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Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize

Gilles-Eric Séralini [a,*], Emilie Clair [a], Robin Mesnage [a], Steeve Gress [a], Nicolas Defarge [a], Manuela Malatesta [b], Didier Hennequin [c], Joël Spiroux de Vendômois [a]

[a] University of Caen, Institute of Biology, CRIIGEN and Risk Pole, MRSH-CNRS, EA 2608, Esplanade de la Paix, Caen Cedex 14032, France
[b] University of Verona, Department of Neurological, Neuropsychological, Morphological and Motor Sciences, Verona 37134, Italy
[c] University of Caen, UR ABTE, EA 4651, Bd Maréchal Juin, Caen Cedex 14032, France

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ABSTRACT

The health effects of a Roundup-tolerant genetically modified maize (from 11% in the diet), cultivated with or without Roundup, and Roundup alone (from 0.1 ppb in water), were studied 2 years in rats. In females, all treated groups died 2–3 times more than controls, and more rapidly. This difference was visible in 3 male groups fed GMOs. All results were hormone and sex dependent, and the pathological profiles were comparable. Females developed large mammary tumors almost always more often than and before controls, the pituitary was the second most disabled organ; the sex hormonal balance was modified by GMO and Roundup treatments. In treated males, liver congestions and necrosis were 2.5–5.5 times higher. This pathology was confirmed by optic and transmission electron microscopy. Marked and severe kidney nephropathies were also generally 1.3–2.3 greater. Males presented 4 times more large palpable tumors than controls which occurred up to 600 days earlier. Biochemistry data confirmed very significant kidney chronic deficiencies; for all treatments and both sexes, 76% of the altered parameters were kidney related. These results can be explained by the non linear endocrine-disrupting effects of Roundup, but also by the overexpression of the transgene in the GMO and its metabolic consequences.
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1. Introduction

There is an ongoing international debate as to the necessary length of mammalian toxicity studies in relation to the consumption of genetically modified (GM) plants including regular metabolic analyses (Séralini et al., 2011). Currently, no regulatory authority requests mandatory chronic animal feeding studies to be performed for edible GMOs and formulated pesticides. However, several studies consisting of 90 day rat feeding trials have been conducted by the biotech industry. These investigations mostly concern GM soy and maize that are rendered either herbicide tolerant (to Roundup (R) in 80% of cases), or engineered to produce a modified *Bt* toxin insecticide, or both. As a result these GM crops contain new pesticide residues for which new maximal residual levels (MRL) have been established in some countries.

If the petitioners conclude in general that there is no major change in genetically modified organism (GMO) subchronic toxicity studies (Domingo and Giné Bordonaba, 2011; Hammond et al., 2004, 2006a,b), significant disturbances have been found and may be interpreted differently (Séralini et al., 2009; Spiroux de Vendômois et al., 2010). Detailed analyses have revealed alterations in kidney and liver functions that may be the signs of early chronic diet intoxication, possibly explained at least in part by pesticide residues in the GM feed (Séralini et al., 2007; Spiroux de Vendômois et al., 2009). Indeed, it has been demonstrated that R concentrations in the range of 10^3 times below the MRL induced endocrine disturbances in human cells (Gasnier et al., 2009) and toxic effects thereafter (Benachour and Seralini, 2009), including *in vivo* (Romano et al., 2012). After several months of consumption of an R-tolerant soy, the liver and pancreas of mice were affected, as highlighted by disturbances in sub-nuclear structure (Malatesta et al., 2008a, 2002a,b). Furthermore, this toxic effect was reproduced by the application of R herbicide directly to hepatocytes in culture (Malatesta et al., 2008b).

Abbreviations: GM, genetically modified; R, Roundup; MRL, maximal residual levels; GMO, genetically modified organism; OECD, Organization for Economic Co-operation and Development; GT, glutamyl-transferase; PCA, principal component analysis; PLS, partial least-squares; OPLS, orthogonal partial least-squares; NIPALS, Nonlinear Iterative Partial Least Squares; OPLS-DA, Orthogonal Partial Least Squares Discriminant Analysis; G, glycogen; L, lipid droplet; N, nucleus; R, rough endoplasmic reticulum (on microscopy pictures only); U, urinary; UEx, excreted in urine during 24 h; APPT, Activated Partial Thromboplastin Time; MCV, Mean Corpuscular Volume; PT, Prothrombine Time; RBC, Red Blood Cells; ALT, alanine aminotransferase; MCHC, Mean Corpuscular Hemoglobin Concentration; A/G, Albumin/Globulin ratio; WBC, White Blood Cells; AST, aspartate aminotransferase.

* Corresponding author. Tel.: +33 (0)231565684; fax: +33 (0)231565320.
E-mail address: criigen@unicaen.fr (G.-E. Séralini).

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Since then, long-term and multi-generational animal feeding trials have been performed with some possibly providing evidence of safety, while others conclude on the necessity of further investigations because of metabolic modifications (Snell et al., 2011). However, none of these studies have included a detailed follow-up of the animals with up to 11 blood and urine samples over 2 years, and none has investigated the NK603 R-tolerant maize.

Furthermore, toxicity evaluation of herbicides is generally performed on mammalian physiology through the long-term study of only their active principle, rather than the formulation used in agriculture, as was the case for glyphosate (Williams et al., 2000), the active herbicide constituent of R. It is important to note that glyphosate is only able to efficiently penetrate target plant organisms with the help of adjuvants present in the various commercially used R formulations (Cox, 2004). When R residues are found in tap water, food or feed, they arise from the total herbicide formulation, which is the most commonly used mixture in agriculture; indeed many authors in the field have strongly emphasized the necessity of studying the potential toxic effects of total chemical mixtures rather than single components (Cox and Surgan, 2006; Mesnage et al., 2010; Monosson, 2005). Even adjuvants and not only glyphosate or other active ingredients are found in ground water (Krogh et al., 2002), and thus an exposure to the diluted whole formulation is more representative of an environmental pollution than the exposure to glyphosate alone in order to study health effects.

With a view to address this lack of information, we have performed a 2 year detailed rat feeding study. The actual guideline 408 of the Organization for Economic Co-operation and Development (OECD) was followed by some manufacturers for GMOs even if it was not designed for that purpose. We have explored more parameters and more frequently than recommended in this standard (Table 1) in a long-term experiment. This allowed us to follow in details potential health effects and their possible origins due to the direct or indirect consequences of the genetic modification itself in GMOs, or due to the formulated herbicide mixture used on GMOs (and not glyphosate alone), or both. Because of recent reviews on GMOs (Domingo and Giné Bordonaba, 2011; Snell et al., 2011) we had no reason to settle at first for a carcinogenesis protocol using 50 rats per group. However we have prolonged the biochemical and hematological measurements or disease status recommended in combined chronic studies using 10 rats per group (up to 12 months in OECD 453). This remains the highest number of rats regularly measured in a standard GMO diet study. We have tested also for the first time 3 doses (rather than two in the usual 90 day long protocols) of the R-tolerant NK603 GM maize alone, the GM maize treated with R, and R alone at very low environmentally relevant doses starting below the range of levels permitted by regulatory authorities in drinking water and in GM feed.

2. Materials and methods

2.1. Ethics

The experimental protocol was conducted in accordance with the regulations of our ethics in an animal care unit authorized by the French Ministries of Agriculture and Research (Agreement Number A35-288-1). Animal experiments were performed according to ethical guidelines of animal experimentations (CEE 86/609 regulation). Concerning field studies of plant species, no specific permits were required, nor for the locations/activities. The maize grown (MON-00603-6 commonly named NK603) was authorized for unconfined release into the environment and use as a livestock feed by the Canadian Food Inspection Agency (Decision Document 2002-35). We confirm that the location is not privately-owned or protected in any way and that the field studies did not involve endangered or protected species. The GM maize was authorized for import into the European Union (CE 258/97 regulation).

2.2. Plants, diets and chemicals

The varieties of maize used in this study were the R-tolerant NK603 (Monsanto Corp., USA), and its nearest isogenic non-transgenic control. These two types of maize were grown under similar normal conditions, in the same location, spaced at a sufficient distance to avoid cross-contamination. The genetic nature, as well as the purity of the GM seeds and harvested material, was confirmed by qPCR analysis of DNA samples. One field of NK603 was treated with R at 3 L ha^{-1} (WeatherMAX, 540 g/L of glyphosate, EPA Reg. 524-537), and another field of NK603 was not treated with R. Corns were harvested when the moisture content was less than 30% and were dried at a temperature below 30 °C. From these three cultivations of

Table 1
Protocol used and comparison to existing assessment, and to non-mandatory regulatory tests.

Treatments and analyses	In this work	Hammond et al., 2004	Regulatory tests
Treatments + controls	GMO NK603, GMO NK603 + Roundup, Roundup, and closest isogenic maize	GMO NK603 + Roundup, closest isogenic maize, and six other maize lines non substantially equivalent	GMOs or chemicals (in standard diet or water)
Doses by treatment	3	2	At least 3
Duration in months	24 (chronic)	3 (subchronic: 13 weeks)	3
Animals measured/group/sex	10/10 SD rats (200 rats measured)	10/20 SD rats (200 rats measured/total 400)	At least 10 rodents
Animals by cage (same sex)	1–2	1	1 or more
Monitoring/week	2	1	1 or more
Feed and water consumptions	Measured	For feed only	At least feed
Organs and tissues studied			For high dose and controls
Histology/animal	34	17/36	At least 30
Organs weighted	10	7	At least 8
Electronic microscopy	Yes	No	No
Behavioral studies (times)	2	1 (no protocol given)	1
Ophtalmology (times)	2	0	2
Number of blood samples/animal	11, each month (0–3) then every 3 months	2, weeks 4 and 13	1, at the end
Blood parameters	31 (11 times for most)	31 (2 times)	At least 25 (at least 2 times)
Plasma sex steroids	Testosterone, estradiol	No	No, except if endocrine effects suspected
Liver tissue parameters	6	0	0
Number of urine samples	11	2	Optional, last week
Urine parameters studied	16	18	7 if performed
Microbiology in feces or urine	Yes	Yes	No
Roundup residues in tissues	Studied	Not studied	Not mandatory
Transgene in tissues	Studied	Not studied	Not studied

The protocol used in this work was compared to the regulatory assessment of NK603 maize by the company (Hammond et al., 2004), and to non mandatory regulatory *in vivo* tests for GMOs, or mandatory for chemicals (OECD 408). Most relevant results are shown in this paper.

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maize, laboratory rat chow was made based on the standard diet A04 (Safe, France). The dry rat feed was made to contain 11, 22 or 33% of GM maize, cultivated either with or without R, or 33% of the non-transgenic control line. The concentrations of the transgene were confirmed in the three doses of each diet by qPCR. All feed formulations consisted in balanced diets, chemically measured as substantially equivalent except for the transgene, with no contaminating pesticides over standard limits. All secondary metabolites cannot be known and measured in the composition. However we have measured isoflavones and phenolic acids including ferulic acid by standard HPLC-UV. All reagents used were of analytical grade. The herbicide diluted in the drinking water was the commercial formulation of R (GT Plus, 450 g/L of glyphosate, approval 2020448, Monsanto, Belgium). Herbicides levels were assessed by glyphosate measurements in the different dilutions by mass spectrometry.

2.3. Animals and treatments

Virgin albino Sprague-Dawley rats at 5 weeks of age were obtained from Harlan (Gannat, France). All animals were kept in polycarbonate cages (820 cm^2, Genestil, France) with two animals of the same sex per cage. The litter (Toplit classic, Safe, France) was replaced twice weekly. The animals were maintained at 22 ± 3 °C under controlled humidity (45–65%) and air purity with a 12 h-light/dark cycle, with free access to food and water. The location of each cage within the experimental room was regularly moved. This 2 year life-long experiment was conducted in a GPL environment according to OECD guidelines. After 20 days of acclimatization, 100 male and 100 female animals were randomly assigned on a weight basis into 10 equivalent groups. For each sex, one control group had access to plain water and standard diet from the closest isogenic non-transgenic maize control; six groups were fed with 11, 22 and 33% of GM NK603 maize either treated or not with R. The final three groups were fed with the control diet and had access to water supplemented with respectively 1.1×10^{-8}% of R (0.1 ppb of R or 50 ng/L of glyphosate, the contaminating level of some regular tap waters), 0.09% of R (400 mg/kg, US MRL of glyphosate in some GM feed) and 0.5% of R (2.25 g/L, half of the minimal agricultural working dilution). This was changed weekly. Twice weekly monitoring allowed careful observation and palpation of animals, recording of clinical signs, measurement of any tumors that may arise, food and water consumption, and individual body weights.

2.4. Biochemical analyses

Blood samples were collected from the tail vein of each rat under short isoflurane anesthesia before treatment and after 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 months: 11 measurements were obtained for each animal alive at 2-years. It was first demonstrated that anesthesia did not impact animal health. Two aliquots of plasma and serum were prepared and stored at −80° C. Then 31 parameters were assessed (Table 1) according to standard methods including hematology and coagulation parameters, albumin, globulin, total protein concentration, creatinine, urea, calcium, sodium, potassium, chloride, inorganic phosphorus, triglycerides, glucose, total cholesterol, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl-transferase (GT), estradiol, testosterone. In addition, at months 12 and 24 the C-reactive protein was assayed. Urine samples were collected similarly 11 times, over 24 h in individual metabolic cages, and 16 parameters were quantified including creatinine, phosphorus, potassium, chloride, sodium, calcium, pH and clairance. Liver samples at the end made it possible to perform assays of CYP1A1, 1A2, 3A4, 2C9 activities in S9 fractions, with glutathione S- transferase and gamma-GT.

2.5. Anatomopathology

Animals were sacrificed during the course of the study only if necessary because of suffering according to ethical rules (such as 25% body weight loss, tumors over 25% body weight, hemorrhagic bleeding, or prostration), and at the end of the study by exsanguination under isoflurane anesthesia. In each case, the following organs were collected: brain, colon, heart, kidneys, liver, lungs, ovaries, spleen, testes, adrenals, epididymis, prostate, thymus, uterus, aorta, bladder, bone, duodenum, esophagus, eyes, ileum, jejunum, lymph nodes, lymphoreticular system, mammary glands, pancreas, parathyroid glands, Peyer's patches, pituitary, salivary glands, sciatic nerve, skin, spinal cord, stomach, thyroid and trachea. The first 14 organs (at least 10 per animal depending on the sex, Table 1) were weighted, plus any tumor that arose. The first nine organs were divided into two parts and one half was immediately frozen in liquid nitrogen/carbonic ice. The remaining parts including other organs were rinsed in PBS and stored in 4% formalin before anatomopathological study. These samples were used for further paraffin-embedding, slides and HES histological staining. For transmission electron microscopy, kidneys, livers and tumors were cut into 1 mm^3 fragments. Samples were fixed in pre-chilled 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M PBS pH 7.4 at 4 °C for 3 h and processed as previously described (Malatesta et al., 2002a).

2.6. Statistical analysis

Biochemical data were treated by multivariate analysis with the SIMCA-P (V12) software (UMETRICS AB Umea, Sweden). The use of chemometrics tools, for example, principal component analysis (PCA), partial least-squares to latent structures (PLS), and orthogonal PLS (OPLS), are robust methods for modeling, analyzing and interpreting complex chemical and biological data. OPLS is a recent modification of the PLS method. PLS is a regression method used in order to find the relationship between two data tables referred to as X and Y. PLS regression (Eriksson et al., 2006b) analysis consists in calculating by means of successive iterations, linear combinations of the measured X-variables (predictor variables). These linear combinations of X-variables give PLS components (score vectors t). A PLS component can be thought of as a new variable – a latent variable – reflecting the information in the original X-variables that is of relevance for modeling and predicting the response Y-variable by means of the maximization of the square of covariance (Max $cov^2(X,Y)$). The number of components is determined by cross validation. SIMCA software uses the Nonlinear Iterative Partial Least Squares algorithm (NIPALS) for the PLS regression. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used in this study (Weljie et al., 2011; Wiklund et al., 2008). The purpose of Discriminant Analysis is to find a model that separates groups of observations on the basis of their X variables. The X matrix consists of the biochemical data. The Y matrix contains dummy variables which describe the group membership of each observation. Binary variables are used in order to encode a group identity. Discriminant analysis finds a discriminant plan in which the projected observations are well separated according to each group. The objective of OPLS is to divide the systematic variation in the X-block into two model parts, one linearly related to Y (in the case of a discriminant analysis, the group membership), and the other one unrelated (orthogonal) to Y. Components related to Y are called predictive, and those unrelated to Y are called orthogonal. This partitioning of the X data results in improved model transparency and interpretability (Eriksson et al., 2006a). Prior to analysis, variables were mean-centered and unit variance scaled.

3. Results

3.1. Mortality

Control male animals survived on average 624 ± 21 days, whilst females lived for 701 ± 20, during the experiment, plus in each case 5 weeks of age at the beginning and 3 weeks of stabilization period. After mean survival time had elapsed, any deaths that occurred were considered to be largely due to aging. Before this period, 30% control males (three in total) and 20% females (only two) died spontaneously, while up to 50% males and 70% females died in some groups on diets containing the GM maize (Fig. 1). However, the rate of mortality was not proportional to the treatment dose, reaching a threshold at the lowest (11%) or intermediate (22%) amounts of GM maize in the equilibrated diet, with or without the R application on the plant. It is noteworthy that the first two male rats that died in both GM treated groups had to be euthanized due to kidney Wilm's tumors that were over 25% of body weight. This was at approximately a year before the first control animal died. The first female death occurred in the 22% GM maize feeding group and resulted from a mammary fibroadenoma 246 days before the first control. The maximum difference in males was 5 times more deaths occurring during the 17th month in the group consuming 11% GM maize, and in females 6 times greater mortality during the 21st month on the 22% GM maize diet with and without R. In the female cohorts, there were 2–3 times more deaths in all treated groups compared to controls by the end of the experiment and earlier in general. Females were more sensitive to the presence of R in drinking water than males, as evidenced by a shorter lifespan. The general causes of death represented in histogram format (Fig. 1) are linked mostly to large mammary tumors in females, and other organic problems in males.

3.2. Anatomopathological observations

All rats were carefully monitored for behavior, appearance, palpable tumors, infections, during the experiment, and at least 10 organs per animal were weighted and up to 34 analyzed post mortem, at the macroscopic and/or microscopic levels (Table 1).

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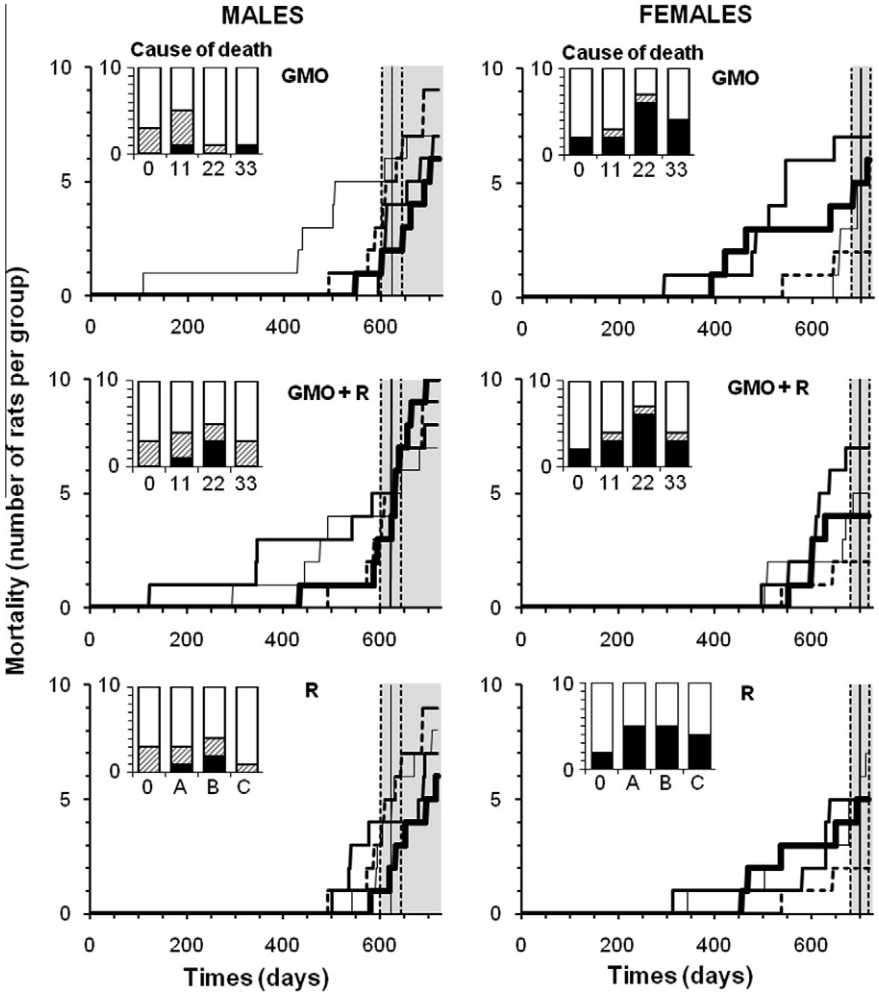


Fig. 1. Mortality of rats fed GMO treated or not with Roundup, and effects of Roundup alone. Rats were fed with NK603 GM maize (with or without application of Roundup) at three different doses (11, 22, 33% in their diet: thin, medium and bold lines, respectively) compared to the substantially equivalent closest isogenic non-GM maize (control, dotted line). Roundup was administrated in drinking water at 3 increasing doses, same symbols (environmental (A), MRL in agricultural GMOs (B) and half of minimal agricultural levels (C), see Section 2). Lifespan during the experiment for the control group is represented by the vertical bar ± SEM (grey area). In bar histograms, the causes of mortality before the grey area are detailed in comparison to the controls (0). In black are represented the necessary euthanasia because of suffering in accordance with ethical rules (tumors over 25% body weight, more than 25% weight loss, hemorrhagic bleeding, etc.); and in hatched areas, spontaneous mortality.

All data cannot be shown in one report, and the most relevant are described here. There was no rejection by the animals of the diet with or without GMOs, nor any major difference in the body weight.

The largest palpable growths (above a diameter of 17.5 mm in females and 20 mm in males) were found to be in 95% of cases non-regressive tumors, and were not infectious nodules. These growths progressively increased in size and number, but not proportionally to the treatment dose over the course of the experiment (Fig. 2). As in the case of rates of mortality, this suggests that a threshold in effect was reached at the lowest doses. They were rarely equal but almost always more frequent than in controls for all treated groups, often 2–3 times more in both sexes. Tumors began to reach a large size on average 94 days before in treated females, and up to 600 days earlier in 2 male groups eating the GM maize (11 and 22% with or without R).

In female animals, the largest tumors were in total 5 times more frequent than in males after 2 years, with 93% being mammary tumors. Adenomas, fibroadenomas and carcinomas were deleterious to health due to a very large size, rather than the grade of the tumor itself. Large tumor size caused impediments to either breathing or nutrition and digestion because of their thoracic or abdominal location and also resulted in hemorrhaging. In addition, one metastatic ovarian cystadenocarcinoma and two skin tumors were identified. Metastases were observed in only 2 cases; one in a group fed with 11% GM maize, and another in the highest dose of R treatment group.

Up to 14 months, no animals in the control groups showed any signs of tumors whilst 10–30% of treated females per group developed tumors, with the exception of one group (33% GMO + R). By the beginning of the 24th month, 50–80% of female animals had developed tumors in all treated groups, with up to 3 tumors per animal, whereas only 30% of controls were affected. The R treatment groups showed the greatest rates of tumor incidence with 80% of animals affected with up to 3 tumors for one female, in each group. A summary of all mammary tumors at the end of the experiment, independent of the size, is presented in Table 2. The same trend was observed in the groups receiving R in their drinking water; all females except one (with metastatic ovarian carcinoma) presented, in addition mammary hypertrophies and in some cases hyperplasia with atypia (Table 2).

The second most affected organ in females was the pituitary gland, in general around 2 times more than in controls for most treatments (Table 2). At this level again, adenomas and/or hyper-

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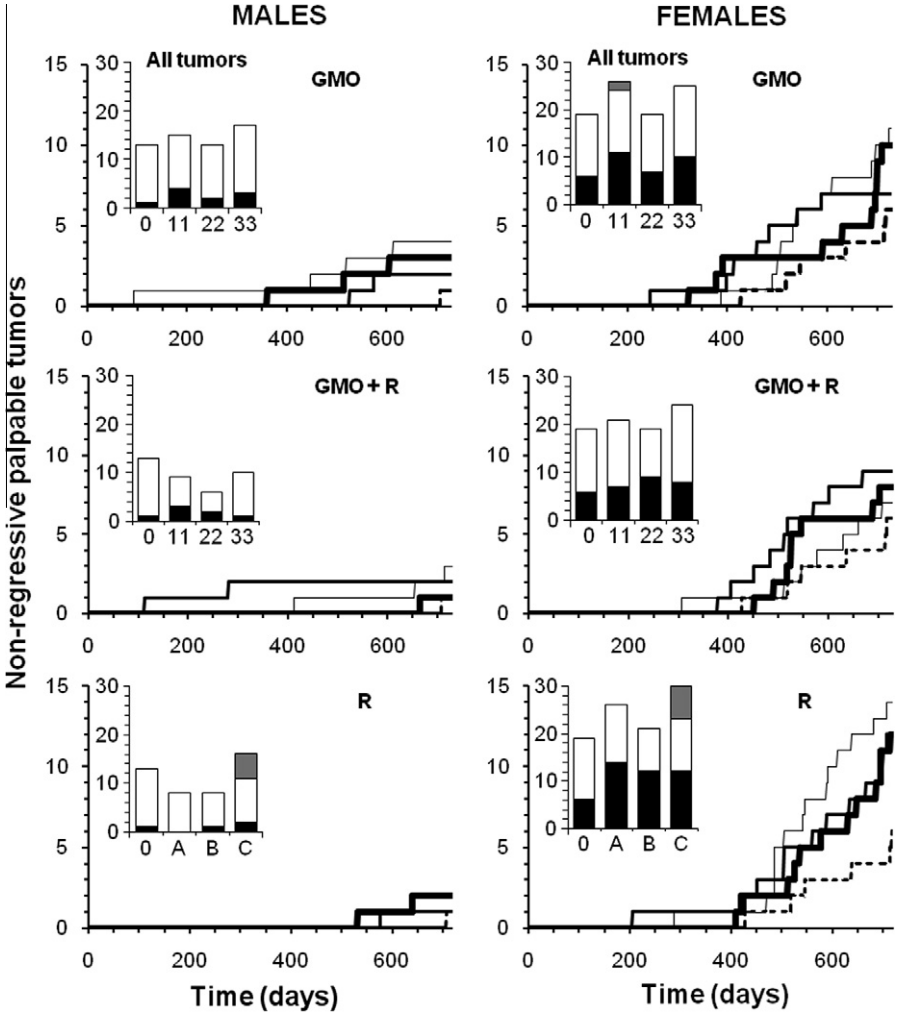


Fig. 2. Largest non-regressive tumors in rats fed GMO treated or not by Roundup, and effects of Roundup alone. The symbols of curves and treatments are explained in the caption of Fig. 1. The largest tumors were palpable during the experiment and numbered from 20 mm in diameter for males and 17.5 mm for females. Above this size, 95% of growths were non-regressive tumors. Summary of all tumors are shown in the bar histograms: black, non regressive largest tumors; white, small internal tumors; grey, metastases.

Table 2
Summary of the most frequent anatomical pathologies observed.

Organs and associated pathologies	Controls	GMO 11%	GMO 22%	GMO 33%	GMO 11% + R	GMO 22% + R	GMO 33% + R	R (A)	R (B)	R (C)
Males, in liver	2 (2)	5 (4)	11 (7)	8 (6)	5 (4)	7 (4)	6 (5)	11 (5)	9 (7)	6 (5)
In hepatodigestive tract	6 (5)	10 (6)	13 (7)	9 (6)	9 (6)	13 (6)	11 (7)	23 (9)	16 (8)	9 (5)
Kidneys, CPN	3 (3)	4 (4)	5 (5)	7 (7)	5 (5)	4 (4)	4 (4)	6 (6)	5 (5)	3 (3)
Females, mammary tumors	8 (5)	15 (7)	10 (7)	15 (8)	10 (6)	11 (7)	13 (9)	20 (9)	16 (10)	12 (9)
In mammary glands	10 (5)	22 (8)	10 (7)	16 (8)	17 (8)	16 (8)	15 (9)	26 (10)	20 (10)	18 (9)
Pituitary	9 (6)	23 (9)	20 (8)	8 (5)	19 (9)	9 (4)	19 (7)	22 (8)	16 (7)	13 (7)

After the number of pathological abnormalities, the number of rats reached is indicated in parentheses. In male animals pathological signs are liver congestions, macroscopic spots and microscopic necrotic foci. Hepatodigestive pathological signs concern the liver, stomach and small intestine (duodenum, ileum or jejunum). Only marked or severe chronic progressive nephropathies (CPN) are listed, excluding two nephroblastomas in groups consuming GMO 11% and GMO 22% + Roundup. In females, mammary fibroadenomas and adenocarcinomas are the major tumors detected; galactoceles and hyperplasias with atypia are also found and added in mammary glands pathological signs. Pituitary dysfunctions include adenomas, hyperplasias and hypertrophies. For details of the various treatment groups see Fig. 1.

plasias and hypertrophies were noticed. For all R treatment groups, 70–80% of animals presented 1.4–2.4 times more abnormalities than controls in this gland.

The big palpable tumors in males (in kidney, and mostly skin) were by the end of the experimental period on average twice as frequent as in controls, in which one skin fibroma appeared during the 23rd month. At the end of the experiment, internal non-palpable tumors were added, and their sums were lower in males than in females. They were not really different from controls, although slightly above in females (Histograms Fig. 2).

The most affected organs in males were the liver, together with the hepatodigestive tract and kidneys (Table 2 and Fig. 3). Hepatic congestions, macroscopic and microscopic necrotic foci were 2.5–5.5 times more frequent in all treatments than in control groups. Gamma GT hepatic activity was increased in particular for GMO + R groups (up to 5.4 times), this being probably due to a liver disorder.

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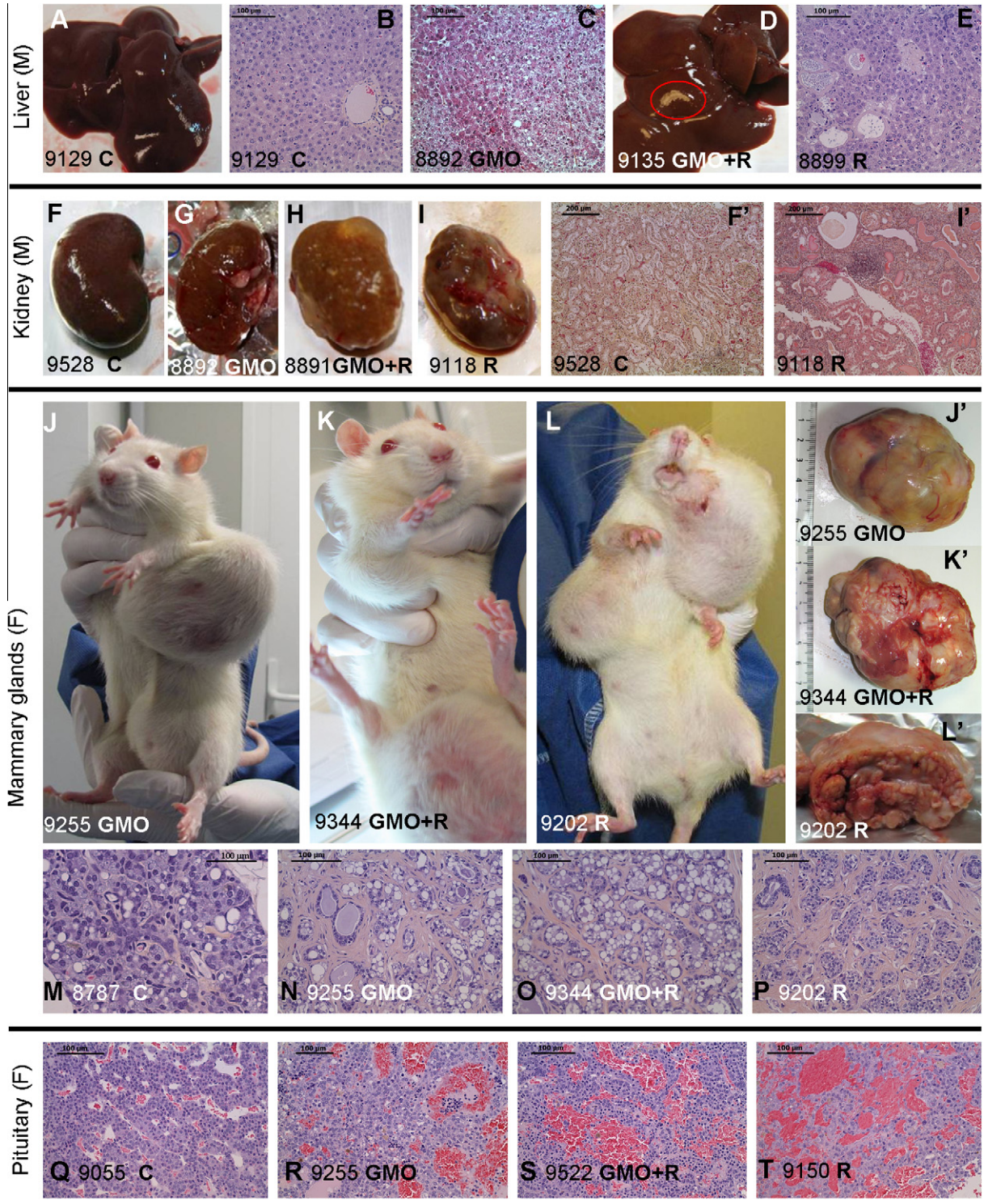


Fig. 3. Anatomopathological observations in rats fed GMO treated or not by Roundup, and effects of Roundup alone. Macroscopic and microscopic photographs show male livers (A–E) and left kidneys (F–I′), female mammary glands (J–P) and pituitaries (Q–T), according to Table 2. The number of each animal and its treatment is specified. Macroscopic pale spots (D) and microscopic necrotic foci in liver (C clear-cell focus, E basophilic focus with atypia), and marked or severe chronic progressive nephropathies, are illustrated. In females, mammary tumors (J,J′,N adenocarcinoma and K,K′,L,L′,O,P fibroadenomas) and pituitary adenomas (R–T) are shown and compared to controls (C after the rat number).

In addition, cytochrome activities also generally increased in the presence of R (in drinking water or GM diet) according to the dose up to 5.7 times at the highest dose. Transmission electron microscopic observations of liver samples confirmed changes for all treated groups in relation to glycogen dispersion or appearance in lakes, increase of residual bodies and enlargement of cristae in mitochondria (Fig. 4). The GM maize fed groups either with or without R application (in plants) showed a reduced transcription in mRNA and rRNA because of higher heterochromatin content, and decreased nucleolar dense fibrillar components. In the GMO + R group (at the highest dose) the smooth endoplasmic reticulum was drastically increased and nucleoli decreased in size,

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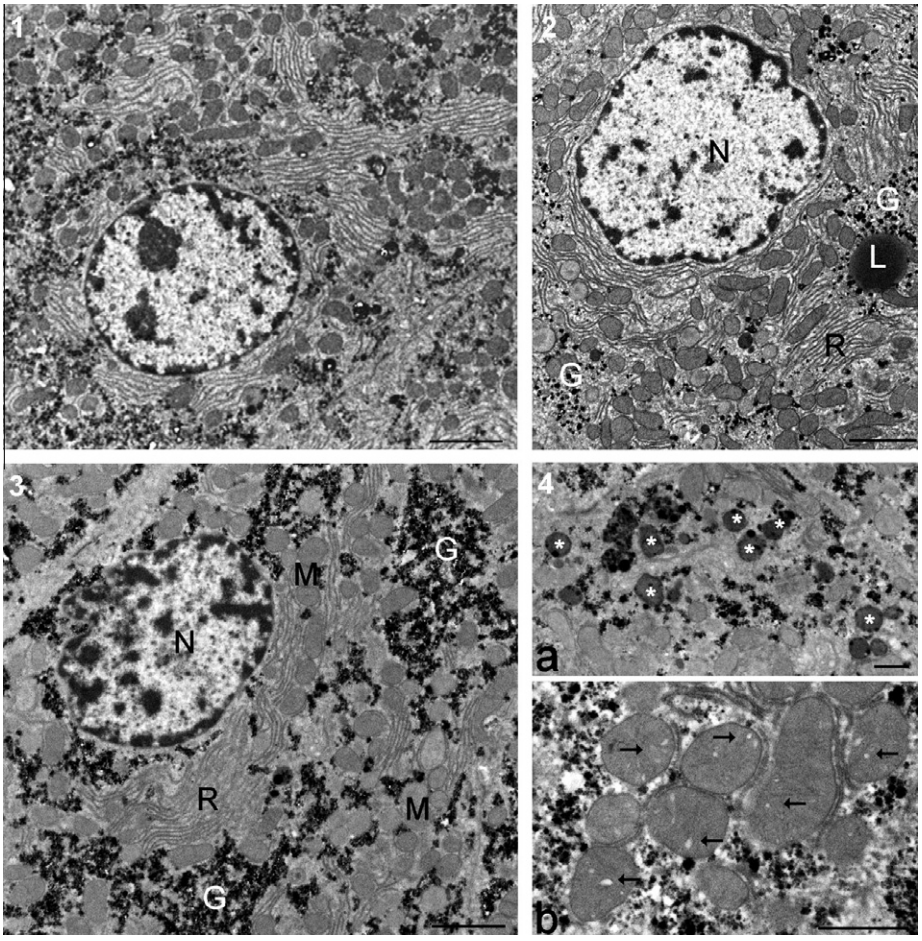


Fig. 4. Ultrastructure of hepatocytes in male rats from groups presenting the greatest degree of liver pathology. (1) Typical control rat hepatocyte (Bar 2 μm except in 4). (2) Effects with Roundup at the lowest dose. Glycogen (G) is dispersed in the cytoplasm. L, lipid droplet; N, nucleus; R rough endoplasmic reticulum. (3) Hepatocytes of animal fed GM maize (GMO) at 22% of total diet. Large lakes of glycogen occur in the cytoplasm. M, mitochondria. (4) Details of treatment effects with 22% dietary GMO (Bar 1 μm). (a) Cluster of residual bodies (asterisks). (b) Mitochondria show many enlarged cristae (arrows).

becoming more compact. For R treatment alone similar trends were observed, with a partial resumption of nucleolar activity at the highest dose.

Degenerating kidneys with turgid inflammatory areas demonstrate the increased incidence of marked and severe chronic progressive nephropathies, which were up to 2-fold higher in the 33% GM maize or lowest dose R treatment groups (Table 2 and Fig. 3).

3.3. Biochemical analyses

For the different corns and diets, the study of the standard chemical composition revealed no particular difference; this is why they were classified as substantially equivalent, except for transgene DNA quantification. For instance, there was no difference between total isoflavones. In addition, other specific compounds not always requested for substantial equivalence establishment were assayed. Among phenolic acids, the only consistent and significant ($p < 0.01$) results concerned ferulic acid that was decreased in both GM and GM + R diets by 16–30% in comparison to the control diet (889 ± 107, 735 ± 89 respectively vs control 1057 ± 127 mg/kg) and caffeic acid by 21–53% (17.5 ± 2.1, 10.3 ± 1.3 vs control 22.1 ± 2.6 mg/kg).

For biochemical measurements in rats, statistical analysis was performed on the results obtained from samples taken at the 15th month time point, as this was the last sampling time when most animals were still alive (in treated groups 90% males, 94% females, and 100% controls). OPLS-DA 2-class models were built between each treated group per sex and controls. Only models with an explained variance $R^2(Y) \geqslant 80\%$, and a cross-validated predictive ability $Q^2(Y) \geqslant 60\%$, were used for selection of the discriminant variables (Fig. 5A), when their regression coefficients were significant at 99% confidence level. Thus, in treated females, kidney failures appeared at the biochemical level (82% of the total disrupted parameters). Ions (Na, Cl) or urea increased in urine. Accordingly, the same ions decreased in serum (Fig. 5B) as did the levels of P, K and Ca. Creatinine or clairance decreased in urine for all treatment groups in comparison to female controls (Table 3). In GM maize treated males (with or without R), 87% of discriminant variables were kidney related, but the disrupted profiles were less obvious because of advanced chronic nephropathies and deaths. In summary, for all treatments and both sexes, 76% of the discriminant variables versus controls were kidney related.

Moreover, in females (Table 3) the androgen/estrogen balance in serum was modified by GM maize and R treatments (at least 95% confidence level, Fig. 5B), and for male animals at the highest R-treatment dose, levels of estrogens were more than doubled.

4. Discussion

This report describes the first life-long rodent (rat) feeding study investigating possible toxic effects rising from an R-tolerant

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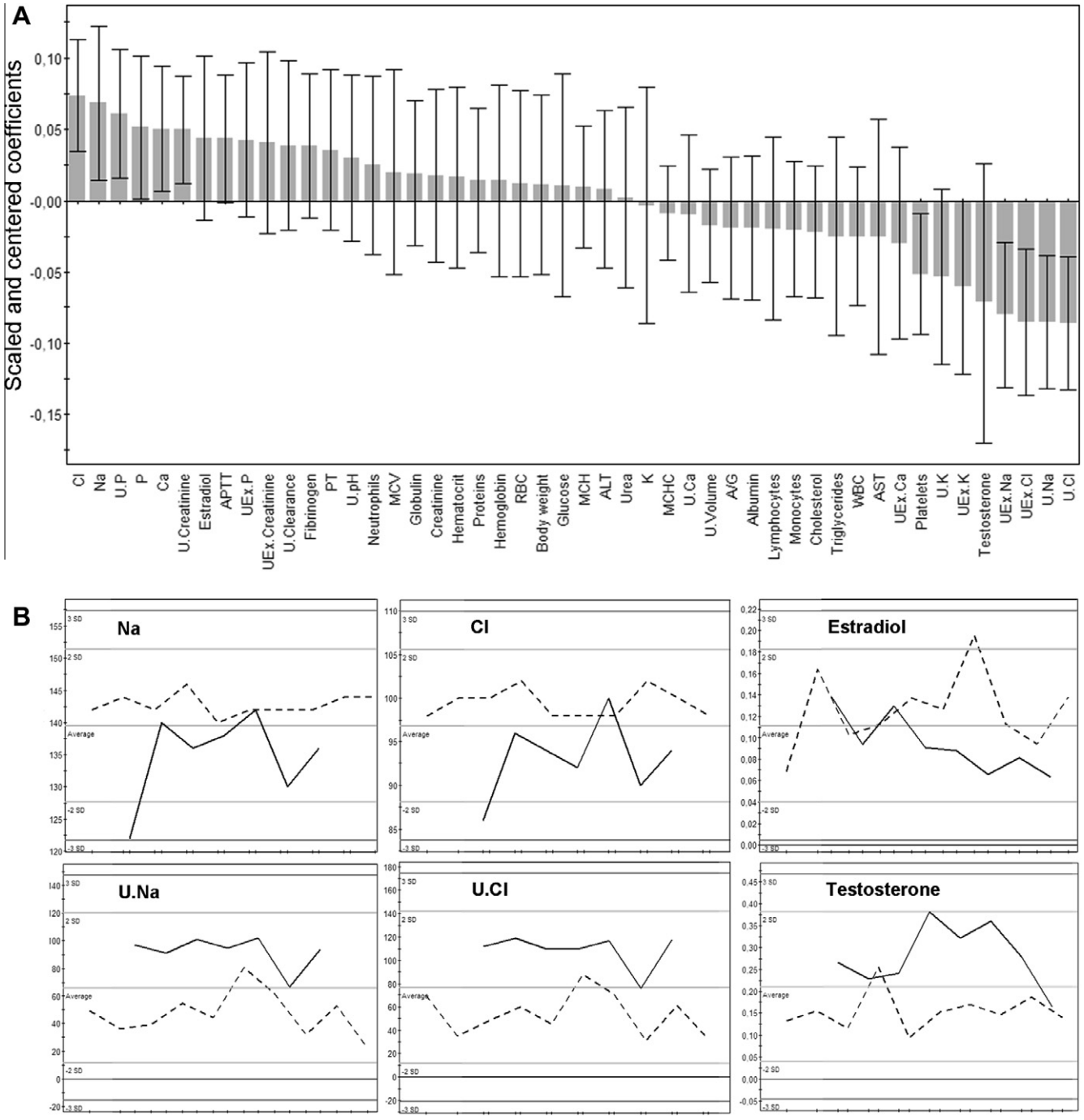


Fig. 5. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) for biochemical data (females fed 33% GMO versus controls). (A) OPLS-DA regression coefficients for predictive component, with jack-knifed confidence intervals at 99% confidence level, indicate discriminant parameters versus controls at month 15 (Abbreviations: U Urinary, UEx Excreted in urine during 24 h, APPT Activated Partial Thromboplastin Time, MCV Mean Corpuscular Volume, PT Prothrombine Time, RBC Red Blood Cells, ALT ALanine aminoTransferase, MCHC Mean Corpuscular Hemoglobin Concentration, A/G Albumin/Globulin ratio, WBC White Blood Cells, AST aspartate aminotransferase). (B) In this case, detailed examples of significant discriminant variables distribution between females fed 33% GMO (bold line) and controls (dotted line). On *x* axis: animals; on *y* axis: serum or urine biochemical values for Na, Cl, estradiol, testosterone. Profiles evidence kidney ion leakages and sex hormonal imbalance versus controls.

GM maize (NK603) and a complete commercial formulation of R-herbicide.

Our data show that, as is often the case for hormonal diseases, most observed effects in this study were not proportional to the dose of the treatment (GM maize with and without R application; R alone), non-monotonic and with a threshold effect (Vandenberg et al., 2012). Similar degrees of pathological symptoms were noticed in this study to occur from the lowest to the highest doses suggesting a threshold effect. This corresponds to levels likely to arise from consumption or environmental exposure, such as either 11% GM maize in food, or 50 ng/L of glyphosate in R-formulation as can be found in some contaminated drinking tap waters, and which fall within authorized limits.

The lifespan of the control group of animals corresponded to the mean rat lifespan, but as is frequently the case with most mammals including humans (WHO, 2012), males on average died before females, except for some female treatment groups. All treatments in both sexes enhanced large tumor incidence by 2–3-fold in com-

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Table 3
Percentage variation of parameters indicating kidney failures of female animals.

Discriminant variables		GMO 11% + R	GMO 22% + R	GMO 33% + R	GMO 11%	GMO 22%	GMO 33%	R (A)	R (B)	R (C)
Urinary decrease	Clairance	−4	−11	−20	**−20**	**−20**	−19	**−20**	**−24**	**−40**
	Creatinine	−5	**−32**	**−37**	−19	−37	**−36**	−43	−23	−1
	Creatinine ex	−5	−11	**−19**	−18	**−17**	−21	**−21**	**−22**	**−39**
Urinary increase	Urea	12	**18**	15	15	12	−1	0	13	**32**
	Na	25	33	30	52	−2	**95**	**62**	65	**91**
	Na ex	24	50	68	50	24	**125**	**108**	51	7
	Cl	14	35	28	46	5	**101**	**67**	56	**94**
	Cl ex	20	63	70	51	31	**138**	**121**	48	13
Serum decrease	Na	2	1	1	−1	**−4**	**−6**	−7	0	−3
	Cl	−1	−2	−2	−5	−7	**−6**	**−8**	−1	−4
	P	−6	−11	−13	−17	**−18**	**−20**	**−32**	−9	−13
	K	4	5	10	2	−4	0	−4	8	**−5**
	Ca	4	3	3	**2**	−2	**−5**	−6	3	**−6**
Gonads	Estradiol	8	−1	2	5	−2	−25	−26	**−73**	39
	Testosterone	5	−9	27	**56**	17	81	**97**	**−72**	10

OPLS-DA was performed on 48 variables at month 15. Here we showed mean differences (%) of variables (discriminant at 99% confidence level, in bold character) indicating kidney parameters of female animals, together with sex hormones. Male kidney pathologies are already illustrated in Table 2.

parison to our controls but also for the number of mammary tumors in comparison to the same Harlan Sprague Dawley strain (Brix et al., 2005), and overall around 3-fold in comparison to the largest study with 1329 Sprague Dawley female rats (Chandra et al., 1992). In our study the tumors also developed considerably faster than the controls, even though the majority of tumors were observed after 18 months. The first large detectable tumors occurred at 4 and 7 months into the study in males and females respectively, underlining the inadequacy of the standard 90 day feeding trials for evaluating GM crop and food toxicity (Séralini et al., 2011).

Suffering inducing euthanasia and deaths corresponded mostly in females to the development of large mammary tumors. These appeared to be clearly related to the various treatments when compared to the control groups. These tumors are generally known to be mostly estrogen-dependent (Harvell et al., 2000). We observed a strikingly marked induction of mammary tumors by R alone, a major formulated pesticide, even at the very lowest dose administered. R has been shown to disrupt aromatase which synthesizes estrogens (Richard et al., 2005), but to also interfere with estrogen and androgen receptors in cells (Gasnier et al., 2009). In addition, R appears to be a sex endocrine disruptor *in vivo*, also in males (Romano et al., 2010). Sex steroids are also modified in treated rats. These hormone-dependent phenomena are confirmed by enhanced pituitary dysfunction in treated females. An estrogen modified feedback mechanism may act at this level (Popovics et al., 2011; Walf and Frye, 2010). The similar pathological profiles provoked by the GM maize containing R residues may thus be explained at least by R residues themselves, knowing that the medium dose of the R treatment corresponds to acceptable levels of this pesticide residues in GMOs.

Interestingly, in the groups of animals fed with the NK603 without R application, similar effects with respect to enhanced tumor incidence and mortality rates were observed. A possible explanation for this finding is the production of specific compound(s) in the GM feed that are either directly toxic and/or cause the inhibition of pathways that in turn generate chronic toxic effects. This is despite the fact that the variety of GM maize used is this study was judged by industry and regulators as being substantially equivalent to the corresponding non-GM closest isogenic line. As the total chemical composition of the GM maize cannot be measured in details, the use of substantial equivalence is insufficient to highlight potential unknown toxins and therefore cannot replace long-term animal feeding trials for GMOs. A cause of the effects of the effects could be that the NK603 GM maize used in this study is engineered to overexpress a modified version of the *Agrobacterium tumefaciens* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Hammond et al., 2004) allowing the R tolerance. The modified EPSPS is not inhibited by glyphosate by contrast to the wild enzyme. This enzyme is known to drive the first step of aromatic amino acid biosynthesis in the plant shikimate pathway; in addition estrogenic isoflavones and their glycosides are also products of this pathway (Duke et al., 2003). They were not disturbed in our study. By contrast, the levels of caffeic and ferulic acids in the GM diets, which are also secondary metabolites from this pathway, but not always measured in regulatory tests, are significantly reduced. This may lower their protective effects against carcinogenesis and even mammalian tumors (Kuenzig et al., 1984; Baskaran et al., 2010). Moreover, these phenolic acids and in particular ferulic acid may modulate estrogen receptors or the estrogenic pathway in mammalian cells (Chang et al., 2006). This does not exclude the action of other unknown metabolites. This explanation also corresponds to the fact that the observed effects of NK603 and R are not additive and reached a threshold. This implies that both the NK603 maize and R may cause hormonal disturbances in the same biochemical and physiological pathway.

As expected, mammary tumors in males occurred far less frequently than in females. Death in male rats was mostly due to the development of severe hepatorenal insufficiencies, confirming the first signs of toxicity observed in 90 day feeding trials with NK603 maize (Spiroux de Vendômois et al., 2009). In females, kidney ion leakages were evidenced at the biochemical levels at month 15, when severe nephropathies were evidenced in dead male animals afterwards, at the anatomopathological level. Early signs of toxicity at month 3 in kidney and liver were also observed for 19 edible GM crops containing pesticide residues (Séralini et al., 2011). As a matter of fact, only elderly male rats are sensitive to chronic progressive nephropathies (Hard and Khan, 2004). The disturbed kidney parameters may have been induced by the reduction of phenolic acids in our study, since caffeic and ferulic acids are beneficial in the kidney as they prevent oxidative stress (Srinivasan et al., 2005; U Rehman and Sultana, 2011). Accordingly, we previously demonstrated that plant extracts containing ferulic and caffeic acids were able to promote detoxification of embryonic kidney cells after R contamination (Gasnier et al., 2011). It is thus possible that NK603 consumption by reducing these compounds may well provoke an early aging of kidney physiology in this study, like R by oxidative stress.

Disturbances that we found to occur in the male liver are characteristic of a chronic intoxication, confirmed by alterations

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in biochemical liver and kidney function parameters. The observation that liver function in female animals is less affected may be due to their physiology being better adapted to estrogen metabolism. Furthermore, liver enzymes have been clearly demonstrated as sex-specific in their expression patterns, including in a 90-day rat feeding trial of NK603 maize (Spiroux de Vendômois et al., 2009). However, in a long-term study, evidence of early liver aging was observed in female mice fed with R-tolerant GM soy (Malatesta et al., 2008a). In the present investigation, deeper analysis at an ultrastructural level revealed evidence of impediments in transcription and other defects in cell nuclear structure that were comparable in both sexes, and dose-dependent in hepatocytes in all treatments. This is consistent with the well-documented toxic effect of very low dilutions of R on apoptosis, mitochondrial function, and cell membrane degradation inducing necrosis of hepatocytes, and other cell lines (Benachour and Seralini, 2009; Benachour et al., 2007; Gasnier et al., 2010; Peixoto, 2005).

The disruptions of at least the estrogen-related pathways and/or enhancement of oxidative stress by all treatments need further investigations. This can be addressed through the application of transcriptomic, proteomic and metabolomic methods to analyze the molecular profiles of kidneys and livers, as well as the GM NK603 maize (Jiao et al., 2010; Zhou et al., 2009; Zolla et al., 2008). Other possible causes of observed pathogenic effects may be due to disturbed gene expression resulting from the transgene insertional, general mutagenic or metabolic effects (Latham et al., 2006; Wilson et al., 2006) as has been shown for MON810 GM maize (Rosati et al., 2008). A consequent disruption of general metabolism in the GMO cannot be excluded, which could lead, for example, to the production of other potentially active compounds such as miRNAs (Zhang et al., 2012) or leukotoxin diols (Markaverich et al., 2005).

In conclusion, it was previously known that glyphosate consumption in water above authorized limits may provoke hepatic and kidney failures (EPA). The results of the study presented here clearly demonstrate that lower levels of complete agricultural glyphosate herbicide formulations, at concentrations well below officially set safety limits, induce severe hormone-dependent mammary, hepatic and kidney disturbances. Similarly, disruption of biosynthetic pathways that may result from overexpression of the EPSPS transgene in the GM NK603 maize can give rise to comparable pathologies that may be linked to abnormal or unbalanced phenolic acids metabolites, or related compounds. Other mutagenic and metabolic effects of the edible GMO cannot be excluded. This will be the subject of future studies, including transgene and glyphosate presence in rat tissues. Reproductive and multigenerational studies will also provide novel insights into these problems. This study represents the first detailed documentation of long-term deleterious effects arising from the consumption of a GM R-tolerant maize and of R, the most used herbicide worldwide.

Altogether, the significant biochemical disturbances and physiological failures documented in this work confirm the pathological effects of these GMO and R treatments in both sexes, with different amplitudes. We propose that agricultural edible GMOs and formulated pesticides must be evaluated very carefully by long term studies to measure their potential toxic effects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Baskaran, N., Manoharan, S., Balakrishnan, S., Pugalendhi, P., 2010. Chemopreventive potential of ferulic acid in 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in Sprague-Dawley rats. Eur. J. Pharmacol. 637, 22–29.

Benachour, N., Seralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Seralini, G.E., 2007. Time- and dose-dependent effects of Roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53, 126–133.

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

Chandra, M., Riley, M.G., Johnson, D.E., 1992. Spontaneous neoplasms in aged Sprague-Dawley rats. Arch. Toxicol. 66, 496–502.

Chang, C.J., Chiu, J.H., Tseng, L.M., Chang, C.H., Chien, T.M., Wu, C.W., Lui, W.Y., 2006. Modulation of HER2 expression by ferulic acid on human breast cancer MCF7 cells. Eur. J. Clin. Invest. 36, 588–596.

Cox, C., 2004. Herbicide factsheet – Glyphosate. J. pestic. reform 24, 10–15.

Cox, C., Surgan, M., 2006. Unidentified inert ingredients in pesticides: implications for human and environmental health. Environ. Health Perspect. 114, 1803–1806.

Domingo, J.L., Giné Bordonaba, J., 2011. A literature review on the safety assessment of genetically modified plants. Environ. Int. 37, 734–742.

Duke, S.O., Rimando, A.M., Pace, P.F., Reddy, K.N., Smeda, R.J., 2003. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. J. Agric. Food. Chem. 51, 340–344.

EPA, 2012. Basic Information about Glyphosate in Drinking Water. <http://water.epa.gov/drink/contaminants/basicinformation/glyphosate.cfm> (Last access June).

Eriksson, L., Johansson, E., Kettaneh-Wold, N., Trygg, J., Wikström, C., Wold, S., 2006a. Multi- and Megavariate Data Analysis Part II Advanced Applications and Method Extensions. Umetrics, Umea, Sweden.

Eriksson, L., Johansson, E., kettaneh-Wold, N., Wold, S., 2006b. Multi and Megavariate Data Analysis Part I – Principles and Applications. Umetrics AB, Umea, Sweden.

Gasnier, C., Benachour, N., Clair, E., Travert, C., Langlois, F., Laurant, C., Decroix-Laporte, C., Séralini, G.-E., 2010. Dig1 protects against cell death provoked by glyphosate-based herbicides in human liver cell lines. J. Occup. Med. Toxicol. 5, 29.

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Seralini, G.E., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262, 184–191.

Gasnier, C., Laurant, C., Decroix-Laporte, C., Mesnage, R., Clair, E., Travert, C., Séralini, G.E., 2011. Defined plant extracts can protect human cells against combined xenobiotic effects. J. Occup. Med. Toxicol. 6, 3.

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

Hammond, B., Lemen, J., Dudek, R., Ward, D., Jiang, C., Nemeth, M., Burns, J., 2006a. Results of a 90 day safety assurance study with rats fed grain from corn rootworm-protected corn. Food Chem. Toxicol. 44, 147–160.

Hammond, B.G., Dudek, R., Lemen, J.K., Nemeth, M.A., 2006b. Results of a 90 day safety assurance study with rats fed grain from corn borer-protected corn. Food Chem. Toxicol. 44, 1092–1099.

Hard, G.C., Khan, K.N., 2004. A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol. Pathol. 32, 171–180.

Harvell, D.M., Strecker, T.E., Tochacek, M., Xie, B., Pennington, K.L., McComb, R.D., Roy, S.K., Shull, J.D., 2000. Rat strain-specific actions of 17beta-estradiol in the mammary gland: correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. Proc. Natl. Acad. Sci. USA 97, 2779–2784.

Jiao, Z., Si, X.X., Li, G.K., Zhang, Z.M., Xu, X.P., 2010. Unintended compositional changes in transgenic rice seeds (*Oryza sativa* L.) studied by spectral and chromatographic analysis coupled with chemometrics methods. J. Agric. Food. Chem. 58, 1746–1754.

Krogh, K.A., Vejrup, K.V., Mogensen, B.B., Halling-Sørensen, B., 2002. Liquid chromatography-mass spectrometry method to determine alcohol ethoxylates and alkylamine ethoxylates in soil interstitial water, ground water and surface water samples. J. Chromatogr. A 957, 45–57.

Kuenzig, W., Chau, J., Norkus, E., Holowaschenko, H., Newmark, H., Mergens, W., Conney, A.H., 1984. Caffeic and ferulic acid as blockers of nitrosamine formation. Carcinogenesis 5, 309–313.

Latham, J.R., Wilson, A.K., Steinbrecher, R.A., 2006. The mutational consequences of plant transformation. J. Biomed. Biotechnol. 2006, 25376.

Malatesta, M., Boraldi, F., Annovi, G., Baldelli, B., Battistelli, S., Biggiogera, M., Quaglino, D., 2008a. A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing. Histochem. Cell Biol. 130, 967–977.

Malatesta, M., Caporaloni, C., Gavaudan, S., Rocchi, M.B., Serafini, S., Tiberi, C., Gazzanelli, G., 2002a. Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. Cell Struct. Funct. 27, 173–180.

Malatesta, M., Caporaloni, C., Rossi, L., Battistelli, S., Rocchi, M.B., Tonucci, F., Gazzanelli, G., 2002b. Ultrastructural analysis of pancreatic acinar cells from mice fed on genetically modified soybean. J. Anat. 201, 409–415.

Malatesta, M., Perdoni, F., Santin, G., Battistelli, S., Muller, S., Biggiogera, M., 2008b. Hepatoma tissue culture (HTC) cells as a model for investigating the effects of low concentrations of herbicide on cell structure and function. Toxicol. In Vitro 22, 1853–1860.

Markaverich, B.M., Crowley, J.R., Alejandro, M.A., Shoulars, K., Casajuna, N., Mani, S., Reyna, A., Sharp, J., 2005. Leukotoxin diols from ground corncob bedding disrupt estrous cyclicity in rats and stimulate MCF-7 breast cancer cell proliferation. Environ. Health Perspect. 113, 1698–1704.

Mesnage, R., Clair, E., Séralini, G.-E., 2010. Roundup in Genetically modified crops: Regulation and toxicity in mammals. Theorie in der Ökologie 16, 31–33.

Monosson, E., 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. Environ. Health Perspect. 113, 383–390.

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122.

Popovics, P., Rekasi, Z., Stewart, A.J., Kovacs, M., 2011. Regulation of pituitary inhibin/activin subunits and follistatin gene expression by GnRH in female rats. J. Endocrinol. 210, 71–79.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.

Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., de Souza, P.B., Viau, P., Bernardi, M.M., Nunes, M.T., de Oliveira, C.A., 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch. Toxicol. 86, 663–673.

Romano, R.M., Romano, M.A., Bernardi, M.M., Furtado, P.V., Oliveira, C.A., 2010. Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch. Toxicol. 84, 309–317.

Rosati, A., Bogani, P., Santarlasci, A., Buiatti, M., 2008. Characterisation of 3′ transgene insertion site and derived mRNAs in MON810 YieldGard maize. Plant Mol. Biol. 67, 271–281.

Séralini, G.-E., Cellier, D., de Vendomois, J.S., 2007. New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch. Environ. Contam. Toxicol. 52, 596–602.

Séralini, G.-E., Mesnage, R., Clair, E., Gress, S., Spiroux De Vendomois, J., Cellier, D., 2011. Genetically modified crops safety assessments: present limits and possible improvements. Environ. Sci. Eur., 23.

Séralini, G.E., Spiroux de Vendomois, J., Cellier, D., Sultan, C., Buiatti, M., Gallagher, L., Antoniou, M., Dronamraju, K.R., 2009. How subchronic and chronic health effects can be neglected for GMOs, pesticides or chemicals. Int. J. Biol. Sci. 5, 438–443.

Snell, C., Bernheim, A., Bergé, J.-B., Kuntz, M., Pascal, G., Paris, A., Ricroch, A.E., 2011. Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: a literature review. Food Chem. Toxicol. 50, 1134–1148.

Spiroux de Vendômois, J., Cellier, D., Velot, C., Clair, E., Mesnage, R., Seralini, G.E., 2010. Debate on GMOs health risks after statistical findings in regulatory tests. Int. J. Biol. Sci. 6, 590–598.

Spiroux de Vendômois, J., Roullier, F., Cellier, D., Seralini, G.E., 2009. A comparison of the effects of three GM corn varieties on mammalian health. Int. J. Biol. Sci. 5, 706–726.

Srinivasan, M., Rukkumani, R., Ram Sudheer, A., Menon, V.P., 2005. Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity. Fundam. Clin. Pharmacol. 19, 491–496.

U Rehman, M., Sultana, S., 2011. Attenuation of oxidative stress, inflammation and early markers of tumor promotion by caffeic acid in Fe-NTA exposed kidneys of Wistar rats. Mol. Cell. Biochem. 357, 115–124.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., Shioda, T., Soto, A.M., Vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.

Walf, A.A., Frye, C.A., 2010. Raloxifene and/or estradiol decrease anxiety-like and depressive-like behavior, whereas only estradiol increases carcinogen-induced tumorigenesis and uterine proliferation among ovariectomized rats. Behav. Pharmacol. 21, 231–240.

Weljie, A.M., Bondareva, A., Zang, P., Jirik, F.R., 2011. ^{1}H NMR metabolomics identification of markers of hypoxia-induced metabolic shifts in a breast cancer model system. J. Biomol. NMR 49, 185–193.

WHO, 2012. World Health Statistics. WHO Press. <http://who.int> (Last access August).

Wiklund, S., Johansson, E., Sjostrom, L., Mellerowicz, E.J., Edlund, U., Shockcor, J.P., Gottfries, J., Moritz, T., Trygg, J., 2008. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal. Chem. 80, 115–122.

Williams, G.M., Kroes, R., Munro, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31, 117–165.

Wilson, A.K., Latham, J.R., Steinbrecher, R.A., 2006. Transformation-induced mutations in transgenic plants: analysis and biosafety implications. Biotechnol. Genet. Eng. Rev. 23, 209–237.

Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J., Bian, Z., Liang, X., Cai, X., Yin, Y., Wang, C., Zhang, T., Zhu, D., Zhang, D., Xu, J., Chen, Q., Ba, Y., Liu, J., Wang, Q., Chen, J., Wang, J., Wang, M., Zhang, Q., Zhang, J., Zen, K., Zhang, C.Y., 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res. 22, 107–126.

Zhou, J., Ma, C., Xu, H., Yuan, K., Lu, X., Zhu, Z., Wu, Y., Xu, G., 2009. Metabolic profiling of transgenic rice with cryIAc and sck genes: an evaluation of unintended effects at metabolic level by using GC-FID and GC-MS. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 877, 725–732.

Zolla, L., Rinalducci, S., Antonioli, P., Righetti, P.G., 2008. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. J. Proteome Res. 7, 1850–1861.

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