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Review

# Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: A literature review

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ABSTRACT

The aim of this systematic review was to collect data concerning the effects of diets containing GM maize, potato, soybean, rice, or triticale on animal health. We examined 12 long-term studies (of more than 90 days, up to 2 years in duration) and 12 multigenerational studies (from 2 to 5 generations). We referenced the 90-day studies on GM feed for which long-term or multigenerational study data were available. Many parameters have been examined using biochemical analyses, histological examination of specific organs, hematology and the detection of transgenic DNA. The statistical findings and methods have been considered from each study. Results from all the 24 studies do not suggest any health hazards and, in general, there were no statistically significant differences within parameters observed. However, some small differences were observed, though these fell within the normal variation range of the considered parameter and thus had no biological or toxicological significance. If required, a 90-day feeding study performed in rodents, according to the OECD Test Guideline, is generally considered sufficient in order to evaluate the health effects of GM feed. The studies reviewed present evidence to show that GM plants are nutritionally equivalent to their non-GM counterparts and can be safely used in food and feed.
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## 1. Introduction

In Europe, GM food and feed safety is assessed by the European Food Safety Authority (EFSA), which recommended that "the safety assessment of GM plants and derived food and feed follows a comparative approach, i.e. the food and feed are compared with their non-GM counterparts in order to identify intended and unintended (unexpected) differences which subsequently are assessed with respect to their potential impact on the environment, safety for humans and animals, and nutritional quality" (EFSA, 2008). With different methods, key elements of the assessment procedure such as molecular, compositional, phenotypic, and agronomic traits are analyzed in both the GM line and its near isogenic counterpart (EFSA, 2008). When "molecular, compositional, phenotypic, agronomic and other analyses have demonstrated equivalence of the GM food/feed, animal feeding trials do not add to the safety assessment" (EFSA, 2009; updated in EFSA, 2011). However, animal feeding studies may provide additional and useful information to complement safety and nutritional value assessments of whole GM food and feed, especially when unintended effects are suspected. The EFSA experts panel recommend that "the use of 90-days studies in rodents should be considered for the detection of possible unintended effects in food and feed derived from GM plants which have been more extensively modified in order to cope with environmental stress conditions like drought or high salt conditions, or GM plants with quality or output traits with the purpose to improve human or animal nutrition and/or health" (EFSA, 2008).

The protocols for *in vivo* toxicological studies are adapted from the 90-day rodent study as described in OECD Test Guideline No. 408 (Organisation for Economic Co-operation and Development, 1998), which defines the experimental material to test and the practical conditions used to test it (target animal species, housing, number of doses administered, gender and number of animals, etc.). The appropriate methods used to measure phenotypic responses (body weight, food consumption, clinical biochemistry, etc.) in test animals throughout the test are also provided. Over the last few decades, these parameters have been refined for an improved toxicological assessment of low molecular weight xenobiotics such as drugs, pesticides or additives, and serve as a foundation for the evaluation of GM-based food or feed. While feeds are identical between animal groups (treated or control) in the normal 90-day rodent study, adaptation of this test to food safety studies raises many specific questions on the strengths and weaknesses of such tests. For example, to assess the potential health effects of GM-based food or feed, 33% GM animal feed is usually incorporated (see recommendations of Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, ANSES, 2011). Feeding experiments using rodent models allow whole GM material to be tested, this assessment being motivated by either a modification in the GM plant composition, or by indications of potential unintended effects (EFSA, 2006, 2008). More precisely, 90-day animal feeding studies do not search for one particular effect of a given molecule, but are supposedly designed to detect most of the changes that may occur, including those potentially generated by the genetic modification as well as those resulting from a compositional change which is directly or not linked to the transgene. Thus, these studies might appear too wide and insufficient to detect weak effects, as EFSA (2008) has already stated "*It is unlikely that substances present in small amounts and/or with a low toxic potential will result in any observable unintended effects*". The key point is that in the case of a chemically defined molecule for which human exposure is very low, one can increase its dose in the classical 90-day feeding studies, whereas one cannot do so with a food or with a quantitatively important constituent in the diet.

Moreover EFSA (2008) states that "the subchronic, 90-day rodent feeding study is not designed to detect effects on reproduction or development, other than effects on adult reproductive organ weights and histopathology. Thus, in some cases, testing of the whole food and feed beyond a 90-day rodent feeding study may be needed. In cases where structural alerts, indications from the subchronic study or other information on the whole GM plant derived food and feed are available that suggest the potential for reproductive, developmental or chronic toxicity, the performance of such testing should be considered".

Possibly, some 90-day rodent feeding studies may be insufficient to reveal the presence of late effects in animals. Therefore, long-term studies, namely those performed for longer than 90 days, as well as multigenerational studies, will evaluate whether unintended effects are only detected by such studies and whether long-term studies have different findings than 90-day studies. In this paper we address the following question: Do recently published studies on long-term effects of GM plants, i.e. studies significantly longer than the 90-day sub-chronic tests, as well as multigenerational studies, present new evidence indicative of some adverse effects? The goal of this review is to compile and discuss the results of recently published studies on long-term effects as well as multigenerational studies for GM plants on which 90-day studies are also available. Twelve long-term and twelve multigenerational studies are examined. This review highlights the knowledge generated by these recently published studies to evaluate the possible existence of long-term health effects. The necessity to renew the current regulatory designs will also be discussed.

### 2. Material and methods

For this systematic review, 55 peer-reviewed references were identified from our database ('BergeRicrochGMLibrary'; Ricroch et al., 2010), which includes 32,000 references on transgenic plants, collected since 1996, using the keywords "*GM plant*", "*health*", "*long term*" and "*multigenerational*". Studies that were neither multigenerational nor longer than 96 days (OECD protocols + 10%), nor concerning animal feeding trials were discarded.

The duration and number of generations in every study concerned were identified. The one-generation long-term studies were manually sorted from the multigenerational ones. All 90-day rodent studies using the same GM line are referenced in Table 1. Twelve studies using a one-generation design, and longer

**Table 1**
90-day Safety Studies Performed on Rats with GM Plant Derived Foods.

| Plant | Trait | Reference | Duration (in days) | Parameters |
|---|---|---|---|---|
| Maize | CP4 EPSPS (*Agrobacterium*) | Hammond et al. (2004) | 90 | Feed consumption. Body weight gain, organ weights. Blood cell count, blood chemistry, urine chemistry. Histopathology |
| Maize | Cry3Bb1 endotoxin (*Bacillus thuringiensis* var kumamotoensis) | Hammond et al. (2006a) | 90 | Feed consumption. Body weight gain, organ weights. Blood cell count, blood chemistry, urine chemistry. Histopathology |
| Maize | Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki) | Hammond et al. (2006b) | 90 | Feed consumption. Body weight gain, organ weights. Blood cell count, blood chemistry, urine chemistry. Histopathology |
| Maize | Cry1F endotoxin (*Bacillus thuringiensis* var aizawai) and phosphinothricin acetyltransferase (*bar* gene, Streptomyces viridochromogenes) | Mackenzie et al. (2007) | 90 | Feed consumption, body weight. Clinical pathology (serum, blood, urine). Anatomical pathology (organ weights, histopathology) |
| Maize | Cry34Ab1 and Cry35Ab1 endotoxins (*Bacillus thuringiensis* Berlinerstrain PS149B1) and phosphinothricin-acetyltransferase (*bar* gene, *Streptomyces viridochromogenes*) | Malley et al. (2007) | 90 | Feed consumption/efficiency, body weight/gain. Neurobehavioural and ophthalmological examinations. Clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis). Pathology (organ weights and gross, microscopic pathology) |
| Rice | GNA lectin (*Galanthus nivalis*) | Poulsen et al. (2007) | 90 | Feed and water consumption, body weight, organ weights. Blood cell count, blood chemistry, blood immunochemistry, splenocyte proliferation. Intestinal microbiology. Histopathology |
| Rice | Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki) | Wang et al. (2002) | 98 | Feed consumption. Body weight, organ weights. Blood chemistry, blood cell count. Histopathology |
| Soybean | CP4 EPSPS (*Agrobacterium*) | Zhu et al. (2004) | 91 | Feed consumption. Body weight, organ weights. Blood chemistry, blood cell count. Histopathology |

than 90 days (Table 2), and 12 multigenerational studies (Table 3) were finally considered in this meta-analysis. Studies which involved the examination of organs are summarized in Table 4.

## 3. Results

### 3.1. 90-day feeding studies

Based on the EFSA (2008) review, we referenced the 90-day studies using GM feed for which long-term or multigenerational studies were conducted (Table 1). Eight 90-day feeding trials conducted using transgenic maize, rice and soybean were performed on rats. The five studies using maize (Hammond et al., 2004, 2006a,b; Mackenzie et al., 2007; Malley et al., 2007) found no differences between the diets containing GM material and the ones which did not. These studies concluded that the maize grain tested were as safe and nutritious as existing commercial maize hybrids. The two studies using rice (Poulsen et al., 2007; Wang et al., 2002) found statistically significant differences between the control and the GM diet groups. However, in both studies most of these observed differences were within the normal biological range and were not indicative of harm. Wang et al. (2002) concluded that the Bt rice flour had no toxic effect on rats. Poulsen et al. (2007) did not conclude on the safety of the GNA lectin-producing rice tested even though no adverse effects were observed: "*In the present study, several significant differences were observed between rats fed diets with genetically modified 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*". Another study using herbicide-tolerant soybean (Zhu et al., 2004) reported no significant difference and therefore concluded that no adverse effects were observed. In summary, analysis of the results from these 90-day rodent feeding trials with GM maize, rice and soybean did not reveal any indication of adverse effects.

### 3.2. Long-term studies

The studies discussed below were carried out for periods longer than 90–96-days and were intended to assess potential hazards that could arise in food or feed derived from GM plants. Studies involving long-term feeding but in which the animals were mated will be examined in the section 'multigenerational studies'. The model mainly used in these studies is rat. However some groups used mice, cows and fish. A variety of criteria have been assessed such as body and organ weight measurements, hematological analyses, enzyme activities, macro- and microscopic (histopathological) observations of particular organs and tissues, and detection of transgenic DNA. The duration of GM-based diet feeding in these studies varies from 182 days (26 weeks) to 728 days (104 weeks).

The studies presented here are listed in the Table 2 in which the various parameters considered and results are reported. Information on transgenic events and cultivars, animal model, diet and number of individuals and groups were considered as crucial parameters for the quality of the experimental protocols. To emphasize the differences that can exist in certain studies, results were presented in three columns: facts, authors' interpretation and the interpretation of others ("Criticisms" column). The following paragraphs discuss the most important parameters that varied and results obtained in the most relevant studies compiled in Table 2.

#### 3.2.1. Insect-resistant maize

No long-term rodent studies are available for GM maize. However, a study in which 36 cows in total were fed a feed based on Bt-maize (event MON810) containing the protein Cry1Ab, or its isogenic not genetically modified counterpart for over 25 months covering two consecutive lactations has been published recently (Steinke et al., 2010). The diets from both the transgenic and isogenic lines were nutritionally equivalent: chemical composition in macronutrients and the estimated net energy content were not different. During this investigation the same cows were submitted to a second lactation and again fed the transgenic line; lactose concentrations in milk were higher and body weight and back fat thickness were lower when compared to cows fed the diet based on the isogenic (non-GM) line. In both groups, the milk yield (i.e. 23.9 and 29.2 kg/cow/d in the first and the second lactation, respectively, for cows fed non-GM maize; 23.7 and 28.8 kg/cow/d in the first and the second lactation, respectively, for cows fed GM maize) was not affected by dietary treatment. Only very small differences in fat, protein and urea concentrations were found, with cows fed the MON810-based diet producing higher levels of these constituents. However, according to the authors, the absolute

**Table 2**
Impact of GM plant diets in long term studies.

| Plant | Trait | Reference and funding | Species | Duration | Parameters | Group ($n$ = number of individuals per group) | Main findings | Authors' interpretation of results | Criticisms |
|---|---|---|---|---|---|---|---|---|---|
| Maize | Bt-MON810 containing Cry1Ab protein | Steinke et al. (2010). Not specific funding mentioned | Dairy cows | 25 months (100 weeks) | Milk composition and yield | 2 groups ($n$ = 18); in total 36 individuals. | Small changes in milk composition and body weight in GM-fed cows but fall within normal ranges | Safe, no long term effects. Bt-MON810 and its isogenic control are equivalent | |
| Soybean | Glyphosate-tolerant soybean (event not mentioned) | Daleprane et al. (2009a). State of Rio de Janeiro Research Assistance Foundation (FAPERJ). National Council for Scientific and Technological Development (CNPq) | Rats | 455 days (65) | Growth. Blood composition | 3 groups ($n$ = 10 male rats); in total 30 individuals. | GM group and organic group weight the same, higher than control group. Lower protein intake in control group. Growth, albumin, serum similar in all three groups | No differences between non-GM and GM groups. Soybean can be used in animal diets as a protein source | No isogenic line used |
| Soybean | Glyphosate-tolerant soybean (event not mentioned) | Daleprane et al. (2010). Brazilian foundations FAPERJ and CNPq | Rats | 455 days (65) | Aorta wall tissue. Cholesterol, triaclyglycerol, insulin, glucose and testosterone | 3 groups ($n$ = 10 male rats); in total 30 individuals. | Lower body weight and fat mass in control group | No differences observed between non-GM and GM groups in all parameters. Non-GM and GM groups substantially equivalent | No isogenic line used |
| Soybean | Glyphosate-tolerant soybean (event not mentioned) | Sakamoto et al. (2007) (complete paper in Japanese). Tokyo Metropolitan Institute of Public Health | Rats | 26 and 52 weeks | Growth. Feed intake. Organ weight. Hematology, serum | 3 (number of individuals not precised) | Differences in growth, feed intake, organ weight between groups. Body weight and feed intake similar between GM and non-GM soybean | Safe, no long term effects | No isogenic line used, event not precised, number of individuals not precised, full article not available in English |
| Soybean | Glyphosate-tolerant soybean (event not mentioned) | Sakamoto et al. (2008) (complete paper in Japanese). Tokyo Metropolitan Institute of Public Health | Rats | 104 weeks | Growth. Feed intake. Organ weight. Hematology, serum | 3 (number of individuals not precised) | Differences in growth, feed intake, organ weight between groups. Body weight and feed intake similar between GM and non-GM soybean | Safe, no long term effects | No isogenic line used, event not precised, number of individuals not precised, full article not available in English |
| Soybean | Glyphosate-tolerant soybean (CP4 EPSPS) | Malatesta et al. (2002a). Not specific funding mentioned | Mice | 240 days (30) | Ultrastructural morphetrical and immunocytochemical analyses of hepatocytes nuclei | 2 groups ($n$ = 12 female mice); in total 24 individuals. | Irregularly shaped nuclei, higher number of nuclear pores, numerous small fibrilla centres and abundant dense fibrillar component, nucleoplasmic and nuclear splicing factor more abundant in GM fed mice | Higher metabolic rate and molecular trafficking. Influence GM soybean intake on hepatocyte nuclear features in young and adult mices (mechanisms unknown) | No isogenic line used. Soybean not grown under the same conditions. International standards not reached. See text |
| Soybean | Glyphosate-tolerant soybean (CP4 EPSPS) | Malatesta et al. (2002b). Not specific funding mentioned | Mice | 240 days (30) | Histocytochemistry pancreatic acinar cells | 2 groups ($n$ = 12 female mice); in total 24 individuals. | No differences in body weight and no macroscopic changes in the pancreas. No structural modifications but quantitative changes in some cellular constituents. Reduction | A diet containing significant amounts of GM food seems to influence the zymogen synthesis and processing in pancreatics incinar cells (reasons remain unknown) | No isogenic line used. Soybean not grown under the same conditions. International standards not reached. See text |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | of α-amylase synthesis | | |
| Soybean | Glyphosate-tolerant soybean (CP4 EPSPS) | Malatesta et al. (2003). Not specific funding mentioned | Mice | 240 days (30) | Ultrastructural morphetrical and immunocytochemical analyses of pancreatic acinar cells nuclei | 2 groups ($n$ = 12 female mice); in total 24 individuals. | Decrease of the shape index and the fibrillar centres density and increase of the pored density, the perichromatin granule density, the percentage of fiibrillar centres in GM-fed mice. Lower labellings for the nucleoplasmic splicing factors | A diet containing significant amounts of GM food seems to influence the pancreatic metabolisms | No isogenic line used. Soybean not grown under the same conditions. International standards not reached. See text |
| Soybean | Glyphosate-tolerant soybean (CP4 EPSPS) | Vecchio et al. (2004). Italian Ministry of University and Research and by the Fondo di Ateneo per la Ricerca, Pavia University | Mice | 240 days (30) | Enzyme chemistry of serum, liver, and pancreas | 2 groups ($n$ = 12 female mice); in total 24 individuals. | Enlarged vesicles of the smooth endoplasmic reticulum, Decrease in the number of nuclear pores. Reduced labelling during the 2–8 month interval. Increase in perichromatin granules in Sertoli cells and in spermatocytes of GM fed mice | A transient transcriptionnal decrease during the 2–8 months intervall. Most of the effects reversible. Causes of the alteration not established, especially because glyphosate residues might influence transcriptionnal process | No isogenic line used. Soybean not grown under the same conditions. International standards not reached. See text |
| Soybean | Glyphosate-tolerant soybean (CP4 EPSPS) | Malatesta et al. (2008). Italian Ministry of Health | Mice | 2 years (104) | Histocytochemistry of hepatocytes | 2 groups ($n$ = 10 female mice); in total 20 individuals. | Different expression of proteins related to hepatocyte metabolism, stress response, calcium signalling and mitochondria in GM-fed mice. Indications of reduced metabolic rate in GM-fed mice | GM soybean can influence some liver features during ageing | No isogenic line used. Soybean not grown under the same conditions. International standards not reached. See text |
| Soybean | Glyphosate tolerant soybean (event not mentioned) | Sissener et al. (2009). Norwegian Research Council | Salmons | 7 months (28) | Growth. Body weight. Organ development. Plasma enzymes and nutrients. Differential white blood cell count | 3 groups ($n$ = 640); in total 1920 individuals | Mid intestine smaller in GM-fed group. Triaclyglcerol increased in GM-fed group. No growth differences between groups | Mid intestine smaller in GM-fed group. Triaclyglcerol increased in GM-fed group. No growth differences between groups | Safe, no term effects. Differences seen due to soybean cultivar non-GM diet |
| Rice | 7Crp#10 (*7Crp* gene derived from cedar pollen Cryj I and Cryj II allergen protein genes) | Domon et al. (2009). Not specific funding mentioned | Macaques | 26 weeks | Gross Necropsy. Histopathology and Absolute and Relative Organ Weights. Blood composition | 3 groups ($n$ = 6; three males and three females); in total 18 individuals. | With few exceptions, no significant differences in hematological or biochemical values between them. Neither pathological symptoms nor histopathological abnormalities observed | With few exceptions, no significant differences in hematological or biochemical values between them. Neither pathological symptoms nor histopathological abnormalities observed | No adverse effects on behavior or body weight, hematological and biochemical variables. No pathological symptoms or histopathological abnormalities |

**Table 3**
Impact of GM plant diets in multigenerational studies.

| Plant | Trait | Reference & Funding | Species | Duration | Parameters | Group ($n$ = number of individuals per group) | Main Findings | Authors' Interpretation of Results | Criticisms |
|---|---|---|---|---|---|---|---|---|---|
| Maize | Bt11 | Brake et al., 2003. No specific funding mentioned | Broiler chickens | 42 days (6 weeks) | Survival. Body weight. Feed efficiency and carcass | 4 groups (100); 2 groups (200 male individuals) and 2 groups (200 female individuals), in total 400 individuals. 3 generations | No differences in all parameters | Safe, no multigenerational effects | |
| Maize | Bt (event not mentioned) | Brake et al., 2004. No specific funding mentioned | Mice | 8, 16, 26, 32, 63, and 87 days after birth | Testicular development. Litter size. Body weight | 2 groups (10 female mice; 6 male mice). 3 male progeny per group sacrificed for each of six time points. 3 generations | No differences in fetal, postnatal, pubertal, or adult testicular development with the GM maize diet | Safe, no multigenerational effects | |
| Maize | Bt176 | Flachowsky et al., 2007. No specific funding mentioned | Broiler chickens, bulls, cows, hens, pigs, quails, sheep | Broiler chickens 35 days (5 weeks),bulls 246 days (35 weeks), cows 246 days (35 weeks approx.), hens 217 days (31 weeks), pigs 91 days (13 weeks), quails 84 days (12 weeks), sheep 91 days (13 weeks) | Digestibility. Feed intake. Health and performance. Meat quality. Reproduction | Broiler chickens: 2 ($n$ = 17), in total 34. Bulls: 2 ($n$ = 20), in total 40. Cows: 2 ($n$ = 20), in total 40. Hens: 2 ($n$ = 6), in total 12. Pigs: 2 ($n$ = 6), in total 12. Quails: 2 (70 male and 75 female, $n$ = 145). Sheep: ($n$ = 4). 10 generations for quails; 4 generations for hens | No recombinant DNA found in organs or tissues. No side effects of GM fed to 2nd generation | Safe, no multigenerational effects | |
| Maize | Bt (event not mentioned) | Kilic and Akay, 2008. No specific funding mentioned | Wistar albino rats | Duration not precised at least 3.5 months (14 weeks) | Histological and biochemical parameters characterizing stomach, duodenum, liver kidney | 3 ($n$ = 9), in total 27 individuals (18 females, 9 males). 3 generations | No differences in organ weights. Some minor histological changes in liver and kidney | The changes are minor and don't threaten the health of rats but long-term feeding studies with GM crops should be performed on other species | Event not mentioned. Lack of precision regarding duration of the study and the event studied |
| Maize | Bt176 | Trabalza-Marinucci et al., 2008. Italian Ministry of Health | Sheep | 44 months (188) | Samples of liver, spleen, pancreas, duodenum, cecal appendix, mesenteric lymph nodes, rumen and abomasum. Immune response, ruminal metabolism, microbial population, meat quality, transgene detection | 2 ($n$ = 53), in total 106 individuals. 3 generations | Changes in cell nuclei of liver and pancreas | Small effects. In general, GM maize is substantially equivalent to conventional maize but more metabolic research is needed in gastrointestinal organs and the immune response mechanisms | No use of isogenic lines. No evidence provided that the cytosolic differences are reproducible in independant biological replicates nor whether they are observed or not at different time points |
| Maize | Bt11 | Haryu et al., 2009. No specific funding mentioned | Mice | 1072 days (153 approx.) | Growth. Gestation, milking periods, reproduction, life span | 2 for each generation (31 female mice, 16 male mice), in total 94 individuals. 5 generations | No differences in all parameters. | Safe, no multigenerational effects | |
| Potato | Phosphinothricin acetyltransferase (*bar* gene) | Rhee et al., 2005. No specific funding mentioned | Rats | 5 generations; 70-day intervals before reproduction. 10 weeks | Presence of DNA. Feed consumption. Body weight. Reproductive performance. | Not specified. 5 generations | No difference in all parameters | Safe, no multigenerational effects | No use of isogenic line. Number of animals not mentioned |

(*continued on next page*)

**Table 3** (*continued*)

| Plant | Trait | Reference & Funding | Species | Duration | Parameters | Group ($n$ = number of individuals per group) | Main Findings | Authors' Interpretation of Results | Criticisms |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Development and viability of progeny. Organ weights. Skeletal and visceral deformations. Histopathology | | | | |
| Soybean | Glyphosate-tolerant soybean (GTS 40-3-2) | Brake and Evenson, 2004. Legislature of the State of South Dakota, Agricultural Experiment Station GMO Grant | Mice | 8, 16, 26, 32, and 63 days after birth (from one week approx. to 12 weeks approx.) | Testicular development. Litter size. Body weight | 2 groups (10 female mice, 6 male mice), in total 32 individuals. Three male progeny chosen for each of six time points. 3 generations | No differences in fetal, postnatal, pubertal, or adult testicular development with the GM soybean diet | Safe, no multigenerational effects | No use of isogenic line. Uncorrect number of animals |
| Soybean | Glyphosate-tolerant soybean (GTS 40-3-2) | Daleprane et al., 2009b. State of Rio de Janeiro Research Assistance Foundation and the National Council for Scientific and Technological Development | Wistar rats | Fed throughout life, exact time unclear | Weight gain, protein intake, ration intake. Food Conversion Ratios | 3 groups ($n$ = 8), in total 24 male mice. 2 generations | Differences between experimental and control | | No use of isogenic line. Uncorrect number of animals |
| Soybean | Glyphosate-tolerant soybean (GTS 40-3-2) | Tudisco et al., 2010. No specific funding mentioned | Goats | 60–67 days (8–9 approx.) | DNA in milk and blood | 2 groups ($n$ = 10) parents, female individuals only; 2 groups ($n$ = 5) kids, male individuals only. 2 generations | Presence of transgenic DNA in milk (parents) and blood (parents and offsprings). A significant difference for the level of LDH enzyme, and substitutions between the isoenzymes | Transgenic DNA detection far less important than chloroplastic DNA. LDH modifications suggest a rise of the cell metabolism. No health issue but further studies should be undertaken | No use of isogenic line. Grown in the same conditions not clear. Overinterpretation of the results. Uncorrect number of animals |
| Triticale | Glufosinate ammonium-tolerant triticale (tolerance to Basta with phosphonithricin as an active substance) | Baranowski et al., 2006. State Committee for Scientific Research | Mice | 91 days then mated/killed (at each generation) | Body weight and growth. Presence of transgenic DNA. Blood, kidneys, spleen, liver, thighs. | 2 (n = 10 sacrificed at each generation; five females and five males). 5 generations. | No presence of transgenic DNA. No weight differences. No pathological manifestations. | Safe, no multigenerational effects. | |
| Triticale | Glufosinate ammonium-tolerant wheat (Basta) (and containing the *β-glucuronisdae* gene) | Krzyowska et al., 2010. No specific funding mentioned | Mice | 120 days then mated/killed (at each generation) | Immune system | 2 ($n$ = 20 sacrificed at each generation). Gender not mentioned. 5 generations | In F5 enlarged inguinal and axillary lymph nodes detected. Decrease in T cells in spleen and lymph nodes and decrease in B cells in lymph nodes and blood | B cell compartment in the secondary lymphoid organs expansion not caused by an allergy or a malignant process but further studies should be undertaken | |

**Table 4**
Organs and parameters examined in long term and multigenerational studies.

| Organ | Parameters | Plant | Reference |
|---|---|---|---|
| Adrenal | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Brain | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Duodenum | Weight and histopathology | Insect-resistant maize (event not specified) | Kiliç and Akay (2008) |
| Gills | Na + K + -ATPase activity | Glyphosate-tolerant soybean (event GTS 40-3-2) | Sissener et al. (2009) |
| Heart | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Heart | Determination of enzymes, detection of chloroplast DNA and transgenic fragment | Glyphosate-tolerant soybean (event GTS 40-3-2) | Tudisco et al. (2010) |
| Heart (aorta) | Histological analysis | Herbicide-tolerant soybean (event not specified) | Daleprane et al. (2010) |
| Intestine | Fat content | Insect-resistant maize (event Bt 176); insect-resistant potatoes (containing Cry5-Bt gene); herbicide-tolerant (Pat) maize (event not specified); herbicide-tolerant (Pat) sugar beet; glyphosate-tolerant soybean (GTS 40-3-2) | Flachowsky et al. (2007) |
| Intestine | Proximal intestine somatic index, mid intestine somatic index, distal intestine somatic index, size | Glyphosate-tolerant soybean (event GTS 40-3-2) | Sissener et al. (2009) |
| Kidney | Detection of transgenic DNA from plant | Glufosinate ammonium-tolerant triticale (event not specified; tolerance to herbicide with phosphonithricin as an active substance) | Baranowski et al. (2006) |
| Kidney | Fat content | Insect-resistant maize (event Bt 176); insect-resistant potato (containing Cry5-Bt gene); herbicide-tolerant (Pat) maize (event not specified); herbicide-tolerant (Pat) sugar beet; herbicide-tolerant soybean (GTS 40-3-2) | Flachowsky et al. (2007) |
| Kidney | Weight and histopathology | Insect-resistant maize (event not specified) | Kiliç and Akay (2008) |
| Kidney | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Kidney | Head kidney-somatic index, size of head kidney | Glyphosate-tolerant soybean (event GTS 40-3-2) | Sissener et al. (2009) |
| Kidney | Determination of enzymes, detection of chloroplast DNA and transgenic fragment | Glyphosate-tolerant soybean (event GTS 40-3-2) | Tudisco et al. (2010) |
| Liver | Histocytochemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Malatesta et al. (2002a,b, 2003) |
| Liver | Enzyme chemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Vecchio et al. (2004) |
| Liver | Detection of transgenic DNA from plant | Glufosinate ammonium-tolerant triticale (event not specified; tolerance to herbicide with phosphonithricin as an active substance) | Baranowski et al. (2006) |
| Liver | Detection of transgenic DNA | Insect-resistant maize (event Bt 176); insect-resistant potato (containing Cry5-Bt gene); herbicide-tolerant (Pat) maize (event not specified); herbicide-tolerant (Pat) sugar beet; herbicide-tolerant soybean (GTS 40-3-2) | Flachowsky et al. (2007) |
| Liver | Weight and histopathology | Insect-resistant maize (event not specified) | Kiliç and Akay (2008) |
| Liver | Protein content, morphology | Glyphosate-tolerant soybean (event GTS 40-3-2) | Malatesta et al. (2008) |
| Liver | Hepato-somatic index | Glyphosate-tolerant soybean (event GTS 40-3-2) | Sissener et al. (2009) |
| Liver | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Liver | Determination of enzymes, detection of chloroplast DNA and transgenic fragment | Herbicide-tolerant soybean (event GTS 40-3-2) | Tudisco et al. (2010) |
| Lung | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Lymphoid | T cell and antibody numbers | Insect-resistant wheat (event not specified) | Krzyzowska et al. (2010) |
| Muscle | Detection of transgenic DNA from plant | Glufosinate ammonium-tolerant triticale (event not specified; tolerance to herbicide with phosphonithricin as an active substance) | Baranowski et al. (2006) |
| Muscle | Detection of promoter | Herbicide-resistant soybean (event not specified; containing *5-enolpyruvylshikimate-3-phosphate* gene) | Suharman et al. (2009) |
| Ovary | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Ovary | Weight | Insect-resistant maize (event Bt 11 N58-D1) | Haryu et al. (2009) |
| Pancreas | Histocytochemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Malatesta et al. (2002a,b, 2003) |
| Pancreas | Enzyme chemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Vecchio et al. (2004) |
| Pituitary | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Placenta | Weight | Insect-resistant maize (event Bt 11 N58-D1) | Haryu et al. (2009) |
| Prostate | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Serum | Enzyme chemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Vecchio et al. (2004) |
| Spleen | Detection of transgenic DNA from plant | Glufosinate ammonium-tolerant triticale (event not specified; tolerance to herbicide with phosphonithricin as an active substance) | Baranowski et al. (2006) |
| Spleen | Detection of transgenic DNA | Insect-resistant maize (event Bt 176); insect-resistant potatoes (containing Cry5-Bt gene); herbicide-tolerant (Pat) maize (event not specified); herbicide-tolerant (Pat) sugar beet; glyphosate-tolerant soybean (GTS 40-3-2) | Flachowsky et al. (2007) |
| Spleen | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Spleen | Spleen-somatic index | Glyphosate-tolerant soybean (event GTS 40-3-2) | Sissener et al. (2009) |
| Spleen | Determination of enzymes, detection of chloroplast DNA and transgenic fragment | Glyphosate-tolerant soybean (event GTS 40-3-2) | Tudisco et al. (2010) |
| Stomach | Fat content | Insect-resistant maize (event Bt 176); Insect-resistant potato (containing Cry5-Bt gene); Herbicide-tolerant (Pat) maize (event not specified); Herbicide-tolerant (Pat) sugar beet; Glyphosate-tolerant soybean (GTS 40-3-2) | Flachowsky et al. (2007) |
| Stomach | Weight and histopathology | Insect-resistant maize (event not specified) | Kiliç and Akay (2008) |
| Testis | Histocytochemistry | Glyphosate-tolerant soybean (event GTS 40-3-2) | Malatesta et al. (2002a,b, 2003) |
| Testis | Cell number, proliferation and differentiation | Insect-resistant maize (event N7070Bt) | Brake et al. (2004) |
| Testis | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Thymus | Weight and histopathology | Rice containing human T-cell epitope from Japanese cedar pollen allergens | Domon et al. (2009) |
| Uterus | Weight | Insect-resistant maize (event Bt11 N58-D1) | Haryu et al. (2009) |

differences were small and within the range of biological variation. Thus, they concluded this long-term study demonstrated the compositional and nutritional equivalence of Bt-MON810 and its isogenic non-transgenic counterpart.

*3.2.2. Herbicide-tolerant soybean*

Daleprane et al. (2009a) compared a diet containing glyphosate-tolerant soybean **(event not mentioned)** with organic soybean diet for 455 days (all diets contained 10% protein) in rats ($n = 10$ per group) to investigate impacts on growth and blood composition. Growth rates were comparable for all groups. Biochemical analyses revealed that albumin concentrations and serum protein were similar in all groups except for hematocrit. It should be mentioned that the authors included, as another control, a casein-based diet but none of these results will be discussed here. The same laboratory went onto further study the protein quality of these soybean diets and effects on growth rate, by performing 'multigenerational studies' which are discussed below (Daleprane et al., 2009b). Furthermore, Daleprane et al. (2010) additionally investigated the aorta (thickness of the three layers composing the aorta and total thickness) as well as growth parameters. No differences were observed between GM soybean and non-GM soybean fed groups in any parameter. Taking into account all these results, the authors stated that it "might" be concluded that the transgenic soybean is supposedly nutritionally equivalent to the non-transgenic variety as no nutritional or functional change is observed in rats fed a glyphosate-resistant soybean-based diet. However it is necessary to highlight that no true isogenic line of soybean was used as an appropriate comparator for transgenic soybean; furthermore the non-GM soybean was organically-grown, implying that plant growth conditions were different, but this apparently did not have any effect on all the measured endpoints in animals considered in this study.

Sakamoto et al. (2008) evaluated the safety of GM glyphosate-tolerant soybeans (**event not mentioned**) in male and female rats. The rats were fed a diet containing GM soybeans or non-GM soybeans (from a related strain but it is not stated whether these were isogenic or not) at a concentration of 30% in diet, both being adjusted to an identical nutrient level. These groups were also compared to a group fed a commercial diet (number of individuals per group not given) to evaluate any effect related to a soybean-based diet. Body weight and food consumption were recorded daily. After 104 weeks, at termination, hematology, serum biochemistry, and pathological examinations were made. Several differences in animal growth, food intake, organ weights and histological findings were observed between the rats fed the GM and/or non-GM soybeans. However, body weight and food intake were similar for the rats fed the GM or the non-GM soybeans. Gross necropsy findings, hematological and serum biochemical parameters, and organ weights showed no difference between rats fed the GM or the non-GM soybeans. Thus, there was neither an increase in incidence nor any specific type of non-neoplastic or neoplastic lesions in the GM soybeans group for both sexes. Long-term intake of GM soybeans at an incorporation level of 30% in diet had no apparent adverse effect in rats. In a previous study (which again did not mention the transgenic event), Sakamoto et al. (2007) found similar results in rats at the intermediate examination (26 weeks), and at the termination (52 weeks).

During a two-year study performed only on female mice, Malatesta et al. (2008) compared a diet consisting of 14% glyphosate-tolerant soybean (**event not stated**) and a control diet prepared from a commercial non-GM soybean. The effect of GM soybean on liver in 24-month old female mice was investigated with body weight also measured. In a liver proteome analysis (proteins related to hepatocyte metabolism, stress response, calcium signalling and mitochondria), 49 differences were reported with 39 proteins present at a higher level and 10 at a lower level in GM soybean diet fed mice than in non-GM soybean diet fed ones. These differences are all quantitatively minor and are in the range of differences observed in other studies (see Ricroch et al., 2011) and can be due to genotypic differences or growth conditions of the plant material used (see below). The electron microscopy results showed changes in nuclei shape and alterations in mitochondrial membrane in GM-fed mice, leading the authors to state that these observations are indicative of the induction of some metabolic disruptions. They concluded that GM-soybean affects the ageing of liver both morphologically and functionally and that more investigations are needed. It should be noted that this paper has been severely criticized (Williams and DeSesso, 2010). According to these authors, the study suffers from six methodological errors, namely (i) "*controlling for potential litter effect*", (ii) "*using an appropriate number of experimental animals per group and acquiring a sufficiently robust sample of independent observations*", (iii) "*establishing the representativeness of observations*", (iv) "*adhering to the principles for stereologic morphometry*", (v) "*using appropriate statistical methods (for study design as well as for data analysis)*", (vi) "*controlling for potential confounding factors, including those related to differences in diet phytoestrogen contents*". It should also be noted that none of the papers published by Malatesta and colleagues explicitly state the exact identity of the soybean lines used. It seems likely that the plant material compared in these studies was not isogenic lines but rather GM material and non-GM material that the authors were able to purchase from different commercial sources. In addition, the authors made comments on the possible involvement of herbicide treatment to explain their results, which confirms that the plant material differs by additional parameters other than just the transgene insertion factor and the fact that the plant materials were not grown side by side in the same field. Therefore, these studies do not match the internationally recommended standards for a proper nutritional or toxicological assessment of a GM line. Concerning previous studies (Malatesta et al., 2002a,b, 2003; Vecchio et al., 2004), members of the UK Advisory Committee on Novel Foods and Processes (2006) commented on reports about ultrastructural changes in liver, kidney and testes of mice given diets containing GM soybean, compared with non-GM soybean controls: "*Members noted that the papers did not state the origin of the GM and non-GM soya used in the feeding studies. There were no details of whether the soya had been grown in a field or under controlled conditions and whether or not the GM and non-GM soya were grown, handled and processed under similar conditions. It was also not clear whether the soya used in the control and GM experiments had a similar genetic background. The Committee was unable to determine whether or not the GM and non-GM soya crops had been treated with the herbicide glyphosate, although the authors had suggested that differences in residual levels of glyphosate might be responsible for the observed differences. The Committee also requested data on the nutritional equivalence of the two diets and as well as confirmation on whether or not the same experimental animals were used in each study*".

In a different animal model, salmon, Sissener et al. (2009) observed the effects of feeding a high level of glyphosate-tolerant soybean for 7 months, comparing it to a non-GM soybean control diet (stated to be a near isogenic maternal line). Various parameters were measured to observe the overall effect on health and performance. Measurements were made when fish were in fresh water and then after their transfer into seawater; a total of 4 samplings were taken. The growth rate and weight, on a whole, gave the same results for both groups though the weight of the mid-intestine of non-GM fed fish was heavier throughout the whole study. Among the parameters studied in hematology and plasma clinical biochemistry analyses, only one showed differences between groups: plasma triacylglycerol levels were higher in the

GM-fed fish throughout the whole study. Levels increased from 2.04 mMol on Dec 13th (124 days of diet) to 2.45 mMol on Feb 28th (200 days of diet) in GM fed fish, compared with 1.71 mMol (124 days of diet) to 2.25 mMol (200 days of diet) in non-GM fed fish). Other changes were seen when fish were transferred to seawater but were comparable between diet groups. The authors claim that the changes in triacylglycerol levels could have been due to the variation between the strains of the soybean used as the nutritional composition may vary slightly between the GM and the near isogenic line as no other previous studies in fish show the same findings. They conclude that the observations were not biologically significant and that GM soybean is nutritionally equivalent and as safe as non-GM soybean, thus it can be included in diets.

*3.2.3. GM rice seeds containing Japanese cedar pollen allergens*

This GM rice produces a hybrid peptide of seven major human T-cell epitopes (7Crp) derived from the *Cry j I and Cry j II* genes from *Cryptomeria japonica* pollen, which encode major human allergens During 26 weeks, cynomolgus macaques were fed with GM rice (Domon et al., 2009). Cv. Kitaake served as a control. The animals were divided into three groups, each comprising three males and three females, and administered a high dose of GM rice, a low dose of GM rice, or a high dose of the parental non-transgenic rice control by oral gavage every day. There were no abnormal clinical signs attributable to administration of GM rice in any group. No adverse effects on behavior or body weight were observed. Serum analysis from animals showed that, with few exceptions, there were no significant differences in hematological or biochemical values between groups. Neither pathological symptoms nor histopathological abnormalities were observed. Repeated oral administration of GM rice has no adverse effects.

*3.2.4. What can be learned from long-term studies?*

Overall, the available long-term studies do not yield new safety concerns and confirm that the studied GM varieties (most of them are major commercial products) are nutritionally equivalent to their non-GM conventional counterparts. It is particularly important to note that the six publications we examined included a large range of animal models (rat, mouse, cow, and salmon) and various durations of feeding. From these, no biologically-significant differences or adverse health effects were observed. However, in contrast to the maize study, the soybean studies do not state whether isogenic non-GM soybean was used as convenient control. In a number of papers we examined, the event is not even mentioned. Therefore it could be argued that if no non-GM isogenic lines were used (which could well explain some of the reported differences) these studies are not up to the required standards to thoroughly compare and examine the effects induced by GM and non-GM soybean.

When taking into account all the results, no long-term feeding study revealed any deleterious health effects and, therefore, did not provide any supplementary information that would already have been provided by the previously performed 90-day feeding studies (see Table 1).

*3.3. Multigenerational studies*

The main data from the publications discussed below are listed in Table 3. Within these studies, although rats and mice have sometimes been used, farm animals (dairy cows and bulls, goats, pigs, sheep, hens, broiler chickens, and quails) have mainly been studied.

Parameters measured include body weight, feed intake, detection of DNA from the GM plant in organs, enzyme concentrations or activities, and some reproductive factors. Within these studies, it is important to note that sometimes animals are fed GM-based diets throughout their life, i.e. on a long-term basis, and some are fed only on a short-term (less than 90 days) basis, but all these animals are bred to produce further generations. As shown below, the number of generations varies from 2 to 10. The main goal of these studies was to determine whether GM plants have a detrimental effect on next generations when the present generation was fed GM plants.

*3.3.1. Insect-resistant maize*

Concerning the general effects of GM plants, such as effects on health, performance and feed intake, on different species, the publication of Flachowsky et al. (2007) is a pivotal one. This review summarizes 18 studies that have been conducted at the Federal Agricultural Research Centre (FAL) in Germany since 1997. The majority (16) of experiments were undertaken using GM feeds based on Bt-maize, Bt-potatoes, glufosinate-tolerant (Pat) maize, glufosinate-tolerant sugar beet, and glyphosate-tolerant soybean. Two other studies were carried out using GM rapeseed which had an altered fatty acids profile (Böhme et al., 2005a), or inulin synthesising potatoes (Böhme et al., 2005b). In all 18 experiments, feeds from the GM lines were compared with their isogenic counterparts. Criteria such as digestibility, feed intake, health, performance, feed quality were studied in cows, bulls, pigs, hens, broiler chicken and quails. The study in hens was conducted for 4 generations and in quails for 10 generations. The other studies (cows, bulls, pigs, broiler chicken) were conducted for 1 generation for a short-term feeding of diet (for a period of 90 days). An additional objective of the study was to look for recombinant DNA in the digestive tract and other animal organs and tissues. No recombinant DNA (between 190 bp and 1000 bp) was found and the results showed that there was no significant difference in the nutritional value of the GM maize and no sign of adverse health effects in animals fed the GM-based diets. For reproductive parameters recorded in quails and hens, no significant difference was found between groups of animals fed diets containing either GM maize, which contains the gene Cry 1A(b)-delta-endotoxin, or non-GM maize.

Trabalza-Marinucci et al. (2008) also conducted a longitudinal study on three generations of sheep fed a Bt176 maize- or non-GM maize-based diet. They evaluated breeding performance, reproductive traits, hematological parameters, antioxidant defenses, lymphocyte proliferative capacity, phagocytosis and intracellular killing of macrophages, and ruminal microbial population and immune response to *Salmonella abortusovis* vaccination for three years. No adverse effect of the GM-based diet was found according to these parameters. No transgenic DNA was detected in tissues, blood or ruminal fluid. However, the authors claimed they observed proliferative activation of basal cells of ruminal epithelium in all GM maize-fed ewes, as well as smaller cell nuclei in hepatocytes and pancreatic acinar cells, which contained increased amounts of heterochromatin and perichromatin granules in GM-fed lambs. They also reported that immune response to *Salmonella* vaccination was more efficient in GM maize-fed sheep advising that it should be necessary to perform new longitudinal studies with a special focus on the effects on the immune system. There are major criticisms to be raised against these results (some of which are termed "preliminary" by the authors themselves). First, it appears that they did not use an isogenic line as a non-GM-comparator and that both lines were not grown under similar conditions. To compensate for these experimental flaws, they measured the composition of the GM and non-GM diets, and they claimed that the compositional differences were "*so minor that they are unlikely to be of any biological significance*". However, this latter comment appears unconvincing. Secondly, there is no evidence provided that the cytosolic differences are reproducible

in independent biological replicates or whether they are observed or not at different time points.

Similar criticisms can be raised for the study of Kiliç and Akay (2008). Wistar albino rats were fed a Bt-maize-based diet for three generations, this cultivar being resistant to the corn borer insect. Unfortunately, the exact variety is not specified and the non-transgenic line is not stated as being truly isogenic. Histological and biochemical parameters were studied in liver, kidneys, stomach and duodenum. Even though there was no difference in organ weights, some minor histopathological changes were found in liver and kidney as well as biochemical changes in creatinine, total protein and globulin. These changes were gender-dependent with females exhibiting increased creatinine levels for example. Changes over the three generations were considered as minor, neither statistically significant nor constituting a health hazard. Nonetheless, the authors suggested that long-term studies should be conducted on other species to confirm their results.

The effects of Bt11 maize (N7070Bt) on breeding traits (survival, body weight, feed efficiency, and carcass yield) were evaluated in broiler chickens in a study performed by Brake et al. (2003). No difference was found between the groups fed GM maize or the control diet.

Concerning the effects of GM maize (Bt11 variety 38PO6 and an isogenic line) diet on reproductive performance, Brake and Evenson (2004) studied the testicular development of mice. By studying the germ cell population at six regular periods throughout the life of mice in four successive generations, they determined that the diet induced no detectable effect on testicular development at any stage of life. Similarly, Haryu et al. (2009) carried out work on female mice. By weighing the fetuses, ovaries, placenta, uterus, assessing lifespan and counting the number of fetuses in five generations of mice, therefore studying growth, gestation and reproduction, no significant difference was found between the GM fed and the non-GM fed groups. Apparently these studies (Brake and Evenson, 2004; Haryu et al., 2009) roughly followed the OECD Test Guideline (OECD, 1998) (see Table 3). Twenty animals (10 female and 10 male) were used at each dose level, of which there were three, and a control was used (OECD, 1998; ANSES, 2011).

### 3.3.2. Herbicide-tolerant soybean

Brake and Evenson (2004) studied the effects of glyphosate-tolerant soybean on the testicular development in three generations of mice with the same methods used with maize as described previously (Brake et al., 2004). No effect of GM soybeans was found on fetal, postnatal, pubertal or adult testicular development.

Daleprane et al. (2009b) studied the use of glyphosate-tolerant soybean and organic soybean (both compared to a control casein diet, which is not discussed here) by measuring the net protein ratio and the protein efficacy ratio on two generations of rats. It should be noted that these parameters are used in nutrition studies, but in this study it is unfortunate that the authors did not adopt conventional parameters such as nitrogen digestibility or apparent or net protein digestibility, and the feed conversion ratio. Some differences were found in body weight ratio, protein intake and quality between the two soybean groups. Some differences between the GM and organic soybean fed groups were found in ration and protein intake, protein intake/weight ratio and calorie intake/weight ratio, and in protein efficacy ratio, net protein ratio and coefficient of alimentary effectiveness of F0 and F1 generations. From one generation to the next, no significant difference in weight was observed within the same group.

Tudisco et al. (2010) studied, in two generations of goats, the effects of glyphosate-tolerant soybean-based feeding and the presence of transgenic DNA fragments in blood and milk during a 15, 30 and 60-day feeding period (60, 30 and 15 days before killing in twenty pregnant dairy goats). The control diet is not precisely characterized (“conventional soybean”). In several organs, the authors found a significant difference in lactic dehydrogenase activity (LDH) and substitutions between the LDH isoenzymes. The LDH activity was measured in liver, heart, kidneys and muscle with an increase in LDH activity observed in liver (LDH1) and kidneys (LDH1), muscle (LDH1 and LDH2), and a decrease in the heart (LDH2 and LDH3) and muscle (LDH5) in kids drinking milk from treated animals. Elevated levels of LDH in tissues could suggest a rise in cell metabolism, but the corresponding enzyme activity in serum remained unchanged, so the effect is not clear and the authors do not consider it as a health issue but state that it should be taken into consideration for future studies. Small plant DNA fragments were detected in milk but also in kids' organs when mothers were fed GM soybean. The detection of transgenic target DNA sequences (35S promoter and CP4 EPSPS) in kids' organs is surprising as these DNA fragments are supposed to originate from the mothers' milk, which contained only low amounts of these DNA fragments. Furthermore, the detection of DNA fragments was not validated by DNA sequencing. In brief, Tudisco et al. (2010) showed some possible effects on metabolism though it is unclear whether this indicates a health risk issue. It is important to note that in this experiment the control group received a diet termed ‘conventional’ soybean, but it is not clear whether this was isogenic and whether the soybean cultivars were grown in the same conditions.

### 3.3.3. Herbicide-tolerant potato

In the studies performed by Rhee et al. (2005) in five generations of rats, the *bar* gene, which provides resistance to phosphinothricin, was not found in any of the reproductive organs of the GM-fed male and female rats to which a low level of potato-based diet (5%) was given. Body weight, food consumption, reproductive performance, and organ weight were all examined with no change. The authors conclude that GM potatoes have no effect on multigenerational reproductive and developmental performance.

### 3.3.4. Herbicide-tolerant triticale

Baranowski et al. (2006) and Krzyzowska et al. (2010) conducted two studies on five generations of mice fed with triticale tolerant to the herbicide glufosinate-ammonium (containing the β-glucuronidase gene (uidA) reporter gene). Baranowski et al. (2006) studied the effects of GM triticale by recording body weight and conducting PCR analysis to detect the presence of transgenic DNA in blood, kidneys, liver, spleen and thigh muscle on five generations. Each generation contained two groups, which were respectively fed either an experimental or a control diet containing 20% (by weight) of conventional triticale grain (except generation F0 that was only fed the control diet) and parameters were measured on mice from the 5 generations (at every generation, mice were sacrificed after 91 days). They also monitored possible pathological effects. They found no weight difference, no presence of transgenic DNA in tissues and no pathological effect.

Krzyzowska et al. (2010) were more concerned with the effects of glufosinate-ammonium tolerant triticale on the immune system. They conducted flow cytometry analysis, histopathological analysis, immunoblot analysis, immunophenotyping and measured the serum levels of cytokines and IgE on mice fed a conventional or a transgenic triticale-based diet containing 20% of the diet for both. These lines are not isogenic but it should be noted that they are the same as the ones used in Baranowski et al. (2006). They found in the fifth generation (only this generation was sacrificed and autopsied) enlarged inguinal and axillary lymph nodes, a decrease in the percentage of T cells in spleen and lymph nodes, increased IL-2 levels (by a factor of 2.5 for the fifth generation compared to controls), and decreased IL-6 levels (by a factor of 0.4 for the fifth generation compared to controls) but no significant changes in

the levels of IgE. The authors showed that this expansion of the B cell compartment in the secondary lymphoid organs was not caused by an allergy or a malignant process. Further studies should investigate the reasons of these changes and whether they are reproducible.

*3.3.5. What can be learned from multigenerational studies?*

Overall, the multigenerational studies on animals fed GM plants do not reveal signs of toxicity or other macroscopic effects on health. Changes in cytological characteristics in some cells and potential differences in metabolism in some organs have been reported (Tudisco et al., 2010), as well as changes in immune responses (Krzyzowska et al., 2010). However, these changes seem to be minor since the authors do not interpret them as potent effects on health. The relevance of the observed differences in some of the parameters is not known and may reflect some natural variation. The authors suggest that additional multigenerational studies should be done in order to study the reproducibility of these results and to try to find the true cause of the detected changes. Unfortunately, it has to be mentioned that, again, these reports suffer from serious weaknesses since they did not use appropriate comparators and this could be the major reason for the changes observed and, hence, the data cannot be interpreted in terms of toxicological effects.

The statistical problem underlying the existence of confounding factors is highlighted in these studies and we also noticed some other recurrent problems in the experimental designs. Some of these issues are poor definition of a control (or group control), weaknesses in the definition of factor levels, lack of a complete combination of factors inside experimental designs, absence of evaluation of the statistical power, and lack of multivariate approaches. Moreover, regulatory agency EFSA (2010) recommended improved methodology for experimental design particularly when statistics are involved.

## 4. Discussion

General principles outlined in the OECD Test Guideline (1998) or discussed by EFSA (2008) have been built or adjusted to get a robust evaluation for the safety of GM-based diet in a case-by-case basis. These are based on i) the substantial equivalence principle, the use of which is intended to compare chemical composition in macro and micro nutrients and known anti-nutrients and natural toxicants of GM lines and near unmodified isogenic lines, and ii) the toxico-nutritional response of animals fed either a GM-based diet or a control diet in sub-chronic toxicity tests, and if necessary long-term or multigenerational studies.

GM lines with no deliberate metabolic modification are usually found to be nutritionally equivalent to their comparator non-GM line. This is not surprising since these GM lines have been selected, from laboratory and field trials, by comparison with known non-GM lines on various phenotypic traits. Thus, it is highly unlikely that such a comparative process would yield GM lines with major unintended chemical differences. Furthermore, these lines are usually backcrossed to elite lines, which also contribute to their equivalence to these comparator lines. Thus, although this has often been overlooked, the whole process of production of GM commercial lines contributes to the food safety of such lines and no other study has been proven really necessary to assess this safety.

Nevertheless, if doubts about this nutritional equivalence still exist, some experts recommend performing sub-chronic toxicity 90-day tests to assess this uncertainty (Aumaitre, com. pers.). Therefore, in this general step-by-step assessment frame, long-term and multigenerational studies would be performed only after such a sub-chronic toxicity 90-day testing.

*4.1. Exploratory studies in the context of a step-by-step approach*

In the present review, most of the studies mentioned were not conducted as part of a regulatory safety assessment process but were exploratory studies performed by public research laboratories. Ten out of the 12 long-term studies examined in this review were all performed within the public sector using public funding. Five studies undertaken at the University of Urbino in Italy (Malatesta et al., 2002a,b, 2003), at the Technical University of Munich in Germany (Steinke et al., 2010) and at the Transgenic Crop Research and Development Center in Japan (Domon et al., 2009) did not mention any specific funding. All of the twelve multigenerational studies were performed within the public sector. Eight out of the twelve multigenerational studies did not mention any specific funding (Brake et al., 2003, 2004; Rhee et al., 2005; Flachowsky et al., 2007; Kiliç and Akay, 2008; Haryu et al., 2009; Tudisco et al., 2010; Krzyzowska et al., 2010).

Despite the exploratory nature of the studies reviewed here, the step-by-step approach is supported by their results. Considering all of them, it is clear that GM food is not revealed to be harmful when the duration of feeding is increased to well over 90 days. Therefore, no evidence is available to show that a duration of 90 days is insufficient to assess the effects of GM food. Studies lasting two years, for example, do not seem necessary except when doubt remains after performing 90-day studies. The concept of nutritional equivalence has been proven to be sufficient to assess the safety of GM food and feed, and it has recently been supported by the use of technologies such as metabolomics, proteomics and transcriptomics (see Ricroch et al., 2011 for a review).

Yet, this review reveals deep weaknesses shared by most long-term studies because of non-adherence to standard procedures outlined in the OECD Test (1998).

*4.2. Standard protocols and quality of the studies*

The studies reviewed here are often linked to an inadequate experimental design that has detrimental effects on statistical analysis as far as the most frequently used statistics are concerned. Internationally agreed test methods should be used for toxicity testing (EFSA, 2011).

The experimental protocol currently used is described in the OECD Test Guideline No. 408, initially designed for assessing the toxicity of chemicals (OECD, 1998). It recommends populations of at least 10 animals per sex and per group, with 3 doses of the test substance and a control group. Six out of the 24 studies examined here used an appropriate number of experimental animals: three long-term studies (Daleprane et al., 2009a, 2010; Sissener et al., 2009) and three multigenerational studies (Brake et al., 2003; Flachowsky et al., 2007; Haryu et al., 2009). It should be mentioned that increasing the number of animals tested increases the statistical power but is more costly. High costs may hinder the public sector from conducting such studies. A balance should be found between robust toxicological interpretations and a reasonable cost (i.e. affordable by the public sector).

Another major problem of the studies examined here is the plant material and its description. Growing GM lines and their comparator side by side can be difficult and even impossible in some countries because of recurrent vandalism or extensive political bans. Furthermore, seventeen out of the twenty-four studies examined did not use isogenic lines for the control diet (or more precisely did not state they used isogenic lines). Comparing two non-isogenic cultivars is problematic when differences are observed since these effects can be caused by the differences between cultivars and not specifically by the transgene. This is simply related to the confounding factor problem and the defective statistical characteristics underlined above, considering the combination

of different controlled factors included in the experimental design. That is why some minor histological and biochemical effects that have been found cannot be causally related to the GM plant itself since they could be due to the conditions of the experiment. Nevertheless, inclusion of commercial cultivars may be useful to check whether the observed values fall within the range of observed values for different parameters. However, inclusion of non-isogenic lines/commercial lines cannot replace the recommended complete experimental design built from different controlled independent variables.

One can mention the studies of Daleprane et al. (2009a,b) which, although it is an interesting study, compared a GM diet and a diet containing organic soybean. Not only were both lines not grown side by side but they were also grown using different agricultural practices. Although few differences were observed, an isogenic line grown side by side with the GM line should have been added in the comparative analysis.

In addition, this systematic review and critical examination of the numerous published studies indicate that those which found changes in some parameters did not follow the required standard protocols. One study sorted from our database did not reach the standard requirement of scientific validity for publication (Velimirov et al., 2008) and therefore is not discussed here.

In summary, the major insufficiencies not only include lack of use of near isogenic lines but also statistical power underestimation, absence of repetitions (see below), over-interpretation of differences, which are often within the normal range of variation, and poor toxicological interpretation of the data. As shown in the present review, the over-90 day and multigenerational studies do not reveal any new effect that has not been found in 90-day studies. Thus, it could be assumed that standard protocols are efficient enough to detect adverse effects and there is no need to design new protocols that cannot lead to sound comparisons. Considering that it is a critical issue surrounding GM evaluations that studies of insufficient quality can still be published, it is our opinion that in the future only publications in peer-reviewed journals devoted to toxicology and nutrition should be considered for a critical analysis of scientific evidence related to such topics.

### 4.3. Cooperation between public laboratories and private firms

Because of recurrent lack of compliance with international standards of many studies, a critical situation has arisen where the private sector may not want to provide plant material for studies. Unfortunately, without such collaboration from the private sector, public laboratories may not always be able to conduct studies using appropriate plants lines. In this context, more rigorous statistical prerequisite and sound toxicological interpretations of the results would encourage a virtuous scientific collaboration between public laboratories and private firms, particularly to access to the different isogenic lines that are true comparator of GM lines.

### 4.4. Fundamental research and harmonization of protocols

If long-term and multigenerational studies would rarely be used in the regulatory safety assessment of GM whole food and feed as they would constitute the third step to assess the safety, such studies could be used in fundamental research aimed to increase basic knowledge. It would be the case, for example, to check whether these specific types of study may provide supplementary observations not displayed in sub-chronic 90-day studies, or to assess the effects of the chemical composition of a particular diet (for example the effects on metabolism of the amount of maize in diet in addition to the factor GM), or to find out the amount of GM material per diet that is the most suitable to assess the whole food and feed or, more generally, to better design such experiments to proceed to a more convenient assessment of GM-based diets.

However, for generating knowledge of indisputable quality, another improvement in the protocols is necessary, namely reproducibility. As shown in Tables 2 and 3, very few studies for a given plant line have been reproduced using the same animal model. Moreover, studies using the same animal model were performed with different parameters, which lead to the fact that no trials have been carried out twice in the same conditions by different research teams. The most used animal model is mouse, for which only two studies were conducted for maize, two for soybean, two for triticale, and one for potato. Other animal models used in such GM food or feed assessment are rats and cows. Tests conducted according to OECD Test Guideline (1998) and a lot of toxicological studies are carried out on rodents, therefore using rats and mice. Studies on cows are mostly used to study effects on milk composition. With such diversity in models, it is difficult to integrate results in meta-analyses and to interpret these results on a large scale.

The same remarks can be made concerning organs according to Table 4, which shows the variety of organs studied but also the variety of parameters studied by organ. The most studied organ is liver, then kidneys and the digestive tract (stomach and intestine), which are relevant since these organs are primary barriers functionally impacted by nutritional factors or possible toxic events. Indeed, the digestive tract is first exposed to any food toxicant, but also some other organs like liver and kidneys are detoxifying organs. Reproductive organs are also studied to evaluate effects, which may not be harmful for the health but would have an effect on reproductive performance. Further harmonization is needed for the statistical approaches as well as previously quoted parameters, as recently proposed by French Agency with responsibility for food safety, concerning statistical analyses of data from 90-day rat feeding studies (ANSES, 2011).

In addition, such exploratory feeding studies could serve to validate future new studies and their methodologies. For example, high throughput techniques performed in a rigorous statistical framework, could be used to better measure low intensity metabolic disruptions induced by a GM-based feeding. This implies that the use of statistical multivariate analyses can also address the longitudinal follow-up of experimental units. At the same time modeling of other controlled factors such as gender or feeding material from various plant cultivars can be pursued in addition to the GM factor *stricto sensu*. These techniques would help to shed light on the inherent metabolic plasticity of the animal model, which could be impacted by variation of diet because of the GM material, the cultivar or other controlled independent variables. Recently, sensitivity of this approach to detect minor but statistically significant homeostatic transitions in metabolically disrupted models has been well demonstrated in nutritional or sub-toxicological studies (Fardet et al., 2007; Domange et al., 2008).

Such basic research would help to establish a clearer view (i.e. with no conflicting advice) on how to combine or harmonize various experimental designs, namely standardized regulatory-oriented experimental designs consisting of either short-term (21–28 days), middle term (91–105 days) or long-term (180–720 days) protocols, or multigenerational studies. They would help to better document all the factors involved in the calculation of the variance (cultivar, event, individual variability in a longitudinal follow-up of the animal model, phenotypic variability of plant and year effect, etc.) and to prioritize the importance of these factors.

## 5. Conclusion

Long-term and multigenerational studies have been used as part of exploratory fundamental research projects. Up to now, none

of them have provided supplementary information indicating that 90-day rodent feeding studies defined by the Guideline No. 408 (OECD, 1998) would not be sufficient to serve as a sound experimental basis for regulatory assessment of new GM traits. Indeed, only the 90-day rodent feeding studies are recommended in some specific cases to detect any hazard as discussed previously (EFSA, 2008). Therefore, long-term and multigenerational studies should only be conducted in a case-by-case approach for GM food safety regulatory assessment if some reasonable doubt remains after a 90-day feeding trial.

The observations of major flaws in some papers highlight the urgent need to improve the reviewing process before publication of papers addressing this subject. This would avoid spreading confusion in the general press, which may not be able to judge the real scientific quality of publications.

Complementary fundamental research should be conducted using different animal models, but a need for harmonization between studies is crucial to provide better results that are more reproducible within a given animal model and more comparable between neighboring animal models. Such research would help to better analyze the physiological differences arising between short, mid and long-term tests and, hence, the conditions of choice of the most appropriate experimental design.

## Conflict of Interest

The authors declare that there are no conflicts of interests.

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