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Carcinogenic effects of long-term exposure from prenatal life to glyphosate and glyphosate-based herbicides in Sprague–Dawley rats

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Abstract

Background Glyphosate-based herbicides (GBHs) are the world's most widely used weed control agents. Public health concerns have increased since the International Agency for Research on Cancer (IARC) classified glyphosate as a probable human carcinogen in 2015. To further investigate the health effects of glyphosate and GBHs, the Ramazzini Institute launched the Global Glyphosate Study (GGS), which is designed to test a wide range of toxicological outcomes. Reported here are the results of the carcinogenicity arm of the GGS.

Methods Glyphosate and two GBHs, Roundup Bioflow used in the European Union (EU) and RangerPro used in the U.S., were administered to male and female Sprague–Dawley (SD) rats, beginning at gestational day 6 (via maternal exposure) through 104 weeks of age. Glyphosate was administered through drinking water at three doses: the EU acceptable daily intake (ADI) of 0.5 mg/kg body weight/day, 5 mg/kg body weight/day and the EU no-observed adverse effect level (NOAEL) of 50 mg/kg body weight/day. The two GBH formulations were administered at the same glyphosate-equivalent doses.

Results In all 3 treatment groups, statistically significant dose-related increased trends or increased incidences of benign and malignant tumors at multiple anatomic sites were observed compared to historical and concurrent controls. These tumors arose in haemolymphoreticular tissues (leukemia), skin, liver, thyroid, nervous system, ovary, mammary gland, adrenal glands, kidney, urinary bladder, bone, endocrine pancreas, uterus and spleen (hemangiosarcoma). Increased incidences occurred in both sexes. Most of these involved tumors that are rare in SD rats

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(background incidence < 1%) with 40% of leukemias deaths in the treated groups occurring before 52 weeks of age and increased early deaths were also observed for other solid tumors.

Conclusions Glyphosate and GBHs at exposure levels corresponding to the EU ADI and the EU NOAEL caused dose-related increases in incidence of multiple benign and malignant tumors in SD rats of both sexes. Early-life onset and mortality were observed for multiple tumors. These results provide robust evidence supporting IARC's conclusion that there is "sufficient evidence of carcinogenicity [of glyphosate] in experimental animals". Furthermore, our data are consistent with epidemiological evidence on the carcinogenicity of glyphosate and GBHs.

Keywords Glyphosate, Glyphosate-based herbicide, Pesticides, Cancer, Sprague–Dawley rat, Acceptable daily intake, Prenatal, Bioassay, DOHaD

Background

Glyphosate [IUPAC chemical name N-(phosphonomethyl) glycine], is a broad-spectrum herbicide used for non-selective weed control both in conventional agriculture and non-agricultural settings. Glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, a component of the shikimate pathway involved in the synthesis of essential aromatic amino acids in plants [1]. Glyphosate's extensive use reflects its effectiveness in controlling a wide range of both broadleaf and grassy weeds. This extensive use of glyphosate-based herbicides (GBHs) has resulted in this compound and its metabolites being readily detected in foodstuffs [2, 3], water [4], and dust [5], indicating a potential for widespread exposure.

In March 2015, the International Agency for Research on Cancer (IARC), the cancer agency of the World Health Organization (WHO), classified glyphosate as *probably carcinogenic to humans* (Group 2A) based on *limited evidence of carcinogenicity* in humans, in particular non-Hodgkin lymphoma, and *sufficient evidence of carcinogenicity in rodents* [6]. In 2015, the IARC Working Group concluded additionally that there is strong evidence that glyphosate is genotoxic and that it induces oxidative stress, including in human cells in vitro [7]. To date, evidence from mechanistic studies in humans and animal models suggests that glyphosate and its formulations possess several of the ten key characteristics of carcinogens [8], including genotoxicity, epigenetic alteration, oxidative stress, chronic inflammation, gut microbiome perturbations, and endocrine disruption [9]. In rats, glyphosate absorption from the gastrointestinal tract to up to 30% of the dose and adjuvants did not seem to exert a major effect on the absorption and excretion of glyphosate [6, 10]. Only limited information is available on human exposure or biological half-life of glyphosate in humans [11, 12].

In 2019, the Ramazzini Institute (RI) launched the Global Glyphosate Study (GGS), a multi-institutional study with the aim of providing the most comprehensive toxicological evaluation of glyphosate and GBHs (glyphosatestudy.org). The GGS is an integrated study [13] designed to test a wide range of toxicological outcomes including carcinogenicity, neurotoxicity, multi-generational effects, organ toxicity, endocrine disruption and prenatal developmental toxicity. Results from different arms of the study and its pilot phase have been already published [9, 10, 14].

In designing the GGS, it was hypothesized that exposure to GBHs may be more toxic than exposure to glyphosate alone because GBHs contain non-herbicidal co-formulants that facilitate glyphosate penetration into plant tissues through the waxy surface of the leaves [15]; hence, it was hypothesized that these co-formulants might increase glyphosate bioavailability in mammals, including humans. Recognizing this potential risk, in 2016 the European Union (EU) banned polyoxyethylene tallow amine (POEA)-type co-formulants from use in GBHs [15–18], but these formulations are still in use outside the EU Following the EU phase-out of POEA, GBH manufacturers have turned to other surfactants.

Here the results are presented on the incidence of neoplastic lesions from the carcinogenicity arm of the GGS. In this investigation, glyphosate and two commercial GBH formulations, the EU formulation Roundup Bioflow (MON 52276) and the US formulation RangerPro (EPA 524–517), were administered long-term to SD rats starting from prenatal life via drinking water to dams at glyphosate concentrations of 0.5, 5, and 50 mg/kg bw/day, and continuing through two years of age. RangerPro contains POEA surfactants [19], while Roundup Bioflow does not contain POEA [20], however the complete co-formulants profile is still currently undisclosed. This dose range encompasses both the EU acceptable daily intake (ADI; 0.5 mg/kg bw/day) and the EU no-observed-adverse-effect level (NOAEL; 50 mg/kg bw/day) for glyphosate [21].

Methods

Test substances

The three test substances administered to female and male SD rats in drinking water were the following:

Glyphosate, CAS 38641-94-0 [N-(phosphonomethyl) glycine, purity 99%, Sigma-Aldrich, Milan, Italy], the commercial GBH formulation Roundup Bioflow [MON 52276, containing 360 g/L of glyphosate acid in the form of 486 g/L isopropylamine salts of glyphosate (41.5%)] and the commercial GBH formulation RangerPro [EPA registration n. 524–517, containing 356 g/L of glyphosate acid in the form of 480 g/L isopropylamine salts of glyphosate (41%)]. Solutions of these three test substances were prepared by the addition of an appropriate volume of tap drinking water, to obtain the desired corresponding concentration of glyphosate, in line with analyses of the solutions stability and concentrations of the test substance [10].

Animals and experimental design

All aspects of the animal experiments were performed according to Italian law regulating the use and treatment of animals for scientific purposes (Legislative Decree n. 26, 2014. Implementation of the directive n. 2010/63/EU on the protection of animals used for scientific purposes. G.U. General Series, n. 61, March 14 th, 2014). All animal study procedures were performed at the Cesare Maltoni Cancer Research Centre of the Ramazzini Institute (RI), Bentivoglio, Italy. The study protocol was approved by the Ethical Committee of the Ramazzini Institute and approved and formally authorized by the ad hoc commission of the Italian Ministry of Health (ministerial approval n. 945/2018-PR).

The RI animal breeding facility was the supplier of SD rats. Female breeder SD rats were placed individually in polycarbonate cages (42 ×26x18 cm; Tecniplast Buguggiate, Varese, Italy) with a single unrelated male (outbred) until evidence of copulation (presence of a vaginal copulation plug or sperm in a daily vaginal lavage) was observed. After mating, the male was removed, and females were housed separately during gestation and delivery. Newborns were housed with their dams until weaning. Weaned offspring were co-housed, by sex and treatment group, no more than three animals per cage. Cages were identified by a card indicating: study protocol code, experimental and pedigree numbers, and dosage group. A shallow layer of white fir wood shavings served as bedding (supplier: Giuseppe Bordignon, Treviso, Italy).

During the experiment, animals had ad libitum access to an organic pellet feed "Corticella bio" supplied by Laboratorio Dottori Piccioni Srl (Piccioni Laboratory, Milan, Italy). In addition, the animals drank fresh municipal tap water from glass bottles ad libitum and bottles were changed daily. The pelleted feed was tested for possible glyphosate contamination in compliance with Commission Regulation (EU) No 293/2013 [maximum residue levels (MRLs) < 1 mg/kg]. Tap drinking water was tested for possible glyphosate contamination in compliance with Directive 2008/105/EC, D.Lgs. 152/2006, Directive2006/118/EC (active substances in pesticides, including their relevant metabolites, degradation and reaction products < 0.1 µg/l). Analyses of chemical characteristics (pH, ashes, dry weight, and specific weight) and possible glyphosate (< 0.01 mg/Kg) and other chemicals contaminations (metals, aflatoxin, polychlorinated biphenyls, organophosphorus and organochlorine pesticides) of the bedding was performed by CONSULAB srl Laboratories (Treviso, Italy).

The cages were placed on racks at room temperature of 22 °C ± 3 °C and 50% ± 20% of relative humidity. Daily checks on temperature and humidity were performed. The light was artificial, and a light/dark cycle of 12 h was maintained.

Sprague–Dawley rat dams (F0) and relative pups (F1) were treated with either glyphosate or Roundup Bioflow or RangerPro diluted in drinking water to achieve the desired glyphosate concentration. The three concentrations selected for each test substance were 5, 50 and 500 mg/L of glyphosate in drinking water: the target exposure levels, based on mean water consumption (40 ml) and a mean body weight (400 g), corresponded to 0.5, 5 and 50 mg/kg bw/day. Control animals were administered drinking water ad libitum. The total number of experimental groups were 10: control group (drinking water), glyphosate groups (3 doses), Roundup Bioflow groups (3 doses), RangerPro groups (3 doses). F0 animals were randomly assigned to treatment groups in order to have no more than 1 male and 1 female per litter per group and were mated outbred. The F0 female breeders received the treatment through drinking water from gestational day (GD) 6, corresponding to early embryo implantation and start of organogenesis to the end of lactation, while the offspring (F1) continued to be exposed after weaning until study termination at 104 weeks of age. After weaning, F1 animals were randomly distributed in order to have no more than 2 males and 2 females per litter per group. Each F1 experimental group was composed of 51 males and 51 females, belonging to the same treatment group as their exposed dams. The total number of animals was 1020 (510 males and 510 females).

Necropsy, histopathology and immunohistochemistry

The in vivo phase of the investigation ended at 104 weeks of age when all surviving animals were euthanized. All animals underwent a complete necropsy including an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs in situ. Histopathology was

performed on the following organs and tissues of all animals: skin and subcutaneous tissue, mammary gland (4 sites: axillary and inguinal, right and left), brain with cerebellum and medulla/pons, pituitary gland, salivary glands, Harderian glands, tongue, esophagus, thyroid and parathyroid, thymus and mediastinal lymph nodes, trachea, lungs, heart, liver (2 lobes for histopathology), spleen, pancreas, kidneys, adrenal glands, stomach (forestomach and glandular stomach), small intestine, large intestine (with the Peyer's patches), bladder, uterus (including cervix), ovaries, vagina, testes and epididymis, seminal vesicles and coagulating glands, prostate, subcutaneous lymph nodes, mesenteric lymph nodes, sternum, cranium and all gross lesions and other tissues when anomalies were present. 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. Complete histopathological evaluations were performed by the pathologists on all organs and tissues from all animals. Histopathology evaluation was performed by at least two pathologists and all lesions were peer-reviewed. The pathological evaluations were performed according to the procedures of the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) [22–29]. All neoplastic lesions are listed in supplementary material (Supplementary materials: Tables 1–4). For rare tumors (historical control incidence < 1%), a comparison with historical control incidences from long-term carcinogenicity studies in SD rats conducted by the RI (lifetime studies) and the US National Toxicology Program (NTP) (two years studies) [30] was performed and incidences exceeding both laboratories were reported. The historical controls of the RI include 7142 animals (3572 males and 3570 females) from studies performed between 1984 and 2008, while NTP historical controls include 1180 animal (590 females and 590 males) from studies performed between 2011 and 2019. RI historical controls data on leukemia are available only for 490 animals, as these were previously classified together with other tumors as "hemolymphoreticular neoplasias" [31].

Immunohistochemistry was used to confirm diagnosis and characterization of malignant Schwannomas and lymphomas. For malignant Schwannomas S-100 (Z0311, Dako Agilent) was used [32]. For lymphomas, a panel of antibodies was used: CD68 (MCA341GA, AbD Serotec), CD3 (ab5690, abcam), CD138 (sc-12765, Santa Cruz), CD45 (ab10558. abcam), CD20 (sc-393,894, Santa Cruz Biotechnology), TdT (BMS14 − 9739–82, eBiosciences), CD33 (orb10316, biorbyt) [33]

Statistical methods

For all analyses, p values < 0.05 were considered statistically significant. Data were collected from both sexes and analyses were conducted separately.

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier and is presented graphically [34]. All animals were considered in the analysis, including those dying from unnatural causes within the observation period (humanitarian sacrifices) and those surviving to the end of the observation period terminated via final sacrifice; their survival time was fixed at 104 weeks.

Dose-related differences and trends were assessed using a log-rank test, first on all experimental groups together, and then separately by exposure group (Glyphosate vs Control, Roundup Bioflow vs Control, RangerPro vs Control). For the subgroup of animals bearing any type of leukemia, dose-related trends were examined with Tarone and Ware's life-table test [35]. All reported p values for the survival analyses are two-sided.

Incidence of neoplastic lesions was calculated as the number of animals with lesions divided by the total number of animals examined microscopically. Tests of significance included pairwise comparisons of each exposed group with the control group using a one-tailed Fisher's exact test (one-sided results were considered because it is well established that only an increase in the disease incidences can be expected from the exposure, and incidences of leukemia in the control group are almost always 0). The Cochran-Armitage trend test was performed to test for dose-related trends within different exposure groups (glyphosate vs Control, Roundup Bioflow vs Control, RangerPro vs Control). Reported p values are one-sided.

Survival differences among groups were considered in assessing the incidence of neoplastic lesions and the Poly-k test was used [36–39]. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to account for survival differences by assigning a reduced risk of neoplasm, proportional to the power of the fraction of time on study, to only those lesion-free animals that did not reach terminal euthanasia. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. Each animal is assigned a risk weight: this value is 1 if the animal had a lesion at the site under consideration or if it survived until terminal euthanasia; if the animal died before terminal sacrifice and did not have a lesion at the site under consideration, its risk weight is the fraction of the entire study time that it survived, raised to the k-th power. A value of k = 3 was used in the

analysis, as recommended by Bailer and Portier [36, 37]. Reported p values for these analyses are one-sided.

Statistical analyses were performed using the German Cancer Research Center software (https://biostatistics.dkfz.de/) and Stata 18 software (StataCorp 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC).

Results

In the in vivo phase of the study, no acute toxic effects were observed during gestation or lactation. All exposure concentrations were well tolerated with no effects on body weight gain or feed and water consumption. Litter sizes were similar among groups. The mean body weight of both dams and pups was in the range of 10% variability relative to controls; food consumption was in the range of 20% variability relative to controls; water consumption was in the range of 20% variability relative to control. After weaning, body weights, water consumption, feed consumption and glyphosate intake were homogenous among groups in both females and males (Fig. 1).

Analysis of survival times in males and females showed no statistically significant differences between treated and control groups. Figure 2 shows the graphical representations of the Kaplan–Meier curves of the survival functions for males and females of the experimental groups.

Leukemia-related incidence rates were significantly elevated in all groups of treated animals compared to control; in particular, no leukemias were observed in concurrent controls.

Most unusually, almost half of the leukemia deaths seen in the glyphosate and GBHs groups occurred in early life—at less than one year of age. Furthermore, analyses with the Tarone-Ware trend test showed increasing frequency of early-onset leukemia related deaths with increasing concentrations of glyphosate and GBHs. In particular, statistically significant increases in early-onset leukemia deaths were observed for males, considering all substances together (Tarone trend test, $p = 0.0150$) and in animals treated with Roundup Bioflow compared to control animals (Tarone trend test, $p = 0.0149$). Thise was accounted for by the poly-3 test, a survival-adjusted statistical analyses of tumor incidence trend that was performed in addition to the Cochran-Armitage trend test. Data on leukemia are summarized in Tables 1, 2 and 3.

No brothers or sisters from the same litter were affected by leukemia. No cases of leukemia were observed in untreated control animals of either sex.

A dose-related increased trend in incidence of lymphoblastic leukemia was observed in males exposed to pure glyphosate ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0335$, poly-3 test); a significantly increased trend in incidence of monocytic leukemia was observed in females ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0411$, poly-3 test). One case of monocytic leukemia was observed in the female animals treated with 50 mg/kg bw/day; while two cases were observed in male animals treated with glyphosate—one treated with 0.5 mg/kg bw/day and the other one with 5 mg/kg bw/day. In females, one case of myeloid leukemia was observed in the group treated with the lowest dose of glyphosate (0.5 mg/kg bw/day) (Table 1).

In the Roundup Bioflow group, a significantly increased trend in incidence of lymphoblastic leukemia was observed in both males ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0425$, poly-3 test) and females ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0439$, poly-3 test). When males and females were considered together, the statistical significance with both the Cochran-Armitage trend test ($p = 0.0072$) and the poly-3 test ($p = 0.0071$) became stronger. A significantly increased trend in incidence of monocytic leukemia was observed in males ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0425$, poly-3 test). A significantly increased trend in incidence of all types of leukemias combined was observed in both males ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0083$, poly-3 test), and females ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0439$, poly-3 test). When males and females were considered together, the observed statistical significance became stronger ($p = 0.0014$, Cochran-Armitage trend test; $p = 0.0016$, poly-3 test) (Table 2).

In the RangerPro group, a significantly increased trend in incidence of lymphoblastic leukemia was observed in both males ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0092$, poly-3 test) and females ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0411$, poly-3 test), When males and females were considered together, the statistical significance observed with both the Cochran-Armitage trend test ($p = 0.0014$) and the poly-3 test ($p = 0.0016$) became stronger. A significantly increased trend in incidence of monocytic leukemia was observed in males ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0459$, poly-3

(See figure on next page.)

Fig. 1 Body weight, water/feed consumption and glyphosate intake in male and female Sprague–Dawley rats after weaning. Legend: Mean body weight (**A** and **B**) and mean water (**C** and **D**) and feed (**E** and **F**) consumption in male and female Sprague–Dawley rats after weaning. Mean male (**A**) and female (**B**) mean body weight; mean male (**C**) and female (**D**) daily water consumption; mean male (**E**) and female (**F**) daily feed consumptions; mean male (**G**) and female (**H**) daily glyphosate intake per kg of body weight. GLY = glyphosate; ROU = Roundup Bioflow; RAN = RangerPro

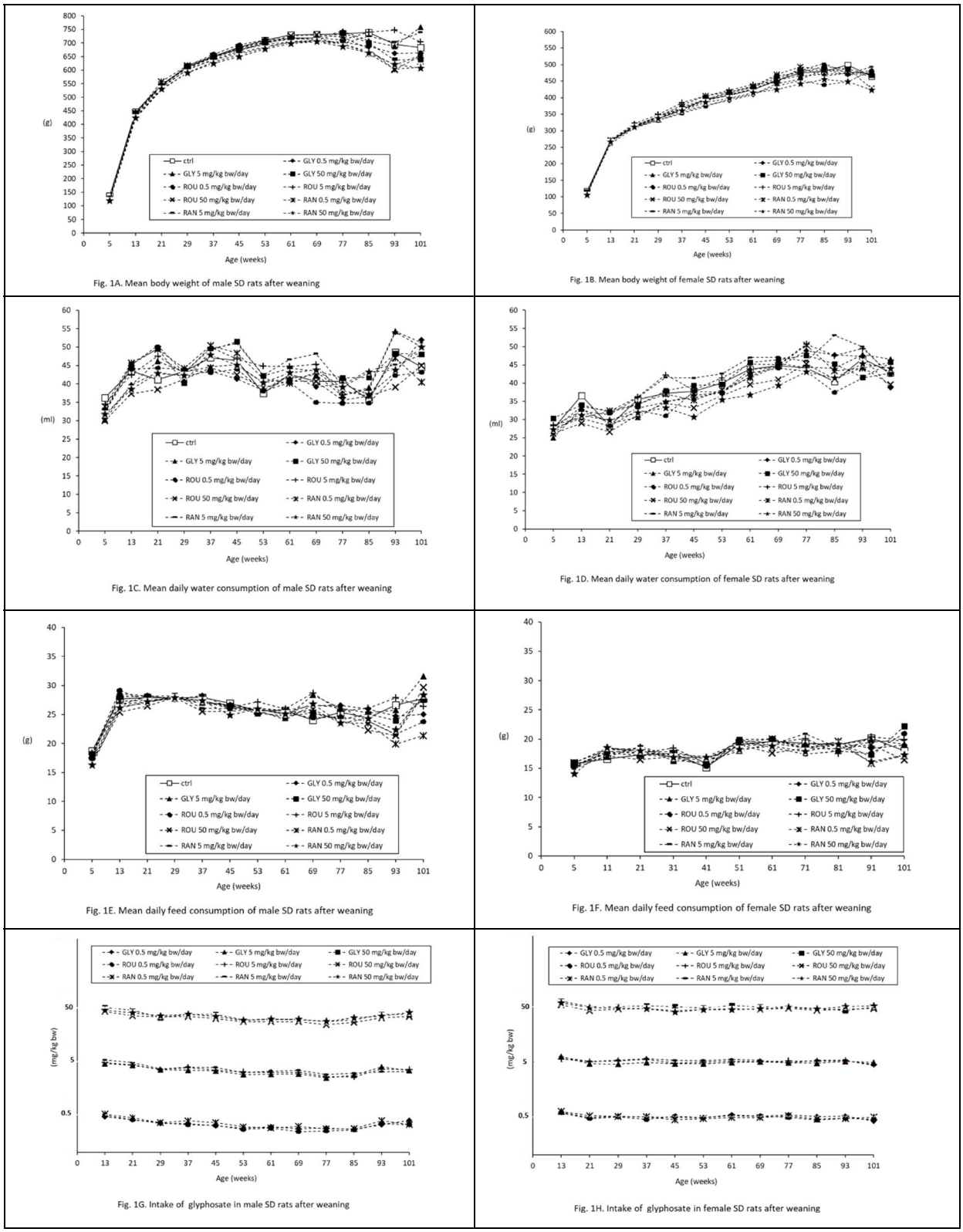

Fig. 1A. Mean body weight of male SD rats after weaning

Fig. 1B. Mean body weight of female SD rats after weaning

Fig. 1C. Mean daily water consumption of male SD rats after weaning

Fig. 1D. Mean daily water consumption of female SD rats after weaning

Fig. 1E. Mean daily feed consumption of male SD rats after weaning

Fig. 1F. Mean daily feed consumption of female SD rats after weaning

Fig. 1G. Intake of glyphosate in male SD rats after weaning

Fig. 1H. Intake of glyphosate in female SD rats after weaning

Fig. 1 (See legend on previous page.)

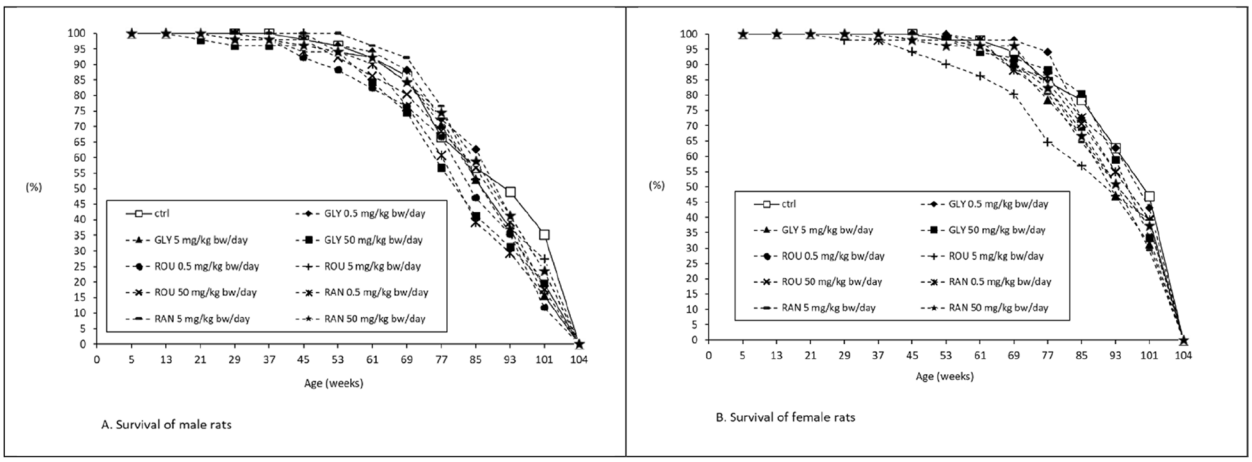

Fig. 2 Survival in male and female Sprague–Dawley rats. Legend: Male (**A**) and female (**B**) survival of Sprague–Dawley rats. GLY = glyphosate; ROU = Roundup Bioflow; RAN = RangerPro

Table 1 Incidence of leukemia: glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Lymphoblastic leukaemias			Monocytic leukaemias			Myeloid leukemias		Total lymphoid leukaemias			Total myeloid leukaemias		All leukaemias[a]	
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	No	%	*P*-value	No	%	No	%
0 (control)	M	51	0	-	[#]0.0419 [*]0.0335	0	-		0	-	0	-	[#]0.0419 [*]0.0335	0	-	0	-
	F	51	0	-		0	-	[#]0.0419 [*]0.0411	0	-	0	-		0	-	0	-
	M + F	102	0	-	[#]0.0421 [*]0.0372	0	-	[#]0.0421	0	-	0	-	[#]0.0421 [*]0.0372	0	-	0	-
0.5 Glyphosate	M	51	0	-		1	1.96		0	-	0	-		1	1.96	1	1.96
	F	51	0	-		0	-		1	1.96	0	-		1	1.96	1	1.96
	M + F	102	0	-		1	0.98		1	0.98	0	-		2	1.96	2	1.96
5 Glyphosate	M	51	0	-		1	1.96		0	-	0	-		1	1.96	1	1.96
	F	51	0	-		0	-		0	-	0	-		0	-	0	-
	M + F	102	0	-		1	0.98		0	-	0	-		1	0.98	1	0.98
50 Glyphosate	M	51	1	1.96		0	-		0	-	1	1.96		0	-	1	1.96
	F	51	0	-		1	1.96		0	-		-		1	1.96	1	1.96
	M + F	102	1	0.98		1	0.98		0	-	1	0.98		1	0.98	2	1.96

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Age at death of animals bearing leukemia (weeks of age): 0.5 glyphosate (male 104, female 104), 5 glyphosate (male 35), 50 glyphosate (male 21, female 101)

test). For all types of leukemia combined, a significantly increased trend in incidence was observed in males ($p = 0.0313$, Cochran-Armitage trend test; $p = 0.0371$, poly-3 test). When males and females were considered together, the statistical significance became stronger ($p = 0.0195$, Cochran-Armitage trend test; $p = 0.0218$, poly-3 test) (Table 3).

The mean age at death among all animals bearing leukemia was 97 weeks (standard deviation ± 13) for the 0.5 mg/kg bw/day groups (all treatments); 68 weeks (standard deviation ± 33) for the 5 mg/kg bw/day groups (all treatments); and 62 weeks (standard deviation ± 29) for the 50 mg/kg bw/day groups (all treatments) (Fig. 3). Notably, 40% of animals bearing leukemias (6/15) died before the first year of age (52 weeks): 5 of these 6 animals were in the highest dose groups (50 mg/kg bw/day), and one was from the intermediate dose group (5 mg/kg bw/day) (Tables 1, 2 and

Table 2 Incidence of leukemia: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Lymphoblastic leukemias			Monocytic leukemias			Myeloid leukemias		Total lymphoid leukemias			Total myeloid leukemias			All leukemias[a]		
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	No	%	*P*-value	No	%	*P*-value	No	%	*P*-value
0 (control)	M	51	0	-	[#]0.0419 [*]0.0425	0	-	[#]0.0419 [*]0.0425	0	-	0	-	[#]0.0419 [*]0.0425	0	-	[#]0.0419 [*]0.0425	0	-	[#]0.0071 [*]0.0083
	F	51	0	-	[#]0.0419 [*]0.0439	0	-		0	-	0	-	[#]0.0419 [*]0.0439	0	-		0	-	[#]0.0419 [*]0.0439
	M + F	102	0	-	[#]0.0072 [*]0.0071	0	-	[#]0.0421 [*]0.0432	0	-	0	-	[#]0.0072 [*]0.0078	0	-	[#]0.0421 [*]0.0432	0	-	[#]0.0014 [*]0.0016
0.5 Roundup Bioflow	M	51	0	-		0	-		0	-	0	-		0	-		0	-	
	F	51	0	-		0	-		0	-	0	-		0	-		0	-	
	M + F	102	0	-		0	-		0	-	0	-		0	-		0	-	
5 Roundup Bioflow	M	51	0	-		0	-		0	-	0	-		0	-		0	-	
	F	51	0	-		0	-		0	-	0	-		0	-		0	-	
	M + F	102	0	-		0	-		0	-	0	-		0	-		0	-	
50 Roundup Bioflow	M	51	1	1.96		1	1.96		0	-	1	1.96		1	1.96		2	3.92	
	F	51	1	1.96		0	-		0	-	1	1.96		0	-		1	1.96	
	M + F	102	2	1.96		1	0.98		0	-	2	1.96		1	0.98		3	2.94	

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Age at death of animals bearing leukemia (weeks of age): 50 Roundup Bioflow (male 47, male 48, female 104)

Table 3 Incidence of leukemia: RangerPro vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Lymphoblastic leukemias			Monocytic Leukemias			Myeloid leukemias		Total lymphoid leukemias			Total myeloid leukemias		All leukemias[a]		
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	No	%	*P*-value	No	%	No	%	*P*-value
0 (control)	M	51	0	-	[#]0.0071 [*]0.0092	0	-	[#]0.0419 [*]0.0459	0	-	0	-	[#]0.0071 [*]0.0092	0	-	0	-	[#]0.0313 [*]0.0371
	F	51	0	-	[#]0.0419 [*]0.0411	0	-		0	-	0	-	[#]0.0419 [*]0.0411	0	-	0	-	
	M + F	102	0	-	[#]0.0014 [*]0.0016	0	-		0	-	0	-	[#]0.0014 [*]0.0016	0	-	0	-	[#]0.0195 [*]0.0218
0.5 RangerPro	M	51	0	-		0	-		1	1.96	0	-		1	1.96	1	1.96	
	F	51	0	-		0	-		0	-	0	-		0	-	0	-	
	M + F	102	0	-		0	-		1	0.98	0	-		1	0.98	1	0.98	
5 RangerPro	M	51	0	-		0	-		1	1.96	0	-		1	1.96	1	1.96	
	F	51	0	-		1	1.96		0	-	0	-		1	1.96	1	1.96	
	M + F	102	0	-		1	0.98		1	0.98	0	-		2	1.96	2	1.96	
50 RangerPro	M	51	2	3.92		1	1.96		0	-	2	3.92		1	1.96	3	5.88	
	F	51	1	1.96		0	-		0	-	1	1.96		0	-	1	1.96	
	M + F	102	3	2.94		1	0.98		0	-	3	2.94		1	0.98	4	3.92	

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Age at death of animals bearing leukemia (weeks of age): 0.5 RangerPro (male 82), 5 RangerPro (male 70, female 100), 50 RangerPro (male 40, male 74, male 76, female 43)

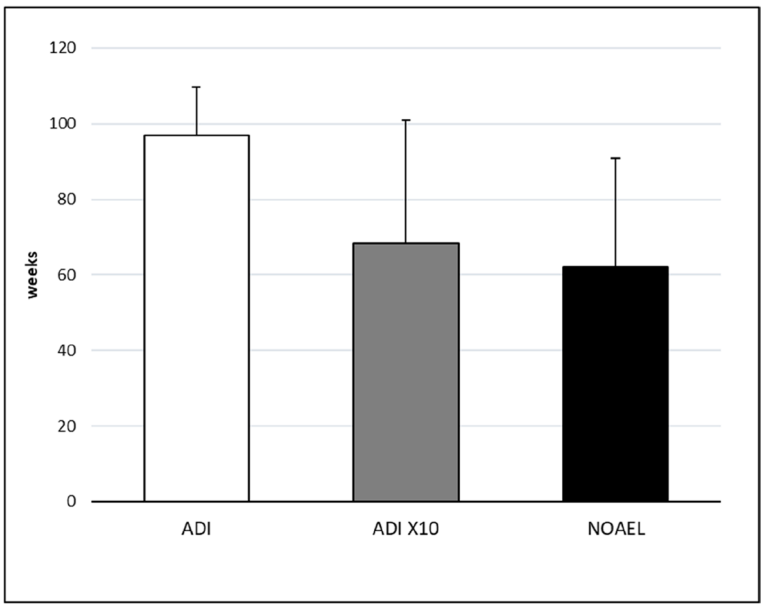

Fig. 3 Weeks of age at death of animals bearing leukemia by glyphosate equivalent doses. Legend: Mean (with standard deviation) weeks of age at death of animals bearing leukemia, divided by doses of 0.5, 5, and 50 mg/kg body weight/day corresponding to EU Acceptable Daily Intake (ADI), ADIx10 and the EU No Observed Adverse Effect Level (NOAEL) for glyphosate. The 3 treatment groups (glyphosate, Roundup Bioflow, RangerPro) are reported combined per dose

3). High prevalence of deaths from leukemia (before 52 weeks of age) in animals bearing these lesions were consistently observed in all three treatment groups: 40% in the glyphosate group (2/5), 66% in Roundup Bioflow (2/3) and 29% in Ranger Pro (2/7).

Because leukemias are rare malignancies in SD rats, we compared the age at death of animals bearing leukemias with historical control data available from the Ramazzini Institute (RI) (490 animals) [31] and the US National Toxicology Program (NTP) (1180 animals) [30]. In 1670 historical controls, only 15 animals were diagnosed with leukemia with an overall incidence rate of 0.90% in comparison to 1.63% of the current study. Study-specific incidence rates were 0.82% at RI (4/490; 1.63% male 4/245, 0% female 0/245) and 0.93% at NTP (11/1180; 1.19% male 7/590, 0.68% female 4/590). Of these 15 cases, none died before the first year of age. These findings contrast sharply with the results observed here, with 40% of the leukemias-related deaths in the glyphosate and GBHs treated groups occurring before the first year of age (Fig. 4).

In addition to leukemia, increased incidences of multiple benign and malignant tumors, including rare tumors (historical control incidence < 1%), were observed in the treated groups, some of these also with early onset [40].

Incidence of benign and malignant skin tumors is summarized in Tables 4, 5 and 6. No cases of skin tumors (malignant or benign) were observed in untreated control animals. No brothers or sisters were affected by skin tumors. In the glyphosate group, a significantly increased trend in incidence of squamous cell papilloma of the skin was observed in males ($p = 0.0392$, Cochran-Armitage trend test; $p = 0.0274$, poly-3 test). At high dose incidence was 3.92% in males, while in males from NTP historical control is 1.02% [30] and from RI historical control only 0.39%. A significantly increased trend in incidence of keratoacanthoma was also observed in males ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0311$, poly-3 test). Also in males, significantly increased trends in incidence were observed considering all benign skin tumors, ($p = 0.0082$, Cochran-Armitage trend test; $p = 0.0046$, poly-3 test) as well as that overall (benign and malignant) skin tumors ($p = 0.0228$, Cochran-Armitage trend test; $p = 0.0120$, poly-3 test). Comparing the high dose and the control group, a statistically borderline increase in the incidence of benign + malignant skin tumors was observed in males treated with glyphosate ($p = 0.059$, Fisher exact test) (Table 4). In the groups treated with Roundup Bioflow a case of trichoepithelioma (1.96% incidence) was observed in high-dose females, with a significantly increased trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0439$, poly-3 test) (Table 5). Trichoepithelioma is a very rare tumor, with no

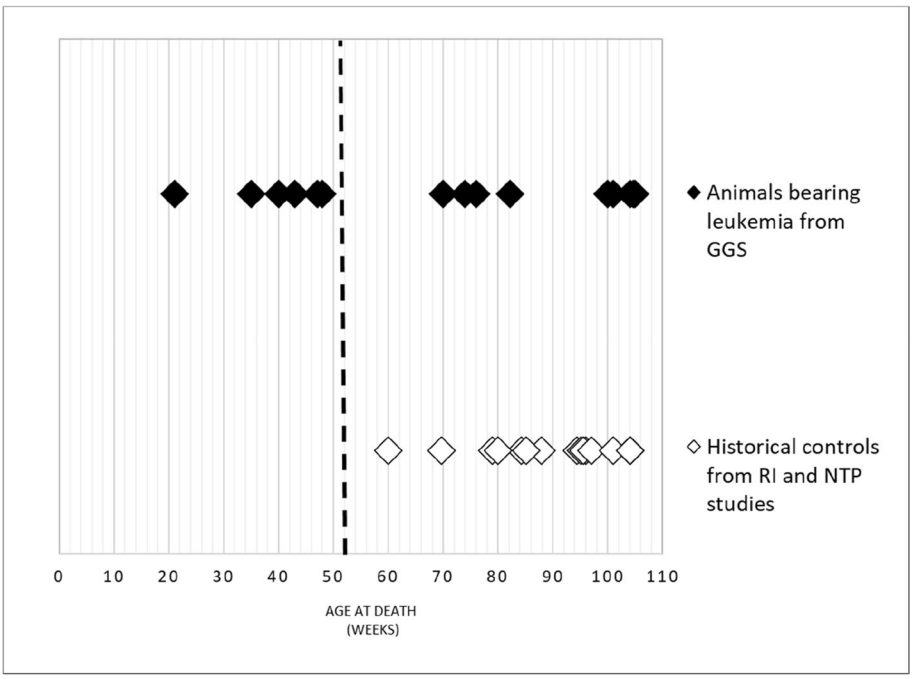

Fig. 4 Age at death of SD rats with leukemia in GGS and NTP-RI historical controls. Legend: Age at death of Sprague–Dawley rats with leukemia from GGS (♦) or from Ramazzini Institute (RI) and US National Toxicology Program (NTP) historical controls (◊). For GGS all animals belong to the treated groups; animals from historical controls belong only to not treated groups from various experiments. Week 52, which corresponds to 1 year of age, is dashed

Table 4 Incidence of benign and malignant tumors of the skin: Glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Squamous cell papilloma			Keratoacanthoma			Benign tumors			Squamous cell carcinoma		Basal cell carcinoma		Sebaceous carcinoma		Malignant tumors		Total benign + malignant		
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	*P*-value	No	%	No	%	No	%	No	%	No	%	*P*-value
0 (control)	M	51	0	-	#0.0392 *0.0274	0	-	#0.0419 *0.0311	0	-	#0.0082 *0.0046	0	-	0	-	0	-	0	-	0	-	#0.0228 *0.0120
	F	51	0	-		0	-		0	-		0	-	0	-	0	-	0	-	0	-	
	M + F	102	0	-	#0.0392 *0.0331	0	-	#0.0421 *0.0361	0	-	#0.0085 *0.0064	0	-	0	-	0	-	0	-	0	-	
0.5 Glyphosate	M	51	0	-		0	-		0	-		1	1.96	0	-	1	1.96	2	3.92	2	3.92	
	F	51	0	-		0	-		0	-		1	1.96	0	-	0	-	1	1.96	1	1.96	
	M + F	102	0	-		0	-		0	-		2	1.96	0	-	1	1.0	3	2.94	3	2.94	
5 Glyphosate	M	51	1	1.96		0	-		1	1.96		0	-	0	-	0	-	0	-	1	1.96	
	F	51	0	-		0	-		0	-		0	-	1	1.96	0	-	1	1.96	1	1.96	
	M + F	102	1	0.98		0	-		1	0.98		0	-	1	0.98	0	-	1	0.98	2	1.96	
50 Glyphosate	M	51	2	3.92		1	1.96		3	5.88		1	1.96	0	-	0	-	1	1.96	4	7.84	♦0.059
	F	51	0	-		0	-		0	-		0	-	0	-	0	-	0	-	0	-	
	M + F	102	2	1.96		1	0.98		3	2.94		1	0.98	0	-	0	-	1	0.98	4	3.92	

♦ *P* values for Fisher exact test

One-sided *p*-values for the Cochran-Armitage trend test

* One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

Table 5 Incidence of benign and malignant tumors of the skin: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Squamous cell papilloma		Kerato-acanthoma		Trichoepithelioma			Benign tumors			Squamous cell carcinoma		Basal cell carcinoma			Malignant tumors		Total benign + malignant	
	Sex	No	No	%	No	%	No	%	*P*-value	No	%	*P*-value	No	%	No	%	*P*-value	No	%	No	%
0 (control)	M	51	0	-	0	-	0	-		0	-		0	-	0	-	[#]0.0419 [*]0.0400	0	-	0	-
	F	51	0	-	0	-	0	-	[#]0.0419 [*]0.0439	0	-	[#]0.0419 [*]0.0439	0	-	0	-		0	-	0	-
	M + F	102	0	-	0	-	0	-	[#]0.0421 [*]0.0428	0	-		0	-	0	-	[#]0.0421 [*]0.0428	0	-	0	-
0.5 Roundup Bioflow	M	51	1	1.96	0	-	0	-		1	1.96		0	-	0	-		0	-	1	1.96
	F	51	0	-	0	-	0	-		0	-		1	1.96	0	-		1	1.96	1	1.96
	M + F	102	1	0.98	0	-	0	-		1	0.98		1	0.98	0	-		1	0.98	2	1.96
5 Roundup Bioflow	M	51	1	1.96	1	1.96	0	-		2	3.92		1	1.96	0	-		1	1.96	3	5.88
	F	51	0	-	0	-	0	-		0	-		0	-	0	-		0	-	0	-
	M + F	102	1	0.98	1	0.98	0	-		2	1.96		1	0.98	0	-		1	0.98	3	2.94
50 Roundup Bioflow	M	51	0	-	0	-	0	-		0	-		0	-	1	1.96		1	1.96	1	1.96
	F	51	0	-	0	-	1	1.96		1	1.96		0	-	0	-		0	-	1	1.96
	M + F	102	0	-	0	-	1	0.98		1	0.98		0	-	1	0.98		1	0.98	2	1.96

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

Table 6 Incidence of benign and malignant tumors of the skin: RangerPro vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Squamous cell papilloma		Keratoacanthoma			Trichoepithelioma		Benign tumors		Squamous cell carcinoma		Sebaceous carcinoma		Malignant tumors		Total benign + malignant	
	Sex	No	No	%	No	%	*P*-value	No	%	No	%	No	%	No	%	No	%	No	%
0 (control)	M	51	0	-	0	-	#0.0419 *0.0489	0	-	0	-	0	-	0	-	0	-	0	-
	F	51	0	-	0	-		0	-	0	-	0	-	0	-	0	-	0	-
	M + F	102	0	-	0	-	#0.0421 *0.0440	0	-	0	-	0	-	0	-	0	-	0	-
0.5 RangerPro	M	51	1	1.96	0	-		1	1.96	2	3.92	0	-	0	-	0	-	2	3.92
	F	51	1	1.96	0	-		0	-	1	1.96	0	-	0	-	0	-	1	1.96
	M + F	102	2	1.96	0	-		1	0.98	3	2.94	0	-	0	-	0	-	3	2.94
5 RangerPro	M	51	0	-	0	-		0	-	0	-	0	0.0	0	-	0	-	0	-
	F	51	0	-	0	-		0	-	0	-	2	3.92	1	1.96	3	5.88	3	5.88
	M + F	102	0	-	0	-		0	-	0	-	2	1.96	1	0.98	3	2.94	3	2.94
50 RangerPro	M	51	0	-	1	1.96		0	-	1	1.96	0	-	0	-	0	-	1	1.96
	F	51	0	-	0	-		0	-	0	-	0	-	0	-	0	-	0	-
	M + F	102	0	-	1	0.98		0	-	1	0.98	0	-	0	-	0	-	1	0.98

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

case observed in females SD rats from NTP [30] and RI historical controls. In a high-dose males, a case of basal cell carcinoma (1.96% incidence) was detected with a significantly increased trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0400$, poly-3 test) (Table 5). As for high-dose males treated with RangerPro of keratoacanthoma (1.96% incidence) was observed, with a significantly increased trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0489$, poly-3 test) (Table 6).

Overall, no statistically significant difference in the incidence of liver tumors was observed between treated and control groups (Tables 7, 8 and 9). However, in several treated male groups, more than one case was observed at different doses (glyphosate 0.5 and 50 mg/kg bw/day, Roundup 0.5 mg/kg bw/day, RangerPro 5 mg/kg bw/day). Hepatocellular carcinoma is a rare malignancy in SD rats. In 590 NTP male historical controls, only 1 animal was diagnosed with hepatocellular carcinoma (overall incidence rate of 0.17%) [30]; considering the RI historical controls, of a total of 3572 controls, 30 males were diagnosed with hepatocellular carcinoma (overall incidence rate of 0.82%). Among the RI lifetime study historical controls, in male animals bearing hepatocellular carcinoma, the average age at death was 118 weeks, while, in the one NTP 104-weeks study historical control male animal died at 94 weeks [30]. In both NTP and RI historical controls, the earlier age when hepatocellular

Table 7 Incidence of benign and malignant tumors of the liver: glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Hepatocellular adenoma		Hepatocellular Carcinoma[a]		Total	
	Sex	No	No	%	No	%	No	%
0 (control)	M	51	0	-	1	1.96	1	1.96
	F	51	0	-	0	-	0	-
	M + F	102	0	-	1	0.98	1	0.98
0.5 Glyphosate	M	51	1	1.96	2	3.92	3	5.88
	F	51	0	-	0	-	0	-
	M + F	102	1	0.98	2	1.96	3	2.94
5 Glyphosate	M	51	1	1.98	1	1.96	2	3.92
	F	51	0	-	0	-	0	-
	M + F	102	1	0.98	1	0.98	2	1.96
50 Glyphosate	M	51	0	-	2	3.92	2	3.92
	F	51	0	-	0	-	0	-
	M + F	102	0	-	2	1.96	2	1.96

[a] Age at death of animals bearing hepatocellular carcinoma (weeks of age): control (104), 0.5 glyphosate (99; 92), 5 glyphosate (74), 50 glyphosate (85; 104)

Table 8 Incidence of benign and malignant tumors of the liver: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Hepatocellular adenoma		Hepatocellular carcinoma[a]		Total	
	Sex	No	No	%	No	%	No	%
0 (control)	M	51	0	-	1	1.96	1	1.96
	F	51	0	-	0	-	0	-
	M + F	102	0	-	1	0.98	1	0.98
0.5 Roundup Bioflow	M	51	0	-	2	3.92	2	3.92
	F	51	0	-	0	-	0	-
	M + F	102	0	-	2	1.96	2	1.96
5 Roundup Bioflow	M	51	0	-	1	1.96	1	1.96
	F	51	0	-	0	-	0	-
	M + F	102	0	-	1	0.98	1	0.98
50 Roundup Bioflow	M	51	0	-	0	-	0	-
	F	51	0	-	0	-	0	-
	M + F	102	0	-	0	-	0	-

[a] Age at death of animals bearing hepatocellular carcinoma (weeks of age): control (104), 0.5 Roundup Bioflow (99; 82), 5 Roundup Bioflow (104)

Table 9 Incidence of benign and malignant tumors of the liver: RangerPro vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Hepatocellular adenoma		Hepatocellular carcinoma[a]		Total	
	Sex	No	No	%	No	%	No	%
0 (control)	M	51	0	-	1	1.96	1	1.96
	F	51	0	-	0	-	0	-
	M + F	102	0	-	1	0.98	1	0.98
0.5 RangerPro	M	51	0	-	0	-	0	-
	F	51	0	-	0	-	0	-
	M + F	102	0	-	0	-	0	-
5 RangerPro	M	51	0	-	2	3.92	2	3.92
	F	51	0	-	0	-	0	-
	M + F	102	0	-	2	1.96	2	1.96
50 RangerPro	M	51	0	-	0	-	0	-
	F	51	0	-	0	-	0	-
	M + F	102	0	-	0	-	0	-

[a] Age at death of animals bearing hepatocellular carcinoma (weeks of age): control (104), RangerPro (94;93)

carcinoma was reported as probable cause of death is 94 weeks. In the present study, a high prevalence of early deaths from hepatocellular carcinoma (before 94 weeks of age) in animals bearing these lesions were consistently observed in all treatment groups: 60% in the glyphosate group (3/5), 33% in Roundup Bioflow (1/3) and 50% in RangerPro (1/2) (Tables 7, 8 and 9). Hepatocellular carcinoma was the only malignant lesion observed in these 5 animals. No brothers or sisters from the same litter were affected by hepatocellular carcinoma. The one control animal bearing hepatocellular carcinoma survived until study termination (104 weeks).

Increased incidences of a number of bone tumors were observed (Tables 10, 11 and 12). In the glyphosate and RangerPro groups, males showed significantly increased trends in incidence of total benign tumors (chondroma and osteoma) (Tables 10 and 12); namely the significance levels were ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0046$, poly-3 test) and ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0379$, poly-3 test) for glyphosate and RangerPro groups, respectively (Table 12). In addition, one case of osteosarcoma (1.96%) was observed in high-dose females treated with Roundup Bioflow, with no cases in controls or at lower dose levels, with a significantly increased trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0439$, poly-3 test) (Table 11).

Thyroid tumors were observed in both sexes, and in two instances the incidence was treatment-related (Tables 13, 14, 15, 16, 17 and 18). Follicular carcinoma of the thyroid gland was observed in males of the highest glyphosate dose group, with a significant increase trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0311$, poly-3 test) (Table 13). No cases were observed in the NTP historical controls [30] and only a 0.09% incidence was observed in RI historical controls. In high-dose females treated with RangePro, one case (1.96%) of C-cell carcinoma was observed, with no cases at lower doses or controls, providing a statistically significantly increased trend ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0390$, poly-3 test) (Table 18). The incidence observed was slightly higher than NTP historical controls (9/589, 1.53%) and RI historical controls (38/3570, 1.06%).

In males, concerning other endocrine tissues, there was 1 case (1.96%) of pancreas islet cell carcinoma present in the high-dose group of RangerPro treatment, with no cases at lower doses or controls: this provided a statistically significantly increased trend in the incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0459$, poly-3 test) (Table 15).

In males, both the central and peripheral nervous system (CNS and PNS) was affected by the treatments (Tables 13, 14 and 15) with two types of malignant tumors observed: malignant granular cell tumor of the central nervous system and malignant Schwannoma of the peripheral nervous system. Schwannomas (benign and malignant) were present also in females in uterus (Tables 16, 17 and 18). A significantly increased trend in incidence of malignant granular cell tumor of central nervous system was observed in males ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0040$, poly-3 test) treated with glyphosate (Table 13). Out of a total of 589 NTP historical controls, only 1 male was diagnosed with brain malignant granular cell tumor (overall incidence rate of 0.17%); considering the RI historical control on a total of 3572 controls 5 males were diagnosed with brain

Table 10 Incidence of bone tumors: glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Chondroma			Osteoma			Total Benign			Osteosarcoma		Total	
														Benign + Malignant	
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	*P*-value	No	%	No	%
0 (control)	M	51	0	-	#0.0419 *0.0311	0	-	#0.0419 *0.0335	0	-	#0.0071 *0.0046	0	-	0	-
	F	51	0	-		1	1.96		1	1.96		0	-	1	1.96
	M + F	102	0	-	#0.0421 *0.0372	1	0.98		1	0.98		0	-	1	0.98
0.5 Glyphosate	M	51	0	-		0	-		0	-		1	1.96	1	1.96
	F	51	0	-		1	1.96		1	1.96		1	1.96	2	3.92
	M + F	102	0	-		1	0.98		1	0.98		2	1.96	3	2.94
5 Glyphosate	M	51	0	-		0	-		0	-		1	1.96	1	1.96
	F	51	0	-		0	-		0	-		0	-	0	-
	M + F	102	0	-		0	-		0	-		1	0.98	1	0.98
50 Glyphosate	M	51	1	1.96		1	1.96		2	3.92		0	-	2	3.92
	F	51	0	-		0	-		0	-		0	-	0	-
	M + F	102	1	0.98		1	0.98		2	1.96		0	-	2	1.96

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

Table 11 Incidence of bone tumors: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Chondroma		Osteoma		Total benign		Osteosarcoma			Total	
												Benign + Malignant	
	Sex	No	No	%	No	%	No	%	No	%	*P*-value	No	%
0 (control)	M	51	0	-	0	-	0	-	0	-		0	-
	F	51	0	-	1	1.96	1	1.96	0	-	#0.0419 *0.0439	1	1.96
	M + F	102	0	-	1	0.98	1	0.98	0	-	#0.0421 *0.0428	1	0.98
0.5 Roundup Bioflow	M	51	0	-	0	-	0	-	0	-		0	-
	F	51	0	-	0	-	0	-	0	-		0	-
	M + F	102	0	-	0	-	0	-	0	-		0	-
5 Roundup Bioflow	M	51	0	-	0	-	0	-	0	-		0	-
	F	51	0	-	0	-	0	-	0	-		0	-
	M + F	102	0	-	0	-	0	-	0	-		0	-
50 Roundup Bioflow	M	51	0	-	0	-	0	-	0	-		0	-
	F	51	0	-	0	-	0	-	1	1.96		1	1.96
	M + F	102	0	-	0	-	0	-	1	0.98		1	0.98

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

malignant granular cell tumor (overall incidence rate of 0.15%). Conversely, in high-dose males of the glyphosate treatment the incidence was 3.92%: no cases were detected at lower doses or in controls. Two cases (3.92%) of malignant Schwannoma of peripheral nervous system were observed in male animals treated with Roundup Bioflow, with no cases in controls or lower dose levels. This finding was associated with a significantly increased trend in incidence ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0080$, poly-3 test) (Table 14). Both animals

Table 12 Incidence of bone tumors: RangerPro vs control group

Dose (mg/kg bw/day of of glyphosate)	Animals		Chondroma		Osteoma			Total benign			Osteosarcoma		Total	
													Benign + Malignant	
	Sex	No	No	%	No	%	*P*-value	No	%	*P*-value	No	%	No	%
0 (control)	M	51	0	-	0	-	[#]0.0419 [*]0.0379	0	-	[#]0.0419 [*]0.0379	0	-	0	-
	F	51	0	-	1	1.96		1	1.96		0	-	1	1.96
	M + F	102	0	-	1	0.98		1	0.98		0	-	1	0.98
0.5 RangerPro	M	51	0	-	0	-		0	-		1	1.96	1	1.96
	F	51	0	-	0	-		0	-		0	-	0	-
	M + F	102	0	-	0	-		0	-		1	0.98	1	0.98
5 RangerPro	M	51	0	-	0	-		0	-		2	3.92	2	3.92
	F	51	0	-	0	-		0	-		0	-	0	-
	M + F	102	0	-	0	-		0	-		2	1.96	2	1.96
50 RangerPro	M	51	0	-	1	1.96		1	1.96		0	-	1	1.96
	F	51	0	-	0	-		0	-		0	-	0	-
	M + F	102	0	-	1	0.98		1	0.98		0	-	1	0.98

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

died very early, at 34 and 67 weeks of age, with no other neoplasm. No case was observed in over 590 male animals in the NTP historical control database and 29 cases over 3572 control animals (0.82%) were observed in RI historical controls. A treatment-related incidence of Schwannomas was observed also in females in their uteri (Tables 16, 17 and 18). In animals treated with glyphosate, benign Schwannomas of the uterus showed a significantly increased trend in incidence due to a single case at the highest dose ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0411$, poly-3 test), whereas malignant Schwannoma showed a non-statistically significant increase (up to 3.92%) in the 5 and 50 mg/kg bw/day doses (Table 16). One case per group was detected in 5 and 50 mg/kg bw/day RangerPro treated groups (Table 18), and one case was detected in 5 mg/kg bw/day in Roundup low treated group (Table 17). The association with treatment of these cases was doubtful but could not be ruled out completely. In fact, no case of benign and malignant Schwannomas was observed in male and female untreated control animals: in the NTP historical controls [30], only 2 cases of uterus malignant Schwannoma (0.40%) were observed in over 449 controls female SD rats, whereas an incidence of 2.74% was detected in RI historical controls. No case of benign Schwannomas was reported in NTP historical controls and only 0.06% was detected in RI historical controls.

One case of hemangiosarcoma of the uterus was found in the highest RangerPro dose treatment group (1.96%), with a significantly increased trend in incidence ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0390$, poly-3 test) (Table 18); no case of hemangiosarcoma of the uterus was observed in NTP historical controls [30] and 4 cases among 3570 females from RI historical control (incidence 0.12%). In males, 1 case of hemangiosarcoma of the spleen (1.96%) was observed in the highest dose treated with RangerPro, with an increase trend in incidence ($p = 0.0419$, Cochran-Armitage trend test, $p = 0.0434$ poly-3 test) (Table 15). Spleen hemangiosarcoma is a rare tumor in rat with an incidence of 0.51% (3/590) in NTP historical control [30] and 0.27% in RI historical control (10/3572). Of note, the treatment-related cases of hemangiosarcoma (uterus and spleen) were detected in the highest-dose group of RangerPro ($p = 0.0532$, Cochran-Armitage trend test). Other sporadic cases were detected in control (1 in female liver) and in the Roundup Bioflow lowest dose group (1 in female soft tissue).

An increased incidence of benign mammary gland tumors in male SD rats was observed in treated groups while no male mammary tumors occurred in the control group (Tables 13, 14 and 15). A significantly increased trend in incidence of mammary gland benign tumors (fibroadenoma and lipoma) was observed in males treated with glyphosate ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0040$, poly-3 test) (Table 13) and with Roundup Bioflow ($p = 0.0071$, Cochran-Armitage trend test; $p = 0.0072$, poly-3 test) (Table 15). In particular, in glyphosate and Roundup Bioflow highest doses 1 case each (1.96% incidence) of lipoma is considered a quite uncommon tumor with no cases observed in NTP

Table 13 Incidence of benign and malignant tumors in Males: glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Mammary gland			Kidney		Urinary Bladder		Thyroid gland			Adrenal gland		Endocrine Pancreas		Spleen		Central nervous system			Peripheral nervous system	
			Benign tumors			Malignant mesenchymal tumor[a]		Transitional cell Carcinoma[a]		Follicular carcinoma[a]			Cortical Carcinoma[a]		Islet cell carcinoma		Hemangiosarcoma[a]		Malignant granular Cell tumor[a]			Malignant Schwannoma[a]	
	Sex	No	No	%	*P*-value	No	%	No	%	No	%	*P*-value	No	%	No	%	No	%	No	%	*P*-value	No	%
0 (control)	M	51	0	-	#0.0071 *0.0040	0	-	0	-	0	-	#0.0419 *0.0311	0	-	0	-	0	-	0	-	#0.0071 *0.0040	0	-
0.5 Glyphosate	M	51	0	-		0	-	0	-	0	-		0	-	0	-	0	-	0	-		0	-
5 Glyphosate	M	51	0	-		0	-	0	-	0	-		0	-	0	-	0	-	0	-		2	3.92
50 Glyphosate	M	51	2	3.92		0	-	0	-	1	1.96		0	-	0	-	0	-	2	3.92		0	-

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

Table 14 Incidence of benign and malignant tumors in Males: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Mammary gland			Kidney			Urinary Bladder			Thyroid gland		Adrenal gland		Endocrine Pancreas		Spleen		Central nervous system		Peripheral nervous system		
			Benign tumors			Malignant mesenchymal tumor			Transitional cell Carcinoma[ab]			Follicular carcinoma[a]		Cortical Carcinoma[a]		Islet cell carcinoma		Hemangio sarcoma[a]		Malignant granular Cell tumor[a]		Malignant Schwannoma[a]		
	Sex	No	No	%	*P*-value	No	%	*P*-value	No	%	P-value	No	%	No	%	No	%	No	%	No	%	No	%	*P*-value
0 (control)	M	51	0	-	[#]0.0071 [*]0.0072	0	-	[#]0.0419 [*]0.0400	0	-	[#]0.0419 [*]0.0325	0	-	0	-	0	-	0	-	0	-	0	-	[#]0.0071 [*]0.0080
0.5 Roundup Bioflow	M	51	0	-		0	-		0	-		0	-	0	-	0	-	0	-	0	-	0	-	
5 Roundup Bioflow	M	51	0	-		0	-		0	-		0	-	1	1.96	0	-	0	-	0	-	0	-	
50 Roundup Bioflow	M	51	2	3.92		1	1.96		1	1.96		0	-	0	-	0	-	0	-	0	-	2	3.92	

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

[b] Age at death of animals bearing urinary bladder transitional cell carcinoma (weeks of age): 50 Roundup Bioflow (61)

Table 15 Incidence of benign and malignant tumors in males: RangerPro vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Mammary gland		Kidney		Urinary Bladder		Thyroid gland		Adrenal gland			Endocrine Pancreas			Spleen			Central nervous system		Peripheral nervous system	
			Benign tumors		Malignant mesenchymal tumor		Transitional cell Carcinoma[a]		Follicular carcinoma[a]		Cortical Carcinoma[a]			Islet cell carcinoma			Hemangiosarcoma[a]			Malignant granular cell tumor[a]		Malignant Schwannoma[a]	
	Sex	No	No	%	No	%	No	%	No	%	No	%	*P*-value	No	%	*P*-value	No	%	*P*-value	No	%	No	%
0 (control)	M	51	0	-	0	-	0	-	0	-	0	-	[#]0.0071 [*]0.0075	0	-	[#]0.0419 [*]0.0459	0	-	[#]0.0419 [*]0.0434	0	-	0	-
0.5 RangerPro	M	51	1	1.96	0	-	0	-	0	-	0	-		0	-		0	-		0	-	0	-
5 RangerPro	M	51	1	1.96	0	-	0	-	0	-	0	-		0	-		0	-		1	1.96	0	-
50 RangerPro	M	51	1	1.96	0	-	0	-	0	-	2	3.92		1	1.96		1	1.96		0	0.0	0	-

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

Table 16 Incidence of benign and malignant tumors in females: glyphosate vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Thyroid gland		Ovary					Uterus						
			C-cell Carcinoma		Fibroma			Malignant Granulosa cell tumor[a]		Benign Schwannoma[a]			Malignant Schwannoma		Hemangiosarcoma[a]	
	Sex	No	No	%	No	%	*P*-value	No	%	No	%	*P*-value	No	%	No	%
0 (control)	F	51	0	-	0	-	[#]0.0419 [*]0.0411	0	-	0	-	[#]0.0419 [*]0.0411	0	-	0	-
0.5 Glyphosate	F	51	2	3.92	0	-		1	1.96	0	-		1	1.96	0	-
5 Glyphosate	F	51	1	1.96	0	-		0	-	0	-		2	3.92	0	-
50 Glyphosate	F	51	1	1.96	1	1.96		0	-	1	1.96		2	3.92	0	-

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

Table 17 Incidence of benign and malignant tumors in females: Roundup Bioflow vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Thyroid gland		Ovary					Uterus					
			C-cell Carcinoma		Fibroma		Malignant Granulosa cell tumor[ab]			Benign Schwannoma[a]		Malignant Schwannoma		Hemangiosarcoma[a]	
	Sex	No	No	%	No	%	No	%	*P*-value	No	%	No	%	No	%
0 (control)	F	51	0	-	0	-	0	-	[#]0.0419 [*]0.0460	0	-	0	-	0	-
0.5 Roundup Bioflow	F	51	0	-	0	-	0	-		0	-	0	-	0	-
5 Roundup Bioflow	F	51	1	1.96	0	-	0	-		0	-	1	1.96	0	-
50 Roundup Bioflow	F	51	0	-	0	-	1	1.96		0	-	0	-	0	-

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

[b] Age at death of animals bearing ovary Malignant Granulosa cell tumor (weeks of age): 50 Roundup Bioflow (43)

Table 18 Incidence of benign and malignant tumors in females: RangerPro vs control group

Dose (mg/kg bw/day of glyphosate)	Animals		Thyroid gland			Ovary				Uterus						
			C-cell Carcinoma			Fibroma		Malignant Granulosa cell tumor[a]		Benign Schwannoma[a]		Malignant Schwannoma		Hemangiosarcoma[a]		
	Sex	No	No	%	*P*-value	No	%	No	%	No	%	No	%	No	%	*P*-value
0 (control)	F	51	0	-	[#]0.0419 [*]0.0390	0	-	0	-	0	-	0	-	0	-	[#]0.0419 [*]0.0390
0.5 RangerPro	F	51	0	-		0	-	0	-	0	-	0	-	0	-	
5 RangerPro	F	51	0	-		0	-	0	-	0	-	1	1.96	0	-	
50 RangerPro	F	51	1	1.96		0	-	0	-	0	-	1	1.96	1	1.96	

[#] One-sided *p*-values for the Cochran-Armitage trend test

[*] One-sided *p*-values for the Cochran-Armitage trend test (Poly-k adjusted)

[a] Rare tumors (incidence < 1%) from NTP and RI historical control

Table 19 Comparison of results observed in animals treated with glyphosate, Roundup Bioflow or RangerPro

Tumors	Glyphosate		Roundup Bioflow		RangerPro	
	Male	Female	Male	Female	Male	Female
HematologicalHematological (Leukemias)	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Skin	↑↑	↑	↑↑	↑↑	↑↑	↑
Liver	↑		↑		↑	
Thyroid	↑↑	↑		↑		↑↑
Nervous System	↑↑	↑	↑↑		↑	
Bone	↑↑			↑↑	↑↑	
Ovary (Sex cord-gonadal stromal tumors)		↑		↑↑		
Mammary gland	↑↑		↑↑			
Adrenal glands			↑		↑↑	
Kidney			↑↑			
Urinary Bladder			↑↑			
Hemangiosarcoma (uterus and spleen)					↑↑	↑↑
Pancreas (endocrine)					↑↑	

↑↑ = Tumors with statistically significantly increased trend in incidence compared to concurrent control

↑ = Rare Tumors in excess compared to NTP and RI historical controls

historical controls [30] and a 1.2% of incidence in RI historical controls.

In males exposed to the highest dose of Roundup Bioflow, a malignant mesenchymal tumor of the kidney (1.96%) was observed, with a statistically significant increasing trend ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0400$, poly-3 test) (Table 14). In NTP historical control [30] this lesion is not reported and in RI historical control only 2 cases in 3752 (0.06%) were detected only in males.

In the males of this same group, an early onset (age at death 61 weeks) transitional cell carcinoma of the urinary bladder was observed (1.96%) with a statistically significant increasing trend ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0325$, poly-3) (Table 14). In NTP [30] and RI historical control this lesion was not observed.

A significantly increased trend in incidence of cortical carcinoma of the adrenal gland was observed in males treated with RangerPro (3.92%; $p = 0.0071$, Cochran-Armitage trend test; $p = 0.0075$, poly-3 test) (Table 15). By contrast, only 1 case was observed among 590 animals (0.17%) in the NTP historical controls [30] and a 0.24% incidence in male RI historical controls.

One case of malignant granulosa cell tumor of ovary (1.96%) was observed in high-dose females treated with Roundup Bioflow (Table 17), driving a significantly increased trend in incidence. of malignant granulosa cell tumor of the ovary was observed in females ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0460$, poly-3 test). Notably the age at death of this case was very low, 43 weeks of age, and no other concomitant lesion was detected. Rare cases (5/588, 0.85%) of this tumor were observed in NTP historical controls [30] and only 2 cases over 3570 (0.06%) in RI historical controls.

In high-dose females of the glyphosate treated group, one case of ovarian fibroma was detected (1.96%), driving a statistically significant increased trend ($p = 0.0419$, Cochran-Armitage trend test; $p = 0.0411$, poly-3 test) (Table 16).

A summary of the different tumors, by site, between glyphosate, Roundup Bioflow and RangerPro treatment groups, in which there was a statistically significant increase in the incidence of benign or malignant tumors or an excess of rare tumors incidence compared to both the NTP and RI historical controls, is provided in Table 19. Hematological tumors (leukemias) were increased in all treatment groups (glyphosate, Roundup Bioflow and RangerPro) in both males and females; skin tumors were increased in the glyphosate (both males and females) and, to a lesser extent, in the Roundup Bioflow and RangePro (males only) groups; liver tumors were increased in all treatment groups in males, thyroid tumors in glyphosate (males) and RangePro (females); central nervous system tumors increased in glyphosate (males, brain granular cell tumors) and peripheral nervous system tumors increased in glyphosate (both sexes, Schwannoma) and possibly in Roundup Bioflow and RangePro (females), bone tumors increased in males treated with glyphosate and RangerPro and in females treated with Roundup Bioflow; ovary tumors (sex cord-gonadal stromal tumors) increased in females treated with glyphosate and Roundup Bioflow, mammary tumors increased in males treated with glyphosate and Roundup Bioflow, adrenal glands tumors increased in

males treated with RangerPro, kidney and urinary bladder tumors increased in males treated with Roundup Bioflow, hemangiosarcoma (spleen and uterus) increased in both sexes treated with RangerPro; and endocrine pancreas tumor increased in males treated with RangerPro (Table 19).

Statistically significant increases in incidence were observed for a number of other non-rare benign and malignant tumors (Supplementary Tables 1–4). In animals treated with glyphosate, a statistically significant increased trend (with Cochran-Armitage trend test) in incidence was detected for lung squamous cell papilloma in males. In animals treated with Roundup Bioflow, a statistically significant increased trend (with Cochran-Armitage trend test) in incidence was detected for lung fibroma in males. In animals treated with glyphosate, a statistically significant increased trend (with Cochran-Armitage trend test) in incidence was observed for osteoma outside the bone (lung) in males. In animals treated with RangerPro a statistically significant increased trend (with Cochran-Armitage trend test) in incidence was detected for soft tissue fibroma in males and lung fibrosarcoma in females. In the female RangerPro lowest dose group, there was a statistically significant increase in incidence (Fisher exact test) of mammary glands tumors (adenoma, fibroma and fibroadenoma).

Discussion

The main finding of this 2-year long-term carcinogenicity study of glyphosate and two commercial GBH formulations was an increased incidence (statistically significant, dose-related and/or in excess compared to both NTP and RI historical controls) of multiple benign and malignant tumors in SD rats at exposure levels corresponding to the EU glyphosate ADI and the EU NOAEL (Table 19).

Dose-related increased incidences of leukemia, and of skin, liver, thyroid, nervous system tumors, compared to concomitant control or historical controls, were observed across all three treatment groups, indicating that glyphosate was sufficient to drive the carcinogenic effects observed in all groups. A statistically significant increase in the incidence of mammary tumors in males was observed in groups treated with glyphosate and Roundup Bioflow, while sporadic cases were recorded in the three doses exposed to RangerPro, similarly to ovary tumors where increases were observed only in glyphosate and Roundup Bioflow, indicating that glyphosate was probably driving the carcinogenic effects observed in all groups. Tumors of the adrenal glands increased in incidence only with exposure to GBHs (Roundup Bioflow and RangerPro) suggesting that these formulations might have either enhanced the carcinogenic effects of glyphosate or had a common mechanism of action other than that related to glyphosate. Tumors of kidney, urinary bladder, hemangiosarcoma and endocrine pancreas increased only with GBHs treatment (Roundup Bioflow or RangerPro), suggesting that mechanisms of action other than those related to glyphosate might be responsible for these increases (Table 19).

In relation to leukemia, the two key findings of our study were, first, a significant dose-related trend in increased leukemia incidence and, second, early onset of leukemia in exposed rats. Dose-related trends in leukemia incidence were observed in both males and females, whereas the majority of significant results were observed in males. When males and females were considered together, the statistical significance consistently became stronger. No case of leukemia was observed in untreated concurrent control animals.

With respect to deaths from leukemia in the glyphosate and GBH treated groups, 40% occurred before the first year of age (52 weeks), which is comparable to less than 35–40 years of age in humans [41]. By contrast, no cases of leukemia death have been observed prior to one year of age in over 1600 SD untreated control rats studied by the NTP and the RI over the past decade (Fig. 4). The early onset of leukemia observed in the glyphosate- and GBH-treated groups appears to be dose-dependent and is likely due to prenatal exposure, as treatment began during pregnancy.

Glyphosate and GBHs induced increased incidence of skin tumors in both males and females. No cases of skin tumors (malignant or benign) were observed in control animals. Portier [42] reported that there is "clear evidence that oral administration of glyphosate in male SD rats causes skin tumors (basal cell tumor and keratoacanthoma). Results obtained by George et al. [43] also suggested that glyphosate has skin carcinogenesis promoting potential in male Swiss mice. Of note, in agricultural workers and pesticide applicators, skin contact is an important route of exposure [6, 44]. These findings are consistent with results from two case–control studies, conducted in different geographical areas (Italy and Brazil) showing an elevated risk of cutaneous melanoma in relation to exposure to pesticides and in particular to herbicides (glyphosate) and fungicides (mancozeb, maneb) [45].

Hepatocellular carcinoma is a rare lesion in SD rats. In the present study, glyphosate and GBHs induced increased incidences of hepatocellular carcinoma compared to historical controls. Although, not reaching statistical significance of incidences compared to the concurrent controls, in many treated groups more than 1 hepatocellular carcinoma was observed at different doses. Furthermore, even though a clear dose–response could not be observed in the present study, this might be

influenced by the length of the experiment (104 weeks), since hepatocellular carcinoma mainly occur in very old animals (average age at death 118 weeks in RI historical control). It should be noted that hepatocellular adenoma has already been considered to be related to glyphosate exposure in male SD rats [42], probably due to the higher doses tested [46]. Glyphosate and GBHs appear to induce oxidative stress in the liver in different rat strains, also at relatively low doses [47–49]. Oxidative stress is a recognized mode of action for genotoxicity and carcinogenicity in the liver, albeit possibly with a threshold [50]. A survey of farm workers as part of the US Agricultural Health Study showed a positive correlation between urine glyphosate levels and markers of oxidative stress, which included elevated levels of 8-hydroxy-2'-deoxyguanosine [51]. Glyphosate may contribute to the development of liver disorders and metabolic syndrome among young people. As part of the CHAMACOS study, Ezkenazi et al. reported these association observing that increased urinary aminomethylphosphonic acid (AMPA) during childhood correlated to an increased risk of elevated hepatic transaminases (14%) and metabolic syndrome (55%); furthermore, living near agricultural applications of glyphosate during early childhood (from birth to age 5 years) was associated with metabolic syndrome at age 18 years [52]. In addition, exposure to GBHs was associated with DNA damage in the liver in an animal study. Molecular profiling of the liver in rats exposed to the GBH Roundup Bioflow or pure glyphosate for 90 days revealed that only the formulations increased hepatic steatosis and necrosis, and that both glyphosate and the formulations altered gene expression, including TP53 activation [47]. In addition, small RNA profiling in liver showed alterations in miRNA profiles with both glyphosate and Roundup Bioflow exposure and also that glyphosate exposure alone resulted in DNA damage as shown by apurinic/apyrimidinic site formation [47]. Furthermore, increased concentration of oxidative stress biomarkers, linked to DNA damage and the potency of endocrine disruptors, were observed proportionally to the concentration of glyphosate detected in blood and seminal plasma [53].

The male rat mammary gland is a sensitive target to evaluate the effects of endocrine disrupting chemicals (EDCs), also considering the recently increased incidence of male breast cancer [54]. The Organization for Economic Co-operation and Development (OECD) considers the evaluation of the mammary gland of the male rat as an EDC-related endpoint in its guidelines for subchronic oral toxicity testing [55]. As a hormone-dependent tissue, mammary gland growth and differentiation is associated with the action of estrogen-pathways as well as other hormonal axes systems [54, 55]. In SD rats, similarly to humans, male mammary tumors are much less frequent than in females [56]. Hence, the presence of several mammary gland tumors in male rats in our study is of interest considering that timing of exposure included the prenatal programming as well as the increased incidences observed for a number of other endocrine-related tumors in the glyphosate and GBHs treated groups (i.e., ovary, thyroid, adrenal gland tumors). Notably, 3 adenocarcinomas of the mammary glands in males treated with RangerPro, were observed, 2 cases at the lowest dose (3.92%) and 1 case in the highest dose group (1.96%) (Supplementary materials: Table 1). Even though statistical significance was not reached in the current study, the incidence of mammary adenocarcinoma in males is in excess of NTP (1/590, 0.17%) [30] and of RI historical controls (14/3572, 0.39%). In a study by Gomez et al. [57] exposure to a GBH during pregnancy and lactation interfered with mammary gland development in male rats disrupting the regulation of gene expression networks; it cannot be ruled out that impaired gene regulation may lead later in life to an increased susceptibility to tumor development after puberty and sexual maturity.

As reported in the INHAND [23], renal malignant mesenchymal tumors are rare and have been shown to be induced in rats only by potent genotoxic chemicals [47]. While they may occur sporadically, for example, in the 2-year carcinogenicity review published by the NTP in 2007, no record of this type of tumor was found in the control rat group of any NTP study. In most studies, renal mesenchymal tumor is present as a single tumor in a single-dose group [58], as it as we report here for male rats exposed to the highest dose of Roundup Bioflow. Other cancer studies in rodents have reported renal tumors from exposure to glyphosate [42, 58]. Agricultural work and occupational pesticide use have been associated with increased risk of renal cell carcinoma, with an elevated risk amongst the highest-level users of several pesticides, including GBHs [59].

Frazier et al. [23] reported also that spontaneous carcinomas of the lower urinary tract and renal pelvis are uncommon tumors in most species and strains of rodents. Spontaneous transitional carcinomas in the mouse urinary bladder or urethra are rare and a few genotoxic chemicals have induced them experimentally. Chandra et al. [60] reported that only 1 male and 1 female SD rat were bearing transitional cell carcinoma among 2669 rats (1340 males and 1329 females) from 17 carcinogenicity studies. In NTP [30] and RI historical controls, no case of kidney transitional cell carcinoma was observed. Due to its rarity in rats, it is quite difficult to assess the importance of this lesion in relation to GBHs exposure. A case–control study, performed on dogs (Scottish Terriers), suggested that

exposure to lawns or gardens treated with herbicides, including GBHs, was associated with an increased risk of urinary bladder transitional cell carcinoma [61].

Our data indicate that glyphosate tumorigenesis may target the endocrine tissues. Adrenal cortical carcinoma is a rare tumor in SD rats. A 'clear evidence' of adrenal carcinogenicity in SD female rats was attributed in the reanalysis by Portier [42], evaluating studies using exposure levels of pure glyphosate compared to the present study. Our results show the presence of cortical carcinoma of adrenal glands in male SD rats exposed to RangerPro and Roundup Bioflow, suggesting that GBH components might either have enhanced the carcinogenic effects of glyphosate or had a common mechanism of action other than that related to glyphosate. In a short-term (2 weeks) study conducted only in male rats and only with Roundup Bioflow, the authors observed a reduction in endogenous adrenocorticotropic hormone receptor (ACTH) regulation, a condition similar to the human condition known as adrenal insufficiency [62]. In contrast, Owagboriaye et al. [63] observed in male rats a significant dose-dependent increase in serum aldosterone and corticosterone following a prolonged (13-week) exposure to a GBH, but not following exposure to equivalent doses of glyphosate. This suggests that GBHs could have an impact on the hypothalamic–pituitary–adrenal axis, which could potentially lead in a lifetime exposure to adrenal cortex cancer [42]. Thyroid tumor increases were shown in male and female SD rats, with a more pronounced effect in females treated with pure glyphosate. This result appeared in line with literature; it has been suggested that environmental risk factors play a crucial role in the increased incidence of thyroid neoplasms in humans and some pesticides have been recognized among these factors [64–66]. In another study in SD rats, a significantly positive trend in the incidence of thyroid C-cell adenoma in female rats was observed [6, 67]; it should be noted that administration started at 8 weeks age and not prenatally as in the present study. Portier [42] also reports "equivocal evidence" for thyroid c-cell adenoma and carcinoma in both sexes and thyroid follicular adenoma and carcinoma in male SD rats treated from adulthood. Our data suggests that pre- and/or postnatal development may be a susceptible window for thyroid cancer elicited by glyphosate exposure.

Toxic effects of glyphosate on the nervous system were described in a recent systematic review, showing that glyphosate produces important alterations in the structure and function of the nervous system of humans, rodents, fish, and invertebrates [68]. Another recent systematic review and meta-analysis on parental exposure to pesticides, including glyphosate, and childhood brain tumors revealed an association between these tumors and exposure before childbirth, after birth, and residential exposure [68, 69]. Moreover pesticide mixture, including glyphosate, have been found to be associated with the overall cancer, brain and other CNS cancers, and leukemia among children, as recently reported by Taiba et al. [70] and also by the French National Institute of Health and Medical Research (Inserm) [71]. In our study, the peripheral and central nervous system was affected to varying degrees, by all 3 types of glyphosate-based treatments and in both sexes. In males, malignant granular cell tumor in the high-dose glyphosate group occurred in the central nervous system while malignant Schwannoma was observed in the peripheral nervous system in association especially with the highest dose of Roundup Bioflow. The role of glyphosate and GBHs in peripheral and central nervous system alteration still needs more thorough investigation, particularly when exposure is initiated early in life or in utero. Recent studies have suggested that glyphosate and GBHs alter cerebellar development, primarily by affecting granule cell migration and differentiation, and that perinatal exposure leads to long-term changes in the nervous system of the adult male rat [72, 73]. In our study, an effect on the PNS it has also been noted in females as we detected several benign and malignant Schwannomas of the uterus, especially in the glyphosate and RangerPro exposure groups. Occasionally, malignant Schwannomas are observed in the uterus/cervix of rats, but usually only upon exposure to direct-acting alkylating agents, such as N-nitrosoethylurea or methylmethane-sulfonate [29]. Exposure to GBHs, early in neuronal development, induces dysregulation of various signaling pathways both in the CNS and PNS; in particular, GBHs alter the expression of S100B protein, a marker of nervous system damage in Wistar rats, inducing downregulation during development and upregulation in adult offspring after chronic exposure [68]. All of these findings on the neurotoxicity of glyphosate are consistent with reports indicating that environmental exposures to pesticides increase risk for human neurodegenerative disorders such as Parkinson's disease [74].

In our study of the carcinogenicity of glyphosate and GBHs, increased incidences of malignant and benign bone tumors were observed with all 3 treatments. Only a few agents (i.e. sodium fluoride, parathyroid hormone and related peptides, ionizing radiation) have been shown in cancer rodent assays to cause osteosarcoma with equivocal or clear evidence [26, 75–77]. In SD rats, even after only seven days administration of glyphosate, the total body burden was primarily concentrated in bone [78]. Alterations of bone and cartilage structure were observed in different models including zebrafish

following early life exposure to glyphosate [79]. An imbalance of bone metabolism (calcium and phosphorus) was observed in rats following sub-chronic exposure to GBHs [80]. Thus, our findings support that the skeletal tissue is a target for glyphosate toxicity and indicate that bone tumors can be a relevant apical effect of long term-exposure.

To evaluate our findings in a broader context, we note that over the past half-century, the incidence of childhood cancers has increased by 35% [81] and epidemiological evidence and mechanistic studies suggest that maternal exposure to pesticides is associated with an increased risk of childhood leukemia [70, 82], as previously suggested also by a working group organized by the European Food Safety Authority [83]. Recent epidemiologic studies have expanded the knowledge of the human carcinogenicity of glyphosate and GBHs. A recent meta-analysis reports that GBHs are associated with a 41% increased relative risk of non-Hodgkin lymphoma among highly exposed individuals [84]. Skidmore et al. [85] showed a significant temporal and geographical relationship between expansion of glyphosate tolerant genetically modified soy cultivation in the Brazilian Amazon and Cerrado regions and deaths from acute lymphoblastic leukemia in children, one of the most common pediatric blood cancers. Hardell et al. [86] published the results of a pooled analysis of three Swedish case–control studies including 1425 cases and 2157 controls, examining exposures to phenoxyacetic acids and glyphosate in relation to non-Hodgkin lymphoma and has shown an association between non-Hodgkin lymphoma and exposure to these herbicides. Another study focusing on fenceline exposure to agricultural pesticides and risk of childhood leukemia in an Italian community, reported an increasing risk in children residing close to arable crops exposed to a mixture of glyphosate and other pesticides [82, 87]. In an updated follow-up report of the United States Agricultural Health Study, a large prospective cohort investigating cancer incidence through 2012 (North Carolina)/2013 (Iowa) with 7290 cases, showed evidence of increased risk of acute myeloid leukemia among the group with highest exposure to glyphosate [59, 88]. Furthermore, in a recent study by Taiba et al. [70] a positive association was observed between agrochemical mixtures containing glyphosate and increased cases of juvenile leukemia (< 20 years of age). Recently it was hypothesized that some of the absorbed glyphosate may move rapidly into the bone marrow and then into the bones, where it may bioaccumulate and contribute to the risk of hematopoietic malignancies (e.g., leukemia) [89].

Animal carcinogenicity studies provide biological plausibility to this expanding epidemiologic literature that links GBHs to cancer by showing glyphosate's ability to cause multiple types of cancer and genotoxic lesions [90]. The main sites of neoplasms previously observed in other glyphosate long-term studies in SD rats were kidney, liver, skin, pancreas, thyroid and testes in males and adrenal and thyroid in females. In other strains of rats,

Table 20 Comparison between the results observed in animals treated with glyphosate in the GGS and previous evaluation

Tumors	Portier 2020[a]		IARC 2017[b]		GGS[c]	
	Male	Female	Male	Female	Male	Female
Hematological (Leukemias)					↑	↑
Skin	CE				↑	↑
Liver	CE		+		↑	
Thyroid	EE	EE		+	↑	↑
Nervous System					↑	↑
Bone					↑	↑
Ovary and Testes (Sex cord-gonadal stromal tumors)	SE					↑
Adrenal glands		CE			↑↑	
Kidney	CE				↑↑	
Urinary Bladder					↑↑	
Hemangiosarcoma (uterus and spleen)					↑↑	↑↑
Pancreas (Endocrine)	EE		+		↑↑	

[a] Adapted from Portier, 2020 [42]: level of evidence for tumors observed in Sprague–Dawley rats: CE Clear Evidence; SE Some Evidence; EE Equivocal Evidence

[b] Adapted from IARC monograph, 2017 [6]: + = significant pairwise or trend test in benign or malignant tumors according to animal carcinogenicity data from Sprague–Dawley rats studies evaluated as adequate by the Working Group

[c] ↑ = statistically significant increased trend in the incidence of benign or malignant tumors in glyphosate treated groups or rare tumors in excess compared to NTP and RI historical control. ↑↑ = statistically significant increased trend in the incidence of benign or malignant tumors only in Roundup Bioflow or RangerPro treated groups or rare tumors in excess compared to NTP and RI historical controls

lesions related to glyphosate exposure were liver and skin tumors, only in males, and mammary and adrenal tumors in females. Kidney tumor incidence was increased also in male CD-1 mice, together with mesenchymal tumors and lymphoma [42]. All studies in rats and mice were, however, based on exposure starting at a standard post-natal early adulthood stage of life and included doses that were generally higher than the those tested in the GGS. The only long-term study (post-natal) performed in SD rats that included similar or lower doses than the GGS was reported by Lankas et al. [91] (~ 3, 10, 31 mg/kg bw/day), in which pancreas (low dose), testes (trend) and thyroid (trend) tumors incidences were significantly increased [6, 42].

Exposure to GBHs, early in neuronal development, induces dysregulation of various signaling pathways leading both in the CNS and PNS [68, 72, 73, 91, 92]. Besides DNA damage, endocrine disruption is a relevant mechanism of tumorigenesis. Glyphosate and GBHs could act as endocrine disruptors in humans and animal models as well documented in the literature [9, 66, 93–95]. In the GGS, glyphosate and Roundup Bioflow showed significant androgen-like effects on reproductive parameters [9]. In the present study, effects potentially associated with endocrine disruption included male mammary, adrenal cortex and thyroid tumors. A further possible endocrine disrupting effect of Roundup Bioflow is the case of ovary malignant granulosa cell tumor, a rare tumor in SD rats, observed in high-dose Roundup group and causing early death at only 43 weeks of age. Chronic exposure to low doses (up to 2.0 mg/kg bw) of glyphosate alter the ovarian proteome and therefore impacts ovarian function in mice; according to the authors, the oxidative stress-related, pathways are the primary target [96]. GBHs were found to alter granulosa cell proliferation in an in vitro models suggesting that GBHs exposure might have more potent effects than glyphosate alone, consistent with GGS findings [9, 96, 97]. It is reported in the literature that chronic exposure to GBH might also alter the endocrine pancreas function and histoarchitecture [98]. Notably we observed a significantly increased trend in islet cell carcinoma in RangerPro.

Bone is another tissue with strong and complex endocrine regulation, including parathyroid, as well as pituitary, thyroid and steroid hormones. Our study indicates that bones are a target of glyphosate and GBHs carcinogenicity. Evidence in GBH-exposed female Wistar rats has associated the observed calcium-phosphorus imbalance and bone structure alterations with impaired thyroid metabolism [90]. It is noteworthy that a recent epidemiological study based on NHANES data linked urinary glyphosate levels to reduced bone mineral density in the U.S. population diseases induced by different chemical agents [26]. Thus, our results suggest that bone might be an additional target of endocrine-related tumorigenesis elicited by glyphosate and GBHs [27].

Our findings also consistent with previous reviews performed by Portier [42] and IARC [6], on the carcinogenicity of glyphosate in SD rats as shown in Table 20. In particular, tumor increases observed in our study are consistent with previous studies in SD rats for skin, liver, thyroid, sex cord-gonadal stroma, adrenal gland, kidney, and endocrine pancreas. Notably, in most cases the tumor increases were not only consistent in terms of target organ, but also in terms of sex. Increases in leukemia, nervous system, bone and hemangiosarcoma were observed only in our study. This is likely associated with the enhanced sensitivity of the study design: the exposure covering also the prenatal and prepubertal development allowed detection of carcinogenic effects caused by early exposures that cannot be effectively explored if the animal's exposure starts from adulthood. Early onset and mortality of leukemia and a number of other rare tumors (liver, ovary, and nervous system) could also be observed in our study because of the sensitivity of the prenatal study design, because adult studies would have longer tumor latencies and might not be as sensitive, particularly for the effects of low doses. Starting with the evidence provided by Prof. Maltoni in its carcinogenicity studies on early exposures to vinyl chloride from in the 1970's [99], until its most recent adoption by the US National Toxicology Program, [100] prenatal design for cancer assays in SD rats are now widely considered a predictive and reliable model for humans. Indeed, it has been clearly shown that chemical exposures even at low doses during pre-natal and developmental phases of life can elicit a number of detrimental and long-term effects, including cancer [81]. This concept, called the developmental origins of health and disease, or DOHaD, is now widely accepted and represents a cornerstone of public health prevention strategies for a variety of diseases including obesity, type 2 diabetes, insulin resistance, asthma, cardiovascular diseases, behavioral disorders, neurodegenerative diseases, reproductive disorders and cancer [101].

Our results provide a new and more comprehensive insight on glyphosate carcinogenicity in rats, and they are consistent with earlier report of increased tumors in experimental animals exposed to glyphosate and GBHs [6, 42]. Our study has limitations. One limitation is that although we compared glyphosate and two of its widely used formulations (Roundup Bioflow and RangerPro), we could not explore the health effects of the specific adjuvants present in the GBHs. The toxicological relevance of increased incidences of rare tumors is difficult to evaluate, particularly when there is borderline statistical

significance or when tumor incidence is found to be in excess only when compared to historical controls. In an effort to overcome this limitation and elucidate the biological plausibility of increases of rare tumors, we compared them with both RI and NTP historical control incidences. In addition, our data do not provide direct information on glyphosate tumorigenic mechanisms and mode(s) of action; however, by examining the available literature, we hypothesize that genotoxicity and endocrine disruption might be involved, but others mechanisms like defective DNA repair or tumor suppressor genes cannot be excluded. Finally, we concentrated on the accurate description of effects without attempting to derive Benchmark Doses, leaving this further elaboration to a future risk characterization phase. However, the EU ADI was established as 0.5 mg/kg bw per day, based on a NOAEL of 53 mg/kg bw per day from a 90-day study in dogs and supported by the NOAEL of 59.4 mg/kg bw per day from a 2-year rat study and covering the NOAEL of 50 mg/kg bw per day for maternal toxicity identified in a rabbit developmental toxicity study [21]. The standard uncertainty factor (UF) of 100 was applied. Notably, in the present study we observed effects at doses equal or lower than the NOAEL in rodents and other animal models.

Conclusion

This report from the carcinogenicity arm of the GGS found that glyphosate and GBHs at exposure levels corresponding to the EU glyphosate ADI and NOAEL caused statistically significant, dose-related increased trends or increased incidences compared to RI and NTP historical controls, of multiple benign and malignant tumors of blood, skin, liver, thyroid, nervous system, ovary, mammary gland, adrenal glands, kidney, urinary bladder, bone, endocrine pancreas and circulatory system. Most of these increases involved tumors that are rare in SD rats (incidence < 1%).

We also observed early onset and early mortality for a number of rare malignant tumors, including leukemia, liver, ovary and nervous system tumors. Notably, approximately half of the deaths from leukemia seen in the glyphosate and GBHs treatment groups occurred at less than one year of age. By contrast, no case of leukemia was observed in the first year of age in more than 1600 SD historical controls in carcinogenicity studies conducted by the RI or the NTP.

Our results provide a comprehensive and accurate overview of the carcinogenicity of glyphosate and GBHs in SD rats. They support the IARC conclusion that there is "sufficient evidence of carcinogenicity [of glyphosate] in experimental animals" [6]. Our findings are consistent also with the epidemiological evidence showing increases in incidence of multiple malignancies in humans exposed to glyphosate and GBHs. Our results indicate that, while glyphosate alone is capable of causing a number of benign and malignant tumors, GBHs co-formulants may enhance the carcinogenicity of glyphosate, particularly in the case of leukemia.

Finally, our findings highlight the importance of considering exposures in early development beginning prenatally during organogenesis through to puberty, in comprehensive evaluations of chemical carcinogenicity in rodents.

Abbreviations

AMPA	Aminomethylphosphonic acid
ADI	Acceptable Daily Intake
CNS	Central Nervous System
DOHaD	Developmental origins of health and disease
EU	European Union
GBH	Glyphosate-Based Herbicide
GGS	Global Glyphosate Study
IARC	International Agency for Research on Cancer
INHAND	International Harmonization of Nomenclature and Diagnostic Criteria
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
PNS	Peripheral Nervous System
POEA	Polyoxyethylene tallow amine
RI	Cesare Maltoni Cancer Research Center, Ramazzini Institute
SD	Sprague–Dawley

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

SP conceptualization, data curation and formal analysis, investigation, visualization, writing the original work, reviewing and editing. ET data curation and formal analysis, investigation, visualization, reviewing and editing. LDA investigation. LF investigation. RG investigation. FG investigation. MI investigation. MM investigation. FM investigation. IM investigation. IME investigation. RM investigation. RN investigation. DS investigation. VS investigation. FT reviewing and editing the work. MNA reviewing and editing the work. JC reviewing and editing the work. GD reviewing and editing the work. SL reviewing and editing the work. AM reviewing and editing the work. RME reviewing and editing the work. MJP reviewing and editing the work. AV investigation. PJL conceptualization, reviewing and editing the work. FB conceptualization, reviewing and editing the work, project administration (until 2019) and resources for study conduct (until 2019). DM conceptualization, investigation, visualization, writing the original work, reviewing and editing, supervision, project administration and resources for study conduct.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Animal carcinogenicity study underwent ethics approval.

Competing interests
The authors declare no competing interests.

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