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Reply to letters to the editor

Answers to critics: Why there is a long term toxicity due to a Roundup-tolerant genetically modified maize and to a Roundup herbicide

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ABSTRACT

Our recent work (Séralini et al., 2012) remains to date the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO). This is true especially for NK603 maize for which only a 90-day test for commercial release was previously conducted using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and Roundup herbicide (R). Our study has limits like any one, and here we carefully answer to all criticisms from agencies, consultants and scientists, that were sent to the Editor or to ourselves. At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable in an electronic format for the whole scientific community to conduct independent scrutiny of the raw data. In our article, the conclusions of long-term NK603 and Roundup toxicities came from the statistically highly discriminant findings at the biochemical level in treated groups in comparison to controls, because these findings do correspond in an blinded analysis to the pathologies observed in organs, that were in turn linked to the deaths by anatomopathologists. GM NK603 and R cannot be regarded as safe to date.

1. Introduction

Our recent publication of research evaluating the long term toxicity of a NK603 Roundup-tolerant genetically modified (GM) maize and of a Roundup (R) herbicide (Séralini et al., 2012) has provoked numerous positive and negative reactions throughout the world. This is the way science moves forward and here we provide a response to this intense debate. Our work is the most detailed study involving the life-long consumption of an agricultural genetically modified organism (GMO), and especially on NK603 for which only a 90-day safety test was previously conducted and using the same rat strain (Hammond et al., 2004). It is also the first long term detailed research on mammals exposed to a highly diluted pesticide in its total formulation with adjuvants. These adjuvants help to stabilize the active principles of pesticides, and promote a better penetration into organisms, and thus more side-effects. R is the most widely used herbicide in the world, which we tested from levels arising in tap water. Indeed in our study, its active principle glyphosate (G) was not studied alone, contrasting with the long term experiments conducted by the manufacturer as part of its application for regulatory approval. As such, the debate in question here is at the cornerstone of science and regulatory issues on this topic. This fact has major economic ramifications for the development of such products, which can explain the severe comments posted within hours of our publication becoming available online. This may explain why 75% of our first criticisms arising within a week, among publishing authors, come from plant biologists, some developing patents on GMOs, even if it was a toxicological paper on mammals, and from Monsanto Company who owns both the NK603 GM maize and R herbicide.

We must firstly focus on science. Our work is a research study; it has not a direct regulatory purpose and should not be considered as a final point in knowing the toxicological effects of NK603 and R. This is a first step in the iterative investigation of the long-term health effects on mammals of these commercial products that should be replicated independently, as well as on developing mammals. It has limits like any study, and here we carefully answer to all criticisms from agencies, consultants and scientists, that

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were sent to the Editor of *Food & Chemical Toxicology* or to ourselves. These challenged our results and the validity of our protocol, some letters even requested the withdrawal of the publication from the journal. All remarks and answers are summarized in Table 1 and with some explanatory details given below.

At this level, a full debate is biased if the toxicity tests on mammals of NK603 and R obtained by Monsanto Company remain confidential and thus unavailable for the scientific community to conduct independent scrutiny of their raw data. This is why, after several exchanges, we requested again from the European Food Safety Agency (EFSA) on September 20th and October 18th 2012 the release on a public website of the raw data on health risks on the basis of which commercialization of these products was granted, in particular results from the longest study of NK603 and Roundup on mammals (Hammond et al., 2004). We ask for a free and transparent exchange of scientific findings, mainly when these are related to public health and environmental risks (Schreider et al., 2010). Examination of industry raw data previously evidenced divergence of regulatory decisions from scientific evidence underestimating toxicological features of G (Antoniou et al., 2012). We recall that the tests on rats are usually considered as a model for mammalian health before clinical trials (for example for pharmaceuticals) or for a direct market release (for novel food and feed, pesticides or chemicals). Moreover, tests on rats are also models for environmental risk assessment, since they are models for other wildlife mammals. The public release of these raw data will reveal if significant differences observed between test and control groups in both studies are coherent and if the statistics are of sufficient power in both cases, thereby allowing the design of appropriate follow-up experiments by others, perhaps through a publically discussed and agreed protocol.

2. Relevance of the scientific context

Some remarks emphasize a lack of context, claiming that the study was performed for non-scientific reasons. The establishment of this protocol was however the consequence of an intense debate about the biological relevance of numerous statistically significant differences compared to controls revealed and admitted to in 90-day feeding studies with agricultural GMOs (Spiroux de Vendomois et al., 2010). This is highly controversial, with companies and regulatory agencies having refuted findings, which were validated by a peer reviewed process in international journals (EFSA, 2007; Séralini et al., 2007). Indeed, regulatory agencies such as EFSA appear to have their own criteria to judge the biological relevance of research findings (Doull et al., 2007), which is markedly at odds with some recent knowledge. This includes cases of sex specific non-linear endocrine disruptions, which were not admitted to as valid at a regulatory level although accepted at a scientific research level (Myers et al., 2009b). In order to overcome the divergence in biological interpretation of early signs of toxicity in blood biochemistry for GMOs, one solution was to prolong 90-day feeding tests to chronic periods. We therefore chose the R tolerant NK603 GM maize because R tolerance is the trait present in approximately 80% of agricultural GMOs (James, 2011) and because statistical differences in the 90-day feeding trial with this maize were admitted to by both the petitioner and regulatory agencies (EFSA, 2009).

3. Originality and limits of the experimental design

Due to the economic and regulatory issues of this topic, it is not surprising that our research study was confounded with pre-commercial regulatory assessments. This is why the most common criticism questions the following of Organization for Economic Co-operation and Development (OECD) guidelines. However, no guidelines exist for GMO toxicity studies in vivo, which are still not mandatory. Published reviews have confirmed that most of the studies conducted to date did not follow specific guidelines or were contradictory (Domingo, 2007; Domingo and Giné Bordonaba, 2011). We compared our design (Table 1 of Séralini et al., 2012) to Hammond et al. (2004) inspired from OECD guideline 408 for chemicals. We have replicated, extended and thus improved the experiments conducted by Hammond and colleagues (Hammond et al., 2004) by measuring outcomes from 3 instead of 2 feed doses and more crucially for a period 8 times longer in duration (90-days vs 2 years), with 11 blood and urine measures of around 50 parameters, 34 organs instead of 17, etc., in order to ascertain if the statistical findings (observed at 90 days; Hammond et al., 2004), were biologically relevant or not in the long term. We thus biochemically measured 10 rats per sex per group as performed by Monsanto. Even for a study of up to two years, we had no reason to monitor biochemical effects on more than 10 animals per sex per group as this is the number recommended in OECD guideline 452 for chronic toxicity testing (OECD 1981 was in application when the study started in 2008), even if 20 animals per group or more are possible.

The purpose of the addition of R treated groups was not to assess R long term carcinogenesis, which needs to follow OECD 453 guideline with at least 50 rats per sex per group (even if 10 rats are then still measured at a biochemical level). The aim of our study was to test R under similar conditions to the GM maize in order to try and understand if residues of R in the feed could explain the possible pathologies that may arise. There were two main potential sources of harm tested in our study: (i) effects from the GM maize itself, treated or not with R, and (ii) herbicide residues alone in drinking water, using 3 doses for each treatment. We recall that the initial investigation published by Hammond and colleagues (Hammond et al., 2004) used 2 doses for each treatment group despite that fact that 3 doses are recommended by OECD guideline 408, which they reported to have followed.

In addition, one of the criteria for biological relevance employed by Monsanto and other critics of our study is the linearity or lack thereof in response to the dose. Such a dose–response relationship cannot be claimed from a trial using only 2 doses of test material as employed in the initial NK603 assessment (Hammond et al., 2004). We therefore find it surprising that the relevance of Monsanto's and the agencies' conclusion of safety was not challenged due to such protocol insufficiencies. A recent review of the literature is often cited as a proof of the safety of GMO consumption on a long-term basis (Snell et al., 2012). However, of the 24 studies they evaluated, only 2 are long-term on rodents, since a 2 year feeding period with pigs or cows do not constitute a life-long experiments. The 2 rodent studies quoted by Snell and colleagues are from Sakamoto et al. (2008) where not all rats fed transgenic soy were analyzed, and Malatesta et al. (2008a) in mice fed again GM soy, which showed at an electronic microscopy level effects of this product on hepatic function. Moreover, of the 24 studies cited, 16 did not mention the use of the closest isogenic non-GM line as a control, many did not describe the methods in detail, and contained additional deficiencies (Snell et al., 2012). However, all these studies were accepted as proof of safety regardless of the inadequacies highlighted here. It would appear that conclusions of safety seem to need fewer requirements than conclusions of toxicity. However, scientifically it is easier to conclude an outcome of toxicity than safety. This was not the first time regulatory agencies used such double standards to minimize independent research findings in regard to industry findings (Hilbeck et al., 2012; Myers et al., 2009a). Our control groups were also questioned and this needs some clarification. Some claimed that controls are lacking for all 4 test groups (GMO+R and GMO alone at 11% and 22%). We compared

Table 1
Summary of criticisms and responses on Séralini et al. long-term NK603 GM maize and Roundup toxicity rat study.

Criticisms	Answers
Relevance of the scientific context	
No scientific context	This study addresses biological interpretations of early signs of toxicity in biochemistry after 90-day feeding trials (Spiroux de Vendomois et al., 2010)
OECD guidelines not respected	No guidelines exist for GMO animal studies. Protocol based and adapted from OECD 408 and 452
Protocol not adapted to tumor findings	This is not a carcinogenesis study, but a long term full toxicological study
GLP violation because of amendments	Research protocols not adapted to GLP agreement because of amendments. The experiment was conducted under a GLP environment and conditions
History of flaw by the authors which are not toxicologists. Previous studies of the group rejected	More than 26 international scientific peer reviewed papers by the team with the lead author on the topic in the last 5 years, and 11 in toxicological journals on the same period only in PubMed. One author, Malatesta, has also published on GMO/pesticide health risks. None of the papers was considered as flawed by the scientific community. Regulatory agencies or Monsanto are not scientific peer reviewed journal systems
Lack of signs in 90 days	Statistical differences in biochemical parameters of liver and kidney function recognized by both industry and agencies
Not the first long term study	First chronic investigation with NK603 GM maize; others of two years in farm animals are not over the entire lifespan; the most detailed study for all agricultural GMOs and a formulated pesticide
Originality and limits of the experimental design	
Choice of the rat strain (sensitivity to mammary tumors and nephropathies in males)	Necessity to have sensitive strains, recommended by the US National Toxicology Program (King-Herbert et al. 2010). Rats and mice have been preferred experimental models because of their susceptibility to tumor induction (OECD guidelines) Relevant comparisons to controls in this work
Number of rats per group	OECD 408 (90-day toxicity study) 10 animals per group OECD 452 (Chronic toxicity study) 20 animals per group but at least 10 animals per group are studied for hematological and clinical biochemical function
Missing data: diet composition and process, PCR analysis of batches, contaminants (mycotoxins, pesticides), storage (R in water, BPA, feed), isogenic line, culture conditions	Normally included in GLP environment studies. No possibility to detail all these data in this scientific study in this journal – in process of publication. Diet equilibrated for substantial equivalence between GMO and the closest isogenic line and other compounds. Other points detailed in the text
No blinding, not the knowledge to interpret tumors, no morphometric analyses, no use of PETO codes, no classification	Independent and blinded analysis by GLP performed by professional regulatory anatomopathologists. Nature of most frequent tumors in Fig. 3 legend and results. A professional report for each rat indicates the cause of mortality
R formulations are different	Depends on the country
Controls not sufficient (number of rats per group, 4 groups 11 and 22%, no drinking water control group)	Number of rats approved in guidelines, best in the world at this level of details for these products. All the animals have eaten 33% of maize and substantially equivalent diets. Only R treated rats had received R in water
No reference groups, no lab historical data	Reference groups add irrelevant variability with non-substantially equivalent diets; historical data contain diets not controlled for pesticides and GMOs, thus not relevant
Ad Libitum feeding	In accordance to guidelines and usual practices
Diurnal variations	All samplings were taken at the same time
Focus on statistics	
Not enough statistical power No Kaplan Meier's curves Variability expected by chance Only raw data in Figs. 1–3 and Table 2	Statistics do not tell the truth, but may help in understanding results. The biological interpretations and the crossing of methodologies are the key. Enough and high statistical power for OPLS-DA, and this is why raw data only were presented in Figs. 1 and 2 and Table 2; no statistical power of Kaplan Meier's analyses for a conclusion demonstrating effects or no effects.
No means and standard deviations in Table 3	OPLS-DA is not a method to compare mean differences which were presented for understanding of biochemical measurements with highly discriminant parameters in bold
Pertinence of the results	
Missing data (Behavioral studies, ophthalmology, microbiology in feces and in infectious nodules, G in tissues, body and organ weights, feed and water consumptions, transgene in tissues, time effects)	All measures cannot be presented in one paper and will be the subject of other publications. The other analyses are not relevant for the conclusions presented
No isoflavones in maize	Testing the diets for phytoestrogens is relevant because the equilibrated diet (non-GM) contains other components
Phenolic acids in the normal range	Used as biomarkers indicative of change in the metabolism of the GMO. This does not exclude the presence of unknown toxic compounds
No incidence / severity Lack of histopathology data	Taken into account as indicated in the legend of Table 2 which consists in a summary of the most relevant data
Endocrine disruption not sufficiently supported	Convergent body of evidence stemming from mammary tumors, pituitary dysfunctions, histopathology and sex hormone biochemistry
Wilm's tumors are only of genetic origin	Promotion by pesticide exposure is plausible and as evidenced by gestational exposure described in the literature
Feeding state explains glycogen in electron microscopy	No difference in feed consumption; experience in the domain by M. Malatesta
Pictures of control rat not shown	Rats representative of each group shown, controls do not present tumors in majority during the experiment, pictures non necessary
Discussion: findings in regard with the contradictory hypotheses	
R is not a sex endocrine disruptor	This is still true at a regulatory but not at a scientific research level. R endocrine disrupting properties are described in vivo and in vitro (references in the text).

Table 1 (*continued*)

Criticisms	Answers
	Regulatory classification should be in process
G is not toxic in two-year tests	G is never used alone in agriculture, but in formulations with G far more toxic than G alone; G tests are not relevant, we used R
G is close in structure to amino acids and surfactant exposure is as soap exposure	This is not supported by the scientific literature; the structural and activity comparisons are not scientifically relevant to predict with certainty toxicological effects or safety
No effects on farm animals and in human population of the USA	No epidemiology, no life-long experimental studies; farms animals are generally killed too young to show development of long term diseases. No traceability and labeling of GMOs in USA, no epidemiological survey can be performed
Sakamoto et al. 2008 not reported	This study does not use the same GMO (soy vs maize) and neither the same strain of rat. No effect for GM soy in F344 rats is claimed but does not imply the same for NK603 GM maize in SD rats
Raw data expected for our study	Raw data also expected for regulatory accepted tests for this GMO and this pesticide to scientifically discuss details
Ethical issues and deontology	
Maize illicitly grown	Not at all; grown and imported with appropriate authorizations
Animal welfare problems, a veterinarian would not authorize such tumor development	The work follows GLP conditions. All rats followed by veterinarians on the site, applying the rules of the ethical committee and guidelines
Conflicts of interests	No conflict for us. Conflicts of interests for companies testing their own products
Role of funders	See acknowledgments, funders identified. No interference in study or results; confidential up to the embargo
Publication released before for journalists	Everything was released on the same day (September 19th), in accordance with the conditions set by the FCT editorial board.
Confidentiality agreement unusual	The confidentiality of the work is a usual practice before embargo
The authors should alert agencies from the end of experiment instead of waiting for a publication	The publication and reviewing of the work is the guarantee of quality with no interference

all treated groups to the control group containing 33% of the closest available isogenic maize, as all diets were equilibrated to 33% maize; that is, for example the 11% GM maize diet was supplemented with non-GM control maize to reach 33%. More accurately the closest available isogenic line was the DKC2675 variety compared to the DKC2978 GM maize (NK603). Regulatory agencies also questioned the conditions under which the maize was grown. One R treatment was applied 4 months before harvest. Fungicides were applied similarly. We were unable to use the same R formulation in the field (Canada) and in the drinking water of the rats (France) because authorized formulations vary between nations. The diet was nutritionally equilibrated from substantially equivalent maize and was then checked by PCR for GMO content. A major concern was the potential presence of mycotoxins. Fumonisin B1 and B2, zearalenone, deoxynivalenol (DON), nivalenol, 3-acetyl-DON, 15-acetyl-DON, fusarenone X, T2 toxin, HT2 toxin and diacetoxyscirpenol were all under recommended limits in food/feed used in this study. We did not present details of each of these substances when no particular changes affecting the understanding of the results were noticed.

As a research protocol, Good Laboratory Practice (GLP, OECD, 1997; 2004/10/EC regulation) was followed, meaning that housing conditions, manufacturing process, diet composition and storage, stability of solutions and dietary contaminants were assessed by approved laboratories. Anatomopathology was performed in a blind manner (without knowing the treatments) by professional anatomopathologists approved for regulatory purposes. An electronic chip was inserted in each rat for identification. However, the technicians employed for the care and sampling of the animals did not know either the nature of the diets or the drinking water prepared independently, or which was the control group. The cages housing the animals were moved within holding rooms regularly and similarly for all animals. The blood (1 mL) and urine samples were coded and the measurement of biochemical parameters also blind, as were the decisions of euthanasia to avoid suffering in accordance with precise regulatory ethical rules (hemorrhages, impossibility to drink and eat, large tumors over 25% body weight because they provoke mortality). All the animals were monitored during the experiment by professional veterinarians. The statistical analysis was also undertaken on coded groups. However, we have made research amendments adding additional analyses (tissue and biochemical parameters) adapted to the findings in order to improve the understanding of the pathologies, thus we are only in a GLP environment. Generally, it is standard practice that a regulatory agency does not take note of research studies because they are not conducted under GLP conditions (Myers et al., 2009a). By its very nature, a research protocol is rarely compatible with GLP agreements. GLP agreement is a good tool to normalize regulatory assessment but research studies need a greater degree of freedom, in test protocols, models, etc.

4. Rat strain

We would like to explain the choice of the strain of rat. This is another redundant remark made by critics of our study design. We recall that OECD norms (408, 452 and 453) are not prescriptive for the strain of rat to be used. Sprague Dawley (SD) rats are subject to spontaneous neoplasms and this property is supposed to invalidate them being used as a model for carcinogenesis. However, on the contrary, the fact that the SD strain develops tumors, hence has led to it is preferentially used by some agencies such as for the National Toxicology Program using it for 2-year carcinogenicity and other long-term studies (King-Herbert et al., 2010). Indeed, it would be a non-sense to study pathologies in a strain insensitive to tumor formation. Long-term OECD guideline 452 even states that rats and mice have been preferred as experimental model systems because of their susceptibility to tumor induction. The same reasoning is used for chronic progressive nephropathies (CPN) developed by SD rats. The fact that the strain developed spontaneous CPN with age (Hard and Khan, 2004) does not invalidate the model as we looked at the difference in the chronology, age, number and severity of CPN in comparison to controls.

To assess the biological relevance of results, many authors make comparisons with historical data of control rats, either within the laboratory or the breeding company from which animals are

sourced. However, this clearly greatly enhances control variability and heightens the risk of false negative findings (Cuffe, 2011). It is now established that this concept should be used with caution. There are several reasons for this. Control diets for rats are generally not monitored, neither for pesticides (Hayes, 2004), nor for chemicals leaching from cages or other environmental sources (Howdeshell et al., 2003). This artificially enhances background effects. The supplier even recognizes that their historical data come from rats potentially fed GMOs since this was not controlled for (Harlan communication), except in our experiment. Thus, it was not appropriate for us to use historical control data. This is also the reason why we did not use reference groups fed different non-substantially equivalent diets, as they increase the standard deviation of the control groups, hiding differential effects due to treatments.

Many non-relevant remarks have also been noticed. Among others, some criticized the use of *ad libitum* feed to explain the increase of tumor incidence. Guidelines on the design and conduct of chronic toxicity studies state that rodents should be fed and watered *ad libitum* (OECD, Guidance Document No. 116). The hormonal imbalances were criticized to be due to diurnal or cyclic variations. However, sampling was performed at the same time each day in the morning.

5. Focus on statistical analytical methods and outcomes

Statistics do not tell the truth, but may assist in our understanding of experimental outcomes. The biological interpretations and the crossing of methodologies are the key (Cooper and Kavlock, 1997). We have applied the most modern statistical methods (OPLS-DA, see below) for multivariate data analysis of approximately 50 parameters measured 11 times for 200 rats. This allowed, in a blinded manner, to obtain results significantly discriminant at 99% confidence levels. These discriminant biochemical markers were, for example in the case of sexual hormones (at 95% for females at month 15), when the differences in hormone-dependent tumor incidence with the control group began. Disability in pituitary function was characteristic of this second most affected organ as certified independently by the pathologists in a blinded manner in treated female groups in comparison to controls. Such a disturbance in hormonal function is known to elicit mammary tumors in rats with the pituitary being a target of endocrine disrupting chemicals (Wozniak et al., 2005). The pathologists employed in our study explained that most of the mortality in females resulted from tumors, which led to euthanasia independently of the grade of cancer. This is why we did not detail the grade of tumors in our research but with the cancerous nature of the major tumor growths described in our study (Fig. 3 legend and results section (Par. 3.2)). These observations together with microscopic analysis reinforced our conclusions.

We believe all this was more pertinent than the study of statistically non-powerful Kaplan–Meiers' curves on survival (because of the groups of 10 animals per sex dying progressively) that cannot allow any conclusion on mortality linked or not to the treatment. Taking into account these limits, we decided to be simply factual in our presentation and thus describe the chronology and incidence of tumors and deaths. In comparison, statisticians from agencies could evaluate the power of the statistical analyses of the tests conducted by Hammond et al. (2004), which gave a score of safety, and that were used for market release. For us, the power of statistics used in Hammond et al. (2004) is extremely low to conclude to safety.

In our study, case PLS-regression (Projections to Latent Structures by means of partial least squares) is of particular relevance because, unlike conventional multivariate data analytical methods, it can analyze data sets with variables more numerous than observations, which can be strongly correlated (Wold et al., 2001). In the case of Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) there is separation between the inter-group variation (represented on the predictive component) and the intra-group variation (variability of the samples, represented on the first Y-orthogonal components). OPLS-DA is thus not an appropriate method with which to compare mean differences. However, for providing biochemical understanding, we have presented and highlighted those in Table 3 of our study, with highly discriminant parameters in bold text. OPLS-DA renders it possible to identify which variables are responsible for the separation of the groups. For instance, we also indicate in Fig. 5B that estradiol and testosterone are significantly discriminant at 95% confidence levels in some groups (not at 99% like other parameters presented).

Moreover, the SIMCA-P (V12) software (UMETRICS AB Umea, Sweden) for the multivariate analysis of biochemical data uses a method of validation of models, which is a k-fold cross-validation. The $Q^2(Y)$ parameter which measures the predictive ability of the models is calculated according to this cross-validation method. Only valid models with a satisfactory predictive quality Q^2 index were retained for the selection of the discriminant variables (bold in figures, Table 3). Furthermore, all models retained are significant (CV-ANOVA test with p-value <5%). One of the authors of our paper (D.H.) previously used this method and published their results in international peer-reviewed journals (Ledauphin et al., 2010; Malzert-Freon et al., 2010a; Malzert-Freon et al., 2010b).

6. Pertinence of the results

The first major criticisms that were raised concerned the results and their format of presentation. A scientific publication is by necessity limited in figures/tables and only shows the data necessary to understand and discuss the conclusions. This is why behavioral studies, ophthalmology, microbiology in feces and in infectious nodules, G in tissues, body and organ weights, feed and water consumptions, transgene in tissues, time effects will be the subject of future publications. The inclusion of these data at this stage would neither add to the main message nor would it improve the understanding of this first publication. Indeed, the peer review process has controlled the logic of the body of data presented. Additional sets of results were included in the revision of the manuscript in response to issues raised by the reviewers prior to publication.

The second major criticism of the results is that we attached too much importance to findings related to mortality and tumor relative to their scientific significance. We are aware of the limitations of these findings as discussed above in relation to the statistical analysis undertaken. The body of evidence for our conclusions comes from the converging methodologies and data (see Focus on Statistics). The variability in rates of mortality can indeed, if looked at in isolation, arise in principle by chance. However, statistical analysis for Figs. 1 and 2 is not of sufficient power to conclude that this is the case or the contrary. This is why we have presented the raw data for these sets of observations. For instance, males presented up to 4 times (2 times of the mean) more large palpable tumors than controls, similarly to that observed in female animals. As these observations may represent a potential risk for the human population, this cannot simply be disregarded so rapidly with non-potent statistics. This is also why we emphasized statistically discriminant biochemical effects at the 15th month, when most of animals were still alive (in treated groups 90% males, 94% females, and 100% controls). The significantly discriminant biochemical markers disrupted do correspond to the organic markers linked to the pathologies in a blinded analysis for the pathologists, who

in turn linked that to the deaths. The two nephroblastomas in GMO fed groups linked to premature deaths was criticized for bringing confusion to the results, because these tumors are often of embryonic and/or genetic origin. However, these tumors are also known to be promoted by pesticide exposure (Fear et al., 1998).

The summary of the major histopathological findings in Table 2 was subject to the same criticisms. In fact, we indicated the severities of the CPN and only marked or severe CPN were shown. Indeed, elderly rats are subject to CPN and taking into account all CPN could hide interesting and important differences. The power of statistics may be discussed as for Figs. 1 and 2. However, all these data need to be seen in the context of all the significant results presented in the paper, as previously underlined.

For the findings obtained from the electron microscopy analysis, it is important to compare our results with those reported previously. Several studies have shown ultrastructural abnormalities in the liver of mice fed with GM soy (Malatesta et al., 2002) and that this structural disturbance was reproduced by adding the herbicide R directly to rat hepatocytes (Malatesta et al., 2008b). We thus wanted to test if the same disruptions can be seen in the liver of the rats in our experiment. This was indeed the case, and furthermore these observations conform with ours and others published in vitro effects of R (Gasnier et al., 2010, 2011). Glycogen dispersion or appearance in lakes found by electron microscopy was attributed to the feeding state by some critics. However, differences in feed consumption were not observed during the course of our study. Not only appearance of glycogen in lakes was noticed, but also a reduced rate of transcription of mRNA and rRNA, which is not normally known to be due to the feeding state, but rather to a toxic insult. Ultrastructural patterns revealed by of electron microscopy were coherent with an increase in detoxifying activity in liver, and this is corroborated by differences in cytochrome enzyme activities.

A major gap in some toxicological assessments is the lack of measurements investigating endocrine disrupting effects (Birnbaum, 2012). As noted previously, the central dogma in toxicology is that effects vary linearly to dose. This is true for standard poison intoxication. However, toxins with endocrine disruptive properties can give response curves that are U, inverted U or J in shape and are frequently observed in the case of exposure to environmental pollutants (Vandenberg et al., 2012). Endocrine disturbance is supported by observations in human (Gasnier et al., 2009) and rat testicular cells for R residues (Clair et al., 2012). In our study it is demonstrated by statistically significant sex hormone imbalances and disabled pituitary function. Moreover, doses varied from 50 ng/L to 2.5 g/L of glyphosate in R; that is, a factor 50 million, from which we cannot expect linear effects with such a wide range of doses tested, characteristic of the range of different kinds of environmental exposures (tap water, GM food and feed, diluted agricultural use). The kidneys and liver are also sensitive to endocrine disruptors. As the two major detoxifying organs, containing cytochrome P450 or other enzymes involved in xenobiotic or sex steroid metabolism, they often react with steroid sex hormone and related compounds (Pascussi et al., 2008).

Last but not least, we have identified phenolic acids as potential biomarkers of metabolic disturbances in the GM diet. We have also measured isoflavones in the diet even though maize does not produce these compounds. Rats indeed did not eat only maize but also other plants in an equilibrated diet. Even OECD 452 guidelines on chronic toxicity ask for testing phytoestrogen content of the diet. Importantly, decrease in phenolic acids is a good indicator of change in the metabolism of the GMO that could in turn lead to a reduced protection against the pathologies observed in the animals fed the NK603 GM maize. However, this does not exclude the possibility of other toxic effects of the GMO alone, which have not been identified in the experiment.

7. Discussion

7.1. Findings in regard with the contradictory hypotheses

Critics have claimed that no argument exists for R to be a sex hormone endocrine disruptor, which is based on a review by Williams et al. (2000), where most of the studies cover G effects alone and not R. We wish to draw attention again to the fact that G is never used as such, but in formulations with other substances allowing toxicity, both of target and non-target species. This is extensively described for G-based herbicides, but also for other pesticides (Eddleston et al., 2012). This is why, in our opinion, all discussion of our study referring to testing of G alone is not relevant. Furthermore, we find it incomprehensible that non-scientific assertions justify R innocuousness by the structural homology of G with non-toxic amino acids. In addition adjuvants in the R formulation cannot be judged harmless by a comparison of their activity to soap. There is no scientific basis to use these assertions to predict with certainty toxicological effects or safety. The fact that G alone is neither a carcinogen nor an endocrine disruptor in regulatory tests is not a proof of the safety of whole R formulations, especially when some formulations contained toxic compounds (Cox, 2004). The unexpected finding of new active principles with human cell toxicity capabilities in G-based herbicides has challenged the relevance of testing G alone as the active principle in R (Mesnage et al., 2012). R has already been demonstrated to be an endocrine disruptor in vivo (Dallegrave et al., 2007; Oliveira et al., 2007; Romano et al., 2010, 2012) with the underlying mechanism understood in vitro.

Several studies have shown significant endocrine disrupting effects of R, such as decrease in progesterone production, decreased levels of Steroidogenic Acute Regulatory (StAR) mRNA production in MA-10 mouse Leydig cells (Walsh et al., 2000), decrease in aromatase mRNA and activity levels in JEG3 cells and placental and equine testicular microsomes (Richard et al., 2005; Benachour et al., 2007), inhibition of transcriptional activities of androgens and of both α- and β-estrogen receptors in cells (Gasnier et al., 2009), and a decrease in testosterone production in rat Leydig cells (Clair et al., 2012). All these studies reinforce the biological relevance of our findings.

Some critics have emphasized that no adverse effects have been reported on either farm animals or in the human population of the USA who have consumed an unknown mixture GMO crop derived food. Such claims are scientifically unsound for the following reasons. First, it is important to note that there have been neither epidemiological studies of the human population nor monitoring of farm animals in an attempt to correlate any ill-health observed with the consumption of a given GM crop. Second, it should be recalled that farm animals are not reared to live for the entire duration of their natural lifespan, and thus usually do not live long enough to develop long-term chronic diseases, which contrasts with the rats in our life-long experiment. If any studies in lactating cows are conducted, biological analyses performed are far less complete than those done in regulatory tests using rodents including in our study. Third, as there is no labeling of GMO food and feed in the USA, the amount consumed is unknown, and no "control group" exists. Thus, without a clear traceability or labeling, no epidemiological survey can be performed.

7.2. Ethical issues

Many critics argue against our refusal to release all the raw data generated in our study. This is a very unusual request when we clearly stated that we plan several other papers out of this data set. Our study was not performed for regulatory purposes. How-

ever, due to the social impact and for full scientific understanding of the potential risks associated NK603 GM maize and R, we will release our raw data if the regulatory agencies that have taken industry data into account in their approval of their products also release the data pertinent for environmental and health risk assessments, in particular their longest toxicological tests on mammals, as we have indicated in our correspondence with EFSA. As a first step to this end, we have communicated the raw data underlying the data presented in Figs. 1 and 2 to the French food safety agency (ANSES), and answered their questions on experimental design and results, including analysis of food composition and mycotoxin content, etc.

Most of the criticisms on the topic of ethical conduct relate to animal welfare, some thinking that we overpassed the threshold in size of tumors above which animals should be euthanized, with the purpose of taking shocking photographs. However, it should be recalled that in a GLP environment, animal welfare is of major concern and that we fully respected the threshold in tumor size before euthanasia. Pictures of every animal and organ were taken. We presented those related to the most observed pathologies, including those of a microscopic nature, for illustrative purposes in Fig. 3, with rats representative of each group.

Some critics raised concerns about the role of the funders of this work, and possible conflicts of interest. Of course, the funders neither played a role in the design and conduct of the experiment, and nor in its interpretation. The data remained confidential to the funders. We recall that in the regulatory assessment of GMOs, chemicals and medicines, tests are conducted by the applying companies themselves, often in their own laboratories. As a result, conflicts of interest exist in these cases. These are even not claimed by authors from the company defending the safety of the tested products (Hammond et al., 2012). Our study does not aim to request commercialization of a new product. In contrast, we wanted to estimate the health risk of these products. It is the most detailed test conducted to date that is also independent from biotechnology and pesticide companies. We encourage others to replicate such chronic experiments, with greater statistical power. What is now urgently required is for the burden of proof to be obtained experimentally by studies conducted independent from industry. This was recommended by regulatory agencies in France that have assessed our work, even though their objective is more to regulate products than to review research. GM NK603 and R cannot be regarded as safe to date.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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