Review of Pesticides by Sub-Committee: Glyphosate

Statement given by the Bayer Crop Science Limited, Head-regulatory Sciences India, Bangladesh, Srilanka as follows Annexure I:

"We would like to state that none of the ongoing litigation and verdicts in the U.S against Glyphosate is final at this stage. We believe that the extensive body of science (hundreds of studies over several decades), more than 40 years of safe use, and the conclusions of regulatory agencies around the world will ultimately determine the outcome of the litigation. We will continue to defend Glyphosate and Glyphosate based formulations based on science. Bayers has not paid any compensation with ongoing litigations against Glyphosate in USA."

Earlier also, it has already deliberated in 410th & 411st RC meeting.
Kind Attention: Dr. J.P Singh – Secretary, CIB&RC
Dr. Sushil Khurana – Chairman, Sub-committee
Dr. Sarita Bhalla – Member Secretary, Sub-committee

Dear Sir/Madam,

Sub: Meeting with Sub-Committee to review continued use of certain pesticides – Additional Information on Glyphosate

Ref: Meeting with Sub-Committee on 4th December 2019

At the outset, we would like to sincerely thank you for providing us an opportunity for discussion on Glyphosate with the Sub-Committee during the meeting called on 4th December 2019.

During the interaction in the said meeting, we appraised the Sub-Committee on submission of certain information on Glyphosate by the industry, in line with the discussion in the first meeting of the Sub-Committee which was held on 11th November 2019.

Further, as advised by the Sub-Committee in the 4th December meeting, we are providing the following information/statement related to Glyphosate court cases in the United States of America:

'We would like to state that none of the ongoing litigations and verdicts in the U.S against Glyphosate are final at this stage. We believe that the extensive body of science (hundreds of studies over several decades), more than 40 years of safe use, and the conclusions of regulatory agencies around the world will ultimately determine the outcome of the litigation. We will continue to defend Glyphosate and Glyphosate based formulations based on science. Bayer has not paid any compensation in connection with ongoing litigations against Glyphosate in USA.'

We shall also submit the original letter in this regard tomorrow. The scanned copy of the letter is attached herewith.

Kindly acknowledge receipt of the letter.

Best Regards,

Kishor Nahar
Head - Regulatory Science India, Bangladesh, Sri Lanka
The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague-Dawley rats: effects on development and endocrine system

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Abstract

Background: Glyphosate-based herbicides (GBHs) are broad-spectrum herbicides that act on the shikimate pathway in bacteria, fungi, and plants. The possible effects of GBHs on human health are the subject of an intense public debate for both its potential carcinogenic and non-carcinogenic effects, including potential effects on the endocrine system. The present pilot study examines whether exposure to GBHs at the dose of glyphosate considered to be "safe" (the US Acceptable Daily Intake - ADI of 1.75 mg/kg bw/day), starting from in utero life, affects development and endocrine system across different life stages in Sprague Dawley (SD) rats.

Methods: Glyphosate alone and Roundup Bioflow, a commercial brand of GBHs, were administered in drinking water at 1.75 mg/kg bw/day to F0 dams starting from gestational day (GD) 6 (in utero) up to postnatal day (PND) 120. After weaning, offspring were randomly distributed in two cohorts: 8 M + 8F/group animals belonging to the 6-week cohort were sacrificed after puberty at PND 73 ± 2; 10 M + 10F/group animals belonging to the 13-week cohort were sacrificed at adulthood at PND 125 ± 2. Effects of glyphosate or Roundup exposure were assessed on developmental landmarks and sexual characteristics of pups.

Results: In pups, anogenital distance (AGD) at PND 4 was statistically significantly increased both in Roundup-treated males and females and in glyphosate-treated males. Age at first estrous (FE) was significantly delayed in the Roundup-exposed group and serum testosterone concentration significantly increased in Roundup-treated females offspring from the 13-week cohort compared to control animals. A statistically significant increase in plasma TSH concentration was observed in glyphosate-treated males compared with control animals as well as a statistically significant decrease in DHT and increase in BDNF in Roundup-treated males. Hormonal status imbalances were more pronounced in Roundup-treated rats after prolonged exposure.

(Continued on next page)
Conclusions: The present pilot study demonstrate that GBHs exposure, from prenatal period to adulthood, induced endocrine effects and altered developmental parameters in male and female SD rats. In particular, it was associated with androgen-like effects, including a statistically significant increase of AGDs in both males and females, delay of FE and increased testosterone in female.

Background
Glyphosate [IFAC chemical name N-(phosphonomethyl)-glycine] is the active ingredient of all glyphosate-based herbicides (GBHs), which is the most widely applied pesticide worldwide including the commercial formulation “Roundup” [6, 31]. Since the late 1970s, the volume of GBHs applied has increased around 100-fold [31]. The widespread exposure of human population to GBHs has raised public health concerns, including potential effects on the endocrine system, for example by inhibiting aromatase enzyme activity [14, 39] and/or by activating estrogen receptors (ERs) [1, 21, 46, 49]. In vitro, the reduction in aromatase activity has been reported in placental and embryonic human cells treated with low concentrations of Roundup [5, 39] and other formulations [14]. In tumor MA-10 Leydig cells, treated with different concentrations of Roundup, the expression of aromatase and steroidogenic acute regulatory protein (STAR) also decreased [52]. GBHs and their adjuvants were able to induce proliferative effects in human hormone-dependent breast cancer cells, further suggesting an endocrine-related mode of action [29, 49]. A more recent in vitro study also showed that human sperm incubation with glyphosate at 1 mg/L reduced sperm motility possibly related to sperm mitochondrial dysfunction [3].

In vivo, sexual development is controlled by hormones and is therefore highly sensitive to exogenous substances with endocrine-related effects. In rats, different studies have investigated the effects of high doses of Roundup administered to rats prenatally and postnatally on sexual maturity. A range of significant effects were observed, including i) both increased or reduced concentration of total testosterone (TT) in males treated with Roundup formulation (Monsanto of Brazil) containing 18% (w/v) polyoxyethyleneamine (surfactant) [11, 41], ii) increased 17β-estradiol (E2) serum concentrations in males treated with Roundup Transorb formulation [41], iii) delayed sexual maturation in females, as indicated by delayed vaginal opening, and iv) decreased spermatogenesis [11]. Similarly, peripubertal exposure to Roundup Transorb retarded sexual maturation, increased alterations of seminiferous tubules and reduced TT in male Wistar rats even at the lowest dose level tested i.e. 5 mg/kg bw/day [42]. Finally, also an alteration in pituitary hormones was observed in adult rats exposed to Roundup [35].

Pure glyphosate might be less potent than GBHs (such as Roundup formulations) in terms of reproductive toxicity. Testicular toxicity and reduced sperm counts, but no hormone variations, were observed in sexually mature male Sprague-Dawley (SD) rats treated active ingredient glyphosate, only at the highest dose level of 500 mg/kg bw/day [10].

In addition, evaluation of glyphosate and GBHs by international agencies is not without controversies. No evidence of interaction of glyphosate with the estrogen pathway was detected in the Endocrine Disruptor Screening Program (EDSP) conducted by the US Environmental Protection Agency (EPA) [50]. However, in Fish Early Life-Stage Toxicity (Threespine Stickleback) assay, EPA dismissed statistically significant differences in plasma vitellogenin, consistent with estrogenic activity, because of a non-monotonic dose response [51]. The European Food Safety Authority (EFSA) concluded in 2017 that the weight of evidence did not support endocrine disrupting properties of GBHs through estrogen, androgen, thyroid or steroidogenesis (EATS) modes of action. However, in a prior 2015 report EFSA noted that ‘signs of endocrine activity could not be completely ruled out’ in some of these assays [51].

Because to date relatively few human health studies have been conducted, the epidemiological evidence of GBH effects on reproductive and developmental health outcomes is too limited to draw conclusions. The Ontario Farm Family Health Study (OFFHS) showed a significant association between preconception exposure to pesticide products containing glyphosate and increased risk of spontaneous abortion [4, 43]. A recent small study found a significant association between urine glyphosate concentration in pregnant women and shorter gestational length [37]. A recent human study also suggested that maternal exposure to organophosphate has been associated with a no significant dose-related elongation of anogenital distance (AGD) in the female newborns at 3 months of age [12]. In rodents and primates, AGD is 50–100% longer in males than females. The increased growth of this region occurs in response to androgens and is related to fetal androgens exposure in early development higher in utero androgen exposure results in longer AGD in both sexes. Many epidemiological studies have reported population data on AGD and have shown links between AGD and testicular function and androgen action across a wide range of clinical outcomes [18]. Therefore, AGD has emerged as an informative and valid biomarker to assess the effects of a sub-optimal hormonal environment.
on human reproductive development from fetal to adult life [48].

Taken together, both in vitro and in vivo published studies to date, present conflicting findings. Glyphosate alone or GBHs exposure combined may relate to adverse developmental or reproductive effects, albeit many studies used very high doses of exposure. In vivo studies have been performed primarily in male rats, from different strains, at different life stages and using different endpoints. It is also not clear whether the possible adverse effects are due to endocrine disruption of GBHs [29]. Interpretation of the available data, particularly for the measurement of circulating hormones which are known to have large variation, should also take into account that different animal models can introduce biological variability, along with no comparable study designs and pre-analytical conditions [7].

The present pilot study examined whether exposure to GBH at a dose of glyphosate considered to be “safe”, i.e. the US Acceptable Daily Intake (ADI) of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (CRD) determined by the US EPA [17], affect the development and endocrine system across different life stages in SD rats. To this purpose, we tested both the active substance and the commercial GBH formulation “Roundup Bioflow”.

Methods

Chemicals

Active ingredient glyphosate (Pestanal® analytical standard, CAS number: 1071–83-6, purity > 99.5%) was supplied from Sigma-Aldrich (Milan, Italy). The commercial formulation Roundup Bioflow (containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salts of glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret) was supplied from a local agricultural consortium (Consorzio Agrario dell’Emilia, Bologna, Italy). The original containers/bottles of glyphosate and Roundup Bioflow were stored in its original container and kept in a ventilated storage cabinet at room temperature (22 ± 3 °C) throughout the study. Suppliers provided purity data for each batch of glyphosate and Roundup Bioflow. The opening and the use date of the different batches of test substances were recorded in the raw data. An aliquot of each lot of the test article is maintained in the ventilated storage cabinet, until 5 years from the end of the main experiment. The solutions of glyphosate and Roundup Bioflow were prepared by the addition of appropriate volume of tap drinking water.

Animals and experimental design

The entire animal experiment was performed following the rules by the Italian law regulating the use and treatment of animals for scientific purposes (Legislative Decree No. 26, 2014. Implementation of the directive no. 2010/63 / EU on the protection of animals used for scientific purposes, G.U. General Series, n. 61, March 14th 2014). All animal study procedures were performed at the Cesare Maltoni Maltoni Cancer Research Centre/Ramazzini Institute (CMCRC/RI) (Bentivoglio, Italy). The study protocol was approved by the Ethical Committee of the Ramazzini Institute. The protocol of the experiment was also approved and formally authorized by the ad hoc commission of the Italian Ministry of Health (ministerial approval n. 710/2015-PR). The CMCRC/RI animal breeding facility was the supplier for the SD rats. Female breeder SD rats were placed individually in polycarbonate cages (42x26x18cm; Tecniplast Buguggiate, Varese, Italy) with a single unrelated male until evidence of copulation was observed. Each of 24 virgin female SD rats (17 weeks old, 270–315 g) was mated outbred with one breeder male rat of the same age and strain. Every day, the females were examined for presence of sperm. After evidence of mating, females were housed separately during gestation and delivery. Newborns were housed with their mothers until weaning. Weaned offspring were co-housed, by sex and treatment group, not more than 3 per each cage. Cages were identified by a card indicating study protocol code, experimental and pedigree numbers, dosage group. A shallow layer of white fine wood shavings were used as bedding (supplier: Giuseppe Bordignon, Treviso, Italy). Analysis of chemical characteristics (pH, ashes, dry weight, and specific weight) and possible contamination (metals, aflatoxin, polychlorinated biphenyls, organophosphorus and organochlorine pesticides) of the bedding was performed by CONSULAB Laboratories (Treviso, Italy). Pellet feed and tap drinking water were tested for possible glyphosate contamination as previously described [36]. The cages were placed on racks, inside a single room prepared for the experiment at 22 ± 3 °C temperature and 50% ± 20% relative humidity. Daily checks on temperature and humidity were performed. The light was artificial and a light/dark cycle of 12 h was maintained. Stress-related husbandry factors were controlled: rats were kept together (same room, same rack, no more than 3 per each cage) and we did not relocate cages. Noise and handling time were minimized.

Two groups of SD rat dams and relative pups were treated with either glyphosate or Roundup Bioflow diluted in drinking water to achieve the desired glyphosate dose of 1.75 mg/kg bw/day. The F0 female breeders received the treatment through drinking water from gestation day (GD) 6 to the end of lactation, while the offspring (F1) continued to be exposed after weaning for additional 6 or 13 weeks. Glyphosate or Roundup solutions were freshly prepared on a daily basis depending on body weight and water consumption of dams or offspring, measured at scheduled time points. Preparation of drinking water solutions, quantification of glyphosate in water, and dosing
adjustments are described in detailed by Panzacchi et al. [36]. During pregnancy and lactation, embryos and offspring (F1) were all retained in the litter and might receive the test compounds mainly through their dams (F0). The day birth occurred was designated as post-natal day 1 (PND 1) for pups and lactation day 1 (LD 1) for dams. After weaning, on PND 28, offspring were randomly distributed in two cohorts: 8 M + 8F/group animals belonging to the 6-week cohort were sacrificed at PND 73 ± 2, i.e. 6 weeks after weaning; 10 M + 10F/group animals belonging to the 13-week cohort were sacrificed at PND 125 ± 2, i.e. 13 weeks after weaning. After weaning, the offspring (F1) were treated through drinking water until sacrifice. Altogether, 108 SD rats (54 males and 54 females) were enrolled in the post-weaning treatment phase.

Measurements in F0 dams and litters prior to weaning
Mean gestational length (duration of pregnancy) was calculated as the number of days from detection of a positive vaginal smear (GD 0) to birth of a litter. Pregnancy was confirmed by the occurrence of parturition. Dams' body weights were recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, dams' body weights were recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Pups' body weight by sex was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. Dams' feed and water consumption were recorded twice weekly during gestation (GD 0, 3, 6, 9, 12, 15, 18, 21), whereas during lactation were measured at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25.

To determine the number of pups born to each dam as accurately as possible, we examined cages at frequent intervals during parturition. Dead pups were removed when found and sexed when possible. Sex was determined on PND 1 and sex ratio data was presented as ratio of males to females. The mean litter size was calculated on PND 0 (within 24 h from delivery), 1, 4, 7, 10, 13, 16, 19, 21, 25. Litter size included dead as well as live offspring. Dead pups were visually examined by floating the lungs in saline, to distinguish if they were stillborn (died in utero) or died shortly after birth. Live birth index was calculated at PND 0 as (number of pups born alive / total number of pups born) x 100. Survival index, calculated as (total number of live pups at designated time point / number of live pups born) x 100, was measured on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. For all the pups, ano-genital distance (AGD), reflecting the linear distance between the genital tubercle and the anus, was measured on PND 4, using a Vernier caliper calibrated with a micrometer stage. Measurement was made from the caudal margin of the anus to the caudal margin of the genital tubercle [22]. Pup body weight was collected on the day the AGD was measured.

Post weaning endpoints up to adulthood
After weaning body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifices, the means of individual body weights were calculated for each group and sex. Daily water and food consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2; the means of individual consumptions were calculated for each group and sex. Time to vaginal opening (VO) was determined by daily inspection of all female pups starting on PND 28. The criterion was met for female rats when a complete rupture of the membranous sheath covering the vaginal orifice was observed [24]. The body weight of each female was recorded on the day that this was observed. Time to bulano-preputial separation (BPS) was determined by daily inspection of all males beginning on PND 35. The criterion for the day complete preputial separation was present when the prepuce was observed to completely retract from the head of the penis [24]. The body weight of each male was recorded on the day that this was observed. The female rats belonging to the developmental cohort (8/ group) were also monitored for the time to first estrous (TE), defined as the first day on which only cornified epithelial cells were observed on a vaginal smear, determined by vaginal cytology for 14 consecutive days, starting 3 days after vaginal opening was observed [32].

Estrous cycle characterization
Starting on approximately PND 95 and for the duration of 3 weeks, daily vaginal lavage was performed on female rats belonging to the 13-week cohort (10 F/group). To reduce variability, vaginal cytology samples were collected by vaginal lavage at the same time of the day over the course of the experiment, in the mid-morning, between 10:00 and 13:00. Collection, processing and vaginal smear evaluation was performed as described by us previously [26].

Necropsy
All the animals were anesthetized by inhalation of a mixture of CO₂/O₂ (70 and 30% respectively), and sacrificed by drawing blood by cava vein. The time and date of necropsy were recorded. Five days after weaning (corresponding to 49 ± 2 days of treatment), dams were sacrificed and the following organs were collected and alcohol fixed during necropsy: mammary glands (4 sites: axillary and inguinal, right and left), adrenal glands, uterus (including cervix), ovaries, vagina. The adrenal glands, uterus and ovaries were also weighed as soon as possible after dissection. For testosterone concentration determination, blood was collected and serum removed by centrifugation and stored at −80°C until analysis.

All male and female pups belonging to both cohorts were sacrificed on PND 73 ± 2 and PND 125 ± 2. The
following organs and tissues were collected and alcohol-fixed: mammary gland (4 sites: axillary and inguinal, right and left), thyroid and parathyroid, adrenal glands, bladder and prostate, seminal vesicle/coagulating gland, left and right testis with epididymis (half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation), uterus (including cervix), ovaries and vagina.

During necropsy, other tissues displaying anomalies and all gross lesions were collected, if present. Adrenal glands, bladder and prostate, seminal vesicle/coagulating gland, left testis, left epididymis, uterus (including cervix) and ovaries were weighed as soon as collected. In case of paired organs, both organs were preserved. The organ weight was related to body weight and was expressed as both absolute and relative organ weight.

Rats were sacrificed randomly across the 4 stages of the estrus cycle. In order to determine and allow correlation with histopathology in reproductive organs and hormone analysis, the stage of estrus cycle was determined by histological appearance of the various components of the reproductive tract for F1 females belonging to the 6-week cohort or by a vaginal smear examined on the day of necropsy for F1 females belonging to the 13-week cohort.

Sperm analysis
Sperm analyses were performed on each male animal from both cohorts, at scheduled necropsies on PND 73 ± 2 and PND 125 ± 2.

Sperm counts, daily sperm production, and sperm transit time through the epididymis
At necropsy, half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation. Spermatozoa resistant to the process of testicular homogenization and spermatozoa present in the caput/corpus and cauda epididymis were counted as previously described by Robb et al. [40] with slide adaptations described as follows. The tunica albuginea was removed from the (half) testicle, and a sample of the parenchyma was weighed and homogenized in 5 ml saline-TritonX-100 0.05%. The samples were then diluted 10-20 times in saline, and the mature spermatozoa resistant to homogenization (step 17-19 spermatozoa) were counted using a Thoma chamber. Four fields per animal were recorded, and the numbers of spermatozoa per gram of testis were calculated. To calculate the daily sperm production (DSP) these values were subsequently divided by 6.7, which is the number of days step 17-19 spermatozoa are present in the seminiferous epithelium [40]. Similarly, the segments of the epididymis (caput, corpus and cauda) were cut with a scissors, weighed, homogenized, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for each segment of the epididymis was calculated. The epididymal sperm transit time through the epididymal caput/corpus and cauda was calculated by dividing the number of spermatozoa present in each portion of the epididymis by the DSP of the associated testis [2].

Sperm morphology
To assess the percentage of morphologically abnormal sperm half of left cauda epididymis of each rat was transferred to a Petri dish containing 2.5 ml (for 70 day old animals) or 3.5 ml (for 120 day old animals) of Dulbecco's phosphate-buffered saline prewarmed to 37 °C, cut in 2-3 pieces and incubated of approximately 3 min at 37 °C, periodically gently swirling the Petri dish and its contents to facilitate release of sperm from the cauda.

Dried smears of epididymal spermatozoa were stained with 1% Eosin Y for 30 min and evaluated at 400 x magnification. Five hundred spermatozoa per rat were evaluated and scored as morphologically normal or abnormal according to the presence or absence of head or tail defects [8, 25].

Histopathology
After fixation, samples were trimmed, processed, embedded in paraffin wax, sectioned to a thickness of 4-5 μm and then processed in alcohol-xylene series and stained with hematoxylin and eosin for microscopic evaluation. Histopathology evaluation was performed by at least two pathologists. At least one senior pathologist peer reviewed all lesions of oncological interest as well as any lesion of dubious interpretation. In the pathological diagnosis, all the pathologists used the same evaluation criteria and the same classification based on international standard criteria (INHAND, NTP) described in the specific Standard Operating Procedures and long adopted at the CMCR/CRI. The diagnoses are reported in the experimental registries.

Hormone analysis
Serum concentration of free (FT) and total testosterone (TT); 5α-dihydrotestosterone (DHT); 17β-estradiol (E2) and Sex Hormone Binding Globulin (SHBG) were measured in duplicates by solid phase enzyme-linked immunosorbent assays (ELISAs). Blood sera, obtained and stored as described above, were used to assess the quantitative measurements in rat serum of E2, FT, TT, DHT and SHBG by ELISA based on the principle of the competitive binding, using the following commercial kits: " Estradiol rat ELISA" (#DEV9999), manufactured by Mededica Diagnostics GmbH (Kiel, Germany), " Rat Free Testosterone (F-TESTO) ELISA" (#CSB-E05979), " Rat Testosterone, T ELISA" (#CSB-E05100R), " Rat dihydrotestosterone (DHT) ELISA" (#CSB-E07879r), and " Rat sex hormone-binding
globulin (SHBG) ELISA* (#CSB-E12118r), manufactured by Cusabio Biotech Co. Ltd. (Houston, TX, USA).

The detection range and the Lower Limit of Detection (LLD) of each ELISA kit was 2.5–1280 pg/mL and 2.5 pg/mL for E2; 0.3–60 pg/mL and 0.15 pg/mL for FT; 0.13–25.6 ng/mL and 0.06 ng/mL for TT; 10–2000 pg/mL and 5 pg/mL for DHT; 375–6000 ng/mL and 375 ng/mL for SHBG. Each kit has been used following the manufacturer’s instructions and absorbance has been measured at 450 nm using a 96-well plate reader (Wallac 1420 VICTOR3™ Multilabel Reader, Perkin Elmer Inc., Waltham, MA; USA).

Plasma pituitary hormones were measured in duplicates using the “Rat Pituitary Magnetic Bead Panel” (CN: RPTMAG-86 K, Milliplex, St. Louis, MO), a Luminex bead-based immunoassay, following manufacturers’ instructions. Using plasma samples from 40 pups (20 females and 20 males) randomly selected from the 6-week cohort (N = 48 total), seven plasma pituitary hormones were measured: adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH). FSH and LH were assessed in 40 pups (20 females and 20 males) randomly selected from the 13-week cohort (N = 60 total). Exploratory analyses of circulating BDNF and TSH results from the 6-week cohort showed marginal differences by exposure groups in male pups; thus we attempted to validate these results by measuring BDNF and TSH in all male pups (N = 30) from the 13-week cohort. Plasma TT was measured in duplicates in all dams (N = 24) using an ELISA kit, the “Testosterone Parameter Assay Kit” (CN: KGE010, R&D Systems, Minneapolis, MN), following manufacturers’ instructions.

Statistical methods

Where data on a particular endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of α = 0.05. For continuous data including body weight, weight gain and organ weights, which are most often normally distributed, one-way ANOVA, followed by a Dunnett’s test was used to compare treatment versus control groups. For hormone data, which are usually non-normally distributed and have high inter-individual variability, a screening for outliers was made, based on a Box and Whisker Plot procedure and considering as outliers the values that were outside the box boundaries by more than 3 times the size of the box itself; in the case of hormone ratios, we have considered the same outliers of the single evaluation. Nonparametric Kruskal-Wallis’ tests, using beta approximation, were used in cases where data were not normally distributed (all hormones). Counting data, not normally distributed, were also analyzed with appropriate regression models. Where the observations were grouped (such as for litter data), fixed and mixed effect models were estimated (litter as random effect) and both reported. For biological parameters related to the body weight (such as the AGD), the statistical analyses were always performed including the body weight of each pup in the regression model. The incidence of pathological lesions, reported as the numbers of animals bearing lesions, were compared using a two-tailed Fisher exact test. The statistical analysis was performed using Stata/IC 10.1 (for all regressions) and Statistix 10 (for all the other tests); graphs were obtained using Microsoft Excel and Statistix 10.

Results

Results for maternal and reproductive outcome of dams are reported in Table 1. In dams, during both gestation and lactation, neither body weight nor weight gain differed between experimental groups. Similarly, we did not observe treatment effects for water or feed consumption during gestation or lactation. All the dams that cohabited with males achieved and maintained pregnancy; gestational period, litter size and sex ratio did not differ significantly between groups. Likewise, the mean live birth index was comparable between groups, although the number of dams with stillbirths was higher in the glyphosate (4/78) group compared to control (2/8). In pups, AGD at PND 4 was statistically significantly increased both in Roundup-treated males (p < 0.01) and females (p < 0.01) and in glyphosate-treated males (p < 0.01) (Table 2). Results were still significantly after running multilevel linear regression models adjusted for body weight and litter as a random effect. Post-weaning body weights as well as water and feed consumption showed no difference in both female and male offspring (Table 2). In female offspring, age and body weight at VO was similar across treatment groups; however, age at FE was significantly delayed (p < 0.05) in the Roundup exposed group (Table 2). The box plots and dot plots of AGD and age at FE were added as supplementary material (Additional file 1: Figure S1 and Additional file 2: Figure S2). Female offspring in the control and glyphosate-treated groups presented the FE within 6 days from the VO, while in the Roundup-treated group two out of ten females presented a more than double interval (12 and 14 days) between VO and FE. In female pups followed up to 13 weeks (N = 30), the percent of time spent in each stage of the estrous cycle did not differ between GBH1-treated animals and controls (Table 3). In male offspring, exposure to glyphosate or Roundup did not affect BPS or sperm parameters (number of mature spermatids in the tests, daily sperm production, number and sperm transit time through caput/corpus and cauda epididymis and morphology) (Tables 2 and 4). There were no treatment-related gross lesions at necropsy in F0 and F1 reproductive organs and endocrine organs in either sex;
### Table 1: Maternal and Reproductive Outcome of Dams Exposed to Glyphosate or Roundup Biowave Throughout Pregnancy and Lactation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Glyphosate</th>
<th>Roundup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational index (%)</td>
<td>100 (8/8)</td>
<td>100 (8/8)</td>
<td>100 (8/8)</td>
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<tr>
<td>Mean gestational length (day)</td>
<td>23.9</td>
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<td>23.0</td>
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<tr>
<td>Relative weight gain during pregnancy (%)</td>
<td>33.1 ± 1.8</td>
<td>32.4 ± 2.2</td>
<td>33.2 ± 1.4</td>
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<tr>
<td>Relative weight gain during lactation (%)</td>
<td>3.1 ± 0.7</td>
<td>2.5 ± 0.5</td>
<td>2.9 ± 0.8</td>
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<tr>
<td>Total pups (n) delivered at PND 0</td>
<td>120</td>
<td>115</td>
<td>124</td>
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<tr>
<td>Litter size (n)</td>
<td>15 ± 1.3</td>
<td>14.4 ± 1.9</td>
<td>15.5 ± 1.7</td>
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<tr>
<td>Sex ratio at birth (%)</td>
<td>53.6 ± 16.9</td>
<td>43.2 ± 9.9</td>
<td>45.4 ± 12.6</td>
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<tr>
<td>Mean live birth index (%)</td>
<td>95.9 ± 9.4</td>
<td>93.9 ± 6.8</td>
<td>96.3 ± 5.8</td>
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<td>Dams with reported stillbirths (n)</td>
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<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Stillborn (n)</td>
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<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Survival index at PND 1 (%)</td>
<td>90.8 ± 106</td>
<td>93.0 ± 8.3</td>
<td>91.5 ± 6.0</td>
</tr>
<tr>
<td>Survival index at PND 2 (%)</td>
<td>90.0 ± 100</td>
<td>91.3 ± 0.3</td>
<td>88.1 ± 8.0</td>
</tr>
</tbody>
</table>

*Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100

*Mean ± standard deviation

*Relative weight gain during pregnancy = relative weight on the last day of pregnancy minus relative weight on the first day of treatment in pregnancy, i.e. GO 0 (weight on GD 6 = 100%)

*Relative weight gain during lactation = relative weight on LD 21 minus relative weight on the first day of lactation, i.e. LD 1 (weight on LD 1 = 100%)

*Live and stillborn pups are considered

*Mean number of pups per litter at PND 0 (within 24 hr of delivery)

*Sex ratio at birth = (no. of male offspring / no. of total offspring) x 100

*Live birth index = (no. of offspring born alive / no. of offspring born) x 100

*Stillborn = no. of pups died in utero

*Survival index = (no. of live offspring at designated time-point / no. of pups born) x 100

### Table 2: Effects of Glyphosate or Roundup Biowave Exposure on Developmental Landmarks and Sexual Characteristics of Pups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Glyphosate</th>
<th>Roundup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of male pups at PND 1</td>
<td>58</td>
<td>48</td>
<td>53</td>
</tr>
<tr>
<td>Male pups weight at PND 1 (g)</td>
<td>68 ± 0.5</td>
<td>71 ± 0.2</td>
<td>68 ± 0.4</td>
</tr>
<tr>
<td>Male pups weaning weight (g)</td>
<td>50.4 ± 4.4</td>
<td>53.5 ± 6.0</td>
<td>51.8 ± 5.8</td>
</tr>
<tr>
<td>Male AGD (mm) at PND 41</td>
<td>402 ± 0.49</td>
<td>426 ± 0.38</td>
<td>434 ± 0.30</td>
</tr>
<tr>
<td>Age (PND) at balano-preputial separation (BPS)</td>
<td>46.33 ± 1.85</td>
<td>46.76 ± 1.73</td>
<td>47.61 ± 2.27</td>
</tr>
<tr>
<td>Body weight at BPS (g)</td>
<td>202.50 ± 10.74</td>
<td>203.89 ± 16.68</td>
<td>207.50 ± 22.70</td>
</tr>
<tr>
<td>Number of female pups at PND 1</td>
<td>51</td>
<td>61</td>
<td>60</td>
</tr>
<tr>
<td>Female pups birth weight (g)</td>
<td>64 ± 0.4</td>
<td>66 ± 0.4</td>
<td>65 ± 0.6</td>
</tr>
<tr>
<td>Female pups weaning weight (g)</td>
<td>48.3 ± 1.1</td>
<td>50.4 ± 5.2</td>
<td>50.5 ± 5.1</td>
</tr>
<tr>
<td>Female AGD (mm) at PND 41</td>
<td>1.70 ± 0.25</td>
<td>1.79 ± 0.21</td>
<td>1.86 ± 0.19</td>
</tr>
<tr>
<td>Age (PND) at vaginal opening (VO)</td>
<td>35.56 ± 1.72</td>
<td>35.39 ± 1.5</td>
<td>35.61 ± 1.14</td>
</tr>
<tr>
<td>Body weight at VO (g)</td>
<td>108.33 ± 6.18</td>
<td>108.06 ± 7.10</td>
<td>109.44 ± 8.73</td>
</tr>
<tr>
<td>Age (PND) at First Estrous (FE)</td>
<td>39.88 ± 1.25</td>
<td>40.13 ± 1.46</td>
<td>42.63 ± 3.25</td>
</tr>
<tr>
<td>Number of days between VO and FE</td>
<td>47.67 ± 0.71</td>
<td>51.37 ± 0.64</td>
<td>7.00 ± 3.78</td>
</tr>
</tbody>
</table>

*Statistically significant (p < 0.01) with multilevel linear regression adjusted for body weight

*Statistically significant (p < 0.05) with Kruskal-Wallis tests

*Mean ± standard deviation

*Weaning weight corresponds to PND 25

*AGD = ano-genital distance

*First estrous (FE) was evaluated only in females belonging to the 6 week cohort
Table 3: Estrous cycle characterization in female rats belonging to the 13-week cohort.

<table>
<thead>
<tr>
<th>Time (%) in Cycle Stages</th>
<th>N. Females</th>
<th>Control</th>
<th>Glyphosate</th>
<th>Roundup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (%) in Diestrus</td>
<td>10</td>
<td>55.24 ± 11.70</td>
<td>51.43 ± 5.41</td>
<td>52.80 ± 5.24</td>
</tr>
<tr>
<td>Time (%) in Proestrus</td>
<td>10</td>
<td>20.95 ± 7.51</td>
<td>23.33 ± 5.24</td>
<td>23.80 ± 3.76</td>
</tr>
<tr>
<td>Time (%) in Estrus</td>
<td>10</td>
<td>23.33 ± 5.24</td>
<td>25.24 ± 3.92</td>
<td>22.83 ± 2.24</td>
</tr>
</tbody>
</table>

Absolute and relative organ weights are presented in Tables 5, 6 and 7.

A panel of seven pituitary plasma hormones was assessed in animals from the 6-week cohort (20 males and 20 females). Most pituitary hormones were unaffected by GBH exposure, with the exception of a statistically significant increase in plasma TSH concentration observed in glyphosate-treated males compared with control animals as well as a statistically significant increase in BDNF in Roundup-treated males compared with control animals (p < 0.05, Tables 8). In female offspring, none of the pituitary hormones was different between treatment groups (Table 9). In light of the results observed in the 6-week cohort, we decided to measure these two pituitary hormones also in male and female offspring from the 13-week cohort (for female only few samples were available for these further analysis). Plasma TSH concentration showed an increase, even if not statistically significant (p = 0.056) in the glyphosate-treated males and a marked and significant increase in Roundup-treated males versus control (p < 0.01). Plasma TSH concentration still showed a borderline significant (p = 0.056) increase in the glyphosate-treated males and a marked and significant increase in Roundup-treated males versus control (p < 0.01).

BDNF plasma concentration was unaffected in this cohort. Sex steroids were measured in all animals of both 6-week and 13-week cohorts, providing data as follows:

- TT serum concentration significantly increased in Roundup-treated female offspring from the 13-week cohort compared to control animals (p < 0.05). TT showed an increase in the glyphosate-treated group, even if not statistically significant (Table 9). However, serum TT concentration did not differ by GBH exposure in the younger female offspring from 6-week cohort (Table 9) or in the male offspring (Table 8).

- In males, serum DHT concentration was markedly and significantly decreased in the Roundup-treated group (13-week cohort) compared to control animals (p < 0.01). The box plots and dot plots of DHT treatment was added as supplementary material (Additional file 3: Figure S3).

- No significant differences in serum FT and SHBG concentrations were observed in males or females belonging to both the cohorts.

- E2 serum concentration did not show statistically significant differences in male offspring exposed to glyphosate or Roundup in both the cohorts. In females, right before sacrifice, the endocrine status (diestrus, proestrus and estrus) for individual rats was assessed by vaginal smears. We could not statistically evaluate E2 (as well as FSH, LH and PRL concentrations) in the different female groups with reference to the stages of the estrous cycle due to insufficient sample size after clustering for endocrine status.

The data on each hormone assay coefficient of variation was provided as supplementary material (Additional file 4: Figure S4 and Additional file 5: Figure S5). Hormone ratios were calculated as indicators of the general balance between hormones (Tables 10 and 11) and, in particular, of the sex steroid hormone bioavailability.

- The ratio between TT and SHBG (e.g., TT/SHBG), currently used as an indicator of testosterone bioavailability and known as the FT index, was significantly increased (p < 0.05) in the Roundup-treated females (13-week cohort), but not 6-week females or in any of the males.

- The E2/SHBG ratio, an indicator of E2 bioavailability known as free estradiol index (FEI), significantly increased in Roundup-treated males belonging to the 6-week cohort (p < 0.05), whereas no effects were observed in E2/SHBG ratio in glyphosate-treated SD male rats.

- The FT/TT ratio significantly decreased in glyphosate- (6-week cohort) and in Roundup-treated males (13-week cohort) (p < 0.05) but not in any of the females.

- Both male and female Roundup-treated animals belonging to the 13-week cohort showed a marked decrease in DHT/TT ratio (p < 0.01). No statistically significant differences were observed in younger males and females (6-week cohort).

- No statistically significant differences were observed the E2/TT ratio in males and females.

Discussion

Roundup Bioflow, when administered to SD rats from in utero through adulthood at a dose level corresponding to the glyphosate RID defined by the US EPA (1.75 mg/
Table 4: Effects of glyphosate or Roundup Bioflow exposure on sperm parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6-week cohort</th>
<th></th>
<th></th>
<th>13-week cohort</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
<td>Roundup</td>
<td>Control</td>
<td>Glyphosate</td>
<td>Roundup</td>
</tr>
<tr>
<td>No of males examined</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sperm numbers (x 10^7/g testis)*</td>
<td>90.3 ± 22.0</td>
<td>83.7 ± 15.6</td>
<td>81.0 ± 12.0</td>
<td>109.4 ± 17.9</td>
<td>107.9 ± 11.3</td>
<td>119.6 ± 20.2</td>
</tr>
<tr>
<td>Daily sperm production (x 10^7/g testis)*</td>
<td>148 ± 36</td>
<td>137 ± 26</td>
<td>133 ± 20</td>
<td>179 ± 29</td>
<td>177 ± 18</td>
<td>196 ± 33</td>
</tr>
<tr>
<td>Cauda epididymal sperm number (x 10^6)*</td>
<td>51.2 ± 10.5</td>
<td>51.9 ± 10.5</td>
<td>50.9 ± 10.7</td>
<td>129.5 ± 28.5</td>
<td>125.9 ± 14.0</td>
<td>122.7 ± 11.0</td>
</tr>
<tr>
<td>Caput/corpus epididymal sperm number (x 10^6)*</td>
<td>67.6 ± 6.1</td>
<td>66.5 ± 7.9</td>
<td>66.5 ± 10.6</td>
<td>470.0 ± 194</td>
<td>933 ± 130</td>
<td>95.2 ± 8.8</td>
</tr>
<tr>
<td>Sperm transit time through caput + corpus of epididymis (days)*</td>
<td>4.8 ± 1.3</td>
<td>5.0 ± 1.1</td>
<td>5.1 ± 1.0</td>
<td>5.5 ± 0.8</td>
<td>5.3 ± 1.0</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>Sperm transit time through cauda of epididymis (days)*</td>
<td>3.5 ± 0.5</td>
<td>3.8 ± 0.8</td>
<td>3.9 ± 0.8</td>
<td>7.4 ± 1.8</td>
<td>7.2 ± 0.9</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>Sperm transit time through epididymis in toto (days)*</td>
<td>8.3 ± 1.7</td>
<td>8.8 ± 1.5</td>
<td>8.9 ± 1.8</td>
<td>12.8 ± 2.2</td>
<td>12.5 ± 1.7</td>
<td>11.2 ± 1.7</td>
</tr>
<tr>
<td>Total abnormal sperm (%)*</td>
<td>63 ± 13</td>
<td>64 ± 18</td>
<td>63 ± 14</td>
<td>46 ± 14</td>
<td>43 ± 19</td>
<td>35 ± 13</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation

kg bw/day), elicited subtle but potentially adverse effects on reproductive development and hormone concentrations. In particular, two apical endpoints were found to be statistically significantly affected:

- AGD was increased in both males (glyphosate group) and females (Roundup group);
- age at FE in females was significantly delayed (Roundup group).

Statistically significant changes in hormone profiles, indicators of hormonal activity, were also observed. In the 6-week cohort, glyphosate and Roundup elicited treatment-related effects only in males (females were not affected in this shorter window of exposure), as follows:

- increased TSH and decreased fT/TT in glyphosate treated rats;
- increased BDNF and E2/SHBG in Roundup treated rats.

In the 13-week cohort, only Roundup and not glyphosate induced sex steroid hormones alterations in both sexes, including:

- decreased DHT; increased TSH; decreased fT/TT in males;
- increased TT and TT/SHBG in females;
- decreased DHT/TT ratio in both sexes.

Overall, these effects indicate an impact on pre- and peri-pubertal sexual maturation. Noticeably, the pattern of effects also indicate specific sex-related and treatment-related differences. In particular, the effects of treatment with glyphosate were essentially limited to increased AGD and TSH concentration, and both changes were specific to males. Conversely, Roundup Bioflow seemed to affect both females and males, resulting in a statistically significant increased AGD and sexual hormones imbalances in both the cohorts.

Considering these outcomes with a weight of evidence approach, statistically significant differences in apical endpoints (AGD and FE) together with changes in hormonal activity detected in both the treatment groups should be taken into account suggesting evidence of concern for reproductive toxicity via an endocrine disruption mechanism [33]. Indeed, a longer AGD at birth in both sexes and an increased age at FE, together with the increased TT in females offspring, are considered endpoints for androgen-mediated activity by

Table 5: Organ weights and testosterone level in dams

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Glyphosate</th>
<th>Roundup</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of dams examined</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)*</td>
<td>302 ± 10.0</td>
<td>308 ± 15</td>
<td>317 ± 13</td>
</tr>
<tr>
<td>Adrenal glands**</td>
<td>0.110 ± 0.030 (0.036 ± 0.009)</td>
<td>0.100 ± 0.013 (0.035 ± 0.005)</td>
<td>0.100 ± 0.045 (0.033 ± 0.005)</td>
</tr>
<tr>
<td>Ureters***</td>
<td>0.087 ± 0.285 (0.0286 ± 0.0091)</td>
<td>0.077 ± 0.129 (0.0233 ± 0.0046)</td>
<td>0.094 ± 0.039 (0.0298 ± 0.0076)</td>
</tr>
<tr>
<td>Ovaries**</td>
<td>0.239 ± 0.087 (0.0797 ± 0.0018)</td>
<td>0.235 ± 0.047 (0.0777 ± 0.0017)</td>
<td>0.247 ± 0.052 (0.0787 ± 0.0016)</td>
</tr>
<tr>
<td>fT (ng/ml)**</td>
<td>3.77 ± 0.52</td>
<td>4.24 ± 1.48</td>
<td>3.90 ± 0.56</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation
**Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)
Table 6 Organ weights of male offspring

<table>
<thead>
<tr>
<th></th>
<th>6-week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No. of males examined</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>321 ± 19</td>
<td>326 ± 16</td>
</tr>
<tr>
<td>Adrenal glands</td>
<td>0.070 ± 0.014</td>
<td>0.071 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>[0.022 ± 0.005]</td>
<td>[0.022 ± 0.004]</td>
</tr>
<tr>
<td>Testes^a^</td>
<td>1.475 ± 0.052</td>
<td>1.474 ± 0.077</td>
</tr>
<tr>
<td></td>
<td>[0.451 ± 0.023]</td>
<td>[0.453 ± 0.023]</td>
</tr>
<tr>
<td>Epididymis^b^</td>
<td>0.370 ± 0.041</td>
<td>0.343 ± 0.040</td>
</tr>
<tr>
<td></td>
<td>[0.115 ± 0.011]</td>
<td>[0.105 ± 0.010]</td>
</tr>
<tr>
<td>Bladder/Prostate^c^</td>
<td>0.563 ± 0.080</td>
<td>0.561 ± 0.099</td>
</tr>
<tr>
<td></td>
<td>[0.176 ± 0.076]</td>
<td>[0.173 ± 0.066]</td>
</tr>
<tr>
<td>Seminal vesicles and coagulating gland^d^</td>
<td>1.129 ± 0.119</td>
<td>1.110 ± 0.291</td>
</tr>
<tr>
<td></td>
<td>[0.353 ± 0.045]</td>
<td>[0.339 ± 0.083]</td>
</tr>
</tbody>
</table>

^Statistically significant with Dunnet’s test (p < 0.05)

^aMean ± standard deviation

^bAbsolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)

the weight of evidence assessment [33]. As already pointed out, the effects of the treatments on hormone concentrations in our study were clearly different between the two sexes. Sex-related differences in toxicological responses are frequently observed with EDCs, associated with differences in hormone regulation in the two sexes [28]. For instance, in terms of an androgenizing mode of action, females have a baseline developmental testosterone exposure lower than males [15]. A number of animal studies have shown that the female reproductive tract is susceptible to virilisation by exogenous androgens, prior to, as well as during, the intrauterine masculinization programming window [13, 53]. The significant increase in AGD and the delay in the appearance of the first estrus cycle observed in Roundup-treated female SD rats is consistent with the increased developmental androgenization. The first ovulation is the true endpoint of a series of morphological and functional changes that affect all levels of the hypothalamic–pituitary–gonadal (HPG) axis, hence, it constitutes the unequivocal sign that puberty has been achieved [20]. We did not observe any difference in the achievement of VO, assessing the pubertal onset. Our findings are consistent with already published data: female Wistar-derived rats exposed to the glyphosate formulation Magnum Super II (Agros S.R.L., Argentina) from GD 9 to weaning, to up to 200 μg/kg bw/day did not show any effect on VO opening, though they observed other noticeable long-term effects, such as a reproductive impairment when mated (lower implantation sites, lower fetal weight) [30] that is not present in our results, probably because our exposure dose was much lower. Our findings on increased TT concentration in Roundup-treated females at 13-weeks are also consistent with studies indicating that intrauterine exposure to androgenizing factors may lead to higher androgen levels later in life [54]. A significant increase in the TT/SHBG ratio (T/T index), an indicator of testosterone bioavailability, was also seen in females exposed to Roundup up to 13 weeks.

In males, a prolonged, albeit of low-intensity, androgenizing effect could eventually evoke a counteracting feedback response from the HPG axis. As apical endpoint, we observed an increased AGD in both the treatment groups.

Table 7 Organ weights (g) of female offspring

<table>
<thead>
<tr>
<th></th>
<th>6-week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No. of females examined</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>225 ± 13</td>
<td>219 ± 11</td>
</tr>
<tr>
<td>Adrenal glands^a^</td>
<td>0.081 ± 0.012</td>
<td>0.073 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>[0.036 ± 0.005]</td>
<td>[0.033 ± 0.004]</td>
</tr>
<tr>
<td>Uterus^b^</td>
<td>0.499 ± 0.114</td>
<td>0.531 ± 0.162</td>
</tr>
<tr>
<td></td>
<td>[0.221 ± 0.050]</td>
<td>[0.241 ± 0.099]</td>
</tr>
<tr>
<td>Ovaries^c,d^</td>
<td>0.181 ± 0.024</td>
<td>0.169 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>[0.081 ± 0.012]</td>
<td>[0.077 ± 0.013]</td>
</tr>
</tbody>
</table>

^aMean ± standard deviation

^bAbsolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)
Table 8: Effects of glyphosate or Roundup Bioflow exposure on hormones in males (mean ± SEM)

<table>
<thead>
<tr>
<th>Serum Hormone</th>
<th>6-week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No. of males examined</td>
<td>8 (8)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>fT (ng/ml)</td>
<td>1.12 ± 0.12</td>
<td>1.02 ± 0.38</td>
</tr>
<tr>
<td>T (pg/ml)</td>
<td>14.53 ± 2.37</td>
<td>7.45 ± 2.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DHT (pg/ml)</td>
<td>761.11 ± 136.21</td>
<td>575.28 ± 238.24</td>
</tr>
<tr>
<td>SHBG (ng/ml)</td>
<td>861.20 ± 30.24</td>
<td>833.24 ± 21.15</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>1.04 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.29 ± 1.85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasma Hormone</th>
<th>6-week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No. of males examined</td>
<td>7 (8)</td>
<td>6 (8)</td>
</tr>
<tr>
<td>fTSH (ng/ml)</td>
<td>7.00 ± 1.38</td>
<td>6.43 ± 1.16</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>1.76 ± 0.79</td>
<td>2.87 ± 0.75</td>
</tr>
<tr>
<td>PR (ng/ml)</td>
<td>3.83 ± 0.64</td>
<td>3.00 ± 0.64</td>
</tr>
<tr>
<td>GH (ng/ml)</td>
<td>6.03 ± 4.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.19 ± 21.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>4.23 ± 0.76</td>
<td>8.17 ± 15.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>346.67 ± 35.32</td>
<td>255.18 ± 43.29</td>
</tr>
<tr>
<td>DBNF (pg/ml)</td>
<td>99.40 ± 25.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>148.85 ± 37.53</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistically significant (p < 0.05) with Kruskal-Wallis' test
<sup>b</sup> Statistically significant (p < 0.01) with Kruskal-Wallis' test
<sup>c</sup> out 8
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 10
<sup>c</sup> out 8
<sup>c</sup> out 8
<sup>c</sup> out 8

Few animal studies have reported an increased male AGD after glyphosate exposure. In utero exposure to persistent polychlorinated biphenyls (PCBs) increased AGD in male SD rats [15], and the dioxin-like coplanar congener PCB118 administered throughout early postnatal development also increased AGD in male SD rats [23]. Hormone profiling in males, revealed a decreased DHT in Roundup treatment group (13-week cohort), suggesting an effect on TT metabolism after a prolonged exposure. In particular, the lower conversion into DHT might indicate a possible reduction in 5α-reductase enzyme activity responsible of the conversion of TT in DHT. In fact, the marked decrease in DHT/TT ratio observed in both Roundup-treated males and females could suggest an overall reduction in the bioconversion of testosterone to 5α-reduced androgen and a possible imbalance of the metabolism of androgens. However, these effects were not observed in 6-week female animals. Also, it should be noted that male gonads showed normal seminiferous tubules and sperm production; this is consistent with the fact that spermatogenesis is heavily regulated by testosterone and FSH [45], both hormones unaffected in Roundup-exposed males.

Finally, we observed a significant increase in TSH in glyphosate-treated males (6-week cohort) and Roundup-treated males (13-week cohort). Due to resource limitation, we could not investigate T3 and T4 yet, we are planning this work in the near future. We did not observe histological changes in the thyroid gland, therefore the altered concentration of TSH together with a normal histological pattern of the thyroid gland are not indicative of a thyroid-related activity. Nevertheless, our findings prompt further detailed investigation on the effect of GBH on thyroid function and development.

BDNF is a neurotrophin playing a fundamental role in survival and differentiation of selected neuronal populations during development, and in the maintenance and plasticity of neuronal networks during adulthood [44]. Our results showed a statistically significant increase in BDNF in Roundup-treated males belonging to 6-week cohort, which was not observed in older animals. BDNF is an explorative and new endpoint for neurodevelopment and the utility of neurotrophins as potential biomarkers is not completely understood. At the moment, any adverse impact of GBH exposure on
Table 9 Effects of glyphosate or Roundup Bioflow exposure on hormones in female (mean ± SEM)

<table>
<thead>
<tr>
<th>Serum Hormones</th>
<th>6 week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No of females examined</td>
<td>8 (8)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>TT (ng/ml)</td>
<td>0.66 ± 0.10</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>FT (pg/ml)</td>
<td>6.49 ± 1.00</td>
<td>6.75 ± 1.89</td>
</tr>
<tr>
<td>DHT (pg/ml)</td>
<td>294.28 ± 50.40</td>
<td>328.34 ± 51.93</td>
</tr>
<tr>
<td>SHBG (ng/ml)</td>
<td>80.48 ± 30.24</td>
<td>95.25 ± 54.98</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>14.95 ± 7.24</td>
<td>32.24 ± 8.77</td>
</tr>
</tbody>
</table>

Plasma Hormones

| No of females examined | 7 (8) | 7 (8) | 7 (10) | 6 (10) |
| FSH (ng/ml) | 3.95 ± 2.50 | 2.67 ± 1.22 | 3.15 ± 1.65 | 1.58 ± 0.51 | 1.73 ± 0.64 | 1.46 ± 0.35 |
| LH (ng/ml)  | 5.75 ± 3.04 | 4.86 ± 1.93 | 4.52 ± 3.38 | 1.83 ± 0.25 | 2.35 ± 1.11 | 2.16 ± 1.28 |
| PRL (ng/ml) | 102.34 ± 164.71 | 70.49 ± 102.32 | 46.49 ± 31.03 | 46.55 ± 25.34 | 48.35 ± 31.03 | 30.03 ± 22.27 |
| GH (ng/ml)  | 12.61 ± 13.30 | 3.85 ± 0.97 | 4.16 ± 2.84 | 4.16 ± 2.84 | 4.16 ± 2.84 | 4.16 ± 2.84 |
| TSH (ng/ml) | 2.70 ± 1.51 | 3.02 ± 2.34 | 3.04 ± 1.53 | 1.29 ± 0.69 | 1.93 ± 0.39 | 3.03 ± 2.27 |
| ACTH (pg/ml) | 331.60 ± 89.59 | 314.09 ± 170.60 | 354.95 ± 104.96 | 25.59 ± 155.77 | 37.70 ± 220.60 | 249.30 ± 14,500 |
| BDNF (pg/ml) | 245.03 ± 155.60 | 436.62 ± 301.02 | 351.33 ± 177.28 | 25.59 ± 155.77 | 37.70 ± 220.60 | 249.30 ± 14,500 |

Statistically significant (p < 0.05) with Kruskal-Wallis’ tests
* Statistically significant (p < 0.01) with Kruskal-Wallis’ tests
7 out 10
6 out 8
5 out 8
4 out 10
3 out 10
2 out 10
1 out 10

Neurodevelopment can only be pointed out as a topic for further investigation.

The present study has some limitations. First, this is a pilot study performed on a limited number of animals where only one dose was used. However, the dose was selected specifically for its relevance to human health risk assessment, as it is the chronic current RfD defined by the USEPA, 1.75 mg/kg bw/day and therefore a dose level expected to be "safe". Several hormones were measured in the dams and offspring, but not all hormones were measured in all the animals, due to insufficient material for a complete data set of hormone profiling after the full-scale hematology and clinical biochemistry (data not yet published at the time this work is presenting).

Table 10 Effects of glyphosate or Roundup Bioflow exposure on hormone ratios in males (Mean ± SEM)

<table>
<thead>
<tr>
<th>Hormone Ratios (ng/mcg</th>
<th>6-week cohort</th>
<th>13-week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No of males examined</td>
<td>8 (8)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>FT/FT (x 10^-12)</td>
<td>1.25 ± 1.35</td>
<td>8.67 ± 0.83</td>
</tr>
<tr>
<td>DHT/FT</td>
<td>0.658 ± 0.081</td>
<td>0.511 ± 0.087</td>
</tr>
<tr>
<td>E2/FT (x 10^-10)</td>
<td>1.01 ± 0.19</td>
<td>2.65 ± 1.15</td>
</tr>
<tr>
<td>TSH/SHPG (x 10^-10)</td>
<td>12.90 ± 1.35</td>
<td>12.23 ± 1.25</td>
</tr>
<tr>
<td>E2/SHPG (x 10^-10)</td>
<td>12.4 ± 2.39</td>
<td>3.90 ± 2.21</td>
</tr>
</tbody>
</table>

*Statistically significant (p < 0.05) with Kruskal-Wallis’ tests
Statistically significant (p < 0.01) with Kruskal-Wallis’ tests
6 out 8
4 out 10
3 out 10
2 out 10
1 out 10

Table 11: Effects of glyphosate or Roundup Bioflow exposure on hormone ratios in females

<table>
<thead>
<tr>
<th>Hormone ratios (ng/ml)</th>
<th>6 week cohort</th>
<th>13 week cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glyphosate</td>
</tr>
<tr>
<td>No of females examined</td>
<td>8 (8)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>TH (TT or 10^-1)</td>
<td>9.17 ± 0.48</td>
<td>8.17 ± 0.55</td>
</tr>
<tr>
<td>DHT (TT)</td>
<td>0.428 ± 0.06</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>E2/TT</td>
<td>0.012 ± 0.00</td>
<td>0.045 ± 0.01</td>
</tr>
<tr>
<td>TT/SHBG (x 10^-1)</td>
<td>7.85 ± 1.02</td>
<td>5.00 ± 1.26</td>
</tr>
<tr>
<td>E2/SHBG (x 10^-1)</td>
<td>1.69 ± 0.78</td>
<td>3.51 ± 1.00</td>
</tr>
</tbody>
</table>

*Statistically significant (p < 0.05) with Kruskal-Wallis’ tests
*Statistically significant (p < 0.01) with Kruskal-Wallis’ tests
17 out 8
14 out 8
16 out 8
Not statistically evaluated due to insufficient sample size after clustering on the basis of the estrous cycle.

Furthermore, the number and timing of blood sample collection was limited to the final sacrifice of animals, considering that this was a pilot study and that in vivo blood sampling could lead to maternal and pupal stress. Another source of uncertainty, which is currently difficult to assess, is the timing of blood sampling during the necropsy session (9.00 am – 3.00 pm); a circadian-dependent modulation of circulating hormones cannot be completely ruled out. Standard errors in different hormone concentrations were wide, in relation to the relatively small group sizes and the physiological variability of hormone concentrations. In females, the estrous cycle status at the time of necropsy is another important source of variability when analyzing sexual hormone profiles. However, even if sacrificing animals on a specific day of the cycle might improve the ability to observe changes in the baseline hormone concentrations, the issue of sacrificing animals in the same cycling period (e.g. estrous) is still controversial. The updated OECD Test Guidelines on reproductive-developmental toxicity do not require the sacrifice of females in the same stage of estrous only the examination of estrous cycle on the day of necropsy is recommend to allow correlation with histopathology in reproductive organs [24]. Finally, we could not study separately the adjuvant(s) present in Roundup Bioflow (corresponding to 16% of the formulation) since the exact ingredients formulation is a trade secret. These are supposed to be surfactants, diluents or adjuvants stabilizing glyphosate and allowing its penetration in 'plants. It is noteworthy that the commercial formulation used in this study, Roundup Bioflow, was the representative formulated product recently evaluated for the renewal of the approval of glyphosate in EU and considered in the European Food Safety Authority peer review (MON 52276) [16]. At the same time, we covered specific windows of susceptibility relevant for the potential androgenic effects of GBHs exposure, for example in utero life and pre-puberty. Indeed, the per-natal and post-natal development, through to puberty, presents different susceptibility windows to EDC modes of action developing organisms with different and changing susceptibility as compared to adulthood [27, 47].

The majority of significant changes observed in hormonal status emerged in the 13-week cohort (animals sacrificed at adulthood) compared to animals in the 6-week cohort (sacrificed after puberty) suggesting that prolonged exposures were more effective in producing imbalances in the hormone concentrations. We have previously reported a possible enhanced retention of GBHs with an increasing pattern of glyphosate excreted in urine in relation to the duration of treatment in these same animals [36]. Finally, in our experimental design, the commercial formulation Roundup Bioflow was definitely more potent than glyphosate alone. Our results confirm previous observations that formulations might have stronger effects than glyphosate alone on endocrine and developmental parameters [9, 14, 19, 38]. Our results corroborate prior mixture studies [14], indicating that technical glyphosate and components of formulations may have cumulative (e.g., additive or synergistic) effects on endocrine-sensitive endpoints. Therefore, ADI calculations and other regulatory experiments should be performed not only on glyphosate, but also on its formulations and their components (that are often undisclosed).

Conclusions
The present study demonstrates that Roundup Bioflow exposure, at a dose level considered as "safe" (1.75 mg/kg bw/day), from prenatal period to adulthood, induced endocrine effects and altered reproductive developmental parameters in male and female SD rats. Roundup
Bioflow exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of AGDs in both males and females, delay of FE and increased testosterone in females. Roundup Bioflow exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week). Overall, the Roundup Bioflow elicited more and more pronounced effects than the active ingredient itself, which only increased AGD and TSH concentration in male rats in the peripubertal window (6-week cohort).

However, considering that retention of any GH in the body may increase with prolonged exposures, a life-course study on GBHs encompassing intrauterine life through to advanced adulthood is needed to confirm and further explore the initial evidence of endocrine-related effects and developmental alterations emerged in this pilot study.

Additional files

Additional file 1: Figure S1. AGD scores and AGD (Mean ± SEM) of all control groups. (.DOCX 53 kb)

Additional file 2: Figure S2. Effects of glycosidase on Roundup Bioflow exposure on hormones in males (Mean ± SEM). (.DOCX 55 kb)

Additional file 3: Figure S3. Male and female DR1: Box plot 1b and dot plot 1b. (.DOCX 86 kb)

Additional file 4: Figure S4: Effects of glycosidase on Roundup Bioflow exposure on hormones in females (Mean ± SEM). (.DOCX 23 kb)

Additional file 5: Figure S5: Effects of glycosidase on Roundup Bioflow exposure on hormones in females (Mean ± SEM). (.DOCX 23 kb)

Abbreviations
ACHT: Adrenocorticotropic hormone; AGD: Anogenital distance; EPS: Baboon prepubertal separation; CAMCRC: Cesare Maltese Cancer Research Centre, Bologna, Italy; DR1: Dosage factors; ESL: Exocrine pancreatic secretion; FGS: Forced Disruptive Screen Program; EPSA: European Food Safety Authority; EUSA: Enzyme-linked immunosorbent assay; EPA: Environmental Protection Agency; ETV: Estrogen receptors; FE: Forced estrus; FSH: Follicle stimulating hormone; F2: Free testosterone; GBH: Glycosidase-based herbicide; GH: Gestation day; GDH: Growth hormone; HD: Hemagglutination day; HUL: Luteinizing hormone; LDL: Low-density lipoprotein; ML: Miscarriage; MPV: Prostatitis; MR: Ramazzini Institute; SD: Sprague-Dawley; ShBG: Sex Hormone Binding Globulin; SHR: Systolic hypertensive rats; TSH: Thyroid stimulating hormone; TT: Testosterone; US-ATF: United States Acceptable Daily intake; VQ: Vaginal opening.

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Availability of data and materials
All raw data recorded and used during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
FM, SP, DM participated in the design of the study, performed the annual experiments, and sample collection, and drafted the manuscript. FE, LF, AM performed the in vivo phase of the study and contributed to the draft. the manuscript. CL drafted the manuscript and performed the analyses on pituitary hormones. TLF drafted the manuscript and performed the analyses on sexual hormones. MM and CG performed the sperm analysis, and helped to draft the manuscript. FM supervised the statistical analysis. All provided critical feedback on endocrine sensitive endpoints. AAM, EMK, SHS aided in interpreting the results on apical developmental endpoints (AGD and FE). JC MP discussed the results and contributed to draft the manuscript. FB was in charge of overall direction and planning of the pilot study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
N/A

Consent for publication
N/A

Competing interests
The authors declare that they have no competing interests.

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34. OECD Test no. 443: Extended one-generation reproductive toxicity study.


50 US E. 2015. Weight of evidence analysis of potential interaction with the estrogen, androgen or thyroid pathways. Glyphosate


Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines

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Endocrine disruptor
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ABSTRACT

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue®, MTT, ToxicLight®), plus genotoxicity (comet assay), anti-estrogenic (on ERα, ERβ) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24 h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell line endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB-231-LB2 cells for the most active formulation (N400). Then, from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited in HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in feed, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

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1. Introduction

Today, the production and dissemination of xenobiotics in the environment increase, and humans are exposed daily to many of these, but also their metabolites, which are present as pollutants (Feron et al., 2002). They act as mixtures having compensatory, multiplicative, or synergistic effects as we have shown (Benachour et al., 2007a). Among them, glyphosate (G)-based herbicides belong to the first herbicides used worldwide, and are major pollutants of rivers and surface waters (Cox, 1998; IFEN, 2006). They can contaminate organisms, including humans, but also food, feed and ecosystems (Takahashi et al., 2001; Acquavella et al., 2004; Contrado-Jara et al., 2008). Their use and presence in the food chain are further increased again with more than 75% of genetically modified edible plants that have been designed to tolerate high levels of these compounds (Clive, 2009), commercialized in various formulations. The question of the active toxic threshold of these substances in vivo is still open; but it is now well demonstrated that mixtures formulated with G and adjuvants are themselves not environmentally safe, in particular for aquatic life (UE classification). They can even enhance heavy metals toxicity (Tsui et al., 2006). Their in vivo carcinogenic, mutagenic and reprotoxic (CMR) actions are discussed in this paper for two reasons. First, in vivo effects on reproduction of G-based herbicides on reproduction, such as sperm production or pregnancy problems and outcomes are already published (Yousef et al., 1995; Savitz et al., 1997; Daruich et al., 2001; Beutet et al., 2005; Dallegrave et al., 2007; Oliveira et al., 2007; Cavalcante et al., 2008). Second, cellular mutagenic and toxic effects are now explained occurring at very low doses in cells involved in reproduction such as embryonic, fetal and placental ones (Marc et al., 2002, 2004; Richard et al., 2005; Dimitrov et al., 2005; Bellé et al., 2007; Benachour et al., 2007b; Benachour and Séralini, 2009). Since numerous CMR are also endocrine disruptors (ED), harmful for the environment and thus the object of specific legislations, the objective of this study was to test for the first time the ED capacities of these major pollutants on human cells. Androgen and estrogen receptors were examined using tran
scripational activation assays, as well as aromatase activity. We have also measured a potential genotoxic activity for the most active formulation.

The human liver cell line HepG2 has been chosen since it constitutes the best characterized human liver cell line, moreover it is used as a model system to study xenobiotic toxicity (Urani et al., 1998; Knasmüller et al., 2004; Westerink and Schoonen, 2007). The defined phase I and phase II metabolism, covering a broad set of enzymes forms in HepG2 cells, offers the best hope for reduced false positive responses in genotoxicity testing (Kirkland et al., 2007). In addition, the liver is the major detoxification organ exposed to food or drink contaminants. It has been demonstrated to damage carp or cat hepatocytes at low levels (Szarek et al., 2008; Malatesta et al., 2008). The objective of this study was also to compare the actions of four mainly used G-based Roundup® formulations, and G alone as control, on different enzymatic pathways and cellular endpoints. The endocrine mechanism was checked not only on three different sexual steroid receptors (estrogen receptors, ERα, ERβ, and androgen receptors AR) but also on aromatase, the enzyme responsible for the irreversible androgen to estrogen conversion (Simpson et al., 1994, 2002). If these parameters are disturbed this will be in turn crucial for sexual and other several cell differentiations, bone metabolism, liver metabolism (Hodgen and Rose, 2007), reproduction, pregnancy and development, but also behaviour and hormone-dependent diseases such as breast or prostate cancer (Seralini and Moslemi, 2001). Few data have thus far been obtained yet at this level (Holtan et al., 2007; Oliveira et al., 2007). This is important since chronic and genetic diseases can be provoked in humans and children by environmental pollution (Edwards and Myers, 2007) as well as by endocrine disruption (Rogan and Ragan, 2007).

2. Materials and methods

2.1. Chemicals

N-Phosphonomethyl glycine (glyphosate, GI, PM 90.97), as well as most other compounds, otherwise specified, were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France (F)). Roundup herbicide formulations (Montanville, Averes, Belgium) were available on the market: Roundup Express® 7.2 g/L of G. homoeolactone 2030323 (21.2%), Bioforce® or Extra 360 at 360 g/L of G. homoeolactone (2600657 [260]), Grands Travaux® 450 g/L of G. homoeolactone 8840425 (8400), Grandes Travaux plus® 450 g/L of G. homoeolactone 2020448 (4500). The 3-(4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Lightstatin at 250 mM, phosphate buffered solution (PBS), EDTA (trypsin (0.025%) was from Invitrogen (F)). Alum Brine was from Biosources (Camarillo, USA). Iysis buffers (RIPA, X), cell lysis reagents and reagents for RT-PCR are from Promega, F. primers from (Eurolab, Les Ulis, F.), DNA from Pharmacia (Orsay, F.), Chromatoxon-Columns (10 μL) medium (Fischer, Illkirch, F) and without antibodies.

The MDA-MB453-kb2 cell line was obtained from ATCC (München, F). This cell line possesses a high level of androgen receptor (Hall et al., 1994) and was stably transfected with the pmMad-Murine Tumor Virus (MMTV) (Wyllon et al., 2003). MDA-MB453-kb2 cells were grown in Dulbecco’s modified Eagle medium (DMEM), and supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA). Cells were cultured at 37°C and the medium was replaced every 48 h. To verify the purity of the different cell lines, the nuclear red solution was performed (Fluorobrite and Pans et al., 1994).

2.3. Toxicity tests

A 25 Roundup solution and an equivalent solution of glyphosate as Roundup Bioforce® were prepared in serum-free medium and adjusted to the pH 5.8 of the 25 Roundup Bioforce®. They were then used for consecutive dilutions up to 10⁻⁷. The mitochodrial activity is measured by the amount of MTT into formazan products (formation) by the mitochondria of enzyme succinate dehydrogenase (Mosmann, 1983; Dimon et al. 1989; Scatena et al., 2004). This assay was used to evaluate human cell viability. MTT was prepared at 2 mg/mL stock solution in PBS, filtered at 0.22 μm, and diluted in 1 mL of a serum-free medium. After treatments, the supernatants were recovered for the Toxicity® bioassay and adherent cells were washed with serum-free medium and incubated with 120 μL MTT per well after each treatment. After 4 h, the 48-well plates, 50,000 cells per well, were incubated for 3 h at 37°C and 120 μL of 0.04 M hydrochloric acid containing isopropanol solution was added to each well. The plates were then vigorously shaken in order to dissolve the formazan crystals formed. The plates were measured at 570 nm using a luminometer Multilab LB 940 (Berthold, Brugge, F).

The bioluminescent Toxicity® bioassay (Lonza, Sainte, Braineuse, F) is a non-destructive cytotoxicity highly sensitive assay designed to measure cell membrane damage. It quantitatively measures the release of alkaline kinase (AK) from the membranes of damaged cells (Crouch et al., 1993; Spickett and Murphy, 1997). AK is a robust protein present in all eukaryotic cells, which is released into the culture medium when cells die. The enzyme activity phosphatase activity and the resulting ALP is then measured using the bioluminescent firstly luciferase reaction with the Toxiclight reagent. The advantage of this assay is that the cell lysis step is not necessary (particularly in 24 h of treatment), of all cells supernatant were deposited in a 96-well plate. Then 50 μL of the ALP Detection Reagent (AKOR) were added by well. The plates (Dusch, Brumsh, M) were then placed under agitation for 15 min safe from the light, and then luminescence was measured using the luminometer Multilab LB 940 (Berthold, Brugge, F). The Toxiclight reagent has the positive control, and a negative control. The positive reaction was the active AKER mixed with cells treated in the serum-free medium to determine the basal activity.

The caspases 3/7 activities were determined with the Caspase-Glo® 3/7 assay (Promega, Paris, F). In 96-well white plates (Dusch, Brumsh, M) it was a luminescent method designed for automated high-throughput screening of caspases activity, at apoptosis induction (O'Brien et al., 2000). The assay provides a provolicent caspase-3/7 substrate, which contains the tetrapeptide sequence (DEVD). This substrate was cleaved to release alanine-huferase, a substrate of luciferase used in the production of light. The Caspase-Glo® 3/7 reagent was added (50 μL/p well) after cell treatments. After 24 h at 37°C. Plates were then again incubated for 45 min at room temperature safe from the light, to stabilize the signal before measuring the glow-type luminescence produced by the caspase cleavage of the substrate. The negative control is the serum-free medium. The positive control is the active reaction mixed with cells treated in the serum-free medium to determine the basal activity of the caspases 3/7. Luminescence was measured using the luminometer Multilab LB 940 (Berthold, Brugge, F).

The AlamarBlue® assay was performed according to the procedure described by O'Brien et al. (2000). About 10,000 HepG2 cells per well were grown in 24 h in 96-well plates and then exposed to 250 μL of different treatments for 24 h, pH adjusted to 7.4. After treatment, 100 μL of the 10X AlamarBlue solution were added in each well and incubated for 2 h at 37°C. Measurement of the optical density at 540 and 620 nm was performed using a spectrophotometer Multilab 120 (Thermo Fisher Scientific, Courtabeuf, F). The viability was expressed as a percentage of the control results (medium only).

The neutral red assay was performed according to the procedure described by Burd and Paumier (1984). About 5000 HepG2 cells per well were seeded in 24-well plates and grown 24 h (37°C). After 24 h of different treatments (1 mL, firstly adjusted to pH 7.4), cells were washed with PBS, then, 1 mL of neutral red solution (50 μg/mL) was added in each well for 1 h (37°C). For the last time, cells were washed and 1 mL acetic/acetonitrile (1/5, v/v) was added in each well, and the plate was stacked for 10min before measuring the neutral red release by fluorescence (excitation filter: 580 nm and emission filter: 535 nm). The viability was expressed as a percentage of controls (medium only).

2.4. Cytotoxicity test

The very sensitive comet assay is also known in the single-cell gel electrophoresis (SCGE) assay. The underlying principle is the ability of damaged DNA fragments to migrate during electrophoresis that can be carried out under highly alkaline conditions (pH 13), in order to detect single-strand and double-strand DNA breaks. The assay was adapted from Singh et al. (1988) with some modifications for cell preparation (Valentin-Sauret et al., 2003). Shortly, after 24 h treatment, cell suspensions were prepared by washing the cells with PBS and reseeding them with trypan/EDTA for 5 min at 37°C. Samples from 8 wells were pooled, centrifuged (100 x g, 5 min, 4°C) and resuspended in 100 μL PBS. Fifty microliters of cells (2 x 10⁶ cells) were prepared for analysis with 5 μL of the cell suspension mixed with 200 μL of 0.9% agarose solution (1.7% agarose) at 37°C. The cell suspension was rapidly spread onto a pre-coated slide, covered with a 25 mm cover slip and placed at 4°C for 5 min. Cover slips were removed and the slides treated with lysis solution for least 1 h at 4°C. After lys, slides were exposed to alkaline electrophoresis buffer (pH 13) for 60 min and
subjected to electrophoresis for 20 min (300 mA, 25 mV). Then, thehalf was neutralized with Tris buffer, the slices rinsed with cold ethyl alcohol, and dried at room temperature. For analysis, slices were recovered with 70% ethanol, then oven-dried and placed under a cover slip. Reading was performed with an ultraviolet microscope (400X). Nuclear observed were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium) and 3 (maximum damage) according to Collins et al. (2004) and Collins et al. (2008).

2.5. Aromatase detection

Aromatase activity was evaluated according to the triinated water release assay (Thompson and Silber, 1994) with a slight modification as previously described (Billinghiuser et al., 1989). This method is based on the specific release of 18-hydroxyestradiol from the aromatogenic compartment, which forms triitated water during aromatization. The HepG2 cells were exposed to non-toxic concentrations of glucose, sodium azide and thiourea, and were washed with serum-free EMEM and incubated for 50 min with 10% FBS and 200 nM ATR on a 37°C (30%, 150 r/min) shaker at 37°C (30%, 150 r/min). The reaction was stopped by centrifugation at 3000 x g at 4°C for 15 min. After adding 0.5 mL of 1% chloroform-T:10% sucrose, the mixture was centrifuged similarly. Supernatant fractions were assessed for radioactivity by scintillation counting (Packard, Liquid scintillation counter 1205L, USA).

Aromatase mRNA levels were measured by semi-quantitative RT-PCR. Total RNA was extracted using Trizol method (Promega, F) from HepG2 cells and checked at 260, 280 nm, and by electrophoresis on agarose gel (1.5%) stained with ethidium bromide. Five microns were reverse-transcribed (RT) using Moloney murine leukemia virus reverse transcriptase) at 42°C for 60 min in the presence of 0.5 μg oligo dT, 200 μg/ml dNTP and 200 μg/ml DTT in a total volume of 40 μl. The cDNA obtained were used for PCR. For each run, a master mix was prepared with 1.5X Taq DNA polymerase in PCR buffer containing 200 mM dNTP (1.5 mM MgCl2), and 25 μg of each primer in a total volume of 30 μl. The PCR primers were External sense, 5'TGA CGT CAA GGA ACA CCA GA 3' and External antisense 5' TCT CCG ACG 3', and for the antisense 5' GGA TCA TGT TCT GCA GAC C 3'. The resulting PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Photographs were taken using a photography apparatus mounted in a gel documentation system, and analyzed with the software Image J computer program.

2.6. Anti-estrogenic activity

Five plasmids were used for the transient transfection of the HepG2 cell line. Plasmids pERE-ERE-Luc, pERE-ERE-Lac and pERE-NH2 were kindly provided by Dr. D. McDonnell (Lipid Pharmaceutical, San Diego, USA); pERE-MycGal and pERE-NH2 were used for the normalization of luciferase activity (Cabanot et al., 2009). ERE-ERE-Luc is a 674 bp expression vector containing a single copy of the estrogen response element of the wirehagenin with a minimal thymidine kinase promoter driving firefly luciferase (Tullman et al., 1993). Pharents HRE and HRE2 are built from the pharent FUS (Fusarium gramineum) promoter (Hart and McDonnell, 1995) and encode the human wild-type estrogen receptor α or β. The pERE-MycGal contains β-galactosidase gene and is used in order to control the transfection efficiency. Finally, pERE-NH2 is used to obtain an appropriate DNA concentration for the transfection.

HepG2 cells were transiently transfected using Exgen 500 procedure (Euromedex, Mundolsheim, France). 100 000 cells per well were grown at 37°C (95% CO2, 5% air) in MEM supplemented with 2 mM glutamine, 1% non-essential amino acid and 10% of donkey-coated charcoal fetal calf serum in 24-well plates. The microplates were then incubated for 24 h for transfections, all plasmids were first diluted to 0.15 M NaCl to a final concentration of 100 μg/mL and then mixed: 100 μg ERE-ERE-Luc, 100 μg HRE and 100 μg pERE-MycGal and 200 μg pERE-NH2. Then 2 μL of Exgen 500 diluted in H2O/0.15 M NaCl were added to DNA. The mix was centrifuged and incubated at least 30 min at room temperature. The mixture was added to DMEM and distributed into the wells (200 μL/well). After 1 h of incubation (37°C, 5% CO2), the medium was removed and replaced by 1 mL of treatment medium without fetal calf serum for 24 h. To observe an anti-estrogenic activity, cells were co-treated with xenobiotics and [17β-estradiol 10-8 M]. [IC50 78 ± 180 (10-8 M)] was used as positive control. At the end of the treatment, cells were lysed with Reporter Lysis Buffer (Promega) and frozen at -80°C for at least 30 min. Then they were scraped and placed into microtubes before being frozen (freezing liquid nitrogen) thawing (37°C water bath) cycles and centrifuged 5 min at 10,000 g.

For luciferase activity measurement, 10 μL of lysate were mixed with 50 μL of luciferase assay reagent (Promega) into a white 96-well plate. The mixtures were immediately analyzed using a luminometer (TopCount NT, PerkinElmer). The β-galactosidase activity was measured using chlorophenol-red β-galactoside (Boehringer, Mannheim, Germany). The chlorophenol-red product was measured with a spectrophotometer at 570 nm (MRX Dynex). Protein concentration determination was performed using 2 μL of the lysate according to Bradford (1976) on a spectrophotometer at 595 nm. Luciferase activity for each treatment group was normalized to β-galactosidase activity and protein level (Luc + Prot/Gal) was compared to the control (17β-estradiol 10-8 M) set at 100%.

2.7. Anti-androgenic activity

MDA-MB-231 cells were seeded in 24-well plates and 50 000 cells per well were grown in L-15 medium without phenol red supplemented with 5% decomposed fetal calf serum for 24 h (37°C without CO2). After 24 h incubation, medium was removed and cells were washed with 500 μL PBS and exposed to compound solutions in co-culture with DHT (4 x 10-10 M) for 24 h in medium without fetal calf serum. Nilsamidate (10-4 M) was used as positive control. For luciferase activity measurement, 20 μL of lysate were mixed with 40 μL of luciferase assay system (Promega) in a white 96-well plate. The mixtures were immediately analyzed using a luminometer (TopCount NT, Packard). Results were expressed as a percentage of the data obtained with the androgen DHT (4 x 10-10 M).

Fig. 1. Dose-dependent effects of glyophosphate (G) and four glyophosphate-based formulations (Roundup containing 7.2-450 μg/L) on HepG2 Cells viability after 24 h of exposure. These effects were evaluated by the MTT test (A) or the TopCount assay (B). The results are presented in % comparatively to non-treated cells (100% viability). % of relative levels to non-treated cells (URL, 1). Cells were grown at 37°C (95% CO2, 5% air) in medium EMEM with 10% Serum during 48 h in 96 wells confluence in 48-well plates for MTT test or 96-well plates for TopCount, and then exposed to the products for 24 h without serum. All experiments were repeated 4 times in replicates.
Table 1
Comparative initial toxicities and LC50 of glyphosate-based formulations measured by three different ways (described in Section 2) on HepG2 cell line.

<table>
<thead>
<tr>
<th>Products</th>
<th>Amaranth blue test (%)</th>
<th>MTT test (%)</th>
<th>Toxicity assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial toxicity</td>
<td>LC50</td>
<td>Initial toxicity</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>2.78</td>
<td>1</td>
</tr>
<tr>
<td>R12</td>
<td>0.2</td>
<td>0.36</td>
<td>0.4</td>
</tr>
<tr>
<td>R250</td>
<td>0.2</td>
<td>0.32</td>
<td>0.5</td>
</tr>
<tr>
<td>R400</td>
<td>0.0005</td>
<td>0.0012</td>
<td>0.005</td>
</tr>
<tr>
<td>R540</td>
<td>0.0005</td>
<td>0.0016</td>
<td>0.008</td>
</tr>
</tbody>
</table>

The initial toxicities correspond to the % of product providing the first significant effects (50% toxicity) for glyphosate alone (G) or at different concentrations (7.2–450 g/L) in different Roundup formulations (R).

Fig. 2. DNA damage (increasing from classes 1–3, Comet assay) after HepG2 cells exposure to R400 during 24 h at different concentrations (0: control C, 1–10 ppm). Benzyl-pyrene (50 μM, BpP) was used as positive control. All experiments are repeated 3 times in duplicate for 100 cells.

2.7.1 Statistical analysis
All data were presented as the mean ± standard error (S.E.M.). Statistical differences were determined by Student t-test using significant levels of 0.01 (**), or 0.05 (*) with GraphPad Prism 4 software.

3. Results
HepG2 cells, in our experiments, generally show a growth rate around 32 h in control medium. All glyphosate-based formulations, by contrast to glyphosate alone (toxic from 1% in MTT assay), induce a rapid decrease in cell viability according to the formulation and the test, within 24 h only (Fig. 1 and Table 1). Several endpoints were reached: mitochondrial respiration and activity (MTT Fig. 1A and Amaranth blue, the most sensitive assay, Table 1) or cellular membrane damage (Fig. 1B). Mortality is dose-dependent for all R in formulations, but there is no dose-dependency to G concentration. This is confirmed for the first time by three specific methods. The most cytotoxic formulation (400 g/L of G) does not contain the highest concentration of G. The two first formulations demonstrate similar middle toxicities (7.2 and 360 g/L of G), the two others show 20–200 times higher toxicity (400 and 450 g/L of G, Fig. 1). The different values of LC50 and initial statistically significant toxicities (around LC10) for the various formulations are in the same range whatever the assay; R400 > R450 > R360 > R7.2 (Table 1).

Effects of R400 on HepG2 DNA after 24 h exposure are illustrated in Fig. 2. In our conditions, we observed around 50% DNA strand breaks at 5 ppm (25% class 1, 11% class 2 and 15% class 3). This effect is dose-dependent with a drastic increase in classes 2 (27%) and 3 (36%), revealing major damages at 10 ppm, corresponding to 24 μM G dissolved in specific adenoviruses. This provokes around 75% DNA fragments in comparison to 35% in negative controls. The positive control, the well-known promutagen Benzaldehyde, induces 95% damages, but at about 2 times higher concentrations (50 μM). This result clearly shows that the DNA of the human hepatoma cell line is damaged by a G-based herbicide.

The caspases 3/7 are significantly activated with non-toxic doses of R450 (60 ppm, Fig. 3) up to 156% in 24 h. Their levels are considerably enhanced to 765% within 48 h, R is able to induce apoptosis.

Fig. 4. Dose-dependent effects of glyphosate (G) and the four Roundup formulations on aromatase activity (bold line) and mRNA levels in HepG2. These effects below toxic levels were evaluated in % controls respectively, by initiated water release during aromatization, and concomitante RT-PCR. Cells were grown as in Fig. 1 and then exposed for 24 h to xenobiotics. All experiments were repeated 3 times in triplicates. Statistically significant differences are indicated for p < 0.01 (**), or p < 0.05 (*).
Fig. 5. Dose-dependent effects of Glyphosate (G) and the four roundup formulations on ERα, ERβ (A, left column) transcriptional activity in HepG2 transiently transfected (ERE-TK-Luciferase) and AR (R, right, measured in MDA-MB-453-kb2 cells). These effects below toxic levels (except last dose on the scale) were evaluated after 24 h in 2% ethanol respectively, activated by 10^{-8} M estradiol (ERα) for ER and 10^{-10} M DHT for AR. All experiments were repeated 3 times in triplicates. Statistically significant differences are indicated for p<0.01** for ERα and AR, # # for ERβ.

We have obtained interferences of G-based herbicides with human cell endocrine activities, below initial toxic doses (which are around 1C10), known for at least two of three cytotoxicity tests. We began to study the gene expression variations of the irreversible sexual steroid conversion, aromatase. Both enzymatic activity and specific mRNA levels were assessed (Fig. 4). C alone is always inactive, while all the formulations inhibited androgen to estrogen conversion, below all LC50 and always in 24h. In the meantime, biphasic effects were seen on the aromatase mRNA levels for all formulations, with increases 130-250% followed by a return to normal in most cases. An inhibition was seen for R400 then followed by the increase. These effects were thus neither linear nor C-proportional.

Furthermore we also observed at lower doses disruptions of estrogen and androgen dependent transcriptional activities. These were quite linear and dose-dependent (for R not for G) in the case of each formulation, in the range of values tested, after 24h of exposure (Fig. 5). The corresponding IC50 were determined (Table 2). For all G-based herbicides, common anti-estrogenic profiles for both ER and anti-androgenic ones were revealed, according to the slopes of the curves (Fig. 5A and B). C alone had no anti-estrogenic activity but was clearly anti-androgenic at sub-agricultural and non cytotoxic dilutions. Even if data showed that both ER transcriptional activities were comparably affected, there were some formulations specificities: R400 is clearly 2 times more active on ERα, and iM50 on ERβ. The most toxic formulations are the...
most inhibitors at lower non-cytotoxic doses, on cell endocrine activities (Fig. 5). All formulations except R450 appeared more anti-androgenic than anti-estrogenic. We can classify the R inhibition efficiencies: from R400 > R450 > R350 > R72, with a 300–800 times difference between the strongest inhibitor and the lowest (Table 2).

4. Discussion

This work evidences the toxic effects of four formulations of the major herbicide worldwide (R) on a human hepatic cell line HepG2, a pertinent model for xenobiotic actions (Knasmüller et al., 2004). This is also because the liver is the first detoxification organ, and very sensitive to dietary pollutants. We tested sub-agricultural dilutions and noticed the first toxic effects at 5 ppm, and the first endocrine disrupting actions at 0.5 ppm, which is 800 times lower than the level authorized in some food or feed (400 ppm, US EPA, 1998). This confirms and enhances the potential toxic action of G-based herbicides that we observed on human placental and embryonic cell lines, and on fresh umbilical cord cells (Richard et al., 2005; Benachour et al., 2007b; Benachour and Séralini, 2009). Their mechanistic time and dose-dependent actions on mitochondria, plasma membrane, caspases 3/7 and DNA fragmentation has been previously demonstrated. Here we obtain for the first time their relative LC50 by three different methods, but also their genotoxicity, and endocrine disruption potentials from lower levels on three different sexual steroid receptors on human cell lines. The mixtures in formulations in this work are always the most toxic in comparison to G alone, as previously underlined (Richard et al., 2005), and also observed in aquatic communities (Reytes, 2008). We confirm that the nature of the adjuvants changes the toxicity more than G itself, not only in embryonic or neonate cells (Benachour and Séralini, 2009) but also in human cell lines (HepG2 and MDA-MB453-k2) from young or adult. This allows deleterious actions at very low levels that have no more herbicide properties. This creates environmental concerns of contaminating authorized amounts found in rivers, soils or food and feed within 24 h only. The time-amplified effects have also been previously described (Benachour et al., 2007b). Our three different methods measuring in particular simultaneously FAD, NAD, and NADPH dehydrogenases, mitochondrial succinate dehydrogenase and plasma membrane degradation gave consistent results with comparable differential toxicities profiles, with the four G-based herbicides, even if one test was obviously more sensitive than the others (Alamar Blue).

We demonstrate here for the first time the DNA damages of a G-based herbicide on a human cell line at residual levels corresponding to 120 nM of G. An association was previously suggested with multiple myeloma incidence in agricultural workers (Defoos et al., 2005). However, there was still a serious doubt about direct genotoxicity in mammals (Williams et al., 2000; Dimitrov et al., 2006), that was recently questioned in mice (Heydons et al., 2008), after contradictory results. G was known to be genotoxic alone on human cells, but at 105 higher levels (M.M. Murray et al., 2005) in comparison to this study. It was similar for AMPA alone, a G metabolite (Mañas et al., 2008). DNA damages were already induced by G and synergistic oxidative stress in human fibroblasts (Leuenen et al., 2004), and thus combined mutagenic effects of adjuvants and G, plus its metabolites, appear obvious at minute doses in the present work. It is noticed that the biotransformation of xenobiotics results in the production of reactive intermediates such as reactive oxygen species which are toxic and can cause oxidative damage to DNA (Cadee et al., 2003). In addition, R, with its adjuvants, has been previously demonstrated to provoke DNA adducts in the kidneys and livers of mice (Peluso et al., 1998) and DNA lesions in tadpoles, bovine cells, drosophila, fish, or cainians (Clément et al., 1997; Li et al., 1998; Kaya et al., 2000; Cavas and Körnen, 2007; Cavalcante et al., 2008; Poletta et al., 2008). The comet is a very sensitive assay but not specific. Two other endpoints must be taken into account using this method: apoptosis and DNA repair. During the apoptotic process, DNA is broken down into nucleosome-sized pieces. Comet equivalent to class 4 (DNA in the tail and small head) can reveal cells in the earliest stages of apoptosis, this class was not taken into account in this study. Caspases 3/7 activations characteristic of apoptosis were demonstrated recently by some of us to be provoked by similar R formulations in other human cells (Benachour and Séralini, 2005). In this study, R450 is able to induce caspases at 80 ppm. As Comet equivalent to class 4 (DNA in the tail and small head) can reveal cells in the earliest stages of apoptosis, this class was not taken into account here (Collins et al., 2008). Experiments are running in the lab to check if at this lower concentration of R400 these DNA damages can be really due to a repair process.

We then tested the potential endocrine disruption below the toxic levels described above in human cells. This was done by measuring not only the capacity of G-based herbicides to disrupt androgenic or estrogenic transcriptional activities, but also to modify a crucial irreversible androgeno-estrogenic steroid metabolism, through aromatase gene expression measurement. A constitutive but low aromatase activity inhibition was observed in this work with all formulations as suggested previously (Richard et al., 2005; Benachour et al., 2007b) due to the combined effects of G plus adjuvants here. Low levels of aromatase decrease resulted in increased gonads and possible female reproductive impairment at adulthood in amphibians (Clément et al., 2008). Comparable hypotheses have been proposed for humans (Séralini and Moslemi, 2001), even for other xenobiotics (Moslemi and Séralini, 2005; Salabert et al., 2009). It becomes obvious that the direct enzymatic effect of G (Richard et al., 2002) does not exclude a transcriptional disruption as it was observed in mouse and urchin eggs (Walsh et al., 2000; Maic et al., 2002, 2005). The biphasic profile of this aromatase transcription disruption could be either due to a direct DNA interaction of G compounds (Peluso et al., 1998) or to a receptor-mediated interaction like it was shown on ER-mediated transcription for other pesticides (Snieezer et al., 2000). In order to test this hypothesis, we studied the interaction with three different steroid receptors able to bind steroid-like structures as well as aromatase, which is indeed regulated by estrogens and androgens in mammals (Bourgiba et al., 2003).

Steroid receptors may be involved in xenobiotic receptor pathways of action (Matthews and Gustafsson, 2005; Rokutanda et al., 2008). They are even disrupted by several xenobiotics, like other ERE or even steroid membrane receptors in various animals (Watson et al., 2006) for instance the pesticide methoxychlor upregulates ERβ in the bass (Blum et al., 2008). Even surfactants, adjuvants, plasticizers or pesticides have been proven to interfere with AR (Paris et al., 2007; Wilson et al., 2008). The in vivo consequences may be obvious for sexual differentiation and reproduction (Sultan et al., 2001; Martin-Skloton et al., 2008). R itself may affect male genital organs in drakes (Oliveira et al., 2007) or estrogen-regulated genes in human cells (Hokanson et al., 2007), it was then logical to test the ED potential on ERα, ERβ and AR. Here we prove for the first time for four G-based formulations their dose-dependent interactions with these receptors. Their IC50 are measured in μM higher than those of well known inhibitors such as Raloxifen or tamoxifen for estrogen receptors (Sibley et al., 2003; Orkan-Arcan and Ozalpán, 2007), and fulvestrant for androgen receptors (Simard et al., 1986), which have IC50 in the nM range. The G in adjuvants has comparable properties than other ED (Xu et al., 2005). Moreover the various adjuvants change obviously the shape or at least the bioavailability, penetration and bioaccumulation of G at this level, and/or anyway its receptor interactions, with the results we have described.
The non-G-positive cytotoxic effects, and at lower levels ED effects, demonstrate all the major role of adjuvants in biological disruptions. Moreover the direct interaction of G with the aromatase catalytic site previously demonstrated (Richard et al., 2005) and confirmed by an aromatase disruption here, has now to be considered with the present interaction demonstration with three steroid receptors. Since G is designed to inhibit in plants the enzyme GPISPS involved in essential aromatic amino acids metabolism (Arnaud et al., 1990), it is possible that G (in R) could fit in a binding site for a molecule with an aromatic cycle, such as those in steroid receptors or steroid metabolizing enzymes (Walsh et al., 2000). It is also possible that, as suggested for other xenobiotics, these herbicides bind more to one site on steroid receptors (Arnold et al., 1997).

In conclusion, according to these data and the literature, G-based herbicides present DNA damages and CMR effects on human cells and in vivo. The direct G action is most probably amplified by vesicles formed by adjuvants or detergent-like substances that allow cell penetration, stability, and probably change its bioavailability and thus metabolism (Benachour and Séralini, 2009). These dormes can also be present in rivers as polluting contaminants. The type of formulation should then be identified precisely in epidemiological studies of G-based herbicides effects (Acquaella et al., 2006). Of course to drive hypotheses in in vivo effects, not only dilution in the body, elimination, metabolism, but also bioaccumulation and time-amplified effects (Benachour et al., 2007b) should be taken into account. These herbicides mixtures also present ED effects on human cells, at doses far below agricultural dilutions and toxic levels on mitochondrial activities and membrane integrity. These doses are around residual authorized levels in transgenic feed, and this paper is the first clear demonstration of these phenomena in human cells. This in vivo ED classification of G-based herbicides with this molecular basis must be now carefully assessed.

Conflict of interest statement

None.

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Roundup® in genetically modified plants: Regulation and toxicity in mammals

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Context

Among the 134 million hectares of genetically modified plants growing worldwide in 2009, more than 99.9% are described as pesticide plants (Clive 2009). Around 80% are tolerant to Roundup, a glyphosate based herbicide. Its use on GMOs is thus amplified, and this phenomenon shed a new light on the problem of herbicide residues in plants. This is because these GM plants have been modified so that they can contain high levels of Roundup. They are modified to behave normally after several treatments with this herbicide, which were not allowed at such levels on regular plants before. The latest generation, like Smartstax crops, even cumulate a tolerance up to 2 herbicides and a production of 6 insecticides. By this worldwide use and the known potential hazards of pesticides, their residues are a major concern for health and the environment. Moreover the new metabolism that they could trigger in GMOs remains to be studied. A debate on international standards is ongoing on their capacity to predict and avoid adverse effects of the herbicide residues at environmental or nutritional exposures, particularly in GMOs.

As far as Roundup is concerned, the formulations of which are mixtures of only one proposed active ingredient (glyphosate) with various adjuvants, up to 400 ppm of residues are authorized in some genetically Modified food and feed (EPA 2008). It is also recognized by regulatory agencies that these residues are found in meat and products generated from livestock fed with glyphosate tolerant soya or maize (EFSA 2009).

Review on Roundup toxicity studies

Surprisingly, more and more studies have revealed unexpected effects of Roundup, including carcinogenic and endocrine disrupting effects. This is at lower doses than those authorized for residues found in Genetically Modified Organisms (GMOs). For example, Roundup altered the spermatogenesis of rats exposed in utero to 50 ppm per day (Dallegreave et al. 2007). Even a tumour promoting potential is observed on mice

exposed to 25 ppm per day (George et al. 2010). Alterations of rat testicular morphology and testosterone levels occur at doses of 5 ppm per day (Romano et al. 2009). In our laboratory, we have observed endocrine disruption on human cell lines; it was a disruption of aromatase, of the androgen and estrogen receptors in 24 hours, starting from 0.5 ppm Roundup. This corresponds to glyphosate concentrations 2000 times less than the authorized levels in GMOs (Gasnier et al. 2009). Furthermore, we have shown that Roundup inhibited cellular respiration, and that it also caused membrane damages. Last but not least, Roundup showed genotoxic effects, as well as it induced apoptosis and necrosis in human cells (Benachour & Séralini 2009). Most of these effects are amplified with time. This is preoccupying, and it does highlight the limits of the Acceptable Daily Intake concept for long-term exposures.

**Debate on health risks**

In all these studies, toxic effects were not detected with the so-called active ingredient glyphosate alone at these doses; they were more related to the formulations of the herbicide and its adjuvants. These remain confidential and their residues are not measured. Of the 20 tests required (or conditionally required) to register a pesticide in the United States, only 7 short-term acute toxicity tests use the whole formulation; the others are done using the sole active ingredient (Cox & Surgan 2006). The problem of pesticide registration is indeed very old, and it is only the active ingredient that is tested in chronic mammalian toxicity tests (generally for 2 years on rats). Moreover, there is generally only one 2-year test worldwide on a mammal per pesticide, performed by the company commercializing this pesticide. Adjuvants are often considered to be inert in the assessment process. This is a major issue. Such a simplistic approach of pesticides hazards bypasses the potential effects of adjuvants and their mixtures with the active ingredient on chronic risks. This issue is even more crucial with GMOs which are designed to tolerate the formulations that enter the edible plant cells.

Nevertheless, it is well known that adjuvants are mixed with the active ingredient in order to increase the efficiency of formulations. In medicine, adjuvants are also used to increase the molecule absorptions, or the effectiveness of vaccines. In chemical products such as pesticides, they are used to increase targeted toxicity (for example, penetration in leaves or insects), but they do have an effect also on non-specific targets too. Some known adjuvants of Roundup such as polyethoxylated tallowamine (or POEA) showed more toxic effects than glyphosate in various models, and even more than Roundup in some cases on aquatic life for example (Tsui & Chu 2003; Marc et al. 2005) or on human cells (Benachour & Séralini 2009).

By only considering the active ingredient, regulatory thresholds seem to guarantee the safety of residues, however we conclude that it is not the case with the whole formulations, in particular those specific to GMOs. In conclusion, confidentiality on the composition of formulations must be lifted, as announced recently by the U.S. Environmental Protection Agency following our work (EPA 2009). People consuming GMOs are thus
exposed to residues of many formulations which are themselves mixtures of different chemicals. The long term combined effects have never been evaluated, not even in laboratory animals. We suggest that regulatory agencies change their paradigms and integrate modern knowledge, in order to guarantee the safety of pesticides residues, in particular when associated with genetically modified plants.

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Analysis of endocrine disruption effect of Roundup® in adrenal gland of male rats

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\section*{ABSTRACT}
The effect of Roundup\textsuperscript{a} on adrenal gland steroidogenesis and signaling pathway associated with steroid production was investigated. Doses of 10, 50, 100 and 250 mg/kg bw/d Roundup\textsuperscript{a} were administered for two weeks to adult male rats. The 10 mg/kg bw/d dose which reduced circulatory corticosterone levels, but did not change food consumption and body weight, was selected for further study. The expression of cholesterol receptors (low density lipoprotein receptor), de novo cholesterol synthesis enzyme (3-hydroxy-3-methylglutaryl-coenzyme A synthase), hormone-sensitive lipase, steroidogenic acute regulatory protein (SIAR) mRNA and phosphorylated form was decreased. Adrenocorticotropic hormone receptor (ACTH), melanocortin-2 receptor, expression was not changed but circulatory ACTH levels and adrenal cortex protein kinase A (PKA) activity were reduced. Surprisingly, exogenous ACTH treatment rescued steroidogenesis in Roundup\textsuperscript{a}-treated animals. Aromatization was evident at 250 mg/kg bw/d, but not at 10 mg/kg bw/d dose. These results suggest that Roundup\textsuperscript{a} may be inhibitory to hypothalamic-pituitary axis leading to reduction in cyclic adenosine monophosphate (cAMP)/PKA pathway, SIAR phosphorylation and corticosterone synthesis in the adrenal tissue.

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1. Introduction

Agricultural advancements have increased production and correspondingly increased the usage and release of herbicides into the environment. Among the herbicides, the glyphosate-based herbicide Roundup\textsuperscript{a} is most extensively used world over [14]. Roundup\textsuperscript{a} is non-selective and broad spectrum herbicide utilized in agricultural fields, gardens, play grounds, road sides etc. [10]. The half life of Roundup\textsuperscript{a} is 47 days in soil and up to 90 days in water with low microbial metabolism and disintegration [18,37,39]. In rats, Roundup\textsuperscript{a} wholly body pharmacokinetics is biphasic for single 10 mg/kg bw dose with half-life of the alpha phase is 6 h and 79–106 h for beta phase [43]. Roundup\textsuperscript{a} and its metabolite, aminomethyl-phosphonic acid, have been detected in water and crops [1,12,26,35]. Therefore, there is increased probability of Roundup\textsuperscript{a} exposure to animals and human, and it becomes of interest to study its toxic effects, if any.

Glyphosate or its formulation Roundup\textsuperscript{a} acts via specific inhibition of plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase which is essential for synthesis of aromatic amino acids [19,34,35] and thus, considered non toxic to animals. However, in recent past, several studies have suggested glyphosate toxic effects such as carcinogen [28,38,40], teratogen [12,13] and as an endocrine disruptor (ED). The endocrine disrupting chemicals (EDC) represent a broad class of exogenous substances that adversely affect the endocrine system by interfering with hormone biosynthesis, metabolism or action [23]. As an EDC, glyphosate and its formulation, Roundup\textsuperscript{a} was reported to decrease testosterone hormone levels in adult rats [9]. The prenatal exposure
of glyphosate disrupted the masculinization process and caused endocrine dysfunction in reproductive parameters of male offspring [32]. Moreover, various formulations of glyphosate including Roundup® were reported to disrupt aromatase activity, enzyme required for estrogen synthesis, in human liver HepG2 cells [15]. The human placental Jeg3 cells treated with Roundup® altered aromatase mRNA levels and enzymatic activity by interacting with the active site of the purified enzyme [31]. A study involving MA-10 Leydig tumor cell line reported down regulation of STAR mRNA levels, a key regulatory steroidogenic gene, and dibutyryl (cAMP-stimulated) progesterone production upon treatment [42]. It has been observed that the commercial formulation had more adverse effects than the active ingredient, i.e., glyphosate [3]. The EDC effects of Roundup® in male reproductive system have been described; however, studies detailing EDC effects on the adrenal gland steroidogenesis have not been reported in animals. In the present study, experiments have been conducted to examine effects of Roundup® on adrenal steroidogenesis at systemic as well as at the tissue level. Different doses of Roundup® were orally administered to adult male rats for 14 days daily although, only the lowest dose required for disrupting the major adrenal gland steroid hormone i.e., corticosterone level was selected for further detailed study. The EDC effect of Roundup® was examined on important regulatory genes for steroidogenesis: STAR and p450, the steroid precursor, cholesterol levels, and cholesterol homeostasis genes and the signaling involved. An ACTH challenge experiment was also performed to evaluate Roundup® action to be via hypothalamic pituitary axis or directly upon the gland.

2. Material and methods

2.1. Chemicals and antibodies

Glyphosate formulation, Roundup® was procured from Monsanto India Ltd., Mumbai, India. Porcine ACTH, TRIp, custom made primers, oligo(dT) and QNTPs were obtained from Sigma-Aldrich Co. (Bangalore, India). The kits purchased for various hormone assays were as follows: rat corticosterone ELISA from Neogen (Lansing, MI), rat corticosterone EIA from Cayman (Ann Arbor, MI), testosterone RIA from Immunotech (Marseille, France) and rat ACTH Ultra-sensitive lumELISA kit from Calbiotech Inc. (Spring Valley, CA). Amplx® red cholesterol assay kit and SYBR Green PCR Master Mix were purchased from Molecular Probes, Life Technologies (Carlsbad, CA). Reverse transcriptase (RevertAid) was from Thermo scientific (Walther, MA). DNase I (RNase free) was from New England Biolabs Inc. (Ipswich, MA). PVDF membrane (Immobilon P) was procured from Millipore (Billerica, MA). Protein molecular weight markers (PageRulerTM Prestained Protein Ladder) and Western blotting detection reagents (SuperSignalTM West Femto Maximum Sensitivity Substrate) were from Thermo Fisher Scientific Inc. (Waltham, MA). Antibody against pCREB (Ser 133) (#9198), CREB (#9197), cleaved Caspase 3 (#9915), goat anti-rabbit IgG, HRP-linked Antibody (#7074) were from Cell Signaling Technology (Danvers, MA) and β-actin (#3700) from Sigma-Aldrich Co. (Bangalore, India). Antibodies for pSTAR and STAR were kind gifts from Professor Steven King (Baylor College of Medicine, Houston, TX) and Professor DM Stocco (Texas Tech University Health Sciences Center, Lubbock, TX). HDL and LDL/VLDL quantitation kit was procured from Sigma-Aldrich Co. (Bangalore, India). All other chemicals, unless otherwise noted were purchased from Sigma-Aldrich Co. (Bangalore, India) or sourced locally.

2.2. Animal experiments

All procedures in animals were approved by the Institutional Animal Ethical Committee, Indian Institute of Science, Bangalore, India. In this study, 2–2.5 months old male Harlan Wistar rats were used. One rat per cage was housed in the room with 12:12 cycle of light/dark each under a controlled temperature of 24–26 °C. Rats were allowed free access to standard chow diet and drinking water ad libitum. For this study, rats were randomly divided into five groups with 5 or more animals per group. Rats were administered with 24.4, 12.19, 24.4 and 60.8 μL of Roundup® (41% w/w) which corresponds to 10, 50, 100 and 250 mg/kg bw/d of glyphosate, respectively. The highest dose tested was well below LD₅₀, 4900 mg/kg bw. Roundup® was dissolved in deionized water to make the total volume of 300 μL except for 250 mg/kg bw/dose, in which Roundup® was administered directly. The vehicle (0 mg/kg bw/d) group received 300 μL of deionized water only. The treatments were oral, once daily (0800–0900h) for 14 days. Body weight and food consumption were monitored twice weekly. Food consumption was calculated by subtracting food pellet weight remaining in the cage mesh from the total food pellets provided to each rat cage mesh. A total 10 rats for control, n = 10 rats for 10 mg/kg, n = 5 rats for 50 mg/kg, n = 10 rats for 100 mg/kg and n = 10 rats for 250 mg/kg bw/dose group were utilized in the study. On day 15 of treatment, animals were weighed and anesthetized with 50 mg/kg bw/d pentobarbital sodium (Sigma Chemical Co., St. Louis, MO) and blood was collected by cardiac puncture. Plasma was separated by centrifugation at 2000 × g for 15 min and stored at −20 °C until analyzed. The anesthetized animals were killed by cervical dislocation. Adrenal glands were dissected out, weighed, transferred to neutral buffered formalin, hydro (NBF) solution or snap frozen in liquid nitrogen and stored in −70 °C freezer until analysis. To examine the effect of exogenous adrenocorticotropic hormone (ACTH) on adrenal gland steroidogenesis, a standardized dose of 5 IU of Porcine ACTH was injected i.v., based on the protocol reported by others [21, 30, 41] to vehicle (n = 4 rats) as well as Roundup® 10 mg/kg bw/d (n = 3 rats) treated animals. Blood sample and adrenal glands were collected after 60 min of ACTH treatment.

2.3. Hormone assays

Two different kits have been utilized for determining plasma corticosterone levels. The steroids extraction from plasma was carried out by using diethyl ether or methylene chloride (Merck, Billerica, MA) as per requirement of the kits. The corticosterone levels obtained from different sources gave similar concentration of corticosterone. The inter- and intra-coefficient of variations of assay were <15%. Plasma concentrations of testosterone were measured by testosterone RIA kit according to the manufacturer’s protocol. ACTH in plasma was measured using luminescence based ELISA kit for rats.

2.4. Cholesterol assay

Adrenal gland tissue lysate was prepared by homogenizing 0.5 mg tissue in unit ml of 10% SDS containing phosphate buffered saline (PBS) (Sigma–Aldrich Co., Bangalore, India). Tissue debris was removed by centrifugation. The tissue lysate and plasma were analyzed for total cholesterol and esterified cholesterol by using the Amplx® red cholesterol assay kit as per the manufacturer’s instructions. Briefly, plasma or tissue sample were mixed with equal volume of Amplx® red working reagent with and without cholesterol esterase. The reaction mixture was then incubated for 30 min at 37 °C in the dark. The fluorescence values were read at an excitation wavelength of 545 nm and an emission wavelength
of 590 nm (Tecan Infinite F200 Microplate Reader, Männedorf, Switzerland). A series of cholesterol standards were prepared that were provided in the kit and ran alongside the plasma and tissue lysates samples. Plasma HDL (high-density lipoprotein) and LDL (low-density lipoprotein) were analyzed by commercially available HDL and LDL/VLDL quantitation kit according to the manufacturer’s instruction.

2.5. qPCR analysis

mRNA expression of key regulatory receptors, enzymes and carrier proteins involved in the cholesterol homeostasis and sterol biosynthesis were determined by qPCR analysis as previously described from the laboratory Byganka et al. [45] using ABI 7500 Real-Time PCR instrument. Briefly, total RNA was isolated from adrenal glands by TRIzol® method, treated with DNase I before performing reverse transcription for cDNA preparation with oligo(dT). Real-time PCR was performed with each reaction carrying 10 ng of sequences, except for gene. The details of primers employed along with the sequence source are provided in Table 1. Expression level of individual gene was normalized to Rp19 expression which was used as calibration (internal control) for each cDNA sample. PCR for each sample was set up in duplicates and the average Ct value was used in the ∆∆Ct equation.

2.6. Hematoxylin and eosin (H&E) staining

The neutral buffered formaldehyde (NBF) fixed adrenal gland was sectioned (5 μm thick) and stained as reported elsewhere [45]. The sections were mounted in DPX (Sigma-Aldrich Co., Bangalore, India) and visualized under light microscope (Olympus IX81 inverted microscope, Tokyo, Japan).

2.7. Oil Red O staining

The 5 μm thick cryosections of frozen adrenal glands were prepared and fixed in NBF, washed with PBS (Sigma-Aldrich Co., Bangalore, India) and stained with Oil Red O (ORO) (Sigma-Aldrich Co., Bangalore, India) for lipid detection. Nuclear staining, sections were equilibrated in McIlwaine’s Citric acid; NaOH buffer and stained with DAPI and the sections were visualized under florescent microscope (Olympus IX81 inverted microscope, Tokyo, Japan).

2.8. Immunoblot analysis

The adrenal gland tissue was homogenized using RIPA buffer with protease inhibitors as per the procedure reported earlier [44] and the lysates were stored at -70°C until further use. Total protein estimation was performed by Bradford method (Bio-Rad Laboratories, Berkeley, CA). Tissue lysates (30 μg protein) were resolved on 12% SDS-PAGE and transferred onto PVDF membrane using a wet transfer unit (Bio-Rad Laboratories, Berkeley, CA). Non specific sites on the membrane were blocked using 10% milk in TBST (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) by incubating it for 1 h at room temperature. The membrane was incubated overnight at 4°C with primary antibody at 1:5000 dilution specific for pCREB (Ser133) CREB, Cleaved Caspase-3, pS6K, Insulin and β-actin. The membrane was washed with TBST and incubated with secondary antibody (horseradish peroxidase labeled anti rabbit IgG) at 1:3000 dilutions. The bands were visualized using Western blot imaging system (Fleischharn FC2, Cell Biosciences Inc., Santa Clara, CA) and the band intensity was quantitated by Gene to tool software (Syngene, Cambridge, UK).

2.9. PKA assay

PKA assays were performed as per the previously published procedure [29] using SigmaTECT® EAMP dependent protein kinase (PKA) assay kit (Promega, Madison, Wisconsin). The activity of PKA was determined by measuring the incorporation of 32P from [γ-32P] ATP into adrenal gland PKA to benzyliated kepide, a highly specific peptide substrate. Briefly, the medullary region of adrenal gland was removed carefully from the decapitated gland under dissecting microscope in ice cold PBS solution. The adrenal medullary region specific gene, PNMT expression was found to be detectable in the dissected corticosterone fraction (data not shown). The cortical fraction was homogenized in cold extraction buffer containing 25 mM Tris–HCl; 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β ME; 1 μg/ml Aprotinin and 1 μg/ml Leupeptin and centrifuged at 4°C, 14,000 × g for 5 min. After protein estimation, 10 μg protein was used to perform the assay by incubating with [γ-32P] ATP at 30°C for 5 min. The reaction was terminated by adding stopping buffer provided with the kit and 10 μl of reaction mixture was spotted onto SAM blot capture membrane. The individual membrane square was dried and then transferred to liquid scintillation vials by counting in β counter (Tri Carb B2950TR liquid scintillation analyzer, Waltham, MA). A control reaction without the substrate was also performed for determining the background activity which was subtracted from the total activity of samples.

2.10. TUNEL assay

Assay was performed using TACS® Tdt DAB in situ apoptosis detection kit ( Trevigen Inc., Gaithersburg, MD) according to manufacturer’s protocol. Briefly, NBF fixed adrenal gland from vehicle, 10 and 250 mg/kg bw drug treated animals were sectioned into 5 μm thickness. Sections were rinsed in xylene and rehydrated using ascending grades of alcohol solution in PBS. Sections were treated with Proteinase K followed by TdT labeling reaction mix and buffer incubation. The reaction was stopped by Tdt Stop Buffer provided with the kit. The sections were washed thoroughly in PBS and treated with HRP conjugated streptavidin in a humid chamber. After PBS washing, the sections were exposed to DAB provided in the kit, till development of brown color, followed by hematoxylin staining for nuclei and the sections were observed under light microscope. The positive and negative controls for the technique were included by addition of nucleases during incubation and removal TdT enzyme from the labeling mix, respectively.

2.11. Statistical analysis

Data were expressed as mean ± SEM. A t-test was used to calculate p value between two groups. Multiple comparisons were made between vehicle and Roundup® treatment groups using Bonferroni’s test after one way ANOVA Prism version 5 (GraphPad, California) was utilized for statistical analysis. A p value <0.05 was considered statistically significant throughout.

3. Results

3.1. Effect of Roundup® on body weight and food consumption

No overt signs of toxicity were observed during oral administration of Roundup® up to dose of 250 mg/kg bw/d for 14 days. However, food consumption and body weight were significantly lower beginning with 50 mg/kg bw/d dose (Table 2A and B). This might be due to toxic effects of Roundup® other than EDC effect, hence doses higher than 50 mg/kg bw/d were not included for further analysis.
2.2. Roundup® effect on circulatory levels of steroid

Plasma testosterone and corticosterone, the representative steroid hormone from major endocrine sources i.e., testis and adrenal gland of male rat, were assayed (Fig. 1A and B). Both hormone concentrations were lowered after Roundup® treatment in a dose dependent manner. The lowest dose 10 mg/kg bw/d itself could decrease corticosterone levels significantly (p < 0.05), therefore this dose was selected for detailed EDC studies.

3.3. Roundup® effect on expression of genes associated with steriodogenesis

The key regulatory steps in steriodogenesis are transport of cholesterol from outer to inner mitochondrial membrane by STAR protein and cholesterol side chain cleavage step by CYP11A1 enzyme. The mRNA expression of P450scc was unchanged, while STAR mRNA and total protein were down regulated in Roundup® (10 mg/kg bw/d) treated animals compared to vehicle group (Fig. 2A and B). Phosphorylated form of CREB, the transcriptional regulator of STAR expression, was down regulated in 10 mg/kg bw/d Roundup® treated rats and phosphorylated STAR protein was also found to be significantly lower (p < 0.05) in the 10 mg/kg bw/d treatment group (Fig. 2C and D).

3.4. Roundup® effect upon circulatory and adrenal gland lipid for cholesterol content

Together with steriodogenesis process, the homeostasis of steriod precursor cholesterol was also examined. The level of cholesterol in the circulation as well as in the adrenal gland was measured in vehicle and Roundup® treated animals. Circulatory levels of total, free and esterified cholesterol were unchanged except for 100 mg/kg bw/d dose (Fig. 3A). A dose-dependent increase in HDL and LDL levels was observed (Fig. 3B and C). Cholesterol was found to be moderately higher in the adrenal gland (Fig. 4A). The weight of adrenal glands (paired weight) was observed to be significantly lower (p < 0.05) in Roundup®-treated animals. H&E and ORO staining indicated moderately higher number of lipid droplets present in the adrenal gland of 10 mg/kg bw/d Roundup®-treated rats (Fig. 4C and D).

3.5. Effect of Roundup® treatment on genes involved in cholesterol intake and de novo synthesis

The RNA expression of receptors for cholesterol uptake i.e., low-density lipoprotein receptor (Ldlr) found to be significantly lower (p < 0.05) however, expression of high-density lipoproteins receptor (Sdh1) was unaltered in the adrenal gland of 10 mg/kg bw/d Roundup®-treated group compared to vehicle group (Fig. 4A and B). Gene expression for enzymes involved in cholesterol de novo synthesis were found to be unchanged (Hmgcr) or significantly

<table>
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(A) Average food consumption (g) per animal per day and (B) body weight (g) during Roundup® treatment.

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<th>Days of treatment</th>
<th>Control (n = 4)</th>
<th>10 mg/kg bw/d (n = 4)</th>
<th>50 mg/kg bw/d (n = 3)</th>
<th>100 mg/kg bw/d (n = 3)</th>
<th>250 mg/kg bw/d (n = 3)</th>
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<td>0-3</td>
<td>16.75 ± 1.2</td>
<td>16.3 ± 1.5</td>
<td>16.7 ± 0.4</td>
<td>16.1 ± 1.0</td>
<td>15.2 ± 0.4</td>
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<td>4-7</td>
<td>21.6 ± 1.8</td>
<td>19.9 ± 1.6</td>
<td>18.55 ± 0.12</td>
<td>15.2 ± 0.4</td>
<td>13.8 ± 0.8</td>
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<td>8-11</td>
<td>22.4 ± 1.9</td>
<td>20.2 ± 1.8</td>
<td>19.15 ± 0.6</td>
<td>12.8 ± 1.1</td>
<td>11.7 ± 0.4</td>
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<td>12-15</td>
<td>23.19 ± 1.9</td>
<td>20.3 ± 1.7</td>
<td>18.4 ± 0.8</td>
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<th>Days of treatment</th>
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<th>50 mg/kg bw/d (n = 3)</th>
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<td>233 ± 15.6</td>
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<td>253.5 ± 15.3</td>
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<td>207.5 ± 8.9</td>
<td>170.4 ± 6.8</td>
<td>176 ± 5.5</td>
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</table>

Values are mean ± SEM. Vehicle group received milliliter water for 14 days and other groups administered with different doses of Roundup® for 14 days. Statistical significance is as follows:

- *p < 0.05
- **p < 0.01
lower (p < 0.05) (Hmgcs) in the adrenal gland of 10 mg/kg bw/d Roundup®-treated animals (Fig. 4C and D). Although expression of genes associated with cholesterol mobilization in the gland was lower, there was a moderately higher accumulation of cholesterol in adrenal cells which could be due to decreased utilization of cholesterol in Roundup®-treated animals.

3.6. Effect of Roundup® treatment on esterified cholesterol (CE) and ester hydrolysis

Stored form of cholesterol i.e., CE was estimated by calculating free from total cholesterol. CE tended to be moderately higher in the adrenal gland of Roundup®-treated animals (Fig. 5A). Cholesterol ester hydrolyase or hormone sensitive lipase (Hsl), catalyzes the hydrolysis of CE into their free form, was found to be lower (Fig. 5B) even though changes in both CE and Hsl, were not statistically significant (p > 0.05).

3.7. Roundup® effect on adrenal gland ACTH receptor expression and circulatory ACTH levels

The ACTH receptor, Mc2r, expression in the adrenal gland was found to be unchanged in 10 and 50 mg/kg bw/d Roundup®-treated rats (Fig. 6A), however the circulatory levels of ACTH were lower in Roundup®-treated rats (Fig. 6B). The results suggest that Roundup® treatment might have decreased the synthesis or release of ACTH from the pituitary gland. Lower ACTH levels might explain observed down regulation in STAR and Hsd expression, expression of both genes regulated by ACTH in the adrenal gland post Roundup® treatment.

3.8. Effect of exogenous ACTH upon circulatory corticosterone levels in Roundup®-treated animals

To examine ACTH responsiveness in the adrenal glands of Roundup®-treated animals, a 5 IU dose [based on previous literature as well as pilot study carried out for the present work (data not presented)] of porcine ACTH administered i.e. to vehicle and Roundup®-treated animals. Administration of exogenous ACTH increased corticosterone levels significantly (p < 0.05) at 60 min in Roundup®-treated rats compared to vehicle treated rats (Fig. 7). Exogenous ACTH treatment also increased STAR, Hsd expression (Fig. 8A and B) and the expression of cholesterol homoeostasis genes (Sri1, Ldr, Hmgcs, Hmgcs, Hsl) in the adrenal gland of Roundup®-treated rats (Fig. 8D–G). The results indicate that responsiveness of the adrenal gland to ACTH was intact in the Roundup®-treated rats.
3.9. Effect of Roundup® on adrenal gland PKA activity

The cAMP/PKA pathway activated by ACTH in the adrenal gland was examined by quantifying PKA activity. Equal amount of gland cortical region total protein from vehicle and 10 mg/kg bw/d Roundup® and/or ACTH treated rats, was utilized for the assay. The PKA activity was significantly lower (p < 0.01) in Roundup®-treated rats, while exogenous ACTH treatment increased the activity (p < 0.001) (Figs. 8C). The results suggested that Roundup® decreased endogenous ACTH levels and in turn cAMP/PKA pathway in the adrenal gland tissue Figs. 9 and 10.
Fig. 5. Expression of genes associated with cholesterol import and de novo synthesis in the adrenal gland of vehicle and Roundup®-treated animals. qPCR analysis was performed to quantify the expression of genes involved in cholesterol transport (Srb1, Ldlr) (A and B) and Cholesterol de novo synthesis (Hmgcs, Hmgcr) (C and D) in the vehicle and Roundup®-treated group. Data presented as mean ± SEM (n = 5 rats per group). Student’s t-test was performed to compare treatment group from vehicle group. *p < 0.05.

Fig. 6. Estriol cholesterol levels (A) of adrenal gland from vehicle and 10 mg/kg bw/d Roundup®-treated animals. Adrenal glands were isolated post treatment and lysis was prepared. Estriol cholesterol levels were obtained by subtracting free cholesterol levels from total cholesterol values. qPCR was performed to detect the gene expression in the adrenal gland of vehicle and 10 mg/kg bw/d Roundup®-treated animals (B). Bmp19 was used as internal control. qPCR values were plotted in relation to the vehicle treated group. Values are presented as mean ± SEM (n = 6 rats per group). Student’s t-test was performed to compare the two groups.

3.10. Analysis of Roundup® effect on cell toxicity

Immunoblot analysis of cleaved caspase 3 (as marker of apoptosis) was performed on the adrenal gland tissue lysate of vehicle and Roundup®-treated animals (Supplementary Fig. 1A and B). It was observed that cleaved caspase-3 levels were up-regulated in 250 mg/kg bw/d dose treated rats while the levels were unchanged in the 10 mg/kg bw/d treated group. TUNEL assay for in vivo apoptosis was performed to determine DNA fragmentation (Supplementary Fig. 1C). The incidence of apoptotic cells in the adrenal gland cortical region was higher in 250 mg/kg bw/d treated rats compared to vehicle and 10 mg/kg bw/d dose of Roundup®-treated rats.

Supplementary material related to this article found in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.07.021.
4. Discussion

4.1. The effect of Roundup® is demonstrable at the low dose of 10 mg/kg bw/d, while toxic effects are evident at the high dose.

The present study was conducted to examine EDC effect of Roundup® on adrenal gland steroidogenesis and to determine its mechanism of action. For this purpose, after determining the effect of ranges of doses on different parameters such as food consumption, body weight etc., the lowest dose which was devoid of obvious toxic effects other than the EDC effect. A dose of 50 mg/kg bw/d Roundup® manifested significant loss in body weight but not in food consumption, while doses higher than 50 mg/kg bw/d caused decrease in food consumption as well as body weight of rats in the second week of the treatment. In previous studies, it was observed that Roundup® exposure did not change body weight of male Spraque-Dawley rats at a dose of 560 mg/kg bw Roundup® for 91 days [4], while female Spraque-Dawley pregnant rats exposed orally to Roundup® with 500 mg/kg bw/d and higher doses for 10 days of pregnancy, reduced food consumption as well as body weight [12]. The discrepancy of the effect of Roundup® observed in the present as well as in other studies may be related to the differences in strains of rats, age of rats and duration of treatment employed. In order to circumvent possible effects of Roundup® on causing stress and other toxicity related effects, the doses of Roundup® higher than 50 mg/kg were not used for studying the endocrine disrupting effect.

Further, circulatory corticosterone levels in the doses 10 and 50 mg/kg bw/d were observed to be lower compared to the vehicle treated group. To verify that the decrease in corticosterone is due to possible EDC effect of Roundup®, another steroid hormone testosterone level was also determined. The Roundup® has previously been reported to inhibit testosterone levels and the results in present study are in agreement with others [9,5,32]. The result observed suggest suitability of the dose 10 mg/kg bw to assess the EDC effect of the Roundup®.

In cancer cell line studies, Roundup® has been reported to have apoptotic effect [7,27]. We examined one of the makers of apoptosis and performed TUNEL assay in adrenal glands and observed evidence for increased apoptosis at higher dose of 250 mg/kg bw/d compared to vehicle but not in 10 mg/kg bw/d treated rats. Therefore, decreased corticosterone level that was seen at low dose may not be attributed to toxicity or cell death induced by Roundup® treatment.
Fig. 9. Expression of SfAR and genes associated with cholesterol homeostasis post vehicle, Roundup® and/or ACTH treatments. qPCR analysis was performed for SfAR (A), Hsd11b2 (B), cholesterol homeostasis-related genes (Slc27a1, Ldlr, Hmgcs1, Hmgcs2) (D–G). The cortisol regulatory pathway was utilized to quantitate PKA activity (C). Values are presented as mean ± SEM (n = 3–4 rats per group). Fisher's test was performed to calculate significance between treatment and vehicle groups. *, ** & *** significantly different from the vehicle group by p < 0.05, p < 0.01, p < 0.001 viz.

Fig. 10. Schematic representation of EDG mechanism of glyphosate formulation on adrenal gland steroidogenesis under in vitro condition. Roundup® appears to act at HPA axis to down regulate endogenous ACTH levels which in turn down regulates cAMP/PKA pathway. Lowered activity of PKA leads to down regulation in CREB and SfAR phosphorylation, leading to down regulation of SfAR function and steroidogenesis.
4.2. Roundup® negatively regulates SAST in the adrenal gland via CAMPP/PKA pathway

The analysis of expression of genes associated with steroidogenesis such as STAR and P450scr, revealed no change in P450scr expression, but decrease in STAR expression, both at mRNA and protein levels. Moreover, phosphorylated STAR expression showed greater degree of down regulation compared to total protein and RNA levels. The difference between total STAR and phosphorylated STAR expression levels post Roundup® treatment, suggests gene regulation at two levels, one at transcription and another at phosphorylation process. Phosphorylation of STAR at serine 194/195 enhances the cholesterol transport capacity to at least 40-50%. Significant down regulation in phosphorylated STAR levels observed in the present study suggest decreased pSTAR could be responsible for down regulation of steroidogenesis in the adrenal gland of Roundup®-treated rats. Interestingly in the present study higher lipid droplet accumulation was observed in Roundup®-treated rats and this is one of the characteristic of the STAR gene knockout mice [17]. The finding that phosphorylated CREB was lower, may be correlated to STAR down regulation, since CREB phosphorylation is involved in STAR transcription [24]. However to what extent that CREB down regulation contributed to decrease in STAR mRNA levels, remains to be explored. It should be pointed out that in the adrenal gland, ACTH upon binding to its cognate receptor MC2r, activates CAMPP/PKA pathway leading phosphorylation of CREB and STAR protein [2]. In the adrenal gland of Roundup®-treated rats, the lowered PKA activity was reversed post ACTH treatment confirming CAMPP/ PKA pathway to be disrupted in the adrenal gland of Roundup®-treated animals.

4.3. Roundup® alters cholesterol homeostasis moderately

Roundup®-treated animals did not show altered total cholesterol levels in circulation at the lowest dose i.e., 10mg/kg b.w/d, but it was observed to be moderately higher in the adrenal gland. With this observation, it can be hypothesized that the cholesterol homeostasis within gland may be altered by increased cholesterol intake and/or de novo synthesis. Interestingly, there was down regulation of genes associated with cholesterol intake (Ldlr, Scarb1) and de novo synthesis (Hmgcs, Hmgcr). Also, analysis revealed higher levels of esterified or stored form of cholesterol in the adrenal gland of Roundup®-treated rats. The data taken together suggest that increased levels of stored cholesterol might be due to lowered utilization and/or lowered hydrolysis of esterified form. Hsl expression was not significantly altered in the present study. Hsl or lip gene is involved in cholesterol ester hydrolysis [22] and reported to be regulated by CAMPP/ PKA pathway [20]. Therefore, Roundup® appears to act via CAMPP/ PKA pathway and regulate STAR phosphorylation negatively leading to decrease in cholesterol utilization and increase in cholesterol stored in adrenal glands. Interestingly, increase in the weight of adrenal gland was observed in Roundup®-treated animals, but its significance is not clear. The study examining diethylnitrosamine effects on the adrenal gland steroidogenesis has suggested steroid metabolic changes to be the contributing factor for increased weight [16].

4.4. Roundup® acts via HPA axis

Since Roundup® treatment at a dose of 10 mg/kg b.w/d decreased corticosterone levels, it became of interest to examine the responsiveness of adrenal gland to exogenous ACTH treatment. The findings that the adrenal gland was responsive to ACTH treatment in Roundup® treated animals suggest that Roundup® acts at a site higher than the adrenal gland and this indirectly sug-

jects that ACTH synthesis and/or release may be affected. Since the adrenal gland responsiveness to external ACTH was found to be similar or higher compared to vehicle-treated animals suggest the process of steroidogenesis in the adrenal gland appears to be intact post herbicide treatment. Therefore, it can be inferred that the stimulation of adrenal gland i.e., ACTH synthesis and release appears to be impaired rather than defects in the steroidogenesis machinery of the adrenal gland. A significantly higher increase in corticosterone levels in response to supraphysiological dose of ACTH was observed in Roundup®-treated rats compared to vehicle treated rats is perhaps due to higher stored cholesterol content in the adrenal gland of Roundup®-treated animals. The mechanism of action may vary with different experimental system e.g., observed higher testosterone, LH and FSH concentrations in second generation or pups of Roundup®-treated Wistar rat dams in contrast to lowered testosterone observed in the present study as well as other studies where adult rats or different cell lines have been studied. Nonetheless, the results are in agreement with a pilot study of photoperiod exposure to Junata fish [6]. In sum, the data suggest Roundup® appears to act at the hypothalamo-pituitary level under in vivo conditions.

4.5. Implications of the study

The recent information regarding Roundup® and its metabolites detection in food, water and in human urine [1] signifies the relevance of toxicological studies as one presented. The findings that Roundup® treatment down regulates endogenous ACTH, is similar to the condition known as adrenal insufficiency in humans. This condition manifests as fatigue, anorexia, sweating, anxiety, shakiness, nausea, heart palpitations and weight loss. Chronic adrenal insufficiency could be fatal, if untreated. A progressive increase in its prevalence has been observed in humans [19], while a few very recent studies relating to xenobiotic exposure and adrenal insufficiency development have been reported. The present study describes one of the possible mechanisms of adrenal insufficiency due to Roundup® and suggests more systematic studies, to investigate the area further.

Acknowledgements

We are grateful to Professor Steven King (Baylor College of Medicine, Houston, TX) and Professor DM Stocco (Texas Tech University Health Sciences Center, Lubbock, TX) for providing phospho STAR and total STAR antibodies. AP was supported by a fellowship from the Council of Scientific and Industrial Research, New Delhi, India. This work was supported by grant from the Department of Science and Technology FIST, Government of India.

References

Review

Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: A meta-analysis and supporting evidence

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2Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, USA
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ABSTRACT

Glyphosate is the most widely used broad-spectrum systemic herbicide in the world. Recent evaluations of the carcinogenic potential of glyphosate-based herbicides (GBHs) by various regional, national, and international agencies have engendered controversy. We investigated whether there was an association between high cumulative exposures to GBHs and increased risk of non-Hodgkin lymphoma (NHL) in humans. We conducted a new meta-analysis that includes the most recent update of the Agricultural Health Study (AHS) cohort published in 2018 along with five case-control studies. Using the highest exposure groups when available in each study, we report the overall meta relative risk (meta-RR) of NHL in GBH-exposed individuals was increased by 41% (meta-RR = 1.41, 95% confidence interval, CI: 1.13-1.75). For comparison, we also performed a secondary meta-analysis using high-exposure groups with the earlier AHS (2005), and we calculated a meta RR for NHL of 1.45 (95% CI: 1.11-1.91), which was higher than the meta-RRs reported previously. Multiple sensitivity tests conducted to assess the validity of our findings did not reveal meaningful differences from our primary estimated meta-RR. To contextualize our findings of an increased NHL risk in individuals with high GBH exposure, we reviewed publicly available animal and mechanistic studies related to lymphoma. We documented further support from studies of malignant lymphoma incidence in mouse treated with pure glyphosate, as well as potential links between glyphosate / GBH exposure and immunosuppression, endocrine disruption, and genetic alterations that are commonly associated with NHL or lymphomagenesis. Overall, in accordance with findings from experimental animal and mechanistic studies, our current meta-analysis of human epidemiological studies suggests a compelling link between exposures to GBHs and increased risk for NHL.

1. Background

1.1 Global usage of glyphosate-based herbicides

Glyphosate is a highly effective broad spectrum herbicide that is typically applied in mixtures known as glyphosate-based herbicides (GBHs) and commonly sold under the trade names of Roundup® and Ranger Pro®. Use of GBHs has dramatically increased worldwide in recent decades (Fig. 1). In the United States alone, usage increased nearly sixteen-fold between 1992 and 2009 [1]. Most of this increase occurred after the introduction of genetically modified glyphosate-resistant "Roundup-ready" crops in 1996 [1]. In addition, there have been significant changes in usage. In particular, the practice of applying GBHs to crops shortly before harvest, so-called "green burnouts," began in the early 2000s to speed up their desiccation; as a consequence, crops are likely to have higher GBH residues [2]. By the mid-2000s, green burnouts became widespread, and regulatory agencies responded by increasing the permissible residue levels for GBHs (3.4).

Abbreviations: AHS, Agricultural Health Study; c-NHEJ, canonical non-homologous end joining pathway; CI, confidence interval; EDC, endocrine disrupting chemical; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; ETS, environmental tobacco smoke; GBHs, glyphosate-based herbicides; IARC, International Agency for Research on Cancer; IFNγ, interferon gamma; IL-2, Interleukin-2; JNFR, Joint Meeting on Pesticide Residues by the Food and Agriculture Organization of the United Nations and World Health Organization; meta-RR, meta-analysis relative risk; mg/kg/day, milligrams per kilogram per day; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; OR, odds ratio; ppm, parts per million; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis; RR, relative risk

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1.2. Ubiquitous exposure in humans

Glyphosate and its metabolites persist in food [5–7], water [8], and dust [9], potentially indicating that exposure in the general population is ubiquitous. Non-occupational exposures occur primarily through consumption of contaminated food, but may also occur through contact with contaminated soil [9], dust [9] and by drinking or bathing in contaminated water [8]. In plants, glyphosate may be absorbed and transported to parts used for food; it has been detected in fish [5], berries [6], vegetables, baby formula [7], and grains [10], and its use as a crop desiccant significantly increases residues. GBH residues in food persist long after initial treatment and are not lost during baking [11].

Limited data exist on internal glyphosate levels among GBH-exposed individuals [12]. Average urinary glyphosate levels among occupationally exposed subjects range from 0.26–7.15 µg/L, whereas levels in environmentally exposed subjects have been reported between 0.13–7.6 µg/L [12]. Two studies of secular trends have reported increasing proportions of individuals with glyphosate in their urine over time [13,14]. Given that more than six billion kilograms of GBHs have been applied in the world in the last decade [3], glyphosate may be considered ubiquitous in the environment [15].

1.3. Controversy surrounding the carcinogenic potential of GBHs

Exposure to GBHs is reportedly associated with several types of cancer, among which the most well-studied in humans is non-Hodgkin...
lymphoma (NHL). Some epidemiological studies have reported an increased risk of NHL in GBH-exposed individuals [17-18], however, other studies have not confirmed this association [19,20]. GBHs have recently undergone a number of regional, national, and international evaluations for carcinogenicity [21-24], resulting in considerable controversy regarding glyphosate and GBHs’ overall carcinogenic potential. Hence, addressing the question of whether or not GBHs are associated with NHL has become even more critical. Here, we evaluated all published human studies on the carcinogenicity of GBHs and present the first meta-analysis to include the most recently updated Agricultural Health Study (AHS) cohort [25]. We also discuss the lymphoma-related results from studies of glyphosate-exposed animals as well as mechanistic considerations to provide supporting evidence for our analysis of the studies of human exposures to GBHs.

2. Current meta-analysis of GBHs and NHL.

2.1. Meta-analysis objective

Epidemiological studies may vary in several ways, such as by study design, sample size, and exposure assessment methods. Results among individual studies vary and may appear to conflict, which poses challenges in drawing an overall conclusion. Meta-analysis is a quantitative statistical tool that is frequently applied to consolidate the results from similar but separate individual studies so that an overall conclusion about the effects of exposure can be drawn. Here, we conducted a meta-analysis using published human studies to better understand whether the epidemiological evidence supports an association between exposures to GBHs and increased NHL risk. Although three previously published meta-analyses have examined the same association and reported positive meta-risks for GBH-associated NHL [23,26,27], our analysis differs from earlier ones by focusing on an a priori hypothesis targeting exposure magnitude and by including the newly updated AHS study [25].

2.2. A priori hypothesis

Our a priori hypothesis is that the highest exposure to GBHs, i.e., higher levels, longer durations and/or with sufficient lag and latency, will lead to increased risk of NHL in humans. The hypothesis is based on the understanding that higher and longer cumulative exposures are likely to yield higher risk estimates, given the nature of cancer development [28]. Hence, when cumulative exposure is higher, either due to higher level or longer duration exposures, an elevated association with the cancer of interest is more likely to be revealed if a true association exists. This a priori approach has been employed to estimate meta-risks for benzene [29] and formaldehyde [30,31], but not in any of the previous meta-analyses exploring the GBH-NHL association [23,26,27].
<table>
<thead>
<tr>
<th>Author/Location</th>
<th>Subject recruitment</th>
<th>Participation</th>
<th>Exposure assessment</th>
<th>Exposure level</th>
<th>Results for NHL</th>
<th>Weaknesses</th>
<th>Adjustments</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrews et al. (2011) (United States)</td>
<td>Agricultural Health Study</td>
<td>Iowa and North Carolina</td>
<td>Not mentioned</td>
<td>Collection: Self-administered and home questionnaire at time of recruitment; 22 specific pesticides application methods, 98% years of use, and days per use</td>
<td>Exposure: Quarters 1-18 calculated by multiplying lifetime exposure-days by an intensity score</td>
<td>None</td>
<td>Adjusted for study site; education, smoking status, alcohol per month, family history of cancer, history of cancer, age at diagnosis, marital status, education, income, occupation, and health status.</td>
<td>Censored on date the subjects left the study.</td>
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<td></td>
<td>Increased RR for multiple myeloma (OR = 2.4, 95% CI = 1.7-3.3) but with large change given adjustment for multiple myeloma.</td>
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</tbody>
</table>

## Notes
1. Censored on date the subjects left the study.
2. Increased RR for multiple myeloma (OR = 2.4, 95% CI = 1.7-3.3) but with large change given adjustment for multiple myeloma.
<table>
<thead>
<tr>
<th>Subject ascertainment</th>
<th>Participation</th>
<th>Exposure assessment</th>
<th>Exposure level</th>
<th>Results for NHL³</th>
<th>Weaknesses</th>
<th>Adjustments</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Similar, demographics (exposed and unexposed): Similar age, sex, smoking, alcohol. Exposed higher education and family history of cancer. Final size: 92 NHL cancers, 36,509 subjects without missing data. Follow-up from enrollment through Dec. 2001 (5-8 years, median = 6.7 years).</td>
<td></td>
<td>sparsely 4088 subjects, agreement on glyphosate ever use = 83%, days per year Q2 = 52% (13%)</td>
<td></td>
<td>n = 36,823 total, 22 cases in the high exposure group. Similar results comparing highest to lowest tertiles. Adjusting for other pesticides did not change RR by more than 20%. Change with adjustment: No.</td>
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</tbody>
</table>

**Table 1 (continued)**

<table>
<thead>
<tr>
<th>Author/Location</th>
<th>Method</th>
<th>Results</th>
<th>Limitations</th>
<th>Data sources</th>
<th>Other results</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Law, S. et al. (2000)</td>
<td>Telephone (Kanses, Nebraska) or in-person (Iowa/Minn) interviews, SIDS, medical history, smoking, and family history. Nebraska: Specific pesticides, number years used, average days used per year. Iowa/Minn: Specific pesticides, first and last year used, method of use, personal application, and PPP. Kansas: Open ended question about pesticides used, duration and days per year only for pesticide groups, and PPP.</td>
<td>Unknown whether there was full case ascertainment in some areas. For example, incidence rates in Nebraska were 76% of those in SIDS, [13]. Few details provided on Minnesota case surveillance system. Only includes White male subjects. Large number of proxy interviews.</td>
<td>No</td>
<td>Telephone or in-person interviews (Kanses, Nebraska) or in-person interviews (Iowa/Minn).</td>
<td>Matched: Race, sex, age, and vital status.</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **Author/Location:** The authors and their location.
- **Method:** The methods used in the study.
- **Results:** The results of the study.
- **Limitations:** The limitations of the study.
- **Data sources:** The sources of data for the study.
- **Other results:** Additional results from the study.

**Notes:**
- **Telephone or in-person interviews (Kanses, Nebraska) or in-person interviews (Iowa/Minn).**
- **Matched: Race, sex, age, and vital status.**
- **1. Farmers: 54.4% among controls.**
- **2. Exclusions: Subjects who did not work on farms after age 18, subjects with missing data on any of 47 pesticides (about 25% of subjects).**
- **3. In Nebraska, large percentage of farmers reported no pesticide use.**
- **4. Overlapping study with Drum, et al. (54) and Lee et al. (37), both of which were evaluated in the sensitivity analysis.**
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Author/Location</th>
<th>Subject assignment</th>
<th>Participation</th>
<th>Exposure assessment</th>
<th>Exposure level</th>
<th>Results for NHL</th>
<th>Weaknesses</th>
<th>Adjustments</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eriksson et al. [17]</strong> (Sweden, mixed)</td>
<td><strong>Whom, Population, and women</strong></td>
<td>Cases: Age 18-74 diagnosed 1999-2002</td>
<td>Randomly selected from the same</td>
<td>Not described</td>
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<tr>
<td><strong>Ylrala et al. [16]</strong> (Sweden, mixed)</td>
<td><strong>Whom, Population, and women</strong></td>
<td>Cases: Age 18-74 diagnosed 1999-2002</td>
<td>Randomly selected from the same</td>
<td>Not described</td>
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</tbody>
</table>

**Exclusions:**
- 134 of 1163 (11.5%) with medical conditions or deceased.
- Interview rates: Cases: 91%; Controls: 92%
- Proxy interviews: 17% of cases.
- Interviewees blinded to case-control status.

**Collection:**
- Mailed questionnaire on work history, specific pesticides, number of years, days per year, hours per day, and number of days per year.
- Interviewers blinded to case-control status.

**Exposures:**
- One full day, or median number of days exposed in the controls.
- Interviewers blinded to case-control status.

**Unadjusted:**
- OR = 2.02 (1.10-3.71) for NHL.
- OR = 3.65 (0.70-17.97) for NHL.

**Adjusted:**
- OR = 2.26 (1.16-4.40) for NHL.

**1.** Matched: Age and sex

**2.** True participation rates may be lower.

**3.** Use of NHL only.

**4.** Adjustment factors not listed in some analyses.

---

**Erlandsson et al. [16]** (Sweden, mixed) | **Whom, Population, and women** | Cases: Age 18-74 diagnosed 1999-2002 | Randomly selected from the same | Not described | Not described | Not described | Not described | Not described |
| **Lundell et al. [17]** (Sweden, mixed) | **Whom, Population, and women** | Cases: Age 18-74 diagnosed 1999-2002 | Randomly selected from the same | Not described | Not described | Not described | Not described | Not described |

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- 134 of 1163 (11.5%) with medical conditions or deceased.
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<th>Workups</th>
<th>Adjustments</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sundby et al. [16]</td>
<td>Norwegian Cancer Registry, men 12 years and older</td>
<td>Cases: 229, 203, 202 (only diagnosed 1991-94)</td>
<td>Case-control</td>
<td>Interview rates: 67.1%</td>
<td>Percent proxy interviewees: 48.5%</td>
<td>Decreased cases excluded</td>
<td>Not provided</td>
<td>Any exposure</td>
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<tr>
<td>McDuffie et al. [17]</td>
<td>Population based, men 12 years and older</td>
<td>Cases: 229, 203, 202 (only diagnosed 1991-94)</td>
<td>Case-control</td>
<td>Interview rates: 67.1%</td>
<td>Percent proxy interviewees: 48.5%</td>
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<td>Weaknesses</td>
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<tr>
<td>Cases: Diagnosed in one of the main hospitals in the 6 cities (CD-03 codes listed in their Table 1)</td>
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<td>Controls: Hospital controls</td>
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<tr>
<td>Source of controls: Men from the same hospitals, mostly orthopedic and rheumatology, unclear if randomly selected.</td>
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</tr>
<tr>
<td>Similar demographics: Similar for SES, education, rural vs urban</td>
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<tr>
<td>Total size: 244 cases and 426 controls</td>
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</tr>
</tbody>
</table>

Exclusions: History of immunosuppression (% unknown), interview rate, cases: 95.7%, Controls: 91.2%, Exposed group: yes, unmentioned.

Exposure: Any, possible definitive duration greater than the median in the exposed.

Unadjusted: OR = 0.89 (0.66-1.18).

Adjusted: OR = 1.0 (0.85-1.18).

Exposed cases for any exposure.

Change with adjustment: No.

1. Decreased cases; probably not included.
2. Private clinic not included.
3. Unknown if control selection is population based.
4. Population based: not a high exposure group or high risk group.

Matched: Center and age adjusted: Age and center.

Other: Rural vs. urban, type of housing, education, infection, family history, skin characteristics, smoking, and alcohol had little impact on results.

1. Also has multiple myeloma results (OR = 2.4; 0.8-7.3).
2. Results for a few subtypes also given but with small numbers.
3. Farm, agriculture, or forestry work in 92 of 426 controls (21.1%).
Risk estimates, including relative risks (RRs) and odds ratios (ORs), in high exposure groups are less likely to be dominated by confounding or other biases compared to RRs or ORs from groups experiencing average or low exposure [12]. Furthermore, including people with very low exposure in the exposed group can dilute risk estimates. Studying the most highly exposed group is also useful to ensure an adequate exposure contrast, given the potential that most people have been exposed either directly or indirectly to GBHs. Because our main goal is to determine whether there is an exposure effect and not to conduct a precise dose-response assessment or to evaluate risks in people with low exposures, we assert that this a priori hypothesis is appropriate for testing whether or not a GBH-NHL association exists.

2.3. Agricultural Health Study (AHS) update

A recently published update [25] from the large AHS cohort of pesticide applicators (N = 50,000) has been included for the first time in our primary meta-analysis. Although the original AHS report [20] was used in previous meta-analyses [23,26,27], the 2018 AHS update [25] contributes 11-12 additional years of follow-up with over five times as many NHL cases (N = 575 compared to N = 92 in the original study [20]), and > 80% of the total cohort was estimated to be exposed to GBHs. As the largest and most recently published study, it adds substantial weight to the new meta-analysis [23]. We also performed a secondary comparison analysis using our a priori hypothesis with the original AHS report [20] for the purpose of comparing results with: 1) our primary analysis (using AHS 2018); and, 2) other meta-analyses published previously.

2.4. Identifying relevant human studies

The literature search was conducted according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [23]. The screening process and results are shown in Fig. 2. We conducted a systematic electronic literature review using PubMed in November 2017, and we updated it in March 2018 and again in August 2018. We used the following keywords: Glyphosate OR pesticide (MesH or herbicides [MesH]) AND (lymphoma, non-Hodgkin [MesH] OR lymphoma [tiab] OR non-Hodgkin [tiab] OR non-Hodgkin's [tiab] OR lymphomas[tiab] OR lymphomas of [tiab] OR NHL or cancer OR cancers) AND (occupational exposure [MesH] OR occupational exposure [tiab] OR occupational exposures [tiab] OR farmers [MesH] OR farmer OR applicators OR applicator OR agricultural workers OR agricultural worker or workers).

Searches included all cohort, case-control, and cross-sectional studies. No language restrictions were applied, although non-English language articles needed to be obtained in full and translated completely in order to be eligible for inclusion. From the PubMed search, we identified 857 studies. Additionally, we identified 52 studies from the IARC [23] review of glyphosate, and the WHO JMPR [22] report on glyphosate, for a total of 909 studies.

After: 43 duplicates were excluded, 866 studies were initially screened by title and abstract, of which 850 were excluded because they were reports, correspondence, reviews, irrelevant studies (animal, mechanistic, para-occupational), or did not include the exposure or outcome of interest (Fig. 2). When the final 16 qualified epidemiological studies of GBHs and NHL were identified, 10 studies were further excluded because: (1) they did not report RRs, ORs, or the data needed to calculate either [34-36], (2) the cohort overlapped with another study [36,37,41-43], or (3) they did not specify whether the lymphomas were specifically NHL [42]. For studies including overlapping cohorts, we used results from the most complete and updated analysis with the greatest number of participants. Although overlapping, we kept the earlier AHS (2006) [15] for comparison with our primary meta-analysis (using the updated AHS 2018 publication) and with previous meta-analyses. The impact of selecting these studies was evaluated in sensitivity analyses (Section 3.3).

2.5. Review and assessment of selected human studies

2.5.1. Data collection and extraction

In total, six studies (one cohort [25] and five case-control controls studies [16,19-41]) with nearly 65,000 participants were eligible for inclusion in the meta-analysis. Two studies were conducted in the United States, one study was from Canada, two studies were from Sweden, and one study was from France. All six studies reported NHL risks (RRs or ORs) above 1.0, three of which were statistically significant in the original analyses (Table 1). From each study, we abstracted information on study design, location, dates, sample size, participation rates, age, sex, case-control source, diagnosis, histologic verification, exposure assessment, results, and statistical adjustments. Table 1 summarizes key aspects of the design and exposure assessment, the results, strengths, and weaknesses of all the studies evaluated in this meta-analysis, including both versions of the AHS report (α = 6 + 1). As described above, the early AHS data [20] were also evaluated in Table 1 and in the secondary comparison meta-analysis described later.

2.5.2. Study quality evaluation

The methodological quality of the cohort (Table 2) and case-control studies (Table 3) included in the meta-analyses was assessed independently by two co-authors using the Newcastle Ottawa Scale (NOS) [44]. Studies were evaluated based on selection, comparability, and outcome or exposure (in nine categories). Cohort studies were evaluated based on (1) representativeness of the cohort, (2) selection of non-exposed, (3) ascertainment of exposure, (4) demonstration that the outcome of interest was not present at the start of study, (5) comparability of cohort on the basis of controlling for other pesticide use and (6) age, (7) assessment of NHL outcome, (8) sufficiency of follow-up length, and (9) response rate.

Case-control studies were evaluated on (1) the validation of cases, (2) representativeness of cases, (3) selection of controls, (4) absence of disease in the controls, (5) whether the study controlled for other pesticide use and (6) age, (7) exposure assessment, (8) concordance of method among cases and controls, and (9) similarity of response rate among both groups. Each study was awarded a maximum of one point for every item that was satisfied, with a total of 9 available points.

According to our quality assessment (Tables 2 and 3), the highest quality study in either design category was the AHS 2018 cohort [25]. The highest quality case-control study was Eriksson et al. [17], while the lowest quality studies were McDuffie et al. [43] and Orr et al. [19].

2.6. Selection of the most highly exposed category

Based on our a priori hypothesis, when multiple RRs or ORs were given in the original studies, we selected estimates in the following order: (1) highest cumulative exposure and longest lag (the time period preceding NHL onset, which is excluded from the exposure estimate) or latency (time between first lifetime exposure and NHL diagnosis); (2) highest cumulative exposure; (3) longest exposure duration and longest lag or latency; (4) longest exposure duration, (5) longest lag or latency; and (6) ever-exposure. The definition of cumulative exposure includes duration and intensity. As we discuss in more detail later in Section 5.2, cumulative exposure in both AHS reports [20,25] was calculated as an intensity-weighted exposure (lifetime exposure days multiplied by an intensity score) [45,46].

We prioritized highest cumulative exposure based on evidence of glyphosate's persistence in the environment [47-49] and because chronic disease, including cancer, is usually the result of cumulative exposures [50]. We selected the longest lag or latency because decades may be needed for the health effects of many environmental toxicants to manifest as detectable cancers. If no high exposure data were
Table 2
Quality assessment of the cohort studies in meta-analysis.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Study</th>
<th>Selection</th>
<th>Compatibility</th>
<th>Outcome</th>
<th>Overall Quality Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Representativeness of Exposed</td>
<td>Selection of Non-Exposed</td>
<td>Exposure Assessment</td>
<td>NHL. Absent at Start</td>
</tr>
<tr>
<td>Andreotti et al. [25]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>De Roos (2009) [30]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The study quality was assessed according to the Newcastle Ottawa Quality assessment scale for cohort studies [43]. One point was awarded for yes, and zero points were awarded for no, unable to determine, or inadequate.

Table 3
Quality assessment of the case-control studies in meta-analysis.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Study</th>
<th>Selection</th>
<th>Compatibility</th>
<th>Exposure</th>
<th>Overall Quality Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adequate Case Definition</td>
<td>Representativeness of Cases</td>
<td>Control Selection</td>
<td>Definition of Controls</td>
</tr>
<tr>
<td>De Roos (2009) [30]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Eriksson et al. [17]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Harrell et al. [18]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>McDuffe et al. [43]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cui et al. [19]</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The study quality was assessed according to the Newcastle Ottawa Quality assessment scale for case-control studies [43]. One point was awarded for yes, and zero points were awarded for no, unable to determine, or inadequate.
available, we used the ever-exposure estimate. Given the relatively few human epidemiological studies published to date on the topic, we made this decision because we did not want to exclude any potentially relevant data. even though the inclusion of minimally exposed individuals in the "exposed" category could attenuate any potential association of interest.

Although there are different perspectives on the best way to account for other pesticide exposures, we selected RR estimates that adjusted for other pesticide use over their unadjusted counterparts to mitigate potential substantially confounding. Five of the seven studies adjusted for a combination of different pesticides [16, 18, 20, 25]. However, if these multiple pesticides acted synergistically or on different points along a pathway, this approach to adjustment may no longer be the appropriate, and alternatives such as interaction analysis should be considered. Reanalysis of the raw data, which is beyond the scope of this paper, would be helpful to address this possibility.

We evaluated the impact of our a priori exposure selection criteria on the sensitivity analyses. We also conducted a separate meta-analysis of all ever-exposed individuals to assess the magnitude of potential bias caused by adding subjects with low exposures (ever-RR from De Roos et al. [20] was used; the ever-RR estimate from Andreotti et al. [25] was not available). In Table 4 we summarize the risk estimates selected from each original study and the study weights used in the meta-analyses.

Table 4

<table>
<thead>
<tr>
<th>Study</th>
<th>Total Number</th>
<th>Exposure Category</th>
<th>Risk Estimate 95% CI</th>
<th>Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHS Cohort</td>
<td>357/575</td>
<td>Ever</td>
<td>3.12 (0.83, 1.51)</td>
<td>34.04</td>
</tr>
<tr>
<td>Be Roos et al.</td>
<td>32/92</td>
<td>&gt; 20 days</td>
<td>2.3 (1.10, 4.00)</td>
<td>1.11</td>
</tr>
<tr>
<td>Case-Control</td>
<td>36/650</td>
<td>Ever, log</td>
<td>2.36 (1.04, 5.37)</td>
<td>7.18</td>
</tr>
<tr>
<td>Fullerton et al.</td>
<td>17/100</td>
<td>Ever</td>
<td>1.85 (0.55, 6.29)</td>
<td>3.09</td>
</tr>
<tr>
<td>Heidel et al.</td>
<td>8/115</td>
<td>Ever</td>
<td>2.12 (1.20, 3.73)</td>
<td>15.05</td>
</tr>
<tr>
<td>Neufeld et al.</td>
<td>23/17</td>
<td>&gt; 2 days</td>
<td>1.60 (0.55, 4.22)</td>
<td>8.82</td>
</tr>
<tr>
<td>Ozor et al.</td>
<td>12/244</td>
<td>Ever</td>
<td>3.71 (1.10, 11.91)</td>
<td>33.73</td>
</tr>
</tbody>
</table>

Abbreviations: AHS, Agricultural Health Study; CI, confidence interval; d, days; I, lifetime; log, logistic regression; y, year.
1 Relative risk (RR) reported in both AHS analyses and odds ratio (OR) reported in all case-control studies.
2 Weight calculated for each study using the fixed-effects model.
3 Intensity-weighted lifetime exposure days (cumulative exposure days multiplied by intensity score).
4 20 years of excess lag (time between study recruitment and NHL onset).
5 Reference group is lowest exposed.

2.7 Statistical methods

We calculated the meta-analysis summary relative risk (meta-RR) and confidence intervals (CI) using both the fixed-effects inverse-variance method [52] and the random-effects method [51]. In the fixed-effects model, the weights assigned to each study are directly proportional to study precision, whereas in the random-effects model, weights are based on a complex mix of study precision, relative risk (RR), and meta-analysis size. One benefit of the random-effects model is the ability to incorporate between-study variance into the summary-variance estimate and confidence intervals, which may help prevent artificially narrow confidence intervals resulting from use of the fixed-effects model in the presence of between-study heterogeneity [52]. However, a feature of the random-effects model is that study weighting is not directly proportional to study precision, and greater relative weight is given to smaller studies, which may result in summary estimates that are less conservative than the fixed-effects model [51]. For these reasons, our primary results focus on the fixed-effects model, although the random-effects model estimates are also reported. We further estimated between-study heterogeneity, defined as the X²-test statistic for heterogeneity being greater than its degrees of freedom (number of studies minus one), using the summary-variance method [52].

We evaluated publication bias through funnel plots, Egger's test, and Begg's test [53, 54]. All statistical analyses were conducted with Stata IC 15.1 [55] and Microsoft Excel 2013 [56].

3. Meta-analysis findings

3.1 Increased meta-relative risk of NHL

Table 5 reports the results from our two meta-analyses, which included the primary analysis using the most recently updated AHS cohort [25] and the secondary comparison analysis using the original study [20]. With the AHS results [56], we observed a meta-RR of 1.41 (95% CI: 1.13-1.75), which indicates a statistically significant increased risk (41%) of NHL following high cumulative GBH exposure. Although our results focus on the fixed-effects model, using the random-effects model resulted in a meta-RR of 1.56 (95% CI: 1.22-2.16) shown in Table 5. With the original AHS 2005 cohort results, we observed a meta-RR of 1.45 (95% CI: 1.11-1.91) for NHL. The results did not change appreciably when comparing the fixed-effects model to the random-effects model.

Forest plots (Fig. 3A, B) and Funnel plots (Fig. 3C, D) from these two major meta-analyses are reported in Fig. 4. We observed little evidence of publication bias in the Funnel plots (Fig. 3C, D); Egger's test (p = 0.183) and Begg's test (p = 0.851).

3.2 Sensitivity analyses

We conducted several sensitivity analyses to evaluate the impact of excluding or including different studies as well as using different RRs/ ORs from original studies (Tables 5 and 6). In general, results were similar across our sensitivity analyses, demonstrating the robustness of our findings.

3.2.1 Alternative exposure criteria

As a sensitivity analysis, we also conducted a meta-analysis using the longest exposure duration results to compare with our primary analysis using the highest cumulative exposure results. When RRs corresponding to exposures with the longest duration were selected from the AHS 2018, the meta-RR remained the same at 1.41 (95% CI: 1.13-1.74). When the AHS 2005 cohort was used, the meta-RRs increased to 1.56 (95% CI: 1.17-2.06) (Table 5).
Table 5
Major findings from current meta-analyses.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>N</th>
<th>Fixed-Effects meta-RR (95% CI)</th>
<th>Random-Effects meta-RR (95% CI)</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest cumulative exposure</td>
<td>6</td>
<td>1.41 (1.13, 1.75)</td>
<td>1.56 (1.12, 2.16)</td>
<td>8.26</td>
</tr>
<tr>
<td>AIS (2018) [23]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIS (2015) [20]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longest exposure duration</td>
<td>6</td>
<td>1.45 (1.11, 1.91)</td>
<td>1.52 (1.00, 2.31)</td>
<td>10.59</td>
</tr>
<tr>
<td>AIS (2018) [23]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIS (2015) [20]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study design</td>
<td>6</td>
<td>1.41 (1.13, 1.74)</td>
<td>1.56 (1.12, 2.16)</td>
<td>8.21</td>
</tr>
<tr>
<td>Case-control [16,19,31,41]</td>
<td>5</td>
<td>1.56 (1.17, 2.06)</td>
<td>1.57 (1.09, 2.26)</td>
<td>7.81</td>
</tr>
<tr>
<td>Cohort AIS (2018) [23]</td>
<td>1</td>
<td>1.84 (1.33, 2.55)</td>
<td>1.86 (1.30, 2.68)</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Abbreviations: AIS, Agricultural Health Study; meta-RR, meta-relative risk; N, number of studies.

a Heterogeneity is present when $\chi^2$ heterogeneity statistic is greater than degrees of freedom (number of studies minus 1).

b De Roos et al. [30] used instead of Ascherio et al. [25] for comparison. See Table 4 for clarifications about the risk estimates used.

Since there was only one cohort study, the RR is presented instead of a meta-RR.

When evaluating studies with only the highest levels of exposure [17,25,43], the meta-RR was 1.36 (95% CI: 1.06–1.75; Table 6). In studies that combined all exposures as ever exposed (16–20,41), the meta-RR was 1.30 (95% CI: 1.03–2.64). Although the higher exposure group was used in the main analysis, Eriksson et al. [17] also provided results for greater than 10 years latency, which contributed to a meta-RR of 1.40 (95% CI: 1.13–1.75). [Note: AIS 2018 did not provide ever exposure, so AIS 2005 was used to calculate this statistic and ever exposure above].

3.2.2. Study inclusion

When we limited our analysis to case-control studies (Table 5), there was little inter-study heterogeneity. We estimated a doubling of the NHL risk (meta-RR = 1.84, 95% CI: 1.33–2.55) from 41% to 84% compared to the estimate that included the cohort study.

To ensure that one individual study was not artificially inflating the meta-risk estimate, we excluded the case-control studies one at a time and found that they all nominally lowered the meta-RR, except for the exclusion of Orsi et al. [19], where the meta-RR increased to 1.46 (1.16–1.83) (Table 6).

3.2.3. NHL vs. cell type specific lymphomas

Although our primary meta-analysis included six studies, there was a possibility to include a seventh study [42]. We excluded this study from the primary analysis since it included all B-cell lymphomas (4 cases), which accounted for approximately 95% of all NHL cases, however, not all four cases were confirmed to be NHL. When we added Cocco et al. [42] to the meta-analysis (n = 7, Table 6), the resulting RR remained fairly similar at 1.45 (95% CI: 1.15–1.88).

Similar to our exclusion of the Cocco et al. [42] study, another cell type specific study evaluated all cases of hairy cell leukemia (HCL), a subtype of NHL [40]. It was one of two studies [39,41] included in the

Fig. 3. Findings from major meta-analyses. A-B: Forest plots for meta-analyses using AHS 2018 (3A) and AHS 2005 (3B); C-D: Funnel plots for meta-analyses using AHS 2018 (3C) and AHS 2005 (3D).
Table 6: Results from sensitivity analyses.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Fixed-effects</th>
<th>Random-effects</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>meta-RR (95% CI)</td>
<td>meta-RR (95% CI)</td>
</tr>
<tr>
<td>Alternative exposure categories</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High level</td>
<td>3</td>
<td>1.36 (1.06, 1.75)</td>
<td>1.63 (0.97, 2.76)</td>
</tr>
<tr>
<td>Low (AHS 2005)</td>
<td>6</td>
<td>1.35 (1.13, 1.57)</td>
<td>1.54 (1.12, 2.13)</td>
</tr>
<tr>
<td>latency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell type specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML (AHS 2005)</td>
<td>18</td>
<td>1.43 (1.15, 1.78)</td>
<td>1.59 (1.16, 2.18)</td>
</tr>
<tr>
<td>Excluding HCL (18)</td>
<td>18</td>
<td>1.43 (1.13, 1.77)</td>
<td>1.61 (1.11, 2.34)</td>
</tr>
<tr>
<td>Only using HCL (18)</td>
<td>18</td>
<td>1.42 (1.14, 1.78)</td>
<td>1.52 (1.14, 2.31)</td>
</tr>
<tr>
<td>Study location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>8</td>
<td>1.38 (1.08, 1.76)</td>
<td>1.61 (0.99, 2.62)</td>
</tr>
<tr>
<td>Europe</td>
<td>8</td>
<td>1.53 (0.92, 2.52)</td>
<td>1.55 (0.88, 2.71)</td>
</tr>
<tr>
<td>Other pesticides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted (AHS 2005)</td>
<td>4</td>
<td>1.46 (1.05, 2.07)</td>
<td>1.43 (1.06, 1.92)</td>
</tr>
<tr>
<td>Unadjusted (AHS 2005)</td>
<td>4</td>
<td>1.69 (1.29, 2.23)</td>
<td>1.70 (1.26, 2.30)</td>
</tr>
<tr>
<td>De Roos et al. (16)</td>
<td>6</td>
<td>1.36 (1.09, 1.70)</td>
<td>1.46 (1.08, 1.96)</td>
</tr>
<tr>
<td>Hierarchical OR</td>
<td>6</td>
<td>1.29 (1.04, 1.54)</td>
<td>1.36 (1.02, 1.87)</td>
</tr>
<tr>
<td>Cantor et al. (8)</td>
<td>6</td>
<td>1.35 (1.11, 1.65)</td>
<td>1.41 (1.19, 1.82)</td>
</tr>
<tr>
<td>Lee et al. (17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>1.23 (0.99, 1.53)</td>
<td>1.30 (0.96, 1.76)</td>
</tr>
<tr>
<td>Excluding one study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andreotti et al. (34)</td>
<td>5</td>
<td>1.84 (1.33, 2.55)</td>
<td>1.86 (1.36, 2.48)</td>
</tr>
<tr>
<td>De Roos et al. (16)</td>
<td>5</td>
<td>1.34 (1.04, 1.69)</td>
<td>1.47 (1.10, 2.11)</td>
</tr>
<tr>
<td>Eriksson et al. (32)</td>
<td>5</td>
<td>1.35 (1.08, 1.70)</td>
<td>1.54 (1.04, 2.07)</td>
</tr>
<tr>
<td>Hardell et al. (9)</td>
<td>5</td>
<td>1.40 (1.12, 1.75)</td>
<td>1.56 (1.08, 2.24)</td>
</tr>
<tr>
<td>McDuffie et al. (47)</td>
<td>5</td>
<td>1.23 (1.02, 1.66)</td>
<td>1.43 (1.07, 2.03)</td>
</tr>
<tr>
<td>Orsi et al. (19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.46 (1.16, 1.83)</td>
<td>1.69 (1.16, 2.45)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; HCL, hairy cell leukemia; meta-RR, meta-relative risk.
1 Heterogeneity is present when the heterogeneity statistic is greater than degrees of freedom (number of studies minus 1).
2 Risk estimates for the most highly exposed group available in the three studies that strongly by exposure level.
3 Eriksson et al. [7] results for any glyphosate exposure > 10 years latency was used instead of the higher exposure group used in the main analysis.
4 The study combined B-cell lymphomas and is added to the analysis on highest cumulative exposure (AHS 2016).
5 Only cell leukemia cases excluded—results presented in Hardell and Eriksson [39].
6 N-1 cases excluded; only HCL results used—results presented in Nordstrom et al. [40].
7 Studies that provided RRs that were both adjusted and not adjusted for other pesticide use for ever exposure, or reported that adjusting for pesticide use had little impact on the RR estimate. AHS (2018) did not report ever exposure, so AHS (2005) was used instead.
8 Hierarchical model RR used instead of the standard logistic regression model RR.
9 Cantor et al. (38) used instead of De Roos et al. (16). Cantor et al. (38) was the only of the three studies combined by De Roos et al. (16) that presented data for glyphosate.
10 Lee et al. (37) used instead of De Roos et al. (16). Lee et al. (37) used same subjects as De Roos et al. (16) but did not adjust for other pesticide exposure, did not exclude those with missing data on other pesticide use, and used only non-athletes.
11 Hohenadel et al. (41) used same subjects as McDuffie et al. (47) but presented results in subjects exposed to glyphosate but not malathion (OR = 0.92; 95% CI: 0.54–1.55).
12 One study excluded at a time to evaluate the impact of each individual study on the overall meta-RR.

Hardell et al. [18] analysis, with the other study examining NHL only [19]. Excluding NHL cases had no effect on the meta-RR (1.41, 95% CI: 1.13–1.77). Similarly, using only hairy cell leukemia cases from Hardell et al. [18] (reported in Nordstrom et al. [40]) did not impact the meta-RR (1.43, 95% CI: 1.14–1.78).

3.2.4. Study location and covariate adjustment

Studies in North America [16,25,43] had a meta-RR of 1.38 (95% CI: 1.08–1.76), whereas European studies [17–19] had a meta-RR of 1.53 (95% CI: 0.93–2.52). On average, when studies were adjusted for other pesticide use [16–18,20], the pooled RR for ever exposure was lower than unadjusted risk estimates from the same studies (meta-RR_adjusted = 1.46, 95% CI: 1.05–2.02; meta-RR_unadjusted = 1.69, 95% CI: 1.29–2.13).

3.2.5. Logistic vs. hierarchical regression

Consistent with the two previous meta-analyses by IARC [23] and Schinasi and Leon [26] discussed in Sections 3.1 below, we selected the RR estimated using the more traditional logistic regression over hierarchical regression in the case-control study by De Roos et al. [16] and found that there was little impact of this selection (meta-RR = 1.36, 95% CI: 1.09–1.70). The De Roos (2002) [16] study included pooled data from two separate studies [17,18]. When Cantor et al. [38] or Lee et al. [37] was used instead of De Roos et al. [16], the meta-RR decreased to 1.29 (95% CI: 1.04–1.59) and 1.35 (95% CI: 1.11–1.65), respectively. Similarly, using Hohenadel et al. [41] instead of McDuffie et al. [47] caused the meta-RR to decrease to 1.23 (95% CI: 0.99–1.53).

4. Comparison with previous meta-analyses

Three meta-analyses of NHL in relation to GBH exposure have been published [23,26,27], all of which report lower, albeit also positive, risk estimates. In contrast to our work, these analyses did not focus on the highest exposed groups. Table 7 summarizes the major results from all GBH-NHL meta-analyses conducted to date, including the current one.

Schinasi and Leon [26] first reported a meta-RR of 1.45 (95% CI: 1.08–1.95). Although their selection criteria stated that they used the most adjusted effect estimate for the dichotomously defined exposure with the greatest number of exposed cases, they did not use adjusted effect estimates in the two Swedish studies [17,18]. The IARC Working
Group subsequently corrected this discrepancy in an otherwise identical meta-analysis \cite{23}, resulting in a meta-RR of 1.30 (95% CI: 1.03–1.65). Although both studies are listed in Table 7 for completeness, we consider IARC 2015 to be the most accurate and updated version of this meta-analysis.

Most recently, Chang and Dezelj \cite{27} reported a meta-RR of 1.27 (95% CI: 1.01–1.59) in their primary analysis (model one). For each included study, the authors selected the most fully adjusted RR from the publication with the most recent and complete study population with the largest number of exposed cases. (In their publication, the meta-RR was rounded to one digit to the right of the decimal point.)

Whereas the three previous meta-analyses focused on general exposure (ever versus never), our new meta-analysis differs primarily because of our a priori selection of risk estimates from the most highly exposed groups when available (from three studies \cite{17,20,43}). In our secondary comparison meta-analysis with the same six studies (including AHS 2005), we documented an additional 0.15–0.18 (or 15–18%) higher NHL RR than previous meta-RRs \cite{23,27} (not including Schiassi and Leon, because it was corrected in IARC 2015). Similarly, in our primary analysis with AHS 2018, our meta-RR estimate adds an additional 0.11–0.14 (11–14%) increase in NHL relative risk to the previous meta-RRs \cite{23,27}. Overall, the meta-RR obtained using our a priori hypothesis, while generally consistent with previous analyses, gave somewhat higher estimates and suggested increased risk of NHL in individuals highly exposed to glyphosate.

5. Strengths and limitations

In this section, we evaluate the strengths and limitations of our meta-analysis, as well as of the cohort and the case-control studies utilized.

5.1. Current meta-analyses

The strengths of this meta-analysis are the inclusion of the updated AHS 2018 study and our novel a priori hypothesis. By using the highest exposure group in each study when it was reported, we maximized the ability to detect the presence of an exposure-disease association. The current meta-analysis is also the first study to include the newly updated AHS 2018.

There are several weaknesses of our analysis that should be noted. First, there were only limited published data available for study \cite{38}, we cannot exclude the potential for publication bias, given balance in study design among the six included studies, five were case-control and one was a cohort. The collection of NHL findings from the cohort study was consistent with a wide range of risks \cite{25}, while, by contrast, most of the case-control studies did suggest an increased risk \cite{16,18,43}. There were also important differences in the comparison group utilized in the studies; some used the lowest exposure group as the reference, while others used the unexposed group. Because of this heterogeneity, and because no statistical tests can confirm elimination of publication bias or heterogeneity in a meta-analysis \cite{59}, our results should be interpreted with caution. Finally, as depicted in Fig. 1 illustrating key milestones related to glyphosate use in society and epidemiological studies, some of the available studies capture the effects of the significant increased usage of glyphosate that began with the introduction of “green burnout” in the mid-2000s.

5.2. AHS cohort study

In general, cohort studies are considered the gold standard among observational studies because of their ability to estimate exposure before disease occurrence (which allows for clarity of temporality and can minimize recall bias). To estimate incidence, to examine multiple outcomes, and for some target populations, to study a large number of exposed subjects. Our current meta-analysis is the first to include the AHS 2018 update, which is the largest, newest, and most heavily weighted study (> 50%, Table 4). Given its importance and because it was the only cohort study in our analyses, we discuss below several aspects of the AHS 2018 \cite{25} and compare it with the results reported in AHS 2005 \cite{20}. Key differences between the AHS 2018 and AHS 2005 are summarized in Table 8.

5.2.1. Exposure assessment and quantification

Exposures were self-reported using questionnaires. AHS 2005 used the exposures reported at baseline only, whereas AHS 2018 supplemented this information with responses to a follow-up questionnaire returned by 69% of AHS participants.

The risk estimates generated from the follow-up AHS 2018 report depended on a “multiple imputation” approach with multiple steps to generate GBH exposure information for the 37% of participants who did not complete the follow-up questionnaire \cite{35}. A standard imputation model captures the full distribution of the exposure by relying on two parts of a model: the regression of the predicted and the residual error part. The validity of the imputed exposures and the resulting risk estimates relies on the validity of both the model and the imputation model. The AHS imputation method for ever/never pesticide use conditioned on the reported pesticide use and other data, including demographics, medical history at baseline, and farming characteristics at enrollment, with some covariates chosen by stepwise regression (see Table 2 of Helsby et al. \cite{60}). Based on their analysis of a 20% holdout dataset, the prevalence of glyphosate use was underestimated by 7.3%,
<table>
<thead>
<tr>
<th>Exposure assessment</th>
<th><strong>AHS 2005 (20)</strong></th>
<th><strong>AHS 2018 (25)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Self-report at baseline</td>
<td>Self-report at baseline &amp; follow-up questionnaire with exposure simulation</td>
</tr>
<tr>
<td><strong>Exposure quantification</strong></td>
<td>Ever/never</td>
<td>Cumulative exposure days</td>
</tr>
<tr>
<td></td>
<td>Ever/never</td>
<td>Cumulative exposure days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intensity-weighted exposure days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unlagged</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ever exposed</td>
</tr>
<tr>
<td>Lag period</td>
<td>Max range: 20–24;</td>
<td>Actual max: 9.3;</td>
</tr>
<tr>
<td>Reference group</td>
<td>Ever/never</td>
<td>Unlagged</td>
</tr>
<tr>
<td>Exposed groups (day)</td>
<td>Max range: 21–27;</td>
<td>Actual max: N/A;</td>
</tr>
<tr>
<td>Exposure duration (year)</td>
<td>Max range: 30–80;</td>
<td>Actual max: 70;</td>
</tr>
<tr>
<td>Potential misclassification</td>
<td>Differential misclassification unlikely; Non-differential misclassification likely</td>
<td></td>
</tr>
<tr>
<td>Follow-up (years)</td>
<td>Multiple myeloma not included in NHL cases</td>
<td></td>
</tr>
<tr>
<td>Outcome Inclusion</td>
<td>Multiple myeloma included in NHL cases</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** AHS, Agricultural Health Study; IQR, interquartile range; max, maximum; N/A, not applicable; NHL, non-Hodgkin lymphoma; T1, tertirole one.

1 This was referred to as "multiple imputation" by study authors; see manuscript text for further details.
2 This algorithm for calculating "intensity-weighted exposure days" was updated between 2005 and 2018. Key differences include rescaling of scores by a factor of 10 and altering the weights for mixing certain pesticide application techniques, and the use of chemically resistant gloves [45]. Therefore, these metrics cannot be directly compared.
3 Ever/never and cumulative exposure days were only presented in the AHS 2005 supplement but are presented here to facilitate comparisons with AHS 2005.
4 Exposure group abbreviations as follows: Tertiles = "T"; Quartiles = "Q".
5 The values provided in this row are based on the subset of individuals who reported using glyphosate.
6 This theoretical maximum exposure duration value was calculated based on the year that glyphosate entered the market (1974) and the end of AHS enrollment (1993–1997), since AHS 2005 used only baseline exposure information.
7 This value was calculated based on the upper bound of the cumulative exposure days tertiles.
8 These theoretical maximum exposure duration values were calculated based on the year that glyphosate entered the market (1974) and the end of AHS follow-up exposure questionnaire (1999–2005), with the appropriate adjustments for the lag times as indicated.
9 These medians were calculated using the information provided in the footnote in Table 3 of the AHS 2018 publication.
10 These follow-up times were calculated based on timing of study enrollment and follow-up.
suggesting some lack of validity in the predictable part of the imputation model that may in turn affect the NHL risk estimates. The imputations of days of use per year and most recent year of farming activity relied upon a stratified sampling with replacement approach, with values sampled from Phase 2 respondents based on strata defined using Phase 1 information.

The imputations did not use the NHL or any other cancer outcome information reported by Andreetti et al. [23]. This approach is problematic because of how the residual error part of the imputation model is handled. It is known that multiple imputation of a covariate (i.e., glyphosate exposure) in a model that omits the outcome variable to be used in the inference leads to attenuation of the effect estimate for that covariate due to lack of correlation with the outcome in the residual error part of the imputed data sets [61]. As we discuss further in the next paragraph, this approach effectively “bakes into the result” the null hypothesis of no increased risk of NHL exposure due to glyphosate risk.

Because the NHL outcome information was not used in the imputation procedure, the exposure “imputation” method used in the AHS 2018 report can be better named “exposure simulation” as described by Gryparis et al. [62]. This term gives a much more accurate understanding of the impact of the imputation of the data on the risk estimates because when exposure is simulated in a model that does not take the outcome into account, the uncertainty in the “imputed” exposure behaves like classical measurement error and, thus, will bias the effect estimate towards the null [63].

AHS 2018 authors argue that their imputation approach “likely did not materially impact risk estimates” [64]. However, their argument has to do with the impact on the average change in the number of predicted events in an outcome-aggregated imputation model and not the role of classical measurement error in the imputed exposure estimates.

There was also a subtle yet important difference in the categorization and quantification of exposure data between AHS 2005 and 2018. As depicted in Table 3, both studies classified exposure based on (1) ever/never, (2) cumulative exposure days, and (3) intensity-weighted exposure days. However, the algorithm utilized to calculate intensity-weighted exposure days was updated between 2005 and 2018. Key differences include re-scaling of scores by a factor of 10 and altering the weights for mixing certain pesticide application techniques, and the use of chemically resistant gloves [59]. Therefore, these metrics cannot be directly compared.

Additionally, it is crucial to highlight the difference in reference groups between these two studies, which further limits the comparability of their estimates. AHS 2005 utilized the lowest exposed tertile as the comparison group for risk estimation. They justified this decision as an attempt to control residual confounding, because of the presence of significant differences in key characteristics between the never-exposed and lowest-exposed groups. By contrast, AHS 2018 utilized the unexposed group as the reference group even though our comparison of the demographics reported in each paper does not suggest there is substantially better compatibility between groups in AHS 2018. Furthermore, because the exposure information by which these groups were classified was based on their imputation procedure, the limitations of which are highlighted above, the actual comparability between groups may differ from the values reported. Not only would it be helpful to be able to compare directly the risk estimates across these two papers, it would also be useful to investigate whether there was residual confounding introduced into the AHS 2018 analysis by the use of the “unexposed” group as the reference.

5.2.2 Exposure misclassification

Differential misclassification is unlikely in a cohort study when exposure is assessed prior to the disease occurrence. In AHS 2018, however, we suspect there is some potential for differential misclassification. Sixty-three percent of the original cohort provided updated exposure information by questionnaire one time between the years of 1999 and 2005. Although details are not provided, it is likely that some of the cases reported their exposure after disease occurrence, allowing for potential differential misclassification in the self-reported exposures in this cohort similar to general concerns with case-control studies. Furthermore, noting large societal trends in glyphosate exposure between initial exposure and the follow-up questionnaire, and the 7.3% under-prediction of glyphosate exposures in the holdout dataset [60], the prediction part of the imputation modeling may be differentially under-predicting exposures.

Non-differential misclassification occurs when exposure status is equally misclassified among exposed cases and unexposed controls [65]. The approach in AHS 2018 to exposure imputation is one theoretically well-understood source of non-differential misclassification. In addition, it may be more problematic in the context of a ubiquitous exposure because it is hard for participants to know what extent or how long they have been exposed. Glyphosate’s ubiquity in the environment leads to profound concern that even “unexposed” individuals in the cohort are likely to have been exposed to glyphosate; consequently, the magnitude of any potential association relative to the unexposed group may be attenuated due to this misclassification. This problem is encountered with other environmental exposures such as environmental tobacco smoke (ETS), never smokers with ETS exposure carry some cancer risk and are not the ideal true reference group in studies of smoking and tobacco-related cancers [66]. As we noted above, non-differential misclassification is likely to attenuate measures of association, biasing the RE towards the null of 1.0 [67]. Although it is difficult to ascertain exactly, the extent of this source of non-differential misclassification can be estimated through smaller-scale validation studies [67].

5.2.3 Disease classification and latency

The updated AHS 2018 included multiple myeloma (MM) in their NHL cases, but the previous AHS 2005 did not. Although MM traditionally did not belong to NHL WHO recently revised the classification of lymphoid neoplasms and suggested some types of MM (e.g., IgA mutation-related MM) are related more closely to lymphomas, including NHL, than to myelomas [68].

There is much uncertainty surrounding the latency period for NHL. The latency period for short-term high-dose exposures to carcinogens may be as short as two years, but it may also be as long as 15 years or more. Long-term low-dose exposures are expected to have longer median latencies between 15 to 20 years for NHL [60,90]. It is possible that different NHL subtypes may have different latencies. Given the uncertainty surrounding NHL latency, it is possible that the follow-up period (median = 6.7 years) in the 2005 AHS study [20], which was unadjusted, may have been too short for a sufficient number of exposure-related cancer events to manifest. Given that participants had been exposed to glyphosate prior to enrolling in the study (median = 8 years; mean = 7.5 years; SD = 5.3 years), participants could have had an exposure duration ranging from as low as 9 years to as high as 32 years at the time of enrollment, assuming a normal distribution. Hence, although some AHS members may have had sufficient exposure durations to develop NHL, many fell short of the median 15-20 years of expected NHL latency.

The 2018 AHS publication added 11-12 further years of follow-up for all study participants, an additional 483 cases of NHL, and considered five, ten, fifteen, and twenty year exposure lags, which was not possible in AHS 2005 due to its short follow-up duration. Epidemiologic studies often lag exposures to account for disease latency under the assumption that recent exposures have little impact on disease development. Theoretically, longer exposure durations and/or lags would present more biologically plausible associations with NHL. For AHS 2018 specifically, not only are the risk estimates associated with longer lag times more plausible than unlagged risk estimates in AHS 2005 and 2018, but the twenty-year exposure lag, specifically, may also be free of the bias caused by exposure imputation described above, given that at this lag exposure information may have been derived exclusively from the baseline questionnaire.

5.2.4 Summary

Overall, the cohort study features highlighted above related to exposure assessment and quantification, misclassification, and latency...
and lag suggest caution in direct comparisons between AHS 2005 and 2018. Additionally, the limitations with AHS 2018 with regard to exposure simulation, potential residual confounding, and misclassification may have accounted for the weaker meta-RR estimate that we obtained when incorporating this study into the meta-analysis.

5.3. Case-control studies

Although cohort studies are the gold standard in observational epidemiology, they are often challenging to conduct due to the small number of incident cases for rare diseases such as NHL. Case-control studies can be more efficient for evaluation of rare diseases. For example, the AHS had to recruit tens of thousands of participants (N = 53,960) and follow them for more than a decade in order to gather 575 new cases of NHL, whereas the 3 case-control studies assembled 2,836 NHL cases among all participants (N = 88,688) in a much shorter period of time (Tables 1 and 4). Though the case-control studies are smaller and carry less weight than the large cohort study, it is worth noting that results from multiple case-control studies displayed little heterogeneity (Table 5) and reported similar findings pointing away from null (Table 4).

However, there are other challenges and concerns relevant to the case-control studies utilized in our meta-analysis, which we briefly discuss below.

5.3.1. Control selection and exposure quantification

Four of the five case-control studies utilized here are population-based, while one is hospital-based [19]. There may be important differences between hospital-based controls and population-based controls that could impact the interpretability and comparability of the resulting risk estimates. Of relevance to this concern is that, as noted above in our sensitivity analyses, exclusion oforsi et al. [19] (the hospital-based case-control study) resulted in an increased meta-RR of 1.46 (95% CI: 1.16-1.83) shown in Table 6, while sequential exclusion of each of the population-based case control studies produced decreased meta-RRs.

Exposure was also quantified differently between the selected case-control studies, further impacting their comparability. While all the studies considered in our meta-analysis conducted exposure assessment based on self-reported questionnaire data, some studies considered ever/never exposure, while others evaluated exposure based on number of days per year (see Tables 1 and 4). Some studies also relied on proxy respondents such as next of kin.

5.3.2. Exposure misclassification

It is always possible for the internal validity of case-control studies to be threatened by recall bias, a form of differential exposure misclassification that occurs when exposures are remembered differently by cases (or their proxies) and controls. Cases may have been more motivated to recall GH1 exposure, and the exposures may be more vivid or meaningful due to awareness of the risk factors for their disease. While differential misclassification can bias the OR in either direction, differential misclassification due to cases being more likely to report exposure tends to artificially inflate the OR.

5.3.3. Latency and lag

As discussed in Section 5.2.3, the latency for NHL is uncertain and could be anywhere from 2 years to greater than 15 years. There were differences in how the case-control studies considered and incorporated latency and lag into their analyses. For example, de Roos et al. [11] and McDuffie et al. [44] do not mention these considerations; by contrast, Hardell et al. [18],orsi et al. [19], and Eriksson et al. [17] each incorporate latency and lag, albeit differently. These differences suggest caution in the integration of these results.

6. Summary of the GH1 and NHL association in humans

Overall, the results from our new meta-analysis employing the a priori hypothesis and including the updated AHS 2005 study (1) demonstrates a statistically significant increased NHL risk in highly GH1-exposed individuals (meta-RR = 1.41, 95% CI: 1.13-1.75; Table 5 and Fig. 3A), (2) are aligned with findings from previous meta-analyses [23,26,27] (Table 7), and (3) reveal an additional 11%-14% and 15%-18% increase in NHL relative risk due to high levels of GH1 exposure (Table 7) when using the AHS 2018 and the AHS 2005 cohort, respectively.

Together, all of the meta-analyses conducted to date, including our own, consistently report the same key finding: exposure to GH1s is associated with an increased risk of NHL.

Because most people in these epidemiological studies were not exposed to pure glyphosate, but rather glyphosate-based formulations (e.g. Roundup® or RangerPro®) within a number of adjacent, it could be argued that the NHL manifested as a result of exposure to the mixture or an ingredient other than glyphosate in the formulation. To investigate causal inference regarding the association between glyphosate exposure and NHL, we discuss briefly whether or not the association identified from epidemiological studies could be supported further by experimental animal and mechanistic studies related to lymphoma.

7. Animal data: lymphoma prevalence in glyphosate-exposed mice

The animal study outcome most closely linked to human NHL is malignant lymphoma. We identified six unpublished glyphosate and lymphoma studies in mice that are in the public domain from two sources: a presentation by the European Food Safety Authority (EFSA) [71] at the EPA FIFRA Scientific Advisory Panel on Carcinogenic Potential of Glyphosate and a report by The Food and Agriculture Organization of the United Nations and World Health Organization Joint Meeting on Pesticide Residues (JMPR) [22]. EFSA [71] reported results from five unpublished studies: four in CD-1 [72-75] and one in Swiss albino mice [76], whereas JMPR [22] also reported data from an additional study in female CD-1 mice [77]. Each study reported four glyphosate doses and corresponding lymphoma incidence in males and females except for Takahashi [77], where the only data available in the public domain was for female mice [22].

7.1. Results of mouse lymphoma studies

Results from all studies (n = 6) of malignant lymphomas in mice available in the public domain are presented in Table 9. Study durations ranged from 18 to 26 months. All studies administered glyphosate through the diet [72-77], and the concentration tested ranged from 100 ppm to 50,000 ppm [22]. EFSA [71] and JMPR [22] reported slightly different doses, with JMPR [22] further stratifying by sex. Lymphoma incidence was abstracted from EFSA [71], with slightly different numbers for one study [22]. Table 9 provides the dietary concentration of glyphosate (reported in ppm), the doses (reported in mg/kg/day) provided by EFSA [71] and JMPR [22], and lymphoma incidence in males and females. One study [74] reported food consumption, which was recorded for each treatment group, and weekly mean achieved dose levels were averaged to calculate actual doses for males and females. Information on how doses were calculated for the other studies [72,73,75,73-77] was not available.

In summarizing these studies, EFSA [71] noted that Sugimoto [79] and Wood et al. [74] showed statistically significant dose-response in males according to the Cochran-Armitage test for linear trend, whereas Kumar [76] showed a statistically significant 2-test for both males and females. In agreement, JMPR [22] noted that Sugimoto [73] and Wood et al. [74] showed a statistically significant trend in males and that Kumar [76] reported statistically significant increases in malignant lymphoma in high-dose groups of both males and females. JMPR [22] further reported Takahashi [77] had a statistically significant increased incidence in lymphoma among females by their trend test. The remaining two studies did not report evidence of a statistically significant dose-response effect.
Table 9
Data from publicly available studies of malignant lymphomas in mice exposed to glyphosate.

<table>
<thead>
<tr>
<th>Study</th>
<th>Strains</th>
<th>Study Duration</th>
<th>Concentration in Diet (ppm)</th>
<th>FESA [71]</th>
<th>JMPR [22]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wunder et al. [74]</td>
<td>CD-1</td>
<td>1.52 years (79 weeks)</td>
<td>0</td>
<td>0/51 (0)</td>
<td>11/51 (22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>500</td>
<td>71, 71.4, 0.7</td>
<td>1/51 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1500</td>
<td>234, 234.2, 298.9</td>
<td>2/51 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000</td>
<td>810, 1010, 1010.2</td>
<td>5/51 (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0, 0, 0</td>
<td>16/50 (20)</td>
</tr>
<tr>
<td>Kemiri [76]</td>
<td>SV mice</td>
<td>1.5 years</td>
<td>100</td>
<td>15</td>
<td>15/50 (30)</td>
</tr>
<tr>
<td></td>
<td>Albino</td>
<td></td>
<td>1000</td>
<td>145, 150.2</td>
<td>16/50 (32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10000</td>
<td>145, 146.8</td>
<td>19/50 (38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0, 0, 0</td>
<td>2/50 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0, 0, 0</td>
<td>4/50 (8)</td>
</tr>
<tr>
<td>Resnick [74]</td>
<td>CD-1</td>
<td>1.5 years</td>
<td>0</td>
<td>0, 0, 0</td>
<td>3/50 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>157, 150, 190</td>
<td>2/40 (4)</td>
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<tr>
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<td>5000</td>
<td>814, 955</td>
<td>2/40 (4)</td>
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<td></td>
<td>10000</td>
<td>4841, 5874</td>
<td>2/40 (4)</td>
</tr>
<tr>
<td>Koeste and Hogen [72]</td>
<td>CD-1</td>
<td>2 years</td>
<td>0</td>
<td>0, 0, 0</td>
<td>3/50 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>0, 0, 0</td>
<td>4/50 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10000</td>
<td>0, 0, 0</td>
<td>4/50 (8)</td>
</tr>
<tr>
<td>Tahmashian [77]</td>
<td>CD-1</td>
<td>1.5 years</td>
<td>0</td>
<td>0, 0, 0</td>
<td>3/50 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5000</td>
<td>0, 0, 0</td>
<td>4/50 (8)</td>
</tr>
</tbody>
</table>

Abbreviations: FESA, European Food Safety Authority; JMPR, Joint Meeting on Pesticide Residues; N/A, not available.

a Number of lymphomas / total mice in group.

b Data for male, female mice.

c Reported slightly differently in JMPR [22] (N = 1).

d P < 0.05 reported by at least one test for trend in FESA [71] or JMPR [22].

7.2. Additional considerations and recommendations

One challenge with these studies is that at face value they appear to be inconsistent because some show statistically significant findings whereas others do not. However, based on EPA’s Cancer Guidelines, evidence of increased lymphoma incidence should not be discounted due to lack of statistical significance in trend and/or pairwise comparison tests. Additional factors that should not be used to exclude study findings are the use of high doses and/or incidence rates that are consistent with levels seen in historical controls [78].

Another consideration is that the study lengths in these animal experiments may have been insufficient for development of lymphoma. There are proposals that the standard timeframe of two years for a cancer bioassay to approximate long-term cancer incidence in humans should be extended to account for potentially longer latencies. Eighty percent of all human cancers occur after the age of sixty. A two-year-old rat approximates a human of 60-65 years, indicating a traditional two-year bioassay may not be sufficient for late-developing tumors [79].

Future work should combine the results from these six studies into an overall pooled analysis to give a more robust assessment of the evidence. A pooled analysis would take into account the varying study durations (of 18 or 24 months) as well as other between-study differences in dose regimens and mouse strains. These studies, in which mice were exposed to only glyphosate, may have underreported incidence of malignant lymphomas given evidence of increased toxicity of GBHs compared to glyphosate alone [80-82]. GBH mixtures, which contain a number of adjuvants, have been reported to exert synergistic toxic effects in mechanistic studies (discussed below). Therefore, we also recommend the evaluation of GBHs in chronic animal carcinogenicity studies to better capture representative exposure of humans.

8. Potential mechanistic context

There are several possible mechanistic explanations for the increased NHL risk in humans and lymphomas in animals. The etiology of NHL remains largely unknown; however, potential risk factors include autoimmune diseases, infection with viruses and/or bacteria, immunosuppressive medications, and exposures to some pesticides [83,84]. Although not a formally recognized risk factor for NHL, endocrine disruptors have been associated recently with risk of B-cell neoplasms [85], most of which are NHL [57]. Furthermore, a genetic hallmark of NHL is the occurrence of chromosomal translocations, such as t(14;18), involving the immunoglobulin heavy chain gene fusion (BCL2-IGH), which are frequently detected in subgroups of NHL patients [86] and in pesticide-exposed farmers [87,88]. Hence, we discuss immunosuppression/inflammation, endocrine disruption, genetic alterations, and oxidative stress as potential underlying mechanisms for the development of lymphomas. Genetic alterations (genotoxicity) and oxidative stress were previously identified as two glyphosate-related key characteristics of carcinogens [126]. Although not specifically linked to NHL, oxidative stress is a general mechanism of carcinogenesis that could contribute to lymphomagenesis.

8.1. Immunosuppression/Inflammation

The strongest factors known to increase NHL risk are congenital and acquired states of immunosuppression [89]. Several studies suggest that glyphosate alters the gut microbiome [80,90] and cytokine IFN-γ and IL-2 production [91]. These changes could impact the immune system.
promote chronic inflammation [92], and contribute to susceptibility of invading pathogens, such as H. pylori [93].

8.2. Endocrine disruption

Disruption of sex hormones may contribute to lymphomagenesis/ NHL [94]. Glycophosphate may act as an endocrine disrupting chemical (EDC) because it has been found recently to alter sex hormone production. Several in vivo studies of male rats exposed to glycophosphate have reported significantly lower testosterone levels [95-97], spermatic numbers [95], altered spermatogenesis and testicular morphology [95,96], greater development of the mammary gland [98], and a surge in mast cell infiltration and proliferation accompanied by increased estrogen receptor (ESR1) [98]. In ovarian granulosa cells, glycophosphate exposure resulted in decreased cell proliferation and estradiol production [99], which may contribute to lymphomagenesis [94].

8.3. Genetic alterations

Several studies report that glycophosphate can induce single- and double-strand DNA breaks [100-102], purine and pyrimidine oxidation [101], increased comet tail moment [104], and activation of the canonical non-homologous end-joining pathway (c-NHEJ) [102] that stimulates DNA repair. Glycophosphate was also reported to induce micro-nuclei [106-111], sister chromatid exchanges [110], and chromosomal aberrations [112], but other studies found no change in these parameters [113-119]. Conclusions on the genotoxicity of glycophosphate remain controversial in the debate on its carcinogenic potential [118]. A recent review reported that this discrepancy could be attributed to differences in the literature analyzed (published versus unpublished), exposure type (glycophosphate versus GB6), and exposure magnitude (low everyday exposures versus higher exposure groups) [119].

8.4. Oxidative stress

Numerous studies indicate glycophosphate causes oxidative stress [120-123]. Biomarkers of oxidative stress have been reported in a number of tissues in rats and mice, including liver, skin, kidney, brain, and plasma. In a study of albino male rats, levels of hepatic reduced glutathione were significantly decreased in GB6-exposed animals (1.64 mmol/g) compared to controls (2.64 mmol/g) [181]. A different study in glycophosphate-exposed Water rats reported increased lipid peroxidation across all tissues studied and reactive nitrogen species in the brain and plasma [120]. A proteomic analysis of Swiss albino mice reported overexpression of carbonic anhydrase 3, a cytoplasmic protein that plays a role in cellular response to oxidative stress [124].

Generally speaking, these mechanisms, among others, provide evidence of biological plausibility for the observed link between glycophosphate exposure and human NHL, though further work is needed to better understand these factors.

9. Conclusions and future directions

The rise of GB6s as the most widely used herbicide raises serious health concerns, given its potential links with NHL. Using our 'a priori' hypothesis and revising the recently updated AHS cohort in a meta-analysis for the first time, we report that GB6 exposure is associated with increased risk of NHL in humans. Our findings are consistent with results reported from prior meta-analyses that show higher risk for NHL because of our focus on the highest exposure groups. However, given the heterogeneity between the studies included, the numerical risk estimates should be interpreted with caution. Additionally, as noted above and depicted in Fig. 1, the available studies do not capture the possible effects of increased population exposures due to secular increases in use. For example, "green burnout" practices that became widespread in the mid-2000s may be a particularly important source of population exposures. The totality of the evidence from six studies of glycophosphate-exposed mice support this association in humans. Although the underlying mechanisms remain unknown, mechanistic studies of glycophosphate-induced immunosuppression/inflammation, endocrine disruption, genetic alterations, and oxidative stress suggest plausibility between GB6 exposure and NHL development. The overall evidence from human, animal, and mechanistic studies presented here supports a compelling link between exposures to GB6s and increased risk for NHL.

Declaration of interest

All authors have no financial conflicts of interest to declare. We disclose Drs. Zhang, Taioli, and Stephens served as Science Review Board Members of the US EPA FIFRA Scientific Advisory Panel (SAP) Meeting that evaluated glycophosphate in December 2016.

Acknowledgements

The authors thank Christina Gilliane, MPH from Icahn School of Medicine at Mount Sinai, New York for carefully checking epidemiological data and Phum Tachachartvanich, PhD for intellectual review and discussion on mechanisms of endocrine disruption. We also thank the anonymous reviewers for their helpful comments. R.M.S. was supported by National Institutes of Environmental Health Sciences (NIEHS) award T32ES056900 and the University of Washington Retirement Association Aging Fellowship. The authors would like to thank Bill Freeman for his helpful information regarding key market milestones for glycophosphate.

References

403  **glyphosate**

Target Site  EPSP synthase inhibition (5-enolpyruvylshikimate-3-phosphate)
HRAC  G  WSSA 9  glycine derivative

![Glyphosate structure](image)

**NOMENCLATURE:**
- **glyphosate**
  - Common name  glyphosate (BSI, E-ISO, (m) F-ISO, ANSI, WSSA, MAF)
  - IUPAC name  N-(phosphonomethyl)glycine
  - Chemical Abstracts name  N-(phosphonomethyl)glycine
  - CAS RN  1071-83-6  EC no. 213-997-4  EPA PC 417300  Development codes  MON 0573 (Monsanto)

**glyphosate-di ammonium**
- IUPAC name  diammonium N-(phosphonomethyl)glycine
  - Chemical Abstracts name  N-(phosphonomethyl)glycine ammonium salt (1:2)
  - CAS RN  69254-40-6  EPA PC 103607

**glyphosate-dimethylammonium**
- IUPAC name  dimethylammonium N-(phosphonomethyl)glycinate; N-(phosphonomethyl)dimethylamine (1:1)
  - Chemical Abstracts name  N-(phosphonomethyl)glycine compound with N-methyl-N,N-dimethyl
dimethylamine (1:1)
  - CAS RN  34494-04-7  EPA PC 103608

**glyphosate-isopropylammonium**
- IUPAC name  isopropylammonium N-(phosphonomethyl)glycinate; N-(phosphonomethyl)isopropylamine (1:1)
  - Chemical Abstracts name  N-(phosphonomethyl)glycine compound with 2-propanamine
  - CAS RN  58641-94-0  EC no. 254-056-8  EPA PC 103601
  - Development codes  MON 77209 (Monsanto);  MON 0139 (Monsanto)

**glyphosate-mono ammonium**
- IUPAC name  ammonium N-(hydroxyphosphinato)methyl)glycine
  - Chemical Abstracts name  N-(phosphonomethyl)glycine ammonium salt (1:1)
  - CAS RN  40465-66-5
  - Other names  glyphosate-ammonium
  - Development codes  MON 8750 (Monsanto)

**glyphosate-potassium**
- IUPAC name  potassium N-(hydroxyphosphinato)methyl)glycine
  - Chemical Abstracts name  N-(phosphonomethyl)glycine potassium salt (1:1)
  - CAS RN  39600-42-5

**glyphosate-sesquiosodium**
- Chemical Abstracts name  N-(phosphonomethyl)glycine sodium salt (2:3)
  - CAS RN  70393-85-0  EPA PC 103603  Development codes  MON 8722 (glyphosate)
  - (Monsanto);  MON 8000 (Monsanto)

590  **glyphosate**
**PHYSICAL CHEMISTRY:**

**Glyphosate**


M.p. (°C) 189.5, V.p. (mPa) 0.0131 (25 °C), Henry (Pa m³ mol⁻¹, calc.) < 2.1 x 10⁻⁷

log Kₐw < 3.2 (pH 5-9), pKa (20–25 °C) 2.34, 5.73, 10.2

**Water solubility (mg/l, 20–25 °C)** 1.05 x 10⁻⁶ (pH 1.9)

Organic solubility (g/l, 20–25 °C): Soluble in acetone (0.078), dichloromethane (0.233), ethyl acetate (0.112), methanol (0.231), isopropanol (0.02), toluene (0.036). F.p. Not flammable. S.g./Bulk density (20–25 °C) 1.704. Stability: Glyphosate and all its salts are non-volatile, do not photochemically degrade in buffered water and are stable in air. Glyphosate is stable to photolysis at pH 3, 6 and 9 (5–35 °C).

**Glyphosate-diammonium**

M.F. C₃H₉N₂O₅P₂. Mol. wt. 203.1

**Glyphosate-dimethylammonium**

M.F. C₃H₈N₂O₅P₂. Mol. wt. 214.2

**Glyphosate-sopropylammonium**

M.F. C₃H₉NO₃P₂. Mol. wt. 228.2. **Physical form** Odourless, white powder.

M.p. (°C) 143–164; 189–223 (dimorphic), V.p. (mPa) 0.0021 (25 °C)

Henry (Pa m³ mol⁻¹, calc.) 4.6 x 10⁻¹⁰, log Kₐw < 3.4. pKa (20–25 °C) 2.18, 5.77

**Water solubility (mg/l, 20–25 °C)** 1.05 x 10⁻⁶ (pH 4.3)

Organic solubility (g/l, 20–25 °C): Soluble in ethyl acetate (0.00004), heptanes (0.00004), ethanol (15.7–28.4). B.p. Decomp. S.g./Bulk density (20–25 °C) 1.482. Stability: Stable at pH 4.5 and 9 (50 °C).

**Glyphosate-normonammonium**

Composition Tech. is 95.2%. M.F. C₃H₈N₂O₅P₂. Mol. wt. 186.1. **Physical form** Odourless, white powder.

M.p. (°C) > 190 (decomp.), V.p. (mPa) 0.009 (25 °C)

Henry (Pa m³ mol⁻¹, calc.) 1.16 x 10⁻⁸, log Kₐw < 3.7. pKa (20–25 °C) 5.5

**Water solubility (mg/l, 20–25 °C)** 1.44 x 10⁻⁶ (pH 3.2)

Organic solubility (g/l, 20–25 °C): Soluble in acetone (0.0023), methanol (0.159). F.p. Not flammable. S.g./Bulk density (20–25 °C) 1.433. Stability: Stable over 5 days at 50 °C.

**Glyphosate-potassium**

Composition in products described as containing glyphosate-potassium, the CAS Registry number for the salt with unspecified potassium content [7909-12-1] is sometimes quoted. M.F. C₃H₈KNOP. Mol. wt. 207.2. M.p. (°C) 219.8. Henry (Pa m³ mol⁻¹, calc.) 3.38 x 10⁻⁷

log Kₐw < 4.0. pKa (20–25 °C) 5.7. **Water solubility (mg/l, 20–25 °C)** 9.187 x 10⁻² (pH 7)

Organic solubility (g/l, 20–25 °C): Soluble in methanol (0.217)

**Glyphosate-sesquidom**

M.F. C₃H₈N₃Na₂O₅P₂. Mol. wt. 405.2. **Physical form** Odourless, white powder.

M.p. (°C) > 260 (decomp.), V.p. (mPa) 0.00756 (25 °C), Henry (Pa m³ mol⁻¹, calc.) 4.27 x 10⁻⁷

log Kₐw < 4.58. **Water solubility (mg/l, 20–25 °C)** 4.14 x 10⁻⁵ (pH 4.2)

Stability: Stable over 5 days at pH 4.7 and 9.50 °C.

APPLICATIONS:
glyphosate
Spectrum and Route of Action Non-selective systemic herbicide, absorbed by the foliage, rapid translocation throughout the plant, inactivated on contact with soil. Uses: Post-emerge control of annual and perennial grass and broad-leaved weeds in genetically engineered, glyphosate-tolerant soybeans, maize, canola, alfalfa, sugar beets, and cotton; post-emergence control of annual and perennial grass and broad-leaved weeds, pre-harvest, in cereals, peas, as oilseed rape, flax, and mustard, at 1500–2000 g/ha; control of annual and perennial grass and broad-leaved weeds in stubble and post-planting/pre-emergence in many crops; control of annual and perennial grass and broad-leaved weeds in vines and olives, at up to 4300 g/ha as a directed spray application; control of annual and perennial grass and broad-leaved weeds in orchards, pastures, forestry and industrial and around households, at up to 4300 g/ha; pre-harvest desiccation in sugar cane and cereals; control of aquatic weeds, at 2000 g/ha.

Formulation types SG, SL Compatibilities Mixing with other herbicides may reduce the activity of glyphosate. Site of Action Inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), enzyme of the aromatic acid biosynthetic pathway. This prevents synthesis of essential aromatic amino acids needed for protein biosynthesis. Selected products Conco (Aul); Gladiol (Aysta India); Glyfall (Hermero); Karda (Lanco); Maxweed (Crop Health); Nasa (Agro); PI G Industries; Piarsato (Pilagrum); Prince (Aak); Rindor (Inquipor); Rhopecide (Rotan); Secherba (Agrimin); Sharp (Baochong). Selected mixtures Flexstar GT (Syngenta); Folar (Syngenta); Halex GT (Syngenta); Ovni (Aak).

glyphosate-sesquisodium
Uses Sugar cane ripener.


EPA Status Registered – Registration Under Review IARC class 2A Water GV (μg/L) Not established Toxicity class: WHO (a.i.) III

592 glyphosate
ENVIRONMENTAL FATE: Animals In mammals, following oral administration, very rapidly excreted unchanged and does not bioaccumulate. Soil/Environment: DT₅₀ 1-130 d, depending on edaphic and climatic conditions in water. DT₅₀ varies 91 d. Photodegradation in natural water occurs. DT₅₀ 33-77 d, no substantial pKₐ soil was recorded over 31 d in a lab. whole system with water and sediment. DT₅₀ (aerobic), 14–22 d (anaerobic). The major metabolite in soil and water is aminox acid. Plants Slowly metabolised to ammoniumphosphoric acid ([(1066-51-9)]), major plant metabolite.

404 guazatine
Target Site Multi-site inhibition
FRAC M7, M guanidine

NOMENCLATURE:
guazatine
Common name guazatine (BSI, E-ISO, (f) F-ISO); guanoctine (former name, BSI)
Chemical Abstracts name guazatine
CAS RN [108173-90-6]

guazatine acetates
Chemical Abstracts name guazatine acetate (1:1)
CAS RN [115044-19-4]
Other names GTA EPA PC 128881 Development codes MC 25 (Murphy), EM

PHYSICAL CHEMISTRY:
guazatine
Composition The approved common name guazatine was originally defined as applying 1,1'-iminodi(octamethylene)diguanidine (BSI) used the name guanoctine from 1970-1972 known that the material marketed commercially is a reaction mixture. Produced by the amidation of tech. iminoct(di(octamethylene)diamine, commercial guazatine contains many guanidines and polyamines; many of these bases are fungicidal. A replacement common is iminoctidine (q.v.), has been established for 1,1'-iminodi(octamethylene)diguanidine.
guazatine acetates
Physical form Yellow to brownish liquid (tech.). V.p. (mPa) <0.01
Henry (Pa m³ mol⁻¹, calc.) 3.8 x 10⁻⁸ log Kow -1.2 (pH 3), -0.9 (pH 10)
The information in this profile may be out-of-date. It was last revised in 1996. EXTOXNET no longer updates this information, but it may be useful as a reference or resource.

Please visit the National Pesticide Information Center (NPIC) to find updated pesticide fact sheets. If you don't find a fact sheet related to your question, feel free to call 1-800-858-7378. NPIC is open five days a week from 8:00am to 12:00pm Pacific Time.

EXTENSION

Extension Toxicology Network

Pesticide Information Profiles

A Pesticide Information Project of Cooperative Extension Offices of Cornell University, Oregon State University, the University of Idaho, and the University of California at Davis and the Institute for Environmental Toxicology, Michigan State University. Major support and funding was provided by the USDA Extension Service/National Agricultural Pesticide Impact Assessment Program.

EXTOXNET primary files maintained and archived at Oregon State University

Revised June 1996

Glyphosate

**Trade and Other Names:** Trade names for products containing glyphosate include Gallup, Landmaster, Pondmaster, Rager, Roundup, Roundup Select, and Touchdown. It may be used in formulations with other herbicides.

**Regulatory Status:** Glyphosate acid and its salts are moderately toxic compounds in EPA toxicity class II. Labels for products containing these compounds must bear the Signal Word WARNING. Glyphosate is a General Use Pesticide (GUP).

**Chemical Class:** Not Available

**Introduction:** Glyphosate is a broad-spectrum, nonselective systemic herbicide used for control of annual and perennial plants including grasses, sedges, broad-leaved weeds, and woody plants. It can be used on non-cropland as well as on a great variety of crops. Glyphosate itself is an acid, but it is commonly used in salt form, most commonly the isopropylamine salt. It may also be available in acetic or trimethylsulfonium salt forms. It is generally distributed as water-soluble concentrates and powders. The information presented here refers to the technical grade of the acid form of glyphosate, unless otherwise noted.

**Formulation:** Glyphosate itself is an acid, but it is commonly used in salt form, most commonly the isopropylamine salt. It may also be available in acetic or trimethylsulfonium salt forms. It is generally distributed as water-soluble concentrates and powders.

**Toxicological Effects:**

- **Acute toxicity:** Glyphosate is practically nontoxic by ingestion, with a reported acute oral LD50 of 5600 mg/kg in the rat. The toxicities of the technical acid (glyphosate) and the formulated product (Roundup) are nearly the same [58,96]. The oral LD50 for the trimethylsulfonium salt is reported to be approximately 750 mg/kg in rats, which indicates moderate toxicity [58]. Formulations may show moderate toxicity as well (LD50 values between 1000 mg/kg and 5000 mg/kg) [58]. Oral LD50 values for glyphosate are greater than 10,000 mg/kg in mice, rabbits, and guinea pigs [96,98]. It is practically nontoxic by skin exposure, with reported dermal LD50 values of greater than 5000 mg/kg for the acid and isopropylamine salt. The trimethylsulfonium salt has a reported dermal LD50 of greater than 2000 mg/kg. It is reportedly not irritating to the skin of rabbits, and does not induce skin sensitization in guinea pigs [58]. It does cause eye irritation in rabbits [58]. Some formulations may cause much more extreme irritation of the skin or eyes [58]. In a number of human volunteers, patch tests produced no visible skin changes or sensitization [58]. The reported 4-hour rat inhalation LC50 values for the technical acid and salts were 5 to 12 mg/L [58], indicating moderate toxicity via this route. Some formulations may show high acute inhalation toxicity [58]. While it does contain a phosphonate functional group, it is not structurally similar to organophosphorus pesticides which contain organophosphorus esters, and it does not significantly inhibit cholinesterase activity [1,96].

- **Chronic toxicity:** Studies of glyphosate lasting up to 2 years, have been conducted with rats, dogs, mice, and rabbits, and with few exceptions, no effects were observed [96]. For example, in a chronic feeding study with rats, no toxic effects were observed in rats given doses as high as 400 mg/kg/day [58]. Also, no toxic effects were observed in a chronic feeding study with dogs fed up to 500 mg/kg/day, the highest dose tested [58,97].

- **Reproductive effects:** Laboratory studies show that glyphosate produces reproductive changes in test animals very rarely and then only at very high doses (over 150 mg/kg/day) [58,96]. It is unlikely that the compound would produce reproductive effects in humans.

- **Teratogenic effects:** In a teratology study with rabbits, no developmental toxicity was observed in the fetuses at the highest dose tested (350 mg/kg/day) [97]. Rats given doses up to 175 mg/kg/day for 6 to 19 of pregnancy had offspring with no teratogenic effects, but
other toxic effects were observed in both the mothers and the fetuses. No toxic effects to the fetuses occurred at 50 mg/kg/day [97]. Glycophosate does not appear to be teratogenic.

- Mutagenic effects: Glycophosate mutagenicity and genotoxicity assays have been negative [58]. These included the Ames test, other in vitro assays, and the Chinese Hamster Ovary (CHO) cell culture. Rat bone marrow cell culture, and mouse dominant lethal assays [58].

- Carcinogenic effects: Rats given oral doses of up to 100 mg/kg/day did not show any signs of cancer, nor did dogs given oral doses of up to 500 mg/kg/day in once-daily fed glycophosphate at doses of up to 4500 mg/kg/day [58]. It appears that glycophosate is not carcinogenic [97].

- Organ toxicity: Some microscopic liver and kidney changes, but no observable differences in function or toxic effects, have been seen after lifetime administration of glycophosate to test animals [97].

- Fate in humans and animals: Glycophosate is poorly absorbed from the digestive tract and is largely excreted unchanged by mammals. After small amounts of glycophosphate had undetectable levels (less than 0.05 ppm) in muscle tissue and fat. Levels in milk and eggs were also undetectable (less than 0.025 ppm). Glycophosphate has no significant potential to accumulate in animal tissue [99].

Ecological Effects:

- Effects on birds: Glycophosphate is slightly toxic to wild birds. The dietary LC50 in both mallards and bobwhite quail is greater than 4500 ppm [1].

- Effects on aquatic organisms: Technical glycophosphate is practically nontoxic to fish and may be slightly toxic to aquatic invertebrates. The 96-hour LC50 is 120 mg/L in bluegill sunfish, 167 mg/L in harlequin, and 86 mg/L in rainbow trout [58]. The reported 96-hour LC50 values for other aquatic species include greater than 10 mg/L in Atlantic oysters, 12 mg/L in pickerel, and 28 mg/L in shrimp [58]. Formulations may be more toxic to fish and aquatic species due to differences in toxicity between the salts and the parent acid or to surfactants used in the formulation [58,96]. There is a very low potential for the compound to bioaccumulate in or build up in the tissues of aquatic organisms [58].

- Effects on other organisms: Glycophosphate is nontoxic to honeybees [1,58]. Its oral and dermal LD50 is greater than 0.1 mg/bee [98]. The reported contact LC50 values for earthworms in soil are greater than 5000 ppm for both the glycophosphate trimethylsulfonium salt and Roundup [58].

Environmental Fate:

- Breakdown in soil and groundwater: Glycophosphate is moderately persistent in soil, with an estimated average half-life of 47 days [58,11]. Reported field half-lives range from 1 to 174 days [11]. It is strongly adsorbed to most soils, even those with lower organic and clay content [11,58]. Thus, even though it is highly soluble in water, field and laboratory studies show it does not leach appreciably, and has low potential for runoff (except as adsorbed to colloidal matter) [3,11]. One estimate indicated that less than 2% of the applied chemical is lost to runoff [99]. Microbes are primarily responsible for the breakdown of the product, and volatilization or photodegradation losses will be negligible [58].

- Breakdown in water: In water, glycophosphate is strongly adsorbed to suspended organic and mineral matter and is broken down primarily by microorganisms [4]. Its halflife in pond water ranges from 12 days to 10 weeks [97].

- Breakdown in vegetation: Glycophosphate may be translocated throughout the plant, including to the roots. It is extensively metabolized by some plants, while remaining intact in others [1].

Physical Properties:

- Appearance: Glycophosphate is a colorless crystal at room temperature [1].
- Chemical Name: N-(phosphonomethyl)glycine [1].
- CAS Number: 1071-83-6
- Molecular Weight: 169.08
- Water Solubility: 12,000 mg/L at 25 °C [1].
- Solubility in Other Solvents: i.e., in common organics (e.g., acetone, ethanol, and xylene) [1].
- Melting Point: 200 °C [1].
- Vapor Pressure: negligible [1].
- Partition Coefficient: 3.2218 - 2.7696 [58].
- Adsorption Coefficient: 24,000 (estimated) [11].

Exposure Guidelines:

- ADE: 0.3 mg/kg/day [12].
- MCL: Not Available.
- RFD: 0.1 mg/kg/day [13].
- PEL: Not Available.
- HA: 0.7 mg/L (lifetime) [98].
- TLV: Not Available.

acetic Manufacturer:

Ontario Company
10 N. Lindbergh Blvd.
St. Louis, MO 63167

- Phone: 314-694-6640
- Emergency: 314-694-4000

References:

nef@ecr.edu/pip/glycophosate.htm
The information in this profile does not in any way replace or supersede the information on the pesticide product labeling or other regulatory requirements. Please refer to the pesticide product labeling.
<table>
<thead>
<tr>
<th>Soybean</th>
<th>Commelina sp, Digeru arvensis, Acalypha indica, Amaranthus viridis.</th>
<th>13.6</th>
<th>125</th>
<th>500</th>
<th>73</th>
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</table>

**Glufosinate Ammonium 13.5% SL (15% w/v)**

<table>
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<tr>
<th>Tea</th>
<th>Panicum repens, Borreria hispida, Imperata cylindrical, Digitaria sanguinalis, Commelina benghalensis, Ageratum conyzoides, Eleusine indica, Paspalum conjugatum</th>
<th>0.375-0.500</th>
<th>2.5-3.3</th>
<th>375-500</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>Echinochloa sp. Cynodon dactylon Cyperus rotundus Digitaria marginata Dactylolcteneum aegyptium</td>
<td>375-450</td>
<td>2.5-3.0</td>
<td>500</td>
<td>96</td>
</tr>
</tbody>
</table>

**Glyphosate 20.2% SL IPA salt**

| Non Crop area | Phyllanthus niruri, Ageratum conyzoides, Parthenium hysterophorus, Sorghum halepense, Amaranthus spinosus, Alternanthera sessilis, Cynodon dactylon, Cyperus rotundus, Echinochloa colonum, Trianthema portulacastrum | 0.82-1.23 kg | 4.1-6.15 | 400-500 | N/A |
| Non Crop area | Cynodon dactylon  
|              | Commelina benghalensis  
|              | Panicum spp.  
|              | Dactyloctenium aegyptium  
|              | Eragrostis major  
|              | Poa annua  
|              | Cyperus rotundus  
|              | Parthenium hysterophorus  
|              | Acalypha indica  
|              | Digeria arvensis  
|              | Phyllanthus niruri  
|              | Euphorbia geniculata  
|              | Corchorus actangularis  
|              | Saccharum sponteum  
|              | Eleusine indica  
|              | Imperata cylindrical  
|              | Ageratum conzoides  |
|             | 4.52-6.79g a.i./litre  |
|             | 20-30ml/lit  |
|             | 300-600  |

**Glyphosate 41% SL: IPA Salt**

| Tea | Arundinella bengalensis  
|     | Axonopus compressus  
|     | Cynodon dactylon  
|     | Imperata cylindrical  
|     | Kalm grass  
|     | Paspalum scrobiculatum  
|     | Polygonum perfoliatum  |
|     | 0.820-1.230kg.  |
|     | 2.0-3.0  |
|     | 450  |
|     | 21  |

| Non-cropped area | Soghum helepense and other dicot & monocot weeds in general  |
|                 | 0.820-1.230kg.  |
|                 | 2.0-3.0  |
|                 | 500  |

**Glyphosate 54% SL: (IPA Salt)**

| Non Crop Area | Ageratum conzoides  
|              | Alternenthera sessilis  
|              | Commimina spp  
|              | Cyperus spp  
|              | Echinochloa sp.  
|              | Eclipta alba  
|              | Iscaemum rogosum  
|              | Setaria spp  |
|              | 1.8 kg  |
|              | 3.33 ltrs.  |
|              | 400-500  |

**Glyphosate Ammonium Salt 5% SL**
<table>
<thead>
<tr>
<th></th>
<th>Ageratum conyzoides</th>
<th>1.5 kg.</th>
<th>30 ltrs.</th>
<th>500</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea</td>
<td>Bidens pilosa</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Borreria latifolia</td>
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<tr>
<td></td>
<td>Cynodon dactylon</td>
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<td></td>
<td>Cyperus rotundus</td>
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<td></td>
<td>Digitaria sanguinalis</td>
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<tr>
<td></td>
<td>Euphorbia spp.</td>
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<tr>
<td></td>
<td>Imperata cylindrica</td>
<td></td>
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<td></td>
<td>Paspalum conjugatum</td>
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<tr>
<td>Nono Crop area</td>
<td>Cynodon dactylon</td>
<td>2 kg.</td>
<td>40 ltrs.</td>
<td>500</td>
<td></td>
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<td></td>
<td>Cyperus rotundus</td>
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<tr>
<td></td>
<td>Digera arvensis</td>
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<tr>
<td></td>
<td>Digitaria sanguinalis</td>
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<tr>
<td></td>
<td>Eragrostis minor</td>
<td></td>
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<tr>
<td></td>
<td>Euphorbia spp.</td>
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<td></td>
<td>Parthenium</td>
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<td>hysterocephorus</td>
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<tr>
<td></td>
<td>Tribulus terrestris</td>
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<tr>
<td></td>
<td>Xantrium stremerium</td>
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<tr>
<td>Glyphosate 71% SG  (Ammonium Salt)</td>
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<tr>
<td>Tea &amp; Nono Crop area</td>
<td>Acalypha indica</td>
<td>2.13 kg</td>
<td>3.0 kg.</td>
<td>500</td>
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</tr>
<tr>
<td></td>
<td>Ageratum conyzoides</td>
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<tr>
<td></td>
<td>Cytorium intybus</td>
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<tr>
<td></td>
<td>Digera arvensis</td>
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</tr>
<tr>
<td></td>
<td>Cynodon dactylon</td>
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<td></td>
<td>Cyperus rotundus</td>
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<td></td>
<td>Digitaria sanguinalis</td>
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<tr>
<td></td>
<td>Eragrostis spp.</td>
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<td></td>
<td>Ipomea digitarea</td>
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<tr>
<td></td>
<td>Paspalum conjugatum</td>
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<td></td>
<td>Sida aculata</td>
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<tr>
<td>Halosulfuron Methyl 75% WG</td>
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<tr>
<td>Sugarcane</td>
<td>Cyperus rotundus</td>
<td>60-67.5</td>
<td>80-90</td>
<td>375</td>
<td>294</td>
</tr>
<tr>
<td>Maize</td>
<td>Cyperus rotundus</td>
<td>67.5</td>
<td>90</td>
<td>375</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Cyperus iria</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bottle gourd</td>
<td>Cyperus rotundus</td>
<td>67.5</td>
<td>90</td>
<td>375</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Cyperus iria</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Haloxytop R Methyl 10.5% w/w EC</td>
<td></td>
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<tr>
<td>Soybean</td>
<td>Brachiaria sp.</td>
<td>108-135</td>
<td>1000-1250</td>
<td>500</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Digitaria sanguinalis</td>
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<td></td>
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<tr>
<td></td>
<td>Dinebra arabica</td>
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</tbody>
</table>
Committee deliberated the agenda and approved the application for grant of registration under section 9(3) observing that now the applicant has submitted manufacturing license issued to M/s BASF for the manufacturing unit where the product is intended to be manufactured.

11.2 **Pesticides to be reviewed as a follow up action of Dr. Anupam Verma Committee recommendation.**

The committee noted that 27 pesticides were recommended to be continued use with the condition of submission of recommended studies and these pesticides were subject to be reviewed. The RC decided that the certificate of registration of these pesticides shall be kept on hold till the decision of review becomes available. RC desired that the review report shall be submitted by the sub-committee expeditiously within a period of one month.

11.3 **Sub-committee report on definition on Non-cropped/Non-crop area**

The RC withdraw the definition of non-cropped area for more clarification as the same was approved in Agenda Item No. 10.25 of 404th RC meeting.

11.4 **Committee to review continued use of certain pesticide.**

Committee decided to constitute a Sub-committee to review the continued use or otherwise of the following pesticides:

1. Glyphosate
2. Profenophos
3. Neonicotenoids
4. 27 pesticides recommended to be continued but to be reviewed after completion of the recommended studies.

The composition of the sub-committee:

1) Dr. Sushil K. Khurana – Chairman
2) Dr. Sandhya Kulshrestha – Member
3) Dr. Archana Sinha – Member
4) Sh. Kiran W. Deshkar – Member
5) Sh. A. K. Reddy – Member
6) Dr. Vandana Pandey – Member
7) Dr. Sarita Bhalla – Member Secretary

11.5 **Request of Joint Director (PP) IPM, DPPQ&S for inclusion of Emamectin benzoate against FAW on Maize and Sorghum on temporary basis.**

The matter was raised by the Plant Protection Advisor as per the request received from Joint Director (PP) IPM division, DPPQ&S, Faridabad dated 04.10.2019 wherein it was requested to consider temporary label claim of Emamectin benzoate against the use of Fall Army Worm (FAW) Spodoptera frugiperda on Maize and Sorghum. This matter was discussed during National Conference Rabi 2019 on 20 September 2019 in open house under the Chairmanship of Secretary (DACFW) with the facts that on the basis of earlier recommendations for use of Emamectin benzoate, as an advance preparatory step, many states include in the package of recommendation and this pesticide was found effective. RC deliberated on the issue and decided to extend label claim till kharif season of 2019 and further sought clarification from ICAR-Indian Institute of Maize Research (IIMR) for its efficacy.
### 1.2 Possibility of registration of insecticides as generic insecticides/pesticides.

Committee deliberated the agenda and accepted the recommendations of the sub-committee which are as under:

1. Committee has recommended for the amendment in the labelling provisions of Insecticides Rules, 1971, so as the common/generic name is given more prominence than the trade name. The generic name should be mentioned above and two font size larger than the trade name.
2. Directions to be given to state agriculture department of all the States/UTs to issue manufacturing license for generic pesticides with prominence.
3. Accordingly, all the stockists/retailers should be directed to keep the separate shelves for display of generic pesticides for sale which should be visible to the end users.
4. Committee also suggested for the training by Central and State agriculture extension functionaries to create awareness regarding purchase and use of generic pesticides by farmers and other end-users.

### 1.3 Revisit of current Guidelines for registration of pesticides u/s 9(3) and 9(4) of the Insecticides Act, 1968.

The committee deliberated the agenda and felt that there is need for simplification of current guidelines to make it more user friendly, harmonize, rationalize, to promote make in India and considering ease of doing business. Accordingly, a sub-committee was constituted with following composition:

1. Dr. Sandhya Kolsrestha, Consultant (Pharma) – Chairperson
2. Dr. Anupama Singh, Principal Scientist & Head, Department of Agro Chemical, IARI – Member
3. Dr. Rohit Mishra, Assistant Industrial Advisor, Shastri Bhawan, New Delhi – Member
4. Dr. Sarita Bhalla, Consultant (Pharma) – Member
5. Sh. Kiran W. Deshkar, DD (E) – Member
6. Sh. A. K. Reddy, DD (WS) – Member
7. Dr. Vandana Pandey, AD (PP) – Member
8. Sh. Avnish Tomar, AD (Pack) – Member
9. Member from IARI, Entomology Division – Member
10. Dr. Archana Sinha, JD (Chem) – Member Secretary

The sub-committee may co-opt any expert to contribute in the endeavor and may submit its report within three months.

### 1.4 Requirement of sample drawl for registration of Technical Indigenous Manufacture u/s 9(3).

RC deliberated the agenda and accepted the proposal of doing away with the requirement of drawl of in process sample u/s 9(3) TIM application and to replace this requirement with the submission of five batch analysis report from GLP laboratory along with application and a sample to CIL. Further, the committee noted that a sub-committee has been formed to review the guidelines (under item no. 1.3 above), hence, this matter may also be considered by the same sub-committee. Meanwhile the existing procedure may continue.

### 1.5 Review of pesticides with respect to agenda item no. 11.2 and 11.4 of 409 RC meeting (twenty-seven pesticides recommended to be continued but to be reviewed after completion of the studies recommended by Dr. Anupam Varma/RC, Neonicotinoids, Glyphosate and Profenofos).

The status w.r.t. 35 pesticides was presented. RC decided that the sub-committee should invite concerned pesticide associations and stakeholders to discuss the deficiencies in submission of required data/information as per recommendations of Dr. Anupam Varma Committee /RC and also have their view. Also directed sub-committee for submission of final report within 4 weeks time.
Documents to be submitted by the Valid Registration Certificate Holders (online mode):
Applications may be received either company wise or product wise with following information:-

i. Documents for Authorized Signatory or BOD resolution.
ii. Duly notarized copy of Valid Certificate of Registration.
iii. Duly notarized copy of already approved Label and leaflet.
iv. Draft new label and leaflet.
v. Revised manner of packing for small and ultra small packing (as applicable).
vi. Affidavit duly notarized regarding endorsement made earlier in respect of Certificate of Registration.
vii. Information/undertaking by 9(3)/9(4) registrants for non-submission of draft label and leaflets and revised manner of packing for secondary packing with justification/reason within six months from the date of publication of the notification.

A separate link to be created by NIC (CROP software) to enable applicants for submitting the applications online for amendment of label and leaflets.

RC further decided that the above decision be conveyed to DAC&FW and also be hoisted on the website of the Sectt. of CIB&RC for information and compliance by the stake holders.

3.2 Sub-committee report on definition on Non-crop area/Non-cropped area.

Committee deliberated the agenda and constituted a sub-committee under the chairmanship of ADG (PP), ICAR for defining Non-crop/non-cropped area with more clarification with following members :-

1. Representative from Directorate of Weed Research, Jabalpur - Member
2. Representative nominated by PPA - Member

The committee shall submit its report within a month.

3.3 Follow up action of agenda Item No. 1.5 [Review of pesticides with respect to agenda Item No. 11.2 and 11.4 of 409th RC meeting (Twenty seven pesticides recommended to be continued but to be reviewed after completion of the studies recommended by Dr. Anupam Varma Committee/RC, Neonicotinoids, Glyphosate and Profenofos)] of 410th RC held on 05.11.2019.

The RC was apprised about the progress of the sub-committee. As directed in 410th meeting the sub-committee had discussions with pesticides associations/stake holders on 11.11.2019 and thereafter the relevant data/information submitted by them were also scrutinized. The information sought by the Dr. Anupam Varma committee / RC was found deficient therefore, it was decided that one more opportunity should be given to them before presenting the final report to the RC.
Minutes of 412th RC meeting held on 18.12.2019

<table>
<thead>
<tr>
<th>8. Agenda Item No. 10.5 &amp; 10.14</th>
<th>List of closed file is enclosed as Annexure - III, respectively.</th>
</tr>
</thead>
</table>

### 2.0 Presentation

**NIL**

### 3.0 Government Business

#### 3.1 Members may kindly recall that the Registration Committee in its 408th meeting constituted a sub-committee in view of some miscellaneous issues being faced while considering various application under different category.

RC deliberated upon the report of sub-committee and decided that the same shall be uploaded on the website for inviting comments from the stake holders within 30 days from the date of uploading of the public notice. The comments received from the stake holders will be presented to the RC for further deliberation and acceptance.

#### 3.2 Follow up action of agenda Item No. 1.5 [Review of pesticides with respect to agenda Item No. 11.2 and 11.4 of 409th RC meeting (Twenty seven pesticides recommended to be continued but to be reviewed after completion of the studies recommended by Dr. Anupam Varma Committee/RC, Neonicotinoids, Glyphosate and Profenofos)] of 410th RC held on 05.11.2019.

RC deliberated, the analysis and comments pertaining to each listed pesticide (27 + 6 + 2) which were provided by subcommittee. The recommendations in the form of report will be submitted to DAC&FW for further necessary action.

### 4.0 Export Cases

#### 4.1 List under section 9(3) Export applications

Committee deliberated the agenda and approved the case as per Annexure 4.1.1, 4.1.2, 4.1.3 & 4.1.4. The committee decided that the application of import for export category shall be subject to the decision taken by the committee in its 356th meeting. It was also directed to recheck case and should strictly follow Gazette Notification SO 3951 (E) dated 08.08.2018 published on 09.08.2018 of Ministry of Agriculture and Farmers Welfare while issuing approvals.

### 5.0 9(3B) Cases

#### 5.1 Consideration of application of M/s Oshnic Crop Science Ltd., for grant of registration for indigenous manufacture of *Pseudomonas fluorescens* 1.0% WP under section 9(3b) (Strain: IIHR, PF-2, Accession No. ITCC No. B0034).

RC deliberated the agenda and approved subject to submission of sample ICAR-NBAIM, Mau Nath Bhanjar, UP for confirmation of microbial culture at molecular level on the strain for 16s ITS sequencing and the DNA of the strain (old application).

#### 5.2 Consideration of application of M/s Dharti Biotech, for grant of registration for indigenous bio-pesticides manufacturing of *Trichoderma viride* 1.50% WP under section 9(3b) (Strain: IIHR, TV-5, Accession No. ITCC No. 6889).