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

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\begin{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 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 necroses 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.
\end{abstract}

\section{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 metabolical analyses (Séràlini 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 (Séràlini 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 (Séràlini 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 Séràlini, 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).
Since then, long-term and multi-generational animal feeding trials have been performed with some 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 Sargun, 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 dilute 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 (Weather-MAX, 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

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

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.

Please cite this article in press as: Séralini, G.-E., et al. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem. Toxicol. (2012), http://dx.doi.org/10.1016/j.fct.2012.08.005
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 high-performance liquid chromatography (HPLC). All reagents used were of analytical grade. The herbicide dilute 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 cm², Genestil, France) with two animals of the same sex per cage. The litter (Tuplit classic, Safe, France) was replaced twice weekly. The animals were maintained at 22 ± 1 °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 CQL environment according to OECD guidelines. After 20 days of acclimatization, 100 male and 100 female rats per group 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 × 10⁻³ % of R (0.1 g phr of R or 50 mg/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 isoflu- ranesthesia 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 cholesterol, triglycerides, glucose, total cholesterol, alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase (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 59 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, hemorrhetic 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, stomach, thyroid, stomach, thymus and thyroid. 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/carboxic 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 HE histological staining. For transmission electron microscopy, kidneys, livers and tumors were cut into 1 mm³ 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, Umeå, 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 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 cov2(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 (Wolff 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 plane 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 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).
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-
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.

Table 2

Summary of the most frequent anatomical pathologies observed.

<table>
<thead>
<tr>
<th>Organs and associated pathologies</th>
<th>Controls</th>
<th>GMO 11%</th>
<th>GMO 22%</th>
<th>GMO 33%</th>
<th>GMO 11% + R</th>
<th>GMO 22% + R</th>
<th>GMO 33% + R</th>
<th>R (A)</th>
<th>R (B)</th>
<th>R (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males, in liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in hepatodigestive tract</td>
<td>2 (2)</td>
<td>5 (4)</td>
<td>11 (7)</td>
<td>8 (6)</td>
<td>5 (4)</td>
<td>7 (4)</td>
<td>6 (5)</td>
<td>11 (5)</td>
<td>9 (7)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Kidneys, CPN</td>
<td>6 (5)</td>
<td>10 (6)</td>
<td>13 (7)</td>
<td>9 (6)</td>
<td>9 (6)</td>
<td>13 (6)</td>
<td>11 (7)</td>
<td>16 (8)</td>
<td>9 (5)</td>
<td></td>
</tr>
<tr>
<td>Females, mammary tumors</td>
<td>3 (3)</td>
<td>4 (4)</td>
<td>5 (5)</td>
<td>7 (7)</td>
<td>5 (5)</td>
<td>4 (4)</td>
<td>4 (4)</td>
<td>6 (6)</td>
<td>5 (5)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>in mammary glands</td>
<td>8 (5)</td>
<td>15 (7)</td>
<td>10 (7)</td>
<td>15 (8)</td>
<td>10 (6)</td>
<td>11 (7)</td>
<td>13 (9)</td>
<td>20 (9)</td>
<td>16 (10)</td>
<td>12 (9)</td>
</tr>
<tr>
<td>Pituitary</td>
<td>9 (6)</td>
<td>23 (9)</td>
<td>20 (8)</td>
<td>8 (5)</td>
<td>19 (9)</td>
<td>9 (4)</td>
<td>19 (7)</td>
<td>22 (8)</td>
<td>16 (7)</td>
<td>13 (7)</td>
</tr>
</tbody>
</table>

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.
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.
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 ($889 \pm 107, 735 \pm 89$ respectively vs control $1057 \pm 127$ mg/kg) and caffeic acid by 21–53% ($17.5 \pm 2.1, 10.3 \pm 1.3$ vs control $22.1 \pm 2.6$ 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 $R^2(Y) > 80\%$, and a cross-validated predictive ability $Q^2(Y) > 60\%$, 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
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-

![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.](image-url)
parison to our controls but also for the number of mammary tu-
mors 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 oc-
curred 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 (Seralini
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 ob-
erved 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 syn-
thesizes estrogens (Richard et al., 2005), but to also interfere with
estrogen and androgen receptors in cells (Gasnier et al., 2011).
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 pro-
voked by the GM maize containing R residues may thus be ex-
plained 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 with-
out R application, similar effects with respect to enhanced tumor
incidence and mortality rates were observed. A possible explana-
tion for this finding is the production of specific compound(s) in
the GM feed that are either directly toxic and/or cause the inhibi-
tion 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 de-
tails, 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) (Ham-
mond 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 bio-
synthesis 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 con-
trast, 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 mam-
malian 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 fre-
fently 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, kid-
ney 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 (Seralini
et al., 2011). As a matter of fact, only elderly male rats are sensitive to
chronic progressive nephropathies (Hard and Khan, 2004). The dis-
turbed 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 (Srivasan
et al., 2005; U Rehman and Sultana, 2011). Accordingly, we previ-
ously demonstrated that plant extracts containing caffeic and caf-
feic 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
provocate 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

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

<table>
<thead>
<tr>
<th>Discriminant variables</th>
<th>GMO 11% + R</th>
<th>GMO 22% + R</th>
<th>GMO 33% + R</th>
<th>GMO 11%</th>
<th>GMO 22%</th>
<th>GMO 33%</th>
<th>R (A)</th>
<th>R (B)</th>
<th>R (C)</th>
</tr>
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<tr>
<td>Urinary decrease</td>
<td>−2</td>
<td>−11</td>
<td>−20</td>
<td>−20</td>
<td>−20</td>
<td>−19</td>
<td>−20</td>
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<tr>
<td>Creatinine</td>
<td>−5</td>
<td>−32</td>
<td>−37</td>
<td>−19</td>
<td>−37</td>
<td>−36</td>
<td>−43</td>
<td>−23</td>
<td>−1</td>
</tr>
<tr>
<td>Creatinine ex</td>
<td>−5</td>
<td>−11</td>
<td>−19</td>
<td>−18</td>
<td>−17</td>
<td>−21</td>
<td>−21</td>
<td>−22</td>
<td>−39</td>
</tr>
<tr>
<td>Urea</td>
<td>12</td>
<td>18</td>
<td>15</td>
<td>15</td>
<td>12</td>
<td>−1</td>
<td>0</td>
<td>13</td>
<td>32</td>
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<tr>
<td>Na</td>
<td>25</td>
<td>33</td>
<td>30</td>
<td>52</td>
<td>24</td>
<td>95</td>
<td>62</td>
<td>65</td>
<td>91</td>
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<tr>
<td>Na ex</td>
<td>24</td>
<td>50</td>
<td>68</td>
<td>50</td>
<td>24</td>
<td>125</td>
<td>108</td>
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<td>Cl</td>
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<td>5</td>
<td>101</td>
<td>67</td>
<td>56</td>
<td>94</td>
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<tr>
<td>Cl ex</td>
<td>20</td>
<td>63</td>
<td>70</td>
<td>51</td>
<td>31</td>
<td>138</td>
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<td>Serum decrease</td>
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<td>−6</td>
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<tr>
<td>Cl</td>
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<td>K</td>
<td>4</td>
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<td>10</td>
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<td>−4</td>
<td>0</td>
<td>−4</td>
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<td>5</td>
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<tr>
<td>Ca</td>
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<td>3</td>
<td>3</td>
<td>2</td>
<td>−2</td>
<td>−5</td>
<td>−6</td>
<td>3</td>
<td>−6</td>
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<tr>
<td>Gonads</td>
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<td>−1</td>
<td>2</td>
<td>5</td>
<td>−2</td>
<td>−25</td>
<td>−26</td>
<td>−73</td>
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<tr>
<td></td>
<td>Testosterone</td>
<td>5</td>
<td>−9</td>
<td>27</td>
<td>56</td>
<td>17</td>
<td>81</td>
<td>97</td>
<td>72</td>
</tr>
</tbody>
</table>

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.
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 (Spiroix 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; Gascier 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 leukotriene 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 formulation, at concentrations well below official 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 acid 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.

Acknowledgments

We thank Michael Antoniou for English assistance and constructive comments on the manuscript, as well as Herrade Hemerdinger for proofreading. We gratefully acknowledge the Association CERES, the Foundation “Charles Leopold Mayer pour le Progrès de l’Homme”, the French Ministry of Research, and CRIGEN for their major support.

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