scaturita non adeguate.

Anche l'Istituto federale tedesco per la valutazione del rischio (BfR) ha pubblicato il 1° ottobre 2012 il proprio parere in merito, ritenendo che "lo studio mostra carenze sia nella progettazione che nella presentazione dei dati raccolti".

Alla luce dei risultati perciò, l'EFSA non ritiene necessario un riesame della sua precedente opinione scientifica sul mais GM NK603.

E' comunque prevista una seconda parte della revisione sullo studio francese che dovrebbe concludersi per fine ottobre e che terrà conto delle eventuali ulteriori informazioni fornite dai ricercatori.

Premesso quanto sopra, tenuto conto che l'EFSA ha richiesto informazioni su eventuali review che gli Stati membri stiano conducendo sullo studio in questione, si chiede a codesti Istituti, ciascuno per le parti di competenza, di fornire un parere in merito allo studio francese al fine di riferire tali osservazioni e commenti anche all'organo politico.

IL DIRETTORE GENERALE
(Dott. Silvio Borrello)

EC

National Institute of Health (ISS) assessment on the Gilles-Eric Séralini et al study: "Long term toxicity of Roundup Herbicide and Roundup- tolerant Genetically Modified maize"

A multi-disciplinary group of ISS experts was asked to review the article "Long term toxicity of Roundup Herbicide and Roundup- tolerant Genetically Modified maize" by Gilles-Eric Séralini et al., published in Journal Food and Chemical Toxicology, 50 (2012): 4221- 4231, that raised concerns about the potential toxicity of genetically modified (GM) maize NK603 and of a herbicide containing glyphosate (Roundup herbicide). One of the members of the expert group, Dr. Alberto Mantovani, as member of EFSA Panel on Plant Protection Products and their Residues (PPR), was peer reviewer of the EFSA statement published on EFSA Journal 2012; 10(10): 2910. In this statement, EFSA concluded that this paper is of insufficient scientific quality to be considered as valid for risk assessment.

The expert group agreed with the EFSA statement. In particular, the review of the article focused on the study design and the statistical evaluation of data.

It was pointed out that the study objectives were not clearly stated by the authors and therefore it was difficult to conclude whether the study design and the statistical evaluation of the results are fit for purpose. This starting deficiency has a consistent impact on the study conclusions that are further invalidated by the choice of protocols being different from the internationally accepted ad hoc protocols for sub-chronic, chronic toxicity and carcinogenicity studies (e.g. OECD 408, OECD 451, OECD 452 and OECD 453).

The review of the study showed the following critical aspects:

- The strain of rats chosen is known to be genetically prone to development of spontaneous tumours. Therefore, the use of this strain could significantly influence the results.
- The study design makes impossible to draw conclusions on carcinogenicity as the number of animals per treatment per sex (10 rats) is far below the number (50) recommended in the relevant international guidelines on carcinogenicity testing (i.e. OECD 451 and OECD 453);
- No suitable controls for all treatment groups were present
- No detailed information on either the composition of the various diets used in the experiment or the possible presence of harmful substances (e.g. mycotoxins and heavy metals) in the feeds used in the study was provided.

As a conclusion, ISS considers that results of the Séralini's study cannot be regarded as evidence of toxic and carcinogenic effects attributable to maize NK 603 treated or non treated with Roundup® due to their insufficient scientific quality for risk assessment.

ISTITUTO ZOOPRTOFILATTICO SPERIMENTALE LAZIO E TOSCANA BIOTECNOLOGIE – GMO NATIONAL REFERECE LABORATRY.

In reference to the request of this General Directorate (protocol n ° 0034022-P-10.9.2012) to provide an opinion on the French study reported in the subject, taking into account that the National Reference Laboratory shall carry out its tasks as defined by the European and national legislation, as part of the official analytical control of genetically modified food and feed and that, therefore, has no specific expertise in the field of toxicology designed to carry out a detailed analysis of the long-term toxicity study performed by Séralini and colleagues, it is considered to be able to express an opinion on the general methodology adopted in the recently published work.
In order to provide its opinion, the National Reference Laboratory has considered not only the information and data contained in the article by Séralini, but also other documents, relevant publications and guidelines currently available, in particular :

- EFSA Statement pubblicato su EFSA Journal 2012; 10(10): 2910, “Review of the Séralini et al. (2012) publication on a 2-year rodent feeding study with glyphosate formulations and GM maize NK603 as published online on 19 September 2012 in Food and Chemical Toxicology”

- EFSA Teleconference with Member States on Séralini et al. study, 28 Settembre 2012, 1st Meeting Report (included addendum 4 “Full text of The Netherlands (NVWA) preliminary conclusions”), available at: http://www.rijksoverheid.nl/bestanden/documenten-en-publicaties/notas/2012/10/03/advies-vwa-bij-onderzoek-naar-gezondheidsgevolgen-ggo-mais-en-roundup/advies-vwa-bij-onderzoek-naar-gezondheidsgevolgen-ggo-mais-en-roundup.pdf

- German Federal Institute for Risk Assessment (BfR) opinion 037/2012 of 1 October 2012 on Feeding study in rats with genetically modified NK603 maize and with a glyphosate containing formulation (Roundup) published by Séralini et al. (2012), available at: http://www.bfr.bund.de/en/press_information/2012/29/a_study_of_the_university_of_caen_neither_constitutes_a_reason_for_a_re_evaluation_of_genetically_modified_nk603_maize_nor_does_it_affect_the_renewal_of_the_glyphosate_approval-131739.html

- *OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects.* Test No. 451: Carcinogenicity Studies

- *OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects.* Test No. 453: Combined Chronic Toxicity/Carcinogenicity Studies

- Domingo J.L., Ginè Bordonaba J. (2011) A literature review on the safety assessment of genetically modified plants. *Environment International* **37**, 734-742

- Snell C., Bernheim A., Bergé J.B., Kuntz M., Pascal G., Paris A., Ricroch A.E. (2012) Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: a literature review. *Food and chemical toxicology* **50**, 1134-1148

In the light of the methodological approach adopted in the French study and the information provided in specific international guidelines, we agree with the opinion expressed by EFSA, in particular for the following aspects:

- The study was not conducted in accordance with specific international guidelines of the Organization for Economic Cooperation and Development, in particular with regard to the number of animals in each treated group. In fact, the authors have carried out an assessment of the potential carcinogenicity of NK603 and of Roundup herbicide in commercial formulation, using groups of 10 animals subjected to different treatments. This number is considerably lower than that one recommended in international guidelines OECD 451 and OECD 453, requiring 50 animals of each sex for each treatment and each control group. The reference "Because of recent reviews on GMOs (Domingo and Giné Bordonaba, 2011, Snell et al., 2011) we had no reason to settle at first for a carcinogenesis protocol using 50 rats per group" reported the introduction of Article Séralini et al., is not an adequate justification, even in two works cited by the same authors.

- An insufficient number of animals in the different groups tested, especially when you consider that the strain of rats used spontaneously develop tumors later in life, can mislead that the differences in the frequency and time of onset of cancer are due to the effect of the treatments under study, without reliable scientific evidence, instead of the natural variability of individual;

- The study also provided a single control group for all 9 groups subjected to different treatments. The chance to identify, in scientific studies and particularly in toxicology, a cause-effect attributable to a particular treatment requires that each treatment group is compared with a specific control group treated in the same way except for the dose of the substance considered subject of study;

- The information in the article Séralini et al. on the composition of the diet given to the rats are indeed, as pointed out by EFSA, rather limited. We agree that the lack of data regarding the possible presence of mycotoxins and a possible different concentrations of the same food given to the different groups over the two years of study, is a weak point of the work, taking into account in particular the well-known toxicity of these substances;

- Actual levels of exposure to the herbicide Roundup in the different groups of animals fed with NK603 treated in the field with the herbicide, are not clearly indicated in the work, since it was not measured the content of glyphosate in NK603 treated with Roundup and fed to animals. These levels should be known in order to identify a possible cause-and-effect attributable to the herbicide under study.

- EFSA has stated that, in order to give a more complete assessment of the study conducted by Séralini et al., needs to have access to a wider documentation on the study and the procedures followed, including the original study protocol and any

changes introduced, the statistical analysis plan, the ratio of the statistical analysis and the complete final report of the study. Also in view of the still open debate in European and international level on the need to perform studies on the in vivo toxicity and how such studies should be conducted to assess the safety of GM plants for food uses, it is desirable that the EFSA and the entire scientific community can have more data and information on this study, in order to reach a scientific assessment as solid and shared on this controversial topic.

Mandate NL NVWA

On 24 September 2012 the Office for Risk Assessment and Research (BuRO) of the Netherlands Food and Consumer Product Safety Authority (NVWA) was asked by the Dutch Competent Authorities (the Ministry of Economic Affairs, Agriculture and Innovation and the Ministry of Health, Welfare and Sport) to provide an opinion and advice on the paper entitled "*Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize*" by the team of Professor Gilles-Eric Séralini, and published in the journal *Food and Chemical Toxicology*.

The Competent Authorities of the Netherlands requested BuRO, by this mandate, to undertake an analysis (review) of the French study reported by this paper in order to determine whether or not it is likely to cast doubt on findings of this GM crop by Séralini and co-authors and, in particular, whether the previous EFSA opinion(s) may be considered conclusive regarding the safety of food derived from GM maize containing event NK603 and of Roundup, a glyphosate-based herbicide.

The BuRO was asked to deliver an opinion on this paper by 3 October 2012, and to advice on the validity of health risk assessment procedures and to propose adjustments to current GMO guidelines, if necessary.

Following preliminary work by experts of the Office of Risk Assessment and Research (BuRO) a multidisciplinary task force of experts was set up by the institutes RIKILT and RIVM, to examine the paper by Séralini et al. BuRO requested the task force to determine whether or not their review of the paper is likely to cast doubt on the preliminary assessment of NVWA (BuRO). Moreover, they were requested to assess whether the study's protocol of Séralini et al. (2012) and their findings call into question the guidelines for health risk assessment of GM plants, and the herbicide Roundup.

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Opinion of the director of the Office for Risk Assessment & Research (BuRO) concerning the assessment of the article of Séralini et al. (2012)

Aan de staatssecretaris van EL&I en de minister van VWS

OPINION

This document provides the scientific assessment of the publication of Séralini and co-authors on health risks for humans and animals after feeding of Roundup-tolerant GM maize (NK603) and Roundup herbicide to rats.

Background

On 19 September 2012, the scientific journal Food and Chemical Toxicology published an article written by Séralini and co-authors on the possible harmful effects of a particular variant of genetically modified maize, NK603, and the herbicide Roundup whose active ingredient is glyphosate. Given the nature of the reported adverse health effects in a two-year rat feeding trial with these products various food safety authorities in Europe have proposed to critically evaluate the paper. On 26 September 2012 the European Commission has subsequently requested the European Food Safety Authority (EFSA) to deliver an EFSA opinion of this study. Furthermore, the European Commission asked EFSA whether the French study contains new scientific insights that give rise to review the previous assessments of EFSA's GMO panel on GM maize (NK603). Earlier, on 19 September 2012, EFSA approached all EU Member States and announced the execution of a scientific review focused on the possible implications of the paper for the food safety of genetically modified crops and herbicides based on glyphosate.
In the publication[1] the authors write that a lifetime exposure to the Roundup-tolerant genetically modified (GM) maize (NK603) and the herbicide Roundup caused severe and lethal diseases in the rat. The genetic modification makes the corn resistant to treatment with the

[1] Séralini, G-E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., Hennequin, D., Spiroux de Vendômois, J. (2012) Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food and Chemical Toxicology. http://dx.doi.org/10.1016/j.fct.2012.08.005

herbicide Roundup and is approved for import into the European Union (Regulation 258/97). The claimed potential of inducing significant risks to public and animal health was reason for the Dutch Ministries of Economic Affairs, Agriculture and Innovation (EL&I) and Health, Welfare and Sport (VWS) and the Office for Risk Assessment & Research (BuRO) of the Netherlands Food and Consumer Product Safety Authority (NVWA) to release on the short-term an opinion of the scientific quality and toxicological interpretation of the pre-publication of Séralini and co-authors (2012). This judgment of NVWA is in anticipation of and supportive to the upcoming EFSA opinion, end of October 2012. The scientific assessment by NVWA and research institutes RIVM and RIKILT focuses on the validity of the conclusions of the study, and aims to request clarifications from the authors as needed and to indicate if EFSA's opinion on GM-maize NK603 and its stacks needs to be reconsidered, and whether there are possible consequences for the health of humans and animals and the environment.

Actions undertaken

Immediately after the pre-publication of the article of Séralini and co-authors NVWA, based on the information available, edited a preliminary review of the scientific quality of the article and its potential impact on the current strategy of safety assessment of genetically modified food and feed crops and herbicides containing glyphosate as the active substance.

On 24 September 2012, the Ministries of EL&I and VWS asked the Office for Risk Assessment & Research of NVWA to assess the quality of the article based on its preliminary assessment of the pre-publication. To this end, the provisional review and a number of questions have been forwarded to the Front Office Food Safety of the institutes RIVM and RIKILT. On 27 September, a first draft of the risk assessor's reply was received and a final response on 1 October 2012.

On 26 September 2012, the director of the Office of Risk Assessment and Research of NVWA discussed a first draft scientific assessment of the quality and meaning of the article of Séralini and co-authors (2012) in the plenary meeting of the EFSA Advisory Forum.

For identification and avoidance of divergent opinions NVWA participated on September 28, 2012 in a teleconference between representatives of EU Member States and EFSA. Particularly, Germany, France, Belgium and the Netherlands have performed (preliminary) assessments of the study of Séralini and co-authors. Germany has sought to obtain further information from the researchers, such as the raw data, their log books etc. Séralini's institute has not yet responded to this request of Germany. Additionally, France requested the supplier of the tested rats, Harlan, to deliver control historical data (HCD) of the strain purchased by Séralini et al. On 1 October 2012, NVWA received this latter documentation from the Haut Conseil des Biotechnologies (HCB).

The scientific review of the Front Office Food Safety of RIVM and RIKILT and the exchange of information with EU Member States and EFSA, formed the basis for this opinion of the director of the Office for Risk Assessment & Research of NVWA.

Review

NVWA-BURO requested the Front Office RIVM-RIKILT Food Safety to critically review the scientific quality of the paper of Séralini et al. (2012). More specifically, the request included a judgment of the experimental design, the performance of research, the interpretation of results, the quality of the feeding trial and the statistical analyses. It was also asked to estimate the potential consequences of the currently applied approach

of risk assessment of genetically modified crops and glyphosate containing pesticides including the application of the so-called 90-day feeding study. The institutes RIVM and RIKILT should also estimate whether there are possible health risks for humans and animals. Thereto, a multidisciplinary team of experts (i.e. laboratory animal specialists, risk assessors, toxicologists, GMO experts, statisticians etc.) has subjected the article of Séralini et al. (2012) following a critical review process (Annex 1). In addition, NVWA, Office for Risk Assessment & Research, re-explored the available scientific literature about the testing of potential adverse effects to humans and animals upon exposure to GM crops and studied the compliance of regular and proper carcinogenicity testing.

Conclusions

Following the scientific review of the study of Séralini and co-authors (2012) the Office for Risk Assessment and Research of NVWA concludes that this paper is of poor quality both in terms of reporting and clarity. The French researchers have identified cause-and-effect relationships that are not scientifically substantiated and, based on the paper, there is no evidence for a need for re-evaluation of GM maize (NK603) and glyphosate.

NVWA criticizes, for instance, the absence of data relating to compositional analysis of GM maize (NK603) and control maize, rodent diets, feed and water consumption or growth rate, and information about whether or not the study was blinded. A statistical analysis of amongst others, consumption, growth, mortality, and cancer/tumour incidence and multiplicity has not been carried out or published and essential data about the statistical analysis of the biochemical measurements are lacking in the paper (Annex 1).

In particular, the following applies.

- The rat strain, Harlan Sprague Dawley (SD), used in combination with a too small study population (only 10 animals/sex/dose), and no comparison with an up-to-date (i.e. relating to the last 5-7 years of use in studies) database of control historical data (HCD) and the absence of a proper statistical analysis, there is a high probability that the investigators have given a false image of the results of their two-year feeding trial in rats. Because of the increased incidence of spontaneous cancers and tumours in SD-rats and other health problems after two years of exposure the observed differences between control and dose groups may be coincidental. A risk that increases as the groups of rats tested were not greater than ten animals per group (Annex 1).

- The ratio of GM maize (NK603)/standard rodent chow was not equal in all dose groups. Therefore, it can also mean that adverse effects might have been caused by differences in their diets and not by the properties of the GM maize (NK603) or the herbicide glyphosate.

- Fractions of animals with neoplastic lesions do not clearly increase with increasing doses of GM maize (NK603) or glyphosate. Authors indicated that there is a 'threshold response' triggered by an influence on the hormone balance. This is not a scientifically meaningful conclusion because thresholds with ten animals per dose group are completely outside the statistically observable range.

- The substance glyphosate, the active ingredient in Roundup, or its metabolites, in crops has not previously been shown to be carcinogenic.

- The research is in terms of design, performance and reporting unsuitable to comment on the correctness of a proposed non-dose-related endocrine disruption by exposure

to the GM maize (NK603) or the substance glyphosate. Such a conclusion requires much more animals and a proper statistical analysis.

- The biochemical changes between nine dose groups and the single control group are unverifiable: underlying data are not published and the statistical method (i.e. two-class discriminant analysis) for data analysis seems to be aimed at finding differences rather than investigating whether or not a difference between a dose group and the controls can be detected.

Finally, the commentary of the teleconference between EFSA and the Member States Belgium, Germany and France on 28 September 2012 led to the conclusion that preliminary assessments of the paper of Séralini et al. are in line with the aforementioned Dutch findings (Annex 2).

Advice

Given the poor scientific quality of the article, as judged by NVWA-BuRO, the Front Office RIVM-RIKILT Food Safety, BfR in Germany, ANSES and HCB in France, EFSA in Italy and WIV-ISP in Belgium, I recommend not to change the methodology of the food and feed safety assessment of GM crops (e.g. guidance EFSA 2011[a], EFSA 2011[b], EFSA 2011[c]) and the risk assessment of the herbicide glyphosate (EFSA 2009[a], EFSA 2009[b]). Furthermore, I recommend to await the final opinion of EFSA that will appear at the end of October 2012.

For further explanation and substantiation of my scientific assessment of the study of Séralini et al. I refer to the second part of the opinion including Annexes 1 and 2.

What else will happen

EFSA has announced to release in the beginning of October 2012, a first draft of EFSA's opinion.

EFSA has announced to bring out at the end of October 2012 a final opinion of the article by Séralini and co-authors.

In October 2012 NVWA will try, like BfR in Germany and ANSES in France, to obtain further detailed information from the French researchers involving, among others, the raw data of their study, the study protocols and log books, the qPCR analysis of DNA samples from GM maize (NK603), the chemical composition of the glyphosate formulations GT Plus and WeatherMAX, the neoplastic lesions and histopathology of individual animals, and the chemical analysis of, inter alia, animal feed and drinking water.

The colleagues of HCB from France have pledged to forward historical control data of the Sprague-Dawley rats purchased by Séralini et al. from Harlan in France. On 1 October 2012 these control data were received.

If there is any reason for it, then NVWA will write an additional opinion on the scientific assessment of the paper of Séralini and co-authors (2012).

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Substantiation of NVWA's assessment of the publication of Séralini and co-authors on health risks for humans and animals after feeding of Roundup-tolerant GM maize and Roundup herbicide to rats

Background

On 19 September 2012 EFSA (European Food Safety Authority) issued a press release that the Authority urgently will study the pre-publication of Séralini and co-authors in the journal Food and Chemical Toxicology on consequences for the food safety of genetically modified crops and glyphosate. In the pre-publication entitled "Long term toxicity of a herbicide Roundup and Roundup-tolerant genetically modified maize" the French researchers determined that the daily exposure to Roundup-tolerant genetically modified (GM) maize NK603 and the herbicide Roundup after two years caused severe and lethal diseases in the SD-rat. The genetic modification, over-expression of the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) transgene in NK603, ensures that the GM-maize (NK603) is resistant to treatment with the herbicide Roundup (the active substance is glyphosate), which is admitted for import into the European Union (EC Regulation 258/97 and Commission Regulation EC 1829/2003). The research paper had a worldwide media attention and controversy caused in relation to its design to determining the food safety of genetically modified crops and the testing of glyphosate formulations on safety for humans, animals and the environment. In particular, the use of the so-called 90-day study with the whole GM crop has been severely criticized in the article (see also Séralini et al. 2011). The press release of EFSA has given rise to conduct a critical review of the article contents by the Office for Risk Assessment & Research (BuRO) of the Netherlands Food and Consumer Product Safety Authority (NVWA).

Questions asked

What is the scientific judgement of RIVM and RIKILT on the study by Séralini and co-authors (2012)?

This involves the following sub-questions:

1. Are the arguments provided by Séralini et al. for not using an experimental set up with a group size of n=50 scientifically valid?

Quote: "we had no reason to settle at first for a carcinogenesis protocol using 50 rats per group. However we have prolonged the biochemical and haematological measurements or disease status recommended in combined chronic studies using 10 rats per group (up to 12 months in OECD 453). This remains the highest number of rats regularly measured in a standard GMO diet study."

2. Do RIVM and RIKILT consider the criticism by Séralini et al. of the recommended 90-day feeding trial justified and substantiated?

For example, see the publication by Snell et al.: Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: A literature review. Food and Chemical Toxicology, Volume 50, Issues 3–4, March–April 2012, Pages 1134-1148 (below) and the EFSA opinion 'Safety and Nutritional Assessment of GM plant derived Foods/Feed. The role of animal feeding trials' (2007).

3. Are glyphosate and/or its residue carcinogenic?

4. What is the opinion of RIVM and RIKILT on the quality of the experimental set up, the reporting of the results, and the statistical analysis of the endpoints in relation to the generally accepted toxicological operating method?

5. What is the opinion of RIVM and RIKILT on the researchers' explanations regarding the cause–and-effect relationships, such as on the one hand a nonlinear endocrinedisrupting effect due to glyphosate or on the other hand a non-linear effect due to
the transgene and/or metabolic disturbances as a result of the insertion?

Design

NVWA has asked the Front Office RIVM-RIKILT Food Safety to assess the scientific quality of the article Séralini et al (2012) and to verify the conclusions drawn by the French researchers. In addition, NVWA critically studied the available scientific literature about possible adverse effects on human and animal health upon exposure to GM crops, as well as the usual toxicological methods regarding carcinogenicity as prescribed by various authorities (e.g. OECD, EPA, NTP, EMA and ECHA).

NVWA - Office for Risk Assessment & Research participated in a Europe-wide exchange of preliminary judgments of the paper of Séralini et al (2012), and pre-investigated possible consequences for the authorization of GM maize (NK603) and Roundup. To this end, EFSA organized a teleconference with Member States that were already actively engaged in this issue (Annex 2).

Subsequently, a multi-disciplinary team of scientists employed at RIVM and RIKILT subjected the first findings of NVWA including additional questions to a critical review process (assessment) (Annex 1).

Based on the scientific assessment by the Front Office RIVM-RIKILT Food Safety and the consultation between Member States and EFSA the opinion of NVWA was finalised.

Results

The study of Séralini and co-authors (2012) is complex in nature and does not follow unequivocally OECD guidelines for laboratory animal research. In general, the study is incompletely published. The article misses a multitude of essential information needed for a fair assessment of the results and the conclusions drawn on the basis thereof (Annexes 1 and 2). For instance, the cultivation/field trial of GM maize (NK603) was done using the glyphosate formulation WeatherMAX (540 g/L glyphosate) whereas GT Plus (450 g/L glyphosate) was added to the drinking water of three dose groups. Rightly authors note that such formulations may contain for example adjuvants or other active ingredients which have not been tested under conditions of a lifetime exposure to laboratory animals. However, the publication omits necessary details on the composition or does not show that both formulations were equivalent.

Other essential details needed for a proper assessment of the science of this French study are missing, such as data of the analysis of the GM maize (NK603) and control maize, rat diets, feed and water consumption, growth and body weights, and whether the study was or was not blinded. A statistical analysis of food intakes, growth, mortality, and cancer/tumour incidence and multiplicity has not been performed. And data on the statistical analysis of the biochemical parameters were missing.

EFSA, guidance of its GMO panel, recommends to perform only a 90-day feeding study with the whole GM crop if prompted by previous toxicological research (EFSA 2011[a],

EFSA 2011[b], EFSA 2011[c]). This is not the case if a genetically modified crop has been convicted to be 'substantially equivalent' to the non-GM isogenic counterpart (EFSA 2011). In the article Séralini and fellow researchers express their criticisms on the EFSA guidance for research into the safety of genetically modified food or feed (see also Séralini et al. 2011). Correctly, the authors note that animal testing has not be required for the adoption of 'substantially equivalent' GMOs. Notwithstanding this adopted principle, Séralini et al. consider this approach a bug in the risk assessment of GMOs. They reject the common use of a feeding trial that lasts 90 days as recommended by EFSA's GMO panel. It is the authors vision (Séralini et al. 2011, Séralini et al. 2012) that an exposure for only three months is highly insufficient to judge whether there are effects on human and animal health. In contrast to the 'guidance' document of the GMO panel (EFSA 2011[a], EFSA 2011[b], EFSA 2011[c]) Séralini and co-authors hold the opinion that the food safety of the whole GM crop should be examined with the help of chronic studies, for example corresponding to the life span of the experimental animal.

The research team of Gilles-Eric Séralini of the University of Caen in collaboration with the committee CRIIGEN at Paris therefore has conducted a two-year feeding study in rats in which they made use of the genetically modified NK603 Roundup Ready maize from Monsanto (U.S.) and the herbicide glyphosate (Roundup). Their study design can be described in short as a chronic study performed with Harlan Sprague-Dawley rats and applying mainly physiological and biochemical assays as are usual for a 90-day feeding trial in order to study the effects in laboratory animals. Only one control group that consumed ordinary, non-genetically modified, maize (33% in standard rodent chow) and clean tap water, has been compared with six dose groups that received GM maize (NK603) grown with or without Roundup in their feed (11%, 22% and 33%) and with three dose groups that received daily a diet of non-genetically modified maize ate (33% in standard rodent feed) and the glyphosate product with brand name GT Plus in their drinking water (50 ng/L, 400 mg/kg and 2.25 g/L glyphosate). The composition of the glyphosate formulations are not given, which are needed because it is not inconceivable that a prolonged exposure to the other (auxiliary) materials in the glyphosate formulation GT Plus could induce negative effects on the health of the animal. It is curious that authors expressed the dosing of the middle dose group as mg glyphosate per kilogram of body weight. How was this analyzed?

Control groups fed respectively with 11 and 22% ordinary maize were not incorporate in the trial protocol. Also missing are the results of the qPCR analysis of DNA samples from the GM maize (NK603), although authors mentioned that such tests have been carried out. This has the result that obviously the ratio of maize and standard diet was not equal among the dose groups. Any effect could thus be caused by differences in composition of the diet. For example, it is known that food intake has an influence on tumour growth (Tucker, 1979).

It is found that essential details needed for proper risk assessment of the content and the research in the article by Séralini et al. are missing.

The authors observed that the GM maize (NK603)-fed rats died earlier with an increasing effect on mortality if the GM maize was treated with Roundup (WeatherMAX?). Their results also indicated that the genetically modified maize with resistance to glyphosate induced up to five times more mammary tumours (females) and four times more palpable kidney and/or skin tumours (males) following lifetime exposure. However, the number and size of neoplastic lesions for each individual rat are not published. The authors mentioned only relative percentages or ratios of benign and malignant cancers/tumours per group. The publication of Séralini and co-authors lacks a histopathological characterization of the neoplasia per animal as is common in toxicological publications.

In order to permit an accurate assessment of the results of a carcinogenicity study the incidence of spontaneous common tumour types in the Sprague-Dawley (SD) rat must be taken into account, since the percentage of mammary gland tumours, for example, can reach up to 50% in the control group (e.g. Mann et al. 1996, Nakazawa et al. 2001) or pituitary tumours (adenomas) to 49% (males) and 75% (females) according to Baldrick (2005), and even over time the incidence can vary within one laboratory (i.e. genetic drift).

It is a good toxicological procedure using control historical data (HCD) in order to investigate whether the tumour responses in a study have been unusual or were similar to what is normal. This is done by comparison with data of the numbers and types of neoplasia in strain-specific control animals by evaluating a large number of earlier performed studies (Baldrick 2005, Peddada and Elmore 2009). Also Séralini et al. (2012) applied this principle to their effects in the treated groups; however, NVWA criticizes the references to the papers of Chandra et al. (1992) and Brix et al. (2005). Both the National Toxicology Program (NTP) of the Department of Health and Human Services (NIEHS) in the U.S. and the European Agency for Medicines (EMA) recommend the use for this purpose of HCD's compiled from studies of respectively the last seven and the last five years. It is a failure not having consulted a valid database on control historical data of the Harlan SD-rat to further study the biological significance of observed effects.

Finally, it should be mentioned that a nutritionist from the supplier Harlan in the U.S. recently indicated in an interview with Tim Worstall of Forbes Magazine that Harlan does not distinguish between GM maize and non-genetically modified varieties as regards the composition of rodent chow (Worstall 2012). Therefore, it is quite conceivable that, at least in the U.S., herbicide-tolerant GM maize has already been fed to rats without observing adverse effects on animal health.

Conclusions: answers to questions

1. Are the arguments provided by Séralini et al. for not using an experimental set up with a group size of n=50 scientifically valid?

No, the number of 10 animals/sex/dose is too low to be able to conclude on differences in tumour incidences between the various test groups, i.e. rats with or without cancer. In a chronic toxicity study, this number of animals would be sufficient (see OECD Guideline 453), if supported by data from groups of 50 animals/sex/dose in the combined carcinogenicity test. Therewithal the French researchers used the Sprague-Dawley rat that is known to be sensitive to the 'spontaneous' development - higher incidence - of mammary gland cancer and pituitary tumours and a greater risk of many health problems at the end of their lifetime (i.e. after two years of exposure). Related to this Séralini and co-authors have not published accurate values of useful control historical data (HCD) of the rat species used. It is also important to note that no statistical analysis of the effects of different treatments on mortality and cancer/tumour incidence has been done.

2. Do RIVM and RIKILT consider the criticism by Séralini et al. of the recommended 90-day feeding trial justified and substantiated?

No, the criticism of Séralini and co-authors is not well-founded. The researchers are selective in citing only those publications showing potential adverse effects of GMOs in laboratory animal studies or of glyphosate formulations in cell culture. Thereby the authors ignored the proof of more than 100 publications demonstrating no adverse effects of GMOs and glyphosate on human and animal health.

3. Are glyphosate and/or its residue carcinogenic?

No, the substance glyphosate has been reviewed in 2000 (EU) and 2004 (JMPR) and carcinogenicity studies conducted in mice and rats indicated that glyphosate is not carcinogenic. The main metabolite of glyphosate in plants and environment exhibits a lower toxicity if compared to the parent compound.

4. What is the opinion of RIVM and RIKILT on the quality of the experimental set-up, the reporting of the results, and the statistical analysis of the endpoints in relation to the generally accepted toxicological method?

The criticism of NVWA-BuRO is shared by scientists and risk assessors of RIVM and RIKILT (Annex 1). The most important fact is that Séralini and co-authors omitted a proper statistical evaluation with respect to the observed mortality and tumour incidence. Had such an analysis been conducted, the commonly used analysis (testing dose groups paired with the control group) for these parameters would not have indicated any statistically significant differences. Such statistically significant differences cannot be expected from a study that uses such a small number of animals per dose group. Because of the small number of animals per group it is very likely that observed differences were merely coincidental. Results for animals that had tumours were summarised (Table 2 of the paper) by adding up the number of tumours per animal, with the number of animals that had one or more tumours being indicated between brackets. Usually, carcinogenicity studies indicate the number of animals with tumours and not the total number of tumours.

A statistical analysis of the biochemical data was carried out by the authors. However, the underlying data are not provided in the paper and the chosen statistical method (two-class discriminant analysis) used for the data analysis seems to be aimed at finding differences, rather than at investigating whether or not differences in biochemical parameters between experimental groups can be detected. The conclusions regarding the biochemical changes in the animals cannot be verified.

5. What is the opinion of RIVM and RIKILT on the researchers' explanations regarding the cause–and-effect relationships, such as on the one hand a nonlinear endocrine disrupting effect due to glyphosate or on the other hand a non-linear effect due to the transgene and/or metabolic disturbances as a result of the insertion?

The study of Séralini in its execution, methodology and reporting is unsuitable for drawing any conclusion on the supposed non-dose-related endocrine disruption. Such a conclusion would require a large number of animals as well as a proper statistical analysis. The authors make connections between cause-and-effect relationships that are not scientifically substantiated.

It is for example known that spontaneous pituitary tumours often occur in Sprague-Dawley rats in conjunction with mammary gland fibroadenomas. Percy and Barthold (1993) reported that 90% of the rats with mammary gland fibroadenomas (approximately 80-95% of all breast cancers) do also have pituitary tumours. This suggests a connection between the two malignancies due to exposure to elevated concentrations of circulating growth hormones (Pecceu 2010).

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Annex 1
NVWA/BuRO/2012/3569

Rijksinstituut voor Volksgezondheid en Milieu
Ministerie van Volksgezondheid, Welzijn en Sport

RIKILT WAGENINGEN UR

RIVM-RIKILT FRONT OFFICE FOOD SAFETY

ASSESSMENT REGARDING ARTICLE SÉRALINI et al. 2012 in FOOD and CHEMICAL TOXICOLOGY

Risk assessment requested by:	Hub Noteborn (NVWA, BuRO)
Risk assessment drafted by:	*RIVM and RIKILT*
Date of request:	24-09-2012
Date of risk assessment:	02-10-2012
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Project numbers:	V/320110/12/AA and V/320800/12/AA

Subject

A research article was published in the scientific journal Food and Chemical Toxicology, written by Séralini G-E, Clair E. et al. (2012), entitled: 'Long term toxicity of a RoundUp herbicide and a RoundUp-tolerant genetically modified maize'. According to the publication it has been proven that lifelong exposure to a glyphosate-resistant GM maize (NK603) and/or a commercial formulation containing glyphosate (RoundUp) leads to very serious disease symptoms in Sprague Dawley rats. The publication has caused worldwide turmoil and within the European Union there is talk about closing the borders to genetically modified crops.

Questions

What is the scientific judgement of RIVM-RIKILT on the study by Séralini and co-authors (2012)?

This involves the following sub-questions:

1. Are the arguments provided by Séralini et al. for not using an experimental set up with a group size of n=50 scientifically valid?

Quote: 'We had no reason to settle at first for a carcinogenesis protocol using 50 rats per group. However we have prolonged the biochemical and hematological measurements or disease status recommended in combined chronic studies using 10 rats per group (up to 12 months in OECD 453). This remains the highest number of rats regularly measured in a standard GMO diet study.'

2. Does RIVM-RIKILT consider the criticism by Séralini et al. of the recommended 90-day feeding trial justified and substantiated?

For example, see the publication by Snell et al.: Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: A literature review. Food and Chemical Toxicology, Volume 50, Issues 3–4, March–April 2012, Pages 1134-1148 (below) and the EFSA opinion 'Safety and Nutritional Assessment of GM Plant derived Foods/Feed. The role of animal feeding trials' (2007).

3. Is glyphosate and/or its residue carcinogenic?

4. What is the opinion of RIVM-RIKILT on the quality of the experimental set up, the reporting of the results, and the statistical analysis of the endpoints in relation to the generally accepted toxicological operating method?

5. What is the opinion of RIVM-RIKILT on the researchers' explanations regarding the cause–effect relationships, such as on the one hand a non-linear endocrine-disrupting effect due to glyphosate or on the other hand a non-linear effect due to the transgene and/or metabolic disturbances as a result of the insertion?

Conclusion

1) The experimental set up used by Séralini is considered not suitable for drawing conclusions on the carcinogenicity of a particular substance, product or genetically modified organism (GMO). For that purpose, a larger number of rats per group would have had to be used. Because of the small group sizes used in this study, the differences observed between the control group and the treated groups could have been coincidental. A further shortcoming of this publication is that it does not include a statistical analysis of the numbers of animals in which tumours (and a number of other effects) were observed.
2) The publication by Séralini et al. (2012) does not mention the EFSA. However, it does state the following: 'Currently, no regulatory authority requests mandatory chronic animal feeding trials to be performed for edible GMOs and formulated pesticides'. The EFSA recommends that a 90-day study of the full GMO product is performed only if and when there is a reason to do so. Such a reason does not exist if a GMO product is found to be 'substantially equivalent' to a non-GMO isogenic counterpart (EFSA, 2011). The statement by Séralini et al. that no animal testing is required with respect to substantially equivalent GMOs is therefore correct, but there is also no reason for changing this.
3) According to the Joint FAO/WHO Meeting on Pesticide Residues (JMPR, 2004) and the European Commission (EC, 2002), glyphosate and/or glyphosate residues are not carcinogenic.
4) Because of the small number of animals per group, it is very likely that the observed differences were merely coincidental. The fraction of animals with such tumours does not clearly increase with increasing dosage.
5) With respect to set up, method and reporting, this study is considered not suitable for drawing conclusions on the presumed non-dose-related endocrine disruption. To arrive at such a conclusion would require the testing of a very large number of animals as well as a proper statistical analysis. Another conclusion concerns the biochemical changes in the animals involved in the toxicity experiment. This conclusion could not be verified, as the underlying data were not provided in the publication. The authors have drawn a connection between treatment and effects that, on the basis of the results, is not scientifically substantiated.

Explanation

Question 1
The number of laboratory animals of 10/sex/dose is too low to justify a conclusion about whether or not an agent is carcinogenic. This number does suffice for a study on chronic toxicity (OECD 453), provided the study is supported with data on the 50 animals/sex/dose in a combined study on carcinogenicity. 'For a thorough biological and statistical evaluation of the study each dose group should at least contain 50 animals of each sex. Each dose group and concurrent control group intended for the chronic phase of this study (OECD 453 and not TG 452, which requires a higher number) should contain at least 10 animals/sex.' Conclusion: using 10 animals/sex/dose is insufficient to comment on differences between numbers of animals with and without cancer.
Furthermore, the publication by Séralini does not include a statistical analysis of the effects of the various treatments on mortality and tumour incidence. The conclusions by Séralini regarding tumours and other reported effects, therefore, have no foundation.

Question 2
Séralini's criticism of the study by Snell et al. (2011) is not properly founded. The author selectively mentions a few studies that have found effects of GMOs in laboratory animals or RoundUp formulations on cell cultures, but completely disregards the many (estimated over 100) publications in which GMOs were fed to laboratory animals for 90 days or more and in which no effects were found. In addition, in the same paragraph, he suggests that, if studies were conducted according to the protocol as described in his own publication, chronic GMO feeding trials would indicate health effects. However, as this study is not suitable for drawing conclusions on this subject, this is no valid argument to undermine the conclusions in the study by Snell et al.
The study by Séralini et al. (2012) does not mention the EFSA, but does state the following: 'Currently, no regulatory authority requests mandatory chronic animal feeding trials to be performed for edible GMOs and formulated pesticides'. The EFSA recommends conducting a 90-day study only if there are reasons to do so. This is not the case if a GMO is found to be 'substantially equivalent' to its non-GMO isogenic counterpart (EFSA, 2011). The statement by Séralini et al. that currently no animal testing is required with respect to substantially equivalent GMOs is therefore correct, but there is also no reason for changing this.

Question 3
The herbicide that is commercially known as RoundUp contains the active ingredient glyphosate. The other ingredients of this compound are not indicated. On the basis of this study no conclusion can be drawn about the carcinogenicity of glyphosate (or residues thereof) (in the form of RoundUp), or of the genetically modified maize in combination with RoundUp, or of RoundUp by itself (see the answers to Questions 1 and 5 regarding the quality of this study).

The toxicity of the active ingredient glyphosate was assessed in the year 2000 in the EU (EU, 2000), and in 2004 by the JMPR (JMPR, 2004). Both assessments concluded that glyphosate is not carcinogenic, according to properly conducted carcinogenicity studies in mice and rats.
In addition, the JMPR concluded in 2011 that aminomethylphosphonic acid (AMPA) (the main metabolite in plants and in the environment) is less toxic than glyphosate (JMPR, 2011). The acceptable daily intake (ADI) of glyphosate and AMPA has been set by the

JMPR at 1 mg/kg bw/day, which was based on the effects on salivary glands as seen in chronic toxicity/carcinogenicity studies (no observed adverse effect level (NOAEL) of 100 mg/kg bw/day).

It is noted that the authors aim at effects that are due to exposure through food and/or water to a glyphosate-containing product with the commercial name of GTplus, by the authors identified as RoundUp (which is something different than the active ingredient alone), and effects due to exposure via consumption of genetically modified NK603 maize. They thus do not directly dispute the conclusions regarding the active ingredient glyphosate itself.

Question 4
Séralini et al. did not report results from a statistical analysis of the effects on mortality and tumour incidence. Had such an analysis been conducted, the commonly used analysis (testing dose groups paired with the control group) for these parameters would not have indicated any statistically significant differences. Such statistically significant differences cannot be expected from a study that uses such a small number of animals per dose group. Because of the small number of animals per group it is very likely that the observed differences were merely coincidental. Results for animals that had tumours were summarised (Table 2) by adding up the number of tumours per animal, with the number of animals that had one or more tumours being indicated between brackets. Usually, carcinogenicity studies indicate the number of animals with tumours and not the total number of tumours.

A statistical analysis of the biochemical data was indeed conducted. However, the underlying data are not provided in the publication and the chosen statistical method (two-class discriminant analysis) used for the data analysis seems to be aimed at finding differences, rather than at investigating whether or not differences in biochemical parameters between experimental groups can be detected. The conclusions regarding the biochemical changes in the animals in the toxicity experiment cannot be verified.

The fractions of animals with tumours do not clearly increase with increased dosage. Séralini et al. argue that the lack of a dose–response relationship is caused by an effect on the hormone balance, without offering any further explanation for this fact. Using such small numbers of animals, variability in the observed shares of animals with a response is to be expected – even if there indeed would be a normal dose–response relationship.

Furthermore, it is deemed highly unlikely that NK603 maize would have the same effect as the glyphosate formulation that was used in this study.

Some additional points of criticism:

- Materials and methods show that the control animals as well as those that received the highest dosage of GMO maize received respective amounts of 33% control maize and 33% GMO (NK603) maize in their standard diet. Animals that received a lower dosage of GMO maize had either 11% or 22% GMO maize added to their standard diet. Control groups with 11% and 22% of control maize in their diets are lacking. Thus, the maize–standard diet ratio was not equal for all groups. Any effects, therefore, could have also been caused by differences in the diet that had nothing to do with GMO maize.
- The study's reporting is very inadequate. Many details that would be required for arriving at a proper judgement are lacking; for example, data on analysis results for the GMO maize and the control maize, rat feed, feed and water consumption,

and on the growth rate of the rats, as well a on whether or not the study was a blind experiment. A statistical analysis of, for instance, consumption, growth, mortality, cancer incidence and multiplicity was not conducted, or at least not included in the article, and essential data are lacking on the statistical analysis of the biochemical data that indeed was conducted.

- The authors claim an effect on the hormone balance. In the description of the materials and methods used for conducting the study, it appears that the phase of the females' estrous cycle at the time of blood extraction was not taken into account. This fact, in combination with a very low number of animals per group, means that the observed differences in estradiol levels could very well be based on coincidence.

Question 5
With regard to method and reporting, the study by Séralini is unsuitable for drawing any conclusion about the assumed non-dose-related hormone disruption. Such a conclusion would require a large number of animals as well as a proper statistical analysis. The authors have drawn a connection between treatment and effects that, on the basis of the results, is not scientifically substantiated.

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Abbreviations:
GM = Genetically Modified
GMO = Genetically Modified Organism