Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXXIII

Settore Concorsuale: 07/H1 – ANATOMIA E FISIOLOGIA VETERINARIA

Settore Scientifico Disciplinare: VET 02 – FISIOLOGIA VETERINARIA

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY STUDY USING SPRAGUE-DAWLEY RATS EXPOSED UNDER VARIOUS CALENDARS TO THE WEEDKILLER GLYPHOSATE AND COMMERCIAL FORMULATIONS GLYPHOSATE-BASED

Presentata da: Fabiana Manservisi

Coordinatore Dottorato **Supervisore**

Prof. Arcangelo Gentile **Prof.ssa Giovanna Galeati**

Esame finale anno 2021

"Leave this world a little better than you found it"

Robert Baden Powell

INDEX:

ABSTRACT

ABSTRACT

Glyphosate-based herbicides (GBHs) are the most globally used herbicides raising the risk of environmental exposition. Carcinogenic effects are only one component of the multiple adverse health effects of Glyphosate and GBHs that have been reported. Questions related to hazards and corresponding risks identified in relation to endocrine disrupting effects are rising. The present study investigated the possible reproductive/developmental toxicity of GBHs administered to male and female Sprague-Dawley rats under various calendars of treatment. Assessments included maternal and reproductive outcome of F0 and F1 dams exposed to GBHs throughout pregnancy and lactation and developmental landmarks and sexual characteristics of offspring. The study was designed in two stages. In the first stage Glyphosate, or its commercial formulation Roundup Bioflow, was administered to rats at the dose of 1.75 mg/kg bw/day (Glyphosate US Acceptable Daily Intake) from the prenatal period until adulthood. In the second stage, multiple toxicological parameters were simultaneously assessed, including multigeneration reproductive/developmental toxicity of Glyphosate and two GBHs (Roundup Bioflow and Ranger Pro). Man-equivalent doses, beginning from 0.5 mg/kg bw/day (ADI Europe) up to 50 mg/kg bw/day (NOAEL Glyphosate), were administered to male and female rats, covering specific windows of biological susceptibility. The results of stage 1 and preliminary data from stage 2 experiments characterize GBHs as probable endocrine disruptors as suggested by: 1) androgen-like effects of Roundup Bioflow, including a significant increase of anogenital distances in both males and females, delay of first estrous and increased testosterone in females; 2) slight puberty onset anticipation in the high dose of Ranger Pro group, observed in the F1 generation treated from in utero life until adulthood; 3) a delayed balano-preputial separation achievement in the high dose of Ranger Pro-treated males exposed only during the peri-pubertal period, indicating a direct and specific effect of GBHs depending on the timing of exposure.

ABBREVIATIONS

ABBREVIATIONS

ADI	Acceptable Daily Intake
AGD	Anogenital distance
AMPA	Aminomethylphosphonic acid
BPS	Balano-Preputial Separation
cRfD	Chronic Reference Dose
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FE	First Estrus
GBH	Glyphosate Based Herbicides
GD	Gestational Day
GMO	Genetic Modified Organism
IARC	Agency for Research on Cancer
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
PND	Post Natal Day
POEA	Polyoxyethyleneamine
PPPs	Plant Protection Products
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
SD	Sprague Dawley
US ADI	United States Acceptable daily intake
VO	Vaginal Opening
WHO	World Health Organization
WOS	Windows of susceptibility

INTRODUCTION

I. INTRODUCTION

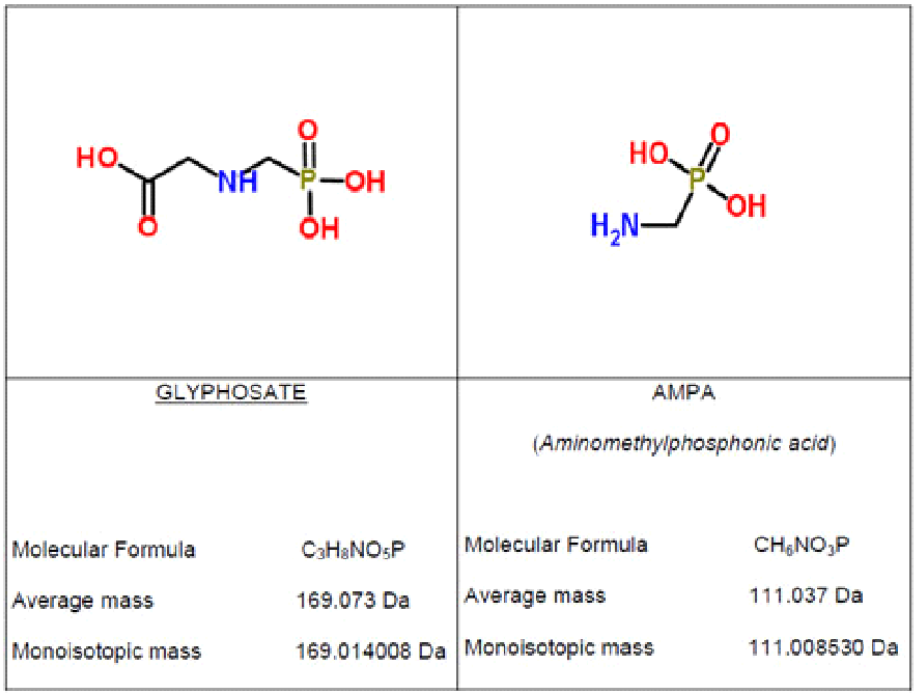
1. Glyphosate: chemical profile, mode of action and uses

Glyphosate (CAS # 1071-81-6), ISO common name for N-(phosphonomethyl)glycine (IUPAC), is a broad spectrum, non-selective, systemic herbicide patented in 1974 by the Monsanto Company and now manufactured and sold by many companies in hundreds of products, representing the most widely used herbicide worldwide. Glyphosate is a phosphonic acid resulting from the formal oxidative coupling of the methyl group of methylphosphonic acid with the amino group of glycine (*Figure* 1). It is used as an active ingredient in commercial formulations referred as Glyphosate Based Herbicides (GBHs), which include other chemical additives that enhance its efficiency as a weedkiller, by promoting toxicity and improving the plant's ability to take up the herbicide. These additives are considered to be 'inert diluents' by manufacturers and are classified as confidential for regulatory purposes (Mesnage *et al.*, 2014). The most common GBH is known with the trade name Roundup™, manufactured by Monsanto, in 2018 acquired by Bayer.

Among a number of surfactants used in the GBHs and in general in Plant Protection Products (PPPs), the polyethoxylated tallowamine (POEA) is added to Glyphosate to allow uptake of the water-soluble active ingredient across plant cells, affecting membrane transport and to reduce the wash-off effect after spray application.

Glyphosate or GBHs act via specific inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is present in plants and some microorganisms and essential for synthesis of three aromatic amino acids (tyrosine, tryptophan, and phenylalanine) (Rubin *et al.*, 1984; Schönbrunn *et al.*, 2001). As the EPSPS-driven pathway does not exist in vertebrate cells, many scientists and environmental regulating agencies attested that Glyphosate would impose minimal risks to mammals, in particular, humans (EFSA, 2015; ECHA, 2017; EPA, 2020). Glyphosate is absorbed through foliage and minimally through roots, meaning that it is only effective on actively growing plants and cannot prevent seeds from germinating. After application, Glyphosate is readily transported around the plant to growing roots and leaves and this systemic activity is important for its effectiveness. Inhibiting the enzyme causes shikimate to accumulate in plant tissues and diverts energy and resources away from other processes, eventually killing the plant. While

growth stops within hours of application, it takes several days for the leaves to begin turning yellow. The primary degradation product of Glyphosate in plants, soil, and water, is aminomethylphosphonic acid (AMPA), whose chemical structure is very similar to that of Glyphosate (*Figure* 1).



GLYPHOSATE		AMPA (*Aminomethylphosphonic acid*)	
Molecular Formula	$C_3H_8NO_5P$	Molecular Formula	CH_6NO_3P
Average mass	169.073 Da	Average mass	111.037 Da
Monoisotopic mass	169.014008 Da	Monoisotopic mass	111.008530 Da

***Figure 1**- Molecular formula and structure of Glyphosate and its metabolite AMPA.*

Glyphosate is used in agriculture, forestry, aquatic environments and in urban and domestic settings. It is often used to clear railroad tracks and get rid of unwanted aquatic vegetation. In many cities, Glyphosate is sprayed along the sidewalks and streets, as well as crevices in between pavement where weeds often grow. Glyphosate is also used for crop desiccation to increase harvest yield and uniformity. Glyphosate itself is not a chemical desiccant; rather Glyphosate application just before harvest kills the crop plants so that the food crop dries from environmental conditions ("dry-down") more quickly and evenly. On a global scale, about 50% of GBHs used in agriculture are used on genetically engineered crops (e.g. maize, cotton, soya beans, oilseed, sugar beet), known as Genetically Modified crops or GM, that

have been genetically engineered to be resistant to Glyphosate, allowing it to target weeds while leaving crops unaffected.

GBHs are widespread in the environment, today their market has reached over 750 products. Between 1974 and 2014 the amount of Glyphosate used worldwide went from 3,200 to 825,000 tons per year and is now found in over 140 countries (Benbrook, 2016). The increase is due to increasingly widespread adoption of GM crops (USDA, 2020). Based on application, the global Glyphosate market has been bifurcated into GM crops and conventional crops. According to the global organisation Transparency Market Research, Europe held around 16.6% of the global Glyphosate market in 2012 and, according to its manufacturers, Glyphosate accounted for 25% of the global herbicide market in 2012 (Transparency Market Research, 2014). Overall, the worldwide market for Glyphosate is estimated at 5.4 billion dollars and it is projected to reach 9.91 billion dollars by 2022 (MarketsandMarkets™, 2017).

As a consequence, Glyphosate has been detected in air, soil, foodstuffs, water (it has even been detected in the coral reef), as well as in man's urine (EFSA, 2016; ISPRA, 2016; Expert Committee on Pesticide Residues in Food, 2016; USDA, 2014; Battaglin *et al.*, 2014; Mercurio *et al.*, 2014).

Urinary levels of both Glyphosate and AMPA were also detected from a repository of urine samples collected from United States farmers in 1997–98, demonstrating that Glyphosate exposures among US farmers were occurring 20 years ago (Perry *et al*., 2019).

Different countries have established a range of "acceptable" daily intake levels of Glyphosate-herbicide exposures for humans, generally referred to in the U.S. as the chronic Reference Dose (cRfD), or in the E.U. as the Acceptable Daily Intake (ADI). The current U.S. Environmental Protection Agency (EPA) cRfD is 1.75 mg of Glyphosate per kilogram body weight per day (mg/kg/ bw/day) (EPA, 1993). In contrast, the current E.U. ADI is more than 3-fold lower at 0.5 mg/kg/day, a level adopted in 2015 after an increase from 0.3 mg/kg/day (EFSA, 2015). The data upon which these exposure thresholds are based were supplied by manufacturers during the registration process, are considered proprietary, and are typically not available for independent review. The German Federal Institute for Risk Assessment is the lead regulatory authority currently conducting an EU reassessment of GBHs (Myers *et al.*, 2016).

At the present time, the subject of Glyphosate is still debated by the research community, control organizations and companies which claim it is either dangerous or not, as the case may be.

2. Health concern and decision-making process in the re-registration of Glyphosate

Pesticides are regulated chemicals and require pre-marketing authorization in most jurisdictions. The EU system also includes a renewal process, requiring all pesticides to be regularly re-assessed in the light of new scientific developments and information requirements. Pesticides, such as Glyphosate, must be approved for use in the EU by the European Commission, according to the EU plant protection products regulation (Regulation: (EC) No 1107/2009). In 2011 the patent owned by the first multinational Glyphosate manufacturer, Monsanto, expired. The representative formulated product for the evaluation in the framework of the renewal of the approval of Glyphosate and also considered in the current study is 'MON 52276', a soluble concentrate (SL) containing 360 g/L Glyphosate as isopropylammonium salt (486 g/L). In light of new scientific evidence, the use and risks of GBHs have been widely discussed, and after a heated vote, Glyphosate was allowed for use in Europe until 15 December 2022 (Szeká and Darvas, 2018). However, that decision has been put off several times. The scientific literature and regulatory conclusions regarding Glyphosate and GBHs show a mix of findings, making the safety of the herbicide a hotly debated subject.

The main phases in the risk assessment and policy-making process that led to the current debate on **cancer** concerns are summarised in chronological order:

- March 2015: The International Agency for Research on Cancer (IARC), organism of the World Health Organization (WHO), after reviewing years of peer-reviewed scientific studies, defined Glyphosate as "probably carcinogenic for humans", group 2A. The team of international scientists found there was a particular association between Glyphosate and non-Hodgkin lymphoma (IARC, 2015).
- November 2015: the European Food Safety Agency (EFSA) stated it was "improbable" that Glyphosate could "constitute a cancer risk to man". The conclusion was based on the evaluation report for renewing Glyphosate (RAR) presented in January 2014 by the German Federal Institute for Risk Assessment (BfR- Bundesinstitut für

Risikobewertung). The EFSA pronouncement was contrary to the IARC (German Federal Institute for Risk Assessment, 2015).

- March 2017: after a heated dispute over the weedkiller's safety and numerous deferments of the European vote, the EU delegated the job of ascertaining whether Glyphosate is toxic to the European Chemical Substances Agency (ECHA). The ECHA Risk Assessment Committee (RAC), after analysing a huge bulk of scientific data, concluded that *"the scientific evidence available to date does not meet the criteria for classifying Glyphosate as a carcinogen, mutagenous agent or toxic for reproduction"*. According to ECHA, Glyphosate can cause serious eyes damages and be toxic for aquatic organisms with long lasting effects. Its toxicity and carcinogenicity for humans have not been demonstrated by the available scientific evidence (ECHA, 2017).
- November 2017: decision by the Member States of the European Union to renew the license to use Glyphosate for 5 years as the active substance in herbicides. Italy nonetheless kept up the ban brought in by the Health Ministry in August 2016, *"on using Glyphosate in areas frequented by the public or by vulnerable groups, such as parks, gardens, sports fields and recreation areas, children's playgrounds, courtyards and enclosed green areas in school complexes and health facilities, as well as in the countryside prior to harvesting with a view simply to optimizing harvesting and threshing".*
- January 2020: in an interim Registration Review Decision on Glyphosate, the US Environmental Protection Agency (EPA) concluded that *"Glyphosate poses no risks to human health when used according to instructions on the label and that it is not a carcinogen"* (EPA, 2020). The evaluation is part of a routine re-registration process that the agency conducts every 15 years for pesticides in the US marketplace. The findings are consistent with those of the US Department of Agriculture, the European Food Safety Authority, and Canada's Pest Management Regulatory Agency. However, they are in contrast to the conclusion of the World Health Organization's cancer agency, which declared in 2015 that Glyphosate is "probably carcinogenic" to humans.

Carcinogenic effects are only one component of the multiple effects of Glyphosate and GBHs that have been reported. Questions related to hazards and corresponding risks identified in relation to **endocrine disrupting effects** divide the scientific community and

official health and environmental authorities, and touch upon fundamental aspects of risk assessment and product regulation.

- The US EPA Endocrine Disruptor Screening Program reported that *"Glyphosate demonstrates no convincing evidence of potential interaction with the oestrogen, androgen or thyroid pathways in mammals or wildlife*" (EPA, 2015). This conclusion was drawn from a battery of Tier-1 tests, composed of in vitro and short-term in vivo tests, on Glyphosate alone. US EPA did not take into consideration any of the findings from studies that tested the formulations of Glyphosate-based herbicides, which is what people and the environment are exposed to.
- In 2015, the EFSA was asked by the European Commission to prepare a statement on the co-formulant POEA based on the toxicological evaluation of POE-tallowamine presented by the rapporteur Member State Germany (EFSA, 2015). While EFSA found that information inadequate to perform a full risk assessment, it observed that the few available animal studies indicated that POEAs exhibited acute oral, dermal and ocular toxicity; genotoxicity; and reproductive impacts on males and females that triggered the need for investigations into their endocrine-disruption potential. In consequence of this review, member states backed a proposal by the European Commission to ban the use of POEA in all Glyphosate-based herbicides, including Roundup and the European Commission enacted this ban soon thereafter (European Commission, 2016).
- In September 2016, EFSA received a mandate from the European Commission to consider information on potential endocrine activity of Glyphosate in accordance with Article 31 of Regulation (EC) No 178/2002. EFSA was requested to assess the available information on potential endocrine activity of Glyphosate. The endocrine disruption potential of Glyphosate was discussed during the Pesticides Peer Review Experts' Meeting 159 in June 2017. The report concluded that the only effect that could be related to a possible endocrine-mediated mode of action in apical studies (level 4 and 5 of the OECD Conceptual Framework) is an isolated marginal (but statistically significant) delay in balano preputial separation (BPS), observed in males at the limit dose of ca. 1000 mg/kg body weight (bw) per day in the first generation (F1 generation) of a two-generation reproductive toxicity study in rats. This effect was not reproduced in the second generation (F2 generation) of the same study or in another study

investigating the same endpoint, and general toxicity has been shown at this dose level in other studies (reduced parental and offspring's body weight). In addition, studies on short- and long-term toxicity, carcinogenicity, developmental toxicity, one-generation range-finding and five other two-generation reproductive toxicity studies did not show any evidence of endocrine disruption potential. On this basis, EFSA concluded that Glyphosate shows no endocrine-mediated adverse effects (EFSA, 2017).

This discrepancy between the conclusions of the regulatory bodies brought reactions from the scientific community around the world and has raised concerns in the general population about how much Glyphosate we are actually exposed to, and what are its potential health effects.

The main scientifically debated topics of dispute concern the studies examined for risk assessment (Vandenberg *et al*., 2017; Myers *et al*., 2016). The main critical issues that still need clarifying are here summarised:

1. Year of publication of studies used for assessment. Many scientific studies examined in the various risk assessment dossiers date back more than 30 years; many have not been scientifically reviewed or not published, while in some cases the experiment conduction criteria contain deficiencies.
2. Doses tested. Seeing that use of Glyphosate increased one hundred-fold from 1974 (year of registration as herbicide in USA) to 2014, most of the studies examined do not experimentally reproduce the current human exposure scenario by man-equivalent doses (Gasnier *et al.*, 2009). Moreover, since this herbicide is considered a potential endocrine disruptor, the dose-response ratio may be inverted, non-monotonic in type, with more acute effects at low doses than high doses.
3. Windows of biological susceptibility. We now know that various windows of biological susceptibility are toxicologically relevant: pregnant women, new-borns and growing infants and adolescents are at high level biological risk and can suffer worse health effects than the rest of the population, exposure being equal. Epidemiological studies on populations residing in South America, where use of Glyphosate-based weedkiller in soy plantations is very high, have recorded an increase in miscarriages and malformations in child development (Benitez-Leite *et al.*, 2009; Campaña *et al*., 2010). The current exposure limits were fixed for healthy adults, not taking account of growing

organisms or the more susceptible sectors of the population. Few case studies have addressed this aspect.

4. Glyphosate-based commercial products. So far only the IARC report has considered another point concerning commercial weedkiller products whose active principle is Glyphosate, such as Roundup. The agencies EFSA and ECHA have only monitored studies testing Glyphosate on its own as an active principle, without considering the GBHs products. Such an approach has caused considerable perplexity since the human population is generally exposed to commercial products containing not just Glyphosate but other adjuvants and co-formulants not always specified (non-ionic tensioactives including alkypolyglycosides and ethoxylated fatty amines). Actually, the scientific literature contains very few studies where Glyphosate and a commercial product based on it were tested simultaneously, so as to compare the pure active principle effects with those of the commercial product. In such studies as have been conducted, including the present study, the commercial product has been associated with stronger effects.
5. Assessment and regulation of POEA and similar surfactants. Available data on the toxicity of surfactants is limited largely to POEAs. In Europe, the use of POEA in commercial formulations based on Glyphosate is banned following studies demonstrating cellular toxicity in vitro (Mesnage *et al*., 2013) in vivo on rats (Adam *et al*., 1997) and in other test systems in vivo such as sea urchins (Marc *et al*., 2005) microorganisms (bacteria, microalgae, protozoa) and crustaceans (Tsui *et al*., 2003). A more recent two-phase study of the life of the Pacific oyster shows that POEA-based adjuvants can be very toxic to embryonic and larval development (Mottier *et al*., 2014). The data available in literature reinforce the need to perform toxicological studies that contemplate the presence of co-formulants in GBHs mainly sold on a global scale, especially in the light of the trade liberalization between Europe and the United States. Indeed, the marketing of GBHs containing POEA is authorized in the United States, widely used by farmers for the increase in efficacy that it confers to Glyphosate (Tush *et al*., 2016) with widespread diffusion in the food chain and in feed and whose health effects are not still properly explored.
6. Regulatory toxicological studies conducted to support the authorisation of Glyphosate follow a standardised study design with a wide, but still limited range of endpoints,

mainly focused on the oral route of exposure. These limitations can contribute to overlooking important aspects of toxicity and underestimating risks and hazards.

7. Independent and comprehensive assessment is needed. Proprietary studies conducted on behalf of the manufacturers often represent a limited investigation of the various toxicological effects. There is a need to conduct independent studies, under strict conditions of experimentation and traceability and avoiding conflicts of interest (Landrigan and Belpoggi, 2018).

II. REPRODUCTIVE TOXICITY RISK ASSESSMENT

Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females as well as developmental toxicity in the offspring. The distinction between developmental and reproductive toxicity is somewhat arbitrary in that developmental exposures can result in effects on reproduction, and vice versa (OECD, 2008). The potential of some chemicals to adversely affect development is largely determined from epidemiological data or from studies conducted in laboratory animals and applying testing guidelines published by regulatory agencies and authorities such as the US EPA, US National Toxicology Program (NTP) and the Organisation of Economic Co-operation and Development (OECD). In the framework of the revision of the test guidelines for the screening and testing of potential Endocrine Disrupting Chemicals (EDCs), both the OECD and NTP recently updated their study guidelines for reproductive and developmental toxicity adding various functional endpoints for assessing how an agent can affect the reproductive and endocrine status of animals.

The reproductive toxicity guideline studies characterize the adverse effects against the reproductive system attributable to the exposure to chemicals for the following purposes: 1) hazard identification; 2) dose-effect, dose-response evaluation and 3) risk characterization. Subchronic, chronic, multigeneration and teratogenic studies provide the majority of data used for hazard identification and dose-effect, dose-response evaluation. Genetic, pharmacokinetic, metabolism, and specially designed investigative studies provide data on the mode of action, target site, delivered dose, primary versus secondary effects, etc. required for characterization of risk.

For the evaluation of hazard, following exposure to chemical substances, a number of OECD Test Guidelines for reproductive/developmental toxicity are available, these include the prenatal developmental toxicity study OECD Test Guideline (TG) n. 414 (OECD, 2018), the one-generation reproduction study OECD TG n. 415 (OECD, 2019), the two-generation reproduction study OECD TG n. 416 (OECD, 2001) the developmental neurotoxicity study OECD TG n. 426 (OECD, 2007), two reproductive/developmental toxicity screening tests OECD TG n. 421 and 422 (OECD, 2016) (OECD, 1996) and the more recent extended one-generation reproductive toxicity study (EOGRTS) OECD TG n. 443 (OECD, 2018).

A new NTP guideline for reproductive and developmental toxicity is the NTP's Modified One-Generation (MOG) Reproduction Study which is able to generate large and robust data sets on sub-chronic toxicity and reproductive/developmental toxicity including early-life exposure and teratogenicity (NTP, 2011).

A range of methods exists to study the possible effects of chemicals on fertility and development. These methods examine effects on a wide range of biological endpoints in both the parental generation and the offspring, including effects on fertility, sexual behaviour, embryo implantation, embryonic/foetal development, parturition, postnatal adaptation, and subsequent growth and development into sexual maturity. An enormous variety of mechanisms at the molecular, cellular and tissue levels cooperate in a concerted and genetically programmed way to regulate these processes. Moreover, different temporal windows of susceptibility (WOS) such as intrauterine, perinatal, juvenile periods and puberty may result in different adverse outcomes at any time point during the exposure period and/or later in life.

For regulatory purposes, the official original and updated guidelines must be followed for performing assays. Under the current EU Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) legislation, screening studies for developmental toxicity are required under Annex VIII for all substances manufactured or imported in quantities of 10 tons or more (Article 12(1)(c)). Data may be available from a wide variety of animal studies even if REACH strongly promotes alternative validated methods to traditional in vivo testing thus reducing the number of animals used in the assessment and improving the predictability for identification of human health hazards.

1. Choice of testing models for reproductive/developmental toxicity testing

Over the last three decades, a wealth of in vivo and in vitro assays has been proposed as test systems for testing toxic effects on the various processes in reproduction and development. The use of animal models to assess hazard and risk to humans from exogenous substances continues to be the standard for protecting human health. Animal tests assessing reproductive toxicity are designed to examine the entire reproductive cycle, either as a series of single tests that evaluate specific stages of the reproductive cycle (reproduction/fertility, prenatal development, postnatal development), or as a protocol (two-generation test). These tests

evaluate structure and function from gametogenesis through embryonic and postnatal development to adulthood.

Animal tests are the current tool to predict the potential for chemicals to cause reproductive deficits in humans. This goal, however, must be weighed considering constraints on costs, ethics, and resources. Animal studies are expensive and, in most cases, may not provide information on the proper mechanism of action.

Ideally, the species of choice should have the same pharmacokinetic profile as in humans. It is thus apparent that the selection of the most sensitive species for evaluating the safety of the substance is important. Advantages and disadvantages of species (strains) should be considered in relation to the substance to be tested, the selected study design and to the subsequent interpretation of the results.

1.1. Mammalian Reproductive Toxicity Testing

The laboratory **rat** has been, and continues to be, a mainstay in reproductive and developmental toxicity studies. Research on the reproductive physiology and endocrinology of the rat as an experimental animal began in the 1930s. Since then, the species has been more thoroughly characterized in these research fields than any other laboratory animal model, and it has been the species of choice for multigenerational testing studies for several decades (Gray *et al.*, 2004). The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) formed by the EPA recommended the laboratory rat as the species of choice for the endocrine screening and testing assays. The main advantages of the rat as a species for reproductive and developmental toxicity tests are that it is inexpensive when compared with bigger mammals and, also, that it produces a satisfactory number of offspring. The rat is very useful in teratology studies because of its short reproductive cycle, large litter size, and relatively few spontaneous congenital anomalies. A disadvantage is that the rat and small rodents do not provide enough quantities of test material during sampling and must frequently be euthanized. Gray et al. (Gray *et al.*, 2004) reported a summary of reproductive physiology similarities among humans and rats and examples in which the reproductive strategy of the rat differs from that of the human (*for details see Table 1 and 2*). A correlation between age of laboratory rats and humans in relation to life stages is outlined in Table 3 (Suckow *et al.*, 2005).

Table 1 *- Examples of reproductive physiology similarities among humans and rats by Gray et al. (2004)*

● Steroid hormone control of reproductive function relies on testosterone, dihydrotestosterone, estradiol, and progesterone.
● CNS-hypothalamic secretion of GnRH controls pituitary release and synthesis of FSH and LH.
● FSH and LH regulate germ cell development after puberty, LH surges induce spontaneous ovulation in the female, LH regulates testis Leydig cell testosterone production.
● Placental support of embryos. Placenta and fetal unit also produce hormones critical for pregnancy maintenance after the first week.
● Hormonal regulation of uterine function and onset of delivery.
● Androgens required to maintain male spermatogenesis and secondary sex characteristics.
● Hormone-dependent mating and other sexually dimorphic behavior. "Rough and tumble" play behavior is sexually dimorphic behavior being imprinted by early androgens.
● Lactation under complex hormonal regulation.
● Dramatic endocrine changes resulting from CNS-HPG maturation responsible for puberty in males and females. Females generally attain puberty at an earlier age than males of the same species.

CNS, central nervous system; GnRH, gonatropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; HPG, hypothalamic-pituitary-gonadal axis.

Table 2 *- Examples in which the reproductive strategy of the rat differs from that of the human by Gray et al. (2004).*

● The rat is a short (22.5-day) gestation species. Pregnancy in humans is 9 mo.
● The rat placenta lacks aromatase; estrogen is produced during pregnancy by the ovary. Human placental tissue expresses high levels of aromatase.
● In the rat, sexual differentiation of the reproductive tract is perinatal, whereas central nervous system (CNS) sexual differentiation is a postnatal event, regulated to a great degree by aromatization of testosterone to estradiol (play behaviour, an exception, is androgen dependent in both rats and humans). In nonhuman primates and presumably humans, more CNS events are prenatal, and androgens are more important than in rats.
● The rat has a 4- to 5-day estrous cycle, with no functional corpus luteum. The estrous cycle can be monitored easily by examining daily cytology. The female rat displays sexual receptivity only during estrus after “lights out” after a proestrus vaginal smear. This behaviour is dependent on estrogen followed by progesterone. Humans have a menstrual cycle approximately 28 days in duration and do not display periods of peak behavioural estrus during the cycle. Corpora luteal function is sustained for approximately 10 days by mating-induced cervical stimulatory prolactin surges in rats, whereas the human menstrual cycle has a spontaneous luteal phase of 10 to 14 days after ovulation.
● Male rat sex behaviour can be induced by estrogens and involves multiple series of ejaculations in a single mating. Mating involves approximately 10 mounts, with intromission before each ejaculation, followed by a postejaculatory interval before the onset of the next series. In

nonhuman primates and presumably humans, male sex behaviour is androgen mediated.
● Both ovaries spontaneously release several ova in response to a luteinizing hormone surge into separate uterine horns, each with a separate cervix in the rat; whereas in women, a single ovum is typically ovulated during each cycle.
● Pregnancy is easily disrupted by estrogens in rats, but not in humans. Rats, unlike humans, are a litter-bearing species. Most strains used for toxicology testing have litters of 10 to 12 pups. Spontaneous reproductive malformations are very rare in the rat, whereas in humans, some malformations such as cryptorchidism occur in 3% of newborn boys.
● Spermatogenesis begins at approximately 5 days of age in the rat; the spermatogenic cycle is about 53 days of age, and sperm appear in the epididymis at about 55 days of age. In humans, spermatogenesis begins during puberty at 10 to 14 years of age, and the entire spermatogenic cycle is approximately 75 days.
● Puberty in the rat (as measured by the age at vaginal opening and the onset of estrous cyclicity) occurs at about 32 days of age in females and 42 days of age (as measured by preputial separation an androgen-dependent event) in male Sprague-Dawley and Long Evans rat strains. In humans, puberty occurs at 9 to 12 years of age in girls, and 10 to 14 years of age in boys.
● Fertility begins to decline in the female rat at about 6 months of age, especially if never mated and allowed to cycle continuously. Fertility begins to decline in women at about 35 years of age, and at 40 years of age, approximately 50% of women are infertile.

Table 3 *- Correlation post-natal days (PND) age of rats against human by Suckow et al. (2004)*

Stage in rat/human	Rat[a]	Human[b]
Neonatal/newborn	PND 0 to 7	0 to 28 days
Infantile/infant	PND 8 to 20[c]	1 to 23 months
Juvenile/child	PND 21 to 32	2 to 12 years
Peripubertal	PND 33 to 37	ND
Puberty/adolescent	PND 38 to 46	12 to 16 years

Note. PND = postnatal day; ND = stage not defined.
[a]Ojeda, Advis, and Andrews (1980).
[b]Barrow, Barbellion, and Stadler (2011).

If another mammalian species other than the rat is used, it is urged, in most test guidelines, that there should be a justification for its selection and a description of the modifications that will be necessary.

The **rabbit** has certain advantages as a non-rodent (lagomorphs) and second model in reproductive and developmental toxicity studies. It has been well characterized, can provide enough quantities of test material during sampling, semen can be obtained easily and repeatedly, and its visceral yolk sac and extra-embryonic membranes resemble the equivalent

histological elements in humans more closely than do rodents. On the other hand, rabbits are more expensive and space consuming and require larger amounts of chemical test compounds (Foote and Carney, 2000).

The **dog** has been commonly used as the non-rodent species for reproductive toxicity testing. The dogs used should be of any defined breed but it is common practice to use the beagle. The latter has many advantages, e.g., medium size and length of hair coat, shows adaptability to living in group housing, and it is most easily handled. It also has some disadvantages, e.g., the number of litters is not as high as in rodents, it is costly, it needs exercise and has special housing requirements, it varies in body weight and size, it has a natural tendency to vomit and requires larger amounts of test material than rodents and, in addition, its use is ethically questionable. A strong case for favouring the dog as a non-rodent test species is the extensive knowledge on the physiology of its reproductive system (Faqi, 2011).

Other species, e.g., the **swine** (Rocca and Wehner, 2009) and **minipigs** (Svendsen, 2006) may also be used, especially in cases where traditional animal models are not relevant. The **cynomolgus monkey** is the non-human primate species used most commonly for reproductive studies (Meyer *et al.*, 2006). Although menstrual cycles and gestation periods are long and affect the length of the studies, the cynomolgus monkeys breed all year round, in contrast to **rhesus macaques**. However, their pregnancy rate is lower than in rodents and they have only one offspring. Ethical issues demand the use of a minimum number of animals.

1.2. Non-Mammalian Reproductive Toxicity Testing

National and international government agencies have defined a need to reduce, refine or replace mammalian species in toxicological testing with alternative testing methods and non-mammalian models. It has now become abundantly clear that some non-mammals are not only convenient materials but also are endowed with physiological and pharmacological properties common to humans. The suitability of alternative species will depend on the reproductive endpoints to be assessed.

Avian test batteries represent a potentially rapid, cost-effective, ethical alternative to the currently available means of assessing developmental toxicity using higher vertebrates. Since the embryos of common Galliformes such as the chicken *(Gallus gallus domesticus*) and Japanese quail (*Coturnix japonica*) can easily be observed and directly manipulated during

embryogenesis, they have become the model organisms most widely used in developmental biology. Avian culture techniques using embryonic tissues may offer certain advantages over *in vivo* experiments. The avian models are advantageous at three points in comparison to the conventional mammalian model for the assessment of the developmental toxicity: (1) direct manipulation, (2) continuous observation, and (3) reduction of unnecessary sacrifices of the pregnant individuals. Avian models also have ethical advantages because mammalian toxicity tests usually require sacrifice of the pregnant animals prior to examination of embryonic development, whereas avian models do not (Kawashima *et al.*, 2016). The validated **avian reproduction test** OECD TG n. 206 (OECD, 1984) determines mortality in adults, egg production, egg-shell thickness, viability, hatchability of eggs, and the effects on young birds. In this test, the birds are fed a diet containing the test substance for 20 weeks at least. Eggs are collected over a 10-week period, incubated and hatched, and the young maintained for observation for 14 days.

Fishes have been used as vertebrate models in developmental biology for a long time, but they are now gaining increasing importance as toxicological models. The most used laboratory fish model species in aquaculture research are zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*). Reproductive peculiarities that make fish particularly vulnerable to toxic impact are ovipary and external fertilization. Endpoints typically measured to assess reproductive toxicity of chemicals to mature fish include fecundity (number of eggs ovulated per female, possibly corrected for female size), clutch size, spawning frequency, age to maturation, fertilization success, reproductive behaviour, or gonadosomatic index (the ratio of gonad to body weight). In addition to these apical endpoints, also molecular and physiological parameters are frequently measured, e.g. circulating levels of reproductive hormones, vitellogenin levels, or gonad histopathology. Each of these parameters may vary with the species-specific reproductive strategy. As fish are a vertebrate group with external fertilization, waterborne toxicants can directly influence this process. A sperm property that is particularly sensitive to toxic exposure is sperm motility, toxicants can also affect reproductive behaviour, in particular courtship behaviour and parental care The validated **fish short-term reproduction assay** OECD TG n. 229 (OECD, 2012) is an in vivo screening assay were sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of

their life cycle (21 days). The recommended species is the fathead minnow *Pimephales promelas*. Two endpoints are measured in males and females as indicators of endocrine activity of a test chemical: vitellogenin and secondary sexual characteristics. Gonads of both sexes are also preserved and histopathology may be evaluated to assess the reproductive fitness of the test animals and to add to the weight of evidence of other endpoints. The validated **21-day fish assay** OECD TG n. 230 (OECD, 2009) is a short-term screening test for certain endocrine active substances on estrogenic and androgenic activity, and aromatase inhibition. During this test sexually mature male and spawning female fish are kept together and exposed to a chemical during a 21-day part of their life cycle. After a 21-day exposure period, vitellogenin is measured in fathead minnow, Japanese medaka, and zebrafish. Secondary sex characteristics are measured in the fathead minnow and Japanese medaka only. In particular, the zebrafish (*Danio rerio*) test model is being used more often due to the increasing amount of molecular and genetic information available for this species (Briggs *et al*., 2002).

Amphibians represent a suitable model for monitoring reproductive performance, early embryo-larval development and advanced development, including metamorphosis and sexual maturation (Fort *et al*., 2004). The validated **amphibian metamorphosis assay** OECD TG n. 231 (OECD, 2009) recommends the use of the species X*enopus laevis.* After a 21-day exposure period, the end points assessed are the developmental stage, snout-to-vent length, and hind limb.

On a different scale the reproductive status of invertebrates in both freshwater and coastal ecosystems may be assessed for the effects of potential endocrine disruptors to develop robust invertebrate chronic test methodologies.

Daphnia magna is a freshwater aquatic invertebrate and a well-established model organism for toxicological studies. Because it is a filter feeder, it is rapidly responsive to suspended or dissolved substances, allowing for simple and efficient toxicological testing of chemicals. The ***Daphnia magna* reproduction test** OECD TG n. 211 (OECD, 2012) is designed to examine the effects of chemicals on the reproductive output *of D. magna Straus*. The test duration is 21 days. End points include the total number of living offspring produced per parent alive, sex ratio, parent mortality, oxygen concentration, temperature, hardness and pH values, and the determination of the concentrations of the test substance.

REPRODUCTIVE TOXICITY RISK ASSESSMENT

Soil biota play an important role in soil functioning, also providing a practical tool for assessing soil quality status. The arthropod ***Folsomia candida,*** a member of the order Collembola (colloquially called springtails), is one of the most used species in ecotoxicological testing since it has a key position in the soil food web as a prey and consumer (Fountain *et al*., 2005). Cultures of this species are very easy to maintain on a diet of granulated dry yeast and they are excellent for laboratory experiments due to their short reproductive cycle (duration 1.5 days) alternating with longer nonreproductive instars (duration 8.5 days). Populations of *F. candida* consist exclusively of parthenogenetic females. The **collembolan reproduction test in soil** OECD TG n. 232 (OECD, 2016) tests chemicals on effects on the reproduction of collembolans in soil. The parthenogenetic *Folsomia candida* and *Folsomia fimetaria* are the recommended species for use. The duration of a reproduction test is 4 weeks for *F. candida* and 3 weeks for *F. fimetaria*. The number of surviving springtails and the offspring of the springtails of the test item groups is compared to the numbers of the control group.

III. GLYPHOSATE AND GBHS REPRODUCTIVE/DEVELOPMENTAL TOXICITY IN MAMMALIAN SPECIES

The safety profile of the herbicide Glyphosate and its commercial formulations is still controversial. Many studies performed by contract laboratories, commissioned by the registrant and submitted to regulatory agencies indicate minimal mammalian toxicity. However, several studies, some described below, now show that GBHs can adversely affect mammalian biology via multiple mechanisms. Glyphosate-based herbicides can interfere with numerous mammalian organs and biochemical pathways, including inhibition of numerous enzymes, metabolic disturbances and oxidative stress leading to excessive membrane lipid peroxidation, and cell and tissue damage (Myers *et al*., 2016).

1. Rats

In rats, different studies have investigated the effects of Glyphosate alone and/or GBHs administered prenatally and postnatally on sexual maturity. Many studies have demonstrated that both Glyphosate and GBHs do disrupt oestrogen, androgen, and other steroidogenic pathways. In particular, these studies indicate that the effects of GBHs in comparison to the active ingredient Glyphosate are either significant or more pronounced (Defarge *et al.*, 2018; Mesnage *et al*., 2014).

One study authored by Dallagrave et al. (2007) showed that Roundup (containing 360 g/l of Glyphosate (N-phosphonomethylglycine) and 18% (w/v) of the surfactant polyoxyethyleneamine- POEA) administered to Wistar rats from the perinatal period to lactation at 0, 50, 150 or 450 mg/kg bw did not induce maternal toxicity but caused reproductive problems in male offspring, including a decrease in the number of sperm in the cauda epididymis affecting the daily production of sperm in adult life. In this study, an increase in the sperm morphological pathology and lower levels of testosterone at puberty was observed (Dallagrave *et al*., 2007).

Similar reproductive effects have been observed in Wistar rats treated only during the pubertal phase from the Post Natal Day (PND) 23 until the PND 53 with Roundup Transorb (containing 480 g/L of Glyphosate, 648 g/L of isopropylamine salt and 594 g/L of inert ingredients) at 5, 50 and 250 mg/kg bw. A significant reduction in serum testosterone

concentrations and changes in the testicular morphology of male have been documented (Romano *et al*., 2010).

The effects of gestational maternal exposure from Gestational Day (GD) 18 to PND 5 to Roundup Transorb (NOAEL 50 mg/kg) on the sexual development of male Wistar rats were investigated (Romano *et al*., 2012).

Behavioural changes in mating, early onset of puberty in male offspring, as well as increases in testosterone and estradiol concentrations were detected. LH and FSH mRNA expression showed increased levels in treated animals which were accompanied by higher amounts of only LH protein in both pituitary and serum. In addition, perinatal Glyphosate exposure increased the total and daily sperm production in the testes. Significant alterations in the level of all the reproductive hormones and oxidative stress markers, reductions in sperm count, percentage motility and significant and increased in abnormal sperm were also observed in adult male albino rats orally exposed to Roundup at 3.6, 50.4 and 248.4mg/kg bw of Glyphosate equivalent for 12 weeks (Owagboriaye *et al*., 2017).

The most adverse effect of GBH on the reproductive tract has been found in male rats, but there are also few reports in females. Female offspring rats born to mothers exposed to different doses of GBH (50, 150 and 450 mg/kg bw Glyphosate equivalent) during pregnancy and lactation showed a delay in vaginal opening, a landmark of sexual maturity (Dallagrave *et al*., 2007).

Recent studies demonstrated alterations in endometrial decidualization in adult rats that received low dose of Magnum Super II (a water-soluble formulation containing 54% w/v of Glyphosate acid) at 2 mg/kg bw of Glyphosate on PND 1, 3, 5 and 7 (Ingaramo *et al*., 2016) and altered uterine development in rats neonatally exposed to the GBH Roundup FULL II (a water-soluble formulation containing 54% w/v of Glyphosate acid) (Schimpf *et al*., 2017).

The same dose and a higher one (200 mg/kg bw of Glyphosate equivalent) were used to assess the effects of both in utero and lactational exposure to the GBH Magnum Super II administered by feed to F0 mothers in Wistar rats (Milesi *et al*., 2018). The percentage of pre-implantation loss (i.e., number of oocytes not fertilized or embryo loss before implantation) was significantly increased in both GBH-exposed groups. Furthermore, a statistically

significant correlation was found between perinatally GBH high dose exposure and fetal anomalies in F2 offspring providing further evidence that GBHs might prompt long-term adverse effects on female reproductive capability (Milesi *et al*., 2018).

On the contrary, the German Federal Institute for Occupational Safety summarized in its report that a very large database submitted by different applicants and from published scientific literature to evaluate the reproductive and developmental toxicity of Glyphosate in rats did not provide evidence of reproductive toxicity or teratogenicity. The institute also claimed that the few observed effects were small, of equivocal relevance and confined to parentally toxic dose levels (EFSA, 2017).

2. Rabbits

Seven developmental toxicity studies have been submitted to regulatory agencies in support of the registration of Glyphosate. Kimmel et al. analyzed the information from these 7 unpublished developmental studies in rabbits (Kimmel *et al*., 2013). These studies enrolled three different rabbit strains (New Zealand white, Japanese white and Dutch belted) and covered a broad range of 15 Glyphosate doses, ranging from 10 to 500 mg/kg/day (Brooker *et al*., 1991a; Coles and Doleman, 1996; Hojo, 1995; Moxon, 1996; Suresh, 1990; Tasker *et al*., 1980a; Bhide and Patil, 1989). Apart from mortality in some studies, maternal toxicity was characterised by gastrointestinal signs, lower body weight (gains) and reduced food consumption and, occasionally, abortion. Generally, it occurred at doses of 150 mg/kg/day or higher. Post-implantation loss was quite variable across studies. Coles and Doleman (1996) reported an increase in post-implantation loss at 200 mg/kg/day; Brooker et al. (1991a) reported increased post-implantation loss at doses of 50 mg/kg/day and above. Examination of the data from the rabbit studies showed a variety of malformations of the heart and great vessels. These included: dilated aorta/narrow pulmonary artery; narrow aorta/dilated pulmonary artery; hypoplasia of the pulmonary artery; interventricular (IV) septal defect; cardiomegaly; single ventricle, thickened ventricle walls; dilated ventricle; retro-esophageal right subclavian artery; interrupted aorta; right subclavian artery arising from aortic arch; "seal-shaped" heart. Two of the studies (Brooker *et al*., 1991a; Suresh, 1990) suggested a possible association of cardiovascular anomalies (interventricular septal defects, dilated hearts) with treatment. In addition, two studies (Hojo, 1995; Moxon, 1996) reported an

increase in skeletal defects at the high dose of 300 mg/kg/day. These anomalies appeared to be the result of reduced ossification, which is likely related to delayed development (evidenced by reduced fetal body weights observed at the high dose).

3. Pigs

Glyphosate has been found in malformed piglets. The research study was conducted by a team of researchers from Germany and Egypt in collaboration with the Danish pig farmer Ib Pedersen, whose pigs were analysed for Glyphosate content. The rate of malformations increased to one out of 260 born piglets if sow feeds contained 0.87-1.13 ppm Glyphosate in the first 40 days of pregnancy. In the case of 0.25 ppm Glyphosate in sow feeds, one out of 1432 piglets was malformed. In this case, therefore, a higher dose of Glyphosate led to more malformations. The piglets showed different abnormalities, including ear atrophy, spinal and cranial deformations, hole in the skull, and leg atrophy. In one piglet, one eye was not developed; it had a single large one (cyclopia, a malformation observed in Argentine populations exposed to Roundup spraying). There were piglets without a trunk, with an "elephant tongue", and a female piglet with testes. One malformed piglet had a swollen belly and the foregut and hindgut were not connected (Krüger *et al.*, 2014).

IV. GLYPHOSATE AND GBHS REPRODUCTIVE/DEVELOPMENTAL TOXICITY IN NON-MAMMALIAN SPECIES

Evidence is accumulating with regard to the potentially negative effects of Glyphosate and GBHs on the development, phenotype, and fitness of most non-target animal taxa from invertebrates to vertebrates, yet, exposure levels (natural exposure load vs. levels used in experimental studies) need to be carefully accounted for (Gill *et al*., 2018). Non-target organisms are commonly exposed to GBH residues in the food chain because residues can persist in soil, water, and plants (Bai *et al*., 2016; Helander *et al*., 2012). Consequently, different regulatory authorities heatedly debate the effects of GBH in our ecosystems.

1. Birds

Birds are highly underrepresented in studies testing the adverse effects of GBH residues on non-target taxa (Gill *et al*., 2018), although they have recently been suggested as a key group for biomonitoring with regard to the effects of GBHs (Kissane *et al.*, 2017). Indeed, birds offer considerable potential in this role because they span agricultural and urban environments, coastal, inland, and wetland ecosystems where Glyphosate residues are known to be present (Kissane *et al.*, 2017). The effects of Glyphosate on bird reproduction evaluated for the EU assessment of Glyphosate only included avian reproduction studies with bobwhite quail and mallard duck following the validated OECD TG n. 206 (OECD, 1984). From the regulatory side, the risk to birds from the intended uses of Glyphosate was considered to be acceptable (German Federal Institute for Risk Assessment, 2015). However, one of the issues with the standard regulatory guideline for reproductive toxicity in birds is that it may not represent realistic exposure in the field. Many substances will not persist in relevant food items for such a long period (i.e. 20 weeks) and not all life-cycle stages are covered in the bird reproductive toxicity study (Brooks *et al*., 2017). Other non-standard in vivo studies on birds offered insight about possible reproductive/developmental effects of GBHs in avian species. A direct injection of a relatively high concentration of Roundup (10 mg/kg Glyphosate) was found to decrease hatchability, induce oxidative stress and cause damage to lipids in the exposed chicks, as compared to the control group (Fathi *et al*., 2019). GBHs and Glyphosate itself interfere with key molecular mechanisms, including endocrine mechanisms, which regulate early development in chickens leading to congenital malformations (Paganelli *et al*.,

2010). Exposure to Roundup caused disruption of the male genital system in mallard ducks: it altered the structure of the testis and epididymis, serum levels of testosterone and oestradiol, and the expression of androgen receptors in the testis (Olivera *et al*., 2007). In a recent study, a parental generation of Japanese quails (*Coturnix japonica*) was exposed to GBHs (200 mg/kg feed) or respective controls. Glyphosate residues were found in eggs (ca 0.76 kg/mg). Embryonic development tended to be poorer in the eggs of GBH-exposed parents compared to control parents. Embryonic brain tissue from GBH-exposed parents tended to express more lipid damage, yet other biomarkers showed no apparent differences. No differences in egg quality (egg, yolk, or shell mass, egg hormone concentration) across the treatment groups were detected (Ruuskanen *et al*., 2020).

2. Fishes

Fishes are exposed to a wide range of environmental stressors throughout their life cycle, including fluctuations in temperature, water chemistry, dissolved oxygen, nutrients, and predator/prey abundance. Fishes are inherently well evolved to respond to changes in their natural environment through compensatory physiological and behavioural alterations. Because Glyphosate has high water solubility, and both it and its metabolite AMPA are increasingly found in the aquatic environment, effects on aquatic organisms are of growing concern (Contardo-Jara *et al*., 2009).

The presence of chemical anthropogenic stressors, such as GBHs in the water, can alter physiological and behavioural endpoints critical to maintaining normal function, and cause adverse effects ranging from the cellular to the population level. Given the extensive usage of GBHs, there is a clear potential for the environmental exposure of fish populations to Glyphosate together with associated formulation products, which may modify its toxicity. The majority of GBHs are not approved for application in aquatic environments; however, with the current widespread use there are multiple routes through which exposure of aquatic organisms may occur. Surface runoff, direct overspray or drift during herbicide application can result in significant quantities of Glyphosate entering the aquatic environments. The application, by untrained individuals without proper precautions for safe herbicide applications, may also contribute to surface and groundwater contamination (ISPRA, 2016).

In a standard test guideline fish short-term reproduction assay (FSTRA) with fathead minnow (*Pimephales promelas*), a not statistically significant decrease in vitellogenin was seen only at mid-treatment; however this effect was observed in isolation in the absence of any treatment-related effects in the other estrogen-related endpoints such as gonado-somatic index, gonadal staging, fecundity and fertilization. In addition, there was no notable gonadal histopathology (Schneider *et al*., 2012).

Developmental teratogenic effects and adult-onset reproductive effects of exposure to environmentally relevant concentrations of Glyphosate and Roundup were also investigated in Japanese medaka fish (*Oryzias latipes*). Hd-rR strain medaka embryos were exposed to 0.5 mg/L Glyphosate, 0.5 and 5 mg/L Roundup (Glyphosate acid equivalent) for the first 15 days of their embryonic life and then allowed to sexually mature without further exposure. Roundup (0.5 mg/L) and Glyphosate decreased cumulative hatching success, while Glyphosate exposure increased developmental abnormalities in medaka fry. Fecundity and fertilization efficiency were not altered due to exposure. The authors concluded that Roundup and its active ingredient Glyphosate can induce developmental, reproductive, and epigenetic effects in fish (Smith *et al*., 2019).

Java medaka adults were cultured in the laboratory and the fertilized eggs of the F2 generation were exposed to different concentrations of Glyphosate-based herbicide (100, 200, 300, 400 and 500 ppm) until they hatched. The survival and hatching rates of the embryos, changes in the heart rate and morphological impairments were recorded (Yusof *et al.*, 2014).

Ovaries of **zebrafish** (*Danio rerio*) were exposed for 15 days to Glyphosate at 65 μg/L, the permissible concentration of Glyphosate in Brazilian inland waters. No apparent changes were noted in general morphology. However, there were significant adverse ultrastructure effects on oocytes and greater expression of steroidogenic factor-1, a major regulator of steroid hormone synthesis, in the oocytes. The authors expressed concern about the impact of these subtle adverse effects on fish reproduction (Armiliato *et al*., 2014). Sperm quality was assessed in zebrafish after 96 hours of exposure to Glyphosate at concentrations of 5 and 10 mg/L, with reduction of sperm motility and period of motility observed at both concentrations (Lopes *et al*., 2014). The effects of realistic concentrations of GBHs on spermatozoa motility and viability were tested in yellowtail tetra fish, *Astyanax lacustris.* Viability of sperm cells

was impaired at 300 $\mu g\, l^{-1}$, a concentration that is within legal limits in U.S.A. waterbodies, while motility was impaired at 50 $\mu g\, l^{-1}$, which is the more stringent limit set in Brazilian law (Gonçalves *et al*., 2018). In a study of egg production, zebrafish were exposed for 21 days to 0.01, 0.5, and 10 mg/L Roundup and a treatment of 10 mg/L Glyphosate, with some adverse effects on embryo survival and hatching observed at the highest doses of 10 mg/L. The gonadosomatic index indicating gonadal weight adjusted for body weight was significantly decreased and the number of eggs laid per female per day during the exposure period was significantly reduced with Glyphosate treatment and a non-significant trend in reduction of eggs laid by females in all of the Roundup-treated females (Uren Webster et al., 2014).

At an ultralow concentration of 0.01 mg l− 1 Glyphosate damaged the primary motoneurons in zebrafish resulting in abnormal effects on larval locomotor activities (Zhang et al., 2017). Exposure of zebrafish embryos to higher concentrations of Roundup® Classic (50 mg l^{-1}) resulted in developmental problems including forebrain, midbrain and eye damage (Roy *et al*., 2016).

3. Amphibians

Amphibians may be particularly susceptible to the effects of GBHs because their preferred breeding sites are often shallow ephemeral pools that, by virtue of the small amount of water, can contain high concentrations of herbicides (Mann *et al*., 2009). Studies show them to be particularly susceptible to formulations containing POEA. Sublethal effects include metabolic disturbance, oxidative stress, DNA damage, endocrine disruption, malformations, and behavioural changes that make them more vulnerable to predators. Roundup can kill testicular cells, reduce sperm numbers, increase abnormal sperm, retard skeletal development, and cause deformities in amphibian embryos.

Effects of chronic exposure to Roundup ® were investigated at non-acute levels in a static renewal test on *Rana cascadae* larval metamorphosis and development. Larvae were evaluated daily for 43 days for mortality, feeding behaviour, swimming activity, morphological abnormalities and behavioural alterations. Slightly larger body sizes of tadpoles were observed with some of the Glyphosate concentrations tested. However, according to technical guideline OECD 231, an increase in growth should never solely be

relied on to determine thyroidal effects. No significant effects were observed on developmental stage, morphometry (hind limb length normalized to snout vent length) and thyroid histology. Therefore, it was concluded that the study does not provide an indication of thyroidal activity (Cauble *et al.*, 2005).

In 2009, Argentinean researchers led by Argentinean scientist, Professor Carrasco of the University of Buenos Aires Medical School, demonstrated significant consistent and systematic malformations in amphibian embryos resulting from very low dose exposure to Glyphosate, and warned that comparable effects can happen in humans (Paganelli *et al*., 2010). In the first part of the study amphibian embryos were immersed in a solution of Roundup Classic, containing 48% w/v of a Glyphosate salt, diluted to 1/5000 (equivalent to 430 μM of Glyphosate). The embryos suffered head deformities. In the second part, the embryos were injected with Glyphosate alone at 8 and 12 μM per injected cell): the impact was even more severe, demonstrating that it is the active ingredient, not the adjuvants that are the problem. The Glyphosate caused marked alterations in cephalic and neural crest development and shortening of the anterior-posterior axis in tadpole embryos, resulting in deformities in the cranial cartilages at the tadpole stage. Other effects included shortening of the trunk, reduced head size, eye defects, genetic alterations in the central nervous system, increased death of cells that help form the skull, deformed cartilage, eye defects, and undeveloped kidneys. Carrasco also stated that the Glyphosate was not breaking down in the cells but was accumulating. The authors concluded their results were "*compatible with the malformations observed in the offspring of women chronically exposed to Glyphosate-based herbicides during pregnancy*" (Paganelli *et al*., 2010).

4. Collembolan species

In soils, Gyphosate is absorbed quickly onto soil particles and inactivated. However, Glyphosate can become unbound again in small amounts. The impact of pesticide application to reproductive capacity in non-target soil organisms, simulating what happens following pesticide application in agricultural fields, is mainly tested in the collembolan species *Folsomia candida*, a model organism demonstrated to be more sensitive to Glyphosate formulations than, for instance, the earthworm *Eisenia Andrei* and the isopod *Porcellio dilatatus*, after following single and multispecies avoidance tests (Santos *et al*., 2012; Santos

et al., 2010; Niemeyer *et al*., 2018). Effects on reproduction were examined by many authors using commercial formulations with the recommended application rates (Casabé *et al*., 2017; Yasmin *et al*., 2007; Kaneda *et al.,* 2009). Behavioural abnormalities were described in terms of reduced casting production (Kaneda *et al.,* 2009) reduced cocoon viability, a reduction in the feeding activity (Casabé *et al*., 2017) or reduced body weight (Yasmin *et al*., 2007). In a reproduction test with *Eisenia fetida*, which was conducted with the active substance Glyphosate itself (Correia *et al*., 2010), earthworms were kept in treated soil and were classified as alive after the evaluation period, but showed significant reduction in mean weight at all test concentrations. Moreover, morphological abnormalities like elevating the body, coiling, and curling were observed in all specimens exposed to the highest concentrations of Glyphosate (1000 mg/kg). On the opposite, other studies revealed that the tested products did not seem to affect earthworms reproduction (Zhou *et al*., 2012; Santos *et al*., 2012; Fusilero *et al*., 2013; Garcia-Torres *et al*., 2014) at least when the recommended field dose was tested. In a more recent study (Niemeyer *et al*., 2018), the effects in reproductive fitness of *F. candida* were tested for four commercial Glyphosate formulations, also using a natural soil collected in the field. The authors did not find significant changes on reproduction for any of the tested formulations until the concentration of 69.8 mg (a.i.) kg^{-1}. The discrepancy of results reported in different studies is most probably related to the influence of different soil types on activities of contaminants. This highlights the importance of measuring soil properties, which may affect pesticide activities. It cannot be excluded that with repeated applications of Glyphosate containing plant protection products during the season or year by year will have negative effects on the biotic soil community. It is considered that herbicide application did not directly affect the mortality or reproduction but instead the biological activity of the animals.

OBJECTIVES

V. OBJECTIVES

To clarify the many critical scientific points on GBHs safety creating uncertainty over the differing results of the current literature, in 2016 the Ramazzini Institute planned an integrated experimental approach to a long-term project by which to monitor many parameters bearing on human health (Manservisi *et al*., 2017). The aim was to test, in male and female Sprague-Dawley (SD) rats, substances deemed of extreme importance to public health, such as Glyphosate and GBHs via a toxicological approach on a broad front. The idea was to perform a single study, using animals from one and the same generation, and simultaneously evaluate key parameters regarding sub-chronic and chronic toxicity, carcinogenesis, developmental and reproductive toxicity, possible neurotoxic effects and alterations to the microbiome.

As previously reported, the use of rodent models for research and testing chemicals needs an awareness of several laboratory animal science issues so as to standardize methods of monitoring, thus facilitating the reproducibility of results among laboratories. In order to provide background data on some endocrine sensitive endpoints for interpreting experimental results in subsequent studies (particularly for developmental/reproductive bioassays) we also preliminary monitored the background data on some endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Cesare Maltoni Cancer Research of the Ramazzini Institute colony (SD-CMCRC/RI) (***Paper 1*** *– Manservisi et al.*, 2018).

On the basis of the published integrated experimental design and after setting the procedures for monitoring reproductive/developmental endpoints, in 2016 we started the following project named “Global Glyphosate study” (https://Glyphosatestudy.org/it/) characterized by two-stages:

1- STAGE 1: THE RAMAZZINI INSTITUTE 13-WEEK STUDY ON GLYPHOSATE-BASED HERBICIDES AT HUMAN EQUIVALENT DOSE IN SPRAGUE-DAWLEY RATS

The aims of this first stage were:

- to investigate some critical endpoints and organizational aspects necessary to plan and perform the integrated experimental study on Glyphosate and GBHs (***Paper 2*** *- Panzacchi et al., 2018*);

OBJECTIVES

- to examined whether the exposure to GBHs affect the development and endocrine system across different life stage of treated Sprague-Dawley rats (***Paper 3*** *- Manservisi et al., 2019*).

The study was performed without any regulatory purposes and did not follow any specific OECD guideline, but it followed the principles of them. In order to ensure the reliability of the experimental findings, a system of quality assurance was established. In this study were tested both Glyphosate and Roundup Bioflow (MON 52276) in a single dose, considered to be safe and corresponding to the Acceptable Daily Intake currently allowed in the United States (ADI USA) equal to 1.75 mg/kg bw/day.

2- STAGE 2: INTEGRATED EXPERIMENTAL STUDY ON SUB-CHRONIC TOXICITY, CARCINOGENICITY, REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

This second stage, based on the integrated experimental design by Manservisi *et al.* (2017) **,** used Sprague-Dawley rats exposed under various calendars to the weedkiller Glyphosate and two commercial formulations Glyphosate-based (Roundup and Ranger Pro) and was aimed to:

- go deeper into studying the most important parameters, emerged from the preliminary phase, associated with the toxicity of Glyphosate, Roundup Bioflow **(**without POEA), and Ranger Pro containing POEA (a surfactant additive)**,** which is one of the most sold GBHs in USA;
- test man-equivalent doses beginning from the lowest Glyphosate dosage corresponding to 0.5 mg/kg bw/day (ADI Europe) up to a maximum dose of 50 mg/kg bw/day (NOAEL Glyphosate, equal to 100 times the ADI Europe);
- assess simultaneously multiple toxicological parameters combining and integrating the guidelines for sub-chronic toxicity and carcinogenesis with the latest guidelines for developmental and reproductive toxicity, in a single study, with animals from the same F1 generation. Indeed, the long-term study is divided into two arms:

    A. a sub-chronic toxicity and carcinogenicity study (arm A);

B. a **reproductive and developmental toxicity study (arm B), whose design and preliminary data are presented in the present thesis**.

The protocol gives us the opportunity to compare the results among each arm by minimizing variables and to spare animals and exploring windows of susceptibility that are currently not addressed in the other guidelines design. Furthermore, a biomonitoring approach across animal lifespan allows examination of dynamic and persistent changes after exposure.

To set this study in motion, the Ramazzini Institute built up a network of authoritative partners including:

- ✔ University of Bologna (Department of Agricultural and Food Science and Dep. of Statistical Sciences)
- ✔ Institute for Cancer Research, Genova
- ✔ Istituto Superiore di Sanità (ISS), Roma
- ✔ Icahn School of Medicine at Mount Sinai, New York, USA
- ✔ George Washington University, Washington, DC.

To preserve independence from the pesticide manufacturing industry and from its competitor (i.e. organic food industry), the long-term integrated study is supported through a global crowd-funding campaign that is open to the world's citizens, non-governmental organizations (NGOs) and national/international institutions. Details of this campaign are available at: https://Glyphosatestudy.org/

MATERIAL AND METHODS

VI. MATERIALS AND METHODS

1. STAGE 1: THE RAMAZZINI INSTITUTE 13-WEEK STUDY ON GLYPHOSATE-BASED HERBICIDES AT HUMAN EQUIVALENT DOSE IN SPRAGUE-DAWLEY RATS

The Materials and Methods are reported in paper 1, 2 and 3.

Additional evaluation not included in papers 2 and 3 are reported below.

1.1. Pathology: necropsy, sperm evaluation and histopathology

At different time points, dams (after weaning) and their offspring, were anesthetized by mixture CO_2/O_2 (70% and 30% respectively) inhalation and sacrificed drawing blood by cava vein. All animals that died or were sacrificed during the 13-week pilot study were subjected to a complete necropsy. The gross necropsy included an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs in situ. The examination included: external and internal portions of some hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic abdominal and pelvic cavities with their associated organs and tissues; muscular/skeletal carcass. All pathological lesions were described, recorded and signed.

Five days after weaning (corresponding to 49 ± 2 days of treatment), dams were sacrificed and the following organs were collected during necropsy:

- mammary glands (4 sites: axillary and inguinal, right and left), brain with cerebellum and medulla/pons*, pituitary gland, cranium, tongue, thyroid and parathyroid gland, kidneys*, adrenal glands*, liver* (2 lobes for the histopathology: caudate and main), uterus (including cervix)*, ovaries*, vagina.

For testosterone concentration determination, blood was collected, and serum removed by centrifugation and stored at − 80 °C until analysis.

At PND 73 ± 2 and PND 125 ± 2, all the male and female animals belonging to the two cohorts, were sacrificed and the following organs and tissues were collected:

- 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, cranium, tongue, esophagus, thyroid and parathyroid, thymus and mediastinal lymph nodes*, trachea, lungs, heart*,liver* (2 lobes for the histopathology: caudate and main), spleen*, pancreas, kidneys*, adrenal glands*, stomach (forestomach and glandular stomach), small intestine, large intestine (with the Peyer's patches), bladder, prostate*, seminal vesicles whit coagulating gland*, testis and epididymis*, uterus (including cervix)*, ovaries*, vagina, subcutaneous lymph nodes, mesenteric lymph nodes, sternum (bone marrow), spinal cord (3 levels: cervical, thoracic and lumbar), skeletal muscle of the leg with sciatic nerve, all gross lesions and other tissues only if anomalies are present.

During necropsy, all gross lesions and other tissues only if anomalies were present, were collected. In the animals sacrificed, the whole starred organs (*) were weighed, as soon as collected. In case of paired organs, both organs were preserved.

At necropsy, portions of about 30-100 mg of liver, kidney, adrenal gland, mammary gland, uterus, testes and prostate were snap frozen in liquid nitrogen and stored at -70°C for molecular profiling purposes. A pair of mammary glands was also collected from the same position in each animal; one was snap frozen in liquid nitrogen and stored at -70°C for molecular profiling purposes and the other one was fixed in alcohol 70° for different histopathology evaluation. From the blood, 750ul of serum was collected from all the experimental animals for metabolome and hormonal analysis.

All organs and tissues were preserved for the histopathological evaluations in alcohol 70%, apart from bone tissues which were preserved in formalin 10%. The organs and tissues previously described were microscopically examined for pathology as reported in Manservisi *et al*. 2019 (*see* ***Paper 3***).

1.2. Haematological, biochemical blood analysis and urinalysis

At the end of each time point (6- and 13-week cohort), all animals were located individually in a metabolic cage for around 16 hours. During this time, the animals had free access to the test solutions and food. The day after, in the morning, samples of spontaneous individual

urine were collected for standard urinalysis (appearance, volume, specific gravity, pH, total protein, glucose, ketone bodies, urobilinogen, bilirubin and occult blood).

After urine collection, in the morning, plasma was obtained individually from all the experimental animals. Blood was withdrawn from the cava vein before sacrifice of the animal that was anesthetized by inhalation using a mixture of CO_2/O_2 (70% and 30% respectively). The blood was collected in a tube containing EDTA, paying attention to volume levels, minimum and maximum, specified by the manufacturer. The samples were gently inverted for 30 second to mix contents. To obtain plasma samples, the tubes were centrifuged at 1500 rpm for 10 minutes at room temperature.

The parameters evaluated were:

- Biochemical: sodium, potassium, chlorine, glucose, inorganic phosphates, calcium, globulins, total cholesterol, triglycerides, blood urea nitrogen, creatinine, total protein, albumin, alaninaminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, total bilirubin.
- Hematological: hematocrit, hemoglobin, erythrocyte, reticulocyte, total and differential leukocyte count, platelets, platelets distribution width: the degree of variation in size of the platelet population , mean platelet volume, plateletcrit value , mean erythrocyte volume in total sample, mean hemoglobin volume per red blood cell count, mean hemoglobin concentration of erythrocytes, calculated distribution width of erythrocytes, coefficient of variation, calculated distribution width of erythrocytes, standard deviation.

1.3. Statistical analysis

Where data on a specific endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of $\alpha = 0.05$. A general screening for outliers was made, based on a Box and Whisker Plot procedure and considering as outliers the values that were outside the box boundaries by more than 3 times the size of the box itself. For continuous data including body weight and organ weights, which are most often normally distributed, one-way ANOVA, followed by a Dunnett's test, was used to compare treatment versus control groups. For clinical chemistry and

haematological data, which are usually non-normally distributed and have high inter-individual variability, Nonparametric Kruskal-Wallis' tests followed by Dunn's test, were used to compare treatment versus control groups. Urine analysis was treated as a score and was analyzed by non-parametric procedures. Incidence of non-neoplastic lesions, was evaluated with a Fisher's exact test (one and two-tailed; one-sided results were also considered, since it is well established that only an increase in the incidences can be expected from the exposure, and incidences in the control group are almost always 0). Statistical analysis was performed using Stata 10.

2. STAGE 2: INTEGRATED EXPERIMENTAL STUDY ON SUB-CHRONIC TOXICITY, CARCINOGENICITY, REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

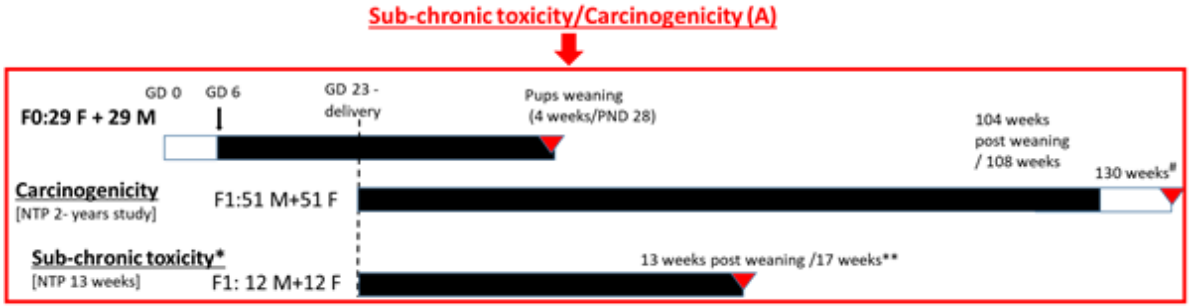
2.1. Study design

Glyphosate and two GBHs (Roundup Bioflow and Ranger Pro) were administered *ad libitum* diluted in SD rats' drinking water over various phases of development at Glyphosate doses of 0.5 mg/kg bw day (ADI Europe) - 5 mg/kg bw day - 50 mg/kg bw day (NOAEL Europe). The project is divided into two research arms (A and B) outlined in Figure 2 and 3, exploring different endpoints:

✔ **Sub-chronic toxicity and Carcinogenicity study (ARM A)**

The ARM A of the integrated experimental study started in November 2019 and is still ongoing. Treatment begins on the mother's (F0) at GD 6 and goes on to apply to the offspring (F1). From the same parent generation F0, F1 animals are distributed across the carcinogenicity study and the sub-chronic toxicity study. In the carcinogenicity cohort, the animals are treated for at least 104 weeks after weaning and then monitored until survival of the control group animals reaches the limit of 25% per sex (upper threshold for sacrificing animals) and in any case not beyond 130 weeks of age. In the sub-chronic toxicity cohort, animals are scheduled for sacrifice at 13 weeks after weaning (prenatal sub-chronic toxicity). From each litter no more than 2 brothers and 2 sisters are allocated for the carcinogenicity study; the remaining brothers and sisters are destined for the sub-chronic

toxicity study and distributed in such a way that there are no more than one brother and one sister per group (*for details see Figure 2*).



Termination of the study is considered when the number of survivors in the control group falls below 25% or at the latest 130 weeks of age, considering the survival of each sex separately, as reported in the OECD Guidance 116

* Micronuclei assays will be performed in all experimental groups according to the OECD TG 474

Dark bars represent the duration of the treatment

Scheduled sacrifice

Figure 2 *- Schematic view of the timeline of the integrated experimental study design Arm A (sub-chronic toxicity/carcinogenicity)*

✔ Reproductive and developmental toxicity study (ARM B)

One single parent generation F0 was to generate animals (F1) destined for assessment of any effects on development from exposure during specific windows of susceptibility (WOS), namely: prenatal, pubertal, adult (animals mated once adult). Mating of females from the adult WOS generated F2. The animals in ARM B followed a different treatment schedule according to the WOS being studied, partly replicating a protocol assessing the effects of endocrine disrupting chemicals already performed in our laboratory in collaboration with the Icahn School of Medicine at Mount Sinai in New York (grant no. 5U01ES019459) (Manservisi *et al.*, 2015). Treatment of animals according to different exposure calendars was designed to show the effects of exposure during gestation and lactation (prenatal WOS: GD 6-PND 21); the period of sexual development (pubertal WOS: PND 28-PND 63); and

adulthood (adult WOS: GD 6-PND 180). From each litter generated from breeders no more than 1 brothers and 1 sister were allocated for each WOS. On reaching adulthood (~PND 120, following the NTP MOG guideline) the animals were mated so as to generate F2 offspring (*for details see Figure 3*).

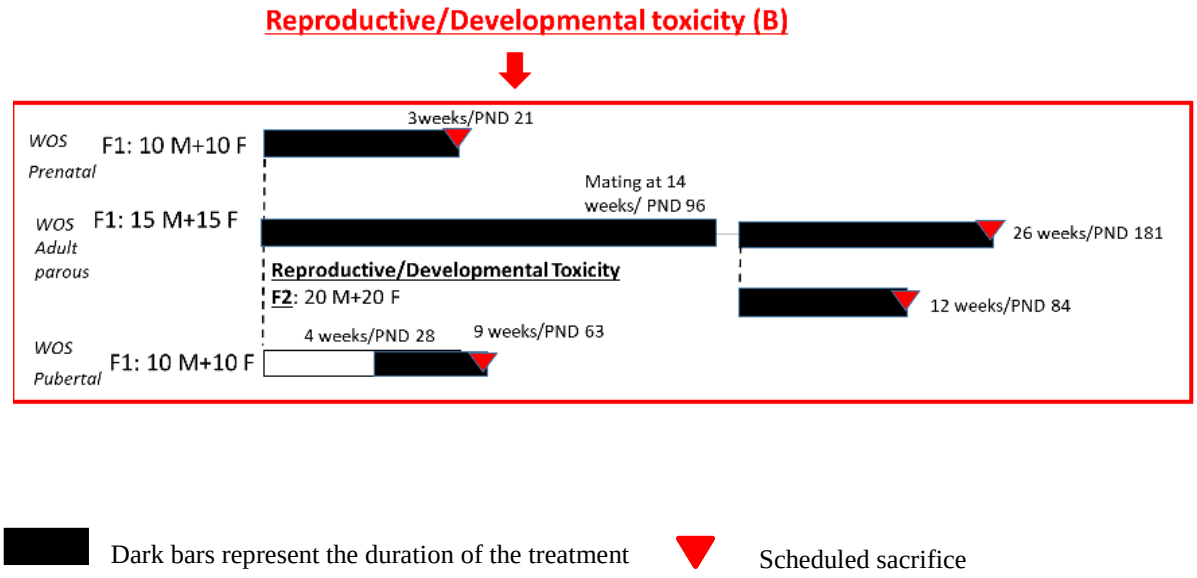


Figure 3 *Schematic view of the timeline of the integrated experimental study design Arm B (Reproductive/Devtable 5elopmental toxicity)*

The Figure 4 represents a schematic view of the integrated experimental design and main objectives addressed.

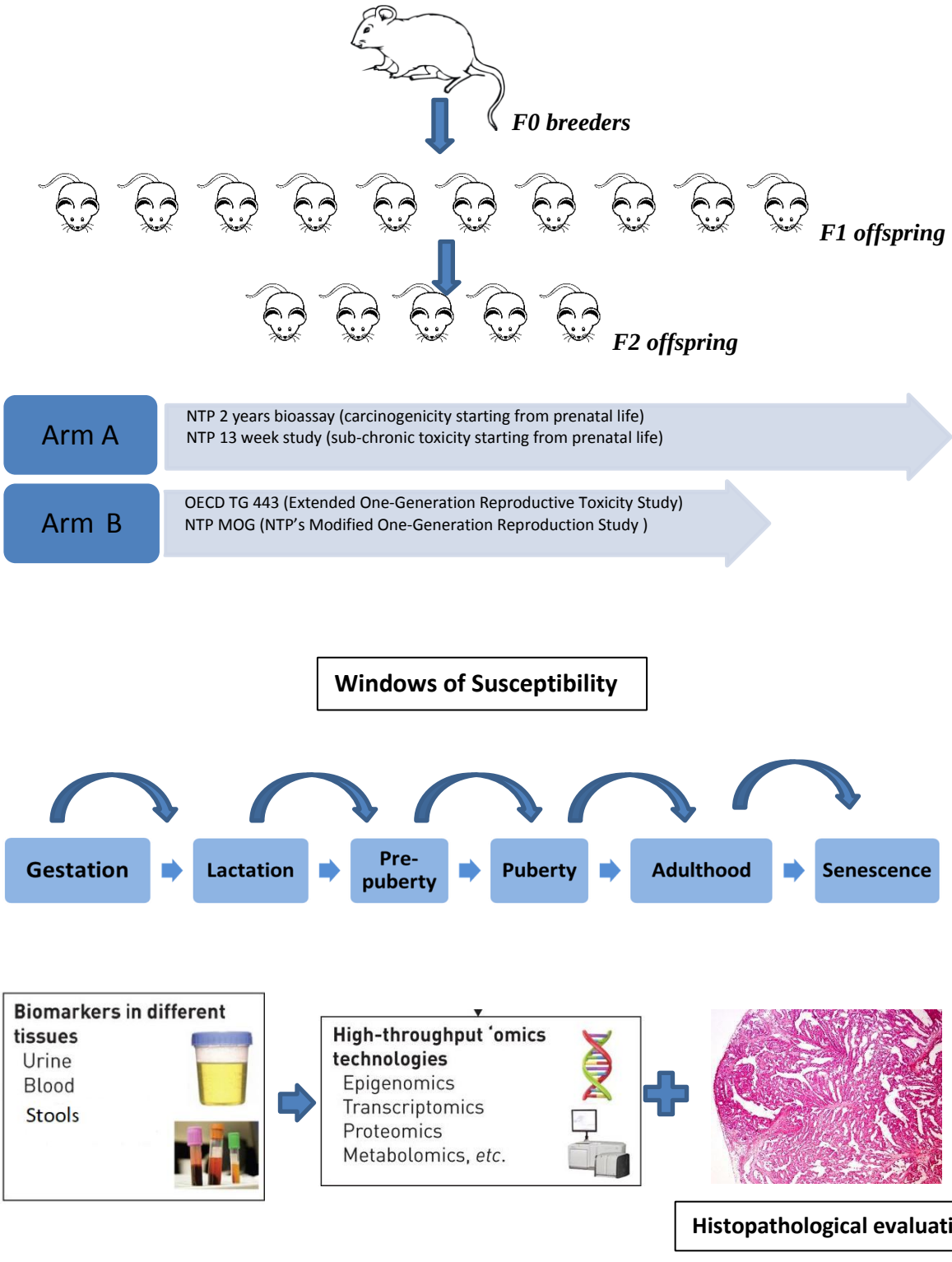


Figure 4 *- Schematic view of the integrated experimental design and main objectives addressed*

2.2. Reproductive and developmental toxicity study (ARM B)

2.2.1. Experimental animals and housing

Rats were housed not more than 3 in polycarbonate cages (41x25x18 cm) with stainless wire tops and a shallow layer of white wood shavings as bedding and staying in the same room, maintained at the temperature of 22±3°C and relative humidity of 50±20%. Lighting was provided by natural and artificial light and a 12-hour light/dark cycle was maintained. No deviations from the above-mentioned values were detected. Cages were identified by a card inserted in a cardholder, specifying animal species, pedigree number and experimental number.

Female SD rats, from the CMCRC breeding facility, were used. The animals were generated in-house, following an outbreed plan. All the experimental animals were identified by ear punch according to the Jackson Laboratory system. After weaning, before the start of the experiment, totally 312 female animals (F0) were randomized, distributed in 10 groups in order to have 29 dams per treated group and 51 for control. The female rats were randomized in order to have only one sister of each litter per group; homogeneous body weight within the various groups and for both sexes is ensured. Animals were single housed in polycarbonate cages in the room destined for the experiment at least two weeks before the start of the treatment in order to acclimatize. After 1 week of acclimation, females of 18 weeks of age were matched outbred with 312 males of same age and generation. On the day of positive evidence of mating the male was removed and this was considered the Gestation Day 0 (GD 0) for the female. The day on which parturition occurs was Lactating Day 0 (LD 0) for the dams, and Postnatal Day 0 (PND 0) for the offspring. After weaning (~PND 24-28), the offspring, identified by ear punch according to the Jackson Laboratory system, were located in the same treatment group of their dams, in order to have no more than two males and two females from the same litter, in the carcinogenicity sub-group, and one male and one female per group in the other sub-groups and Arm. The total number of F1 animals were 1260 (Arm A) and 700 (Arm B).

2.2.2. Diet

Animals were fed *ad libitum* on standard feed in "Corticella" –type pellets supplied by the firm Laboratorio Dottori Piccioni s.r.l (n. Ric. Reg. Lombardia αIT200009MI). Test

compounds were administered via drinking water. Tap water from the local water-main supplier, alone or with a test compound, was administered to animals in glass bottles ad libitum. After 24/48 hours, the drinking water was discarded, and the bottles cleaned and refilled. Both feed and water were periodically analyzed to identify possible contaminants or impurities; the analyses are included in the documentation of the experiment. Drinking water was controlled for eventual contaminations of pesticides according to Dir. 2008/105/EC, D.Lgs. 152/2006, Dir. 2006/118/EC.

2.2.3. Test substances

The following test substances were administered to SD rats:

- Glyphosate Sigma Aldrich with purity ≥ 95%;
- Roundup Bioflow: commercial formulation containing containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret)
- Ranger Pro: commercial formulation containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of glyphosate (41.5%), and surfactant (59%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret).

Certificates of analysis including the chemical-physical characteristics and purity were provided by suppliers. These certificates are included in the study documentation. The test substances were diluted in tap water at the programmed concentrations. Stability of the test compounds in water was assessed through HPLC-MS by Neotron laboratory, Modena, Italy.

2.2.4. Treatment

The treatment included ten experimental groups:

I. Untreated control group (tap drinking water);

II. Glyphosate diluted in drinking water at a concentration of 0.5 mg/kg bw day (ADI Europe);

III. Glyphosate diluted in drinking water at a concentration of 5 mg/kg bw day;

IV. Glyphosate diluted in drinking water at a concentration of 50 mg/kg bw day (NOAEL Europe);

V. Roundup Bioflow diluted in drinking water at a concentration of 0.5 mg/kg bw day Glyphosate equivalent;

VI. Roundup Bioflow diluted in drinking water at a concentration of 5 mg/kg bw day Glyphosate equivalent;

VII. Roundup Bioflow diluted in drinking water at a concentration of 50 mg/kg bw day Glyphosate equivalent;

VIII. Ranger Pro diluted in drinking water at a concentration of 0.5 mg/kg bw day Glyphosate equivalent;

IX. Ranger Pro diluted in drinking water at a concentration of 5 mg/kg bw day Glyphosate equivalent;

X. Ranger Pro diluted in drinking water at a concentration of 50 mg/kg bw day Glyphosate equivalent;

The beginning and duration of exposure to test substances varies between the WOS as outlined in Figure 3. The plan of the developmental/reproductive toxicity study is presented in Table 4.

Table 4 *– Experimental plan reproductive and developmental toxicity study (Arm B)*

Experimental plan Reproductive/Developmental toxicity study (Arm B)

Group	Sex	Animal			Treatment[a]					
		Breeders (F0)	Offspring (F1 and F2)[b]				Age at start		Age at final sacrifice (week)	
			Reproductive/ Developmental tox (F1)	Reproductive/Developmental toxicity and Immuno-neuro toxicity(F2)	Test Compound	Dose (mg/kg bw/day of Glyphosate)[c]	Reproductive/ Developmental tox (F1)	Immuno-Neuro tox (F2)°	Reproductive/ Developmental tox (F1)	Reproductive/ Developmental toxicity and Immuno-neuro toxicity. (F2)
		N	N	N						
I	M	-	35	20	Tap drinking	0	GD 6, PND28	GD0	21,63,181	84
	F	51	35	20						
		-	70	40						
II	M	-	35	20	Glyphosate	0.5 (ADI EU)	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
III	M	-	35	20	Glyphosate	5	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
IV	M	-	35	20	Glyphosate	50	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
V	M	-	35	20	Roundup Bioflow	0.5 (ADI EU)	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
VI	M	-	35	20	Roundup Bioflow	5	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
VII	M	-	35	20	Roundup Bioflow	50	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
VIII	M	-	35	20	Ranger Pro	0.5 (ADI EU)	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
IX	M	-	35	20	Ranger Pro	5	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
X	M	-	35	20	Ranger Pro	50	GD 6, PND28	GD0	21,63,181	84
	F	29	35	20						
	M+F	-	70	40						
TOTAL	M	-	350	200						
	F	312	350	200						
	M+F	-	700	400						

[a] Test substances administered ad libitum in drinking water

[b] The offsprings are distributed in order to have and no more than one brother and one sister per group

[c] The dose in mg/kg bw/day are calculated considering a mean body wieght of 400 gr and a daily mean solution consumption of 40 ml both for male and female rats.

2.2.5. Conduct of the study

Every animal in the experiment was checked 3 times per day on weekdays, twice on Saturday and Sunday/public holidays. The presence of spermatozoa in vaginal smears was registered as GD0 and used for the calculation of the index of gestation in dams. Pregnant females with sperm-positive smears were housed separately and pregnancy was confirmed by the occurrence of parturition. Dams were examined daily for evidence of normal maternal behaviour. The following parameters relating to reproductive outcome were assessed:

- **Fertility index (%)**: number of pregnant females /number of females mated × 100

- **Gestation index (%)**: number of females with live pups/number of pregnant females × 100.
- **Gestation length**: number of days between GD 0 (day of positive evidence of mating) and day of parturition.

All the dams generating the offspring of Prenatal and Adult WOS were weighed one day before the start of treatment (GD 5) and thereafter every 3 days during gestation (GD 6, 9, 12, 15, 18, 21) and for the first week of lactation (lactation day LD 1, 4, 7); as of the second week of lactation the measurement routine is weekly (LD 14, 21 and 28) until the point of weaning. In the Prenatal and Adult WOS, pup weight was recorded per sex and per litter at PND 1, 4, 7, 14, 21 and 28.

For the assessment of correct sexual development, body weight, general clinical features together with measurement of the AGD was assessed on all members of each litter on PND 4. After weaning, the offspring's body weight was recorded once a week until 13 weeks of age, and once every 2 weeks thereafter until final sacrifice. In the Pubertal WOS, body weight was recorded once a week. In all cases, body weight measurement was accompanied by individual clinical observation for fur, skin and subcutis (appearance of any nodules), mucous secretions, locomotion, breathing, large organ function and behaviour.

The mean 24-hour water and food intake by mothers was measured one day before the start of treatment (GD 5) and thereafter every 3 days during gestation (GD 6, 9, 12, 15, 18, 21) and for the first week of lactation (lactation day LD 1, 4, 7); as of the second week of lactation the measurement routine was weekly (LD 14, 21 and 28) until the point of weaning. After weaning, the 24-hour water and food intake per cage was measured once a week until 13 weeks of age, and thereafter every two weeks until final sacrifice.

The following parameters relating to sexual development were assessed:

- **Ano-genital distance (AGD)**: measured at PND 4 in the pups belonging to the prenatal and adult WOS as well as the F2 generation. Measurements were made using a digital Vernier caliper calibrated with a micrometer stage from the caudal margin of the anus to the caudal margin of the genital tubercle. Pup body weight was collected on the day the AGD was measured.

- **Vaginal opening (VO)**: monitored in the animals belonging to the pubertal and adult WOS, as well as the F2 females. Time to VO was determined by daily inspection of all female pups starting after weaning (~ PND 28). The criterion was met for female rats when a complete rupture of the membranous sheath covering the vaginal orifice was observed. The body weight of each female was recorded on the day that this was observed.

- **Evaluation of first estrus (FE)**: this was done by daily vaginal swab for 14 days beginning from the day after the vaginal opening, in females belonging to the adult WOS, as well as the F2 females.

- **Estrous cycle pattern**: this was monitored by daily vaginal swab beginning from the 13th week of age for 3 weeks, on animals belonging to the adult WOS.

- **Balano-preputial separation (BPS):** monitored in male rats belonging to the pubertal and adult WOS, as well as in the F2 generation. Time to BPS was determined by daily inspection of males beginning on PND 35. The criterion for the day complete preputial separation was present when the prepuce was observed to completely retract from the head of the penis. The body weight of each male was recorded on the day that this was observed.

2.2.6. Statistical analysis

Where data on a particular endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of $\alpha = 0.05$.

Fertility and gestion indices were analyzed using the Chi-square test. Continuous data including body weight, weight gain and organ weights, which are most often normally distributed, were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed significant ($p < 0.05$) intergroup variance, Dunnett's test was used to compare the test substance-treated groups to the control group. *Post hoc* one-way nonparametric ANOVA (Kruskal-Wallis' tests) was used in cases where data were not normally distributed (AGD, BPS and VO and vaginal cytology endpoints) and a Wilcoxon's test was used to compare, in a pairwise fashion, each exposed group to the control group.

For biological parameters related to the body weight (such as the AGD, BPS and VO), the statistical analyses were always performed including the body weight of each pup in the regression model. The statistical analysis was performed using Stata/IC 10.1 (for all regressions) and Statisti× 10 (for all the other tests); graphs were obtained using Microsoft Excel and Statisti× 10.

RESULTS

VII.RESULTS

RESULTS OF STAGE 1: THE RAMAZZINI INSTITUTE 13-WEEK STUDY ON GLYPHOSATE-BASED HERBICIDES AT HUMAN EQUIVALENT DOSE IN SPRAGUE-DAWLEY RATS

The results of STAGE 1 are published in:

Paper 1: Manservisi et al., 2018
CONTROL DATA ON ENDOCRINE SENSITIVE ENDPOINTS FOR UNTREATED SPRAGUE-DAWLEY RATS FROM THE RAMAZZINI INSTITUTE COLONY.

Paper 2: Panzacchi et al., 2018
THE RAMAZZINI INSTITUTE 13-WEEK STUDY ON GLYPHOSATE-BASED HERBICIDES AT HUMAN EQUIVALENT DOSE IN SPRAGUE-DAWLEY RATS STUDY DESIGN AND FIRST IN-LIFE ENDPOINTS EVALUATION
Henvironmental Health 17:52

Paper 3: Manservisi et al., 2019
THE RAMAZZINI INSTITUTE 13-WEEK PILOT STUDY GLYPHOSATE-BASED HERBICIDES ADMINISTERED AT HUMAN EQUIVALENT DOSE TO SPRAGUE-DAWLEY RATS: EFFECTS ON DEVELOPMENT AND ENDOCRINE SYSTEM

Additional results of stage 1 not published in paper 3 (see page 96).

Research

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 00-00, 2018

Control data on endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Ramazzini Institute colony

Fabiana Manservisi[1,2], Laura Falcioni[1], Luciano Bua[1], Ilaria Menghetti[1], Daniele Mandrioli[1,3], Giovanna Galeati[2], Marcella Spinaci[2], Carlo Tamanini[2], Fiorella Belpoggi[1]

[1] Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bentivoglio, Bologna, Italy; [2] Department of Veterinary Medical Sciences, University of Bologna, Italy, [3] Department of Agricultural Sciences, University of Bologna, Italy

Summary. *Background and aim:* Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes. Rats have been used extensively for developmental and reproductive physiology and endocrinology research and a number of endocrine sensitive endpoints have been well established in a variety of regulatory guidelines on rodent bioassays. We monitored the background data on some endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Cesare Maltoni Cancer Research of the Ramazzini Institute colony (SD-CMCRC/RI). *Materials and methods:* General reproductive indices from dams and data for the entire litter were recorded. All the littermates were retained until the achievement of puberty and balanopreputial separation (BPS) was monitored in all the males; estrous cycle length and pattern were also evaluated in one female/litter. We compared our data with those provided by the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI). *Results:* Overall, reproductive indices and pre-post weaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI. *Conclusions:* Procedures for monitoring and physiological biological variations in our SD-CMCRC/RI rats fall within the range of values typically obtained for the selected endpoints. Further investigations are suggested in order to verify whether retaining all pups to sexual maturation can improve the sensitivity to discriminate between natural variation and treatment effects. A more comprehensive analysis of other relevant endocrine sensitive endpoints should be performed in order to provide a representation of the normal developmental landmarks and endocrine values at different ages.

Key words: endocrine endpoints, historical control data, Sprague-Dawley rats

Introduction

Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes, including abnormal puberty, irregular estrous cycle, reduced semen quality, testicular dysgenesis syndrome and other adverse effects involving disruption of the Hypothalamus-Pituitary-Gonadal (HPG) and/or Hypothalamus-Pituitary-Thyroid (HPT) axis (1).

The laboratory rat is widely used as the traditional animal model of choice for research on developmental and reproductive toxicity testing, conducted to support human health hazard identification and risk assessment. Considering the substantial conservation of reproductive process across rat and human, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the laboratory rat as the species of choice for the endocrine screening and testing assays (2, 3).

RESULTS

PAPER 1

A number of endocrine-related endpoints has been well established in a variety of regulatory guidelines on rodent bioassays, including areola/nipple retention and anogenital distance in pups at birth; balano-preputial separation (BPS) in males and day of vaginal opening (VO) in females as primary landmarks of sexual development. Information on the integrity and performance of both male and female reproductive systems, including gonadal function, estrous cycle, mating behavior, conception, gestation, lactation, and the growth and development of the offspring are also addressed by current test methods focusing on developmental and reproductive toxicity. All these endpoints, sensitive to endocrine disruption, are well recorded in many Organization for Economic Co-operation and Development Test Guidelines (OECD TGs) such as the Two-Generation Reproduction Study (OECD TG 416) (4), the Extended One-Generation Reproduction Study (OECD TG 443) (5) and the Developmental Neurotoxicity (DNT) study (OECD TG 426) (6). The National Toxicology Program (NTP) has also developed a range of techniques and testing regimes to evaluate the potential of environmental and occupational substances to affect development and damage reproductive systems. The NTP's Modified One-Generation Reproductive study design (MOG) provides information on the effects of substances on prenatal development, postnatal development, and reproduction (7). Recently, many OECD TGs have been revised placing additional emphasis on endocrine endpoints; the need for careful clinical observations of the animals, so that to obtain as much information as possible, is also stressed (8, 9).

The use of rodent models for research and testing on EDCs needs an awareness of a number of laboratory animal science issues in order to standardize methods of monitoring thus facilitating the reproducibility of results among laboratories (10).

We monitored untreated Sprague-Dawley (SD) rats belonging to the colony of the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (CMCRC/RI) in order to provide background data on some endocrine sensitive endpoints for interpreting experimental results in developmental/reproductive studies.

General reproductive indices of the dams were recorded for subsequent interpretation of reproductive health effects of a tested substance. Indeed, changes in reproductive indices can be due to several factors, including alteration in hormone levels and fetal growth retardation (11).

Data for the entire litter, including litter size and sex ratio, were reported as an important endpoint in the overall evaluation of reproductive performance. A decreased litter size may indicate an adverse reproductive effect and can be used as a nonspecific indicator of reproductive toxicity. Altered sex ratios may be related to several factors, including selective loss of male or female offspring, sex-linked lethality (genetic germ cell abnormalities), abnormal production of X or Y chromosome-bearing sperm, or hormonal alterations that result in intersex conditions (masculinized females or feminized males) (11).

All the littermates were retained until the achievement of puberty without performing culling (reduction of litter size) a widely used procedure in reproductive toxicity studies (12). The balano-preputial separation (BPS) was monitored in males. Cleavage of the balano-preputial gland is an apical measure of the progression of puberty and it has been used as the primary endpoint of puberty onset in the male rat as it is an androgen dependent event (13, 14).

In one female per litter the estrous cycle pattern was determined by observing changes in the vaginal smear cytology. Vaginal cytology is known to be dependent upon the hormonal balance and to respond rapidly to the administration of chemical possessing hormonal activity as, for example, oestrogenic agonist or antagonist activity. The inclusion of an assessment of estrous cyclicity by examination of vaginal smears or washes offers a quick and easy way to measure the sex hormone status within the female and is of value in interpreting other findings (for example, weight or pathological data for the female reproductive organs). Potentially this technique could also act as a simple initial marker of changing reproductive capacity with age in chronic studies (11).

A comparison with the laboratory's historical control data is an important aid to determine whether small increases or decreases (including not statistically significant ones) in an endpoint might constitute a treatment-related effect. As part of this evaluation, we compared our data with those provided by the Health

RESULTS

PAPER 1

and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) that provided a retrospective analysis of 43 multi-generation studies (16 in Wistar rats, 27 in Sprague-Dawley rats) conducted according to the United States Environmental Protection Agency (U.S. EPA) Reproduction and Fertility Effects Test Guideline (OPPTS 870.3800/OECD 416) (15).

Materials and methods

Male and female SD rats belonging to the colony used in the laboratory of the CMCRC/RI for over 40 years were used in the experiment. All the animals were kept in a single room at 23±3°C and at 40-60% relative humidity. The light/dark cycle was 12 hours. Rat feed (Dr. Piccioni Laboratory, Milan, Italy) and tap water were available *ad libitum*. Each lot of feed and tap water was periodically analyzed for biological (bacteria) and chemical (mycotoxins, pesticides, arsenic, lead, mercury, selenium) contaminants.

Eleven virgin female rats were cohabited with 11 breeder male rats of the same strain, one male per female, never brother and sister. Every day, the females were examined for presence of sperm by vaginal cytology. The day in which sperm was found in vaginal canal was defined as Day 0 of pregnancy (GD 0). The fertility index was defined as the number of animals inducing pregnancy or becoming pregnant divided by the number of mating sets. The gestation index was reported as the percentage of pairs with confirmed mating that have produced at least one pregnancy within a fixed period. Mean gestational length (duration of pregnancy) was the time from GD 0 to parturition. The day birth occurred was designated as post natal day 1 (PND). Each dam and delivered litter were housed in a common nesting box during the postpartum period. Newborns were housed with their mothers until weaning at PND 28. Sex was determined on PND 1 and sex ratio data was presented as percentage of males to total number of offspring. The mean litter size, including dead as well as live offspring, was calculated on PND 1. We totally evaluated 136 pups, 67 males and 69 females.

All the littermates were observed until the achievement of sexual maturity.

Starting on PND 35 until completion, all the males were examined daily (between 9:00 A.M. and 12:00 P.M.) for BPS. Each male rodent was removed from its cage and held in a supine position. Gentle digital pressure was applied to the sides of the prepuce, and the criterion was met when the prepuce completely retracts from the head of the penis. Each male rodent was examined daily until acquisition.

Starting from young adulthood (approximately PND 120) and for the duration of 3 weeks, daily vaginal lavage was performed on one female/litter. The female rat was removed from the cage and approximately 0.25 ml of physiological saline solution were drawn into a new clean dropping pipette. The tip of the pipette was gently inserted into the vaginal canal, the pipette bulb was firmly but gently depressed to expel the saline into the vagina and the saline was drawn back into the dropping pipette which was removed from the vaginal canal. A spray fixative (Cytofix™ Fixation Buffer, BD Biosciences, supplied by Di Giovanni srl, Bologna, Italy) was applied onto the slide prior to Papanicolaou stain. By using Papanicolaou staining, the maturity of nucleated epithelial cells can be distinguished with less mature cells stained turquoise and more mature cells pink- or orange-stained. Briefly, slides were successively submerged in alcohol 95%, 80%, 70% and water, then stained with Harris' Hematoxylin solution (Labochimicha srl, Padua, Italy). After a brief dipping in diluted hydrochloric acid and water to remove excess stain, the cells were dehydrated prior to immersion in the Orange G (Labochimicha srl,), an alcohol based cytoplasmic counterstain which stains keratin in brilliant orange. Slides were raised off in 95% alcohol and stained with the second counterstain, Eosin-Azure (E.A.) 50 (Labochimicha srl,), and rinsed off in 95% again. Finally, slides were immersed in absolute alcohol to dehydrate completely and in xylene. Slides were mounted with the Permount, then coverslipped and observed under a light microscope. The cytology of the vaginal smears allowed a classification in the following estrous stages: diestrus (D), predominance of leukocytes and a few scattered cornified epithelial cells; proestrus (P), predominance of round nucleated epithelial cells that may be dispersed or clumped; or estrus (E), all cornified cells (16). All the vaginal smear slides were evaluated by two pathologists in blind and

RESULTS

PAPER 1

any discrepancy was solved by final consensus. For each female, measurement of estrous cycle length was performed by selecting the estrous stage and counting until the recurrence of the same stage. An analysis of estrous cycle pattern was also performed and reported as percentage of time in each stage.

Results

Results for dams and pre-weaning pups of SD-CMCRC/RI rats are reported in Table 1. The female's ability to achieve pregnancy, calculated as fertility index, turned out to be 91.6%. All the pregnant dams maintained pregnancy and delivered live pups (gestational index equal to 100%). The eleven dams displayed a similar gestational length (22.9±0.8 days). The mean litter size was 12.4±2.2 and sex ratio at birth (% males/total offspring) was 48.5±9.8.

Data on post-weaning endpoints are presented in Table 2. Balanopreputial separation, evaluated in all the littermates, was achieved at PND 45.0±1.9. The mean estrous cycle length, evaluated in one female/litter, was 4.9±0.3 days. Estrous cycle pattern, evaluated over a 3-week monitoring period, revealed a percentage of 51.4±9.2 days in diestrus; 24.8±6.3 in proestrus and 23.8±4.5 in estrus. The comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and inter-Laboratory control SD- derived rats data provided by ILSI is reported in Table 3.

Table 1. Dams and pre-weaning litter data from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Fertility index (%)[a]	91.6
Gestational index (%)[b]	100 (11/11)
Mean gestational length (day)[c,d]	22.9±0.8
Total pups (n) delivered at PND 1[e]	136
Litter size (n)[d,f]	12.4±2.2
Total male pups (n) at PND 1	67
Total female pups (n) at PND 1	69
Sex ratio at birth (%)[d,g]	48.5±9.8

[a:] Fertility index = (number of pregnant females/number of females cohabitated) x 100
[b:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[c:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[d:] Mean ± standard deviation
[e:] Live and stillborn pups are considered
[f:] Mean number of pups per litter at PND 1 (within 24 hours from delivery)
[g:] Sex ratio at birth = (no. of male offspring/no. of total offspring) x 100

Table 2. Post-weaning landmarks of pups from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Age (PND) at balano-preputial separation (BPS)[a]	45.0±1.9
Estrous cycle length (days)[a]	4.9±0.3
Time in diestrus (%)[a]	51.4±9.2
Time in proestrus (%)[a]	24.8±6.3
Time in estrus (%)[a]	23.8±4.5

[a:] Mean standard deviation

Table 3. Comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and SD-derived rats*.

Parameter	SD- CMCRC/RI	SD-derived*
Fertility index (%)[a, b]	91.6	89.8±5.9
Gestational index (%)[b, c]	100	99.2±2.6
Mean gestational length (day)[b, d]	22.9±0.8	22.1±0.4
Litter size (n)[b, e]	12.4±2.2	13.7±0.9
Sex ratio at birth (%)[b, f]	48.5±9.8	52
Age (PND) at balano-preputial separation (BPS)[b]	45.0±1.9	45.3±2.1
Estrous cycle length (days)[b]	4.9±0.3	4.2±0.4

* Crl:CD®(SD)IGS BR, Crl:CD® (SD)IGS BR–VAF/Pluss, Crl:CD (SD), Crl:CD® (SD) BR, Crl:CD® BR, Crl:CD® BR–VAF/Pluss, CD®
[a:] Fertility index = (number of pregnant females / number of females cohabitated) x 100
[b:] Mean ± standard deviation
[c:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[d:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[e:] Mean number of pups per litter at PND 0 (within 24 hours from delivery)
[f:] Sex ratio at birth= (no. of male offspring/no. of total offspring) x 100. Standard deviation for control values is not reported by Marty MS et al. 2009

RESULTS

PAPER 1

Discussion

Comprehensive historical control data are important in toxicity studies, as comparisons of data from study controls with historical ones may help to distinguish treatment-induced changes from spontaneously occurring background changes specific to species and strains (17).

Caution should be taken particularly when comparing certain endpoints, such as endocrine-related endpoints, with historical control databases from other laboratories, owing to possible inter-laboratory differences in procedures and classification schemes. Furthermore, subtle changes in species occur over time, owing to genetic alterations in strains or stocks of species and to change in environmental conditions, both in breeding colonies and in individual laboratories (17).

In our work, the reproductive indices and preweaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI.

Interestingly, for SD-derived rats, data were separated by ILSI into litters that were standardized (i.e., culled) or not. In our work, pups were not culled, all the littermates were retained until the time of puberty. Culling is a procedure of artificial equalization of the number of offspring in litter used in rodent experiments to control litter size (18). The rationale for unculling litters is based on the possibility to explore the litter variability and to improve the sensitivity of the statistical analysis in detection of statistically significant and biologically important differences in maturational endpoints. Further statistical analysis on individual data from different laboratories could help to demonstrate whether culling evaluation of all the littermates *vs* one or two pups/sex/litter influences the outcome of data by reducing the probability of identifying a false negative result.

We also evaluated some endocrine relevant endpoints that are currently required by the OECD TGs, i.e BPS in males and estrous cyclicity in females.

The mean ages at BPS in SD-derived rat, reported by ILSI, ranged between 41.2 and 49.0 days, with a mean of 45.0±1.9 days. These values were remarkably closed to those obtained by the SD-CMCRC/RI male rats (45.3±2.1), indicating that the assessment was conducted in a consistent manner within and between studies. It is also noteworthy that BPS was monitored by the examination of all littermates. This procedure represents a new and interesting perspective, indeed, sexual maturation assessments are usually performed on only one weanling rat per sex after litter standardization or culling. For these reasons, the reported historical control values are usually based on observations for one weanling pup/sex/litter. Concern is expressed that culling could affect many health-related endpoints, including the onset of developmental landmarks and sexual maturation (12, 18).

In females, estrous cycle data are used to complement other data and do not typically indicate an adverse effect alone. Cooper and Goldman (19) reported that estrous cycle pattern is an important parameter in order to detect changes that might be masked when only examining estrous cycle length. Altered estrous cyclicity or complete cessation of vaginal cycling in response to toxicants should be considered an adverse female reproductive effect. In our work, data on estrous cycle length were within the expected range (e.g., 4-5 days). While estrous cycle length was reported in the ILSI review, estrous cycle pattern was not included due to the lack of the evaluation or of an agreed-upon method for correctly assessing cycle normality and duration (15). Consequently, a comparison for this endpoint was not possible.

Conclusions

Overall, the data for endocrine sensitive endpoints from our untreated SD-CMCRC/RI rats are comparable to the value reported in the scientific literature, suggesting that procedures for monitoring and physiological biological variations in our SD rats fall within the range of values typically obtained for the selected endpoints. In particular, BPS values were comparable for unculled SD-CMCRC/RI rats and other culled SD-derived rats. Further data on other relevant endocrine sensitive endpoints, such as anogenital distance, vaginal opening, first estrus and relative body weight at the time of acquisition, sperm analysis need to be investigated in our SD colony in the future, in order to provide a more comprehensive perspective

for interpreting data from treated animals, particularly with regard to reproductive and developmental toxicity bioassays.

Author contribution:
Fabiana Manservisi, Laura Falcioni, Luciano Bua, Ilaria Menghetti: concept and design of study, data collection, data interpretation and analysis, drafting, revision, approval of final manuscript; Daniele Mandrioli, Giovanna Galeati, Marcella Spinaci, Carlo Tamanini and Fiorella Belpoggi: data interpretation and analysis, critical revision of the entire text, approval of final manuscript.

References

1. Manibusan MK, Touart LW. A comprehensive review of regulatory test methods for endocrine adverse health effects. Critical Reviews in Toxicology 2017; 47: 440-488.
2. Gray LE, Jr., Wilson V, Noriega N, et al. Use of the laboratory rat as a model in endocrine disruptor screening and testing. ILAR J 2004; 45: 425-437.
3. Manservisi F, Marquillas CB, Buscaroli A, et al. An Integrated Experimental Design for the Assessment of Multiple Toxicological End Points in Rat Bioassays. Environ Health Perspect 2017; 125: 289-295.
4. OECD. Test No. 416: Two-Generation Reproduction Toxicity. 2001.
5. OECD. Test No. 443: Extended One-Generation Reproductive Toxicity Study. 2018.
6. OECD. Test No. 426: Developmental Neurotoxicity Study. 2007.
7. NTP. NTP's Modified One-Generation Reproduction Study. In. 2011.
8. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. 2018.
9. OECD. Test No. 414: Prenatal Developmental Toxicity Study. 2018.
10. Everitt JI, Foster PMD. Laboratory Animal Science Issues in the Design and Conduct of Studies with Endocrine-active Compounds. ILAR Journal 2004; 45: 417-424.
11. Hood R. Developmental and reproductive toxicology - a practical approach. 2006.
12. Palmer AK, Ulbrich BC. The cult of culling. Fundam Appl Toxicol 1997; 38: 7-22.
13. Lyons W. R. BI, Friedlander S. Cornification of balano-preputial epithelium in normal rats and in castrated rats treated withm testosterone propionate. . Endocrinology 1942; 31: 659-663.
14. OECD. Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. Series on Testing and Assessment. No. 150. Paris : OECD Publishing, 2014.
15. Marty MS, Allen B, Chapin RE et al. Inter-laboratory control data for reproductive endpoints required in the OPPTS 870.3800/OECD 416 reproduction and fertility test. Birth Defects Res B Dev Reprod Toxicol 2009; 86: 470-489.
16. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol 2007; 80: 84-97.
17. Kuwagata M, Sakai Y, Tanaka S, et al. Historical control data on developmental toxicity studies in rats. Congenit Anom (Kyoto) 2018.
18. Suvorov A, Vandenberg LN. To Cull or Not To Cull? Considerations for Studies of Endocrine-Disrupting Chemicals. Endocrinology 2016; 157: 2586-2594.
19. Cooper RL GJ. Vaginal cytology. In Daston G KC (ed) An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington, DC: ILSI Press. 1999; p 42-56.

Correspondence:
Fabiana Manservisi,
Cesare Maltoni Cancer Research Center, Ramazzini Institute
Via Saliceto 3, 40010 Bentivoglio, Bologna, Italy
Tel. +39 051 6640460
Fax +39 051 6640223
E-mail: manservisif@ramazzini.it

RESULTS

PAPER 2

Panzacchi *et al. Environmental Health* (2018) 17:52
https://doi.org/10.1186/s12940-018-0393-y

Environmental Health

RESEARCH Open Access

The Ramazzini Institute 13-week study on glyphosate-based herbicides at human-equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation

Simona Panzacchi[1†], Daniele Mandrioli[1,2†], Fabiana Manservisi[1,3], Luciano Bua[1], Laura Falcioni[1], Marcella Spinaci[3], Giovanna Galeati[3], Giovanni Dinelli[2], Rossella Miglio[4], Alberto Mantovani[5], Stefano Lorenzetti[5], Jianzhong Hu[6], Jia Chen[7], Melissa J. Perry[8], Philip J. Landrigan[9] and Fiorella Belpoggi[1*]

Abstract

Background: Glyphosate-based herbicides (GBHs) are the most widely used pesticides worldwide, and glyphosate is the active ingredient of such herbicides, including the formulation known as Roundup. The massive and increasing use of GBHs results in not only the global burden of occupational exposures, but also increased exposure to the general population. The current pilot study represents the first phase of a long-term investigation of GBHs that we are conducting over the next 5 years. In this paper, we present the study design, the first evaluation of in vivo parameters and the determination of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in urine.

Methods: We exposed Sprague-Dawley (SD) rats orally via drinking water to a dose of glyphosate equivalent to the United States Acceptable Daily Intake (US ADI) of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA, starting from prenatal life, i.e. gestational day (GD) 6 of their mothers. One cohort was continuously dosed until sexual maturity (6-week cohort) and another cohort was continuously dosed until adulthood (13-week cohort). Here we present data on general toxicity and urinary concentrations of glyphosate and its major metabolite AMPA.

Results: Survival, body weight, food and water consumption of the animals were not affected by the treatment with either glyphosate or Roundup. The concentration of both glyphosate and AMPA detected in the urine of SD rats treated with glyphosate were comparable to that observed in animals treated with Roundup, with an increase in relation to the duration of treatment. The majority of glyphosate was excreted unchanged. Urinary levels of the parent compound, glyphosate, were around 100-fold higher than the level of its metabolite, AMPA.

Conclusions: Glyphosate concentrations in urine showed that most part of the administered dose was excreted as unchanged parent compound upon glyphosate and Roundup exposure, with an increasing pattern of glyphosate excreted in urine in relation to the duration of treatment. The adjuvants and the other substances present in Roundup did not seem to exert a major effect on the absorption and excretion of glyphosate. Our results demonstrate that urinary glyphosate is a more relevant marker of exposure than AMPA in the rodent model.

Keywords: Glyphosate, Roundup, 13-week, Sprague-Dawley rat, Glyphosate based herbicides, GBH

* Correspondence: belpoggif@ramazzini.it
†Simona Panzacchi and Daniele Mandrioli contributed equally to this work.
[1]Cesare Maltoni Cancer Research Center (CMCRC), Ramazzini Institute (RI), Via Saliceto, 3, 40010 Bentivoglio, Bologna, Italy
Full list of author information is available at the end of the article

Background

Glyphosate [IUPAC chemical name N-(phosphonomethyl)glycine] is the most widely applied pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including in the formulation "Roundup" [1, 2]. It is mainly marketed as a *broad-spectrum* systemic *herbicide* and crop desiccant [3]. The Asia-Pacific region represents the largest supplier of glyphosate active ingredient worldwide in terms of production.. In 2016, China contributed the largest share in the Asia Pacific, and is likely to remain a dominant market for years to come. The United State trails behind the Asia-Pacific market in the production of GBHs. Latin America, Middle East and Africa are expected to grow in terms of use at a significant rate during 2017–2025 [4]. Production and use of glyphosate have risen dramatically with the introduction in 1996 of genetically modified (GM) glyphosate tolerant crop varieties. In the United States (US) glyphosate is contained in over 750 products, particularly herbicides used for intensive GM crops that have built-in tolerance to glyphosate, but also in other products used in agriculture, forestry, urban, and home applications [5]. In 2015, 89% of corn, 94% of soybeans, and 89% of cotton cropped in the US were genetically modified to be glyphosate-tolerant [6]. Only a few data on the use of individual pesticides are available for certain countries in the European Union (EU), making it difficult to find out how much glyphosate is being used by farmers [7]. However, surveys in individual countries give some indication. Glyphosate is the top ranked herbicide in United Kingdom arable crop production [8]. In Denmark, glyphosate accounts for 35% of all pesticides used in agricultural production [9]. In Germany, it has been estimated that glyphosate is used on 4.3 million hectares (39%) of agricultural land each year, with nearly two thirds applied to just 3 crops - oilseed rape, winter wheat and winter barley [10]. The EU has a strict regulation regarding the planting of GM crops (Directive EU 2015/412) [11] and GBHs are mainly applied to cereals for post-harvest desiccation purposes (wheat, rye, triticale, barley and oats), oilseeds (rapeseed, mustard seed and linseed), orchards and vineyards [12].

The massive and increasing use of GBHs leads to a global burden of occupational exposures in manufacturing workers and GBH applicators (farmers), as well as increasing exposures in the general population, as demonstrated by environmental contamination from glyphosate residues found in air [13], groundwater [14, 15], drinking-water [16], crops [17, 18], food [19, 20] and animal feed [21]. Microbial biodegradation of glyphosate occurs in soil, aquatic sediment and water. The main pathway of biodegradation of glyphosate appears to be by splitting the C–N bond to produce aminomethylphosphonic acid (AMPA), the major microbial metabolite [22]. In humans, the main exposure routes to glyphosate are inhalation and dermal exposure in the occupational setting and consumption of water and food for the general population [22]. The results of oral studies with [^{14}C] glyphosate in rats, rabbits and goats indicate that absorption from the gastrointestinal tract is incomplete and amounts to up to 30% of the dose [23–25]. The most relevant routes of excretion following oral administration of glyphosate [^{14}C] are feces (70–80%) and urine (20–30%) [26]. In rats, after a single oral administration of [^{14}C] glyphosate, almost all radioactivity was detected in urine and feces, and the radiolabeled detected chemical was present as the unchanged parent compound [27–29]. Elimination through exhaled air was very low. AMPA was the only metabolite detected, accounting for only 0.2–0.3% of the applied dose of [^{14}C] glyphosate [30]. The limited data currently available on glyphosate pharmacokinetics in vertebrates are insufficient to predict transport and fate of glyphosate in different mammalian tissues, organs and fluids in the body, and to determine whether or where bioaccumulation occurs, although animal metabolism studies indicate kidney and liver as target tissues [1].

The possible effects of GBHs on human health is the topic of intense public debate, for both its potential carcinogenic and non-carcinogenic effects, including endocrine disruption, neurotoxicity, developmental and reproductive toxicity, which might occur even at doses much lower than the ones considered for risk assessment, in particular during sensitive periods of life (such as fetal development) [5, 12, 31, 32]. Glyphosate, as the pure active substance, and GBHs may not be quite the same from the toxicological standpoint. Glyphosate formulations contain a number of so-called 'inert' ingredients or adjuvants to facilitate the uptake by plants, most of which are patented and not publicly known (in many countries the law does not require a full disclosure of pesticide ingredients). GBHs that contain surfactants and adjuvants might act differently than glyphosate alone [33, 34]. In fact, adjuvants might potentiate the toxic effects of glyphosate [35–38].

The Ramazzini Institute 13-week pilot study: aims and experimental design

The present pilot study is the first phase of an integrated long-term project on GBHs that we are conducting during the next 5 years [39]. The initial focus of our pilot study is to assess techniques and methods for glyphosate detection in different matrices (results presented here), then to evaluate target organ toxicity, genotoxicity and endocrine disrupting activities, together with omics and microbiome alterations (not presented here). In our pilot study, we exposed Sprague-Dawley (SD) rats to either glyphosate or Roundup, one of the most popular

branded GBHs, with a dosage considered to be "safe", the United States Acceptable Daily Intake (US ADI) of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA [40]. The design of the pilot study derives from the 13-week cohort protocol of the National Toxicology Program (NTP) guideline Modified One-Generation study (MOG) [39, 41]. It incorporates exposure during the perinatal period (i.e., gestation and lactation) and later for 13 weeks after the pups are weaned, evaluating standard sub-chronic toxicity and functional endpoints (e.g., sperm analysis, vaginal cytology, indices of puberty and sexual differentiation) to investigate possible effects on the reproductive and endocrine systems. In order to provide more information about specific modes of action, we further integrated the 13-week cohort NTP MOG design with transcriptome analyses of potential target tissues and gut microbiome evaluation at different time-points and life stages in both dams and their offspring. The whole-transcriptome analysis can provide important mechanistic information and support the pathological evaluation of target organs and hormone analysis. The gut microbiome evaluation is a novel endpoint representing the potential role of altered balance in the gut microbiota that relate to several health disorders such as metabolic diseases, hepatic, coronary and gastrointestinal diseases (e.g., inflammatory bowel disease) [32]. The experimental plan and the endpoints investigated in the study are presented in Table 1 and Table 2.

The protocol of the pilot study commences with exposure from gestation day (GD) 6 (implantation) continuously through pregnancy and lactation. To satisfy the need to consider multiple effects across multiple life stages, at weaning the offspring were assigned to two testing cohorts at *random*, so as to have minimal differences in body weight among groups (standard deviation < 10% of the average). The first cohort (6-week cohort) was continuously dosed until full sexual maturity (Post Natal Day-PND 73 ± 2), then sacrificed. The second cohort (13-week cohort) was continuously dosed until adulthood (PND 125 ± 2), then sacrificed. Both cohorts were analyzed for post-natal developmental landmarks, microbiome, target organs toxicity and clinical pathology.

The design of the pilot study has been developed by the Ramazzini Institute in collaboration with all Institutions taking part in the overall Glyphosate Study. All of the in vivo experimental phases of the study were performed at the Ramazzini Institute, while the other collaborating Institutions have independently assessed different outcomes and endpoints of interest. In this paper, we present the study design, the first evaluation of in vivo parameters and the determination of glyphosate and its major metabolite AMPA in urine.

Methods

Experimental model

The study was conducted following the rules established by the Italian law regulating the use and humane treatment of animals for scientific purposes [Decreto

Table 1 Experimental plan

Breeders			Offspring							End of the experiment	
Group	Animals		Group	Animals[a]			Treatment[b]				
	Sex	No.	N.	Sex	Cohort		Compound	Dose[c]	Age at start[d]	Cohort	
					6-week (No.)	13-week (No.)				6-week (PND)	13-week (PND)
I	F	8	I	M	8	10	Control (drinking water)	0	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F + M	16		M + F	16	20					
II	F	8	II	M	8	10	Glyphosate	US ADI	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F + M	16		M + F	16	20					
III	F	8	III	F	8	10	Roundup	US ADI Glyphosate equivalent	GD 6	70[e]	120[f]
	M	8		M	8	10					
	F + M	16		F + M	16	20					
Total	M + F	48		M + F	48	60					

[a]No more than 2 sisters and 2 brothers per litter
[b]Test compounds are administered ad libitum in drinking water
[c]Doses are calculated considering the Glyphosate US ADI defined as the chronic Reference Dose (cRfD) determined by the US EPA (1.75 mg/kg bw/day)
[d]Solutions are admistered to dams starting from the 6th day of pregnancy
[e]Animals are treated until the landmarks of sexual development are acquired (PND 73 ± 2)
[f]Animals are treated from embryonic life (GD 6) indirectly from dams milk until PND 28 ± 2, then directly for 90 days after weaning (until PND 125 ± 2)

RESULTS

PAPER 2

Table 2 Summary of the endpoints and relative monitoring time points evaluated in the study, in dams and offspring (6-week and 13-week cohorts)

Endpoints	Time points	Dams	Offspring 6-week cohort	Offspring 13-week cohort
Gestation length	GD0-delivery	✓	–	–
AGD and body weight in male and female pups	PND 1	–	✓	✓
Litter size	PND 1, 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Live-birth index	PND 1	–	✓	✓
Survival index	PND 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Age and body weight at BPS in male pups	PND 35	–	✓	✓
Age and body weight at VO in female pups	PND 28	–	✓	✓
First estrous in female pups	3 days after VO	–	✓	–
Estrous cycle length and percentage of days in each stage	PND 95 - PND 116	–	–	✓
Estrous cycle prior to necropsy	PND 125 ± 2	–	–	✓
Serum hormone measures	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Clinical biochemistry	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Urinalysis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Glyphosate and AMPA detection in urine	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Sperm counts	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Daily Sperm production	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm transit time through the epididymis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm morphology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm aneuploidy	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Partial histopathology (reproductive organs, brain, liver, kidney)	End of lactation (dams)	✓	–	–
Complete histopathology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Organ weight	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Micronuclei test (bone marrow)	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Transcriptome on mammary glands	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on brain	PND 125 ± 2	–	–	✓
Transcriptome on liver	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on kidneys	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Microbiome analysis in dams	Before mating, GD 5 (before treatment), GD 13, LD 7, LD 14	✓	–	–
Microbiome analysis in offspring	PND 7, PND 14, PND 31 (before puberty), PND 57 (after puberty), PND 125 ± 2 (adulthood)	–	✓	✓

GD gestation day, *LD* lactation day, *PND* postnatal day, *AGD* anogenital distance, *VO* vaginal opening, *BPS* balano preputial separation

Legislativo (D.Lgs.) N. 26, 2014. Attuazione della direttiva n. 2010/63/UE in materia di protezione degli animali utilizzati a fini scientifici. - G.U. Serie Generale, n. 61 del 14 Marzo 2014]. Before starting, the protocol was examined by the Internal Ethical Committee for approval. The protocol of the experiment was also approved and formally authorized by the ad hoc commission of the Italian Ministry of Health (ministerial approval n. 710/2015-PR). The experiment was performed on both male and female SD rats, which belong to the colony used at the Cesare Maltoni Cancer Research Center laboratories of the Ramazzini Institute (CMCRC/RI) for over 40 years. An animal disease screening program enforced by the Italian Health Authority and Research Organization for Animal Health is in place and ongoing on sentinel animals belonging to the RI colony.

Female breeders SD rats were placed individually in Polycarbonate cage (42x26x18cm; Tecniplast Buguggiate, Varese, Italy) with a single unrelated male until evidence of copulation was observed. After mating, matched females were housed separately during gestation and delivery. Newborns were housed with their

mothers until weaning. Weaned offspring were housed, by sex and treatment group, not more than 3 per each cage. Cages were identified by a card indicating: study protocol code, experimental and pedigree numbers, dosage group. A shallow layer of white fir wood shavings served as bedding (supplier: Giuseppe Bordignon, Treviso, Italy). Analysis of chemical characteristics (pH, ashes, dry weight, specific weight) and possible contamination (metals, aflatoxin, polychlorobiphenyls, organophosphorus and organochlorine pesticides) of the bedding was performed by CONSULAB Laboratories (Treviso, Italy). The cages were placed on racks, inside a single room prepared for the experiment at 22 °C ± 3 °C temperature and 50 ± 20% relative humidity. Daily checks on temperature and humidity were performed. The light was artificial and a light/dark cycle of 12 h was maintained.

During the experiment SD rats received ad libitum the standard "Corticella" pellet feed supplied by Laboratorio Dottori Piccioni Srl (Piccioni Laboratory, Milan, Italy). The constituents of the diet are: ground corn (23%), barley milled (15%), soybean meal extract (20.6%), wheat middling (24%), wheat bran (2%), spray dried whey (2. 5%), di-calcium phosphate (2%), calcium carbonate (1. 1%), chicken meal (6%), carob bean gum (3%), sodium chloride (0.5%), mixed vitamins (0.3%). Every day, the animals drank fresh municipal tap water from glass bottles ad libitum. Both feed and water were periodically analyzed to identify possible chemical or microbiological contaminants or impurities; the analyses are included in the documentation of the experiment. 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).

Active ingredient glyphosate (Pestanal™ analytical standard, CAS number 1071–83-6, purity > 99,5%) was supplied from Sigma-Aldrich (Milan, Italy). The commercial formulation Roundup Bioflow (containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret) was supplied from a local agricultural consortium (Consorzio Agrario dell'Emilia, Bologna, Italy). The original containers/bottles of glyphosate and Roundup were stored in its original container and kept in a ventilated storage cabinet at room temperature (22 °C ± 3 °C) throughout the study. Purity data for each batch of glyphosate and Roundup were provided by the supplier. The opening and the use date of the different batches of test substances were recorded in the raw data. An aliquot of each lot of the test article is maintained in the ventilated storage cabinet, until 5 years from the end of the main experiment. The solutions of glyphosate and Roundup were prepared by the addition of appropriate volume of tap drinking water.

Experimental plan

Each of twenty-four virgin female SD rats (17 weeks old, 270-315 g) was cohabited outbred with one breeder male rat of the same age and strain. Every day, the females were examined for presence of sperm. Gestational day (GD) 0 was defined as the one in which the sperm was found in vaginal smears. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and PND 0 for the offspring. Each dam and delivered litter was co-housed in common nesting box during the postpartum period. Following the NTP MOG design, on PND 28, thus 28 days after the last litter was delivered, the offspring were weaned and identified by ear punch according to the Jackson Laboratory system. Sequentially, they were allocated in the same treatment group of their mother in order to have 18 males (8 for the 6-week cohort and 10 for the 13-week cohort) and 18 females (8 for the 6-week cohort and 10 for the 13-week cohort) for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 108 SD rats (54 males and 54 females) were enrolled in the post-weaning treatment phase. The experimental plan of the pilot study is outlined in Table 1. A summary of the endpoints and relative monitoring time points evaluated in the pilot study, both in dams and in the offspring (6-week and 13-week cohorts) is presented in Table 2.

Two groups of SD rats were treated with either glyphosate or Roundup diluted in tap water administered ad libitum and one group received only tap water as control. Roundup was diluted in tap water in order to obtain an equivalent dose of glyphosate of 1.75 mg/kg bw/day. During gestational and lactational periods, embryos and newborns (F1) received the test compounds mainly through their dams (F0). Glyphosate and Roundup water formulations during these periods were freshly prepared on a daily base depending on individual body weight and water consumption of dams as measured at each scheduled time point (see below). After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), the test substances were administered in tap water to F1 animals on the basis of the average body weight and average water consumption *per* sex and *per* experimental group, as measured at each scheduled time point (see below). Males and females were considered separately because of their difference in weight gain, body weight and water consumption.

At least every week, the exposure doses were recalculated and registered. The actual levels of test compounds that reached the fetus during gestation or that were ingested postnatally by the offspring during the period of lactation were not estimated in the present study.

Animals were monitored during the entire experimental period. The following procedures were performed:

Health status control: from the start of the experiment, animals were checked three times daily, except on Sundays and non-working days, when they were only checked twice. All observed variations from normal status were recorded.

Clinical control: status, behavior and clinical observation on the experimental animals were checked before the start of the treatment, and at least every two days until the end of the experiment. Any findings listed below were then recorded: alterations of skin, hair, eyes and mucosa; modification in production of secretions or excretions and in autonomic activity; respiratory symptoms; postural changes or changes in walk; presence of tonic or clonic contractions; unusual stereotypes and behavior.

Dams' body weights were recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, dams' body weights were recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Pups' body weight by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, the body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifices; the means of individual body weights were calculated for each group and sex.

Dams' feed and water consumption were recorded twice weekly during gestation (GD 0, 3, 6, 9, 12, 15, 18, 21), whereas during lactation were measured at LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28.

After weaning the daily water and feed consumption *per* cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2; the means of individual consumptions were calculated for each group and sex.

The day before the terminal sacrifices, all the animals were located individually in metabolic cages and starved for around 16 h. During this time, the animals had free access to water alone or to the programmed test compound solutions. The day after, in the morning, samples of at least 5 ml of spontaneous urine from each animal were collected and put in separate labelled tubes. Urine samples for analysis of glyphosate and AMPA excretion were obtained from 3 dams/group and from 10 (5 males + 5 females) rats/group belonging to the 6-week and 13-week cohorts.

Glyphosate and aminomethylphosphonic acid (AMPA) detection

Analyses of glyphosate and its metabolite AMPA in drinking water, feed and urine were performed by Neotron Laboratories (Modena, Italy), an officially accredited laboratory by Accredia (Lab. N. 0026) according to European regulation UNI CEI EN ISO/IEC 17025:2005. The specification and results are maintained in the experimental documentation. The analytical method is based on liquid chromatography tandem mass spectrometry (LC-MS/MS) [42–45]. The limit of quantification (LQ) for glyphosate and AMPA corresponded to 0.10 μg/l in water, 50 μg/kg in feed, and 1 μg/kg in urine.

Statistical analysis

Summary statistics, means ± standard deviations (sd), were calculated for continuous variables. For body weight, water and feed consumption over time further analyses were performed using multilevel mixed-effect linear regression models, to control for within subject correlation across time; moreover we have considered also the litter effect during the lactation period. Analysis of variance and Dunnett's tests (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods.

All tests were two tailed, with alpha set at 0.05. Statistical analyses were perfomed by using STATA version10 (Stata Corporation, College StationTexas, USA).

Results

In dams, during both gestation and lactation, body weight and weight gain were not statistically different among the different groups (Fig. 1 a-b). In both female and male offspring, post weaning body weights were homogenous and no statistically significant differences in body weight gain were observed among groups (Fig. 1 c-f). All 24 dams and 108 SD rats from the 6-week (48/48) and 13-week (60/60) cohorts survived until sacrifice.

Water and feed consumption during gestation and lactation were no different across the groups (Fig. 2 a-b and Fig. 3 a-b). Litter sizes were fully comparable among groups, with mean number of live pups: control group 13.6 (range 10–16); glyphosate group 13.3 (range 11–17); Roundup group 13.9 (range 11–16). Post weaning water and feed consumption were not affected by the treatment (Fig. 2 c-d and Fig. 3 c-d).

No unexpected clinical signs or symptoms were observed in the experimental animals during the in vivo phase. In particular, there was no clinical evidence of alterations in activity or behavior, reflexes, the eye or skin, or the respiratory, gastrointestinal, genito-urinary and cardiovascular systems.

The results of glyphosate and AMPA urinary concentrations are reported in Table 3 and Fig. 4. The urinary concentration of both glyphosate and AMPA of SD rats treated with 1.75 mg/kg bw/day of glyphosate were comparable to the ones observed in SD rats treated with

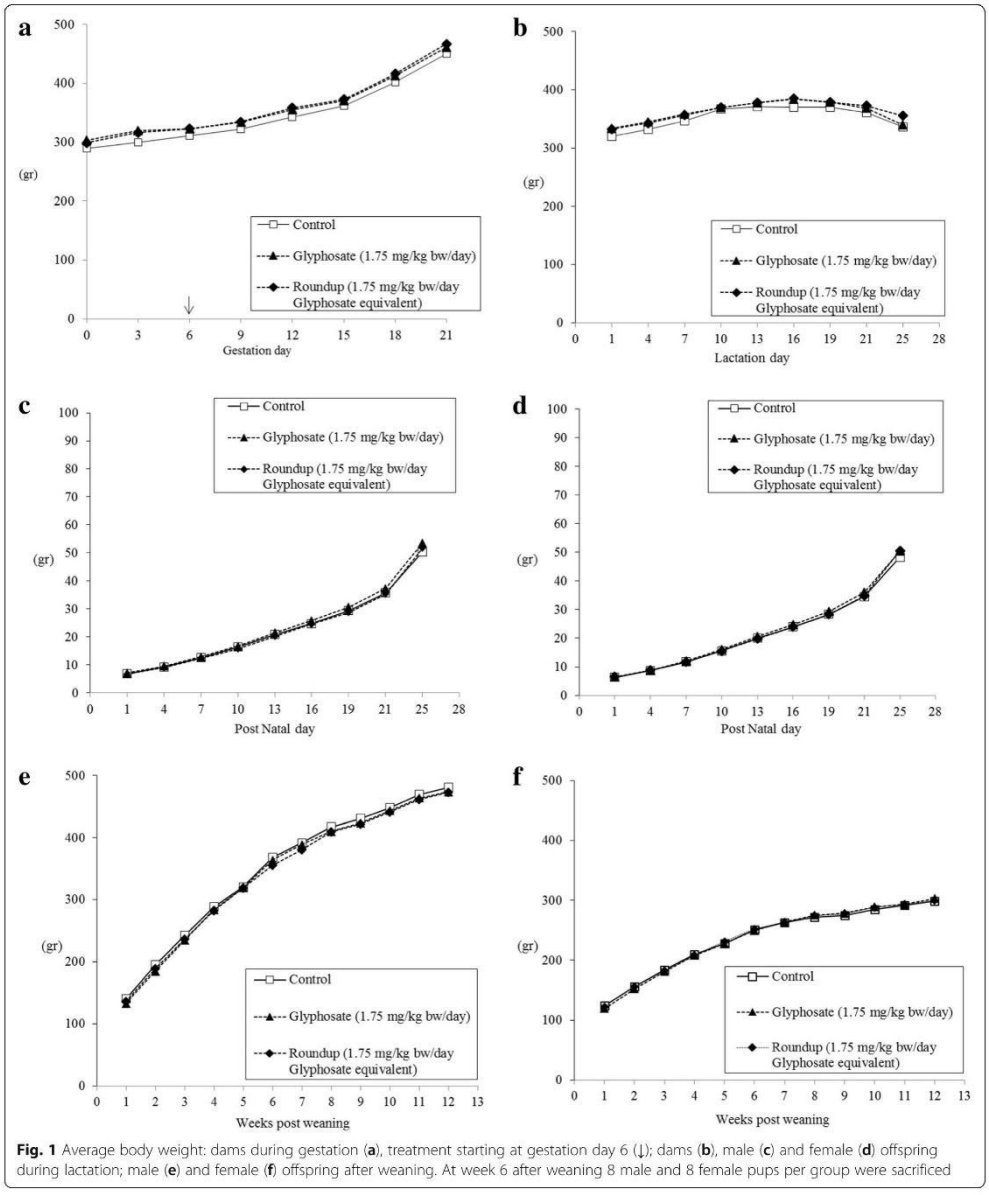


Fig. 1 Average body weight: dams during gestation (**a**), treatment starting at gestation day 6 (↓); dams (**b**), male (**c**) and female (**d**) offspring during lactation; male (**e**) and female (**f**) offspring after weaning. At week 6 after weaning 8 male and 8 female pups per group were sacrificed

Roundup dose equivalent to 1.75 mg/kg bw/day, despite limited sample size and the large standard deviations. In the control group, as expected, the glyphosate and AMPA urinary levels were all below or close to the limit of quantitation (0.001 mg/kg). In the treated SD rats, the majority of glyphosate was excreted unchanged (as parent compound), with urinary levels about 100-fold higher than that of its metabolite AMPA. For example,

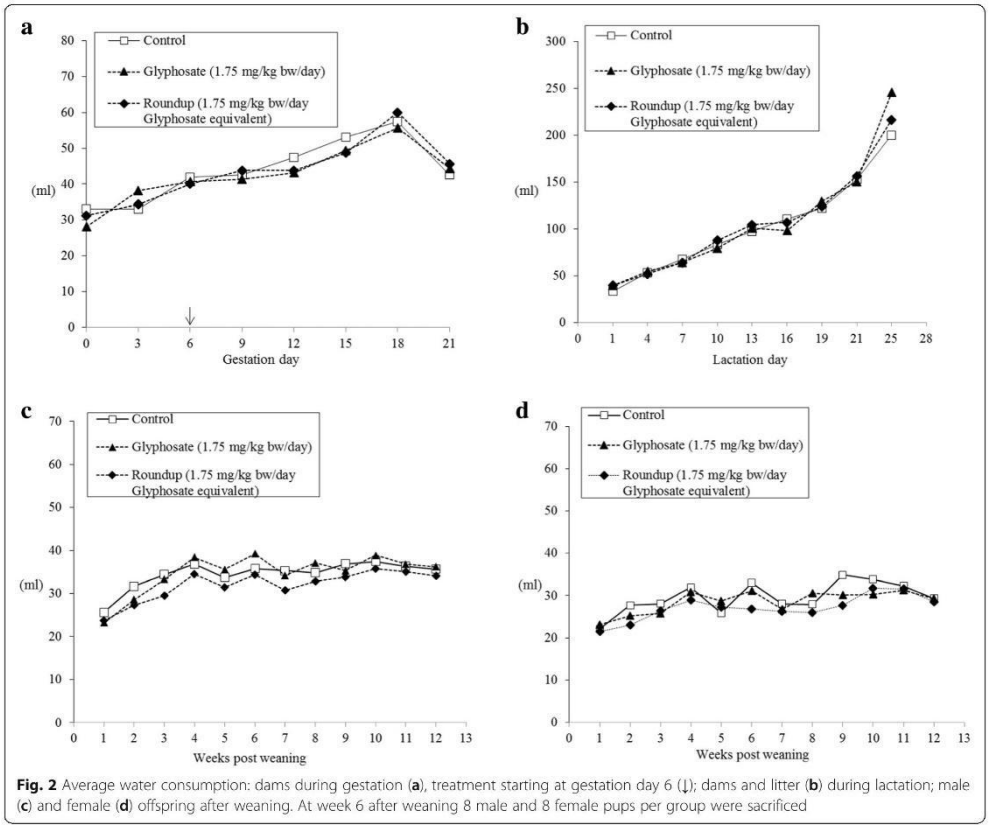


Fig. 2 Average water consumption: dams during gestation (**a**), treatment starting at gestation day 6 (↓); dams and litter (**b**) during lactation; male (**c**) and female (**d**) offspring after weaning. At week 6 after weaning 8 male and 8 female pups per group were sacrificed

glyphosate and Roundup treated females in the 13-week cohort presented mean urinary levels of glyphosate respectively of 1.354 mg/kg and 1.524 mg/kg, while the AMPA levels were respectively 0.013 mg/kg and 0.021 mg/kg. In glyphosate and Roundup treated SD rats, a time-dependent increase in the mean urinary concentration of glyphosate was observed. In glyphosate and Roundup treated males, an approximate 2-fold increase of mean urinary concentration of glyphosate in the 13-week cohort (animals exposed prenatally until 125 ± 2 days after birth) compared to the 6-week cohort (animals exposed prenatally until 73 ± 2 days after birth) was observed. In glyphosate treated females, the 6-week cohort (animals exposed prenatally until 73 ± 2 days after birth) showed a 2-fold higher value of mean urinary concentration of glyphosate than the dams after weaning (exposed for 49 ± 2 days), while the 13-week cohort (animals exposed prenatally and 125 ± 2 days after birth) showed a 1.5-fold increase compared to the 6-week cohort. In the Roundup treatment group, the increase was less steep, but the time-dependent pattern was still evident. In glyphosate and Roundup treated SD rats, the levels of AMPA were comparable at the different time points in both males and females. In these animals, large standard deviations of the values of AMPA concentrations in urine have been observed, in particular for values close to the limit of quantitation as in the control groups.

Discussion

Survival, body weights, food and water consumption of SD rats were not affected by the treatment with glyphosate and Roundup. Clinical changes in the animals were not observed in the various groups. Overall, both glyphosate and Roundup treatments seemed to be well tolerated, which is consistent with previous experiments performed by the US NTP [26].

Glyphosate and Roundup exposure led to comparable concentrations of glyphosate and AMPA in urine,

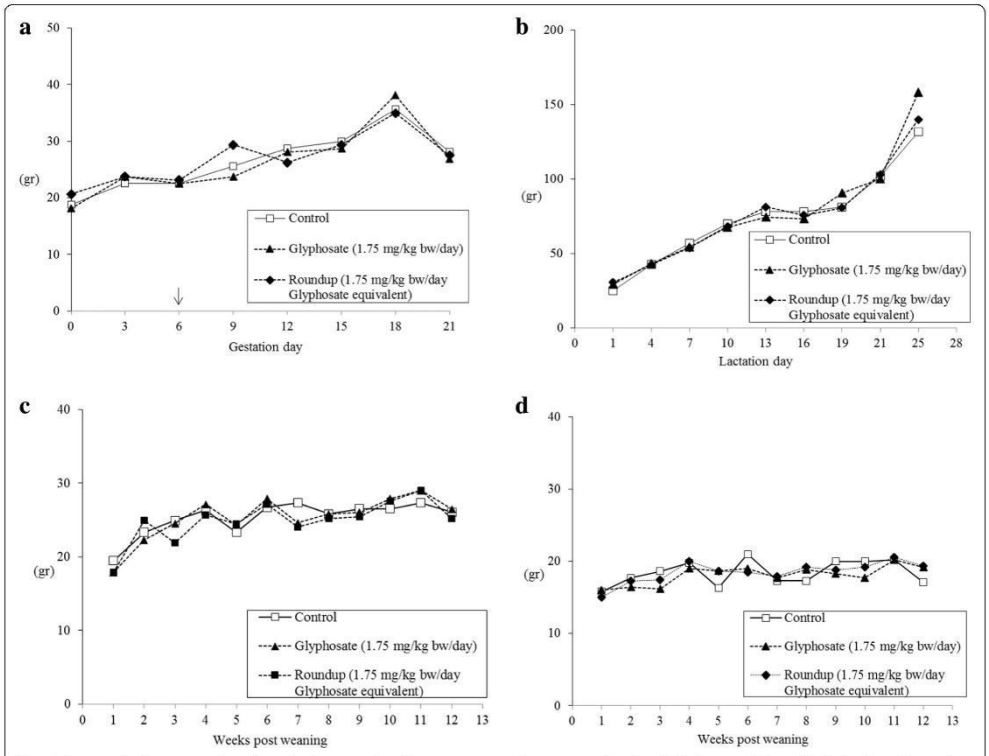


Fig. 3 Average feed consumption: dams during gestation (**a**), treatment starting at gestation day 6 (↓); dams and litter (**b**) during lactation; male (**c**) and female (**d**) offspring after weaning. At week 6 after weaning 8 male and 8 female pups per group were sacrificed

indicating that systemic exposure does occur at the selected exposure level of 1.75 mg//kg bw/day, corresponding to the US ADI. The bioavailability of glyphosate in our study is also supported by the evident increase of glyphosate concentration in urine in relation to the length of treatment. The adjuvants and the other substances present in Roundup did not seem to exert a major effect on the absorption and excretion of glyphosate, even though mean values of glyphosate seem to be somewhat higher in the Roundup treated group. The levels in urine were also comparable between the two sexes; however, a consistent inter-individual variability was observed. In rats, glyphosate in urine appears to be the most accurate biomarker of exposure to GBHs. In

Table 3 Glyphosate and AMPA concentration in urine. Results are reported as mean ± standard deviations

		Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
	Treatment	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	_	_	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

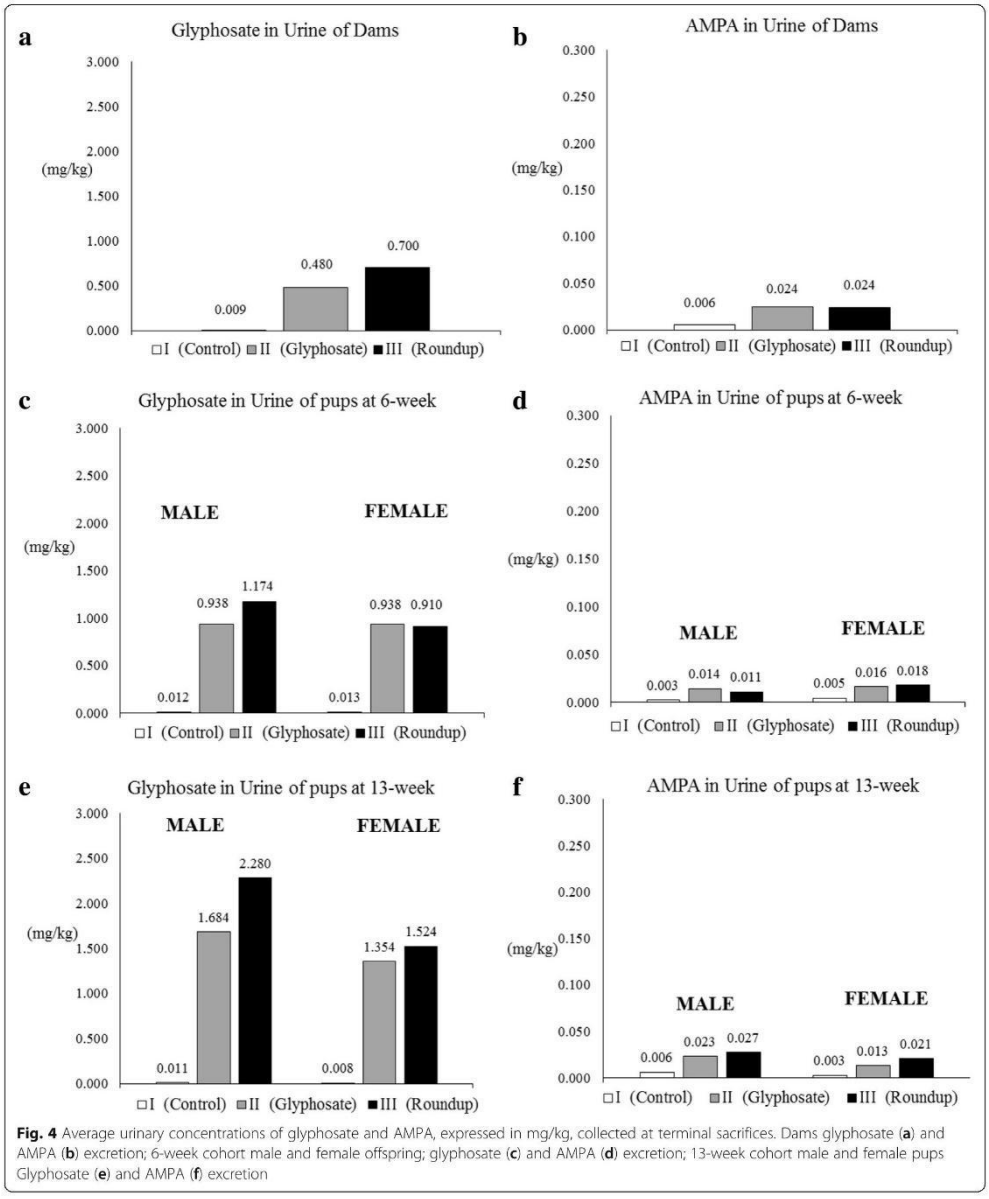


Fig. 4 Average urinary concentrations of glyphosate and AMPA, expressed in mg/kg, collected at terminal sacrifices. Dams glyphosate (**a**) and AMPA (**b**) excretion; 6-week cohort male and female offspring; glyphosate (**c**) and AMPA (**d**) excretion; 13-week cohort male and female pups Glyphosate (**e**) and AMPA (**f**) excretion

fact, our results confirm previous evidence that in rodents most of the administered dose of glyphosate (98%) is excreted as unchanged parent compound, whereas the metabolite AMPA in urine is at around 0.2–0.3% of the administered dose [46]. Furthermore, with the level of exposure to glyphosate used in this pilot study, AMPA urinary values of treated animals (0.011–0.027 mg/kg) were already close to the chromatographic LQ (0.001 mg/kg)

and this might limit the reliability of the measures. On the other hand, glyphosate concentration in urine of treated animals (0.480–2.280 mg/kg) resulted up to 100-fold higher than the AMPA concentration and at least 500-fold higher than the chromatographic LQ (0.001 mg/kg). Therefore, in order to assess exposure to glyphosate in rats, in particular at doses that are equal or lower than the one used in this pilot study (1.75 mg/kg bw/day), glyphosate appears to be the biomarker of choice.

The presence of negligible levels of glyphosate (0.003–0.013 mg/kg), close to the chromatographic LQ (0.001 mg/kg), in some of the urine of the control groups might reflect an ubiquitous environmental contamination at ultra-low doses of glyphosate, which is consistent with previous reports from other authors [21]. As the current limit of quantitation of glyphosate in HPLC for pelleted animal feed is 0.050 mg/kg, this represents a technical limiting factor for testing ultra-low doses of glyphosate. As reported by a recent inter laboratory comparative study on the quantitative determination of glyphosate at low levels, caution should be taken when interpreting results if the tested doses of glyphosate are close to the LQ of HPLC [47].

It is noteworthy that the commercial formulation used in this study, Roundup Bioflow, was the representative formulated product recently evaluated for the renewal of the approval of glyphosate in EU and considered in the European Food Safety Authority peer review (MON 52276) [48].

Our results seem particularly relevant in light of the massive global burden of exposure to glyphosate, as shown by the exponential increase in the last 20 years of the levels of glyphosate and AMPA measured in the urine of the general population in Germany [49] and in the US [50].

Conclusion

We performed a pilot study on the health effects of glyphosate and its formulation Roundup administered at currently admitted doses (US ADI = 1.75 mg/kg bw/day) to SD rats. In this paper, we described the study design, the first evaluation of in vivo parameters and the determination of glyphosate and its major metabolite AMPA in urine. The treatment with either glyphosate or Roundup seemed to be overall well tolerated, consistently with previous experiments performed by the US NTP [26]. Both glyphosate and Roundup exposure led to comparable urinary concentrations of glyphosate and AMPA with an increasing pattern of glyphosate excreted in urine in relation to the duration of treatment, indicating the systemic bioavailability of the active substance and a possible mechanism of bioaccumulaton. The adjuvants and the other substances present in Roundup did not seem to exert a major effect on the absorption and excretion of glyphosate. Our results confirm that, in rodents, glyphosate in urine is the much more relevant marker of exposure than AMPA in particular at doses that are equal or lower than the one used in this pilot study (1.75 mg/kg bw/day). The evaluation of different outcomes and endpoints of interest (i.e., pathology of target organs, molecular toxicity, genotoxicity, endocrine disrupting activities, microbiome, developmental toxicity, etc.) is currently ongoing in the different partner laboratories of the project.

Abbreviations

AMPA: Aminomethylphosphonic acid; CMCRC: Cesare Maltoni Cancer Research Center; EU: European Union; GBH: Glyphosate-based herbicides; GD: Gestational day; GM: Genetically modified; LC-MS/MS: Liquid chromatography tandem mass spectrometry; LD: Lactating day; LQ: Limit of Quantification; MOG: Modified One-Generation study; NTP: National Toxicology Program; PND: Post Natal Day; RI: Ramazzini Institute; SD: Sprague-Dawley; US ADI: United States Acceptable Daily Intake

Acknowledgements

We thank the over 30,000 associates and volunteers of the Ramazzini Institute that made this pilot study possible through their commitment and generosity. We thank the Municipality of Bologna, the Emilia-Romagna Region, and the International Society of Doctors for Environment for organizing several events to promote this pilot study; "Coop Reno" and "Coopfond Fondo Mutualistico Legacoop" for supporting our research activity.

Funding

This work was funded by Institution fund of the Ramazzini Institute, Bologna, Italy.

Availability of data and materials

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

Authors' contributions

All authors provided substantial contributions to the conception/design of the work, acquisition, analysis or interpretation of the data, revised the manuscript critically, and approved the final version for submission. FM, SP, DM participated in the design of the study, performed the animal experiments and sample collection, and drafted the manuscript. LB and LF performed the animal experiments and sample collection. FB supervised the study, participated in the design of the study and helped to draft the manuscript. MS, GG, GD, RM, AM, SL, JH, JC, MJP, PJL, helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

N/A

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Cesare Maltoni Cancer Research Center (CMCRC), Ramazzini Institute (RI), Via Saliceto, 3, 40010 Bentivoglio, Bologna, Italy. [2]Department of Agricultural Sciences, University of Bologna, Viale Fanin 44, 40127 Bologna, Italy. [3]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna, Italy. [4]Department of Statistical Sciences, University of Bologna, Via Belle Arti 41, 40126 Bologna, Italy. [5]Department of Food safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. [6]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, 1425 Madison Ave, New York, NY 10029, USA. [7]Department of Environmental Medicine and Public Health, Icahn School of Medicine at

RESULTS

PAPER 2

Mount Sinai, New York, USA. [8]Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, 950 New Hampshire Ave, Washington, DC 20052, USA. [9]Arnhold Institute for Global Health, Icahn School of Medicine at Mount Sinai, 1216 Fifth Avenue, New York, NY 10029, USA.

Received: 2 February 2018 Accepted: 10 May 2018
Published online: 29 May 2018

References

1. Myers JP, Antoniou MN, Blumberg B, Carroll L, Colborn T, Everett LG, et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environmental health : a global access science source. 2016;15:19. https://doi.org/10.1186/s12940-016-0117-0.
2. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. Environ Sci Eur. 2016;28(1):3. https://doi.org/10.1186/s12302-016-0070-0.
3. Smith EA, Oehme FW. The biological activity of glyphosate to plants and animals: a literature review. Vet Hum Toxicol. 1992;34(6):531–43.
4. Research TM. "Herbicides Market by product (Acetohlor, 2,4-D, Glyphosate, Atrazine), by application (Oilseeds & Pulses, Cereals & grains, Fruits & vegetables) - Global Industry Analysis, Size, Share, Growth, Trends, and Forecast, 2017–2025". Available at: https://www.transparencymarketresearch.com/herbicides-market.html. 2017.
5. IARC. Glyphosate. In: Some organophosphate insecticides and herbicides: diazinon, glyphosate, malathion, parathion, and tetrachlorvinphos. Vol 112. IARC Monogr Prog, 1–92. 2015.
6. USDA. Adoption of Genetically Engineered Crops in the U.S.. Economic Research Service 2015;Retrieved 20 December 2017..
7. Garthwaite D, Sinclair C, Glass R, Pote A, Trevisan M, Sacchettini G, et al. Collection of pesticide application data in view of performing environmental risk assessments for pesticides. EFSA Supporting Publications. 2015;12(7)
8. Garthwaite D, Barker I, Parrish G, Smith L, Chippindale C, Pietravalle S. Pesticide usage survey report 235. Arable Crops in UK 2010 (Including Aerial Applications 2010). 2010.
9. DEPA. Annual Pesticides Statistics 2009. 2009.
10. Steinmann HH, Dickeduisberg M, Theuvsen L. Uses and benefits of glyphosate in German arable farming. Crop Prot. 2012;42:164–9.
11. EU. Directive (EU) 2015/412 of the European Parliament and of the Council of 11 March 2015 amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory. 2015.
12. EFSA. European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA J. 2015;13:4302.
13. Majewski MS, Coupe RH, Foreman WT, Capel PD. Pesticides in Mississippi air and rain: a comparison between 1995 and 2007. Environ Toxicol Chem. 2014;33(6):1283–93. https://doi.org/10.1002/etc.2550.
14. ISPRA. National Report of Pesticides in Water (2013–2014) [in italian, "Rapporto Nazionale pesticidi nelle acque dati 2013–2014"] Rapporti. 2016; 244/2016.
15. Battaglin WA, Meyer MT, Kuivila KM, Dietze JE. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. J Am Water Resour Assoc. 2014;50 https://doi.org/10.1111/jawr.12159.
16. Rendón-von Osten J, Dzul-Caamal R. Glyphosate residues in groundwater, drinking water and urine of subsistence farmers from intensive agriculture localities: a survey in Hopelchén, Campeche, Mexico. Int J Environ Res Public Health. 2017;14(6):595. https://doi.org/10.3390/ijerph14060595.
17. Cuhra M. Review of GMO safety assessment studies: glyphosate residues in roundup ready crops is an ignored issue. Environ Sci Eur. 2015;27 https://doi.org/10.1186/s12302-015-0052-7.
18. USDA. Agricultural Marketing Service. Pesticide data program annual summary, program year 2011. In: Appendix C distribution of residues in soybean by pesticide. Washington, D.C: U.S. Department of Agriculture; 2013.
19. EFSA. The 2014 European Union report on pesticide residues in food. EFSA journal. 2016;14(10):e04611-n/a) https://doi.org/10.2903/j.efsa.2016.4611.
20. PRIF. Expert Committee On pesticide residues in food (PRiF): annual report for 2016. Available at: https://www.gov.uk/government/publications/expert-committee-on-pesticide-residues-in-food-prif-annual-report.
21. Mesnage R, Defarge N, Rocque LM, Spiroux de Vendomois J, Seralini GE. Laboratory rodent diets contain toxic levels of environmental contaminants: implications for regulatory tests. PLoS One. 2015;10(7):e0128429. https://doi.org/10.1371/journal.pone.0128429.
22. WHO. Glyphosate and AMPA in drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality copyright World Health Organization. 2005:9.
23. Powles P. 14C-glyphosate: absorption, distribution, metabolism and excretion following repeated oraladministration to the dairy goat. Unpublished report no. 676/9–1011, dated 7 November 1994, from Hazleton Europe, Harrogate, England. Submitted to WHO by Cheminova a/S, Lemvig, Denmark. 1994.
24. Colvin L, Miller J. CP 67573 Residue and metabolism. Part 9: the metabolism of N-phosphonmethylglycine-14C (CP 67573-14C) in the rabbit. Unpublished report. 1973(298).
25. Brewster DW, Warren J, Hopkins WE. Metabolism of glyphosate in Sprague-Dawley rats: tissue distribution, identification, and quantitation of glyphosate-derived materials following a single oral dose. Fundam Appl Toxicol. 1991;17(1):43–51.
26. NTP. NTP technical report on the toxicity studies of Glyphosate (CAS No. 1071-83-6) Administered In Dosed Feed To F344/N Rats And B6C3F1 Mice. Toxicity report series. 1992;16:1–D3.
27. Davies D. Glyphosate acid: excretion and tissue retention of a single oral dose (1000mg/kg) in the rat. In: Unpublished report no CTL/P/4942; 1996.
28. Davies D. Glyphosate acid: excretion and tissue retention of a single oral dose (10 mg/kg) in the rat. Unpublished report no CTL/P/4940, Dated. 1996.
29. Davies D. Glyphosate acid: excretion and tissue retention of a single oral dose (10 mg/kg) in the rat following repeat dosing. Unpublished report no CTL/P/4944, Dated. 1996.
30. Howe R, Chott R, McClanahan R. Metabolism of glyphosate in Sprague–Dawley rats. II. Identification, characterization, and quantitation of glyphosate and its metabolites following intravenous and oral administration. Unpublished report. St Louis, MO: Monsanto Environmental Health Laboratory; 1988.
31. ECHA. ECHA's Committee for Risk Assessment (RAC). https://echa.europa.eu/-/echa-s-opinion-on-classification-of-glyphosate-published.. 2017.
32. Shehata AA, Schrodl W, Aldin AA, Hafez HM, Kruger M. The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol. 2013;66(4):350–8. https://doi.org/10.1007/s00284-012-0277-2.
33. Mullin CA, Fine JD, Reynolds RD, Frazier MT. Toxicological risks of agrochemical spray adjuvants: Organosilicone surfactants may not be safe. Front Public Health. 2016;4:92. https://doi.org/10.3389/fpubh.2016.00092.
34. Landrigan PJ, Benbrook C. GMOs, herbicides, and public health. N Engl J Med. 2015;373(8):693–5. https://doi.org/10.1056/NEJMp1505660.
35. Coalova I, Rios de Molina Mdel C, Chaufan G. Influence of the spray adjuvant on the toxicity effects of a glyphosate formulation. Toxicology in vitro : an international journal published in association with BIBRA. 2014; 28(7):1306–11. https://doi.org/10.1016/j.tiv.2014.06.014.
36. Mesnage R, Bernay B, Seralini GE. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology. 2013;313(2–3):122–8. https://doi.org/10.1016/j.tox.2012.09.006.
37. Defarge N, Takacs E, Lozano VL, Mesnage R, Spiroux de Vendomois J, Seralini GE, et al. Co-Formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. Int J Environ Res Public Health. 2016;13(3) https://doi.org/10.3390/ijerph13030264.
38. Williams GM, Kroes R, Munro IC. Safety evaluation and risk assessment of the herbicide roundup and its active ingredient, glyphosate, for humans. Regulatory toxicology and pharmacology : RTP. 2000;31(2 Pt 1):117–65. https://doi.org/10.1006/rtph.1999.1371.
39. Manservisi F, Babot Marquillas C, Buscaroli A, Huff J, Lauriola M, Mandrioli D, et al. An integrated experimental Design for the Assessment of multiple toxicological end points in rat bioassays. Environ Health Perspect. 2016; https://doi.org/10.1289/ehp419.
40. EPA. Re-registration Eligibility Decision (RED) Glyphosate: EPA-738-R-93-014. Washington, DC: US Environmental Protection Agency, Office of Pesticide Programs and Toxic Substances; 1993.
41. NTP. NTP's Modified One-Generation Reproduction Study 2011.
42. Granby K, Johannesen S, Vahl M. Analysis of glyphosate residues in cereals using liquid chromatography-mass spectrometry (LC-MS/MS). Food Addit Contam. 2003;20(8):692–8. https://doi.org/10.1080/0265203031000109477.

RESULTS

PAPER 2

43. FDA. Pesticide analytical manual vol. II -method I: "method of analysis for residues of 2-chloroethylphosphonic acid (ethephon) in pinneapples. Pesticide Reg. Sec 180:300. 2002.
44. FDA. Pesticide Analytical Manual Vol. II -Method I. "gas chromatographic determination of residues of Fosetyl-Al and phosphorous acid in pineapples". Pesticide Reg. Sec. 2002;180:415.
45. Kubilius DT, Bushway RJ. Determination of maleic hydrazide in pesticide formulations by capillary electrophoresis. Journal of AOAC International (USA). 1998;
46. IPCS. Glyphosate. Geneva, world health organization, International Programme on Chemical Safety (Environmental Health Criteria 159). 1994.
47. Simonetti E, Cartaud G, Quinn RM, Marotti I, Dinelli G. An Interlaboratory comparative study on the quantitative determination of glyphosate at low levels in wheat flour. J AOAC Int. 2015;98(6):1760–8. https://doi.org/10.5740/jaoacint.15-024.
48. European Food Safety A. Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate. EFSA Journal 2017; 15(9):e04979-n/a doi:102903/jefsa20174979.
49. Conrad A, Schroter-Kermani C, Hoppe HW, Ruther M, Pieper S, Kolossa-Gehring M. Glyphosate in German adults - time trend (2001 to 2015) of human exposure to a widely used herbicide. Int J Hyg Environ Health. 2017; 220(1):8–16. https://doi.org/10.1016/j.ijheh.2016.09.016.
50. Mills PJ, Kania-Korwel I, Fagan J, McEvoy LK, Laughlin GA, Barrett-Connor E. Excretion of the herbicide glyphosate in older adults between 1993 and 2016. JAMA. 2017;318(16):1610–1. https://doi.org/10.1001/jama.2017.11726.

RESULTS

PAPER 3

Manservisi *et al. Environmental Health* (2019) 18:15
https://doi.org/10.1186/s12940-019-0453-y

Environmental Health

RESEARCH Open Access

The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system

Fabiana Manservisi[1,2†], Corina Lesseur[3†], Simona Panzacchi[1], Daniele Mandrioli[1,4], Laura Falcioni[1], Luciano Bua[1], Marco Manservigi[1], Marcella Spinaci[2], Giovanna Galeati[2], Alberto Mantovani[5], Stefano Lorenzetti[5], Rossella Miglio[6], Anderson Martino Andrade[7], David Møbjerg Kristensen[8], Melissa J. Perry[9], Shanna H. Swan[3], Jia Chen[3] and Fiorella Belpoggi[1*]

Abstract

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

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

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

(Continued on next page)

* Correspondence: belpoggif@ramazzini.it
†Fabiana Manservisi and Corina Lesseur contributed equally to this work.
[1]Cesare Maltoni Cancer Research Center (CMCRC), Ramazzini Institute (RI), Via Saliceto, 3, 40010, Bentivoglio, Bologna, Italy
Full list of author information is available at the end of the article

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

Background

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

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

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

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

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

on human reproductive development from fetal to adult life [48].

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

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

Methods

Chemicals

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

Animals and experimental design

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

Two groups of SD rat dams and relative pups were treated with either glyphosate or Roundup Bioflow diluted in drinking water to achieve the desired glyphosate dose of 1.75 mg/kg bw/day. The F0 female breeders received the treatment through drinking water from gestation day (GD) 6 to the end of lactation, while the offspring (F1) continued to be exposed after weaning for additional 6 or 13 weeks. Glyphosate or Roundup solutions were freshly prepared on a daily basis depending on body weight and water consumption of dams or offspring, measured at scheduled time points. Preparation of drinking water solutions, quantification of glyphosate in water, and dosing

adjustments are described in detailed by Panzacchi et al. [36]. During pregnancy and lactation, embryos and offspring (F1) were all retained in the litter and might receive the test compounds mainly through their dams (F0). The day birth occurred was designated as post-natal day 1 (PND 1) for pups and lactation day 1 (LD 1) for dams. After weaning, on PND 28, offspring were randomly distributed in two cohorts: 8 M + 8F/group animals belonging to the 6-week cohort were sacrificed at PND 73 ± 2, i.e. 6 weeks after weaning; 10 M + 10F/group animals belonging to the 13-week cohort were sacrificed at PND 125 ± 2, i.e. 13 weeks after weaning. After weaning, the offspring (F1) were treated through drinking water until sacrifice. Altogether, 108 SD rats (54 males and 54 females) were enrolled in the post-weaning treatment phase.

Measurements in F0 dams and litters prior to weaning

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

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

Post weaning endpoints up to adulthood

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

Estrous cycle characterization

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

Necropsy

All the animals were anesthetized by inhalation of a mixture of CO_2/O_2 (70 and 30% respectively), and sacrificed by drawing blood by cava vein. The time and date of necropsy were recorded. Five days after weaning (corresponding to 49 ± 2 days of treatment), dams were sacrificed and the following organs were collected and alcohol fixed during necropsy: mammary glands (4 sites: axillary and inguinal, right and left), adrenal glands, uterus (including cervix), ovaries, vagina. The adrenal glands, uterus and ovaries were also weighed as soon as possible after dissection. For testosterone concentration determination, blood was collected and serum removed by centrifugation and stored at − 80 °C until analysis.

All male and female pups belonging to both cohorts were sacrificed on PND 73 ± 2 and PND 125 ± 2. The

following organs and tissues were collected and alcohol-fixed: mammary gland (4 sites: axillary and inguinal, right and left), thyroid and parathyroid, adrenal glands, bladder and prostate, seminal vesicle/coagulating gland, left and right testis with epididymis (half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at − 80 °C until evaluation), uterus (including cervix), ovaries and vagina.

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

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

Sperm analysis

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

Sperm counts, daily sperm production, and sperm transit time through the epididymis

At necropsy, half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at − 80 °C until evaluation. Spermatids resistant to the process of testicular homogenization and spermatozoa present in the caput/corpus and cauda epididymis were counted as previously described by Robb et al. [40] with slide adaptations described as follows. The tunica albuginea was removed from the (half) testicle, and a sample of the parenchyma was weighed and homogenized in 5 ml saline-TritonX-100 0.05%. The samples were then diluted 10–20 times in saline, and the mature spermatids resistant to homogenization (step 17–19 spermatids) were counted using a Thoma chamber. Four fields *per* animal were recorded, and the numbers of spermatids *per* gram of testis were calculated. To calculate the daily sperm production (DSP) these values were subsequently divided by 6.1, which is the number of days step 17–19 spermatids are present in the seminiferous epithelium [40]. Similarly, the segments of the epididymis (caput, corpus and cauda) were cut with a scissor, weighed, homogenized, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for each segment of the epididymis calculated. The epididymal sperm transit time through the epididymal caput/corpus and cauda was calculated by dividing the number of spermatozoa present in each portion of the epididymis by the DSP of the associated testis [2].

Sperm morphology

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

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

Histopathology

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

Hormone analysis

Serum concentration of free (fT) and total testosterone (TT); 5α-dihydrotestosterone (DHT); 17β-estradiol (E2) and Sex Hormone Binding Globulin (SHBG) were measured in duplicates by solid phase enzyme-linked immunosorbent assays (ELISAs). Blood sera, obtained and stored as described above, were used to assess the quantitative measurements in rat serum of E2, fT, TT, DHT and SHBG by ELISA based on the principle of the competitive binding, using the following commercial kits: "Estradiol rat ELISA" (#DEV9999), manufactured by Demeditec Diagnostics GmbH (Kiel, Germany), "Rat Free Testosterone (F-TESTO) ELISA" (#CSB-E0597r), "Rat Testosterone, T ELISA" (#CSB-E05100R); "Rat dihydrotestosterone (DHT) ELISA" (#CSB-E07879r), and "Rat sex hormone-binding

globulin (SHBG) ELISA" (#CSB-E12118r), manufactured by Cusabio Biotech Co. Ltd. (Houston, TX, USA).

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

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

Statistical methods

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

Results

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

RESULTS

PAPER 3

Table 1 Maternal and reproductive outcome of dams exposed to glyphosate or Roundup Bioflow throughout pregnancy and lactation

Parameter	Control	Glyphosate	Roundup
Gestational index (%)[a]	100 (8/8)	100 (8/8)	100 (8/8)
Mean gestational length (day)[b]	22.9	23.0	23.0
Relative weight gain during pregnancy (%)[d, e]	33.1 ± 1.8	32.4 ± 2.2	33.2 ± 1.4
Relative weight gain during lactation (%)[d, f]	3.1 ± 0.7	2.5 ± 0.5	2.9 ± 0.8
Total pups (n) delivered at PND 0[g]	120	115	124
Litter size (n)[d, h]	15 ± 1.3	14.4 ± 1.9	15.5 ± 1.7
Sex ratio at birth (%)[d, i]	53.6 ± 16.9	43.2 ± 9.9	45.4 ± 12.6
Mean live birth index (%)[d, l]	95.9 ± 9.4	93.9 ± 6.8	96.1 ± 5.8
Dams with reported stillbirths (n)	2	4	3
Stillborn (n)[m]	5	7	5
Survival index at PND 1 (%)[n]	90.8 ± 10.6	93.0 ± 8.3	91.5 ± 9.0
Survival index at PND 21 (%)[n]	90.0 ± 10.0	91.3 ± 8.3	88.1 ± 8.0

[a]Gestational index = (number of females with live born / number of females with evidence of pregnancy) × 100
[b]Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[d]Mean ± standard deviation
[e]Relative weight gain during pregnancy = relative weight on the last day of pregnancy minus relative weight on the first day treatment in pregnancy, i.e. GD 6 (weight on GD 6 = 100%)
[f]Relative weight gain during lactation = relative weight on LD 21 minus relative weight on the first day of lactation, i.e. LD 1 (weight on LD 1 = 100%)
[g]Live and stillborn pups are considered
[h]Mean number of pups per litter at PND 0 (within 24 h from delivery)
[i]Sex ratio at birth = (no. of male offspring / no. of total offspring) × 100
[l]Live birth index = (no. of offspring born alive / no. of offspring born) × 100
[m]Stillborn = no. of pups died in utero
[n]Survival index = (no. of live offspring at designated time-point / no. of pups born) × 100

Table 2 Effects of glyphosate or Roundup Bioflow exposure on developmental landmarks and sexual characteristics of pups

Parameter	Control	Glyphosate	Roundup
Number of male pups at PND 1	58	46	53
Male pups weight at PND 1 (g)[a]	6.8 ± 0.5	7.1 ± 0.2	6.8 ± 0.4
Male pups weaning weight (g)[a, b]	50.4 ± 4.4	53.5 ± 6.0	51.8 ± 5.8
Male AGD (mm) at PND 4[a, c]	4.02 ± 0.49	4.26 ± 0.38**	4.34 ± 0.30**°
Age (PND) at balano-preputial separation (BPS)	46.33 ± 1.85	46.78 ± 1.73	47.61 ± 2.77
Body weight at BPS (g)	202.50 ± 10.74	203.89 ± 16.68	207.50 ± 22.70
Number of female pups at PND 1	51	61	60
Female pups birth weight (g)[a]	6.4 ± 0.4	6.6 ± 0.4	6.5 ± 0.6
Female pups weaning weight (g)[a, b]	48.3 ± 5.1	50.4 ± 5.2	50.5 ± 5.1
Female AGD (mm) at PND 4[a, c]	1.70 ± 0.25	1.79 ± 0.21	1.86 ± 0.19**°
Age (PND) at vaginal opening (VO)[a]	35.56 ± 1.72	35.39 ± 1.5	35.61 ± 1.14
Body weight at VO (g)[a]	108.33 ± 6.18	108.06 ± 7.10	109.44 ± 8.73
Age (PND) at First Estrous (FE)[a, d]	39.88 ± 1.25	40.13 ± 1.46	42.63 ± 3.25#
Number of days between VO and FE[a]	4.75 ± 0.71	5.13 ± 0.64	7.00 ± 3.78

**Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight
°Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight and litter (random effect)
#Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
[a]Mean ± standard deviation
[b]Weaning weight corresponds to PND 25
[c]AGD = ano-genital distance
[d]First estrous (FE) was evaluated only in females belonging to the 6 week cohort

Table 3 Estrous cycle characterization in female rats belonging to the 13-week cohort

Time (%) in cycle stages	N. females	Control	Glyphosate	Roundup
Time (%) in Diestrus	10	55.24 ± 11.70	51.43 ± 5.41	52.86 ± 5.24
Time (%) in Proestrus	10	20.95 ± 7.51	23.33 ± 5.24	23.86 ± 3.76
Time (%) in Estrus	10	23.33 ± 5.24	25.24 ± 3.92	23.81 ± 2.24

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

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

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

- TT serum concentration significantly increased in Roundup-treated female offspring from the 13-week cohort compared to control animals ($p < 0.05$); TT showed an increase in the glyphosate-treated group, even if not statistically significant (Table 9). However, serum TT concentration did not differ by GBH exposure in the younger female offspring from 6-week cohort (Table 9) or in the male offspring (Table 8).
- In males, serum DHT concentration was markedly and significantly decreased in the Roundup-treated group (13-week cohort) compared to control animals ($p < 0.01$). The box plots and dot plots of DHT was added as supplementary material (Additional file 3: Figure S3).
- No significant differences in serum fT and SHBG concentrations were observed in males or females belonging to both the cohorts.
- E2 serum concentration did not show statistically significant differences in male offspring exposed to glyphosate or Roundup in both the cohorts. In females, right before sacrifice, the endocrine status (diestrus, proestrus and estrus) for individual rats was assessed by vaginal smears. We could not statistically evaluate E2 (as well as FSH, LH and PRL concentrations) in the different female groups with reference to the stages of the estrous cycle due to insufficient sample size after clustering for endocrine status.

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

- The ratio between TT and SHBG (e.g. TT/SHBG), currently used as an indicator of testosterone bioavailability and known as the fT index, was significantly increased ($p < 0.05$) in the Roundup-treated females (13-week cohort), but not 6-week females or in any of the males.
- The E2/SHBG ratio, an indicator of E2 bioavailability known as free estradiol index (FEI), significantly increased in Roundup-treated males belonging to the 6-week cohort ($p < 0.05$), whereas no effects were observed in E2/SHBG ratio in glyphosate-treated SD male rats.
- The fT/TT ratio significantly decreased in glyphosate- (6-week cohort) and in Roundup-treated males (13-week cohort) ($p < 0.05$) but not in any of the females.
- Both male and female Roundup-treated animals belonging to the 13-week cohort showed a marked decrease in DHT/TT ratio ($p < 0.01$). No statistically significant differences were observed in younger males and females (6-week cohort).
- No statistically significant differences were observed the E2/TT ratio in males and females.

Discussion

Roundup Bioflow, when administered to SD rats from in utero through adulthood at a dose level corresponding to the glyphosate RfD defined by the US EPA (1.75 mg/

RESULTS

PAPER 3

Table 4 Effects of glyphosate or Roundup Bioflow exposure on sperm parameters

Parameter	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Sperm number ($\times 10^6$/g testis)[a]	90.3 ± 22.0	83.7 ± 15.6	81.0 ± 12.0	109.4 ± 17.9	107.9 ± 11.3	119.6 ± 20.2
Daily sperm production ($\times 10^6$/g testis)[a]	14.8 ± 3.6	13.7 ± 2.6	13.3 ± 2.0	17.9 ± 2.9	17.7 ± 1.8	19.6 ± 3.3
Cauda epididimal sperm number ($\times 10^6$)[a]	51.2 ± 10.5	51.9 ± 10.5	50.9 ± 10.7	129.5 ± 28.5	125.9 ± 14.0	122.7 ± 11.0
Caput/corpus epididimal sperm number ($\times 10^6$)[a]	67.6 ± 6.1	66.5 ± 7.9	66.5 ± 10.6	97.0 ± 16.4	93.3 ± 13.0	93.2 ± 8.8
Sperm transit time through caput + corpus of epididymis (days)[a]	4.8 ± 1.3	5.0 ± 1.1	5.1 ± 1.0	5.5 ± 0.8	5.3 ± 1.0	4.8 ± 0.7
Sperm transit time through cauda of epididymis (days)[a]	3.5 ± 0.5	3.8 ± 0.8	3.9 ± 0.8	7.4 ± 1.8	7.2 ± 0.9	6.4 ± 1.1
Sperm transit time through epididymis in toto (days)[a]	8.3 ± 1.7	8.8 ± 1.5	8.9 ± 1.8	12.8 ± 2.2	12.5 ± 1.7	11.2 ± 1.7
Total abnormal sperm (%)[a]	6.3 ± 1.3	6.4 ± 1.8	6.3 ± 1.4	4.6 ± 1.4	4.3 ± 1.9	3.5 ± 1.3

[a]Mean ± standard deviation

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

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

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

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

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

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

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

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

Table 5 Organ weights and testosterone level in dams

	Control	Glyphosate	Roundup
No. of dams examined	8	8	8
Body weight (g)[a]	302 ± 10	306 ± 15	317 ± 13
Adrenal glands[a, b]	0.110 ± 0.030 [0.036 ± 0.009]	0.106 ± 0.013 [0.035 ± 0.005]	0.106 ± 0.045 [0.033 ± 0.005]
Uterus[a, b]	0.867 ± 0.285 [0.286 ± 0.091]	0.772 ± 0.129 [0.253 ± 0.046]	0.994 ± 0.239 [0.298 ± 0.076]
Ovaries[a, b]	0.239 ± 0.62 [0.079 ± 0.018]	0.235 ± 0.047 0.077 ± 0.017]	0.247 ± 0.052 [0.078 ± 0.016]
TT (ng/ml)[a]	3.77 ± 0.53	4.24 ± 1.48	3.90 ± 0.56

[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)

Table 6 Organ weights of male offspring

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Body weight (g)[a]	321 ± 19	326 ± 16	317 ± 23	458 ± 19	454 ± 19	449 ± 15
Adrenal glands[a, b]	0.070 ± 0.014 [0.022 ± 0.005]	0.071 ± 0.015 [0.022 ± 0.004]	0.063 ± 0.009 [0.020 ± 0.002]	0.085 ± 0.025 [0.019 ± 0.005]	0.083 ± 0.024 [0.018 ± 0.005]	0.144 ± 0.190 [0.032 ± 0.041]
Testis[a, b]	1.475 ± 0.052 [0.461 ± 0.023]	1.474 ± 0.077 [0.453 ± 0.023]	1.459 ± 0.100 [0.460 ± 0.017]	1.568 ± 0.065 [0.342 ± 0.013]	1.529 ± 0.076 [0.337 ± 0.013]	1.562 ± 0.068 [0.347 ± 0.012]
Epididymis[a, b]	0.370 ± 0.041 [0.115 ± 0.011]	0.343 ± 0.040 [0.105 ± 0.010]	0.368 ± 0.038 [0.116 ± 0.006]	0.595 ± 0.039 [0.130 ± 0.008]	0.563 ± 0.043 [0.124 ± 0.008]	0.553 ± 0.026* [0.123 ± 0.006]
Bladder/Prostate[a, b]	0.563 ± 0.080 [0.176 ± 0.026]	0.561 ± 0.099 [0.173 ± 0.036]	0.506 ± 0.090 [0.159 ± 0.022]	0.967 ± 0.086 [0.211 ± 0.024]	0.897 ± 0.160 [0.197 ± 0.033]	0.906 ± 0.190 [0.202 ± 0.045]
Seminal vesicles and coagulating gland[a, b]	1.129 ± 0.129 [0.353 ± 0.045]	1.110 ± 0.291 [0.339 ± 0.083]	1.235 ± 0.135 [0.389 ± 0.029]	2.049 ± 0.418 [0.446 ± 0.083]	1.879 ± 0.298 [0.414 ± 0.063]	2.055 ± 0.404 [0.457 ± 0.090]

*Statistically significant with Dunnett's test ($p < 0.05$)
[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio *100)

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

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

Table 7 Organ weights (g) of female offspring

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Body weight (g)[a]	225 ± 13	219 ± 11	223 ± 11	280 ± 20	283 ± 13	283 ± 13
Adrenal glands[a, b]	0.081 ± 0.012 [0.036 ± 0.005]	0.073 ± 0.009 [0.033 ± 0.004]	0.079 ± 0.012 [0.036 ± 0.005]	0.094 ± 0.019 [0.033 ± 0.005]	0.084 ± 0.019 [0.030 ± 0.007]	0.086 ± 0.018 [0.031 ± 0.006]
Uterus[a, b]	0.499 ± 0.114 [0.221 ± 0.059]	0.531 ± 0.162 [0.241 ± 0.069]	0.619 ± 0.221 [0.277 ± 0.099]	0.539 ± 0.082 [0.192 ± 0.025]	0.575 ± 0.137 [0.202 ± 0.047]	0.589 ± 0.111 [0.209 ± 0.043]
Ovaries[a, b]	0.181 ± 0.024 [0.081 ± 0.012]	0.169 ± 0.027 [0.077 ± 0.013]	0.172 ± 0.034 [0.077 ± 0.014]	0.192 ± 0.029 [0.068 ± 0.009]	0.182 ± 0.025 [0.064 ± 0.009]	0.186 ± 0.036 [0.065 ± 0.012]

[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)

Table 8 Effects of glyphosate or Roundup Bioflow exposure on hormones in males (mean ± SEM)

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
Serum Hormones						
No. of males examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
TT (ng/ml)	1.12 ± 0.12	1.02 ± 0.28	0.84 ± 0.11[a]	8.16 ± 2.86	7.65 ± 2.86	3.76 ± 0.90
fT (pg/ml)	14.53 ± 2.37	7.45 ± 2.23[b]	13.12 ± 3.74[b]	296.70 ± 123.70[c]	724.24 ± 419.22[c]	90.40 ± 29.44
DHT (pg/ml)	761.11 ± 136.21	575.28 ± 238.24	554.29 ± 145.16[a]	15,709.0 ± 5547.20	16,711.8 ± 6724.5	1980.2 ± 664.68[c,**]
SHBG (ng/ml)	861.20 ± 30.24	833.24 ± 21.15	856.78 ± 32.39	917.58 ± 16.94	906.36 ± 21.62	906.51 ± 18.89
E2 (pg/ml)	1.04 ± 0.21[a]	3.29 ± 1.85	6.19 ± 2.28[b]	3.66 ± 2.57[c]	1.08 ± 0.02[d]	6.00 ± 1.11
Plasma Hormones						
No. of males examined	7 (8)	6 (8)	7 (8)	10 (10)	10 (10)	10 (10)
FSH (ng/ml)	7.00 ± 1.38	6.43 ± 1.16	7.18 ± 0.68	2.32 ± 0.40[e]	2.18 ± 0.16[f]	2.90 ± 0.28[f]
LH (ng/ml)	3.76 ± 0.79	2.87 ± 0.63	4.41 ± 0.62	1.20 ± 0.17[e]	1.25 ± 0.24[d]	1.40 ± 0.18[f]
PRL (ng/ml)	3.83 ± 0.64	3.00 ± 0.64	4.31 ± 1.32	–	–	–
GH (ng/ml)	6.03 ± 4.32[g]	23.19 ± 21.17[h]	4.38 ± 1.94[i]	–	–	–
TSH (ng/ml)	4.23 ± 0.76	8.17 ± 1.58[*]	5.57 ± 0.31[b]	1.89 ± 0.20	2.53 ± 0.25	3.69 ± 0.42[**]
ACTH (pg/ml)	346.67 ± 35.52	255.18 ± 43.29	292.26 ± 26.22	–	–	–
BDNF (pg/ml)	99.49 ± 25.32[b]	148.85 ± 37.53	171.79 ± 14.65[*]	53.83 ± 14.77[c]	58.07 ± 13.83	45.15 ± 14.64

[*]Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
[**]Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]7 out 8
[b]6 out 8
[c]9 out 10
[d]8 out 10
[e]6 out 10
[f]7 out 10
[g]5 out 8
[h]3 out 8
[i]4 out 8

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

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

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

Table 9 Effects of glyphosate or Roundup Bioflow exposure on hormones in female (mean ± SEM)

	6 week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
Serum Hormones						
No. of females examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
TT (ng/ml)	0.66 ± 0.064	0.75 ± 0.12	0.68 ± 0.11[a]	0.51 ± 0.06	0.72 ± 0.10	0.72 ± 0.07[b] *
fT (pg/ml)	6.49 ± 1.00[c]	6.74 ± 1.89[d]	7.70 ± 1.35[a]	9.18 ± 2.49	12.04 ± 1.25	12.52 ± 1.76[b]
DHT (pg/ml)	294.28 ± 50.40	328.34 ± 51.93[a]	488.94 ± 114.68[a]	382.93 ± 52.14	460.09 ± 60.06	268.84 ± 45.56[b]
SHBG (ng/ml)	864.82 ± 30.24	952.75 ± 54.98	903.07 ± 29.61	968.27 ± 21.39	993.44 ± 32.79	964.81 ± 27.20
E2 (pg/ml)[g]	14.95 ± 7.24	32.24 ± 8.77	66.96 ± 25.17	18.08 ± 8.49	28.48 ± 13.71	43.91 ± 9.92
Plasma Hormones						
No. of females examined	7 (8)	7 (8)	6 (8)	7(10)	5(10)	6(10)
FSH (ng/ml) [g]	3.95 ± 2.50	2.67 ± 1.22	3.15 ± 1.65	1.58 ± 0.51	1.73 ± 0.64	1.46 ± 0.35
LH (ng/ml)[g]	5.75 ± 3.04	4.86 ± 1.93	4.52 ± 3.38	1.83 ± 0.25	2.35 ± 1.11	2.16 ± 1.28
PRL (ng/ml)[g]	102.34 ± 164.71[c]	27.49 ± 30.23	46.49 ± 31.03	–	–	–
GH (ng/ml)	12.61 ± 13.30	3.85 ± 0.97[c]	4.16 ± 2.84	–	–	–
TSH (ng/ml)	2.70 ± 1.13	3.02 ± 2.00	3.04 ± 1.53	1.29 ± 0.69[e]	1.93 ± 0.89[f]	3.03 ± 2.22[e]
ACTH (pg/ml)	331.60 ± 89.59	314.09 ± 170.60	354.95 ± 104.96	–	–	–
BDNF (pg/ml)	245.03 ± 155.68	483.62 ± 301.02	351.33 ± 177.28	25,399 ± 155.77[e]	377.79 ± 226.30[f]	249.39 ± 14,566[e]

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]7 out 8
[b]9 out 10
[c]6 out 8
[d]5 out 8
[e]4 out 10
[f]2 out 10
[g]Not statistically evaluated due to insufficient sample size after clustering on the basis of the estrous cycle

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

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

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

Hormone ratios (ng/ml)	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
fT/TT (x 10^{-3})	12.5 ± 1.35	8.67 ± 0.83[a] *	10.8 ± 0.45[b]	53.0 ± 11.1[c]	85.0 ± 27.5[c]	24.7 ± 4.03*
DHT/TT	0.658 ± 0.081	0.511 ± 0.087	0.614 ± 0.122[d]	2.195 ± 0.37	2.490 ± 0.70	0.65 ± 0.15[c] **
E2/TT (× 10^{-3})	1.01 ± 0.19[d]	2.65 ± 1.15	10.1 ± 5.17[d]	1.59 ± 1.23[c]	0.52 ± 0.14[e]	1.84 ± 0.42[c]
TT/SHBG (× 10^{-4})	12.90 ± 1.35	12.23 ± 3.25	10.1 ± 1.37[d]	88.30 ± 31.86	86.59 ± 33.21	40.68 ± 9.36
E2/SHBG (× 10^{-6})	1.24 ± 0.23[d]	3.90 ± 2.21	7.18 ± 2.66*	3.87 ± 2.69[c]	1.20 ± 0.003[f]	6.66 ± 1.25

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]6 out 8
[b]5 out 8
[c]9 out 10
[d]7 out 8
[e]8 out 10
[f]7 out 10

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

Hormone ratios (ng/ml)	6 week cohort			13 week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	9 (10)
fT/ TT (x 10^{-3})	9.17 ± 0.48^{a}	8.12 ± 0.55^{b}	11.90 ± 2.37^{c}	19.3 ± 5.20	19.0 ± 2.80	17.0 ± 1.66
DHT/ TT	0.428 ± 0.036	0.481 ± 0.060^{c}	0.718 ± 0.173^{c}	0.794 ± 0.11	0.702 ± 0.10	$0.383 \pm 0.07^{d**}$
E2/ TT[e]	0.012 ± 0.0003	0.045 ± 0.014	0.066 ± 0.028^{c}	0.019 ± 0.005	0.035 ± 0.012	0.068 ± 0.015^{d}
TT/SHBG (× 10^{-4})	7.85 ± 1.02	8.00 ± 1.26	7.81 ± 1.35^{c}	5.35 ± 0.62	7.27 ± 0.98	$7.5 \pm 0.61^{*}$
E2/SHBG (× 10^{-5})[e]	1.69 ± 0.78	3.51 ± 1.00	7.28 ± 2.55	1.90 ± 0.89	2.89 ± 1.41	4.87 ± 1.10^{d}

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]6 out 8
[b]5 out 8
[c]7 out of 8
[d]9 out 10
[e]Not statistically evaluated due to insufficient sample size after clustering on the basis of the estrous cycle

Furthermore, the number and timing of blood sample collection was limited to the final sacrifice of animals, considering that this was a pilot study and that in vivo blood sampling could lead to maternal and pups stress. Another source of uncertainty, which is currently difficult to assess, is the timing of blood sampling during the necropsy session (9.00 am – 3.00 pm); a circadian-dependent modulation of circulating hormones cannot be completely ruled out. Standard errors in different hormone concentrations were wide, in relation to the relatively small group sizes and the physiological variability of hormone concentrations. In females, the estrous cycle status at the time of necropsy is another important source of variability when analyzing sexual hormone profiles. However, even if sacrificing animals on a specific day of the cycle might improve the ability to observe changes in the baseline hormone concentrations, the issue of sacrificing animals in the same cycling period (e.g estrous) is still controversial. The updated OECD Test Guidelines on reproductive-developmental toxicity do not require the sacrifice of females in the same stage of estrous, only the examination of estrous cycle on the day of necropsy is recommend to allow correlation with histopathology in reproductive organs [34]. Finally, we could not study separately the adjuvant(s) present in Roundup Bioflow (corresponding to 16% of the formulation) since the exact ingredients formulation is a trade secret. These are supposed to be surfactants, diluents or adjuvants stabilizing glyphosate and allowing its penetration in plants. It is noteworthy that the commercial formulation used in this study, Roundup Bioflow, was the representative formulated product recently evaluated for the renewal of the approval of glyphosate in EU and considered in the European Food Safety Authority peer review (MON 52276) [16]. At the same time, we covered specific windows of susceptibility relevant for the potential androgenic effects of GBHs exposure, for example in utero life and pre-puberty. Indeed, the pre-natal and post-natal development, through to puberty, presents different susceptibility windows to EDC modes of action developing organisms with different and changing susceptibility as compared to adulthood [27, 47].

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

Conclusions

The present study demonstrates that Roundup Bioflow exposure, at a dose level considered as "safe" (1.75 mg/kg bw/day), from prenatal period to adulthood, induced endocrine effects and altered reproductive developmental parameters in male and female SD rats. Roundup

Bioflow exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of AGDs in both males and females, delay of FE and increased testosterone in females. Roundup Bioflow exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week). Overall, the Roundup Bioflow elicited more and more pronounced effects than the active ingredient itself, which only increased AGD and TSH concentration in male rats in the peripubertal window (6-week cohort). However, considering that retention of any GBH in the body may increase with prolonged exposures, a life-course study on GBHs encompassing intrauterine life through to advanced adulthood is needed to confirm and further explore the initial evidence of endocrine-related effects and developmental alterations emerged in this pilot study.

Additional files

Additional file 1: Figure S1. AGD index and AGD (Mean per litter) box plot (A) and dot plot (B). (DOCX 83 kb)

Additional file 2: Figure S2. First estrous box plot (A) and dot plot (B). (DOCX 33 kb)

Additional file 3: Figure S3. Male and female DHT box plot (A) and dot blot (B). (DOCX 86 kb)

Additional file 4: Figure S4. Effects of glyphosate or Roundup Bioflow exposure on hormones in males (mean ± SEM); coefficient of variation in square brackets. (DOCX 23 kb)

Additional file 5: Figure S5. Effects of glyphosate or Roundup Bioflow exposure on hormones in females (mean ± SEM); coefficient of variation in square brackets. (DOCX 23 kb)

Abbreviations

ACTH: Adrenocorticotropic hormone; AGD: Anogenital distance; BPS: Balano preputial separation; CMCRC: Cesare Maltoni Cancer Research Center; cRfD: Chronic Reference Dose; DHT: 5α-dihydrotestosterone; DLS: Daily sperm production; EDSP: Endocrine Disruptor Screening Program; EFSA: European Food safety Authority; ELISA: Enzyme-linked immunosorbent assays; EPA: Environmental Protection Agency; ERs: Estrogen receptors; FE: First estrous; FSH: Follicle stimulating hormone; fT: Free testosterone; GBH: Glyphosate-based herbicides; GD: Gestational day; GH: Growth hormone; LD: Lactating day; LH: Luteinizing hormone; LLD: Lower Limit of Detection; PND: Post Natal Day; PRL: Prolactin; RI: Ramazzini Institute; SD: Sprague-Dawley; SHBG: Sex Hormone Binding Globulin; StAR: Steroidogenic acute regulatory protein; TSH: Thyroid stimulating hormone; TT: Testosterone; US ADI: United States Acceptable Daily Intake; VO: Vaginal opening

Acknowledgements

We thank the over 30,000 associates and volunteers of the Ramazzini Institute that made this pilot study possible through their commitment and generosity. We thank the Municipality of Bologna, the Emilia-Romagna Region, and the International Society of Doctors for Environment for organizing several events to promote this pilot study; the Susan G. Komen Italia ONLUS for supporting this pilot study; "Coop Reno" and "Coopfond Fondo Mutualistico Legacoop" for supporting our research activity. We would like to thank Dr. Michela Polazzi, cytologist at the Ramazzini Institute Clinical Facility (Bologna, Italy), and Dr. Angela Lenzi, cytologist at the Department of Pathology, Histology and Cytology, F. Addari Institute, S. Orsola Malpighi Hospital (Bologna, Italy) for their technical assistance respectively in staining and evaluating animals vaginal smears.

Funding

This work was funded by Institutional funds of Ramazzini Institute, Bologna, Italy; Fondazione del Monte di Bologna e Ravenna (Bank Foundation), Bologna, Italy; Institutional funds of the Istituto Superiore di Sanità, Rome, Italy. CL and JC were supported by the NIH/NIEHS P30ES023515.

Availability of data and materials

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

Authors' contributions

FM, SP, DM participated in the design of the study, performed the animal experiments and sample collection, and drafted the manuscript. LF, LB, MM performed the in vivo phase of the study and contributed to the draft the manuscript. CL drafted the manuscript and performed the analyses on pituitary hormones. SL drafted the manuscript and performed the analyses on sexual hormones. MM and GG performed the sperm analysis and helped to draft the manuscript. RM supervised the statistical analysis. AM provided critical feedback on endocrine sensitive endpoints. AMA, DMK, SHS aided in interpreting the results on apical developmental endpoints (AGD and FE). JC, MJP discussed the results and contributed to draft the manuscript. FB was in charge of overall direction and planning of the pilot study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

N/A

Consent for publication

N/A

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Cesare Maltoni Cancer Research Center (CMCRC), Ramazzini Institute (RI), Via Saliceto, 3, 40010, Bentivoglio, Bologna, Italy. [2]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy. [3]Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York, USA. [4]Department of Agricultural Sciences, University of Bologna, Bologna, Italy. [5]Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy. [6]Department of Statistical Sciences, University of Bologna, Bologna, Italy. [7]Department of Physiology, Division of Biological Sciences, Federal University of Paraná, Curitiba, Paraná, Brazil. [8]Danish Headache Center, Department of Neurology, Rigshospitalet, University of Copenhagen, 1165 Copenhagen, Denmark. [9]Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, Washington, DC, USA.

Received: 26 November 2018 Accepted: 8 February 2019
Published online: 12 March 2019

References

1. Altamirano GA, Delconte MB, Gomez AL, Ingaramo PI, Bosquiazzo VL, Luque EH, et al. Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in wistar male rats. Food Chem Toxicol. 2018;118:111–8.
2. Amann RP, Johnson L, Thompson DL Jr, Pickett BW. Daily spermatozoal production, epididymal spermatozoal reserves and transit time of spermatozoa through the epididymis of the rhesus monkey. Biol Reprod. 1976;15:586–92.
3. Anifandis G, Katsanaki K, Lagodonti G, Messini C, Simopoulou M, Dafopoulos K, et al. The effect of glyphosate on human sperm motility and sperm DNA fragmentation. Int J Environ Res Public Health. 2018;15:1117.

4. Arbuckle TE, Lin Z, Mery LS. An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. Environ Health Perspect. 2001;109:851–7.
5. Benachour N, Seralini GE. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem Res Toxicol. 2009;22:97–105.
6. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. Environ Sci Eur. 2016;28:3.
7. Bielohuby M, Popp S, Bidlingmaier M. A guide for measurement of circulating metabolic hormones in rodents: pitfalls during the pre-analytical phase. Mol Metab. 2012;1:47–60.
8. Chapin RE, Heindel JJ. Methods for evaluation of rat epididymal sperm morphology; 1993.
9. Clair E, Linn L, Travert C, Amiel C, Seralini GE, Panoff JM. Effects of roundup((r)) and glyphosate on three food microorganisms: Geotrichum candidum, lactococcus lactis subsp. Cremoris and lactobacillus delbrueckii subsp. Bulgaricus. Curr Microbiol. 2012;64:486–91.
10. Dai P, Hu P, Tang J, Li Y, Li C. Effect of glyphosate on reproductive organs in male rat. Acta Histochem. 2016;118:519–26.
11. Dallegrave E, Mantese FD, Oliveira RT, Andrade AJ, Dalsenter PR, Langeloh A. Pre- and postnatal toxicity of the commercial glyphosate formulation in wistar rats. Arch Toxicol. 2007;81:665–73.
12. Dalsager L, Christensen LE, Kongsholm MG, Kyhl HB, Nielsen F, Schoeters G, et al. Associations of maternal exposure to organophosphate and pyrethroid insecticides and the herbicide 2,4-d with birth outcomes and anogenital distance at 3 months in the Odense child cohort. Reprod Toxicol (Elmsford, NY). 2018;76:53–62.
13. Dean A, Smith LB, Macpherson S, Sharpe RM. The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. Int J Androl. 2012;35:330–9.
14. Defarge N, Spiroux de Vendômois J, Séralini GE. Toxicity of formulants and heavy metals in glyphosate-based herbicides and other pesticides. Toxicol Rep. 2018;5:156–63.
15. Dickerson SM, Cunningham SL, Patisaul HB, Woller MJ, Gore AC. Endocrine disruption of brain sexual differentiation by developmental pcb exposure. Endocrinology. 2011;152:581–94.
16. EFSA EFSA. 2017. Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate.
17. EPA. 1993. Re-registration eligibility decision (red) glyphosate: Epa-738-r-93-014.
18. Foresta C, Valente U, Di Nisio A, Cacco N, Magagna S, Cosci I, et al. Anogenital distance is associated with genital measures and seminal parameters but not anthropometrics in a large cohort of young adult men. Hum Reprod (Oxford, England). 2018;33:1628–35.
19. Gasnier C, Dumont C, Benachour N, Clair E, Chagnon M-C, Séralini G-E. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009;262:184.
20. Gaytan F, Morales C, Leon S, Heras V, Barroso A, Avendaño MS, et al. Development and validation of a method for precise dating of female puberty in laboratory rodents: the puberty ovarian maturation score (pub-score). Sci Rep. 2017;7:46381.
21. Guerrero Schimpf M, Milesi MM, Luque EH, Varayoud J. Glyphosate-based herbicide enhances the uterine sensitivity to estradiol in rats. J Endocrinol. 2018;239(2):197-213.
22. Hood R. Developmental and reproductive toxicology - a practical approach; 2006.
23. Kuriyama SN, Chahoud I. In utero exposure to low-dose 2,3',4,4',5-pentachlorobiphenyl (pcb 118) impairs male fertility and alters neurobehavior in rat offspring. Toxicology. 2004;202:185–97.
24. Lewis EM, Barnett JF, Freshwater L, Hoberman AM, Christian MS. Sexual maturation data for crl Sprague-dawley rats: criteria and confounding factors. Drug Chem Toxicol. 2002;25:437–58.
25. Linder RE, Strader LF, Slott VL, Suarez JD. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. Reprod Toxicol (Elmsford, NY). 1992;6:491–505.
26. Manservisi F, Falcioni L, Bua L, Menghetti I, Mandrioli D, Galeati G, Spinaci M, Tamanini C, Belpoggi F. Control data on endocrine sensitive endpoints for untreated Sprague-dawley rats from the ramazzini institute colony. Eur J Oncol. 2018;23(2):80–5.
27. Mantovani A, Fucic A. Puberty dysregulation and increased risk of disease in adult life: possible modes of action. Reprod Toxicol (Elmsford, NY). 2014;44:15–22.
28. Marino M, Masella R, Bulzomi P, Campesi I, Malorni W, Franconi F. Nutrition and human health from a sex-gender perspective. Mol Asp Med. 2011;32:1–70.
29. Mesnage R, Phedonos A, Biserni M, Arno M, Balu S, Corton JC, et al. Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents. Food Chem Toxicol. 2017;108:30–42.
30. Milesi MM, Lorenz V, Pacini G, Repetti MR, Demonte LD, Varayoud J, et al. Perinatal exposure to a glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in wistar rats. Arch Toxicol. 2018;92:2629–43.
31. Myers JP, Antoniou MN, Blumberg B, Carroll L, Colborn T, Everett LG, et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ Health. 2016;15:19.
32. NTP. Multigenerational reproductive study of genistein (cas no. 446-72-0) in Sprague-dawley rats (feed study). Natl Toxicol Program Tech Rep Ser. 2008;539:1–266.
33. OECD. Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption, Series on testing and assessment. No. 150. Paris: OECD Publishing; 2014.
34. OECD. 2018. Test no. 443: Extended one-generation reproductive toxicity study.
35. Owagboriaye FO, Dedeke GA, Ademolu KO, Olujimi OO, Ashidi JS, Adeyinka AA. Reproductive toxicity of roundup herbicide exposure in male albino rat. Exp Toxicol Pathol. 2017;69:461–8.
36. Panzacchi S, Mandrioli D, Manservisi F, Bua L, Falcioni L, Spinaci M, et al. The ramazzini institute 13-week study on glyphosate-based herbicides at human-equivalent dose in Sprague dawley rats: study design and first in-life endpoints evaluation. Environ Health. 2018;17:52.
37. Parvez S, Gerona RR, Proctor C, Friesen M, Ashby JL, Reiter JL, et al. Glyphosate exposure in pregnancy and shortened gestational length: a prospective Indiana birth cohort study. Environ Health. 2018;17:23.
38. Perego MC, Caloni F, Cortinovis C, Schutz LF, Albonico M, Tsuzukibashi D, et al. Influence of a roundup formulation on glyphosate effects on steroidogenesis and proliferation of bovine granulosa cells in vitro. Chemosphere. 2017;188:274–9.
39. Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect. 2005;113:716–20.
40. Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil. 1978;54:103–7.
41. Romano MA, Romano RM, Santos LD, Wisniewski P, Campos DA, de Souza PB, et al. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch Toxicol. 2012a;86:663–73.
42. Romano RM, Romano MA, Bernardi MM, Furtado PV, Oliveira CA. Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch Toxicol. 2010;84:309–17.
43. Savitz DA, Arbuckle T, Kaczor D, Curtis KM. Male pesticide exposure and pregnancy outcome. Am J Epidemiol. 1997;146:1025–36.
44. Schinder AF, Poo M. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci. 2000;23:639–45.
45. Sofikitis N, Giotitsas N, Tsounapi P, Baltogiannis D, Giannakis D, Pardalidis N. Hormonal regulation of spermatogenesis and spermiogenesis. J Steroid Biochem Mol Biol. 2008;109:323–30.
46. Sritana N, Suriyo T, Kanitwithayanun J, Songvasin BH, Thiantanawat A, Satayavivad J. Glyphosate induces growth of estrogen receptor alpha positive cholangiocarcinoma cells via non-genomic estrogen receptor/erk1/2 signaling pathway. Food Chem Toxicol. 2018;118:595–607.
47. Swan SH, Sathyanarayana S, Barrett ES, Janssen S, Liu F, Nguyen RH, et al. First trimester phthalate exposure and anogenital distance in newborns. Hum Reprod (Oxford, England). 2015;30:963–72.
48. Thankamony A, Pasterski V, Ong KK, Acerini CL, Hughes IA. Anogenital distance as a marker of androgen exposure in humans. Andrology. 2016;4:616–25.
49. Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J. Glyphosate induces human breast cancer cells growth via estrogen receptors. Food Chem Toxicol. 2013;59:129–36.

50. US E. 2015. Weight of evidence analysis of potential interaction with the estrogen, androgen or thyroid pathways. Glyphosate.
51. Vandenberg LN, Blumberg B, Antoniou MN, Benbrook CM, Carroll L, Colborn T, et al. Is it time to reassess current safety standards for glyphosate-based herbicides? J Epidemiol Community Health. 2017;71:613.
52. Walsh LP, McCormick C, Martin C, Stocco DM. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (star) protein expression. Environ Health Perspect. 2000;108:769–76.
53. Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE Jr. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. Toxicol Sci. 2002;65:71–86.
54. Wu XY, Li ZL, Wu CY, Liu YM, Lin H, Wang SH, et al. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-dawley rats. Endocr J. 2010;57:201–9.

ADDITIONAL RESULTS OF STAGE 1

Pathology:

Macroscopically, not detectable pathological differences among treated groups and controls were observed during the final sacrifices. **In dams, absolute brain and liver weight was increased in the Roundup group**. The ratio was not significantly affected due to a higher (even if not statistically significant with the ANOVA method) dam body weight. Kidney and adrenal glands weight was not affected by the treatment (*see Table 5*). No significant differences in organ weight were observed in both cohorts of male and female offspring.

***Table 5**- Dam's organ weight.*

Dams	**Control**	**Glyphosate**	**Roundup**
No.	8	8	8
Body weight (g) [a]	302.50 ± 10.35	306.25 ± 15.53	317.50 ± 12.82
Brain and cerebellum [a, b]	1.899 ± 0.119 [0.628 ± 0.042]	1.956 ± 0.121 [0.639 ± 0.038]	**2.058 ±0.050**** [0.649 ± 0.023]
Liver [a, b]	9.760 ± 0.118 [3.220 ± 0.261]	10.502 ± 0.882 [3.427 ± 0.178]	**10.907 ± 0.949*** [3.434 ± 0.240]
Kidneys [a, b]	2.065 ± 0.167 [0.682 ± 0.032]	2.089 ± 0.088 [0.683 ± 0.034]	2.164 ± 0.242 [0.682 ± 0.073]
Adrenal glands[a, b]	0.110 ± 0.030 [0.036 ± 0.009]	0.106 ± 0.013 [0.035 ± 0.005]	0.106 ± 0.045 [0.033 ± 0.005]

[a]: Mean ± standard deviation

[b]: Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio *100)

*: Statistically significant with Dunnett's test (p≤0.05)

**: Statistically significant with Dunnett's test (p≤0.01)

In **dams**, we observed a general statistical significant increase in animals bearing kidney non-neoplastic lesions in particular **renal tubule degeneration** (p=0.041) (*Figure 5 A*) and **focal minimal inflammation** (p=0.007) (*Figure 5 B*), only in the **Roundup-treated** animals compared to control group (*Table 6*).

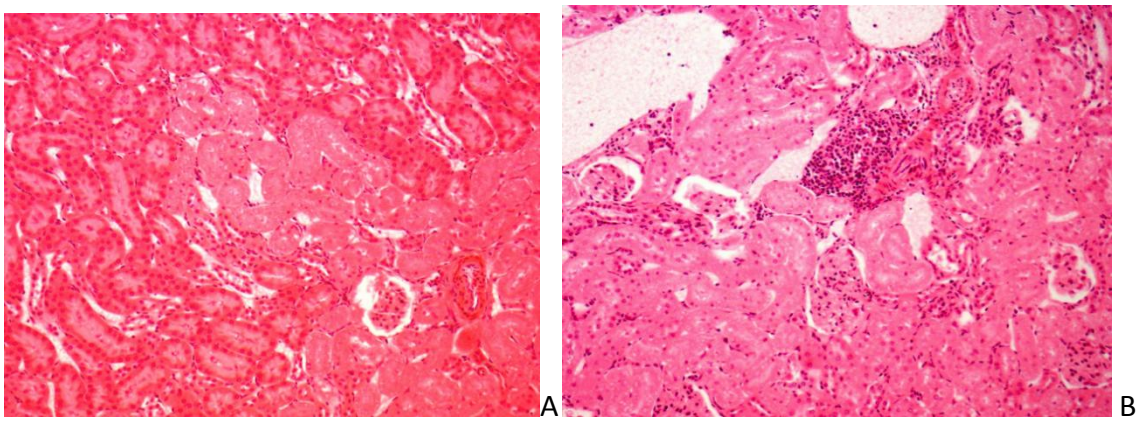
Table 6 *- Main kidney pathological non-neoplastic lesions in dams (animal bearing lesions)*

		Control		Glyphosate		Roundup	
		No.	%	No.	%	No.	%
Urinary system							
Kidney	Hyalinization, moderate	1	12.5	2	25.0	1	12.5
	Degeneration, mild, renal tubule	2	25.0	3	37.5	7	**87.5***
	Inflammation, mild, peritubular	2	25.0	2	25.0	2	25.0
	Inflammation, focal, minimal	0	-	1	12.5	6	**75.0****
	Total [a]	3	37.5	5	62.5	8	**100.0***

[a] The same animal can bear one or more types of lesions in the same organ, and it is plotted only once.

* Statistically significant (p≤0.05) using Fisher's exact test (two-tail)

** Statistically significant (p≤0.01) using Fisher's exact test (two-tail)



A B

Figure 5 *- Kidney lesions in dams (renal tubule degeneration -A 20X and minimal focal inflammation-B 20X)*

In the offspring, no significant histopathological differences related to the treatment were observed among groups. Two sporadic neoplastic lesions recorded in the female-Glyphosate treated group deserve attentions: 1) a lipoma of the peritoneum in the 6-week cohort (*see Figure 6*) a mammary gland adenocarcinoma in the 13-week cohort (*see Figure 7*). Both lesions are not statistically significant if related to the control group.

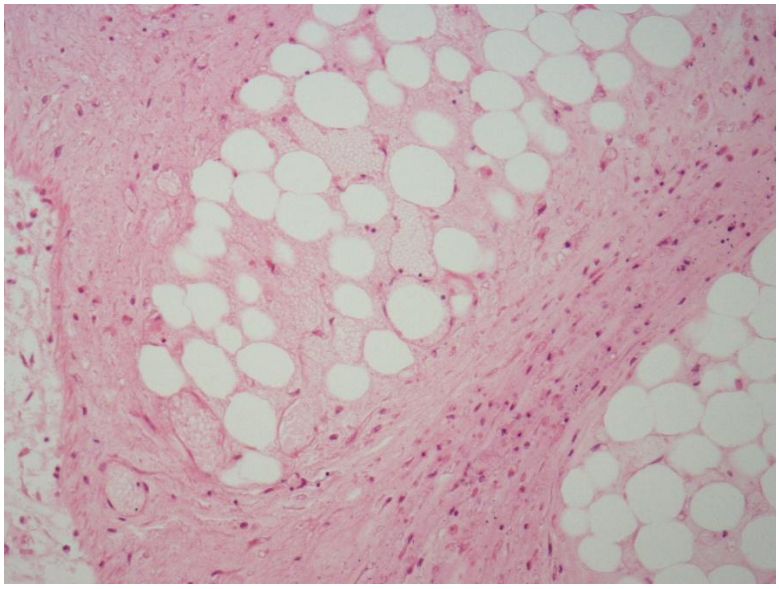


Figure 6*- Lipoma of the peritoneum in a female rat 10 weeks old and Glyphosate-treated.*

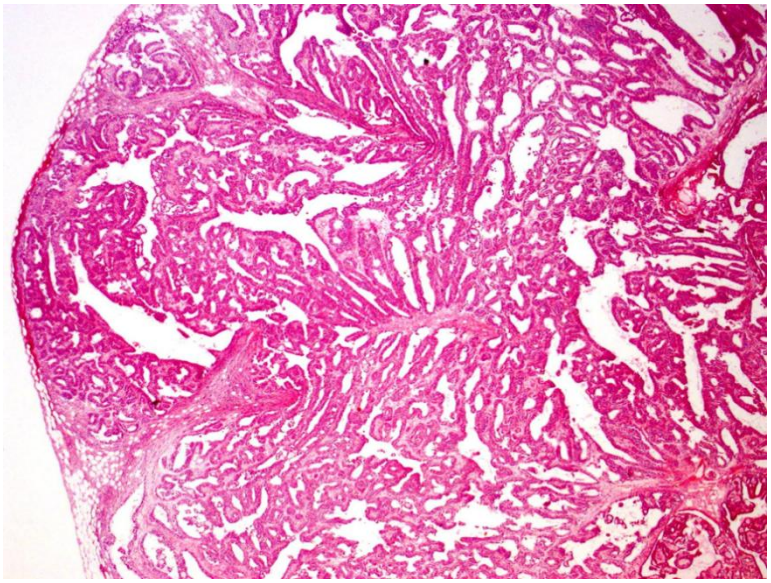


Figure 7 - *Mammary gland adenocarcinoma in a female rat 17 weeks old and Glyphosate-treated.*

Haematological biochemical blood analysis and urinalysis

Data of haematological and biochemical blood analysis are given in Tables 7-12.

Few statistically significant differences in both haematological and biochemical analysis were observed among groups belonging to the **6-week cohort** (*see details in Table 7-12*):

- a decrease ($p<0.05$) in mean blood glucose level in females treated with Roundup, even if this slight difference was not of biological importance because rats were not fasted before blood collection.
- **A decrease ($p<0.05$) in percentage of monocyte** in both male and female rats **Roundup-treated** compared to controls; this result was also confirmed in female rats belonging to the 13-week cohort (*Tables 11-12*).

In the **13-week cohort**, we observed, mainly in the Roundup treatment group, the following statistically significant differences in biochemical parameters:

- **Inorganic Phosphate** was statistically significantly **increased** in both **male ($p<0.05$)** and **female ($p<0.01$)** belonging to the **Roundup** group (*Tables 7-8*).
- A **decrease in creatinine concentration** in **Roundup-treated females** ($p<0.001$); even if this fluctuation is very close to the CMCRC range of SD rats belonging to the historical controls (*Table 8*).
- A **decrease in total protein** in **Roundup-treated male** rats ($p<0.01$) together with a slight **decrease in globulins concentration** in the same group ($p<0.01$) (*Table 7*).
- A small but statistically significant **decrease** ($p<0.05$) in **sodium concentration** in **Glyphosate-treated males** (*Table 7*) even if sodium level fluctuations are common and often associated, such as glucose, with non-fasting of animals.

Statistically significant changes were also observed in haematological parameters in the **13-week cohort**:

- A modest but statistically significant increase for the **total number of platelets** ($p<0.05$), the **Calculated Distribution Width of Erythrocytes, Coefficient of Variation** ($p<0.05$) and the **Plateletcrit Value** ($p<0.01$) in **Roundup-treated males** (*Table 9, part II*). All the other parameters related to the red blood cell population did not differ as compared with those of rats in the normal control group.
- Total Number of Leucocytes was generally higher in Roundup-treated animals belonging to both the cohorts compared to the control group. In **Roundup-treated**

female rats the **increase in the number of leucocytes** was statistically significant ($p<0.05$) (*Table 12*).

- **Lymphocytes were increased**, both in absolute concentration (male: $p<0.05$; female $p< 0.01$) and in percentage (female: $p<0.01$) in **Roundup-treated** rats (*Table 12*).
- **Neutrophil count and percentage decreased** in both in **Glyphosate and Roundup-treated female** rats (respectively $p<0.05$ and $p<0.01$) (*Table 12*).
- **Eosinophil percentage** was statistically significantly **decreased in Roundup-treated females** ($p<0.05$) (*Table 12*)**.**
- **Monocyte percentage** was statistically significantly **decreased** in both **male** ($p<0.05$; *Table 11*) **and female** rats **Roundup-treated** ($p<0.01$; *Table 12*)**.**

No statistically significant differences among treated and control groups were observed in urinalysis parameters (data not shown).

Table 7 *- Male offspring clinical chemistry (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Glucose (mg/dL)	99.63 ± 29.06	82.25 ± 10.43	84.63 ± 6.63	120.50 ± 18.76	**108.70 ± 17.19***	141.20 ± 27.26
Blood Urea Nitrogen (mg/dL)	12.75 ± 1.49	12.25 ± 1.04	13.88 ± 1.25	13.10 ± 1.29	11.80 ± 1.03	**11.50 ± 0.71***
Creatinine (mg/dL)	0.41 ± 0.04	0.44 ± 0.05	0.40 ± 0.00	0.35 ± 0.08	0.31 ± 0.06	0.32 ± 0.04
Inorganic Phosphate (mg/dL)	12.46 ± 0.90	12.93 ± 1.22	12.85 ± 0.78	10.06 ± 0.66	10.10 ± 0.78[a]	**10.92 ± 0.59***
Total Protein (g/dL)	6.58 ± 0.31	6.40 ± 0.19	6.41 ± 0.38	7.04 ± 0.22	6.79 ± 0.29	**6.58 ± 0.25****
Calcium (mg/dL)	10.11 ± 0.27	10.18 ± 0.32	10.25 ± 0.26	10.03 ± 0.25	8.78 ± 2.94[a]	9.97 ± 0.25
Albumin (g/dL)	3.15 ± 0.17	3.05 ± 0.11	3.03 ± 0.18	3.23 ± 0.19	3.13 ± 0.24	3.16 ± 0.16
Globulins (g/dL)	3.43 ± 0.28	3.35 ± 0.25	3.39 ± 0.34	3.81 ± 0.26	3.66 ± 0.21	**3.42 ± 0.26****
Alanine Aminotransferase (U/L)	46.63 ± 8.43	46.75 ± 5.55	51.13 ± 6.90	54.10 ± 7.39	53.70 ± 6.58	50.50 ± 7.65
Alkaline Phosphatase (U/L)	195.75 ± 13.59	192.25 ± 8.38	199.25 ± 25.56	109.80 ± 14.38	103.00 ± 19.36	104.20 ± 12.31
Gamma Glutamyl Transferase (U/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total Bilirubin (mg/dL)	0.19 ± 0.08	0.23 ± 0.07	0.23 ± 0.14	0.36 ± 0.10	0.36 ± 0.11	0.30 ± 0.07
Cholesterol (mg/dL)	23.88 ± 3.14	25.75 ± 5.50	28.50 ± 9.43	20.90 ± 2.60	20.70 ± 3.77	18.40 ± 4.62
Sodium (mmol/L)	146.13 ± 1.55	144.50 ± 2.83	146.75 ± 4.27	146.10 ± 1.91	**143.80 ± 1.48***	145.50 ± 2.59
Potassium (mmol/L)	6.78 ± 1.16	6.68 ± 1.27	7.11 ± 1.01	7.40 ± 1.09	8.12 ± 0.65	7.76 ± 0.91
Chloride (mmol/L)	104.00 ± 1.69	104.38 ± 1.30	105.50 ± 1.60	104.50 ± 1.08	104.30 ± 1.42	104.10 ± 1.20

[a]: analysis performed on 9 samples of 10

*: Statistically significant ($p \leq 0.05$) with Dunn's test

**: Statistically significant ($p \leq 0.01$) with Dunn's test

***Table 8**- Female offspring clinical chemistry (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Glucose (mg/dL)	95.88 ± 6.03	91.88 ± 18.11	**81.63 ± 10.16***	115.80 ± 23.58	**94.10 ± 12.08***	93.00 ± 11.34
Blood Urea Nitrogen (mg/dL)	17.25 ± 3.77	15.50 ± 2.39	17.00 ± 3.02	13.10 ± 1.52	13.00 ± 2.67	11.90 ± 1.29
Creatinine (mg/dL)	0.48 ± 0.05	0.44 ± 0.05	0.45 ± 0.05	0.40 ± 0.07	0.34 ± 0.05	**0.31 ± 0.03****
Inorganic Phosphate (mg/dL)	11.40 ± 0.82	11.65 ± 0.81	11.76 ± 0.62	8.38 ± 1.07	9.84 ± 1.20	**10.31 ± 0.58****
Total Protein (g/dL)	6.75 ± 0.39	6.71 ± 0.33	6.90 ± 0.16	7.55 ± 0.31	7.22 ± 0.35	7.45 ± 0.22
Calcium (mg/dL)	9.99 ± 0.32	10.15 ± 0.47	10.13 ± 0.49	10.10 ± 0.23	10.08 ± 0.28	10.17 ± 0.39
Albumin (g/dL)	3.58 ± 0.23	3.55 ± 0.26	3.54 ± 0.18	3.75 ± 0.16	3.66 ± 0.26	3.71 ± 0.15
Globulins (g/dL)	3.18 ± 0.37	3.16 ± 0.29	3.36 ± 0.26	3.80 ± 0.22	3.56 ± 0.18	3.74 ± 0.18
Alanine Aminotransferase (U/L)	38.63 ± 4.44	39.75 ± 6.45	43.38 ± 4.66	39.40 ± 9.57	42.30 ± 8.78	37.70 ± 3.89
Alkaline Phosphatase (U/L)	121.38 ± 14.37	126.13 ± 18.52	130.38 ± 13.23	80.80 ± 9.60	87.60 ± 13.88	75.70 ± 17.90
Gamma Glutamyl Transferase (U/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total Bilirubin (mg/dL)	0.25 ± 0.13	0.24 ± 0.12	0.29 ± 0.14	0.32 ± 0.09	0.33 ± 0.11	0.38 ± 0.08
Cholesterol (mg/dL)	30.50 ± 4.60	29.75 ± 6.58	30.63 ± 4.14	48.40 ± 6.17	42.90 ± 6.49	48.00 ± 4.64
Sodium (mmol/L)	144.75 ± 3.85	142.38 ± 2.77	144.25 ± 2.71	145.40 ± 2.50	146.50 ± 2.22	146.60 ± 2.84
Potassium (mmol/L)	7.26 ± 1.28	8.40 ± 0.75	7.70 ± 1.68	7.89 ± 1.37	8.37 ± 0.99	8.42 ± 0.80
Chloride (mmol/L)	105.13 ± 1.96	104.88 ± 2.23	105.13 ± 1.36	104.40 ± 1.58	105.30 ± 1.57	105.30 ± 2.00

*: Statistically significant ($p \leq 0.05$) with Dunn's test

**: Statistically significant ($p \leq 0.01$) with Dunn's test

***Table 9 (part I)**- Male offspring haematology (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Hematocrit Value: erythrocyte ratio of total blood volume (%)	45.10 ± 1.46	45.20 ± 1.56	45.53 ± 1.13	47.66 ± 1.40	47.88 ± 1.24	48.54 ± 1.92
Hemoglobin Concentration (g/dL)	15.13 ± 0.43	15.18 ± 0.50	15.25 ± 0.35	16.00 ± 0.41	16.16 ± 0.42	16.36 ± 0.58
Total Number of Erythrocyte (T/L)	7.69 ± 0.25	7.77 ± 0.27	7.79 ± 0.18	8.65 ± 0.16	8.71 ± 0.21	8.85 ± 0.37
Reticulocyte (%)	4.44 ± 0.75	4.26 ± 0.55	4.52 ± 0.30	3.66 ± 0.72	3.45 ± 0.87	3.16 ± 0.93
Reticulocyte (K/uL)	340.25 ± 53.33	329.85 ± 39.93	351.65 ± 22.95	315.52 ± 59.42	299.53 ± 73.25	279.57 ± 81.87
Mean Erythrocyte Volume in Total Sample (fL)	58.69 ± 0.75	58.21 ± 0.43	58.46 ± 0.72	55.11 ± 0.94	54.97 ± 0.50	54.86 ± 0.56
Mean Hemoglobin Volume per Red Blood Cell (pg)	19.70 ± 0.19	19.54 ± 0.15	19.58 ± 0.17	18.50 ± 0.24	18.55 ± 0.21	18.50 ± 0.16
Mean Hemoglobin Concentration of Eythrocytes (g/dL)	33.54 ± 0.31	33.59 ± 0.29	33.51 ± 0.25	33.58 ± 0.34	33.75 ± 0.22	33.70 ± 0.28
Calculated Distribution Width of Erythrocytes, Standard Deviation (fL)	30.25 ± 0.64	30.16 ± 0.29	30.36 ± 0.34	31.93 ± 1.34	32.34 ± 1.04	32.59 ± 0.74

*: Statistically significant ($p \leq 0.05$) with Dunn's test

**: Statistically significant ($p \leq 0.01$) with Dunn's test

***Table 9 (part II)**- Male offspring haematology (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Calculated Distribution Width of Erythrocytes, Coefficient of Variation (%)	17.18 ± 0.68	17.71 ± 0.76	17.74 ± 0.83	21.41 ± 0.54	21.79 ± 0.41	**22.02 ± 0.36***
Total Number of Platelets (G/L)	987.00 ± 76.62	1048.88 ± 49.95	1057.63 ± 62.14	983.60 ± 68.97	1049.10 ± 119.24	**1084.20 ± 69.53***
Platelet Distribution Width: Degree of Variation in Size of Platelet Population (fL)	7.90 ± 0.24	7.95 ± 0.15	7.80 ± 0.14	8.28 ± 0.16	8.44 ± 0.21	8.45 ± 0.34
Mean Platelet Volume (fL)	7.10 ± 0.17	7.08 ± 0.10	7.00 ± 0.13	7.17 ± 0.13	7.30 ± 0.18	7.34 ± 0.18
Plateletcrit Value (%)	0.70 ± 0.05	0.74 ± 0.04	0.74 ± 0.04	0.70 ± 0.05	0.77 ± 0.10	**0.80 ± 0.06****

*: Statistically significant ($p \leq 0.05$) with Dunn's test

**: Statistically significant ($p \leq 0.01$) with Dunn's test

Table 10 (part I) *- Female offspring haematology (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Hematocrit Value: erythrocyte ratio of total blood volume (%)	44.10 ± 1.65	45.61 ± 1.89	46.06 ± 1.52	46.77 ± 1.83	47.72 ± 1.78	46.42 ± 2.30
Hemoglobin Concentration (g/dL)	15.01 ± 0.52	15.59 ± 0.64	15.68 ± 0.49	15.99 ± 0.42	16.07 ± 0.54	15.65 ± 0.63
Total Number of Erythrocyte (T/L)	7.49 ± 0.27	7.73 ± 0.35	7.74 ± 0.24	8.08 ± 0.29	8.21 ± 0.25	8.01 ± 0.36
Reticulocyte (%)	4.79 ± 0.40	4.44 ± 0.85	4.76 ± 0.84	4.58 ± 0.64	4.50 ± 0.77	4.49 ± 0.61
Reticulocyte (K/uL)	358.26 ± 27.99	342.40 ± 62.73	369.18 ± 71.72	370.51 ± 53.27	369.31 ± 64.89	359.15 ± 48.97
Mean Erythrocyte Volume in Total Sample (fL)	58.88 ± 0.66	59.03 ± 0.68	59.50 ± 0.49	57.88 ± 0.54	58.11 ± 0.78	57.92 ± 0.66
Mean Hemoglobin Volume per Red Blood Cell (pg)	20.05 ± 0.21	20.18 ± 0.25	20.24 ± 0.11	19.79 ± 0.73	19.55 ± 0.19	19.52 ± 0.17
Mean Hemoglobin Concentration of Eythrocytes (g/dL)	34.05 ± 0.29	34.16 ± 0.28	34.04 ± 0.37	34.24 ± 1.41	33.69 ± 0.28	33.72 ± 0.37
Calculated Distribution Width of Erythrocytes, Standard Deviation (fL)	28.70 ± 0.45	28.79 ± 0.47	29.01 ± 1.04	30.84 ± 0.73	31.15 ± 1.00	31.33 ± 1.00

[a]: analysis performed on 9 samples of 10

Table 10 (part II) *- Female offspring haematology (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Calculated Distribution Width of Erythrocytes, Coefficient of Variation (%)	15.74 ± 0.48	16.21 ± 0.90	16.41 ± 1.09	19.27 ± 0.65	19.45 ± 0.61	19.38 ± 0.76
Total Number of Platelets (G/L)	1065.75 ± 69.15	1047.88 ± 83.23	1029.88 ± 83.96	980.50 ± 102.75	888.70 ± 227.26	914.40 ± 323.33
Platelet Distribution Width: Degree of Variation in Size of Platelet Population (fL)	7.88 ± 0.24	7.98 ± 0.21	8.04 ± 0.40	8.19 ± 0.30	8.44 ± 0.44	8.20 ± 0.27[a]
Mean Platelet Volume (fL)	7.03 ± 0.16	7.11 ± 0.11	7.13 ± 0.21	7.26 ± 0.21	7.55 ± 0.55	7.27 ± 0.13[a]
Plateletcrit Value (%)	0.75 ± 0.05	0.74 ± 0.06	0.74 ± 0.05	0.71 ± 0.07	0.66 ± 0.14	0.74 ± 0.03[a]

[a]: analysis performed on 9 samples of 10

***Table 11**- Male offspring white blood cell count (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Total Number of Leucocytes (G/L)	18.73 ± 4.34	19.79 ± 2.53	21.59 ± 2.75	19.47 ± 2.65	19.54 ± 3.50	21.87 ± 1.89
Basophil Count (K/Ul)	0.04 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.02
Basophil Percent (%)	0.19 ± 0.10	0.10 ± 0.11	0.08 ± 0.05	0.17 ± 0.08	0.16 ± 0.08	0.15 ± 0.11
Eosinophil Count (K/Ul)	0.11 ± 0.04	0.12 ± 0.02	0.14 ± 0.03	0.15 ± 0.04	0.19 ± 0.03	0.18 ± 0.04
Eosinophil Percent (%)	0.60 ± 0.11	0.64 ± 0.18	0.66 ± 0.09	0.76 ± 0.22	0.97 ± 0.18	0.81 ± 0.18
Neutrophil Count (K/Ul)	3.13 ± 0.52	3.40 ± 0.89	3.57 ± 0.71	4.69 ± 1.82	4.50 ± 0.91	4.36 ± 0.70
Neutrophil Percent (%)	17.19 ± 3.53	17.06 ± 3.56	16.54 ± 2.60	23.63 ± 5.83	23.28 ± 4.17	19.99 ± 3.10
Lymphocyte Count (K/Ul)	12.99 ± 4.38	14.36 ± 2.14	16.15 ± 2.45	12.58 ± 1.89	13.31 ± 2.78	**15.49 ± 1.74***
Lymphocyte Percent (%)	68.29 ± 9.45	72.51 ± 4.37	74.68 ± 4.27	65.07 ± 8.91	67.87 ± 4.19	70.76 ± 4.24
Monocyte Count (K/Ul)	2.44 ± 0.87	1.90 ± 0.55	1.71 ± 0.48	2.02 ± 0.88	1.51 ± 0.40	1.81 ± 0.32
Monocyte Percent (%)	13.74 ± 6.61	9.69 ± 2.72	**8.05 ± 2.29***	10.37 ± 3.98	7.72 ± 1.66	8.29 ± 1.60

*: Statistically significant ($p \leq 0.05$) with Dunn's test

***Table 12** - Female offspring white blood cell count (mean ± standard deviation)*

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Total Number of Leucocytes (G/L)	16.47 ± 1.63	16.23 ± 3.77	17.05 ± 3.10	14.74 ± 3.01	17.46 ± 2.45	**18.13 ± 3.29***
Basophil Count (K/Ul)	0.03 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.05 ± 0.03	0.03 ± 0.01	0.03 ± 0.02
Basophil Percent (%)	0.20 ± 0.19	0.21 ± 0.14	0.15 ± 0.09	0.34 ± 0.22	0.20 ± 0.05	0.18 ± 0.10
Eosinophil Count (K/Ul)	0.17 ± 0.03	0.16 ± 0.04	0.16 ± 0.03	0.16 ± 0.03	0.20 ± 0.06	0.16 ± 0.05
Eosinophil Percent (%)	1.03 ± 0.18	1.03 ± 0.26	1.00 ± 0.21	1.12 ± 0.19	1.15 ± 0.30	**0.87 ± 0.17***
Neutrophil Count (K/Ul)	2.34 ± 0.37	2.31 ± 0.48	2.27 ± 0.49	2.44 ± 0.29	**2.97 ± 0.59***	2.23 ± 0.50
Neutrophil Percent (%)	14.23 ± 1.71	14.43 ± 2.34	13.44 ± 3.02	16.94 ± 3.23	17.00 ± 1.99	**12.21 ± 1.77****
Lymphocyte Count (K/Ul)	12.51 ± 1.15	12.52 ± 2.93	13.50 ± 2.57	10.87 ± 2.68	13.03 ± 1.90	**14.65 ± 2.68****
Lymphocyte Percent (%)	76.04 ± 2.19	77.21 ± 3.56	79.08 ± 3.92	73.22 ± 3.37	74.60 ± 2.51	**80.88 ± 2.46****
Monocyte Count (K/Ul)	1.41 ± 0.30	1.20 ± 0.72	1.10 ± 0.45	1.22 ± 0.30	1.23 ± 0.24	1.07 ± 0.35
Monocyte Percent (%)	8.51 ± 1.05	7.13 ± 2.67	**6.34 ± 1.79***	8.31 ± 1.35	7.05 ± 1.19	**5.86 ± 1.57****

*: Statistically significant ($p \leq 0.05$) with Dunn's test

**: Statistically significant ($p \leq 0.01$) with Dunn's test

RESULTS OF STAGE 2
INTEGRATED EXPERIMENTAL STUDY ON SUB-CHRONIC TOXICITY, CARCINOGENICITY, REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Pregnancy outcome data in F0 and F1 dams

There were no treatment-related effects on pregnancy outcome in all dose groups in both F0 and F1 generations, as assessed by fertility and gestational index. Mean gestational length in all dose groups in both F0 and F1 generations was approximately 22 days. The body weight gain of the dams during pregnancy was similar in all groups in both F0 and F1 generations (Table 13-14).

Table 13 *– Pregnancy outcome of F0 dams.*

Group	Treatment	Fertility index (%) [a]	Gestational index (%) [b]	Mean gestational length (day) [c]	Relative weight gain during pregnancy [d]
I	Control	45/51 (88.2)	45/45 (100)	21.8 ± 0.7	31.5 ± 3.5
II	Glyphosate 0.5 mg/Kg bw/day	24/29 (82.8)	24/24 (100)	22.0 ± 0.5	31.6 ± 2.2
III	Glyphosate 5 mg/Kg bw/day	28/29 (96.6)	27/28 (96.4)	21.8 ± 0.5	29.9 ± 6.4
IV	Glyphosate 50 mg/Kg bw/day	24/29 (82.8)	23/24 (95.8)	21.9 ± 0.5	30.9 ± 3.1
V	Roundup 0.5 mg/Kg bw/day Gly eq.	24/29 (82.8)	24/24 (100)	22.0 ± 0.4	30.8 ± 2.9
VI	Roundup 5 mg/Kg bw/day Gly eq.	24/29 (82.8)	24/24 (100)	22.0 ± 0.4	30.1 ± 2.4
VII	Roundup 50 mg/Kg bw/day Gly eq.	26/29 (89.7)	26/26 (100)	22.0 ± 0.2	30.3 ± 2.5
VIII	Ranger Pro 0.5 mg/Kg bw/day Gly eq.	28/29 (96.6)	28/28 (100)	21.6 ± 1.2	31.3 ± 3.0
IX	Ranger Pro 5 mg/Kg bw/day Gly eq.	27/29 (93.1)	27/27 (100)	22.0 ± 0.4	31.0 ± 2.9
X	Ranger Pro 50 mg/Kg bw/day Gly eq.	25/29 (86.2)	20/25 (80.0)	21.9 ± 0.3	28.9 ± 6.7

[a:] No. of pregnant females/No. of females with confirmed mating.
[b]: Number of female that delivered at least one live pup/total females with evidence of pregnancy
[c]: Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition (mean ± standard deviation)
[d]: Relative weight gain during pregnancy = relative weight on the last day of pregnancy minus relative weight on the first day of treatment in pregnancy, i.e. GD 6 (weight on GD 6 = 100%) (mean ± standard deviation)

Table 14 *– Pregnancy outcome of F1 dams belonging to WOS adult.*

Group	Treatment	Fertility index (%) [a]	Gestational index (%) [b]	Mean gestational length (day) [c]	Relative weight gain during pregnancy [d]
I	Control	15/15 (100)	15/15 (100)	22.0 ± 0.4	28.5 ± 2.1
II	Glyphosate 0.5 mg/Kg bw/day	13/15 (86.7)	13/13 (100)	22.1 ± 0.4	28.6 ± 1.8
III	Glyphosate 5 mg/Kg bw/day	15/15 (100)	14/15 (93.3)	22.0 ± 0.7	27.6 ± 3.3
IV	Glyphosate 50 mg/Kg bw/day	15/15 (100)	15/15 (100)	22.1 ± 0.3	28.8 ± 2.3
V	Roundup 0.5 mg/Kg bw/day Gly eq.	15/15 (100)	15/15 (100)	22.0 ± 0.5	28.5 ± 2.1
VI	Roundup 5 mg/Kg bw/day Gly eq.	13/15 (86.7)	13/13 (100)	22.2 ± 0.4	26.7 ± 2.8
VII	Roundup 50 mg/Kg bw/day Gly eq.	14/15 (93.3)	14/15 (93.3)	21.8 ± 0.4	28.2 ± 2.4
VIII	Ranger Pro 0.5 mg/Kg bw/day Gly eq.	14/15 (93.3)	14/15 (93.3)	22.1 ± 0.3	27.4 ± 2.8
IX	Ranger Pro 5 mg/Kg bw/day Gly eq.	11/15 (73.3)	9/11 (81.8)	22.1 ± 0.3	27.0 ± 2.1
X	Ranger Pro 50 mg/Kg bw/day Gly eq.	13/15 (86.7)	13/13 (100)	22.1 ± 0.3	26.7 ± 2.9

[a]: No. of pregnant females /No. of females with confirmed mating.

[b]: Number of female that delivered at least one live pup/total females with evidence of pregnancy

[c]: Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition (mean ± standard deviation)

[d]: Relative weight gain during pregnancy = relative weight on the last day of pregnancy minus relative weight on the first day of treatment in pregnancy, i.e. GD 6 (weight on GD 6 = 100%) (mean ± standard deviation)

Sexual development in F1 and F2 generations

The onset of puberty in male F1 belonging to the WOS adult, analysed using linear regression model adjusted for body weight, showed a slight anticipation of the age at BPS in the highest dose of Ranger Pro (50 mg/Kg bw/day of Glyphosate equivalent), even if it was borderline statistically significant (p=0.059) (Table 15). In the F1 male rats belonging to the WOS pubertal, the age at BPS was delayed (again with a borderline significance, p=0.058) in the highest dose of Ranger Pro, after running linear regression model adjusted for body weight (Table 17). In F2 male offspring there were no treatment-related effects on BPS achievement (Table 19).

The onset of puberty in F1 female rats belonging to the WOS adult showed a significant reduction in the age at vaginal opening in the highest dose of Ranger Pro group (50 mg/Kg bw/day of Glyphosate equivalent) (two-sample Wilcoxon rank-sum test, p=0.0097). This group also showed a statistically significant lower body weight at VO (one-way ANOVA, p=0.0001). Differences in the age at VO were not statistically significant if evaluated with a linear regression model adjusted for body weight. Body weight at VO was also decreased in the highest dose group of Glyphosate (50 mg/Kg bw/day); mid (5 mg/Kg bw/day of Glyphosate equivalent) and high (50 mg/Kg bw/day of Glyphosate equivalent) dose of Ranger Pro (p = 0.0249; p=0.0553; p=0.0047 respectively) (Table 16). No significant differences in VO were noted in F1 female rats belonging to the WOS pubertal (Table 18) nor F2 females, with the exception of a statistically significant decrease in body weight at VO in F2 female rats treated with Ranger Pro at 5 mg/Kg bw/day of Glyphosate equivalent (One-way ANOVA, p=0.0328) (Table 20).

Table 15 - *Age and body weight at BPS in F1 male rats belonging to WOS adult (mean ± standard deviation).*

Group	Treatment	PND at BPS	Body weight at BPS
I	Control	43,9 ± 2,9	198,6 ± 26,6
II	Glyphosate 0.5 mg/Kg bw/day	43,7 ± 3,2	197,9 ± 21,1
III	Glyphosate 5 mg/Kg bw/day	42,9 ± 1,3	187,5 ± 30,2
IV	Glyphosate 50 mg/Kg bw/day	42,8 ± 1,6	189,1 ± 22,7
V	Roundup 0.5 mg/Kg bw/day Gly eq.	43,7 ± 2,8	198,7 ± 15,7
VI	Roundup 5 mg/Kg bw/day Gly eq.	42,8 ± 2,5	186,9 ± 18,9
VII	Roundup 50 mg/Kg bw/day Gly eq.	42,9 ± 1,4	189,8 ± 9,2
VIII	Ranger Pro 0.5 mg/Kg bw/day Gly eq.	42,9 ± 2,2	192,1 ± 12,6
IX	Ranger Pro 5 mg/Kg bw/day Gly eq.	42,7 ± 2,3	187,1 ± 12,5
X	Ranger Pro 50 mg/Kg bw/day Gly eq.	41,9 ± 1,6	184,8 ± 16,9

Table 16 - *Age and body weight at VO in F1 female rats belonging to WOS adult (mean ± standard deviation).*

Group	Treatment	PND at VO	Body weight at VO
I	Control	36,6 ± 1,9	126,3 ± 10,4
II	Glyphosate 0.5 mg/Kg bw/day	36,4 ± 1,6	120,7 ± 15,8
III	Glyphosate 5 mg/Kg bw/day	36,7 ± 2,6	122,1 ± 14,8
IV	**Glyphosate 50 mg/Kg bw/day**	36,1 ± 1,8	**116,8 ± 11,4***
V	Roundup 0.5 mg/Kg bw/day Gly eq.	37,3 ± 2,0	123,1 ± 11,9
VI	Roundup 5 mg/Kg bw/day Gly eq.	37,6 ± 2,7	128,9 ± 14,8
VII	Roundup 50 mg/Kg bw/day Gly eq.	36,1 ± 1,6	118,7 ± 11,3
VIII	Ranger Pro 0.5 mg/Kg bw/day Gly eq.	35,7 ± 2,1	119,0 ± 9,5
IX	**Ranger Pro 5 mg/Kg bw/day Gly eq.**	35,4 ± 2,1	**112,6 ± 13,7****
X	**Ranger Pro 50 mg/Kg bw/day Gly eq.**	**34,8 ± 1,8**[##]	**106,7 ± 12,9****

*Statistically significant ($p < 0.05$) with one-way ANOVA

**Statistically significant ($p < 0.01$) with one-way ANOVA

[##]Statistically significant ($p < 0.01$) with two-sample Wilcoxon rank-sum test

Table 17 *- Age and body weight at BPS in F1 male rats belonging to WOS pubertal (mean ± standard deviation).*

Group	Treatment	PND at BPS	Body weight at BPS
I	Control	42,3 ± 1,6	192,4 ± 19,6
II	Glyphosate 0.5 mg/kg bw/day	43,4 ± 1,4	190,7 ± 16,4
III	Glyphosate 5 mg/Kg bw/day	42,8 ± 1,4	184,8 ± 14,1
IV	Glyphosate 50 mg/Kg bw/day	42,2 ± 0,8	191,2 ± 13,8
V	Roundup 0.5 mg/Kg bw/day Gly eq.	43,1 ± 1,2	194,2 ± 17,7
VI	Roundup 5 mg/Kg bw/day Gly eq.	43,5 ± 1,2	197,1 ± 8,4
VII	Roundup 50 mg/Kg bw/day Gly eq.	43,2 ± 1,9	192,0 ± 16,6
VIII	Ranger Pro 0.5 mg/Kg bw/day Gly eq.	43,5 ± 2,6	194,0 ± 18,5
IX	Ranger Pro 5 mg/Kg bw/day Gly eq.	43,6 ± 1,7	198,8 ± 13,1
X	Ranger Pro 50 mg/Kg bw/day Gly eq.	43,8 ± 1,7	197,3 ± 20,2

Table 18 *- Age and body weight at VO in F1 female rats belonging to WOS pubertal (mean ± standard deviation).*

Group	Treatment	PND at VO	Body weight at VO
I	Control	36,9 ± 2,0	127,7 ± 15,8
II	Glyphosate 0.5 mg/kg bw/day	37,6 ± 2,0	130,2 ± 9,1
III	Glyphosate 5 mg/kg bw/day	37,7 ± 2,7	128,5 ± 12,0
IV	Glyphosate 50 mg/kg bw/day	36,5 ± 1,6	122,8 ± 15,7
V	Roundup 0.5 mg/kg bw/day Gly eq.	37,2 ± 1,9	129,7 ± 13,2
VI	Roundup 5 mg/kg bw/day Gly eq.	36,7 ± 3,1	127,7 ± 16,4
VII	Roundup 50 mg/kg bw/day Gly eq.	36,9 ± 1,6	128,3 ± 13,6
VIII	Ranger Pro 0.5 mg/kg bw/day Gly eq.	37,2 ± 2,2	128,5 ± 14,0
IX	Ranger Pro 5 mg/kg bw/day Gly eq.	35,9 ± 2,3	119,7 ± 13,5
X	Ranger Pro 50 mg/kg bw/day Gly eq.	36,2 ± 2,3	126,2 ± 14,0

***Table 19**- Age and body weight at BPS in F2 male rats (mean ± standard deviation).*

Group	Treatment	PND at BPS	Body weight at BPS
I	Control	42,3 ± 1,2	207,5 ± 28,9
II	Glyphosate 0.5 mg/kg bw/day	42,1 ± 1,4	194,9 ± 25,5
III	Glyphosate 5 mg/kg bw/day	42,0 ± 2,2	198,8 ± 19,2
IV	Glyphosate 50 mg/kg bw/day	43,2 ± 2,2	194,1 ± 17,0
V	Roundup 0.5 mg/kg bw/day Gly eq.	41,5 ± 1,5	199,4 ± 16,7
VI	Roundup 5 mg/kg bw/day Gly eq.	41,2 ± 1,3	199,7 ± 15,4
VII	Roundup 50 mg/kg bw/day Gly eq.	42,7 ± 1,4	198,2 ± 14,9
VIII	Ranger Pro 0.5 mg/kg bw/day Gly eq.	41,7 ± 0,8	199,2 ± 13,9
IX	Ranger Pro 5 mg/kg bw/day Gly eq.	42,5 ± 1,6	190,7 ± 9,1
X	Ranger Pro 50 mg/kg bw/day Gly eq.	41,8 ± 1,9	198,5 ± 14,9

***Table 20**- Age and body weight at VO in F2 female rats (mean ± standard deviation).*

Group	Treatment	PND at VO	Body weight at VO
I	Control	35,3 ± 2,3	123,4 ± 15,2
II	Glyphosate 0.5 mg/kg bw/day	35,8 ± 1,7	119,2 ± 14,5
III	Glyphosate 5 mg/kg bw/day	35,0 ± 1,7	117,6 ± 13,7
IV	Glyphosate 50 mg/kg bw/day	34,8 ± 1,6	116,8 ± 8,0
V	Roundup 0.5 mg/kg bw/day Gly eq.	35,8 ± 1,7	128,0 ± 10,1
VI	Roundup 5 mg/kg bw/day Gly eq.	35,0 ± 1,3	126,0 ± 11,7
VII	Roundup 50 mg/kg bw/day Gly eq.	35,8 ± 1,5	125,6 ± 13,1
VIII	Ranger Pro 0.5 mg/kg bw/day Gly eq.	34,3 ± 2,3	118,8 ± 12,1
IX	**Ranger Pro 5 mg/kg bw/day Gly eq.**	35,1 ± 1,8	**112,3 ± 7,3***
X	Ranger Pro 50 mg/kg bw/day Gly eq.	34,3 ± 1,8	121,1 ± 9,5

*Statistically significant ($p < 0.05$) with one-way ANOVA

GENERAL DISCUSSION AND CONCLUSIONS

VIII. GENERAL DISCUSSION AND CONCLUSIONS

The "Glyphosate debate" in the framework of the re-registration process is receiving prominent attention due to significant commercial interests and environmental and health concerns. There are profound gaps in the risk assessment of Glyphosate including: 1) discrepancy among the conclusions of different studies available in literature and among the regulatory bodies; 2) numerous GBHs marketed worldwide; 3) the use of unknown surfactants either as components in Glyphosate formulations or as adjuvants which are added prior to application. To follow up on regulatory uncertainty around Glyphosate, the Ramazzini Institute planned a comprehensive project called "Global Glyphosate study" aimed at examining the effects of a range of different environmentally relevant doses of Glyphosate alone and commonly used GBHs. This study extended over two generations of rats that were exposed to Glyphosate alone or to GBHs. The F1 and F2 generations of offspring were exposed during in utero life through their mothers, during weaning through their mothers' milk, and during their lifetimes through drinking water containing the tested substances. A specific window of biological susceptibility from prepubertal until puberty period was also investigated (WOS pubertal). The global project started with a 13-week pilot study (stage 1) that was followed by an integrated experimental study (stage 2). Both the studies are reported in this thesis, the results of the 13-week pilot study are attached as reviewed papers (***Paper 2*** *- Panzacchi et al., 2018;* ***Paper 3*** *- Manservisi et al., 2019*) and further data, not yet published, are presented. This chapter will simply consist in a general discussion on additional results on stage 1 and preliminary data on endocrine-sensitive endpoints of the Reproductive/Developmental toxicity ARM B of the stage 2.

It is well known that environmental contaminants such as pesticides can alter homeostatic parameters in rats and some of these parameters can be used as biomarkers. Thus, biochemical, physiological and histological analysis used as biomarkers become sensitive tools that can be used to assess the adverse effects of several pollutants in laboratory experimental conditions. These biomarkers may be able to provide an early warning signal even before adverse clinical health effects are manifested.

In the 13-week pilot study, the kidney histopathological analysis in Roundup-treated dams revealed statistically significant increase in renal tubule degeneration and focal minimal inflammation. The renal damage in dams, treated only with Roundup formulation, is in line

with other results where only the commercial formulation and not the active ingredient (pure Glyphosate) had an effect on kidneys (Wunnapuk *et al*., 2014; Dedeke *et al*., 2018). Recent epidemiological studies have also confirmed that the kidney represents a susceptible organ to GBHs (Jayasumana *et al*., 2015).

Biomarker responses like circulating alanine transaminase, gamma-glutamyl transferase, serum total protein, cholesterol, glucose, etc., represent the functional status of homeostasis. A few scattered changes occurred in clinical chemistry data. Exposure to Roundup (not Glyphosate alone) induced a statistically significant phosphorus increase in serum, both in male and female rats belonging to the 13 week-cohort. Furthermore, a statistically significant decrease in creatinine concentration was observed in Roundup-treated females belonging to the same cohort; even if the value was very close to the range of the historical control data of SD rats belonging to the CMCRC/RI. The kidneys play a major role in maintaining the proper excretion of phosphorus in the urine, to ensure that their serum levels are adequate for the performance of various functions. Hyperphosphatemia reflects a disparity in phosphate metabolism by renal failure imbalance between intestinal absorption and urinary excretion (Ospina *et al*., 2017). Hyperphosphatemia has also been reported in fish and other animals after exposure to various pesticides, like endosulfan and aldrin (Gill *et al.*, 1991; Singh *et al.*, 1996). Interestingly, changes in serum creatinine and phosphorus (lower and higher levels, respectively) were also detected in a cohort of 106 intensive agriculture workers that were assessed twice during the course of a spraying season for changes in serum biochemistry (Hernández *et al*., 2006). These results provide support for a slight impairment of the kidney function, even if these findings are not supported by clinically significant hepatotoxicity and need to be confirmed by the findings of the integrated experimental study.

A statistically significant decrease in total protein was also observed in Roundup-treated male rats belonging to the 13-week cohort. Organophosphorus pesticides are known to alter serum levels of amino acids (Gomes *et al*., 2004); for instance, carbofuran decreased liver and muscle total protein. Pesticides are suggested to reduce tissue protein content because of glucose production in the gluconeogenesis process and also because of inhibition of protein synthesis (Karami-Mohajeri and Abdollahi, 2011).

Lymphocyte and leukocyte counts were particularly increased in Roundup-treated rats. The increase in the 13-week cohort observed in both sexes, more prominent in female, was in line with the 13-week study performed by the NTP in which they reported a statistically significant increase in lymphocyte and leukocyte only in females treated with pure glyphosate (NTP 1992; IARC 2015). The increased level of lymphocyte and leukocyte could be considered an inflammatory marker, predictive of inflammatory diseases (Chen *at al.*, 2018). Due to the limitations of a pilot study with few animals and short treatment, we cannot relate GBHs to the alteration of the lymphoreticular system, but we might consider this alteration as of interest for the ongoing histopathological and clinical chemistry analysis of animals belonging to the integrated experimental study. In addition, IARC underlines the evidence of positive association in humans, with non-Hodgkin lymphoma, that is related to the alteration of haemolymphoreticular system.

Environmental contaminants such as pesticides are also known to interfere with reproduction and other endocrine-regulated functions. Concern has been expressed that the current testing paradigm does not adequately predict perturbation of the endocrine system due to the lack of experimental designs covering the complete life cycle of a mammal, from conception to old age. The integrated experimental study (stage 2), as planned, allows to monitor animals for any potential adverse health effects resulting from exposure during sensitive biological windows under extensive hormonal regulation (i.e. gestation, lactation, pre-puberty, puberty, development to adulthood until senescence). Extensive assessments of in-life endocrine-sensitive endpoints required in the OECD TG 443 (OECD, 2018) and NTP MOG (NTP, 2011), including AGD, VO and BPS, were addressed in both stages for two generations of rats. These endpoints are under hormonal regulation and therefore warrant specific attention in view of potential endocrine disruption (OECD, 2018).

AGD is an early-life biomarker of fetal androgen exposure in multiple species; AGD length is influenced by body size and is longer in males compared to females (Swan and Kristensen 2018). During early development, androgens regulate masculinization; disruptions during this critical ‘masculinization programming window’ can lead to shorter AGD (feminized) and reproductive tract abnormalities in males and androgen-driven masculinization of females. The 13-week pilot study demonstrates that Roundup Bioflow exposure, at a dose level considered as “safe” (1.75 mg/kg bw/day), from prenatal period to adulthood, was

associated with androgen-like effects, in particular in females, including a statistically significant increase of AGDs in both males and females, delay of FE and increased testosterone in females. Roundup Bioflow exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week). An association between prenatal Glyphosate and AMPA exposure measured by maternal urinary excretion and masculinized (longer) AGD in female infants from the Infant Development and the Environment Study (a multicenter pregnancy cohort) was presented as a poster at the International Society for Environmental Epidemiology 2020 Virtual conference (Lesseur *at al*., 2020) and at the Ramazzini Days 2020 (https://www.collegiumramazzini.org/ramazzini-days/all-abstracts). The findings from this human study are the first to link in utero Glyphosate exposure with masculinized AGD in female offspring in rats and in humans. In a weight of evidence approach, effects in apical mammalian endpoints (e.g. increased in AGD) detected both in laboratory animals and in humans add evidence to the current literature of possible endocrine disrupting effects of glyphosate.

The information obtained from the measurement of VO and BPS can be useful for determining how a tested chemical influences the pubertal process. The age at puberty is an important component of reproductive development and is influenced by sex hormones, which can determine its anticipation or delay. BPS is triggered by the rise of serum testosterone concentrations in the prepubertal period (Korenbrot *et al.* 1977). In the 13-week pilot study, the day of age when BPS or VO occurred in the offspring was unaffected by treatment. In the integrated experimental study, exposure to the highest dose of Ranger Pro (50 mg/Kg bw/day Glyphosate equivalent) affected the time of and body weight at VO in female pups of the F1 generation (WOS adult) such that the time of VO was significantly anticipated (two days earlier) and shifted to lower body weight. This finding is not corroborated by the results presented in Dallegrave (2007) which indicate a delay in VO in female offspring Wistar rats born to mothers exposed to different doses (50, 150 and 450 mg/kg) of Roundup (containing 360 g/l of glyphosate and 18% (w/v) polyoxyethyleneamine) during pregnancy and lactation (Dallegrave *et al.,* 2007). A dose-related decrease of testosterone was also reported at puberty by those authors.

The slight delay, with a borderline significance, in BPS achievement (PND 43.8 ± 1.7) in F1 male rats belonging to the WOS pubertal treated from PND28 until PND63 with Ranger Pro at 50 mg/Kg bw/day Glyphosate equivalent compared to controls (PND 42.3 ± 1.6), was consistent with the study of Romano et al. (2010) who investigated the effects of the herbicide Roundup Transorb in Wistar rats treated from the PND23 until the PND53. The daily exposure to Roundup Transorb caused a significant delay in the pubertal age at 50 and 250 mg/Kg bw/day (Romano *et al.*, 2010). Interestingly, the same authors in 2012 published a study focusing on the same chemical, Roundup Transorb, administered to male Wistar rats at 50 mg/kg bw/day, from in utero life (GD18) until PND5. Daily exposure to Roundup Transorb during late gestational and early postnatal days was reported to be associated with a significant reduction in the age at puberty onset and also in the body weight at puberty (Romano *et al.*, 2012). In our integrated experimental study, early preputial separation (PND 41.9 ± 1.6) in the Ranger Pro high dose F1 males (WOS adult) compared to controls (PND 43.9 ± 2.9) was also noted, even if with a borderline significance. It is noteworthy that, in both studies, the treatment started from in utero life (GD6). Furthermore, the degree of deviation for BPS achievement in Ranger Pro high dose F1 males (WOS adult) from historical controls in SD-CMCRC/RI rats (PND 45.0 ± 1.9) ***(Paper 1** – Manservisi et al., 2018**)*** reinforces the biological relevance of a treatment-related effect.

Other endpoints sensitive to disturbance by endocrine disruptors are still under evaluations such as AGD, sperm parameters, circulating hormone levels and regularity and duration of the oestrous cyclicity, as well as more conventional endpoints such as histopathology and weights of organs of the reproductive tract.

In summary, while the available data does not permit to draw definitive conclusions regarding the reproductive/developmental toxicity of GBHs administered to SD rats under various calendars, the following considerations can be pointed out:

- The toxicity of Roundup Bioflow and even more of Ranger Pro, which contains POEA surfactants, is far higher than the toxicity of the active ingredient Glyphosate.
- The results of the 13-week pilot study and preliminary data on the integrated experimental study might **characterize GBHs as probable endocrine disruptors** as suggested by:

1. **Androgen-like effects of Roundup Bioflow**, in particular in females, including a significant increase of AGDs in both males and females, delay of FE and increased testosterone in females belonging to the 13-week pilot study ***(Paper 3*** *- Manservisi et al., 2019*).

2. **An overall pattern of slight puberty onset anticipation in the high dose of Ranger Pro group**, observed in the F1 generation treated from in utero life until adulthood, as suggested by an early onset of the age at VO and BPS.

3. **A delayed BPS achievement in the high dose of Ranger Pro-treated** F1 males **exposed only during the peri-pubertal period**, suggesting a direct and **specific effect of GBHs depending on the timing of exposure**.

- While this effect cannot be unequivocally associated with the surfactants POEA, the available data on endocrine-sensitive endpoints suggest that Ranger Pro is the GBH which most likely impacts endocrine sensitive endpoints.
- GBHs can alter homeostatic parameters, as indicated by increased serum phosphorus levels, decreased serum total protein levels and impairment of some haematological indices in the 13-week pilot study.
- Kidney has been confirmed as a target organ for GBHs exposure, in particular the 13-week pilot study revealed that GBHs exposure during pregnancy and lactation induce renal damage in dams.

Overall, the data presented in this thesis need to be interpreted relative to all other findings under the ongoing integrated experimental study and with balanced consideration for other apical endpoints in a weight-of evidence approach.

Taken together, these conclusions all indicate that it is essential to understand the adverse and cumulative effects on health and environment of GBHs for a more comprehensive risk assessment, update the EU authorization policies based only on active ingredients, put in place new experimental protocols (encompassing potential carcinogenicity and endocrine disruption effects covering biological windows of susceptibility) and revise the free availability to anyone of co-formulants applied in GBHs, which is also a pressing issue to which the results of this study can contribute.

REFERENCES

IX. REFERENCES

- Adam A, Marzuki A, Abdul Rahman H, Abdul Aziz M. The oral and intratracheal toxicities of ROUNDUP and its components to rats. Vet Hum Toxicol. 1997;39(3):147-151.
- Armiliato N, Ammar D, Nezzi L, Straliotto M, Muller YM, Nazari EM. Changes in ultrastructure and expression of steroidogenic factor-1 in ovaries of zebrafish Danio rerio exposed to Glyphosate. J Toxicol Environ Health A. 2014;77(7):405-414.
- Bai SH. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ Sci Pollut Res Int. 2016; 23(19): 18988–19001.
- Battaglin WA, Meyer MT, Kuivila KM, Dietze JE. Glyphosate and Its Degradation Product Ampa Occur Frequently and Widely in U.S. Soils, Surface Water, Groundwater, and Precipitation. J. Am. Water Resour. Assoc. 2014;50:275–290.
- Benbrook CM. Trends in Glyphosate herbicide use in the United States and globally. Environ Sci Eur. 2016; 28, 3.
- Benítez-Leite S, Macchi ML, Acosta M. Malformaciones congénitas asociadas a agrotóxicos. Pediatría (Asunción). 2007; 34(2):111-121.
- Bhide MB, Patil UM. Rabbit teratology study with Glyphosate technical. Study No. IIT Project No. 1086 1989.
- Briggs JP. The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol. 2002;282(1): R3-R9.
- Brooker AJ, Brennan C, John DM, et al. The effect of Glyphosate on pregnancy of the rabbit (incorporates preliminary investigations). Study/Project No. CHV 45 & 39 & 40/901303 1991a.
- Brooks AC, Fryer M, Lawrence A, et al. Reflections on bird and mammal risk assessment for plant protection products in the European Union: Past, present, and future. Environ Toxicol Chem. 2017;36(3):565-575.
- Campaña H, Pawluk MS, López Camelo JS; Grupo de Estudio del ECLAMC. Prevalencia al nacimiento de 27 anomalías congénitas seleccionadas, en 7 regiones geográficas de la Argentina [Births prevalence of 27 selected congenital anomalies in 7 geographic regions of Argentina]. Arch Argent Pediatr. 2010;108(5):409-417.

REFERENCES

- Casabé N, Piola L, Fuchs J, et al. Ecotoxicological assessment of the effects of Glyphosate and chlorpyrifos in an Argentine soya field. Journal of Soils and Sediments 7, no. 4, 2007: 232-239.
- Cauble K, Wagner RS. Sublethal effects of the herbicide Glyphosate on amphibian metamorphosis and development. Bull Environ Contam Toxicol. 2005;75(3):429-435.
- Chen L, Deng H, Cui H, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017;9(6):7204-7218.
- Coles RJ, Doleman N. Glyphosate technical: oral gavage teratology study in the rabbit. SPL Project No. 434/020 1996.
- Contardo-Jara V, Klingelmann E, Wiegand C. Bioaccumulation of Glyphosate and its formulation Roundup Ultra in Lumbriculus variegatus and its effects on biotransformation and antioxidant enzymes. Environ Pollut. 2009;157(1):57-63.
- Correia FV, Moreira JC. Effects of Glyphosate and 2,4-D on Earthworms (Eisenia foetida) in Laboratory Tests. Bull Environ Contam Toxicol. 2010; 85, 264–268.
- Dallegrave E, Mantese FD, Oliveira RT, et al. Pre- and postnatal toxicity of the commercial Glyphosate formulation in Wistar rats. Arch Toxicol. 2007;81(9):665-673
- Defarge N, Vendômois JS, Séralini GE. Toxicity of formulants and heavy metals in Glyphosate-based herbicides and other pesticides. Toxicology Reports. 2018; 5, 156 - 163.
- Dedeke GA, Owagboriaye FO, Ademolu KO, Olujimi OO, Aladesida AA. 2018. Comparative assessment on mechanism underlying renal toxicity of commercial formulation of roundup herbicide and glyphosate alone in male albino rat. International journal of toxicology 37:285-295
- Fort DJ, Thomas JH, Rogers RL, et al. Evaluation of the developmental and reproductive toxicity of methoxychlor using an anuran (Xenopus tropicalis) chronic exposure model. Toxicol Sci. 2004;81(2):443-453.
- Environmental Protection Agency's (EPA). Re-registration Eligibility Decision (RED) Glyphosate: EPA-738-R-93-014.Washington, DC: US Environmental Protection Agency, Office of Pesticide Programs and Toxic Substances; 1993.
- Environmental Protection Agency (EPA). Endocrine Disruption Screening Program Weight of Evidence Conclusions on the Tier I Screening Assays for the List 1 Chemicals;

Office of Chemical Safety and Pollution Prevention, U.S. Government Printing Office, Washington, DC, 2015.

- Environmental Protection Agency (EPA). Glyphosate. Interim Registration Review Decision. Case Number 0178. January 2020. Available on line at: https://www.epa.gov/sites/production/files/2020-01/documents/Glyphosate-interim-reg-review-decision-case-num-0178.pdf
- European Chemicals Agency (ECHA). Glyphosate not classified as a carcinogen by ECHA. ECHA/PR/17/06. Mar 15, 2017. Available online at: https://echa.europa.eu/da/-/Glyphosate-not-classified-as-a-carcinogen-by-echa
- European Commission (2016b). Implementing Regulation (EU) 2016/1313 of 1 August 2016 Amending Implementation Regulation (EU) No 540/2011 as Regards the Conditions of Approval of the Active Substance Glyphosate. OJ L208:1. Available online at: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2016.208.01.0001.01.ENG
- European Food Safety Authority (EFSA). Conclusion on the peer review of the pesticide risk assessment of the active substance Glyphosate. EFSA Journal 2015; 13:4302.
- European Food Safety Authority (EFSA). Request for the evaluation of the toxicological assessment of the co-formulant POE-tallowamine. EFSA Journal 2015;13(11):4303
- European Food Safety Authority (EFSA). The 2014 European Union Report on Pesticide Residues in Food. EFSA Journal 2016;14(10):4611.
- European Food Safety Authority (EFSA). Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of Glyphosate. EFSA Journal 2017a; 15:497913.
- Expert Committee on Pesticide Residues in Food (PRiF): annual report for 2016. Available online at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/824814/expert-committee-pesticide-residues-food-annual-report-2018.pdf
- Faqi AS. 2011. The Dog as Alternative Species in Developmental Toxicology Studies [PowerPoint presentation]. Retrieved from: https://www.devtox.org/files/WS2011_Faqi.pdf
- Fathi MA, Abdelghani E, Shen D, et al. Effect of in ovo Glyphosate injection on embryonic development, serum biochemistry, antioxidant status and histopathological

changes in newly hatched chicks. J Anim Physiol Anim Nutr (Berl). 2019;103(6):1776-1784.

- Foote RH, Carney E. The rabbit as a model for reproductive and developmental toxicity studies. Reprod Toxicol. 2000; 14(6):477-493.
- Fountain MT, Hopkin SP. Folsomia candida (Collembola): A ‘‘Standard’’ soil arthropod. Ann. Rev. Entomol. 2005; 50:201–222.
- Fusilero MA, Mangubat J, Ragas RE, et. al. Weed management systems and other factors affecting the earthworm population in a banana plantation. European journal of soil biology. 2013; 56:89-94.
- García-Torres T, Giuffré L, Romaniuk R, et al. Exposure assessment to Glyphosate of two species of annelids. Bull Environ Contam Toxicol. 2014;93(2):209-214.
- Gasnier C, Dumont C, Benachour N, et al. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009; 262(3):184-191.
- German Federal Institute for Risk Assessment (2015). Final Addendum to the Renewal Assessment Report. Risk Assessment Provided by the Rapporteur Member State Germany and Co-Rapporteur Member State Slovakia for the Active Substance Glyphosate According to the Procedure for the Renewal of the Inclusion of a Second Group of Active Substances in Annex I to Council Directive 91/414/EEC laid down in Commission Regulation (EU) No. 1141/2010. Available online at: https://echa.europa.eu/documents/10162/13626/renewal_assessment_report_addenda_en.pdf
- Gill JPK, Sethi N, Mohan A, et al. Glyphosate toxicity for animals. Environmental Chemistry Letters. 2018; 16(2):401-426.
- Gill TS, Pande J, Tewari H. Effects of endosulfan on the blood and organ chemistry of freshwater fish, Barbus conchonius Hamilton. Ecotoxicol Environ Safety. 1991;21(1):80–91.
- Gonçalves BB, Nascimento NF, Santos MP, et al. Low concentrations of Glyphosate-based herbicide cause complete loss of sperm motility of yellowtail tetra fish Astyanax lacustris. J Fish Biol. 2018;92(4):1218-1224.
- Gomes J, Dawodu AH, Lloyd O, Revitt DM, and Anilal SV. Hepatic injury and disturbed amino acid metabolism in mice following prolonged exposure to organophosphorus pesticides. Hum Exp Toxicol 1999; 18: 33–37. Begum G. Carbofuran insecticide induced

biochemical alterations in liver and muscle tissues of the fish Clarias batrachus (linn) and recovery response. Aquat Toxicol 2004; 66: 83–92

- Gray LE Jr, Wilson V, Noriega N, et al. Use of the laboratory rat as a model in endocrine disruptor screening and testing. ILAR J. 2004;45(4):425-437.
- Helander M, Saloniemi I, Saikkonen K. Glyphosate in northern ecosystems. Trends Plant Sci. 2012;17(10):569-574.
- Hernández AF, Amparo Gómez M, Pérez V, et al. Influence of exposure to pesticides on serum components and enzyme activities of cytotoxicity among intensive agriculture farmers. Environmental Research. 2006 Sep;102(1):70-76.).
- Hojo H. HR-001: a teratogenicity study in rabbits. Study No. IET 94-0153 1995.
- IARC Working Group. Glyphosate. In: Some organophosphate insecticides and herbicides: diazinon, Glyphosate, malathion, parathion, and tetrachlorvinphos. Vol 112. IARC Monogr Prog, 2015:1–92
- Ingaramo P, Varayoud J, Milesi M, et al. Effects of neonatal exposure to a Glyphosate-based herbicide on female rat reproduction, Reproduction. 2016; 152(5), 403-415.
- Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) (2016). National Report of Pesticides in Water (2013-2014) [in italian, "Rapporto Nazionale pesticidi nelle acque dati 2013-2014"] . Rapporti , 244/2016.
- Jayasumana C, Paranagama P, Agampodi S, et al. Drinking well water and occupational exposure to Herbicides is associated with chronic kidney disease, in Padavi-Sripura, Sri Lanka. Environ. Health, 14 (2015), p. 6
- Kaneda S, Okano S, Urashima Y, et al. Effects of herbicides, Glyphosate, on density and casting activity of earthworm, Pheretima (Amynthas) carnosus. Japanese Journal of Soil Science and Plant Nutrition. 2009; 80(5):469-476.
- Kawashima T, Ahmed WM, Nagino K, et al. Avian Test Battery for the Evaluation of Developmental Abnormalities of Neuro- and Reproductive Systems. Front Neurosci. 2016; 10:296.
- Karami-Mohajeri S, Abdollahi M. Toxic influence of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: A systematic review. Human & Experimental Toxicology. 2011;30(9):1119-1140.

REFERENCES

- Kimmel GL, Kimmel CA, Williams AL, DeSesso JM. Evaluation of developmental toxicity studies of Glyphosate with attention to cardiovascular development. Crit Rev Toxicol. 2013;43(2):79-95.
- Kissane Z, Shephard JM. The rise of Glyphosate and new opportunities for biosentinel early-warning studies. Conserv Biol. 2017;31(6):1293-1300.
- Korenbrot CC, Huhtaniemi IT, Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod. 1977 Sep;17(2):298-303
- Krüger M, Schrödl W, Pedersen Ib, Shehata AA. Detection of Glyphosate in Malformed Piglets. J Environ. Anal Toxicol. 2014; 4: 230.
- Landrigan PJ, Belpoggi F. The need for independent research on the health effects of Glyphosate-based herbicides. Environ Health. 2018;17(1):51.
- Lesseur C., Pirrotte P., Pathak KV et al. In Utero Glyphosate Exposure and Anogenital Distance in Newborns from The Infant Development and Environment Study (TIDES). International Society for Environmental Epidemiology 2020 Virtual conference, poster session.
- Lopes FM, Varela Junior AS, Corcini CD, et al. Effect of Glyphosate on the sperm quality of zebrafish Danio rerio. Aquat Toxicol. 2014; 155:322-326.
- Mann RM, Hyne RV, Choung CB, Wilson SP. Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Pollut. 2009;157(11):2903-2927.
- Manservisi F, Gopalakrishnan K; Tibaldi E, Hysi A; Iezzi M; Lambertini L; Teitelbaum S; Chen J; Belpoggi F. Effect of maternal exposure to endocrine disrupting chemicals on reproduction and mammary gland development in female sprague-dawley rats. Reproductive Toxicology, 2015 Jul;54:110-9.
- Manservisi F, Marquillas CB, Buscaroli A, et al. An Integrated Experimental Design for the Assessment of Multiple Toxicological End Points in Rat Bioassays. Environmental Health Perspectives. 2017; 125(3): 289-295
- Manservisi F, Falcioni L, Bua L, et al. Control data on endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Ramazzini Institute colony. Eur J Oncol Environ Health. 2018; 23(2):80-85.
- Manservisi F, Lesseur C, Panzacchi S, et al. The Ramazzini Institute 13-week pilot study Glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system. Environ Health. 2019;18(1):15.

REFERENCES

- Marc J, Le Breton M, Cormier P, et al. A Glyphosate-based pesticide impinges on transcription. Toxicol Appl Pharmacol. 2005; 203(1):1-8.
- MarketsandMarkets™. (2017). Tratto da Glyphosate Market by Crop Type (Genetically Modified, Conventional), Form (Liquid, Dry), Application (Agricultural (Grains & Cereals, Oilseeds & Pulses, Fruits & Vegetables), Non Agricultural), and Region - Global Forecast to 2022: https://www.marketsandmarkets.com/Market-Reports/Glyphosate-market-8522593.html
- Mercurio P, Flores F, Mueller JF, et al. Glyphosate persistence in seawater. Marine Pollution Bulletin. 2014 Aug;85(2):385-390.
- Mesnage R, Bernay B, Séralini GE. Ethoxylated adjuvants of Glyphosate-based herbicides are active principles of human cell toxicity. Toxicology. 2013;313(2-3):122-128.
- Mesnage R, Defarge N, Spiroux de Vendômois J, Séralini GE. Major pesticides are more toxic to human cells than their declared active principles. Biomed Res Int. 2014; 2014:179691.
- Meyer JK, Fitzsimmons D, Hastings TF, Chellman, GJ. Methods for the prediction of breeding success in male cynomolgus monkeys (Macaca fascicularis) used for reproductive toxicology studies. J. Am. Assoc. Lab. Anim. Sci. 2006; 45 (2), 31e36.
- Milesi MM, Lorenz V, Pacini G, et al. Perinatal exposure to a Glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats. Arch Toxicol. 2018;92(8):2629-2643.
- Mottier A, Pini J, Costil K. Effects of a POEA surfactant system (Genamin T-200(®)) on two life stages of the Pacific oyster, Crassostrea gigas. J Toxicol Sci. 2014;39(2):211-215.
- Moxon ME. Glyphosate acid: developmental toxicity study in the rabbit. Report No. CTL/P/5009 1996.
- Myers JP, Antoniou MN, Blumberg B, et al. Concerns over use of Glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ Health. 2016; 15:19.
- Niemeyer JC, de Santo FB, Guerra N, et al. Do recommended doses of Glyphosate-based herbicides affect soil invertebrates? Field and laboratory screening tests to risk assessment. Chemosphere. 2018; 198:154-160.

REFERENCES

- NTP (2011). GUIDANCE DOCUMENT FOR THE DEVELOPMENTAL NEUROTOXICITY TESTING. Available online at: https://ntp.niehs.nih.gov/ntp/test_info/mog_guidance_508.pdf
- NTP. NTP's Modified One-Generation Reproduction Study 2011.
- NTP Technical Report on Toxicity Studies of Glyphosate Administered in Dosed Feed to F344/N Rats and B6C3F1 Mice. NIH Publication 923135 July 1992.
- OECD (1984), Test No. 206: Avian Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (1996), Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, OECD Publishing, Paris.
- OECD (2001), Test No. 416: Two-Generation Reproduction Toxicity, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- OECD (2007), Test No. 426: Developmental Neurotoxicity Study, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- OECD (2008). Series on testing and assessment number 43 guidance document on mammalian reproductive toxicity testing and assessment.
- OECD (2009), Test No. 230: 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (2009), Test No. 231: Amphibian Metamorphosis Assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (2012), Test No. 211: Daphnia magna Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (2012), Test No. 229: Fish Short Term Reproduction Assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (2016), Test No. 232: Collembolan Reproduction Test in Soil, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
- OECD (2016), Test No. 421: Reproduction/Developmental Toxicity Screening Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- OECD. (2018). Test No. 414: Prenatal Developmental Toxicity Study. Section 4, OECD Publishing, Paris.

REFERENCES

- OECD (2018), Test No. 443: Extended One-Generation Reproductive Toxicity Study, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- OECD (2019), Test No. 415: One-Generation Reproduction Toxicity Study, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
- Oliveira AG, Telles LF, Hess RA, et al. Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reprod Toxicol. 2007; 23(2):182-191.
- Ospina, C., Holguín, M.C., Escobar, D.C., & Valencia, C. Importance of hyperphosphatemia in chronic kidney disease, how to avoid it and treat it by nutritional measures. Revista Colombiana De Nefrología. 2017; 4(1), 38-56.
- Owagboriaye FO, Dedeke GA, Ademolu KO, et al. Reproductive toxicity of Roundup herbicide exposure in male albino rat. Exp Toxicol Pathol. 2017; 69(7):461-468.
- Paganelli A, Gnazzo V, Acosta H, et al. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem Res Toxicol. 2010; 23(10):1586-1595.
- Panzacchi S, Mandrioli D, Manservisi F, et al. The Ramazzini Institute 13-week study on Glyphosate-based herbicides at human-equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. Environ Health. 2018; 17(1):52.
- Perry MJ, Mandrioli D, Belpoggi F, Manservisi F, Panzacchi S, Irwin C. Historical evidence of Glyphosate exposure from a US agricultural cohort. Environ Health. 2019;18(1):42.
- Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32009R1107.
- Rocca MS, Wehner NG. The Guinea pig as an animal model for developmental and reproductive toxicology studies. Birth Defects Res. Part. B 2009. 86, 92e97.
- Romano RM, Romano MA, Bernardi MM, et al. Prepubertal exposure to commercial formulation of the herbicide Glyphosate alters testosterone levels and testicular morphology. Arch Toxicol. 2010; 84(4):309-317.
- Romano MA, Romano RM, Santos LD, et al. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Archives of Toxicology. 2012 Apr; 86(4):663-673.

REFERENCES

- Roy NM, Carneiro B, Ochs J. Glyphosate induces neurotoxicity in zebrafish. Environ Toxicol Pharmacol. 2016; 42:45-54.
- Rubin JL, Gaines CG, Jensen RA. Glyphosate Inhibition of 5-Enolpyruvylshikimate 3-Phosphate Synthase from Suspension-Cultured Cells of Nicotiana silvestris. Plant Physiol. 1984; 75(3):839-845.
- Ruuskanen, S., Rainio, M.J., Uusitalo, M. et al. Effects of parental exposure to Glyphosate-based herbicides on embryonic development and oxidative status: a long-term experiment in a bird model. Sci Rep. 2020; 10, 6349.
- Santos MJ, Ferreira MF, Cachada A, et al. Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei. Ecotoxicology. 2012; 21(8):2113-2122.
- Santos MJ, Soares AM, Loureiro S. Joint effects of three plant protection products to the terrestrial isopod Porcellionides pruinosus and the collembolan Folsomia candida. Chemosphere. 2010; 80(9):1021-1030.
- Schneider SZ (2012). Glyphosate: Fish Short-Term Reproduction Assay with the Fathead Minnow (Pimephales promelas). Unpublished study conducted by Wildlife International, Ltd, Easton, MD 21601. Laboratory report number 707A-102A. Study sponsored by Joint Glyphosate Task Force.
- Schimpf MG, Milesi MM, Ingaramo PI, LuqueNeonatal EH Varayoud J. Neonatal exposure to a Glyphosate based herbicide alters the development of the rat uterus. Toxicology. 2017; 376:2-14.
- Schönbrunn E, Eschenburg S, Shuttleworth WA, et al. Interaction of the herbicide Glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. Proceedings of the National Academy of Sciences of the United States of America. 2001 Feb; 98(4):1376-1380.
- Swan SH and Kristensen DM. Anogenital Distance: A Marker of Steroidal Endocrine Disruption. Human Reproduction. 2018; 30: 963b72.
- Singh NN, Das VK, Singh S. Effect of aldrin on carbohydrate, protein, and ionic metabolism of a freshwater catfish, Heteropneustes fossils. Bull Environ Contam Toxicol. 1996;57(2):204–10

REFERENCES

- Smith CM, Vera MKM, Bhandari RK. Developmental and epigenetic effects of Roundup and Glyphosate exposure on Japanese medaka (Oryzias latipes). Aquat Toxicol. 2019; 210:215-226.
- Suckow MA; Weisbroth SH; Franklin CL. The laboratory rat, 2nd ed. 2005. Elsevier Science.
- Suresh TP. Teratogenicity study in rabbits – test compound: Glyphosate technical (FSG 03090 H/05 March 1990). Study No. TOXI: 884-TER-RB 1993.
- Svendsen O. The minipig in toxicology. Exp Toxicol Pathol. 2006;57(5-6):335-339.
- Székács, András and Béla Darvas. "Re-registration Challenges of Glyphosate in the European Union." Frontiers in Environmental Science 6 (2018): 6, 78.
- Tasker EJ, Rodwell DE, Jessup DC. (1980a). Technical Glyphosate: teratology study in rabbits. Report No. IR-79-018.
- Transparency market research. (2014). From: Glyphosate Market for Genetically Modified and Conventional Crops - Global Industry Analysis, Size, Share, Growth, Trends and Forecast 2013 - 2019: https://www.transparencymarketresearch.com/Glyphosate-market.html
- Tsui MT, Chu LM. Aquatic toxicity of Glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere. 2003; 52(7):1189-1197.
- Tush D, Meyer MT. Polyoxyethylene Tallow Amine, a Glyphosate Formulation Adjuvant: Soil Adsorption Characteristics, Degradation Profile, and Occurrence on Selected Soils from Agricultural Fields in Iowa, Illinois, Indiana, Kansas, Mississippi, and Missouri. Environ Sci Technol. 2016; 50(11):5781-5789.
- Uren Webster TM, Laing LV et al. Effects of Glyphosate and its formulation, roundup, on reproduction in zebrafish (Danio rerio). Environ Sci Technol. 2017; 48(2):1271-9.
- U.S. Department of Agriculture (USDA)'s Agricultural Marketing Service (AMS). 2013 Pesticide Data Program (PDP) Annual Summary. Press Release No. 0276.14. Available on line at: https://www.usda.gov/media/press-releases/2014/12/19/usda-releases-2013-annual-summary-pesticide-data-program
- U.S. Department of Agriculture (USDA). Adoption of Genetically Engineered Crops in the U.S. Last update July 2020. Available online at: https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/

REFERENCES

- Vandenberg LN, Blumberg B, Antoniou MN, et al. Is it time to reassess current safety standards for Glyphosate-based herbicides?. J Epidemiol Community Health. 2017;71(6):613-618.
- Wunnapuk K, Gobe G, Endre Z, et al. Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers. Toxicol Lett. 2014; 225:192–200.
- Yasmin S, D'Souza D. Effect of pesticides on the reproductive output of Eisenia fetida. Bull Environ Contam Toxicol. 2007; 79(5):529-532.
- Yusof S, Ismail A, Alias MS. Effect of Glyphosate-based herbicide on early life stages of Java medaka (Oryzias javanicus): a potential tropical test fish. Mar Pollut Bull. 2014; 85(2):494-498.
- Zhang S, Xu J, Kuang X, et al. Biological impacts of Glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (Danio rerio). Chemosphere. 2017; 181:270-280.
- Zhou CF, Wang YJ, Yu YC, et al. Does Glyphosate impact on Cu uptake by, and toxicity to, the earthworm Eisenia fetida? Ecotoxicology. 2012; 21(8):2297-2305.