Caspase –Glo™ 3/7 assay:	Cytotoxicity assessment, apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96 -well plates
Test conditions:	Before the assay, cells were treated with different dilutions of Roundup Bioforce® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The Caspase-Glo® 3/7 reagent was prepared in a buffer. After 30 min at room temperature, 50 µL of Caspase-Glo® 3/7 reagent was added to 50 µL of culture medium containing the cells previously treated. After shaking the plate 15 min, an incubation period of 45 min at ambient temperature in the dark was required to stabilize the signal before luminescence measurement with a luminometer was performed.
Dose levels:	Not exactly specified, several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	10^5 per well in 96-well plates
Exposure duration:	3, 6, 9, 12, 18, 24 or 48 h
Replicates per dose level:	9
DAPI-labelling:	Apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	24 -well plates
Test conditions:	After 24 h incubation with various dilutions of the test substances, 24-well plates were centrifuged and the medium was removed slowly. Leydig cells were fixed for a day in ab absolute ethanol-chloroform–acetic acid (6:3:1, v/v/v) at -20 °C. The wells were rinsed with PBS (pH7.4) and incubated with 1µg/mL of a solution containing DAPI during 30 min. Each well was washed with water and then observed with a microscope using a fluorescent mode.
Dose levels:	0.05, and 1 % of Roundup Bioforce® and 1% of glyphosate in DMEM/Ham F12 medium
Cells per well:	30000 per well in 24-well plates
Exposure duration:	24 h
Replicates per dose level:	9
3β-hydroxysteroid dehydrogenase (3β-HSD) activity:	Assessment of testosterone production
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96-well plates

Test conditions:	Leydig cells were exposed to different concentrations of the test substances. Afterwards the wells containing the pretreated cells and 3β-HSD reagent containing DHEA (substrate), NAD (cofactor), NBT and nicotinamide were incubated at 37 °C for 45-60 min**.** Subsequently, as soon as the cells were stained, a solution of 10% acetic acid was added to solubilise the previously formed formazan crystals. The 3β-HSD activity was then measured by reading the optical density of each well at 560 nm (formazan) through a plate reader.
Dose levels:	Not exactly specified; several concentrations from 0 – 0.1% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	Not reported
Exposure duration:	24 h
Replicates per dose level:	9
Radioimmunoassay (RIA) of testosterone:	Assessment of testosterone production
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	Not reported
Test conditions:	The RIA was carried out on Leydig cells by competition and stopped using the method of activated charcoal. 200 µL of unlabeled testosterone standard solution, phosphate buffer or culture supernatant were incubated with 100 µL of radioactive testosterone and 100 µL of rabbit anti-testosterone antibody. After 30 min at ambient temperature the mixture was placed at 4 °C until the next day. Afterwards 500 µL of charcoal/dextran (50%/5%) was added and the mix incubated at 4 °C. Finally, the tubes were centrifuged (10 min at 2400 rpm at 4 °C) and the radioactivity counted.
Dose levels:	0, 0.0001, 0.0005, 0.001, 0.0025, 0.005, 0.0075 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium
Cells per well:	Not reported
Exposure duration:	24 h
Replicates per dose level:	9
Real time PCR:	Measurement of mRNA expression of aromatase, androgen receptor and estrogen receptor α- and β.
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	6-well plates
Test conditions:	After exposure of Leydig cells with the test substances cell pellets were treated with Trizol for the cell degradation. The chloroform was added to recover the aqueous phase containing the RNA. RNA precipitation was done by adding isopropanol and washing by adding 70% ethanol.

250 ng of RNA , 200 U of MMLV-RT (Moloney murine leukemia virus reverse transcriptase), 0.2 g of random primers, 500 mM of each dNTP and 20 U of recombinant RNasin® were incubate 90min at 37°C to obtain cDNA, The reaction was stopped by 5 min at 75 °C. The polymerase chain reaction was performed on cDNA using the method GoTaq® qPCR Master Mix (Promega). The PCR conditions were an initial step at 95 °C for 3 min, then 40 cycles of 30 s at 95°C abd 60°C for 60 s. mRNA levels of aromatase, estrogen receptor α and β and androgen receptor were normalized using the L19 control gene.

Dose levels: 0, 0.001, 0.005 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium

Cells per well: Not reported

Exposure duration: 24 h

Replicates per dose level: 9

6. Observations/analyses:

Measurements: Citotoxicity of Roundup Bioforce® or glyphosate measured through adenylate kinase activities; measurements of caspases 3 and 7 (key-caspases of apoptosis) in cell cultures by means of bioluminescence based method; study of chromatin condensation by DAPI-labelling; measurement of 3β-HSD activity; changes in testosterone production secreted from Leydig cells in medium

Statistics: All data are present as means ± SEM. Statistically significant differences from controls were determined by an ANOVA test followed by Bonferroni post-test with $p<0.001$ (****), $p<0.005$ (***), $p<0.01$ (**), and $p<0.05$ (*).

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline *in vitro* test with methodological (i.e. no positive controls included) and reporting deficiencies (e.g. dose levels not always specified).

2. Relevance of study: **Not relevant** (Due to reliability. In addition, *in vitro* data, do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment purposes.)

3. Klimisch code: **3**

Response - GTF

This publication presents no new findings relevant to the current discussions of glyphosate safety. It is clear from the previous work of Seralini and others that surfactants can injure or kill cells when applied to exposed cells living in a Petri-dish environment. It also is not surprising that injured cells demonstrate activation of injury-response systems or suffer from a general decline in a wide variety of cellular functions, including hormone production in cells which normally serve that function. The concentrations used in these experiments are not relevant to human exposures to glyphosate and the experimental system used is not relevant to whole animal outcomes. Importantly, the alleged impacts on endocrine function have not been observed in animal studies of glyphosate or other components of glyphosate formulations at

relevant concentrations. Authors state that the lowest concentration of glyphosate tested was 50 ppm, several orders of magnitude higher than an anticipated human intake (based on pharmacokinetics described in Anadon et al., 2009) following worst case dietary exposure at the ADI.

The experiments reported in this publication involve two additional cell types; Leydig and Sertoli cells from rat testes. However, Petri dish experiments in a laboratory are not representative of exposures to a living animal and are not informative about real-world risks to humans. Instead, these experiments demonstrate what we already know – substances, soaps, detergents of surfactants, can injure unprotected cells *in vitro*.

Author(s)	Year	Study title
Hokanson, R. Fudge, R. Chowdhary, R. Busbee, D.	2007	Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. Human & Experimental Toxicology Volume: 26 Pages: 747-752

Abstract*

Gene expression is altered in mammalian cells (MCF-7 cells), by exposure to a variety of chemicals that mimic steroid hormones or interact with endocrine receptors or their co-factors. Among those populations chronically exposed to these endocrine disruptive chemicals are persons, and their families, who are employed in agriculture or horticulture, or who use agricultural/horticultural chemicals. Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized *in vitro* DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes, determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed *in utero*.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate formulation
Source:	Unknown retail supplier
Purity:	Not reported
Concentration:	15% home use preparation

2. Vehicle and/or positive control: SFBS medium / no positive control

3. Test system/cells:

Cell line:	MCF-7
Source:	American Type Culture Collection (Rockville, MD, USA)
Growing medium:	MEM (minimal essential medium), phenol red-free MEM
Source:	Gibco (Gaithersburg, MD, USA)
Culture conditions:	Not reported
Further materials:	17β-estradiol (E2) (Sigma, St. Louis, USA), fetal bovine serum (FBS) (Summit Biotechnology, USA) RZPD microarray chips (Deutsches Ressourcencentrum für Genomforschung GmbH, Berlin, Germany) Roche´s cDNA synthesis kit (Roche) Real time PCR kit (ABI, NJ, USA); ABI 7500 Real-Time PCR

system thermocycler (ABI, USA)

4. Test method:

Study type:	*In vitro* DNA microarray analysis, quantitative real-time PCR (qrtPCR)
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Dose levels:	0.1, 0.01, 0.001 or 0.0001% dilutions of the glyphosate stock solution containing 15% glyphosate.
Duration of exposure:	18 h
Exposure:	MCF-7 cells were grown in MEM in T-150 vented culture flasks. Upon reaching 60% confluency, the medium was removed and replaced with phenol-red-free MEM containing 10% stripped fetal bovine serum (SFBS), to reduce the E2 availability to the cells. After a growing period of 24 hours the cells were treated with glyposate concentrations at 0.1, 0.01, 0.001 or 0.0001% dilutions of the stock solution (i.e. 15% glyphosate) with or without 3×10^{-10} M E2 for 18 hours.
DNA micro array:	Microarray analysis was performed in commercially available microarray slides. After 18 h exposure cells were harvested and RNA was purified. Closed DNA (cDNA) was generated from the isolated RNA using Roche´s cDNA synthesis kit. Cyanine-5 and cyanine-3 labeled anti-sense RNA was generated and hybridized using Wellmer´s protocol. The labelled RNA was loaded with a labelled control sample onto the array slides. Array slides were scanned in an Axon Genepix 4000B. Details of the hybridisation and scanning procedures were not reported.
qrtPCR:	Test was conducted in semi-skirted 96 well PCR plate using a commercially available PCR system
Replicates per gene of interest:	3

5. Observations/analyses:

Measurements:	Scan of microarray slides, quantitative rt-PCR
Statistics:	Statistical analysis utilized one-way ANOVA followed by Dunnett's test to analyse differences between control and chemically treated samples, with $P < 0.05$ considered to be statistically significant.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Not acceptable *in vitro* methods for test mixtures containing surfactant. Well documented study publication which meets basic scientific principles, but surfactants are inappropriate test substance in cell lines.

2. Relevance of study: **Not relevant** Temporal altered gene expression is not a biomarker for toxicity, but rather, may be within the range of normal biological responses of homeostasis. *In vitro* cytotoxicity of surfactants, however, is a significant confounder in data interpretation. Data do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment

purposes.)

3. Klimisch code: **3**

Response - GTF

- Relevance of altered gene expression in a cell line derived from a breast cancer should not be extrapolated to reflect human health endpoints.
- Altered gene expression should not be confused with adverse health outcomes. Rather altered gene expression may equally be considered a biological response within the range of normal homeostasis.
- The authors describe a "bewildering array" of possible human health endpoints, which are conspicuously absent in the vast glyphosate toxicology data base.
- The concluding sentence, with implications of both adult and foetal cell damage, lack biological plausibility when considering glyphosate *in vivo* ADME, kinetics and toxicology data.

IN VIVO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K.	1995	Toxic Effects of Carbofuran and Glyphosate on Semen Characteristics in Rabbits. Journal of Environmental Science and Health. Part B. Volume: 30 Number: 4 Pages: 513-534

Abstract*

The present study was undertaken to investigate the effect of chronic treatment with two sublethal doses of Carbofuran (carbamate insecticide) and Glyphosate (organophosphorus herbicide) on body weight and semen characteristics in mature male New Zealand white rabbits. Pesticide treatment resulted in a decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality. This was accompanied with increases in the abnormal and dead sperm and semen methylene blue reduction time. The hazardous effect of these pesticides on semen quality continued during the recovery period, and was dose-dependent. These effects on sperm quality may be due to the direct cytotoxic effects of these pesticides on spermatogenesis and/or indirectly via hypothalami-pituitary-testis axis which control the reproductive efficiency.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (N-(phosphonomethyl) glycine)-containing pesticide
	Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate)-containing pesticide
Active substance(s):	Glyphosate
	Carbofuran
Source:	Glyphosate: Monsanto Company, USA
	Carbofuran: Brichima S.P.A., Italy, Brifur
Purity:	Not reported
Lot/Batch #:	Not reported

2. Vehicle: Gelatine capsule

3. Test animals:

Species:	Rabbit
Strain:	New Zealand white
Source:	Not reported
Age of test animals at study initiation:	8 months
Sex:	Male
No. of rats:	20
Body weight:	2863 ± 59.8 g

Acclimation period:	Not reported
Diet/Food:	Ration pellets consisting of 48% berseem hay (Trifolium alexandrinum), 18% wheat bran, 16% ground corn, 14% soybean meal, 3% molasses, 0.5% salt, and 0.5% vitamins. Feed provided *ad libitum*
Water:	Water provided *ad libitum*
Housing:	Individually in cages
Environmental conditions:	Temperature: Not reported Humidity: Not reported Air changes: Not reported Light/dark cycle: Not reported

4. Test system:

Study type:	Toxic Effects of Carbofuran and Glyphosate on Semen Characteristics in Rabbits
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	18 weeks
Pre-exposure period:	6 weeks
Duration of exposure:	6 weeks
Recovery period:	6 weeks
Dose levels:	1st group – control group; 2nd group – 1/100 LD_{50} carbofuran; 3rd group – 1/10 LD_{50} carboruran; 4th group – 1/100 LD_{50} glyphosate; 5th group – 1/10 LD_{50} glyphosate The doses of the pesticides were calculated according to the animals' body weight on the day before dosing. (The LD_{50} values of both pesticides were not reported. Dose levels were not reported as mg/kg bw/day.
Animals per dose group:	4 animals per group
Administration:	Given orally into a gelatine capsule

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	Measured weekly in the morning before access to feed and water
Collection of test material:	Semen was collected once a week from all animals and continued throughout the 18-week experimental period
Measurement:	Volume of each ejaculate; Determination of seminal initial fructose was carried out directly after collection; Methylene blue reduction time (MBRT) was measured using

methylene blue semen mixture in a capillary tube;

Assessment of live, dead and abnormal spermatozoa were performed using an eosin-nigrosine blue staining mixture;

Evaluation of sperm concentration by the improved Neubauer hemocytometer slide using weak eosin solution;

Semen osmolality was determined by measuring the freezing point depression by using Osmete A (Precision Systems Inc., Sudbury, Mass., USA).

Food- and water consumptions: Not reported

Haematology: Not done

Clinical chemistry: Not done

Urine analysis: Not done

Sacrifice/pathology: Not reported

Organ weights: Not reported

Histology and morphometry: Not reported

Statistics: Data were analysed by generalized linear model procedure, Statistical Analysis System (SAS, 1984). The level of significance was reported as $P \leq 0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-GLP, non-guideline study with major reporting deficiencies. Dose-levels poorly defined as 1/10 and 1/100 LD_{50}. Purity of the test substances, source of animals, environmental conditions, mortality, and clinical signs not reported. No testis and epididymis weights were determined or reported and no histopathological examination conducted. In addition, stability and homogeneity assessment of test substance preparations were not done or not reported. Rabbits have low body weights at study start, suggesting impaired health status.

2. Relevance of study: **Not relevant** (Due to very low confidence in study conduct and the inadequacy of reporting)

3. Klimisch code: **3**

Response – summarized from Williams et al. (2000)

- Numerous serious deficiencies in the design, conduct, and reporting of this study which make the results uninterpretable.
- Only four rabbits per treatment group were used, and therefore statistics are questionable.
- Rabbits appeared to be small for their age; at study start (32 weeks) tested animals had 16-25% lower body weight than historical weights for commercially bred animals of the same age and strain.
- Low body weights as study start suggest compromised health status of the animals at initiation.
- Dose levels were not quantified.
- Purity of glyphosate and composition of the glyphosate formulation were not reported.
- Inadequate description of test material administration.
- Improper semen collection technique reported.

- Report is unclear whether control animal sham handling was undertaken, a critical factor in stress related outcomes in this species.
- Food consumption of test and control groups not adequately reported.
- Variability not adequately reported for endpoint measurements in test and control groups, preventing statistical analysis to support the author's conclusions.
- Dose-responses not observed, despite the wide dose spread.
- Sperm concentrations of all groups within normal ranges for this strain of rabbit.
- No meaningful conclusions can be drawn from this publication.

Author(s)	Year	Study title
Daruich, J. Zirulnik, F. Gimenez, M. S.	2001	Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses Environmental Research Volume: 85 Pages: 226-231

Abstract*

To prevent health risk from environmental chemicals, particularly for progeny, we have studied the effects of the herbicide glyphosate on several enzymes of pregnant rats. Glyphosate is an organo-phosphorated nonselective agrochemical widely used in many countries including Argentina and acts after the sprout in a systemic way. We have studied three cytosolic enzymes: isocitrate dehydrogenase NAD+ dependent, glucose-6-phosphate dehydrogenase, and malic dehydrogenase in liver, heart, and brain of pregnant Wistar rats. The treatment was administered during the 21 days of pregnancy, with 1 week as an acclimation period. The results suggest that maternal exposure to agrochemicals during pregnancy induces a variety of functional abnormalities in the specific activity of the enzymes in the studied organs of the pregnant rats and their fetuses.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Herbycigon
Active substance(s): Glyphosate
Source: Herbycigon, M.F. I. S.R.L., Argentinia
Lot/Batch #: Not reported
Purity: Not reported

2. Vehicle: Tap water

3. Test animals:

Species: Rat
Strain: Wistar
Source: National University of San Luis, Argentina
Age of test animals at study initiation: Not reported
Sex: Females
Body weight: 210-230 g
Acclimation period: 1 week
Diet/Food: 20 g of stock laboratory diet (elaborated at Cargill) per day; ingredients: meat flour, bone and meat flour, fish meal, blood flour, soybean meal, toasted soybean, soy expeller, sunflower flour, cotton flour, peanut meal, animal fat, corn, wheat, sorghum, oat, barley, wheat bran, rice bran, gluten meal, vitamins A, E, B, D3, K3, and B12, niacin, pantothenic acid, choline, ascorbic acid, bone ash, salt, calcium carbonate, oyster, manganese oxide, zinc oxide, ferrous sulfate, copper oxide, sodium selenite, iodine, and cobalt.

Water:	35 ml of potable water per day
Food and water for control group:	Low water and food (10 ml and 10 g, respectively)
Housing::	After mating, individually in cages
Environmental conditions:	Temperature: 22-25°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Enzymatic activity of cytosolic enzymes in pregnant rats and fetuses
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	21 days during pregnancy
Dose levels:	0 (tap water), glyphosate solution 0.5% w/v in tap water (dose: 0.2 ml glyphosate/ml water), glyphosate solution 1% w/v in tap water (dose: 0.4 ml glyphosate/ml water)
Animals per test substance group:	8
Animals per control group:	Tap water control group: 8 Low water and low food control group: 6 The latter group received only 10 g food and 10 mL tap water per day. This treatment began in the second week after the high dose group exhibited a decreased water- and foodintake.
Administration:	The test substance was prepared as solution in tap water. 35 mL of the test substance preparations were provided in water bottles per day and animal
Mating:	Female rats at the proeatrus stage were housed for one night with fertile males. Fertilisation was assumed by the presence of spermatozoa in the vaginal smear. That day was designated as gestation day 1.

5. Observations/analyses:

Analyses of test material preparations:	Not reported
Measurements:	Enzymatic activity of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, malic dehydrogenase
Mortality:	Not reported
Clinical signs:	Not reported
Maternal body weight:	Measured daily
Food- and water consumptions:	Measured daily
Test substance intake:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported
Sacrifice/pathology:	On day 21 of gestation, rats were anesthetisied with

	diethylether. Each foetus was delivered by rapid hysterectomy, identified, weighed and then killed by decapitation. Maternal and foetal livers, hearts, and brains were immediately removed, washed in a cold saline solution, and stored at -20°C until analysis. Foetal organs were pooled.
Tissue sample processing:	Livers, hearts, and brains (0.5g/1 ml buffer) were homogenised in an Ultra Turrax with 0.5 M Tris-HClbuffer, pH 7.4 containing 1 mM dithiothreitol. Cytosolic fractions were obtained by ultra centrifugation.
Measurements (enzymatic assays):	Enzymatic activities of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and maic dehydrogenase were measured in the supernant by the determination of the rate of NADPH formation at 340 nm in a spectrometer. The results were expressed as µmol NADP/min/mg protein. Protein concentration was measured by Biuret reaction.
Organ weights:	Liver, hearts and brains of maternal females
Histopathology:	Not done.
Statistics:	Significant differences among means were considered at a level of $P < 0.05$ and identified by one-way ANOVA, Kolmogorov-Smirnov and Newman-Keul procedures. In all the cases the variances were homogeneous.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Basic data given, however, the study is performed with methodological and reporting deficiencies (unknown exposure levels, only cytosolic enzymes measured, inappropriate controls, lack of consistent dose-response data).

2. Relevance of study: **Not relevant** (due to reliability. In addition, study was performed with a glyphosate formulation (commercialised in Argentina) and not with glyphosate)

3. Klimisch code: 4

Response 1 – GTF

- Test substance administration is poorly described, but rough calculations on approximate surfactant intake show excessively high and unrealistic exposures when compared to DART systemic parental and reproductive/developmental NOAEL values for POEA formulation surfactants.
    - For the low dose group, based on 360 g/L glyphosate solution containing 18% surfactant, 0.1 mL glyphosate (conservatively assumed to be the formulation)/mL water = 0.018 mL surfactant/mL water. Assuming water consumption of 10 mL/day surfactant intake = 0.18 mL per rat per day. Assuming surfactant density of 1 g/mL and 250 gram rat, surfactant low dose = 720 mg/kg/day.
    - Conservative high surfactant dose estimate = 1440 mg/kg/day
    - Conservative estimate of surfactant intake is at least one order of magnitude greater than parental and DART NOAEL values reported in Williams et al. (2012).

Response 2 – summarized from Williams et al. (2012)

- Test substance and doses not adequately described.

- Inappropriate control groups.
- Results suggest that the effect of treatment on body and organ weights may be due to reduced food and water intakes.
- A consistent effect of treatment was not observed and dose-response relationships were generally lacking
- The information gathered may be misleading because the enzymes monitored are found in both the cytosol and mitochondria.
- Food restriction affects the activity of many enzymes, including those examined in this study.
- Same comments apply to Bueret et al. (2005; on-line version 2004), in which test group dams showed a 23% reduction in food consumption, 21% reduction in water consumption and 42% reduction in body weight gain versus controls.

Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Coelho, R. S. Pereira, J. D. Dalsenter, P. R. Langeloh, A.	2003	The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats Toxicology letters Volume: 142 Pages: 45-52

Abstract*

The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup(R) (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Cesarean sections were performed on day 21 of pregnancy, and number of corpora lutea, implantation sites, living and dead fetuses and resorptions were recorded. Weight and gender of the fetuses were determined, and fetuses were examined for external malformations and skeletal alterations. The organs of the dams were removed and weighed. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup(R) is toxic to the dams and induces developmental retardation of the fetal skeleton.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup®
Active substance:	Glyphosate
Source:	Monsanto of Brasil
Lot/Batch #:	BS 1096/98
Concentration:	360 g/l
Surfactant Class:	Polyoxyethyleneamine (POEA)
Concentration:	18% (w/v) (POEA)

2. Vehicle: Distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Department of Pharmacology, Instituto de Ciencias Basicas da Saude, Brazil
Age of test animals at study initiation:	90 days
Sex:	Male and virgin female
Body weight:	200-280 g
Acclimation period:	Not reported
Diet/Food:	Laboratory rat chow, *ad libitum*
Water:	Water , *ad libitum*

Housing:	Polyethylene (65 x 25 x 15 cm) home cages, with sawdust-covered floors
Environmental conditions:	Temperature: 22 ± 2°C
	Humidity: not reported
	Air changes: not reported
	12-hour light/dark cycle

4. Test system:

Study type:	Developmental toxicity study
Guideline:	Refers to the EPA (Environmental Protection Agency), 1996. Guidelines for Reproductive Toxicity Risk Assessment-EPA/630/R-96/009, Washington, USA, pp. 1-163. (reproductive toxicity protocols; segment II).
GLP:	no
Guideline deviations:	Reduced allowed mating time
Duration of study:	From day 6 up to 15 of gestation
Dose levels:	0 (water), 500, 750 or 1000 mg/kg glyphosate-Roundup® diluted in water
Animals per dose group	Sixty pregnant rats were divided into four groups (n=15±1 per group).
Administration:	Test substance preparations were prepared by diluting the Roundup formulation with appropriate volumes of distilled water. Applications were done once daily by oral gavage Dosing volume: 10 mL/kg bw
Mating:	3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.

5. Observations/analyses:

Test substance preparations:	Not reported
Mortality:	Assessed, but details (e.g. time points, etc) not specified.
Clinical signs:	Not reported
Body weight:	Maternal body weights were determined daily during pregnancy and lactation periods. Offspring body weights were determined in weekly intervals from lactation to puberty
Body weight gain:	The body weight noted at day 0 (sperm positive smear) in parent females was considered as 100 %. The differences observed during the study with regard to this parameter were expressed as relative weight gain.
Food- and water consumptions:	In three day intervals during pregnancy. Data presented as relative intakes without reference to how data were normalized.
Test substance intake:	Not applicable
Sacrifice/pathology:	On day 21 of gestation dams were anesthetized with a combination of 5 mg/kg bw xylazine and 90 ,g/kg bw ketamine injected intramusculary and subjected to caesarean section. The uterus was removed and weighed with its contents.

Organ weights:	The weights of the following organs were determined and relative organ-to-body weights were calculated.
	Maternal: heart, lungs, liver, spleen and kidney
Developmental parameters:	Number of living and death foetuses, number of implantation sites, corpora lutea, resorptionssex of pups, sex-ratio, external malformations and skeletal alterations.
	Reported errors include more foetuses than implantation sites in one dose group.
	Note artifacts from atypical fixing and staining of foetal skeletons may have caused skeletal damage.
Statistics:	Parametric data, expressed as mean ± S.E.M., were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Duncan test when appropriate. The non-parametric data, expressed as proportion or percentage, were analyzed by the x^2-test. Differences were considered to be statistically significant when $P<0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design similar to US-EPA and OECD 414, with deviations (e.g. group size, inadequate dosing period) and reporting deficiencies. In addition, some methodological deficiencies (e.g. histopathological methods)

2. Relevance of study: Relevant study type for investigating developmental endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product not glyphosate.

3. Klimisch code: **3**

Response 1 – GTF

- This non-guideline prenatal developmental toxicity study with a POEA containing formulation may be compared directly with the test guideline and GLP compliant POEA rat prenatal developmental toxicity study, in which the same POEA surfactant maternal NOAEL was 15 mg/kg/day, and developmental NOAEL was considered the highest dose tested, 300 mg/kg/day.
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
    - If based on dose levels of 500, 750 or 1000 mg/kg formulation, surfactant doses are 90, 135 and 180 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
    - If based on dose levels of 500, 750 or 1000 mg/kg glyphosate technical acid (versus the salt form in the formulated product), surfactant doses are even more extreme, approximately 250, 375 and 5000 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
- This publication reports excessively high and unrealistic exposures to the POEA surfactant in the tested formulation.
- While reporting weight gain in an atypical manner as relative %, actual reported mean body weight gains for mid and high dose groups align with the control group, while the low dose group body weight gain is approximately 20% less than the control group, indicating significant maternal toxicity in the low dose group. This significant non-dose related toxicity brings the quality and accuracy of this study into question.

Response 2 – summarized from Williams et al. (2012)

- Non-guideline prenatal developmental toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- 15 rats per group, significantly lower than the recommended minimum of 20 litters per group in OECD 414.
- High dose group was further reduced to 7 pregnant dams due to maternal deaths.
- Few data presented in the publication.
- Unusual data presentation for body weight, food intake and water consumption, all a relative numbers without any reference to normal values.
- Fetal findings are presented as percentages or unsubstantiated mean values throughout the article, which complicates interpretation.
- Further investigation data presented notes a number of reporting errors (see Williams et al., 2012, Table 3). For example, in the 750-mg/kg/d treatment group, more fetuses than implantation sites were reported.
- Reports a dose-related increased incidence of skeletal alterations.
- Unusual methods described to fix and stain the fetal skeletons for evaluation which may have led to artifacts that were falsely categorized as alterations (use of a proteolytic enzyme which may have digested peptide bonds in the bone matrix). The reported skeletal alterations showed an extremely high prevalence of incomplete ossification of various bone structures, which are signs of a developmental delay that correct themselves within a brief period.
- treatment during gestation days 6-15 rather that to full term as per current test OECD 414 guidelines
- "Based on the use of these questionable methods, and the obviously flawed reporting of data, it is not possible to draw any conclusions regarding the developmental effects of "Roundup" treatment from this article. Furthermore, because a commercial formulation was used, it is not possible to attribute any observed effects to glyphosate specifically."

Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Oliveira, R. T. Andrade, A. J. M. Dalsenter, P. R. Langeloh, A.	2007	Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats Archives of Toxicology Volume: 81 Pages: 665-673

Abstract*

Glyphosate is the active ingredient and polyoxyethyleneamine is the surfactant present in the herbicide Roundup (R) formulation commercialized in Brazil. The aim of this study was to assess the reproductive effects of glyphosate-Roundup (R) on male and female offspring of Wistar rats exposed during pregnancy and lactation. Dams were treated orally with water or 50, 150 or 450 mg/kg glyphosate during pregnancy (21-23 days) and lactation (21 days). These doses do not correspond to human exposure levels. The results showed that glyphosate-Roundup (R) did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis, tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup (R) may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test Item:	Roundup ®
Active substance(s):	Glyphosate
Source:	Monsanto of Brazil
Lot/Batch #:	Not reported
Concentration:	360 g/L
Surfactant:	Polyoxyethyleneamine (POEA)
Concentration:	18% (w/v) POEA

2. Vehicle: Distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Department of Pharmacology, Federal University of Rio Grande do Sul, Brazil
Age of test animals at study initiation:	90 days
Sex:	Male and female
Body weight:	250-350 g
Acclimation period:	Not reported
Diet/Food:	standard lab rat chow (Nuvital®, Curitiba/PR, Brazil), *ad*

libitum

Water: Water, *ad libitum*

Housing:: Polyethylene (65 x 25 x 15 cm) home cages with sawdust-covered floors

Environmental conditions: Temperature: 22 ± 2°C
Humidity: not reported
Air changes: not reported
12-hour light/dark cycle

4. Test system:

Study type: Reproductive toxicity

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 21-23 days during pregnancy;
21 days during lactation

Dose levels: 0 (water), 50, 150, 450 mg/kg glyphosate-Roundup®

Mating: 3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.

Animals per dose group: Sixty primigravid female rats were randomly divided into 4 groups of 15 animals each

Administration: Test substance preparations were prepared by diluting the Roundup-formulation with appropriate volumes of distilled water.
Applications were done once daily by oral gavage
Dosing volume: 10 mL/kg bw

5. Observations/analyses:

Test substance preparations: Not reported

Mortality: Assessed, but details (e.g. timepoints, etc) not specified.

Clinical signs: Assessed, but details (e.g. timepoints, etc) not specified.

Body weight: Maternal body weights were determined daily during pregnancy and lactation periods.
Offspring body weights were determined in weekly intervals from lactation to puberty.

Body weight gain: The body weight noted at day 0 (first period day) in parent females was considered as 100 %, for each period. The differences observed during the study with regard to this parameter were expressed as relative weight gain.

Food- and water consumptions: Not done

Test substance intake: Not applicable

Haematology: Not done

Clinical chemistry: Not done

Hormone levels: For determination of testosterone levels, blood was collected at termination, and the serum was removed. The samples were

	analysed in duplicate using a double-antibody according to the standard protocol for the radioimmunoassay (RIA) with Diagnostic Products Corporation testosterone kits.
Urine analysis:	Not done
Litter data:	Litter size, number of living and dead pups, viable pups, sex ratio (male/female)
Offspring development:	The development of offspring was assessed daily from lactation until puberty. The following characteristics were assessed: ears unstuck, fur emergence, incisor eruption, eye opening, testis descent (by scrotum palpation starting after the 15th postnatal day), preputial separation (by manually retracting the prepuce with gentle pressure after the 30th postnatal day) and opening of the vaginal canal (after the 30th postnatal day)
Sacrifice/pathology:	Males: One male from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65 days) and adulthood (age: 140 days). Selected males were sacrificed by thiopental anaesthesia followed by diaphragm incision. Females: One female from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65-70 days) and adulthood (age: 140 days).
Organ weights:	The weights of the following organs were determined and relative organ-to-body weights were calculated. Males: heart, lungs, liver, spleen, kidneys, adrenal glands and brain, testis, epididymis, seminal vesicle with coagulating glands (without fluid) and prostate Females: heart, lungs, liver, spleen, kidneys, adrenal glands and brain, uterus, oviducts and ovaries
Histopathology:	Five testes per dose group were fixed in Bouin´s solution immediately after removal, embedded in paraffin, sectioned at 3 µm and stained with hematoxylin/eosin. 20 essentially round seminiferus tubules per testis were analysed microscopically. The following parameters were assessed: tubule diameter, percentage of seminiferus tubules with complete spermatogenesis, presence of degenerating, sloughed and/or infiltrating cells, and absence of tubular lumen and of elongated spermatids.
Reproductive toxicity assessment:	Relative weight of the reproductive organs expressed as percentage of body weight and of reproductive indices, including sperm number per epididymis tail, daily sperm production, sperm transit, sperm morphology, testis morphology and serum testosterone level. Spermatid and sperm counts were determined.
Statistics:	Parametric data, expressed as mean ± standard error (SEM), were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Bonferroni test when appropriate. The nonparametric data, expressed as proportion or percentage, were analyzed by the chi-square test. Differences were

considered statistically significant when $P < 0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Study that does not comply with any test guideline. Reporting deficiencies. Conflicting results include decreased testes weights but increased testosterone levels in high dose. Questionable micrograph quality and interpretation may be artifacts of processing techniques. Conclusions not consistent with findings when viewed in light of dose-response or historical data for this strain of rat.

2. Relevance of study: **Not relevant** based on lack of dose response, contradicting findings and unreliable data quality)

3. Klimisch code: **3**

Response 1 – GTF

- This non-guideline reproductive toxicity study with a POEA containing formulation may be compared to the DART NOAEL values for POEA surfactants reported in Williams et al. (2012).
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
- If based on dose levels of 50, 150 or 450 mg/kg formulation (18% POEA), surfactant doses are 9, 27 and 81 mg/kg/day. In this case, doses are in the range of NOAEL values reported by Williams et al. (2012).
- Based on dose levels of 50, 150 or 450 mg/kg glyphosate technical acid (versus the salt form in the formulated product), POEA surfactant doses would be approximately 25, 75 and 225 mg/kg/day. In this case the low/mid doses are in the range of NOAEL values and the high dose exceeds NOAEL values reported by Williams et al. (2012).
- The findings reported by Dallegrave et al. (2007) are contrary to the GLP and guideline compliant studies reviewed by Williams et al. (2012), in which no effects on testis morphology, sperm parameters or testosterone levels were evident.

Response 2 - summarized from Williams et al. (2012)

- Non-guideline prenatal developmental-reproductive toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- Maternal toxicity was not observed.
- Reproductive outcomes (number of pups, sex ratio, etc.) and pup weights unaffected.
- Statistical increased percentage of abnormal sperm in male offspring at the low but not medium or high dose offspring, suggesting a random finding
- Non-dose-related delay in vaginal opening in females within the normal physiological range for the species and in line with historical control data.
- Non-dose-related early preputial separation in the high dose males within the normal physiological range for the species and in line with historical control data.
- Contrary to expected outcome of early preputial separation, a statistical decrease in blood testosterone levels was also observed at puberty for high dose males.
- Decreased testosterone level was no longer evident at adulthood
- No dose-related findings in adult sperm production parameters

- Investigators fail to mention enlarged interstitial cells in the micrographs, suggesting limited experience conducting such histological examinations.
- The other reported histological interpretations, reduction in elongated spermatids and the presence of vacuolization at puberty and degeneration of the tubular lumen at adulthood, may be attributable to an artifact of tissue processing rather than exposure related effects.
- Multiple guideline study types and a subchrionic National Toxicology Program study do not report the testicular anomalies described by Dallegrave et al. (2007).

Author(s)	Year	Study title
Romano, R.M. Romano, M.A. Bernardi, M.M. Furtado, P.V. Oliveira, C.A.	2010	Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Archives of Toxicology Volume: 84 Pages: 309-317

Abstract*

Glyphosate is a herbicide widely used to kill weeds both in agricultural and non-agricultural landscapes. Its reproductive toxicity is related to the inhibition of a StAR protein and an aromatase enzyme, which causes an in vitro reduction in testosterone and estradiol synthesis. Studies in vivo about this herbicide effects in prepubertal Wistar rats reproductive development were not performed at this moment. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis. Results showed that the herbicide (1) significantly changed the progression of puberty in a dose-dependent manner; (2) reduced the testosterone production, in semineferous tubules' morphology decreased significantly the epithelium height ($P < 0.001$; control = 85.8 ± 2.8 µm; 5 mg/kg = 71.9 ± 3.3 µm; 50 mg/kg = 69.1 ± 1.7 µm; 250 mg/kg = 65.2 ± 1.3 µm) and increased the luminal diameter ($P < 0.01$; control = 94.0 ± 5.7 µm; 5 mg/kg = 116.6 ± 6.6 µm; 50 mg/kg = 114.3 ± 3.1 µm; 250 mg kg = 130.3 ± 4.8 µm); (4) no difference in tubular diameter was observed; and (5) relative to the controls, no differences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 ± 12.9 ng/dL; 5 mg/kg = 108.6 ± 19.6 ng/dL; 50 mg/dL = 84.5 ± 12.2 ng/dL; 250 mg/kg = 76.9 ± 14.2 ng/dL). These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L as isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported
Age of test animals at study initiation:	21 days
Sex:	Male
No. of rats:	68

Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats
Water:	Mineral water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Evaluation of endocrine disruption potential of glyphosate formulation by assessment of rats prepubertal reproductive development.
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	From postnatal day (PND) 23 until PND53
Dose levels:	Control group – deionized water; 5, 50 or 250 mg/kg of body weight of glyphosate-Roundup Transorb
Animals per dose group:	4 treatment groups, 17 animals per group
Animal selection	No mention of avoiding selection of siblings within the same group to control for possible litter effects
Administration:	The glyphosate-Roundup Transorb was diluted in a watery suspension and administered once a day, by gavage; Dosing volume: 0.25 mL/100 g of body weight, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The experimental design was composed of random blocks, with the formation factor of these blocks as the body weight at the PND23. All the animals were weighed, and the average and standard deviation were calculated. The animals having body weights lower or higher than two standard deviations from the average were removed from the experiment.
Determination of puberty age:	Evaluation of the balanopreputial separation was made, which consists of the separation of the preputial membrane and the externalization from the glands of the penis. The assessment, which included gentle tissue manipulation, was performed once per day from the PND33 and was completed at the time of the balanopreputial separation. No discussion on whether this was a blinded procedure to avoid bias.
Hormone measurements:	Hormone concentrations of testosterone, estradiol and

	corticosterone in the serum were measured by radioimmuno-assay (RIA) from commercial kits (Testosterone Total Coat-A-Count, Estradiol Coat-A-Count and Coat-A-Count Corticosterone in rats, DPC, Los Angeles, CA, USA).
Food- and water consumptions:	Not reported
Haematology:	Not done
Clinical chemistry:	Not done
Urine analysis:	Not done
Sacrifice/pathology:	On PND 53. No details reported.
Organ weights:	The testes (right and left) and the adrenal glands (right and left) were weighed in absolute values and then transformed to relative weights as mg/100 g of body weight at PND53.
Histology and morphometry:	The testes and adrenal glands of all 68 animals were fixed in Bouin´s solution for 8 h; treated with alcohol, embedded in paraffin and prepared as stained laminas with hematoxylin and eosin. Laminas were analysed by light microscopy (40x and 100 x magnification). The linear morphometry from the seminiferous tubules were analysed by determining the tubular diameter (measured from the basal lamina to the basal lamina in the opposite direction), seminiferous epithelium (from the basal lamina to the neck of the elongated spermatids) and luminal diameter. Micrographs presented are of poor quality with artifacts such as shrinkage. Considered together with the natural variably in spermatogenesis of pubescent rats, the accuracy of morphometric data comes into question. For each tubule, the averages were calculated for the measurements indicated and, then, the average of each Weld was also calculated. The measurement for each animal was obtained through measure of all the analyzed Welds.
Statistics:	The variables under study were first submitted to tests of normality from Kolmogorov–Smirnov and homocedasticity by the test of Bartlet. When some of the premises of parametric testing were not obtained, non-parametric tests were chosen for subsequent averages and tests. Statistical differences were considered significant when the value of *P* was lower than 0.05. The values were expressed in mean (*x*) and standard error of the mean (±SEM). Data analysis of daily weights was performed through the two-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM). The weights were compared between diVerent groups and diVerent ages, considering the evolution expected by the body growth. The day and the weight of the complete balanoprepucial separation were compared among the groups using non-parametric analyses by the Kruskal–Wallis method followed by the post hoc Dun test. The testis and the adrenal weights were analyzed by the Kruskal–Wallis followed by the post hoc Dunn test, or by using a one-way analysis of variance (ANOVA) followed by the post hoc Tukey test. The testis measures of tubular diameter and

epithelium depth, as well as the serum concentrations of testosterone, estradiol and corticosterone, were analyzed by the ANOVA followed by the Tukey test.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study with methodological and reporting deficiencies or conflicting findings. Eg, increased relative testicular weights, but decreased testosterone measurements.

2. Relevance of study: Relevant study type for investigating male reproductive endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product, not glyphosate.

3. Klimisch code: **3**

A comprehensive review, pointing out a significant number of issues with this publication, was undertaken by experts in reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc I, and included in Appendix K and their summary is quoted below.

> "To the uninformed reader, this manuscript by Romano et al. appears to demonstrate that exposure to Roundup Transorb alters testosterone levels and testis morphology. In this respect, the importance of these data to the scientific literature can be grossly over-interpreted by the uninformed reader. Upon closer examination, the authors have failed to provide robust data to support their conclusion that the "commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats". The authors failed to measure many of the key parameters in the validated pubertal male assay protocol by Stoker et al., (2000a) and hence generated data that were internally inconsistent, incomplete or in error. The results lack the scientific rigor necessary to support a definitive scientific conclusion and certainly do not equal or offset previous large, definitive and GLP-compliant studies concluding that Roundup and glyphosate do not affect reproductive development."

Author(s)	Year	Study title
Romano, M.A. Romano, R.M. Santos, L.D. Wisniewski, P. Campos, D.A. de Souza, P.B. Viau, P. Bernardi, M.M. Nunes, M.T. de Oliviera, C.A.	2012	Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression Archives of Toxicology Volume: 86 Number: 4 Pages: 663-673

Abstract*

Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-day-old male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount, testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate (isopropylamine salt)
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported

Age of test animals at study initiation:	90 days
Sex:	Female
No. of rats:	12
Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats, *ad libitum*
Water:	Water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Glyphosate effects on the reproductive development of male offspring
Guideline:	Non-guideline study
GLP:	No
Guideline deviations:	Not applicable
Duration of exposure:	From gestational day 18 to postnatal day (PND) 5
Dose levels:	Control group – deionized water; 50 mg/kg bw of glyphosate
Animals per dose group:	2 treatment groups, animals per group – not reported
Administration:	Roundup Transorb was diluted in a watery suspension and administered once a day by gavage from Gestation Day 18 to Post Natal day 5. Dosing volume: 0.25 mL/100 g bw, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The pups were weighted at PND21 (weaning), PND30, PND40 and PND60 to compare the body growth between the groups.
Sexual partner preference:	The sexual partner preference was assessed at PND 60 by exposing male offspring from treated and non-treated mothers to female stimulus rats (i.e. ovariectomised female rats that were treated with estradiol (50 µg/kg bw s.a. 54 h before the test) and progesterone (2 mg/kg bw s.c., 6 h before test)).
Sexual behaviour:	Sexual behaviour was assessed at PND 60 by exposing the male rats to an oestrus-induced female for 40 min. Several parameters were assessed incl. Number of mounts, intromission, ejaculatory intervals, number of attempted mounts).
Determination of puberty age:	Evaluation of the balanopreputial separation (separation of the preputial membrane and externalization from the glands of the

	penis). The assessment (including gentle tissue manipulation) was performed once per day from the PND33 and was completed at the time of the balanopreputial separation.
Hormone measurements:	Hormone serum concentrations of testosterone, estradiol in the serum were measured by radioimmunoassay-assay (RIA) from commercial kits (Coat-A-Count, DPC, Los Angeles, CA, USA). The serum FSH and LH measurements were determined by chemiluminescence immunoassay using Luminex xMAP technology (Milliplex MAP rat pituitary panel, Billerica, MA, USA).
Pituitary hormone levels:	mRNA-levels of LH, FSH and GH were assessed by real-time PCR in homogenised pituitary tissues. Protein expression of LH, FSH and GH was assessed in homogenised pituitary tissues using Western-blot analysis.
Food- and water consumptions:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported
Sacrifice/pathology:	Not reported
Organ weights:	The testes, epididymides and seminal vesicle were weighed, and the values were converted to relative weights of mg/100 g bw at PND60. The epididymis was previously divided into three segments: caput, corpus and cauda. The seminal vesicle was weighted with fluid (undrained) and after fluid removal (drained).
Sperm evaluation:	At PND 60, the sperm counts were determined. Testes and epididymes (capus, corpus, cauda) were weighed. The tunica albuginea was removed from the testes, and the parenchyma was homogenized. The samples were then diluted 10 times in saline, and the mature spermatids resistant to homogenization were counted using a hemocytometer. Daily sperm production was calculated. The segments of the epididymis were cut with a scissor, homogenized, diluted and counted. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for the parts of the epididymis were calculated. The mean time for sperm transit through the epididymis was calculated.
Histology and morphometry:	The testes were fixed in Bouin's solution for 8 h, treated with alcohol and embedded in paraffin, and were prepared as stained laminas with hematoxylin and eosin. Laminas were analysed by light microscopy (40x and 100 x magnification). Linear morphometry of the seminiferous tubules were analyzed by determining the tubular diameter, seminiferous epithelium length and luminal diameter. For each tubule, the averages were calculated for the measurements indicated, and the average of each field was also calculated. The measurement for each animal was obtained by measuring all the analyzed fields.
Statistics:	First the Kolmogorov–Smirnov tests for normality and the

Bartlett test for homoscedasticity. For analysis of body growth the multi-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM) was used. Weights were compared between different groups and ages, considering the expected changes with age. The sexual behavior and day of PPS were compared among the groups using the Mann–Whitney *U* test. Weights of seminal vesicle (drained and undrained) were compared by paired Student's *t-test.* All other parameters were analyzed by Student's *t-test*. Statistical differences were considered significant when the value of P was < 0.05. Values were expressed as means and the standard error of the mean (±SEM) for parametric and interquartile ranges of nonparametric analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline, non-GLP study meeting scientific principles. Unusual and short dosing regiment commencing towards the end of pregnancy (GD18, rather than GD6 as per OECD Test Guidelines 414) through post natal day 5. *In vivo* study with reporting deficiencies (detailed strain description, source of animals, housing conditions, no information if clinical signs were assessed, stability and homogeneity assessment of test substance preparations, no of male offspring evaluated in individual tests evaluated). A number of atypical endpoints evaluated.

2. Relevance of study: Not relevant (due to questionable dosing regimen and atypical array of endpoints measured

3. Klimisch code: **3**

This quality and value of this follow-up study to Romano et al. (2010) is consistent with their previous publication. Selective literature citations in the introduction frame the basis for this research as endocrine disruption potential, referring mostly to the publications from Seralini laboratory, previously discussed. The concluding sentences inaccurately cite published *in vitro* research (Richard et al., 2005) as evidence that "women occupationally exposed to this herbicide have reproductive disorders".

From the outset, the study design and endpoint selection are not consistent with other research in the field of developmental and reproductive toxicology, suggesting a lack of experience in this well published and studied discipline. Dosing was very limited to dams, starting on gestation day 18, well after organogenisis, through post natal day 5. No controls for litter effects appear to be reported, confounding interpretation of results.

Any glyphosate exposure to offspring either before or after parturition is questionable. ADME studies with glyphosate clearly demonstrate poor absorbtion via the gastrointestinal tract, rapid excretion of systemic doses via urine and a lack of bioaccumulation. Restricted placental transfer for glyphosate is documented in an *ex vivo* human perfusion system, in which the three other compounds tested (caffeine, benzoic acid and antipyrine) demonstrated much greater transfer kinetics across the placenta (Mose et al., 2008). Given the physico-chemical properties and *in vivo* kinetics of glyphosate, exposure to offspring during lactation should be considered negligible, if any.

With the very short window of maternal exposure, biological plausibility of any test substance related effects in the mature offspring is questionable. However, the normal variability of some unusual or atypical endpoint measurements, such as "sexual partner preference" along with mRNA and protein expression, is not known. Of particular concern, however, are differences in critical endpoints for control animals reported in Romano et al. (2010) compared to Romano et al. (2012); these include increased day of preputional sepratarion (PPS) of control male rate (37 days in 2010; 47 days in 2012), body weight at day of PPS (146 grams in 2010; 245 grams in 2012), serum testosterone concentrations (155 ng/dL in 2010; 63 ng/dL in 2012), estradiol concentrations (32 pg/mL in 2010; 1.4 pg/mL in 2012), subular diameter (266 µm in 2010; 479 µm in 2012), epithelial height (86 µm in 2010; 92 µmin 2012) and luminal height (94 µm in 2010; 257µm in 2012). Therefore, results are difficult to interpret, particularly for relevance to human health risk assessment. The merits of this publication should be placed in context with the quality of the authors' previous published research (Romano et al., 2010), as critiqued by experts in DART and ED above.

EPIDEMIOLOGY DART/ED PUBLICATIONS

Author(s)	Year	Study title
Arbuckle, T. E. Lin, Z. Mery, L. S.	2001	An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population Environmental Health Perspectives Volume: 109 Pages: 851-857

Abstract*

The toxicity of pesticides on human reproduction is largely unknown—particularly how mixtures of pesticide products might affect fetal toxicity. The Ontario Farm Family Health Study collected data by questionnaire on the identity and timing of pesticide use on the farm, lifestyle factors, and a complete reproductive history from the farm operator and eligible couples living on the farm. A total of 2,110 women provided information on 3,936 pregnancies, including 395 spontaneous abortions. To explore critical windows of exposure and target sites for toxicity, we examined exposures separately for preconception (3 months before and up to month of conception) and postconception (first trimester) windows and for early (< 12 weeks) and late (12–19 weeks) spontaneous abortions. We observed moderate increases in risk of early abortions for preconception exposures to phenoxy acetic acid herbicides [odds ratio (OR) = 1.5; 95% confidence interval (CI), 1.1–2.1], triazines (OR = 1.4; 95% CI, 1.0–2.0), and any herbicide (OR = 1.4; 95% CI, 1.1–1.9). For late abortions, preconception exposure to glyphosate (OR = 1.7; 95% CI, 1.0–2.9), thiocarbamates (OR = 1.8; 95% CI, 1.1–3.0), and the miscellaneous class of pesticides (OR = 1.5; 95% CI, 1.0–2.4) was associated with elevated risks. Postconception exposures were generally associated with late spontaneous abortions. Older maternal age (> 34 years of age) was the strongest risk factor for spontaneous abortions, and we observed several interactions between pesticides in the older age group using Classification and Regression Tree analysis. This study shows that timing of exposure and restricting analyses to more homogeneous endpoints are important in characterizing the reproductive toxicity of pesticides.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various pesticides (herbicides, insecticides, fungicides, miscellaneous)
Active substance(s):	Dicamba, glyphosate, 2,4-DB, 2,4-D, MCPA, atrazine, cyanazine, carbaryl, captan
Chemical families:	Phenoxy acetic acid (phenoxy herbicides), triazine, organophosphates, thiocarbamate

2. Vehicle and/or positive control: Not applicable

3. Test group:

Number of test persons:	2110
Age:	≤ 44 years
Sex:	Females
Inclusion criteria:	The couple had to be living year round on the study farm; The wife had to be 44 years of age or younger; At least one member of the couple had to be working on the farm

4. Test system:

Type:	Retrospective epidemiological study
Collection of data:	Questionnaire
Guideline:	Non-guideline study
GLP / GCP:	no

5. Observations / analyses:

Information in questionnaires:	Demographic and lifestyle information; Pesticides currently and historically used on the farm and around the home; Medical history; Complete reproductive history farm activities
Outcome of interest in analysis:	Self reported spontaneous abortion of less than 20 weeks' gestation
Subgroups created:	Spontaneous abortions of less than 12 weeks' and 12–19 weeks' gestation
Pesticide use:	Information from the farm operator husband and wife to construct a history of monthly agricultural and residential pesticide use
Identification of pesticides:	Using a database of registered pesticide products in Canada
Dose levels:	Not reported
Grouping of pesticides:	Major classes of use: herbicides, insecticides, fungicides, and miscellaneous others (including those that could not be classified)
Number of reported pregnancies:	3936
Number of reported spontaneous abortions:	395
Number of reported early abortions:	226
Number of reported late abortions:	169
Analysed exposure to pesticides:	For two windows: - pre-conception, the 4-month period from 3 months before conception to the calendar month of conception (consistent with potential sperm-mediated effects); - post-conception, the 3-month period from the first calendar month after conception to the end of the first trimester (consistent with a fetotoxic effect)
Number of pesticide variables:	17
Number of possible risk factors for spontaneous abortions:	21
Statistics:	Crude odds ratios (ORs) using logistic regression for each combination of pesticide unit, exposure window, and gestational age at abortion category. To explore statistical interactions between the various pesticide units and other risk factors for spontaneous abortion, we used the Classification and Regression Tree (CART) method.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: No information about exposure duration, used glyphosate products and application rates. No information, if the subjects used more than one pesticide. Due to study design and evaluation methods, study results are not reliable.

2. Relevance of study: **Not relevant** (Study design is not suitable for assessment of glyphosate exposure).

3. Klimisch code: **3**

Response 1 – GTF

- This publication reports an "exploratory analysis" of pesticide exposure timing as a possible risk factor for spontaneous abortion.
- Pre-conception glyphosate exposure odds ratio for spontaneous abortion is considered of borderline significance (OR = 1.4).
- Post-conception glyphosate exposure was not associated with spontantous abortion (OR = 1.1).
- Authors note multiple limitations of the study relating to exposure
  - likely misclassification of pesticides
  - correct assignment of exposure window to pre- or/and post-conception
- This is one of several publications arising from the Ontario Farm Family Health Study (OFFHS), in which farm couples were asked to recall on-farm activities and pesticide usage over the last 5 years. Participants were also asked to recall all pregnancy outcomes (38% of which occurred more than 10 years earlier). This information was gathered via mail questionnaires with telephone follow-up for non-respondents.
- OFFHS information gathering methodology has high potential recall bias. Blair and Zahm (1993; referenced in Doc L, available in Doc K) report 60% accuracy when comparing self reported pesticide usage with purchasing records.
- OFFHS relied exclusively on maternal self-reports of adverse pregnancy outcomes, not all of which were confirmed via medical or other records.
- Three highly relevant confounding factors were not considered in the OFFHS questionnaire
  - history of previous spontaneous abortion(s);
  - maternal age; and
  - smoking.
- Lack of control for putative pesticide effect(s) and consideration use of multiple pesticides further compromise the utility of the data set.
- Arbuckle et al. (2001) Reported findings linked preconception use of phenosyacetic acids, triazines, glyphosate and thiocarbamates with weak but stastically significant spontaneous abortions.
- Authors considered the findings reported "hypothesis generating", and cautioned that "results should be interpreted with care and tested in other studies".
-

Response 2 – Summarized from Williams et al. (2012)

- 395 spontaneous abortions were reported out of 3936 pregnancies; rate of spontaneous aborting in Arbuckle et al. (2001) was 10%.
- The baseline rate of spontaneous abortions in the general populations is much higher, ranging from 12% to 25%.
- Recall bias is reflected in the recall of spontaneous abortion over the previous 5 years (64% of all spontaneous abortions reported) being much higher than the recall of those greater than 10 years prior to the survey (34% of all spontaneous abortions reported).
- Substantial exposure misclassification may have occurred (pre- versus post-conception) due to likely author extrapolation of exposure data.
- Strong confounding variables are not apparent in previous data analyses published by the authors of the OFFHS, and therefore odds ratios are crude.

- Published results fail to demonstrate a significant association of glyphosate exposure spontaneous abortion risk and therefore must be considered cautiously.

Author(s)	Year	Study title
Savitz, D.A. Arbuckle, T. Kaczor, D. Curtis, K.M.	1997	Male pesticide exposure and pregnancy outcome. American Journal of Epidemiology Volume: 146 Number: 12 Pages: 1025-1036

Abstract*

Potential health effects of agricultural pesticide use include reproductive outcomes. For the Ontario Farm Family Health Study, the authors sampled Ontario farms from the 1986 Canadian Census of Agriculture, identified farm couples, and obtained questionnaire data concerning farm activities, reproductive health experience, and chemical applications. Male farm activities in the period from 3 months before conception through the month of conception were evaluated in relation to miscarriage, preterm delivery, and small-for-gestational-age births. Among the 1,898 couples with complete data (64% response), 3,984 eligible pregnancies were identified. Miscarriage was not associated with chemical activities overall but was increased in combination with reported use of thiocarbamates, carbaryl, and unclassified pesticides on the farm. Preterm delivery was also not strongly associated with farm chemical activities overall, except for mixing or applying yard herbicides (odds ratio = 2.1, 95% confidence interval 1.0-4.4), Combinations of activities with a variety of chemicals (atrazine, glyphosate, organophosphates, 4-[2,4-dichlorophenoxy] butyric acid, and insecticides) generated odds ratios of two or greater. No associations were found between farm chemicals and small-for-gestational-age births or altered sex ratio. Based on these data, despite limitations in exposure assessment, the authors encourage continued evaluation of male exposures, particularly in relation to miscarriage and preterm delivery.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various pesticides (herbicides, insecticides, fungicides, livestock chemicals)
Active substance(s):	Glyphosate, atrazine, 2,4-DB, 2,4-D, MCPA, dicamba, carbaryl, and other pesticides
Chemical families:	Phenoxy herbicides, thiocarbamates, organophosphates, triazines

2. Vehicle and/or positive control: Not applicable

3. Test group:

Number of test persons:	2964 couples (initial inclusion) 1898 couples (complete response)
Age:	Females: ≤ 44 years
Sex:	Males/females
Inclusion criteria:	The couple had to be living year round on the study farm; The wife had to be 44 years of age or younger; At least one member of the couple had to be working on the farm
No. of pregnancies analyzed:	3984
Miscarriage cases due to glyphosate:	17

4. Test system:

Type:	Retrospective epidemiological study
Collection of data:	Questionnaire, telephone interview, interview
Guideline:	Non-guideline study
GLP / GCP:	no

5. Observations/analyses:

Information in questionnaires:	Mother's and father's age, education, jobs outside the farm (classified as potentially hazardous or nonhazardous), tobacco use, alcohol use, caffeine use, mother's language, ethnicity, religion, parity, per capita income, child's sex, interval between conception and the survey, and the month of conception.
Classification of pregnancies:	Singleton live births, multiple gestations (twins, triplets), miscarriage (recognized pregnancy loss before 20 weeks of completed gestation), stillbirth (pregnancy loss at 20 or more weeks of completed gestation), medically induced abortion, currently pregnant, or other (ectopic pregnancy, hydatidiform mole, unknown).
Criteria for classification of pregnancies:	Singleton live births were classified as preterm if they occurred before the completion of 37 weeks of gestation and small for gestational age (SGA) if they fell below the 10th percentile of birth weight for gestational age based on Canadian percentiles. Sex ratio was defined as the proportion of males among singleton live births.
Analyzed outcomes of pregnancies:	Risk of miscarriage (pregnancies ending in miscarriages, singleton live births, induced abortions, and stillbirths, as well as current pregnancies of 20 or more weeks of gestation), preterm delivery (all live births and current pregnancies of 37 or more weeks of gestational age), and SGA births (all live births of known weight and gestational age), as well as sex ratio (all live births of known sex), not addressing stillbirths and other more rare outcomes due to insufficient numbers for analysis.
Farm activities:	Over the past 5 years; for each reported activity, months of the year were asked
Activities that involve direct pesticide exposure:	Mixing or applying crop herbicides, crop insecticides and fungicides, livestock chemicals, yard herbicides, and building pesticides.
Man's exposure classification:	Based on man's experiences in the time window of 3 months before conception to the time of conception. During that time window, specific to each pregnancy, we first determined whether he had engaged in any activities associated with direct pesticide exposure for 1 or more months. Defined 2 groups of activities: - chemical activity; - nonchemical activity + no activity.
Use of protective equipment:	Information gathered on date of use, but not specified to each of the chemical activities.
Pesticide use:	Information from farm operator (who may or may not have been the male partner) regarding the application of specific pesticides on the farm in the time period of interest.

Data analysis: Unadjusted risk ratios between the potential confounders and each of the four outcomes (miscarriage, preterm delivery, SGA, and sex ratio) were calculated, starting with finely stratified exposure variables. Based on the pattern of crude results, variables were eliminated and categories of variables were collapsed to retain only those variables and strata that yielded risk ratios of less than 0.8 or greater than 1.2. For each of the pregnancy outcomes, a logistic regression model was constructed that used the reduced set of variables and category levels. Additional variables were eliminated from the logistic regression models, and categories were collapsed or converted to continuous variables as appropriate.
For each of the four outcomes, risks and relative risks were generated, contrasting exposed to unexposed groups. Men with no activity or no chemical activity served as the referent, with various subsets of men defined by activity, use of protective equipment, and farm chemical use constituting the exposed groups. Adjusted odds ratios were calculated using logistic regression models with all the predictors of each outcome described above along with the exposure of interest. Because multiple pregnancies per woman were included, the variance estimates from the logistic regression are expected to be slightly underestimated on average. Several logistic regression analyses were conducted based on generalized estimating equations, which account for the within-woman correlation across pregnancies.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: No information about exposure duration, used glyphosate products and application rates. No information, if the subjects used more than one pesticide. Due to study design and evaluation methods, study results are not reliable.

2. Relevance of study: **Not Relevant** (Study design is not suitable for assessment of glyphosate exposure).

3. Klimisch code: **3**

Response to Savitz, Arbuckle and the Onratio Farm Family Health Study (OFFHS) taken from monsanto.com
http://www.monsanto.com/products/Documents/glyphosate-background-materials/gly_reprooutcomes_bkg.pdf

Glyphosate is one of many pesticides mentioned in three epidemiological reports that examine possible links between on-farm pesticide use and reproductive outcomes. All three reports - Savitz et *al.* (1997) [category 'E' in this literature review], Curtis et *al.* (1999) [outside the scope of this literature review as per the introduction describing literature review categories], and Arbuckle et *al.* (2001) [previously reviewed publication] - use data from the Ontario Farm Family Health Study (OFFHS) (Arbuckle 1994). Savitz *et al.* (1997) investigated associations between reported pesticide use by males and pregnancy outcomes, specifically: miscarriage, pre-term delivery and small-for-gestational-age birth. Curtis *et al.* (1999) studied whether reported pesticide use by males or females was associated with delayed pregnancy,

while Arbuckle *et al.* (2001) looked for associations between reported pesticide use and spontaneous abortion.

The OFFHS was a questionnaire-type study in which farm couples were asked to recall on-farm activities and pesticide usage on the farm during the previous 5 years. They were also asked to recall all pregnancy outcomes, 38% of which occurred more than 10 years before the survey. The farm couples lived year-round on a farm and the OFFHS investigators employed mail questionnaires to collect information about pregnancy outcomes from the mothers. Telephone follow-up was employed for non-respondents.

In the study by Savitz et *al.,* a number of specific pesticides had weak statistical associations with miscarriages and pre-term deliveries, but pesticides tended not to be associated with small for gestational age births. There were no statistically significant findings for glyphosate. In the study by Curtis *et al.,* for farms on which glyphosate was used, there was no significant association for women being engaged in pesticide activities. For men, glyphosate use was associated with a slight, but statistically significant, decrease in time to pregnancy. The authors dismissed this finding, which was contrary to their hypothesis that pesticide exposure delayed pregnancy, as probably due to uncontrolled factors or chance. Arbuckle *et al.* (2001) found that reported preconception use of phenoxyacetic acids, triazines, glyphosate, and thiocarbamates were weakly, but statistically significantly, associated with spontaneous abortions. Post conception reported use was not associated with increased risk. The authors characterized the associations between pesticides and spontaneous abortions as "hypothesis generating" pending confirmation from other epidemiologic studies.

These studies are not convincing evidence of a relationship between glyphosate exposure and adverse pregnancy outcomes for a number of reasons:

1. Uncertainty about exposure
There was no actual exposure data per se in these three epidemiologic studies. Exposures were assumed based on questionnaire responses by study subjects about farm activities and pesticide use. This type of information can be inaccurate. For example, according to a study by the National Cancer Institute, self-reports of pesticide usage were found to be only 60 percent accurate when compared with purchasing records (Blair & Zahm 1993). Further increasing the potential for inaccuracy is the fact that study subjects were only asked about pesticide use for the 5 years before the OFFS survey. These responses were assumed to be applicable to the entire farming careers of study subjects, an assumption inconsistent with changes in agricultural practice. Lastly, basing exposure estimation on questionnaire responses has the potential to be influenced by what epidemiologists call "recall bias." This refers to the likelihood that families that experienced an adverse reproductive outcome are more likely to remember use of certain pesticides than families that had only normal births.
The most widely used pesticides, like atrazine, glyphosate, and 2,4-D, are most easily recalled and most likely to be over-reported.

2. Low biological plausibility
Biologic plausibility is an important criterion for deciding whether a reported statistical association between a pesticide and a disease is likely to be valid. Glyphosate, even at very high doses in chronic feeding studies, does not cause adverse reproductive outcomes in laboratory animals (USEPA 1993, WHO 1994). This makes statistical associations from epidemiologic studies less plausible.

3. Inaccuracy of reported pregnancy outcomes
The OFFHS study relied exclusively on maternal self-reports of adverse pregnancy outcomes with no medical or other validation. Generally, scientists place less confidence in reports of health outcomes that are not validated with medical records.

4. Confounding
A confounding factor is a cause of a disease that is correlated with another exposure being studied. Failure to control confounding factors, especially those that are strong causes of a disease, can create spurious associations between benign exposures and diseases. In the Arbuckle study, there were at least three

important potential confounding factors that were not controlled: history of previous spontaneous abortion, maternal age, and smoking. Even a weak correlation between these factors and use (or recall of use) of pesticides would produce spurious associations. In addition, in all three studies, the authors did not control the putative effect of one pesticide for the putative effects of other pesticides. So, for example, since farmers tend to use 4 or more pesticides each year, a disease that is associated with one pesticide will likely be associated with all, since their use patterns are correlated. In the absence of an analysis that controls for multiple pesticides, the best that can be said is that the findings for any individual pesticide might be due to its correlation with another pesticide.

In summary, three publications based on data collected in the OFFHS found associations between several pesticides and various adverse reproductive outcomes. There was no actual exposure data per se in these three epidemiologic studies. Exposures were assumed based on questionnaire responses by study subjects about farm activities and pesticide use. This type of information can be inaccurate. Glyphosate was not significantly associated with adverse reproductive outcomes in two of these studies (Savitz *et al.* 1997, Curtis *et al.* 1999). Glyphosate and other pesticides were weakly associated with spontaneous abortion in the study by Arbuckle (2001). However, the author did not control for important personal confounding factors or for multiple exposures and no actual exposure data was used, casting doubt on the validity of the findings in this study.

Biomonitoring data for glyphosate, collected as part of the Farm Family Exposure Study (FFES), provide assurance that human health effects related to glyphosate exposure are very unlikely. In the FFES, researchers from the University of Minnesota collected 5 days of urine samples from 48 farm families before, during, and after a glyphosate application (Mandel *et al.,* accepted for publication). Only 60% of farmers showed detectable exposure to glyphosate with a 3 part per billion limit of detection, and the maximum estimated absorbed dose was 0.004 *mg/kg* (Acquavella *et al.* 2004). For farmers who apply glyphosate 10 times per year for 40 years, this maximum dose is more than 30,000-fold less than the EPA reference dose1 of 2 mg/kg/day. For spouses, only 1% showed detectable exposures and the maximum systemic dose was 0.00004 mg/kg/day. Since glyphosate is not a reproductive toxic in high dose animal studies (USEPA 1993, WHO 1994) and since actual exposures on farms are so low, it is very unlikely that glyphosate would cause adverse reproductive outcomes for farmers or their spouses.

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Author(s)	Year	Study title
Garry, V. F. Harkins, M. E. Erickson, L. L. Long-Simpson, L. K. Holland, S. E. Burroughs, B. L.	2002b	Birth defects, season of conception, and sex of children born to pesticide applicators living in the Red River Valley of Minnesota, USA. Environmental Health Perspectives Volume: 110 Pages: 441-449

Abstract*

We previously demonstrated that the frequency of birth defects among children of residents of the Red River Valley (RRV), Minnesota, USA, was significantly higher than in other major agricultural regions of the state during the years 1989-1991, with children born to male pesticide applicators having the highest risk. The present, smaller cross-sectional study of 695 families and 1,532 children, conducted during 1997-1998, provides a more detailed examination of reproductive health outcomes in farm families ascertained from parent-reported birth defects. In the present study, in the first year of life, the birth defect rate was 31.3 births per 1,000, with 83% of the total reported birth defects confirmed by medical records. Inclusion of children identified with birth or developmental disorders within the first 3 years of life and later led to a rate of 47.0 per 1,000 (72 children from 1,532 live births). Conceptions in spring resulted in significantly more children with birth defects than found in any other season (7.6 vs. 3.7%). Twelve families had more than one child with a birth defect (n = 28 children). Forty-two percent of the children from families with recurrent birth defects were conceived in spring, a significantly higher rate than that for any other season. Three families in the kinships defined contributed a first-degree relative other than a sibling with the same or similar birth defect, consistent with a Mendelian inheritance pattern. The remaining nine families did not follow a Mendelian inheritance pattern. The sex ratio of children with birth defects born to applicator families shows a male predominance (1.75 to 1) across specific pesticide class use and exposure categories exclusive of fungicides. In the fungicide exposure category, normal female births significantly exceed male births (1.25 to 1). Similarly, the proportion of male to female children with birth defects is significantly lower (0.57 to 1; p = 0.02). Adverse neurologic and neurobehavioral developmental effects clustered among the children born to applicators of the fumigant phosphine (odds ratio [OR] = 2.48; confidence interval [CI], 1.2-5. 1). Use of the herbicide glyphosate yielded an OR of 3.6 (CI, 1.3-9.6) in the neurobehavioral category. Finally, these studies point out that a) herbicides applied in the spring may be a factor in the birth defects observed and b) fungicides can be a significant factor in the determination of sex of the children of the families of the RRV. Thus, two distinct classes of pesticides seem to have adverse effects on different reproductive outcomes. Biologically based confirmatory studies are needed.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Herbicides, insecticides, fumigants, fungicides
Active substance(s):	At least 15 different substances that were not further specified. (Only pesticide classes were assessed)
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	Not reported
Sex:	Males and females

4. Test system:

Study type:	Epidemological study for the assessment of birth defects, season of conception, and sex of children born to pesticide applicators living in the Red River Valley of Minnesota, USA.
Collection of data:	Interview and questionnaire
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Inclusion criteria:	Farm families with live births fathered by a pesticide applicator
No. of live births with birth defects:	1532
No. of family participants:	695
No. of family with children:	536
No. of control persons:	None

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	Confounding variables such as maternal smoking, drinking, age, and chronic diseases such as diabetes and hypertension were examined. In this retrospective study, where possible, familial genetic history (pedigree), pregnancy medication use, and nonmedicinal drug use (including vitamins) were assessed in families with birth defects. Each certified pesticide applicator was initially interviewed by phone regarding current and past pesticide use in agriculture with specific attention to product name, years used, and the number of days per year applied. Approximately 6 months later, where possible, the subject was re-interviewed by written questionnaire to document common pesticide use by pesticide class, acreage treated, type of crop, and use of personal protective gear. Overlap between the two questionnaires was intentional to validate use of pesticides by class (herbicides, insecticides, fumigants, fungicides).
Statistics:	Regression analysis, two-sided *t*-tests, and analysis of variance methods were employed. Variables considered for regression analysis included mother's age, smoking status, alcohol use, and season of conception. Chronic diseases such as diabetes, pharmacologically treated hypertension, and arthritis and occupations other than agriculture were considered separately. Specific medication use during pregnancy and dietary information were not considered in our survey. Residence at a rural site (towns with populations <3,000) or on a farm during childhood (<18 years of age) was considered a factor in some

of these statistical analyses.

Conditional logistic regression analysis for matched studies was performed with SAS statistical program. Odds ratios and 95% confidence intervals were obtained. Both univariate and multivariate analyses were done. In the pooled analysis an adjustment was made for study, study area and vital status. When risk estimates for different pesticide exposures were analysed only subjects with no pesticide exposure were taken as unexposed, whereas subjects exposed to other pesticides were disregarded.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemiological study with some methodological / reporting deficiencies (selection of study subjects, no information about exposure duration, exposure concentration, pesticide use frequency)

2. Relevance of study: **Not relevant** (Glyphosate not mentioned.)

3. Klimisch code: **3**

Response 1 – summary from Mink et al. (2011)

- Publication reports on different classes of pesticides and several birth defects and developmental outcomes.
- Paternal use of glyphosate was associated with parent-reported ADD/ADHD in children (OR = 3.6). Six out of 14 children with parent reported ADD/ADHD also reported exposure to glyphosate.
- Diagnoses of ADD/ADHD were not all confirmed. However, overall rate for the sample population (14/1533) was well below ADD/ADHD rates for the general population (7%).
- Variables in statistical model analyses were not reported.

Response 2 – summary from Williams et al. (2012)

- Health data obtained via parent reporting for 695 families via written questionnaire and confirmed where possible.
- Pesticide use information obtained initially via telephone then followed up by written questionnaire.
- Reproductive health outcomes for births occurring between 1968 and 1998 were obtained for 1532 live births. Over half the births occurred prior to 1978, approximately 20 years after study initiation.
- All pesticide use classes (herbicide only; herbicide and insecticide; herbicide, insecticide and fungicide; herbicide, insecticide and fumigant) were associated with birth defects.
- Authors state neurobehavioral disorder would not be considered based lack consistent diagnoses. However, a detailed analysis was conducted for ADD/ADHD.
- 43% (6/14) parent reported children with ADD/ADHD were associated with glyphosate formulation use.
- 14 cases of ADD/ADHD reported out of 1532 live births, which is substantially lower that the diagnosed incidence of 7% for the general population.
- No conclusions regarding glyphosate exposure and ADD/ADHD outcome can be drawn.
- No other glyphosate specific data were reported.

Author(s)	Year	Study title
Garry, V.F., Holland, S.E., Erickson, L.L., Burroughs, B.L.	2003	Male Reproductive Hormones and Thyroid Function in Pesticide Applicators in the Red River Valley of Minnesota Journal of Toxicology and Environmental Health, Part A Volume: 66 Number: 11 Pages: 965-986

Abstract*

In the present effort, 144 pesticide applicators and 49 urban control subjects who reported no chronic disease were studied. Applicators provided records of the season's pesticides used by product, volumes, dates, and methods of application. Blood specimens for examination of hormone levels were obtained in summer and fall. In the herbicide-only applicator group, significant increases in testosterone levels in fall compared to summer and also elevated levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the fall were noted. With respect to fungicide uses in an earlier cross-sectional epidemiologic study, data demonstrated that historic fungicide use was associated with a significant alteration of the sex ratio of children borne to applicators. As before, among current study subjects it was noted that historic fungicide use was associated with increased numbers of girls being born. Lower mean total testosterone concentrations by quartile were also correlated with increased numbers of live-born female infants. A downward summer to fall seasonal shift in thyroid-stimulating hormone (TSH) concentrations occurred among applicators but not among controls. Farmers who had aerial application of fungicides to their land in the current season showed a significant shift in TSH values (from 1.75 to 1.11 mU/L). Subclinical hypothyroidism was noted in 5/144 applicators (TSH values >4.5 mU/L), but not in urban control subjects. Based on current and past studies, it was concluded that, in addition to pesticide exposure, individual susceptibility and perhaps economic factors may play a supporting role in the reported results.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, fungicides, and insecticides
Active substance(s):	Various active substances that were not specified in detail
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	Exposed group to herbicides: 43.5 y; non-exposed (no pesticide use during the relevant application season): 43.0 y; non-exposed (control): 41.8 y
Sex:	Exposed group: 144 males non-exposed group: 49 males

4. Test system:

Study type:	Epidemiological study to determine male reproductive hormones and thyroid function in pesticide applicators
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Data collection:	Interview and questionnaire
Duration of study:	Not specified
Application rate:	Not reported
Persons per group:	144 exposed; 49 non-exposed (control)
Application technique:	Ground, aerial, manual, and custom ground spraying and seed treatment
Test conditions:	The test group consisted of 144 randomly selected applicators residing in the Red River Valley (RRV). Exposure occurred during the applications. The non-exposed group consisted of 49 individuals selected as the volunteers from the community blood bank. Non-exposed controls were matched by age, health, and smoking status with the pesticide applicators. Control samples taken in summer and in fall were from different subjects!
Inclusion criteria:	No chronic disease, no chronic medication, herbicide use frequency >10 day/year, no use of fungicides, or fungicide use of < 5 days/year, no or < 5 agricultural pesticide application during the last year
Blood sampling:	Exposed group: blood samples were collected in summer and fall. Non-exposed group: Blood samples were collected and processed as for the exposed group.

5. Observations/analyses:

Clinical history:	All subjects
Exposure assessment:	Past pesticide use (incl. use frequency, application technique); During and at the end of the application season , detailed assessment of used pesticides, application rates, use frequency, application techniques etc., as well as a reproductive health history of the family.
Clinical signs:	Not performed
Body weight:	Not performed
Haematology:	Not performed.
Clinical chemistry:	LH and FSH levels; Total and free testosterone levels; TSH, total and free T4 levels
Urine analysis:	Not performed.
Other:	Offspring gender ratio
Statistics:	Urban control and pesticide applicator subject comparison groups were matched by age (within 5 yr) and smoking status. Within-group hormonal measurements from summer and fall were compared using paired t-tests. Between-group

comparisons were conducted using two-tailed t-tests for significance. The criterion for significance was set at $p<0.05$. Applicators and control subjects whose values exceeded the established normal clinical range (outliers) for these hormones were treated separately in our analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemiological study with some methodological / reporting deficiencies (e.g. selection of control subjects/samples, no details of exposure). Documentation is insufficient for assessment.

2. Relevance of study: Not relevant (Due to reliability. In addition, no direct assessment of glyphosate exposure was made).

3. Klimisch code: **3**

Response – GTF

- The publication brings little if any information on endpoints attributable to glyphosate.
- Given the subjects were pesticide applicators, little can be drawn from the findings other than perhaps certain endpoints which may be associated with this specific occupation exposed to multiple chemical substances.
- Of the 136 participants volunteering blood samples, only one individual (subject D) was noted with one abnormally high thyroid hormone levels associated with glyphosate use; thyroid stimulating hormone (FSH) was about double the normal range in the fall and thyroid stimulating hormone (TSH) higher than normal in the summer.
- Another individual (subject E) had abnormally high TSH levels associated with multiple pesticide usage of 12 different active ingredients.

Author(s)	Year	Study title
Bell, E.M. Hertz-Picciotto, I. Beaumont, J.J.	2001	A Case-Control Study of Pesticides and Fetal Death Due to Congenital Anomalies Epidemiology Volume: 12 Number: 2 Pages: 148-156

Abstract*

We examined the association between late fetal death due to congenital anomalies (73 cases, 611 controls) and maternal residential proximity to pesticide applications in ten California counties. A statewide database of all applications of restricted pesticides was linked to maternal address to determine daily exposure status. We examined five pesticide chemical classes. The odds ratios from logistic regression models, adjusted for maternal age and county, showed a consistent pattern with respect to timing of exposure; the largest risks for fetal death due to congenital anomalies were from pesticide exposure during the 3rd– 8th weeks of pregnancy. For exposure either in the square mile of the maternal residence or in one of the adjacent 8 square miles, odds ratios ranged from 1.4 (95% confidence interval = 0.8 – 2.4) for phosphates, carbamates, and endocrine disruptors to 2.2 (95% confidence interval = 1.3 – 3.9) for halogenated hydrocarbons. Similar odds ratios were observed when a more restrictive definition of nonexposure (not exposed to any of the five pesticide classes during the 3rd– 8th weeks of pregnancy) was used. The odds ratios for all pesticide classes increased when exposure occurred within the same square mile of maternal residence.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various chemical groups – carbamates, halogenated hydrocarbons, phosphates, pyrethroids, and endocrine disruptors (total of 327 pesticides)
Active substance(s):	Various active substances incl. glyphosate
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	18 - >35y
Sex:	Exposed group: 73 females non-exposed group: 611 females

4. Test system:

Study type:	A Case-Control Study of Pesticides and Fetal Death Due to Congenital Anomalies
Guideline:	None

GLP / GCP:	No
Guideline deviations:	Not applicable
Duration of study:	Not applicable
Application rate:	Not reported
Persons per group:	73 exposed; 611 non-exposed (control)
Application technique:	Ground and aerial spraying
Test conditions:	The exposed group consisted of 73 selected cases which were located in the same square mile or surrounding square miles from an area where the pesticides were applied. Exposure occurred during 1-20, 1-13, and 3-8 weeks of pregnancy by ground or aerial spraying. The non-exposed group consisted of 611 healthy females not exposed to the specific pesticide during the relevant time period. None of the persons (exposed, non-exposed) were involved in application of pesticides.
Case identification:	Exposed group: identified congenital anomalies in foetuses from the death certificates. Late foetal deaths after week 20 were considered. Non-exposed group: normal births defined as livebirths with no congenital malformations.

5. Observations/analyses:

Clinical history:	Exposed and non-exposed persons
Clinical signs:	Exposed persons only
Body weight:	Not performed.
Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Statistics:	Stratified analyses were used to determine which covariates had potential to be confounders. The exposure prevalence among controls and the distribution of covariates by case-control status were assessed. Stratified odds ratios (ORs) were examined to screen for potential effect modifiers. Inclusion criteria for potential effect modifiers required that stratum-specific ORs differ by 100% or more. On the basis of the results of these stratified analyses, we included no interaction term in the model. Adjusted ORs and 95% confidence intervals (CIs) were calculated using logistic regression for those exposed according to the nine-TRS definition, and again for those exposed in the one-TRS definition, separately for each of the five pesticide classes. Separate analyses for ground and aerial modes of application were also completed for those exposed in the nine TRSs. These analyses were limited to those exposed to the specific pesticide class and mode of interest. For those who returned questionnaires, an analysis that adjusted for variables not available from the birth and death certificates was conducted.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemological study with methodological deficiencies (e.g. glyphosate was included in the pesticide class of phosphates, thiophosphates, phosphonates, no differentiation between single and multiple exposures, correlation, if any, only to pesticide classes and not to specific active substances)

2. Relevance of study: **Not relevant** (No glyphosate-specific results.)

3. Klimisch code: **3**

Response – summary from Williams et al. (2012)

- Classes of pesticides were evaluated in this study, with glyphosate included as one of 47 active ingredients in the broad category of "phosphates/triphosphotates/phosphonates".
- Of the 47 active ingredients, many were organophosphate insecticides with known mammalian modes of action. The glyphosate mode of action is on the EPSPS enzyme in plants, which is not present in the animal kingdom.
- Given the very low volatility of glyphosate and the low potential for inhalation exposures to aerosol sprays up to two miles away from the subjects, systemic doses to glyphosate would be considered negligible.
- Mose et al., (2008) demonstrated a low perfusion rate of glyphosate across the placenta. Coupled with the known low dermal and gastrointestinal absorption of glyphosate and the rapid elimination of systemic doses of glyphosate in the urine, human *in utero* exposures would be extremely limited.
- The reported congenital anomalies associated with fetal death in Bell et al. (2001) can in no way be linked to glyphosate exposure.

Author(s)	Year	Study title
Aris, A. Leblanc, S.	2011	Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. Reproductive toxicology Volume: 31 Pages: 528-533

Abstract*

Pesticides associated to genetically modified foods (PAGMF) are engineered to tolerate herbicides such as glyphosate (GLYP) and gluphosinate (GLUF) or insecticides such as the bacterial toxin bacillus thuringiensis (Bt). The aim of this study was to evaluate the correlation between maternal and fetal exposure, and to determine exposure levels of GLYP and its metabolite aminomethyl phosphoric acid (AMPA), GLUF and its metabolite 3-methylphosphinicopropionic acid (3-MPPA) and Cry1Ab protein (a Bt toxin) in Eastern Townships of Quebec, Canada. Blood of thirty pregnant women (PW) and thirty-nine nonpregnant women (NPW) were studied. Serum GLYP and GLUF were detected in NPW and not detected in PW. Serum 3-MPPA and CryAb1 toxin were detected in PW, their fetuses and NPW. This is the first study to reveal the presence of circulating PAGMF in women with and without pregnancy, paving the way for a new field in reproductive toxicology including nutrition and utero-placental toxicities.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test items / active substances: Glyphosate;
Gluphosinate;
Bacillus thuringiensis;
AMPA (aminomethyl phosphoric acid)
3-MPPA (3-methylphosphinicopropionic acid)
Cry1Ab protein (Bt toxin)

Purity: Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species: Human
Sex: Female
Age: Pregnant woman: 32.4 ± 4.2 yr (mean)
Non-pregnant women: 33.9 ± 4.0 yr (mean)
Number of test persons (pregnant): 30
Number of control persons (non-pregnant): 39

4. Test system:

Study type: Maternal and foetal exposure to pesticides associated to genetically modified foods
Guideline: Non-guideline study
GLP: No

Guideline deviations: Not applicable

Duration of study: Not reported

Collection of data:

Inclusion criteria: Subjects were pregnant and non-pregnant women living in Sherbrooke, an urban area of Eastern Townships of Quebec, Canada. No subject had worked or lived with a spouse working in contact with pesticides.

Eligible groups were matched for age and biody mass index (BMI)

BMI: Pregnant woman: 24.9 ± 3.1 kg/m^2 (mean)

Non-pregnant women: 24.8 ± 3.4 kg/m^2 (mean)

Exposure conditions: It was assumed that the subjects were exposed due to the diet of herbicide-tolerant genetically modified crops.

Diet: The diet taken is typical of a middle class population of Western industrialized countries. A food market basket, representative for the general Sherbrooke population.

Additional factors: Participants were not known for cigarette or illicit drug use or for medical condition (ie: diabetes, hypertension or metabolic disease).

5. Observations/analyses:

Sampling: Blood sampling was done before delivery for pregnant women or at tubal ligation for nonpregnant women and was most commonly obtained from the median cubital vein, on the anterior forearm.

Measurements: Levels of GLYP, AMPA, GLUF and 3-MPPA were measured using gas chromatography–mass spectrometry (GC–MS).

Cry1Ab protein levels were determined in blood using a commercially available double antibody sandwich (DAS) enzyme-linked immunosorbent assay.

Statistics: PAGMP (pesticides associated to genetically modified plants) exposure was expressed as number, range and mean ± SD for each group. Characteristics of cases and controls and PAGMP exposure were compared using the Mann–Whitney U-test for continuous data and by Fisher's exact test for categorical data. Wilcoxon matched pairs test compared two dependent groups. Other statistical analyses were performed using Spearman correlations. Analyses were realized with the software SPSS version 17.0. A value of $P < 0.05$ was considered as significant for every statistical analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Exact levels of PAGMF, glyphosate or AMPA in the diets were not determined. It is not clear if the measured concentrations could have been resulted from other exposure routes.

2. Relevance of study: **Relevant with restrictions** (Provides real life actual exposure concentrations in humans. Data are limited due to the absence of any information on applied pesticides, application rates, etc.)

3. Klimisch code: 3

Response – Monsanto Letter to the Editor

Comment: Aris and Leblanc "Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada"

To the Editor,

We have reviewed the publication of Aris and Leblanc entitled "Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada", and wish to provide comment. The study has also been the subject of a regulatory review (FSANZ) which reached conclusions similar to our own. Findings for glyphosate and AMPA are consistent with previous publications (cquavella et al., 2003; Curwin et al., 2007), and levels detected are consistent with intakes far below any level of concern (Curwin et al., 2007). Glyphosate has not demonstrated reproductive or developmental toxicity in repeated mammalian studies. The recent inclusion of glyphosate in Tier 1 endocrine disrupter screening is the result of exposure potential, not evidence of endocrine disruption as implied by Aris and Leblanc.

Attempts to detect Cry proteins in the blood of GM-fed animals have been limited by methodological challenges and commercial immunoassay kits (as used in this study) did not produce valid results in porcine blood. An assay system validated for use in bovine blood failed to detect Cry1Ab (LOD 1 ng/mL) despite very much higher intake (as % diet or per kg body weight) than humans, making assay validation essential. The authors did not provide validation information for the Cry1Ab assay in human blood. A standard curve was said to span a range of 0.1-10 ng/mL, but no statistical limit of detection is reported. It appears that the authors have reported all signals above baseline as confirmed "detects", despite the fact that many samples have concentrations below the likely detection limit of this assay system based on our own experience. Thus, the number of Cry1Ab detects is likely overstated, probably significantly.

The antibody in the Agdia immunoassay kit is known to bind to other cry proteins, and can also bind to fragments derived from the intact protein. While protein digestion and absorption primarily takes place as mono to tri-peptides, small quantities of proteins or larger protein fragments are absorbed as a part of normal human physiology.

Cry1Ab and related proteins (which may interact in this assay system) are widely used in organic agriculture on foods intended for direct human consumption. Cry1Ab is present in GM maize intended primarily for animal feed and processing to food ingredients (corn syrup, starch, etc.), and human consumption is expected to be quite low. Further, very little corn is consumed by humans in a raw state, and cooking denatures Cry1Ab protein eliminating its biological (insecticidal) activity.

Although we believe that the reported rate of detection is elevated, it is possible that Cry1Ab (or fragments) can be found in some individuals with a sufficiently high intake and sensitive assay system. This must be put in proper perspective. Cry proteins as a class are exempt from tolerance (i.e. no maximal intake levels were set), indicating that any potentially achievable exposure raises no safety concern. The no-effect level for purified Cry1Ab in acute animal testing is 4000 mg/kg (highest level tested). For a theoretical 50 kg female, this is the equivalent of 200,000,000 _g of Cry1Ab protein. Detection of 1 ng/mL of Cry1Ab in the blood of a 50 kg female (assuming 20% extracellular fluid volume, as proteins generally do not distribute intracellularly) is crudely equivalent to 10 _g of total Cry1Ab – 20-million times less than a dose which has no discernable effect.

In short, results for glyphosate are unsurprising and raise no health concerns. Detections of Cry1Ab appear to be over-reported. Based upon the limited intake of Cry1Ab and the fact that little protein is absorbed intact, reported detections may be technical artifacts and at best represent protein fragments in addition to intact protein – the vast majority of which are expected to be biologically inactive after processing.

Cry1Ab has been subjected to extensive safety assessment accounting for human exposure with a large margin of safety. Contrary to Aris and Leblanc, available traits are approved for human consumption, even if not the primary intent of cultivation. Mammalian toxicity has not been demonstrated with Cry1Ab or related Cry proteins, and all of the women and infants were normal. The reported findings, even if they should prove to be correct, raise no safety concerns.
The authors are full-time employees of Monsanto company, a manufacturer of products incorporating glyphosate and Cry1Ab.

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Author(s)	Year	Study title
Benítez-Leite, S. Macchi, ML and Acosta, M.	2009	Malformaciones congénitas asociadas a agrotóxicos. Arch Pediatr Urug Volume: 80 Number: 3 Pages: 237-247

Abstract*

Introduction: exposure to pesticides is a known risk for human health. This paper describes the relationship between parental exposure and congenital malformations in the newborn. Objective: to study the association between exposure to pesticides and congenital malformations in neonates born in the Regional Hospital of Encarnación, in the Department of Itapúa, Paraguay. Materials and methods: a prospective case-controlled study carried out from March 2006 to February 2007. Cases included all newborns with congenital malformations, and controls were all healthy children of the same sex born immediately thereafter. Births outside the hospital were not counted. Exposure was considered to be any contact with agricultural chemicals, in addition to other known risk factors for congenital defects. Results: a total of 52 cases and 87 controls were analyzed. The average number of births each month was 216. The significantly associated risk factors were: living near treated fields (OR 2,46, CI95% 1,09-5,57, p<0,02), dwelling located less than 1 km (OR 2,66, CI95% 1,19-5,97, p<0,008), storage of pesticides in the home (OR 15,35, CI95% 1,96-701,63), p<0,003), direct or accidental contact with pesticides (OR 3,19, CI95% 0,97-11,4, p<0,04), and family history of malformation (OR 6,81, CI95% 1,94-30,56, p<0,001). Other known risk factors for malformations did not show statistical significance. Conclusion: the results show an association between exposure to pesticides and congenital malformations. Further studies are required to confirm these findings.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Several pesticides were assessed but not specified.
Active substance(s):	Several active substances were assessed but not specified.
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	Newborn babies (The exposed mothers had an average age of 25 years (range: 12-45 years)) (Age of mothers from the control group not specified)
Sex:	Males and females

4. Test system:

Study type:	Epidemiological study for developmental toxicity
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Duration of study:	11 months (between March 28, 2006 and February 28, 2007)
Collection of data:	Questionnaire
Test group:	2414 newborn babies
Control group:	Controls were all healthy children of the same sex born immediately after the study period (February 28, 2007): up to 87 newborns
Application rate:	The concentration of the pesticides to which the mothers had been exposed was not specified.
Exposure frequency:	Not assessed.
Application technique:	Mainly fumigation.

5. Observations/analyses:

Clinical history:	Not performed.
Clinical signs:	All persons during pregnancy.
Body weight:	Not performed.
Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Evaluation:	The test group consisted of all newborns recorded in the Regional Hospital of Encarnación during the observation period. A total of 2414 cases were recorded (mean value: 216/month). The mothers of the newborn were asked several questions such as where they live, if they store pesticides at home, if they work with pesticides, etc…. The region of Itapúa has mainly soya cultivation. Paraguay was declared by the FAO as a place of concern, since big amounts of pesticides are yearly used (approx. 24 million L of pesticides per year). Population living in the area are exposed to these agrochemicals via many pathways (mother's home proximity to treated fields, workplace, or private use of pesticides). According to the statistic conducted with the mothers 55% of them lived in urban areas, 82% worked as housewife.
Exposure situation:	19.9% of the mothers had had direct contact or accidentally with pesticides, 28.8% of the fathers had been exposed. 42.3% of the asked mothers lived near treated fields. 15.3% had pesticides at home for private use.
Record of malformations:	22 different types of congenital malformations were recorded and statistically assessed, such as ear, hand or arm malformation.
Clinical history:	Not performed.
Clinical signs:	All mothers during pregnancy.
Body weight:	Not performed.

Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Malformation assessment:	All newborns were examined for malformations
	22 different types of congenital malformations were recorded and statistically assessed, such as ear, hand or arm malformation.
Statistics.	Yes, odds ratio statistic.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design of epidemiological study for developmental toxicity insufficient for assessment, as well as methological and reporting deficiencies (no assessment to which pesticides / active substances the mothers were exposed, use frequency not specified, selection of control group after study period is questionable, no information on exposure situation of mother for this control group assessed, etc.).

2. Relevance of study: **Not relevant** (The exposure to several pesticides was assessed in general, but no pesticide or active substance, including glyphosate, was specified or assessed)

3. Klimisch code: **3**

2. Literature Review of Neurotoxicity Publications

Several publications over the last decade have evaluated glyphosate with respect to neurotoxicity endpoints. Three papers report a total of two human cases of Parkinson's disease. The first case followed acute exposure to a glyphosate formulation while spraying a garden (Barbosa et al., 2001; da Costa et al, 2003). The second case reported chronic exposures to a factory worker in China, where a variety of pesticides including glyphosate were produced (Wang et al, 2011). Several questions arise in attempting to link glyphosate exposures with each case of Parkinson's disease. Firstly, significant systemic exposures to glyphosate in each instance are questionable, given the poor dermal absorption and low volatility of the compound. Secondly, if glyphosate was a causative agent of this fairly common disease, a significant number of cases associated with either acute and/or chronic exposures would be evident. Glyphosate formulations are sometimes readily accessible for suicide attempts, which are usually unsuccessful, as less than 10% of glyphosate self administered ingestions result in death. No reports of Parkinson's disease in survivors following very acute ingestions of glyphosate products have been documented. Glyphosate has been manufactured and widely used in agriculture and consumer markets for approximately 40 years, so a single case of a pesticide factory worker developing Parkinson's disease, while unfortunate, does not constitute cause and effect; there is no evidence of a higher frequency of Parkinson's disease in glyphosate production workers.

Multiple long term animal studies with glyphosate have failed to demonstrate any evidence of neurotoxicity, and certainly have not shown evidence of Parkinson's-like abnormalities. While some studies have suggested statistical associations with general pesticide exposure (Kenborg et al., 2011) or general insecticide or herbicide exposure (Engel et al., 2001), there is no evidence suggesting a specific association between glyphosate and Parkinson's disease. In the largest study to date of US Farmers (Agricultural Health Study), no increased risk of Parkinson's disease was found in association with reported glyphosate use (Kamel et al, 2007). Human non-cancer epidemiologic outcomes related to glyphosate have recently been reviewed (Mink et al, 2012), and there is no convincing evidence for an increased incidence of Parkinson's disease or other neurological disorders in individuals reporting glyphosate exposure.

Several publications open with the premise that pesticide exposures are linked with Parkinson's disease, and then proceed to report *a priori* research linking glyphosate with a measurable endpoint. This endpoint is then extrapolated to link with Parkinson's disease in humans. Despite the lack of compelling human associations between glyphosate exposure and Parkinson's disease, such research continues to be published. Astiz et al., (2009), Negga et al. (2011) and Gui et al. (2012) all conducted glyphosate research in the above mentioned manner all in very different test systems. Negga et al. (2011) notes neurodegeneration in *Caenorhabditis elegans* worms following exposure to glyphosate (trimesium form, which has a different toxicology profile than glyphosate) uses concentrations equal to the LD25, LD50 and LD75, or actual concentrations of glyphosate of 3 to 10 percent, i.e.- the high concentration is approximately 10-fold HIGHER than concentrations applied directly in the field. The relevance of such high-dose exposures to the trimesium salt in this experimental model to human Parkinson's disease is highly questionable and irrelevant to the Annex 1 renewal of glyphosate technical acid. Atiz et al. (2009) and Gui et al. (2012) both affirm their test models (in rats and in PC-12 cells respectively) for evaluating neurodegenerative disorders, then directly link their research results to Parkinson's disease in humans; these two studies are addressed below.

Cole et al. (2003) evaluated 15 different pesticides for neurotoxic endpoints in *C. elegans* with analytical grade active ingredients, noting reduced cholinesterase for pesticides with this mode of action, but not glyphosate. Interestingly, the authors report a low pH effect resulting in reduced cholinesterase activity in the high dose of glyphosate and a plant growth promoter. Glyphosate formulations contain salt forms of glyphosate, not the technical acid and thus do not have a low pH. Additionally, human incidents of self induced glyphosate poisonings do not report the common symptoms of acute acetylcholinesterase inhibition; salivation, lacrimation, urination and defecation (SLUD).

Author(s)	Year	Study title
Barbosa, E.R. Leiros da Costa M.D. Bacheschi, L.A. Scaff M.	2001	Parkinsonism After Glycine-Derivate Exposure Movement Disorders Volume: 16 Number: 3 Pages: 565-568

Abstract*

This 54-year-old man accidentally sprayed himself with the chemical agent glyphosate, a herbicide derived from the amino acid glycine. He developed disseminated skin lesions 6 hours after the accident. One month later, he developed a symmetrical parkinsonian syndrome. Two years after the initial exposure to glyphosate, magnetic resonance imaging revealed hyperintense signal in the globus pallidus and substantia nigra, bilaterally, on T2-weighted images. Levodopa/benserazide 500/125 mg daily provided satisfactory clinical outcome.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate
Purity: Not reported

2. Vehicle and/or positive control: Not applicable

3. Test person:

Sex: Male
Age: 54 years
Body weight: Not reported

4. Test system:

Study type: Case report
Guideline: Non-guideline study
GLP/GCP: No
Guideline deviations: Not applicable
Duration of study: 2 years
Exposure: Acute accidental exposure of a 54-year-old male during spraying glyphosate in the garden. The man did not wear any protective gear (e.g. gloves, face mask). Exposure occurred as the breeze blew the spray back into his trunk, arms, legs, and face. The substance residues were washed off his body 30 minutes after exposure.
Application parameters: Concentration – not reported;
Total amount handled – not reported;
Personal protection equipment – not worn
Additional factors: Medical history – not reported;
Lifestyle factors (smoking etc.) – not reported;
Use of prescribed drugs – not reported

5. Observations/analyses:

Observations: Six hours after exposure – severe conjunctival hyperemia and a generalised cutaneous rash
One week after exposure – skin lesions became blistered;
One month – rigidity and slowness in extremities;
One year – slow tremor of hand, impaired short-term memory;
Further on parkinsonian syndrome.

Statistics: Not applicable

KLIMISCH EVALUATION

1. Reliability of study: **Not assignable**

Comment: Medical case report

2. Relevance of study: **Relevant with restrictions** (Data are limited due to the absence of any information on purity and application concentrations of glyphosate formulation, as well as co-formulations.)

3. Klimisch code: **4**

GTF Comment

- Single incident report of acute exposure to a glyphosate formulation while spraying a garden.
- See opening paragraph of this section.
- See Medical section 5.9.4: "Clinical signs and symptoms of poisoning and details of clinical tests"

Author(s)	Year	Study title
Astiz, M. de Alaniz, M.J. Marra, C.A.	2009	Effect of pesticides on cell survival in liver and brain rat tissues Ecotoxicology and Environmental Safety Volume: 72 Pages: 2025-2032

Abstract*

Pesticides are the main environmental factor associated with the etiology of human neurodegenerative disorders such as Parkinson's disease. Our laboratory has previously demonstrated that the treatment of rats with low doses of dimethoate, zineb or glyphosate alone or in combination induces oxidative stress (OS) in liver and brain. The aim of the present work was to investigate if the pesticide-induced OS was able to affect brain and liver cell survival. The treatment of Wistar rats with the pesticides (i.p. 1/250 LD50, three times a week for 5 weeks) caused loss of mitochondrial transmembrane potential and cardiolipin content, especially in substantia nigra (SN), with a concomitant increase of fatty acid peroxidation. The activation of calpain apoptotic cascade (instead of the caspase-dependent pathway) would be responsible for the DNA fragmentation pattern observed. Thus these results may contribute to understand the effect(s) of chronic and simultaneous exposure to pesticides on cell survival.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Active substance(s):	**Zineb** (zinc ethylene-bis-dithiocarbamate) **Glyphosate** (N-phosphonomethyl-glycine) **Dimethoate** (O,O-dimethyl-S-methyl-carbamoyl-methyl phosphorodithioate)
Source:	Unknown local commercial sources & Instituto Nacional de Tecnologica Agropecuaria (INTA, Castelar, Argentina)
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle: Polyethylene-glycol (PEG-400)

3. Test animals:

Species:	Rat
Strain:	Wistar, SPF-free
Source:	Not reported
Age of test animals at study initiation:	Not reported
Sex:	Male
Body weight:	190 ± 20 g
Acclimation period:	1 week
Diet/Food:	Standard Purina chow from Ganave Co. (Santa Fe, Argentina)
Water:	Water, *ad libitum*
Housing::	Not reported
Environmental conditions:	Temperature: 25 ± 2°C

Humidity: Not reported
Air changes: Not reported
12-hour light/dark cycle

4. Test system:

Study type:	Sub-chronic study
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	5 weeks
Dose levels:	Untreated control; Vehicle control: polyethylene-glycol 400 (PEG-400); 15 mg zineb/kg body weight (b.w.) in PEG-400; 10 mg glyphosate/kg b.w. in PEG-400; 15 mg dimethoate/kg b.w. in PEG-400 15 mg zineb/kg b.w.+10 mg glyphosate/kg b.w. in PEG-400; 15 mg zineb/kg b.w.+15 mg dimethoate/kg b.w. in PEG-400; 10 mg glyphosate/kg b.w.+15 mg dimethoate/kg b.w. in PEG-400 15 mg zineb/kg b.w.+10 mg glyphosate/kg b.w.+15 mg dimethoate/kg b.w. in PEG-400
Animals per dose group:	9 groups of 4 rats each
Exposure route:	i.p. injection
Dose volume:	Not reported
Frequency of administration:	3 times a week
Animal maintenance and handling:	According with the NIH guide for the care and use of laboratory animals

5. Observations/analyses:

Test substance preparations:	Not reported. There are also no information on achieved concentrations, homogeneity and stability of test substance preparations.
Mortality:	Not reported
Clinical signs:	Noted weekly
Body weight:	Measured weekly
Food- and water consumptions:	Assessed, but not reported
Haematology:	Not performed
Clinical chemistry:	Not performed
Urine analysis:	Not performed
Pathology:	No gross pathology performed
Organ weights:	Liver, cerebral cortes and substantia nigra of brains
Histopathology:	Not performed
Sample preparations:	Livers and brains (cerebral cortex (ventromedial areas directly connected with substantia nigra) and substantia nigra) each

homogenized in HEPES 50 mM pH 7.4 with CHAPS 5 mM, dithiotreitol 5 mM, and aprotinin10 µg/ml, in a proportion of 300 µl buffer to 50 mg tissue. Cytosolic fractions for caspase and calpain measurements was prepared by homogenate centrifugation (20000 g, 15 min, 4°C). All samples were stored at -80°C until analyses.

Mitochondrial fractions were prepared from all tissues by homogenisation with HEPES 10 mM, pH 7.5 containing ´mannitol 200 mM, sucrose 70 mM, and EGTA 1 mM followed by a combination of low- and high-speed centrifugation procedures. Samples of mitochondrial suspensions were treated with glutaraldehyde, impregnated and included in epoxyde-polymer for electron microscopy.

DNA-samples were purified according to the Qiagen kit protocol after homogenisation of tissue.

Analytical methods: To assess the integrity of the inner mitochondrial membrane (IMM), the electrochemical proton gradient (ΔΨ) was tested using a membrane potential-sensitive probe (JC-1) using MITO-ISO1 test kit, (Sigma Co.);

The integrity of the outer mitochondrial membrane (OMM) was measured by determining the cytochromec (Cyt_c) oxidase activity, in the presence and absence of the detergent n-dodecyl β-D-maltoside using CYTOCOX1 kit (Sigma Co.);

Milli- (m-) and micro- (µ-) calpain activities were measured in the cytoplasmic fractions. The assay involves the hydrolysis of whole ultra pure casein (Sigma, Chem. Co.) by calpain activity and the subsequent detection of trichloroacetic acid (TCA)-soluble peptidic fragments at 280 nm;

Caspase-3 activieties were measured in tissue homogenates using a colorimetric assay kit (CASP-3-C).

Total mitochondrial glutathione was measured by an adaptation of the Ellman's method using the purified mitochondrial suspension as sample;

DNA fragmentation patterns were also analyzed using the DNA laddering technique;

Protein content was determined according to the method of Lowry et al. (1951).

Lipid analysis: Mitochondrial cardiolipin (CL) content was quantified by means of phosphorous measurement using the method of Chen et al. (1956). Colorimetric reactions were performed on lipid extracts previously obtained by the method of Folch et al. (1957). Samples were separated by high-performance thin layer chromatography (HPTLC).

Statistics: The results were expressed as the mean ± standard error of four independent experiments. They were statistically analyzed by one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test, and were considered different with respect to control data at two levels of significance:*P<0.05 and **P<0.01.Linear and non-linear correlation coefficients were calculated.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Unsuitable test system (i.p exposure route is not relevant for human exposure). No information on purities of test substances used. Small group size (4 males/dose group), reporting deficiencies

2. Relevance of study: **Not relevant** (intraperitoneal injection is a non-relevant route of exposure for humans)

3. Klimisch code: **3**

Response – GTF

- This non-guideline study utilized very small group numbers (4 rats/group), and therefore is not sufficiently robust to appropriately identify changes attributable to the test material administration.
- Route of administration via intraperitoneal injection is not an appropriate route of exposure for human health or environmental risk assessment.
- The test materials are not well described, without indication of whether a glyphosate salt form or acid was used and purity was not reported.
- The publication focuses on the post necropsy data analysis and reporting. Data on animal husbandry, clinical observations, feed and water intake, weekly body weight were not reported, but the authors note there were no adverse observations.
- No statistically significant effects were noted for liver endpoints, yet the liver is in close proximity to test material administration via intraperitoneal injection.
- Statistically significant effects were noted for brain tissue endpoints in the substantia nigra and cerebral cortex. However, there is a lack of biological plausibility for brain exposures to glyphosate, given the necessity to pass the blood-brain barrier and the known rapid elimination kinetics of this polar molecule via urine.

Author(s)	Year	Study title
Gui, Y.X., Fan, X.N., Wang, H.M., Wang, G., Chen, S.D.	2012	Glyphosate induced cell death through apoptotic and autophagic mechanisms. Neurotoxicology and teratology Volume: not specified (*accepted manuscript*) Pages: not specified

Abstract*

Herbicides have been recognized as the main environmental factor associated with human neurodegenerative disorders such as Parkinson's disease (PD). Previous studies indicated that the exposure to glyphosate, a widely used herbicide, is possibly linked to Parkinsonism, however the underlying mechanism remains unclear. We investigated the neurotoxic effects of glyphosate in differentiated PC12 cells and discovered that it inhibited viability of differentiated PC12 cells in dose-and time-dependent manners. Furthermore, the results showed that glyphosate induced cell death via autophagy pathways in addition to activating apoptotic pathways. Interestingly, deactivation of Beclin-1 gene attenuated both apoptosis and autophagy in glyphosate treated differentiated PC12 cells, suggesting that Beclin-1 gene is involved in the crosstalk between the two mechanisms.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Active substance(s):	Glyphosate
Source of test items:	Sigma Aldrich (St. Louis, MO, USA)
Lot/Batch #:	Not specified
Purity:	Not specified

2. Vehicle and/or positive control: DMSO

3. Test system / cells:

Primary cell culture:	Differentiated PC12 cells
Source:	Not reported
Culture conditions:	Grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, PAA), 100 U/ml penicillin, and 100 µg/ml Streptomycin (GIBCO). All cells were maintained in a humidified 5% CO_2 containing atmosphere at 37°C. For transfection experiments, PC12 cells were plated the day before transfection to achieve ~50% confluency.

4. Test methods:

Guideline:	Non-guideline assays (for all tests)
GLP:	No (for all tests)
Guideline deviations:	Not applicable (for all tests)

Preparation of test substance: Glyphosate was dissolved in DMSO as a stock solution at −20◦C and diluted with culture medium to various working concentrations.

siRNA mediated silencing of Beclin-1

Conditions: Cells were transfected with Beclin-1 siRNA or scrambled siRNA (Santa Cruz, CA, USA) using Lipofectamine 2000 reagent according to the manufacturer's instructions. After 48 h, cells were subjected to various treatments that were not further specified.

MTT assay: Assessment of cell viability

Conditions: PC12 cells after various treatment were plated in 96-well culture plates (1.0×10^4 cells per well). 5 g/l MTT solution was added to the culture medium 4 h before the end of treatments.

Dose concentrations: 0, 5, 10, 20, 40 mM glyphosate

Exposure duration: 12, 24, 48, 72 h

Replicates: Not reported

Apoptosis detection

Conditions: Nuclei were stained with DAPI to detect chromatin condensation or nuclear fragmentation, which were characteristics of apoptosis. Following various treatments, cells were fixed with 4% paraformaldehyde for 20 min and then stained with DAPI for 10 min away from light at room temperature.

Dose concentrations: Not reported

Exposure duration: 0, 24, 48, 72 h

Replicates: 3 per treatment

Autophagy detection

Conditions: For LC3 punctate analyses experiments, cells were transiently transfected with EGFP empty vector or GFP-LC3 expression vector.

Dose concentrations: Not reported

Exposure duration: Not reported

Replicates: Not reported

Western blot analysis

Conditions: Cells after various treatment were lysed in ice-cold RIPA lysis buffer (1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 50 mM TrisHCl, pH 7.4) supplemented with protease inhibitor cocktail and phenylmethanesulfonyl fluoride for 30 min, followed by centrifugation at 12,000g for 30 min at 4 °C before collecting the supernatants.

Dose concentrations: Not reported

Exposure duration: Not reported

Replicates: 3 per treatment

5. Observations/analyses:

Measurements: Cell viability, apoptosis and autophagy induction

siRNA mediated silencing of Beclin-1: Microscopy: In order to evaluate the effects of Beclin-1 siRNA or scrambled siRNA on GFP-LC3 puncta formation, cells were cotransfected with GFP-LC3 and either Beclin-1 siRNA or scrambled siRNA following various treatments and observed by confocal laser scanning microscopy. The Beclin-1 siRNA sequence (5'-CAGTTTGGACAATCAATA-3') efficiently targeted.

MTT assay: Detection: After DMSO was added to each well to dissolve the dark blue crystals the absorbance at 570 nm was interpreted on a microplate reader (Safire-TECAN).

Apoptosis detection: Microscopy: 24 h after transfection, the cells were subjected to various treatments before being observed under confocal laser scanning microscopy. In each experiment, no fewer than 200 transfected cells were counted.

Analysis: The cell apoptosis was further determined using FITC Annexin V Apoptosis Detection Kit according to the manufacturer's recommendations. Cells were then analyzed by flow cytometry (FCM) on a linear scale, to detect apoptosis using a Becton Dickinson FACS Array.

Autophagy detection: Microscopy: 24 h after transfection, the cells were subjected to various treatments before being observed under confocal laser scanning microscopy. In each experiment, no fewer than 200 transfected cells were counted.

Analysis: Only cells with at least five dots were scored as GFP-LC3-positive. The percentage of the positive cells was thus determined and expressed as the mean of four independent experiments. For western blot analyses, LC3-II/ LC3-I ratio was evaluated by band density analysis as the marker of cell autophagy.

Western blot analysis: Analysis: Protein extracts were quantified and equal amounts of lysates were resolved by SDS-PAGE, and then transferred into PVDF membrane (Millipore). After blocking with 3% BSA, appropriate primary antibodies and secondary antibodies were applied. The signals were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Band density values of interested bands were normalized to loading control and quantitative analyses were performed by imageJ software (Wayne Rasband, NIH).

Statistics: Statistical analyses were done by SPSS software using a one-way ANOVA, followed by a two-tailed Student's *t*-test or multiple comparison test where appropriate. A $P < 0.05$ was considered significant for all analyses.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Documentation insufficient for assessment (not clearly stated dose levels and duration of exposure, as well as treatment conditions for all tests. In addition, tested doses were much higher than real in vivo concentrations)

2. Relevance of study: Not relevant (Due to reliability)

3. Klimisch code: **3**

Response - GTF

- In this paper, the authors apply glyphosate to adrenal cancer cells in culture at concentrations sufficient to cause cell death. Two major interacting pathways leading to cell death (autolysis and apoptosis) are evaluated, and the results are hardly surprising – the cells do indeed die via known mechanisms leading to cell death. The authors use these observations, and the fact that Parkinson's disease involves the death of certain nerve cells in the brain, to try and create a link between glyphosate and Parkinson's disease. There are, however many problems with this extrapolation.
- The cells used are not the neurons involved in Parkinson's, but rather a cell line derived from an adrenal gland cancer (pheochromocytoma), and the doses used are very high- the high dose killed nearly 50% of cells in 72 hours, and the low dose was ¼ this level. The high dose equates to approximately 1/10 the concentration applied directly in the field, and is far higher than any internal glyphosate concentration that could ever occur following glyphosate use. A sufficiently high dose of anything will kill cells - but this does not mean that everything causes Parkinson's disease.
- There is no evidence that glyphosate causes Parkinson's disease. The authors cite two case reports of Parkinson's disease, discussed in the introduction of this neurotoxicity literature review.
    - The cited letter by Wang et al. (2011) reports a single patient, a 44 year old woman, who had worked in a glyphosate production unit for three years prior to developing Parkinson's. This provides no evidence for causation whatsoever.
    - The cited letter by Barbosa et al. (2001) is similarly a single case report, in this instance a 54 year old man who had dermal contact with a glyphosate formulation six months prior to developing Parkinson's disease.
- Unprotected cells in culture are highly susceptible to changes in pH and other non-specific effects, and it is not clear that the researchers assessed or accounted for these possible effects. This being said, the concentrations of glyphosate used (40 mM) are known to kill other cell types in culture (Koller et al., 2012; Heu et al., 2012b) via induction of apoptosis. Thus, no particular specificity or neuronally-specific susceptibility exists for the cell line tested. While 40 mM glyphosate is toxic to cells in culture, the LD-50 in rodents is over 5000 mg/kg and *C. elegans* will have a 25% survival following exposure to a 10% solution of glyphosate. In-vitro results do not appear to reflect *in vivo* events.
- Anadon et al. (2009) dosed rates with 400 mg/kg of glyphosate, a massive dose relative to any environmental exposure, and achieved glyphosate peak modeled plasma concentrations of approximately 5 ug/ml (5 ppm). Assuming linear kinetics, the current maximum allowable EU daily intake (0.3 mg/kg/day) would give an approximated blood concentration of 0.17 ppm (170

ppb). This is conservative, as McQueen et al (2012) recently evaluated glyphosate exposure to pregnant women and concluded that estimated exposures based on actual measurements in food were only 0.4% of the current European acceptable daily intake.

The lowest glyphosate concentration used in this experiment is 5mM (830 ppm), or 5000 times higher than the estimated blood concentration following the current EU maximum allowable daily exposure. It is also 166 times higher than the concentrations Anadon et al. (2009) achieved using doses of 400 mg/kg glyphosate. In short, the concentrations used in this work are massively higher than any concentration is blood (let alone brain tissue) that can be achieved following normal human exposures.

3. Literature Review of Carcinogenicity Publications

Over the 40 year product history of glyphosate based herbicides, regulatory expert and other authoritative review panels have evaluated multiple data sets to evaluated glyphosate safety, including potential for carcinogenicity. These multiple reviews over the decades have consistently drawn the same conclusion; glyphosate is not carcinogenic. These conclusions include those of the U.S. Environmental Protection Agency in 1993 and 1997 (Category E, evidence of non-carcinogenicity for humans -- based on the lack of convincing evidence of carcinogenicity in adequate studies); the European Commission's Health and Consumer Protection Directorate-General in 2002 (no evidence of carcinogenicity); the U.S. Forest Service (based on standard animal bioassays for carcinogenic activity *in vivo*, there is no basis for asserting that glyphosate is likely to pose a substantial risk); Canadian regulators (no evidence that glyphosate causes cancer); the World Health Organization and Food and Agriculture Organization of the United Nations in 2004 (long-term studies of toxicity and carcinogenicity were conducted in mice and rats. In the study of carcinogenicity in mice, no toxic effects were observed at up to the highest dose tested (1000 mg/kg bw per day), and there was no evidence of carcinogenicity.

A number of epidemiology studies over the last decade have focused on pesticide exposure and associated health outcomes. Publications vary in the specificity of their conclusions regarding pesticides in general, classes of pesticides and in some cases individual insecticides, herbicides or fungicides. While some of these publications specifically mention glyphosate, few draw tenable associations with any specific cancer outcome. Publications suggesting glyphosate is associated with any cancer outcome are discussed below.

One publication (George et al., 2009) utilized a 2-stage cancer model in mice to evaluate a glyphosate formulation for tumor promotion. A known tumor promoter, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) was used for a positive control/comparator after exposure to a tumor initiator, 7, 12-dimethylbenz[a]anthracene. Proteomics were later applied to extrapolate a basis for glyphosate formulation tumor promotion. This study is discussed in more detail below.

An essential consideration in both risk assessment and interpreting the relevance of toxicology data is exposure assessment. An inherent low level of confidence exists for epidemiological studies where tenuous links to exposure exist. Suggested associations between health outcomes and any possible causative agent are merely speculation if exposures are not identifiable. Pivotal to the understanding of glyphosate exposure are data published by Acquavella et al. (2004; 2005), which quantified human systemic glyphosate exposure levels in farmer applicators and their families. The geometric mean systemic dose for farmers applying glyphosate, some of whom applied glyphosate to areas up to 400 acres, was 0.0001 mg/kg/day, approximately 0.03% of the current EU glyphosate acceptable operator exosure Level (AOEL). The highest systemic dose, skewed well above the geometric mean, was 0.004 mg/kg/day, which is 1.95% of current EU glyphosate AOEL and 1.3% of the current EU glyphosate attapcable daily intake (ADI). Not surprisingly, even lower systemic doses were determined for spouses and children, 0.00004 mg/kg and 0.0008 mg/kg, respectively. Interestingly, the current European ADI is based on the NOAEL (highest dose tested) in an old 2-year rat carcinogenicity study; multiple carcinogenicity studies have since been conducted by numerous glyphosate registrants demonstrating NOAELs of at least ten-fold higher than the highest dose tested in the study driving the current EU ADI calculation.

The largest epidemiological study of pesticide exposure and health outcomes in the United States is the Agricultural Health Study (AHS), which included glyphosate. Dozens of publications have resulted from data generated in this study of approximately 57,000 enrolled farmer applicators. Blair et al. (2009) provided an overview of cancer endpoints associated with different agricultural chemicals reported in earlier AHS publications. Glyphosate was not reported to be associated with leukemia, melanoma, or cancers of the prostate, lung, breast, colon or rectum. De Roos et al. (2005) reported AHS data evaluating glyphosate use and multiple cancer endpoints; no association was noted for glyphosate with all cancers, including cancer of the lung, oral cavity, colon, rectum, pancreas, kidney, bladder, prostate, melanoma, all lymphohematopoietic cancers, non-Hodgkin's lymphoma (NHL) and leukemia. In an earlier publication based on another data set, however, De Roos et al., (2003) reported an association between NHL and glyphosate use. McDuffie et al. (2001) reported a non-significant positive association between self-

reported glyphosate exposure and NHL in a Canadian study. Blair et al. (2009) did not report an association between glyphosate use and NHL in the AHS data, but a "possible association" between glyphosate use and multiple myeloma was mentioned. The AHS publication reporting this refers to a "suggested association" between glyphosate use and multiple myeloma (De Roos et al., 2005), yet it did not demonstrate significant increase in relative risk for multiple myeloma. Both De Roos papers will be discussed in more detail below. Interestingly, a subsequent AHS review paper for the President's Cancer Panel (Freeman, 2009) specifically references De Roos (2005) as providing no observed incidents of cancers of any type being associated with glyphosate.

Lee et al. (2005) reported a glyphosate association with gliomas, with the odds ratio differing between self-respondents (OR = 0.4) and proxy respondents (OR = 3.1). The authors expressed concern that higher positive associations observed for proxy respondents with glyphosate and several other pesticides, and suggested perhaps more accurate reporting of proxies for cases, and underreporting by proxies for controls; proxy respondents were spouses in 62% of cases versus 45% of controls, lending to lower reported incidents in the control group.

The follow epidemiology publications report a lack of association between glyphosate and specific cancer types.

- Alavanja et al. (2003) reported on prostate cancer associations with specific pesticide exposures in the AHS; glyphosate did not demonstrate a significant exposure-response association with prostate cancer.
- Multigener et al, (2008) also reported a lack of association between glyphosate use and prostate cancer. This data appears to have also been reported by Ndong et al. (2009).
- The lack of association between glyphosate use and prostate cancer was also supported recently in an epidemiology study of Farmers in British Columbia, Canada by Band et al. (2011).
- Lee et al. (2004) reported a lack of association between glyphosate use and stomach and esophageal adenocarcinomas.
- Carreon et al. (2005) reported epidemiological data on gliomas and farm pesticide exposure in women; glyphosate had no association with gliomas.
- Engel et al. (2005) reported AHS data on breast cancer incidence among farmers' wives, with no association between breast cancer and glyphosate.
- Flower et al (2004) reported AHS data on parental use of specific pesticides*a* and subsequent childhood cancer risk among 17280 children, with no association between childhood cancer and glyphosate.
- Andreotti et al. (2009) reported AHS data where glyphosate was not associated with pancreatic cancer.
- Landgren et al. (2009) reported AHS data on monoclonal gammopathy of undetermined significance (MGUS), showing no association with glyphosate use.
- Karunanayake et al. (2011) reported a lack of association between glyphosate and Hodgkin's lymphoma.
- Pahwa et al. (2012) reported a lack of association between glyphosate and multiple myeloma.

In summarizing AHS publications, Weichenthal et al. (2010) noted that increased rates in the following cancers were not associated with glyphosate use; overall cancer incidence, lung cancer, pancreatic cancer, colon or rectal cancer, lymphohematopoietic cancers, leukemia, NHL, multiple myeloma, bladder cancer, prostate cancer, melanoma, kidney cancer, childhood cancer, oral cavity cancers, stomach cancer, esophagus cancer and thyroid cancer.

Monge et al (2007) investigated associations between parental pesticide exposures and childhood Leukaemia in Costa Rica. Results are not interpretable for glyphosate as exposure was estimated with "other pesticides", including paraquat, chlorothalanil and "others". No association was noted for paternal exposures, but elevated leukaemias were associated with maternal exposures to "other pesticides" during

pregnancy. Similarly, glyphosate is captured under "other pesticides" being associated with NHL by Fritschi et al. (2005) and therefore should not be interpreted as an association with glyphosate.

Non-Hodgkin's Lymphoma (NHL)

Non-Hodgkin's lymphoma is not a specific disease, but rather a grouping of all lymphoma types, other than Hodgkin's lymphoma. This is a large group of different cancers of the immune system including Burkitt lymphoma, diffuse large B-cell lymphoma (NLPHL), follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, mycosis fungoides, anaplastic large cell lymphoma, and precursor T-lymphoblastic lymphoma (National Cancer Institute, http://cancer.gov/cancertopics/wyntk/non-hodgkin-lymphoma.pdf). Risk factors associated with NHL include weakened immune system (such as from an inherited condition or certain drugs used after an organ transplant), infections (Epstein-Barr virus, EBV; Human immunodeficiency virus, HIV; *Helicobacter pylori* bacteria; Human T-cell leukemia/lymphoma virus, HTLV-1; Hepatitis C virus; age). There are many different types of Non-Hodgkin's lymphomas, which are different lymphomas arising from different pathogeneses, and as such, should not be clustered together as a single disease with a common etiology for epidemiological investigation. When clustered together in epidemiological studies, further investigation to identify both the specific type of lymphoma and any underlying risk factors associated with individual reports of HNL is necessary.

Author(s)	Year	Study title
Hardell, L. Eriksson, M.	1999	A Case-Control Study of Non-Hodgkin Lymphoma and Exposure to Pesticides. Cancer Volume: 85 Number: 6 Pages: 1353-1360

Abstract*

BACKGROUND. The incidence of non-Hodgkin lymphoma (NHL) has increased in most Western countries during the last few decades. Immunodefective conditions are established risk factors. In 1981, the authors reported an increased risk for NHL following exposure to certain pesticides. The current study was designed to further elucidate the importance of phenoxyacetic acids and other pesticides in the etiology of NHL.
METHODS. A population-based case–control study in northern and middle Sweden encompassing 442 cases and twice as many controls was performed. Exposure data were ascertained by comprehensive questionnaires, and the questionnaires were supplemented by telephone interviews. In total, 404 cases and 741 controls answered the questionnaire. Uni-variate and multi-variate analyses were performed with the SAS statistical data program.
RESULTS. Increased risk for NHL was found for subjects exposed to herbicides (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.0 –2.5) and fungicides (OR, 3.7; 95% CI, 1.1–13.0). Among herbicides, the phenoxyacetic acids dominated (OR, 1.5; 95% CI, 0.9–2.4); and, when subclassified, one of these, 4-chloro-2-methyl phenoxyacetic acid (MCPA), turned out to be significantly associated with NHL (OR, 2.7; 95% CI, 1.0–6.9). For several categories of herbicides, it was noted that only exposure during the most recent decades before diagnosis of NHL was associated with an increased risk of NHL. Exposure to impregnating agents and insecticides was, at most, only weakly related to NHL.
CONCLUSIONS. Exposure to herbicides in total, including phenoxyacetic acids, during the decades before NHL diagnosis resulted in increased risk for NHL. Thus, the risk following exposure was related to the latency period. Fungicides also increased the risk for NHL when combined, but this group consisted of several different agents, and few subjects were exposed to each type of fungicide.
* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, insecticides, fungicides, impregnating agents, organic solvents
Active substance(s):	Glyphosate, phenoxyacetic acid, MCPA, 2,4-D, 2,4,5-T, DDT, Pyrethrins, mercurial seed dressing, chlorophenols, pentachlorophenol, arsenic, creosote
Description:	Not reported
Source of test medium:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human

Age of test persons:	≥25
Sex:	Males

4. Test system:

Study type:	A Case-Control Study of Non-Hodgkin Lymphoma and Exposure to Pesticides
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Collection of data:	Questionnaire
Total No. of cases analysed:	442
Total No. of controls:	741
No. of exposed cases to glyphosate:	4
No. of controls for glyphosate	3

5. Observations/analyses:

Working history:	All subjects
Additional information:	Smoking habits, previous diseases, and certain food habits were assessed.
Detailed assessment of exposure:	Years and total number of days for exposure to various pesticides were assessed for all subjects.
Parameters determined:	Tumour induction period (time from first exposure to diagnosis), time span (time from last exposure to diagnosis). NHLs with different pathogeneses were not distinguished.
Statistics:	Conditional logistic regression analysis for matched studies was performed with the SAS statistical program. Thereby, odds ratios (OR) and 95% confidence intervals (95% CI) were obtained. All 95% CIs were rounded outward, e.g., a 95% CI of 1.07 – 4.52 is written 1.0 – 4.6. Both uni-variate and multi-variate analyses were performed. When exposure to different pesticides was analyzed, subjects with no pesticide exposure were taken as unexposed.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comments:	Study prone to selection and recall bias. No evidence of relevant glyphosate exposures. Medical history was assessed, but not reported.
2. Relevance of study:	**Not relevant** (Exposure to multiple chemicals and though glyphosate exposure data were convincing (7/1145 subjects) and statistically non-significant positive associations reported.
3. Klimisch code:	**3**

Response 1 – Review by Mark R. Cullen, MD, Professor of Medicine and Epidemiology, Yale University School of Medicine, June 21 1999

This study is part of an ongoing effort of the investigators and their team to unravel the cause(s) of NHL, which has been increasing in incidence in Sweden and most developed countries for at least 2 decades. The premise, that the increase suggests an environmental cause or causes, is certainly correct.

The basic approach, the case control study using the superb existing tumor and population registries of Sweden, is appropriate to this challenge, and the investigators seem to have a clear grasp of the basic approach to such studies. Inclusion criteria for cases appear well considered, and the ability to recruit almost all is a strong plus for the study. The criteria for including controls, including the matching on vital status for comparability of information regarding past exposures is laudable, though, as discussed below, possibly unsuccessful despite careful consideration. The response of the subjects is encouragingly high.

Unfortunately the approach to exposure assessment for agricultural chemicals is very problematic. First, as I believe the data themselves ultimately demonstrate, it is not at all clear that even living subjects, let alone relatives of dead ones, can meaningfully assess or quantify exposure to herbicides and pesticides. It appears from the small number of phone interviews conducted (itself a problem, see below) that almost every subject provides different information or expanded information when directly contacted by phone. It is not at all obvious that the respondents can easily evaluate their exposures, which in many cases amount to an occasional use of a product many years before the survey, nor is it obvious that the surrogate measure of dose, i.e., days of use, is meaningful, especially given the remarkable difference which exists in actual biological exposure depending on how the products are used, information which was not even attempted here. In other words, the first problem is the degree to which this study classifies subjects in any biologically relevant way, or validly.

As if this were not problematic enough, there is evidence within the study results to suggest significant information or recall bias. When they were contacted because of ambiguous or missing information, a high proportion, possibly all subjects reported a positive history of exposure - it is unclear from the report just how many such were contacted overall, but it appears that most were contacted to confirm positive histories, despite the evidence that the negative histories were more likely unreliable. I would worry greatly that cases, clearly aware of their disease status even if not the underlying hypothesis here, might be more thorough in their recollection of these distant events, whose recall is likely more subtle than recall of major industrial chemicals which likely would have involved (unforgettable) daily work exposures, unlike the chemical use with doses averaging about a month! The authors would have done well to interview everybody given this spareness, and the ubiquity of recall bias in such studies.

The third problem with the exposure assessment relates to co-linearity. For obvious reasons people exposed to one agricultural chemical have a non-independent (true) chance of exposure to another, and that recollection of one is likely to interact with recollection of others. The data presented are consistent with this, though the actual degree of overlapping exposures in the data are not fully disclosed. In any event, the effort to tease them apart using multi-variate regression unlikely gets at the fundamental issue, which is that information is hopelessly confounded. Even if one were not concerned about the other issues vitiating the exposure assessment, the attempt to distinguish one exposure from another within the herbicide category is, in my view, fatuous, though the investigators have drawn some rather sweeping inferences from it, and from the latency analysis which I believe suffers from the same recall issues.

One final comment, which I fear may betray a range of the authors preconceived ideas, is the inclusion of glyphosate in the uni-variate and multi-variate analyses, despite the fact that only 7 of 1145 subjects in the study gave exposure histories to this agent, and for a mean duration of what appears to be a few days! Since there is zero possibility that exposure to glyphosate could explain the Swedish excess of NHL which is the premise of the study, and since it is biologically absurd to imagine a few days exposure to virtually any short lived compound, let alone one with so little oncogenic potential based on its toxicologic profile, the inclusion of these data and the highlighting of them in the discussion - with a very biased review of the tox literature-- undermines even further the report.

In the end I think this study adds little to our overall knowledge of the cause(s) of NHL, though it continues to appear that farmers have increased risk, certainly an important clue for follow-up. However, it is unlikely that the roles of infection, other biological factors, UV light, diet and lifestyle issues or agricultural chemicals will be successfully unraveled by studies of this design. In particular, the evidence

regarding glyphosate in relation to NHL is meaningless, and it would be highly inappropriate to construe this as a positive study in that regard.

Response 2 – Review by Hans-Olav Adami, Professor of Epidemiology, Harvard School of Public Health and Dimitrios Trichopoulos, Vincent L. Gregory Professor of Cancer Prevention, Department of Epidemiology, Harvard School of Public Health.

We have classified our comments into those concerning study design and those concerning data analysis and interpretation, and we have concluded our evaluation with a short commentary and overall assessment.

Study design

The study base comprises men 25 years of age or older and living in any of seven Swedish counties from January 1, 1987 to December 31, 1990. The cases were divided according to their vital status at a time when the actual data collection took place. Of the 442 cases, 192 were deceased. The date of vital status ascertainment is not clearly indicated, as it should have been. Since, however, data were collected from 1993 to 1995, we assume that vital status was determined in 1993 or earlier.

The authors state that they have conducted a population-based study, but they have chosen their controls in a way that violates the defining characteristics of these studies. Sampling from the population register took place sometime after 1990, so that people who had migrated out of the area after the diagnosis of the corresponding case would have been incorrectly ineligible, whereas those who had migrated into the area after the diagnosis of the corresponding case would have been incorrectly eligible. Migration is generally related to socio-economic status, which is a plausible predictor of exposure to pesticides. Thus, important bias may have been introduced.

There are other issues that should have been addressed in the study design. Is it really possible to blind interviewers as to the case or control status of the interviewed person, so as to minimize interviewer-related information bias? And, what assurance is there that the substantial difference in response proportion between cases and controls did not introduce interviewee-related selection bias? It is certainly disturbing that all 17 reported odds ratios (Table 1 of the authors) were higher than the null value of 1, even though only marginally significant results were reported. It is also astonishing that there is no category of missing or unknown in any of the tables, even though about half of the exposure information was provided by proxy responders and this information was concerning compounds as complicated as 2,4-D/2,4,5-trichlorophenoxyacetic acid.

Analysis

The analysis is in many ways superficial and shows a surprising disregard to confounding. The authors appear so eager to report significant results, that when multi-variate analysis, *that is the proper analysis*, reduces all reported odds ratios to essentially non-significant values (table 7), they make the amazing statement that "regarding lymphomagenesis, the uni-variate analysis may be more informative than the multi-variate analysis". Moreover, they pay little attention to the multiplicity of comparisons and they attempt causal inferences with unacceptable disregard of the statistical limitations of their study. For example, for glyphosate, the p value is no less than 0.35 and for phenoxyacetic acids the multi-variate odds ratio has a p value of 0.25.

There are several other issues in the analysis. Although most of them are trivial, one deserves more attention. Non-Hodgkin lymphoma has been reported to be more common in some rural occupations. Exposure to pesticides is a possible explanation, but there are other plausible explanations, including exposure to infectious agents of animal origin and delayed establishment of herd immunity with concomitant increase in the average age at exposure to possible critical agents (the classical paradigm of paralytic polio has been invoked by several investigators in the study of the etiology of multiple sclerosis,

leukemias and lymphomas). In the latter two instances, occupation should be adjusted for in the analysis, in order to control for confounding.

Conclusion

This is a study that has limited power, was inadequately designed, poorly analysed and confusingly reported. Every epidemiological investigation should meet basic standards concerning selection bias, information bias, confounding and power. The investigation by Hardell and Eriksson does not provide reasonable confidence that it is free of information and selection bias, shows clear signs of uncontrolled confounding and lacks the power necessary to document agent-specific effects when several agents are inter-correlated, as they are in this situation. There is also evidence that the results were selectively interpreted by the investigators. For these reasons, the study cannot provide reliable information concerning possible associations between exposures to pesticides and risk for non-Hodgkin lymphoma

Response 3 – Monsanto Review by John Acquavella, PhD and Donna Farmer, PhD

Executive Summary

Hardell and Erikkson conducted a case control study to look for associations between reported pesticide use and non-Hodgkin's lymphoma (NHL). The study included 404 NHL cases and 741 controls. The measure of association in this study was the odds ratio (OR), a statistic that estimates of the ratio of disease rates (in this case NHL rates) for exposed and unexposed populations.

The authors reported statistically significant associations for NHL with: reported use of any herbicide (OR = 1.6), reported use of any fungicide (OR = 3.7), and reported use of 4-chloro-2-methylphenoxyacetic acid (OR = 2.7). The major limitations of this study were: the reliance on reported pesticide use (not documented exposure) information, the small number of subjects who reported use of specific pesticides, the possibility of recall bias, the reliance on secondary sources (next-of-kin interviews) for approximately 43% of the pesticide use information, and the difficulty in controlling for potential confounding factors, given the small number of exposed subjects.

The authors also reported a moderately elevated OR of 2.3 for glyphosate. This OR was not statistically significant and was based on only four "exposed" cases and three "exposed" controls. This finding needs to be evaluated in light of the limitations of the study, mentioned above, and the wealth of toxicologic information that has resulted in glyphosate being judged to be non-mutagenic and noncarcinogenic by the U.S. Environmental Protection Agency and the World Health Organization. Systematic error or chance seem the most likely explanations for the findings reported for glyphosate in this study.

Hardell and Eriksson[1] conducted an epidemiologic study to look for associations between self-reported pesticide use and non-Hodgkin's lymphoma (hereafter NHL). The rationale for conducting this research was previous studies by the first author and by investigators at the U.S. National Cancer Institute[4,5], which found associations between reported use of phenoxyacetic acids (primarily 2,4-D) and NHL. The results of these studies were determined to be inconclusive by a special Science Advisory Panel convened in the early 1990s by the U.S. Environmental Protection Agency (EPA).[6]

The present study presents new data about phenoxyacetic acids and other commonly used pesticides. Herein, we review the methods and results of this recent study.

Study design

Hardell and Eriksson employed a case control design for their research. In case control studies, subjects are selected on the basis of their disease status. Those with the disease of interest (in this case those with NHL) are the cases; disease free study participants are the controls. Information about presumptive etiologic factors are collected from cases and controls using similar methodology.

The controls in a case control study provide an estimate of the exposure prevalence (in this case the prevalence of self-reported pesticide use) in the base population that gave rise to the cases and controls[7]. The exposure odds for the cases is then compared to the exposure odds for the controls. The resulting ratio of exposure odds - called the odds ratio (OR) - estimates the ratio of disease rates for exposed versus unexposed subjects[8]. The ratio of disease rates is the fundamental measure of association in epidemiologic studies.

The interpretation of the OR is straightforward. An OR of 1.0 implies that the disease rate (in this case the rate of NHL) is the same for exposed members of the base population and for unexposed members and indicates no association between exposure and disease. An OR greater than 1.0 or less than 1.0 implies that the disease rate is different for the exposed population than for the unexposed population and, if valid, may indicate an exposure disease relationship. Exposure disease relationships can be "positive" (viz. the OR is greater than 1.0) - where exposure is associated with increased rates of disease - or inverse (viz. the OR is less than 1.0) - where exposure is associated with decreased rates of disease (viz. exposure prevents disease). For example, an OR of 2.0 is consistent with a disease rate among exposed persons that is twice the disease rate for unexposed persons; likewise, an OR of 0.5 is consistent with a disease rate for exposed persons that is half the disease rate for unexposed persons.

Interpreting ORs at face value requires the assumption that there is no confounding or other bias in a study. Much of the evaluation of epidemiologic studies hinges on whether there are discernible sources of bias or potential for bias, which, if present, compromise the validity of findings. Often it is not possible to pinpoint specific sources of bias, but methodologic limitations can usually be identified and the results interpreted accordingly.

A major validity concern in case control studies is recall bias: that is when cases or their next-of-kin are more likely to recall (real or imagined) specific exposures than are controls. This can result in differential exposure misclassification whereby cases are more likely to be classified as exposed than are controls, despite no real difference in exposure prevalence. Recall bias is particularly an issue in cancer studies; cancer being a disease that stimulates introspection about presumptive causes. Other important validity concerns are selection bias (cases or controls as selected are unrepresentative) or uncontrolled confounding factors. Proper reporting of an epidemiologic study requires consideration of potential biases and their likely impact on study results.

Finally, findings are also evaluated according to how likely they are to have occurred by chance alone if there is not, in fact, a true relationship between exposure and disease. This is evaluated by calculating a probability (called a p-value) for seeing results at least as extreme as those observed if the null hypothesis of no true effect is true. By convention, only findings where the p value is less than 0.05 are considered "statistically significant." Hardell and Eriksson did not actually calculate p values in their study. Instead, they calculated 95% confidence intervals for the OR. The 95% CI is defined as the range of values that are consistent with the data observed in a study with 95% confidence. For example, a CI of 0.4 to 13.0 means the data are consistent with an OR as low as 0.4 (implying a 60% reduced rate with exposure) or as high as 13.0 (implying a 13-fold elevated rate with exposure). A finding is statistically significant when the OR of 1.0 is not included in the 95% CI.

Study subjects

The study included 404 NHL cases, diagnosed during the period 1987-1990, from the four most northern counties of Sweden. These cases (or their next-of-kin when cases were deceased) and 741 controls (or their next-of-kin when controls were deceased) were sent a mailed 18 page questionnaire that addressed a variety of (self-reported, viz. undocumented) factors including pesticide use, work history and chemical exposures, smoking habits, previous diseases, and certain dietary habits.

Controls were selected to be similar to cases in terms of age and vital status (i.e. living cases were matched to living controls and deceased cases were matched to deceased controls). Matching subjects on vital status was intended to minimize recall bias to the extent that the fact of death, but not death from a

specific cause, might affect recollections of pesticide use. Approximately 43% of cases were deceased, hence next-of-kin information a significant component of this study.

Exposure Assessment

There was no exposure assessment, per se, in this study. Exposure was presumed based on reported use of specific pesticides. This can be an inaccurate indicator of exposure for two reasons: 1) inaccurate recall or 2) negligible exposure from use. An example of the latter would be glyphosate which has very low skin penetrability[9], so reported use is not equivalent to (meaningful) exposure. A recent study of forestry sprayers by Lavy et al. found indications of significant dermal exposure, but no indication, based on biomonitoring, of an absorbed dose of glyphosate.[10]

Statistical analysis

The data analysis involved standard techniques to estimate the OR and control, in a very limited sense, for coincident pesticide exposures as potential confounding factors. These statistical techniques included uni-variate and multi-variate logistic regression analysis. The analysis was primarily restricted to a crude dichotomous classification of reported pesticide use (ever use versus never use). There were too few "exposed" subjects to conduct dose response analyses for most specific chemicals. The authors also estimated 95% CIs as a measure of the statistical variability of the ORs.

Results

The authors found modest, though statistically significant, associations between NHL and reported use of any herbicide (OR = 1.6, 95% CI 1.0-2.5) reported use of any fungicide (OR = 3.7, 95%
CI 1.1-13.0) and reported use of 4-chloro-2-methyl phenoxyacetic acid (MCPA) (OR = 2.7, 95% CI 1.07.0).
Through various analyses, the authors concluded that only exposure in the two decades preceding diagnosis was associated with increased risk.

The authors also reported findings for glyphosate, none of which were statistically significant. The overall OR for glyphosate was 2.3 (95% CI 0.4-13.0) based on 4 cases (1% of cases) and 3 controls (0.4% of controls) reporting glyphosate use. The authors also mentioned an additional analysis where glyphosate and phenoxyacetic acids were considered jointly in attempt to control for confounding from phenoxyacetic acids on the glyphosate/NHL association. In this instance, the OR for glyphosate was 5.8 (95% CI 0.6-54.0) and the OR for phenoxyacetic acids was 1.4 (95% CI 0.8-2.2). The description of this analysis was insufficient to know what the authors actually did or even to know the number of cases who reported using glyphosate. But it was clear that there was no systematic attempt to assess the association between glyphosate and NHL while controlling for exposures other than phenoxyacetic acids.

Authors' conclusions

The authors interpreted their results as supportive of a role for chemical pesticides in the etiology of NHL. They speculated, since NHL is known to be related to immunosuppression from studies of transplant patients[11], that phenoxyacetic acids might produce NHL by an immunosuppressive mechanism. In fact, they interpreted selected papers from the literature as supportive of an immunotoxic effect for phenoxyacetic acids and chlorophenols.[12,13,14]

The authors reached less definite conclusions about other pesticides and specifically about glyphosate. They noted the elevated OR for glyphosate, an elevated OR for glyphosate from another study of theirs[15] concerning hairy cell leukemia (OR = 3.1, 95% CI 0.8-12.0, based on 4 cases who reported use of glyphosate), and selected toxicologic data[16-21] as indicative that glyphosate is, at least, deserving of further epidemiologic study.

The authors considered several potential biases in interpreting their results. They ruled out selection bias by arguing that they had good response rates from cases and controls and included most cases who were diagnosed during the study period. They felt they minimized recall bias by matching cases and controls on vital status and collecting information from all study subjects using similar (blinded) methodology.

Critique

This study has several important limitations: no exposure assessment, dependence on next-of-kin's recollections of study subjects' pesticide use for approximately 43% of study subjects, potential recall bias, and the very small number of subjects who reported using specific herbicides. The latter leads to findings that are statistically imprecise. Due to the potential for bias and the statistical imprecision, the results of this study are not convincing.

In epidemiologic studies results can be:

- real (viz. disease is due to exposure)
- biased (viz. the results are invalid)
- due to chance (viz. the association is unbiased, but non causal)

It is by exclusion of the latter two possibilities and application of generally-accepted criteria for causality[22] that scientists come to believe that an exposure-disease association is causal. The most important causal criteria are strength of association (judged by the size of the OR), dose-response (judged by whether the OR increases or decreases with increasing exposure), temporality (exposure should precede the onset of disease by an appropriate induction/latent period), consistency of findings across studies, and biological plausibility. I'll return to each of these criteria subsequently.

The major potential sources of bias in this study are recall bias, confounding bias, and selection bias. Recall bias is a major concern in cancer case control studies because cancer cases, and especially their next-of-kin, tend to scrutinize their lives hoping to understand the cause(s) of their disease. Hardell and Eriksson's matching of study subjects on vital status does not address the specific recall bias issue for cancers. Other investigators have found elevated ORs for the popular herbicide 2,4-D based on next-of-kin responses, but not based on responses of direct informants.[23] Results based on a substantial number of next-of-kin respondents are usually considered less persuasive than data from actual study subjects. It would have been informative had Hardell and Eriksson analyzed their data separately for next-of-kin respondents to see whether the elevated ORs were determined primarily by next-of-kin responses. That would be difficult in the present study due to the limited number of cases who reported using most specific pesticides.

A second important limitation of the study was the inability to control for potential confounding factors. Confounding refers to finding spurious exposure-disease associations resulting from other correlated factors. The confounding factor must also be a risk factor for the disease in question. Relatively little is known about the etiology of NHL, other than there seems to be a relationship with immunosuppression.[24] It is difficult to control for confounding factors when little is known about etiologic factors. In addition, in light of the high correlation between reported use of various pesticides, it is difficult in such a study, given the small number of exposed subjects, to separate the putative effects of one pesticide from another. Therefore, associations reported for any specific pesticide might be due to effects from other pesticides.

The final source of bias to be considered is selection bias. There is no way to know whether the cases or controls who participated in the study were a biased sample, but the relatively high participation rates for cases and controls would make selection bias a less likely explanation for the findings in this study.

Specific results in an epidemiology study can be due to chance, especially when many statistical associations have been evaluated. The convention is that a p value of 0.05 or less is considered unlikely to have occurred by chance and is therefore "statistically significant." The p values for the glyphosate findings are well in excess of 0.05, approximately 0.30 or greater by my estimation, so neither of the

elevated ORs for glyphosate are close to the conventional criterion for statistical significance. They could easily be chance findings. It is noteworthy that if even one exposed case was misclassified, the OR would be approximately 1.8 (95% CI 0.6-9.9, p value 0.43); two misclassified exposed cases would give an OR of 1.2 (95% CI 0-6.2, p value 0.99). Hence, the elevated OR for glyphosate hinges on the classification of a single case or two and an exposure assessment methodology of questionable accuracy.

It is helpful at this point to assess how the findings in the present study for glyphosate (and for most of the other herbicides) match up with the causal criteria generally accepted by epidemiologists.
Specifically:

- strength of association - the findings of the present study show a weak to moderate non significant association between glyphosate use and NHL. The association is statistically imprecise and, even assuming an absence of bias, is not convincing.
- temporality - in this study, the presumed exposures would precede disease onset satisfying, in general, the temporality criterion. However, the authors did not have enough exposed subjects to consider issues of disease induction/latency as they tried to do for the phenoxyacetic acids.
- dose response - there was insufficient data in this study to consider dose response. Also, in light of glyphosate's very low skin penetrability9, one can question whether any meaningful range of
- exposure occurred among study subjects.
- consistency - there are no other studies that have reported an association between glyphosate and NHL. Hence the consistency criterion cannot be met.
- biological plausibility - Hardell and Eriksson characterized the available glyphosate toxicologic data as showing: excess mutations and chromosome aberrations in studies with mouse lymphoma cells[16-19], excess sister chromatid exchanges (SCEs) in cultures of human lymphocytes[20], and a somewhat increased incidence of various cancers in one carcinogenicity study of mice.21 However, five of the six references cited did not use glyphosate as the test material.[16-19,21] In these studies the test material was sulfosate, the trimesium salt of glyphosate. Sulfosate has a somewhat different toxicology profile than glyphosate. Nonetheless, it is worth pointing out that Hardell and Erikkson's assessment of these studies is not shared by regulatory agencies. For example, the U.S. Environmental Protection Agency (EPA) considered the mouse lymphoma findings[16-19] to be false positives due to sulfosate's acidity; sulfosate was not mutagenic in this assay when the pH was adjusted to a physiological level.[25] Also, EPA characterized the sulfosate mouse carcinogenicity study[21] as showing "… no evidence of carcinogenicity … at the doses tested" and classified sulfosate as category E – no evidence for carcinogenicity in humans.[25]

The one glyphosate toxicology study cited[20] showed weak positive findings for sister chromatid exchange in human lymphocytes in vitro. This study had many limitations and numerous, more specific, mutagenicity assays have not shown positive results for glyphosate.[26] Extensive reviews of the available toxicologic data have been completed recently by the U.S. Environmental Protection Agency[27,28] (EPA) and the World Health Organization.[29] These agencies concluded that glyphosate is not mutagenic or carcinogenic. EPA classified glyphosate as category E.[27,28] This would argue against the biological plausibility of the findings reported by Hardell and Erikkson.

In conclusion, the study by Hardell and Eriksson found a modest association between NHL and several chemical pesticides - most notably for MCPA and the collective group of fungicides. The reported weak to moderate associations for glyphosate are not statistically significant and could be due to chance or to recall or confounding bias. It is clear, however, that the widespread use of glyphosate and concerns about pesticide related health effects for farmers and their families will raise the "index of concern" for glyphosate in future agricultural epidemiologic studies.

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Hardell, L. Eriksson, M. Nordstrom, M.	2002	Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: Pooled analysis of two Swedish case-control studies. Leukemia & Lymphoma Volume: 43 Number: 5 Pages: 1043-1049

Abstract*

Increased risk for non-Hodgkin's lymphoma (NHL) following exposure to certain pesticides has previously been reported. To further elucidate the importance of phenoxyacetic acids and other pesticides in the etiology of NHL a pooled analysis was performed on two case-control studies, one on NHL and another on hairy cell leukemia (HCL), a rare subtype of NHL. The studies were population based with cases identified from cancer registry and controls from population registry. Data assessment was ascertained by questionnaires supplemented over the telephone by specially trained interviewers. The pooled analysis of NHL and HCL was based on 515 cases and 1141 controls. Increased risks in uni-variate analysis were found for subjects exposed to herbicides (OR 1.75, CI 95% 1.26-2.42), insecticides (OR 1.43, CI 95% 1.08-1.87), fungicides (OR 3.11, CI 95% 1.56-6.27) and impregnating agents (OR 1.48, CI 95% 1.11-1.96). Among herbicides, significant associations were found for glyphosate (OR 3.04, CI 95%

1.08-8.52) and 4-chloro-2-methyl phenoxyacetic acid (MCPA) (OR 2.62, CI 95% 1.40-4.88). For several categories of pesticides the highest risk was found for exposure during the latest decades before diagnosis. However, in multi-variate analyses the only significantly increased risk was for a heterogeneous category of other herbicides than above.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, insecticides, fungicides, impregnating agents, organic solvents
Active substance(s):	Glyphosate, phenoxyacetic acid, MCPA, 2,4-D, 2,4,5-T, DDT, Pyrethrins, mercurial seed dressing, chlorophenols, pentachlorophenol, arsenic, creosote
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

(in the following data only presented for exposures to glyphosate and total number of subjects)

Species:	Human
Age of test persons:	~25
Sex:	Males

4. Test system:

Study type:	Epidemiological study for Non-Hodgkin´s Lymphoma (NHL) and Hairy cell Leukemia (HCL)
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Collection of data:	Questionnaire & telephone interviews
No. of exposed persons with NHL or HCL:	NHL study: 404 HCL study:111 Total: 515
No. of control persons:	NHL study: 404 HCL study:111 Total: 515
No. of persons with NHL or HCL exposed to glyphosate:	8
No. of persons in control group:	8

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	Years and total number of days for exposure to various pesticides were assessed for all subjects. For analysis only subjects with a minimum exposure of 1 working day (8h) and a

tumour induction period of at least one year were included.

Parameters determined: Tumour induction period (time from first exposure to diagnosis), time span (time from last exposure to diagnosis). NHLs with different pathogeneses were not distinguished.

Statistics. Conditional logistic regression analysis for matched studies was performed with SAS statistical program. Odds ratios and 95% confidence intervals were obtained. Both uni-variate and multi-variate analyses were done. In the pooled analysis an adjustment was made for study, study area and vital status. When risk estimates for different pesticide exposures were analysed only subjects with no pesticide exposure were taken as unexposed, whereas subjects exposed to other pesticides were disregarded.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: This publication combines the results of two previous studies by the authors on HNL (Hardell and Eriksson, 1999) and HCL (Nordstrom et al., 1998). No information about exposure duration, exposure concentration, as well as medical history, lifestyle factors (e.g. smoker, use of prescribed drugs etc). Study documentation is insufficient for assessment.

2. Relevance of study: **Not relevant** (Due to reliability of data set drawn from Hardell and Eriksson, 1999)

3. Klimisch code: **3**

Response – GTF

- This study pools NHL data from the previously reviewed publication by Hardell and Eriksson (1999) with HCL data from Nordstrom et al. (1998). Therefore the responses to Hardell and Eriksson (1999), the methodology and data issues, also apply to the NHL data set used in Hardell et al. (2002). It is of interest to note that Hardell was also a coauthor of Nordstrom et al. (1998).
- Each individual study reported non-statistically significant associations between glyphosate and NHL or HCL.
- Each study was based on few exposed cases, 4 each. The pooled analysis combined these cases.
- The uni-variate odds ratio was similar to those in the two individual studies (OR = 3.04; 95% CI: 1.08–8.52), the multi-variate adjusted odds ratio was attenuated (OR = 1.85; 95% CI: 0.55–6.20)
- These data fail to demonstrate convincing evidence for an association between glyphosate and NHL or HCL.

Author(s)	Year	Study title
Fritschi, L. Benke, G. Hughes, A. M. Kricker, A. Turner, J. Vajdic, C. M. Grulich, A. Milliken, S. Kaldor, J. Armstrong, B. K.	2005	Occupational exposure to pesticides and risk of non-Hodgkin's lymphoma American Journal of Epidemiology Volume: 162 Pages: 849-857

Abstract*

Pesticide exposure may be a risk factor for non-Hodgkin's lymphoma, but it is not certain which types of pesticides are involved. A population-based case-control study was undertaken in 2000–2001 using detailed methods of assessing occupational pesticide exposure. Cases with incident non-Hodgkin's lymphoma in two Australian states (n = 694) and controls (n = 698) were chosen from Australian electoral rolls. Logistic regression was used to estimate the risks of non-Hodgkin's lymphoma associated with exposure to subgroups of pesticides after adjustment for age, sex, ethnic origin, and residence. Approximately 10% of cases and controls had incurred pesticide exposure. Substantial exposure to any pesticide was associated with a trebling of the risk of non-Hodgkin's lymphoma (odds ratio = 3.09, 95% confidence interval: 1.42, 6.70). Subjects with substantial exposure to organochlorines, organophosphates, and "other pesticides" (all other pesticides excluding herbicides) and herbicides other than phenoxy herbicides had similarly increased risks, although the increase was statistically significant only for "other pesticides." None of the exposure metrics (probability, level, frequency, duration, or years of exposure) were associated with non-Hodgkin's lymphoma. Analyses of the major World Health Organization subtypes of non-Hodgkin's lymphoma suggested a stronger effect for follicular lymphoma. These increases in risk of non-Hodgkin's lymphoma with substantial occupational pesticide exposure are consistent with previous work.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Organophosphates, organochlorines, phenoxy herbicides, other herbicides, and other pesticides
Active substance(s):	Glyphosate and others
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	20-74
Sex:	Males and females

4. Test system:

Study type:	Occupational exposure study to assess exposure to pesticides and risk of non-Hodgkin's lymphoma

Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Collection of data:	Questionnaire Histopathological confirmation of NHL was done by an experienced pathologist.
No. of exposed persons with NHL:	694
No. of control persons:	694
Pesticide use frequency:	Not reported

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	The questionnaire included a diary with a detailed lifetime history of each job the subject had held for 1 year or more. Information obtained on each job included job title, employer, industry, start and finish years, number of hours worked per day, and number of days worked per week.
Parameters determined:	A pesticide-crop matrix was developed for assistance with exposure assessment. Levels of exposure were considered according to time-weighted average threshold limit values. Frequency of exposure was allocated as number of 8-hour days per year and was calculated using responses to the task questions. If no data on frequency of exposure were available (n=4), subjects were assumed to have been exposed for 2 days per year.
Statistics:	Logistic regression was used to calculate odds ratios (as estimates of relative risk) for non-Hodgkin's lymphoma associated with exposure to any pesticide and exposure to each pesticide subtype in each amount category (substantial or nonsubstantial), with adjustment for age, sex, ethnic origin, and state of residence. In addition, logistic regression analyses were carried out for exposure to any pesticide after restricting the sample to males only and after excluding cases that were not on the electoral roll. We also examined the odds of non-Hodgkin's lymphoma using the following metrics of exposure to any pesticide: maximum exposure level (low, medium, high); ever being exposed before 1985 (yes, no); maximum frequency of exposure (0, ≤4, or >4 days/year); and total number of years exposed (0, ≤5, or >5 years). For the latter two metrics, 4 days per year and 5 years were the median frequency and duration, respectively, in control subjects. All *p* values were two-sided.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: No information about exposure duration, used glyphosate products, exposure duration and application rates. Documentation is insufficient for assessment.

2. Relevance of study: **Not relevant** (Multiple pesticide exposures. No definitive association between NHL and glyphosate can be made.)

3. Klimisch code: **3**

Author(s)	Year	Study title
De Roos, A. J. Zahm, S. H. Cantor, K. P. Weisenburger, D. D. Holmes, F. F. Burmeister, L. F. Blair, A.	2003	Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men. Occupational and Environmental Medicine Volume: 60 Number: 9 Pages: -E11

Abstract*

Background: An increased rate of non-Hodgkin's lymphoma (NHL) has been repeatedly observed among farmers, but identification of specific exposures that explain this observation has proved difficult.

Methods: During the 1980s, the National Cancer Institute conducted three case-control studies of NHL in the midwestern United States. These pooled data were used to examine pesticide exposures in farming as risk factors for NHL in men. The large sample size (n = 3417) allowed analysis of 47 pesticides simultaneously, controlling for potential confounding by other pesticides in the model, and adjusting the estimates based on a prespecified variance to make them more stable.

Results: Reported use of several individual pesticides was associated with increased NHL incidence, including organophosphate insecticides coumaphos, diazinon, and fonofos, insecticides chlordane, dieldrin, and copper acetoarsenite, and herbicides atrazine, glyphosate, and sodium chlorate. A subanalysis of these "potentially carcinogenic" pesticides suggested a positive trend of risk with exposure to increasing numbers.

Conclusion: Consideration of multiple exposures is important in accurately estimating specific effects and in evaluating realistic exposure scenarios.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, insecticides (in total 47)
Active substance(s):	Glyphosate and 46 others
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	≥ 21
Sex:	Males

4. Test system:

Study type:	Epidemiological studies for Non-Hodgkin´s Lymphoma (NHL) in male farm workers exposed to pesticides

	Pooled data from three population based cased control studies conducted in Nebraska, Iowa and Minesota and Kansas.
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Selection of test persons:	Nebraska: Persons identified by Nebraska Lymphoma Study Group and area hospitals (Time of diagnosis: July 1983 – June 1986). Iowa and Minesota: Ascertained from records of the Iowa State Health Registry; Surveillance system of Minnesota hospitals and pathology laboratories (Time of diagnosis: 1980 - 1983). Kansas: A random sample of cases from the statewide cancer registry run by the University of Kansas Cancer Data Service (Time of diagnosis: 1979 – 1981)
Selection of control persons:	Randomly: Same geographical areas as the cases; Frequency matched to cases by race, sex, age, and vital status at the time of interview
Collection of data:	Questionnaire / Interview
(in the following data only presented for exposures to glyphosate and total number of subjects)	
No. of exposed persons with NHL:	870
No. of control persons:	2569
No. of persons with NHL or HCL exposed to glyphosate:	36
No. of persons in control group:	61
Pesticide use frequency:	> 20 per person

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	Years and total number of days for exposure to various pesticides were assessed for all subjects. For analysis only subjects with a minimum exposure of 1 working day (8h) and a tumour induction period of at least one year were included. No analysis of actual exposure duration or frequency was included.
Parameters determined:	Tumour induction period (time from first exposure to diagnosis), time span (time from last exposure to diagnosis)
Analyses and statistics:	Standard logistic regression (maximum likelihood estimation); Hierarchical regression, calculating odds ratios to estimate the relative risk associated with each pesticide Models included variables for age (coded as a quadratic spline variable with one knot at 50 years) and indicator variables for study site Other factors known or suspected to be associated with NHL, including first degree relative with haematopoietic cancer, education, and smoking, were evaluated and found not to be important confounders of the associations between NHL and pesticides

Conditional logistic regression analysis for matched studies was performed with SAS statistical program. Odds ratios and 95% confidence intervals were obtained. Both uni-variate and multi-variate analyses were done. In the pooled analysis an adjustment was made for study, study area and vital status. When risk estimates for different pesticide exposures were analysed only subjects with no pesticide exposure were taken as unexposed, whereas subjects exposed to other pesticides were disregarded.

The standard logistic regression models did not assume any prior distribution of pesticide effects, in contrast to the hierarchical regression modelling

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: No useful information about exposure duration, exposure concentration, as well as medical history, lifestyle factors (e.g. smoker, use of prescribed drugs etc were reported. Specific lymphomas are not identified (NHL captures all types of lymphoma other than Hodgkin's lymphoma). Documentation is insufficient to associate exposures with specific NHL diseases.

2. Relevance of study: **Not relevant** (no reports of identifying various types of lymphoma under the NHL umbrella; no definite association between specific NHL diseases and glyphosate can be made)

3. Klimisch code: **3**

Response – GTF

- The authors pooled data from three case-control studies conducted in Iowa and Minnesota, Nebraska, and Kansas
- The data available in this study did not permit analyses of duration or frequency of use.
- No consideration of types of NHL of varying pathogeneses was presented.
- The reported logistic regression analysis noted, a statistically significant odds ratio for ever use of glyphosate and NHL (OR = 2.1; 95% CI: 1.1–4.0).
- The reported hierarchical regression did not find a statistically significant odds ratio for ever use of glyphosate and NHL (OR = 2.1; 95% CI: 1.1–4.0) (OR = 1.6; 95% CI: 0.9–2.8).
- Authors introduce the phraseology "a possible increase" in NHL incidence establishing their criteria for this category as OR >1.3 and lower confidence limit >0.8.

Author(s)	Year	Study title
De Roos, A.J. Blair, A. Rusiecki, J.A. Hoppin, J.A. Svec, M. Dosemeci, M. Sandler, D.P. Alavanja, M.C.	2005	Cancer Incidence among Glyphosate-Exposed Pesticide Applicators in the Agricultural Health Study Environmental Health Perspectives Volume: 113 Number: 1 Pages: 49-54

Abstract*

Glyphosate is a broad-spectrum herbicide that is one of the most frequently applied pesticides in the world. Although there has been little consistent evidence of genotoxicity or carcinogenicity from *in vitro* and animal studies, a few epidemiologic reports have indicated potential health effects of glyphosate. We evaluated associations between glyphosate exposure and cancer incidence in the Agricultural Health Study (AHS), a prospective cohort study of 57,311 licensed pesticide applicators in Iowa and North Carolina. Detailed information on pesticide use and other factors was obtained from a self-administered questionnaire completed at time of enrolment (1993–1997). Among private and commercial applicators, 75.5% reported having ever used glyphosate, of which >97% were men. In this analysis, glyphosate exposure was defined as *a*) ever personally mixed or applied products containing glyphosate; *b*) cumulative lifetime days of use, or "cumulative exposure days" (years of use × days/year); and *c*) intensity-weighted cumulative exposure days (years of use × days/year × estimated intensity level). Poisson regression was used to estimate exposure–response relations between glyphosate and incidence of all cancers combined and 12 relatively common cancer subtypes. Glyphosate exposure was not associated with cancer incidence overall or with most of the cancer subtypes we studied. There was a suggested association with multiple myeloma incidence that should be followed up as more cases occur in the AHS. Given the widespread use of glyphosate, future analyses of the AHS will allow further examination of long-term health effects, including less common cancers.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various pesticides
Active substance(s):	Glyphosate and 50 others
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	Up to 70 years
Sex:	Males and females

4. Test system:

Study type:	Prospective cohort study
Data collection:	Self-administered enrolment questionnaire
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
No. of persons analyzed:	54315

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	Collected comprehensive-use data on 22 pesticides, ever/never use information for 28 additional pesticides, and general information on pesticide application methods, personal protective equipment, pesticide mixing, and equipment repair. Data were also collected on basic demographic and lifestyle factors. Glyphosate exposure metrics for this analysis: *a*) ever personally mixed or applied products containing glyphosate (ever/never); *b*) cumulative lifetime days of use, or "cumulative exposure days" (years of use × days per year, categorized in tertiles among users: 1–20, 21–56, 57–2,678); and *c*) intensity-weighted cumulative exposure days (years of use × days per year × intensity level, categorized in tertiles: 0.1–79.5, 79.6–337.1, 337.2–18,241).
Parameters determined:	The median time of follow-up for occurring cancers was 6.7 years.
Statistics:	Differences between the exposure groups were tested using the chi-square statistics and associated *p*-values. Poisson regression analyses were carried out for all cancers combined and specific cancer sites to estimate rate ratios (RRs) and 95% confidence intervals (CIs) associated with glyphosate exposure metrics; the effect of each metric was evaluated in a separate model for each cancer. Tertile exposure variables were analyzed in separate models using either the lowest tertile–exposed or never-exposed subjects as the reference category. For each exposure metric, RRs were adjusted for emographic and lifestyle factors, including age at enrolment (continuous), education (dichotomous: ≤ high school graduate or GED/education beyond high school), pack-years of cigarette smoking [indicator variables: never, pack-years at or below the median (12 pack years), pack-years above the median], alcohol consumption in the past year [indicator variables: none, frequency at or below the median (72 drinks), frequency above

the median], family history of cancer in first-degree relatives (dichotomous: yes/no), and state of residence (dichotomous: Iowa/North Carolina).

Potential confounding from exposure to other pesticides was explored by adjusting for the five pesticides for which cumulative exposure-day variables were most highly associated with glyphosate cumulative exposure days [(2,4-dichlorophenoxy)acetic acid (2,4-D), alachlor, atrazine, metolachlor, trifluralin].

Tests for trend across tertiles were conducted by creating a continuous variable with assigned values equal to the median value of cumulative exposure days (or intensity weighted exposure days) within each tertile; the *p*-value for the trend test was that from the Poisson model coefficient for this continuous variable. **P**-values < 0.10 were considered as indicative of a trend.

Additional analyses were conducted for cancers for which we observed elevated RRs, and for NHL (non-Hodgkin lymphoma) because of its association with glyphosate in previous studies. These included analyses stratified by state and analyses across quartiles and quintiles (where numbers allowed) of exposure day's metrics.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable without restrictions**

Comment: Well documented publication. Study included glyphosate exposure, as well as demographic and lifestyle factors. However, adjusted relative risk calculations eliminated a significant proportion of the data set without justification.

2. Relevance of study: **Relevant** (Evaluation focussed on glyphosate, although other pesticides were also considered in the data evaluation)

3. Klimisch code: **2**

Response 1 – summary from Letter to the Editor by Donna Farmer, PhD (Monsanto), Timothy Lash, PhD (Boston University) and John Acquavella PhD (Monsanto)

- Authors provided an incomplete genotoxicity review which was inconsistent with opinions of regulatory agencies and experts around the world, that glyphosate is not genotoxic. An extensive toxicology review of glyphosate was cited by the authors, mentioning a lack of carcinogenicity with glyphosate exposures, yet neglected to cite the extensive genotoxicity review in the same publication by Williams et al. (2000)
- Biological plausibility of a cancer effect should be considered in the light of exposure. Acquavella et al (2004) reported the maximum systemic dose to resulting from application of glyphosate to areas as large as 400 acres was 0.004 mg/kg, and the geometric mean systemic dose was 0.0001 mg/kg in farmers. If these glyphosate applications and exposures continued daily over the course of a lifetime, the systemic dose would be at least 250,000-fold lower than the cancer no-effect level in rodents.

- The authors were requested to further evaluate their models for confounding and selection bias in the multiple myeloma analysis.

  Note: Farmer et al. (2005) is referenced in Doc L Table 3 and included in Doc K.

Response 2 – summary from Lash (2007)

- Table 2 of De Roos et al. (2005) noted 32 cases of multiple myeloma associated with "ever-use" of glyphosate and when compared with "never-use" (adjusted for age only) yielded a rate ratio of 1.1 (95% CI 0.5-2.4). However, when the data set was adjusted for age, demographic and lifestyle factors and other pesticide use, the rate ratio increased to 2.6 (95% CI 0.7-9.4).
- The adjusted estimate merits careful inspection and can only be undertaken with access to the primary data, not made available by the authors.
- Bias analysis was conducted, accounting for confounding and exposure misclassification.
- Adjustment for confounders in De Roos et al. (2005), which resulted in limiting the data set by 25% because of missing data on the adjustment variables, likely introduced selection bias and produced the a rate ratio of 2.6 that was substantially biased.

  Note: Lash (2007) was captured in the literature search, is referenced in Doc L Table 2 and included in Doc K.

Author(s)	Year	Study title
Eriksson, M. Hardell, L. Carlberg, M. Akerman, M.	2008	Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis International Journal of Cancer Volume: 123 Pages: 1657-1663

Abstract*

We report a population based case-control study of exposure to pesticides as risk factor for non-Hodgkin lymphoma (NHL). Male and female subjects aged 18-74 years living in Sweden were included during December 1, 1999, to April 30, 2002. Controls were selected from the national population registry. Exposure to different agents was assessed by questionnaire. In total 910 (91%) cases and 1016 (92%) controls participated. Exposure to herbicides gave odds ratio (OR) 1.72, 95% confidence interval (CI) 1.18-2.51. Regarding phenoxyacetic acids highest risk was calculated for MCPA, OR 2.81, 95% CI 1.27-6.22, all these cases had a latency period >10 years. Exposure to glyphosate gave OR 2.02, 95% CI 1.10-3.71 and with >10 years latency period OR 2.26, 95% CI 1.16-4.40. Insecticides overall gave OR 1.28, 95% CI 0.96-1.72 and impregnating agents OR 1.57, 95% CI 1.07-2.30. Results are also presented for different entities of NHL. In conclusion our study confirmed an association between exposure to phenoxyacetic acids and NHL and the association with glyphosate was considerably strengthened.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, insecticides, fungicides, rodenticides, and impregnating agents
Active substance(s):	Glyphosate and others
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

(in the following data only presented for exposures to glyphosate and total number of subjects)

Species:	Human
Age of test persons:	18-74
Sex:	Males and females

4. Test system:

Study type:	Epidemological study for pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Collection of data:	Questionnaire

No. of exposed persons with NHL:	910
No. of control persons:	1016
No. of persons with Non-Hodgkin lymphoma (NHL) exposed to glyphosate:	29
No. of persons in control group:	18
Pesticide use frequency:	Glyphosate exposed / control group ≤ 10 days: 1/9 persons ≥ 10 days: 17/9 persons
Application rates:	Not reported

5. Observations/analyses:

Working history:	All subjects
Other:	Smoking habits, medications, leisure time activities, proximity from home to certain industrial installations (these factors were not reported)
Detailed assessment of exposure:	Questionnaire included a total work history with in depth questions regarding exposure to pesticides, organic solvents and several other chemicals. For all pesticides not only numbers of years and numbers of days per year, but also approximate length of exposure per day were questioned. Since most work with pesticides was performed in an individualized manner, no job-exposure matrix was judged to be applicable.
Parameters determined:	Regarding phenoxy herbicides and glyphosate an analysis was made taken the latency period for exposure into account
Statistics:	Unconditional logistic regression analysis (Stata/SE 8.2 for Windows) was used to calculate odds ratios (OR) and 95% confidence intervals (CI). Adjustment was made for age, sex and year of diagnosis (cases) or enrolment (controls). In the uni-variate analysis, different pesticides were analyzed separately and the unexposed category consisted of subjects that were unexposed to all included pesticides. When analyzing subgroups of NHL all controls were used in the separate analyses. In the dose-response calculations made for agents with at least 20 exposed subjects, median number of days of exposure among controls was used as cut-off. Latency period calculations and multi-variate analyses included agents with statistically significant increased OR, or with an OR > 1.50 and at least 10 exposed subjects

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Multiple avenues for bias were introduced in study design, execution and data processing. No information about exposure duration, used glyphosate products and application rates. Other factors (i.e. smoking habits, medication etc.) were assessed but not included in the evaluation.

2. Relevance of study: **Relevant** with reservation

3. Klimisch code: 3

Response –Review by Professor Pamela Mink, PhD, Rollins School of Public Health, Emory University, Atlanta Georgia, USA

Study Overview and Main Findings

The authors (Eriksson et al. 2008) conducted a population-based case-control study of exposure to a variety of pesticides and non-Hodgkin lymphoma (NHL), including separate analyses of histopathological categories of NHL. Study subjects were males and females, ages 18-74, living in Sweden between December 1, 1999 and April 30, 2002. The final study group included 910 cases and 1016 controls. Exposure, ascertained via an interviewer-administered questionnaire, focused on pesticide and other chemical agents, and included a total work history (although a job-exposure matrix was not used). For pesticide exposure, information on number of years, number of days per year, and approximate length of exposure per day was also obtained. A minimum of one full day of exposure was required for categorization as "exposed."

The authors reported a statistically significant positive association between "herbicide exposure" and NHL (OR = 1.72; 95% CI: 1.18-2.51). Glyphosate exposure was reported by 29 cases and 18 controls, and the corresponding odds ratio (OR) was 2.02 (95% CI: 1.10-3.71). The ORs for glyphosate exposure of <10 days and >10 days were 1.69 (95% CI: 0.70-4.07) and 2.36 (1.04-5.37), respectively. The ORs for glyphosate were 1.11 (95% CI: 0.24-5.08) and 2.26 (95% CI: 1.16-4.40) for "latency" periods of 1-10 years and >10 years, respectively. In analyses of glyphosate and type of NHL, statistically significant positive associations were observed for small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) (OR = 3.35; 95% CI: 1.42-7.89) and for "unspecified NHL" (OR = 5.63; 95% CI: 1.44-22.0). Odds ratios for the other types (total B-cell lymphomas, grade I-III follicular lymphoma, diffuse large B-cell lymphoma, other specified B-cell lymphoma, unspecified B-cell lymphoma, and T-cell lymphomas) were above 1.0, but were not statistically significant (i.e., the 95% confidence intervals were relatively wide and included the null value of 1.0).

The authors concluded, "Glyphosate was associated with a statistically significant increased OR for lymphoma in our study, and the result was strengthened by a tendency to dose-response effect…" (p. 1662). The authors suggested that their findings are consistent with results of a previous case-control study (Hardell and Eriksson 1999) and pooled analysis (Hardell et al. 2002) that they conducted. In the case-control study, an OR of 2.3 (95% CI: 0.4-13.0), based on 4 exposed cases and 3 exposed controls, was reported for glyphosate and NHL. In the pooled analysis of two case-control studies, which included data from Hardell and Eriksson (1999), an OR of 3.04 (95% CI: 1.08- 8.52) was reported, based on 8 exposed cases and 8 exposed controls. The authors also cited three studies (De Roos et al. 2003; McDuffie et al. 2001; De Roos et al. 2005) by other groups as being consistent with their results in that they "also associate glyphosate with different B-cell malignancies such as lymphomas and myelomas." It should be noted, however, that the relative risk (RR) reported by De Roos et al. (2005) for the highest versus lowest category of cumulative exposure days of glyphosate and NHL in the prospective Agricultural Health Study was 0.9.

Interpretation Issues

Identification of Cases and Potential Referral Bias. It is noteworthy that the cases in the current analysis were identified from some of the same hospitals as the authors' prior publication; thus, referral bias may have been an issue. In particular, the researchers approached the patients after diagnosis if the physicians deemed it appropriate. Therefore, if the physicians were concerned that their patient's NHL was associated with agricultural exposures, they may have suggested participation in the study.

Participation Rates and Potential Selection Bias. The authors report a participation rate of 91% and 92% for cases and controls, respectively; however, these figures are based on completed questionnaires out of those who had previously said they would participate in the study. The number of eligible patients (i.e., prior to physician approval to "approach") was not reported, so the computation of an exact participation

rate is difficult. Based on information provided in the paper, participation among cases is estimated to be about 80%. Nonparticipation is a concern for several reasons. First, in a case-control study, an odds ratio will be an accurate representation of the exposure-disease association when the cases are representative of all cases and the controls are representative of the exposure experience of the population that gave rise to the cases. If the final study sample is not representative of this "target population" then measures of effect (e.g., the odds ratio) may not be valid. In addition, one must be concerned about selection bias. Selection bias occurs in a case-control study when the exposure distribution for cases and controls differ for those who participate in the study compared to those who are eligible but do not participate in the study. It is not possible to determine whether there is selection bias without information about nonparticipants.

Strengths and Limitations of Using Living Cases Only versus All Cases (Living + Dead).
The authors noted that 88 potential cases died before they could be interviewed and were therefore excluded from the study. It is also stated in the Discussion that restricting the study to living cases and controls was an "advantage" of the study, as interviewing cases and controls directly compared to interviewing next-of-kin was preferable. While it is generally true that this would be an advantage, the following statement by the authors, therefore, is not accurate, "The study covered all new cases of NHL during a specified time" (p. 1660). The study did not include all new cases; it included only those cases who survived until the time of the interview. Thus, while there may have been an advantage to restricting the study to living cases, there was a trade-off in that the study population did not represent all cases, specifically those cases with more aggressive disease. This disadvantage was not discussed by the authors, nor was the potential bias that could have resulted from excluding many eligible cases.

Exposure Measurement and Information Bias. Exposure was ascertained via a questionnaire oriented towards pesticide and other chemical agents. In addition, interviewers collected information by telephone if "important" data were lacking, incomplete, or unclear. It is unknown what is meant by "important," and the proportion of cases and controls who received phone calls was not reported. Thus, information bias may be a concern. Even though interviewers were blinded to case and/or control status, they may have been able to determine this information during the course of the interview. Furthermore, recall bias may be an issue because exposure information was based on participant response and cases and controls may recall and/or report past pesticide exposures differently. No exposure validation techniques were implemented, nor did an industrial hygienist (or any other type of personnel trained in assessing occupational exposures) independently validate/estimate the frequency and/or intensity of exposure. The authors assumed that "some misclassification regarding quantity of exposure has probably occurred, but such misclassification would most probably be nondependent of case/control status, and therefore only weaken any true risks" (p. 1660). They do not provide any explanation as to why they believe that exposure misclassification would be "most probably" nondifferential. If NHL cases believe that pesticides may be related to their disease, then it is certainly possible that they may recall and/or report pesticide exposure differently than NHL-free controls, which could result in odds ratios that are inflated as a result of bias.

Interpretation of "dose-response" analyses. The referent group in the statistical analyses consisted of participants who were unexposed to all pesticides. The dose-response analyses were based on a dichotomy of the median number of days exposed to a particular agent. It is difficult to analyze "dose-response" when only two exposure categories are considered. Furthermore, the dose-response analyses were based on median values of exposure but heterogeneity of cut-points is evident across agents. For example, glyphosate was analyzed as < 10 days and > 10 days, whereas, "other" herbicides were analyzed as < 32 days and > 32 days. Although analytical cut-points were data driven, interpretation across the wide variety of exposures is complicated by the variability in exposure cut-points. In addition, even though the OR for the higher category of exposure days was greater than the OR for the lower category, the two 95% confidence intervals were wide and overlapped considerably (0.70-4.07 and 1.04-5.37).
Thus, it is not clear whether the two point estimates reported (1.69 and 2.36) are significantly different from each other. Finally, this result cited in the "dose-response" analyses may have been confounded by exposure to other herbicides. In Table II (Eriksson et al. 2008), the authors observed elevated associations for other herbicides, including MCPA, 2,4,5-T and/or 2,4-D. The correlation between exposure to glyphosate and other herbicides was not provided nor were analyses of glyphosate-exposed individuals

after accounting for the collinear relation between this agent and other agents. The odds ratio for "ever" exposure to glyphosate was attenuated after additional adjustment for other pesticides (Table VII, Eriksson et al. 2008), but multi-variate -adjusted estimates for the "dose-response" odds ratios were not reported.

Unusual Pattern of Positive Associations. The authors conducted multiple comparisons, and one would expect a certain proportion of their findings to be statistically significant (whether in the positive or inverse direction) simply as a result of chance. It is somewhat surprising, therefore, that the vast majority of the ORs presented in this manuscript are greater than 1.0, regardless of the statistical significance. The authors do note that for some of the analyses (e.g., latency), only chemicals for which ORs were greater than 1.5 and for which there were at least 10 exposed cases, or for which there was a statistically significant OR were evaluated. On the other hand, dose-response was evaluated based on the number of exposed subjects and not on the strength or significance of the findings. The authors do not address this directly, but do state in their Discussion, "…several pesticides are chemically related and may exert their effects on humans through a similar mechanism of action, which may explain the wide range of pesticides that have been related to NHL over time in different countries and with different exposure conditions" (p. 1661). On the other hand, this pattern of positive findings could be a result of bias, including recall bias (or other information bias), selection bias, uncontrolled confounding, or a combination of these and other factors.

Interpretation of Eriksson et al. (2008) in Context of Other Studies. Despite the statement by the authors that, "Recent findings from other groups also associate glyphosate with different B-cell malignancies such as lymphomas and myeloma" (p. 1662), most multi-variate analyses of glyphosate and NHL do not report statistically significant associations (De Roos et al. 2005; Cantor et al. 1992; De Roos et al. 2003; Hardell and Eriksson 1999; Hardell et al. 2002; Ives et al. 2004; McDuffie et al. 2001; Nordstrom et al. 1998) (Tables A and B). It is notable that Hardell et al. (2002) reported a significant positive association between glyphosate association and NHL, but the multi-variate -adjusted odds ratio was attenuated and not statistically significant. Similar findings were reported by Eriksson et al. (2008). Specifically, the association reported by the authors in the abstract (OR = 2.02; 95% CI: 1.10-3.71) was adjusted for age, sex and year of diagnosis or enrollment. When other pesticides were added to that model (i.e., agents with statistically significant increased odds ratios, or with an odds ratio greater than 1.5 and with at least 10 exposed subjects), the adjusted odds ratio was 1.51 (95% CI: 0.77-2.94). Thus, the authors' final statement, "Furthermore, our earlier indication of an association between glyphosate and NHL has been considerably strengthened" is questionable. Their previous findings showed a non-significant association after multi-variate adjustment (OR = 1.85; 95% CI: 0.55-6.20). The 2008 study similarly reported a statistically non-significant association between glyphosate and NHL after multi-variate adjustment (OR = 1.51; 95% CI: 0.77-2.94). The results reported for analyses of duration of exposure and latency of exposure did not adjust for other pesticides, and one would expect that those ORs would also be attenuated.

Summary of Findings: Cohort and Case-Control Studies of Exposure to Glyphosate and Non-Hodgkin Lymphoma

Table A. Cohort Studies

Author Year	Description	No. of Exposed Cases	Type of Relative Risk Estimate	Relative Risk Estimate	95% Confidence Limits	Variables Included in Statistical Model
De Roos et al. 2005	57-2,678 vs. 1-20 Cumulative Exposure Days[a]	17	RR	0.9	0.5-1.6	Age at enrollment, education, pack-years of cigarette smoking, alcohol consumption in the past year, family history of cancer in first-degree relatives, and state of residence Also adjusted for other pesticides
	337.2-18,241 vs. 0.1-79.5 Intensity-Weighted Exposure Days[b]	22	RR	0.8	0.5-1.4	

[a] Years of use x days per year; categorized by tertiles
[b] Years of use x days/year x estimated intensity level; categorized by tertiles

Table B. Case Control Studies

Author Year	Exposure Evaluated	Subgroup Description	No. of Exposed Cases	No. of Exposed Controls	OR	95% CI	Variables Included in Statistical Model
Cantor et al. 1992	Agricultural exposure based on ever living or working on a farm	Nonfarmer	266	547	1.0	Referent	Vital status, state, age, smoking, family history of lymphopoietic cancer, high-risk occupations, and high-risk exposures
		Farmer	356	698	1.2	1.0-1.5	
	Farmers with specific pesticide exposures (ever mixing, handling, or applying) compared to nonfarmers	Glyphosate	26	49	1.1	0.7-1.9	
De Roos et al. 2003	Ever exposure to specific pesticide; men only (all 47 pesticides were regressed simultaneously)	Glyphosate (Logistic Regression)	36	61	2.0	1.1-4.0	Age, study site and other pesticides
		Glyphosate (Hierarchical Regression)	36	61	1.6	0.9-2.8	Second-level model incorporated what was known about each true effect parameter prior to seeing the study data
Hardell and Eriksson 1999	Exposure to specific pesticides (ever/never exposed to the specific pesticide vs. no exposure to any pesticide)	Glyphosate (conditional logistic regression; uni-variate analysis)	4	3	2.9	0.4-13	Age and country (matching factors)
		Glyphosate (conditional logistic regression; multi-variate analysis)	4	3	5.8	0.6-54	Multi-variate variables not listed by authors
Hardell et al. 2002	Exposure to specific pesticides (ever/never exposed to the specific pesticide vs. no exposure to any pesticide)	Glyphosate (conditional logistic regression; uni-variate analysis)	8	8	3.04	1.08-8.52	Age and county (matching factors); study, study area (county), and vital status
		Glyphosate (conditional logistic regression; multi-variate analysis)	8	8	1.85	0.55-6.20	Multi-variate variables not listed by authors
Lee et al. 2004a	Exposure to individual pesticides	Glyphosate use, Non-asthmatics	53	91	1.4	0.98-2.1	Age, state, vital status
		Glyphosate use, Asthmatics	6	12	1.2	0.4-3.3	

McDuffie et al. 2001	Exposure to individual active chemicals	Glyphosate (Round-Up)	51	133	1.26	0.87-1.80	Strata for age and province of residence
		Glyphosate (Round-Up)	NR	NR	1.20	0.83-1.74	Plus statistically significant medical variables
Nordstrom et al. 1998	Exposure to specific herbicides, insecticides, and fungicides	Glyphosate	4	5	3.1	0.8-12	Age and country (matching factors)
Eriksson et al. 2008	Exposure to specific herbicides regardless if they also had been exposed to phenoxyacetic acids or not	Glyphosate	29	18	2.02	1.10-3.71	Age, sex, and year of diagnosis or enrollment
			29	18	1.51	0.77-2.94	Age, sex, and year of diagnosis or enrollment and pesticides with statistically significant increased odds ratios, or with an odds ratio greater than 1.5 and with at least 10 exposed subject
	Exposure to herbicide stratified by median number of days among exposed controls	Glyphosate ≤ 10 days	12	9	1.60	0.70-4.07	Age, sex, and year of diagnosis or enrollment
		Glyphosate >10 days	19	9	2.36	1.04-5.37	
	Exposure to specific herbicides according to different lymphoma entities	Glyphosate: B-Cell lymphomas	NR	NR	1.87	0.998-3.51	Age, sex, and year of diagnosis or enrollment
		Lymphocytic lymphoma/B-CLL	NR	NR	3.35	1.42-7.89	
		Follicular grade I-III	NR	NR	1.89	0.62-5.79	
		Diffuse large B-cell Lymphoma	NR	NR	1.22	0.44-3.35	
		Other specified B-cell lymphoma	NR	NR	1.63	0.53-4.96	
		Unspecified B-cell Lymphoma	NR	NR	1.47	0.33-6.61	
		T-cell lymphomas	NR	NR	2.29	0.51-10.4	
		Unspecified NHL	NR	NR	5.63	1.44-22.0	

Author(s)	Year	Study title
George, J. Prasad, S. Mahmood, Z. Shukla, Y.	2010	Studies on glyphosate-induced carcinogenicity in mouse skin: A proteomic approach Journal of Proteomics Volume: 73 Pages: 951-964

Abstract*

Glyphosate is a widely used broad spectrum herbicide, reported to induce various toxic effects in non-target species, but its carcinogenic potential is still unknown. Here we showed the carcinogenic effects of glyphosate using 2-stage mouse skin carcinogenesis model and proteomic analysis. Carcinogenicity study revealed that glyphosate has tumor promoting activity. Proteomic analysis using 2-dimensional gel electrophoresis and mass spectrometry showed that 22 spots were differentially expressed (>2 fold) on glyphosate, 7, 12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) application over untreated control. Among them, 9 proteins (translation elongation factor eEF-1 alpha chain, carbonic anhydrase III, annexin II, calcyclin, Fab fragment anti-VEGF antibody, peroxiredoxin-2, superoxide dismutase [Cu–Zn], stefin A3, and calgranulin-B) were common and showed similar expression pattern in glyphosate and TPA-treated mouse skin. These proteins are known to be involved in several key processes like apoptosis and growth-inhibition, anti-oxidant responses, etc. The up-regulation of calcyclin, calgranulin-B and down-regulation of superoxide dismutase [Cu–Zn] was further confirmed by immunoblotting, indicating that these proteins can be good candidate biomarkers for skin carcinogenesis induced by glyphosate. Altogether, these results suggested that glyphosate has tumor promoting potential in skin carcinogenesis and its mechanism seems to be similar to TPA.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test Item:	Roundup Original ®
Active substance(s):	Glyphosate
Source:	Monsanto Company St. Louis, USA (obtained from a local market)
Lot/Batch #:	Not reported
Purity:	360 g/L glyphosate salt equivalent as the isopropylamine salt
Co-formulants:	The formulation contained 15% POEA (polyethoxylated tallow amine)

2. Vehicle and positive controls:

50% ethanol
12-o-tetradecanoylphorbol-13-acetate (TPA);
7, 12-dimethylbenz[a]anthracene (DMBA).

3. Test animals:

Species:	Mice
Strain:	Swiss albino
Source:	Indian Institute of Toxicology Research (IITR)
Age of test animals at study initiation:	Not reported
Sex:	Male
Body weight:	12-15 g
Acclimation period:	1 week

Diet/Food:	Synthetic pellet basal diet (Ashirwad, Chandigarh, India), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 2°C
	Humidity: 55 ± 5%
	Air changes: Not reported
	Light/dark cycle Not reported

4. Test system:

Study type:	**Proteomic study in mouse skin**
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	32 weeks
Dose groups:	**Group I** – Untreated control (No treatment). **Group II** – Glyphosate alone (25 mg/kg bw, topically 3 times per week). **Group III** – DMBA+TPA (Single topical application of DMBA, 52 µg/mouse followed 1 week later by thrice a week application of TPA, 5 µg/mouse). **Group IV** – Glyphosate (s)+TPA (Single topical application of glyphosate, 25 mg/kg bw followed 1 week later by TPA application as in group III). **Group V** – Glyphosate (m)+TPA (Thrice a week topical application of glyphosate, 25 mg/kg bw for 3 weeks [total of 9 applications], followed 1 weeklater byTPA application as in group III). **Group VI** – DMBA (Single topical application of DMBA, 52 µg/ mouse). **Group VII** – TPA (Thrice a week topical application of TPA, 5 µg/mouse). **Group VIII** – DMBA+glyphosate (Single topical application of DMBA [as in group III], followed 1 week later by topical treatment of glyphosate, 25mg/kg bw thrice per week).
Animals per dose group:	8 groups of 20 animals each

Study type:	**Proteomic study**
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Dose groups:	**Group I** – Untreated controls (No treatment). **Group II** – Glyphosate (Single topical application, 50 mg/kg bw/mouse). **Group III** – DMBA (Single topical application of DMBA, 104 µg/mouse). **Group IV** – TPA (Single topical application of TPA, 10 µg/mouse).
Animals per dose group:	4 groups of 4 animals each

Sampling and sample preparation:	24 h after application animals were sacrificed and skin tissues from the treatment site were excised. Hair and subcutaneous fat was removed, and small pieces of cleaned skin tissues of each mouse from all the groups were then homogenised (10 % w/v) individually, in 2-DE lysis buffer. The lysed samples were soniocated, centrifuged and pooled for the respective group. After quantification of proteins by Lowry´s method, the supernants were stored at -80°C until use fro electrophoresis.

5. Observations/analyses:

Carcinogenicity study in mouse skin

Body weight:	Measured weekly
Development:	Examined weekly
Gross morphological changes:	Volume of squamous cell papillomas (tumors) locally on the skin was examined during the entire study period. Tumors larger than 1mm diameter were included in the total number of tumors.
Mortality:	Not reported
Clinical signs:	Not reported
Food- and water consumptions:	Not reported
Test substance intake:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported
Sacrifice/pathology:	Not reported
Organ weights:	Not reported
Histopathology:	Not reported

Proteomic study

Protein quantification:	Quantification of proteins in the supernants prepared for 2-DE by Lowry's method.
Protein expression profile:	2-D electrophoresis (2-DE) IEF was carried out using commercially dedicated equipment, Protean IEF. IEF was performed for each individual sample to a total of 45.5 kVh. All IEF steps were carried out at 20 °C. After the first-dimensional IEF, focused IPG strips were placed in an equilibration solution. Separation in the second dimension was carried out using Protean II xi electrophoresis equipment. Each experiment was performed in triplicate to obtain the reproducible results. After completion of the second-dimension electrophoresis, the gels were fixed and stained by using a fast silver staining protocol with neutral silver nitrate. Analysis of the 2D-gels including background subtraction, spot detection, volume normalization and differences in protein

	expression levels among samples were analyzed by using PDQuest software Ver. 7.4.0. To determine the variation, 3 gels were prepared for each sample. The protein spots that varied >2 fold change and were specific for the test groups and the control group were manually labeled and considered for MS analysis.
Protein identification	Matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF/TOF) and liquid chromatography mass spectrometry (LC-MS) Differential protein spots of interest were excised manually and washed with deionised water. After in-gel digestion, trypsinised samples were dissolved and mixed with matrix (α-cyano-4-hydroxy cinnamic acid). Following drying the peptides were spotted on ground steel plate and subjected to Bruker Ultraflex MALDI-TOF/TOF and 2100 Nano LC-ESI-Trap (Agilent) for mass spectrometric identification. Data acquisition and analysis was performed using flex control and flex analysis/biotools version 2.2 software, respectively. Data was acquired in reflectron positive mode using 15–18% laser power. Mass tolerance and monoisotopic values (50 ppm/100 ppm for peptide mass fingerprint and peptide mass tolerance of 2 Da for MS/MS spectra) were used for searching. The datasets of the MS spectra, including peptide sequence information, were searched against the SWISS-PROT and NCBInr database using Mascot Daemon as a client attached to the Mascot search protocol.
Verification of calcyclin, calgranulin-B and SOD 1	The differential proteins screened with 2-DE were confirmed by Western blot analysis. Skin tissue samples were lysed in lysis buffer, resolved on a 12-15% polyacrylamid gel, and electro-transferred onto polyvinyliden fluoride membranes. After blocking, the membranes were immunoblotted with antibodies of calcyclin, calgranulin-B and superoxide dismutase (SOD 1) and beta-actin at dilutions recommended by the suppliers. Horseradish conjugated secondary antibodies and chemiluminescence kit, were used for detection. Protein expression was visualized by Versa Doc Imaging System. The intensity was given in terms of relative pixel density for each band normalized to band of beta-actin. The intensity of the bands was measured using software UNSCAN-IT automated digital system version.
Statistics:	The skin tumour incidence was analyzed by one-way analysis of variance (ANOVA) test in untreated control and treated groups, $p<0.05$ value was considered as significant. Protein expression data for untreated control and treated groups are expressed as the mean±SD of 3 replicate gels for fold changes of normalized spot volumes. For the statistical analysis of data, Student-t-test was used and $p<0.05$ was considered as significant. Hierarchical clustering analysis using Ward's minimum variance was performed by NCSS software.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Non-guideline mechanistic study. Scientifically acceptable study with deficiencies (controls with glyphosate alone, and co-formulants were not included)

2. Relevance of study: **Relevant with restrictions** (Glyphosate formulation not glyphosate alone was tested.)

3. Klimisch code: **2**

Response – GTF

It is important to note that the authors use glyphosate as a synonym for what is really a glyphosate based formulated product. Doses in this study are not representative of human exposures to glyphosate or glyphosate based formulations. Mice in the tumor promoting group VIII received topical applications of concentrated glyphosate formulated product three times per week for over thirty weeks without washing after an initial treatment with the potent tumor initiator DMBA. Glyphosate had been shown to have very low dermal absorption, even in formulated products, and since is non-volatile, would likely accumulate on mouse skin. Surfactants are typically irritating and non-volatile. Given the irritation potential of the unwashed exposed mouse skin over the course of thirty or more weeks, tumor promotion may be a physical response to substantial localized dermal irritation. Epidemiological studies reported above note no association with glyphosate and either skin or lip cancers.

Label directions outline appropriate personal protective equipment such as gloves and long sleeves. Furthermore, any dermal exposure of concentrated product to human skin would prove irritating and prompt handlers to wash off soon after dermal exposure.

Human *in vitro* dermal absorption studies reported in Section IIA 5.9.9 for a range of glyphosate based formulations containing different surfactant systems all demonstrate extremely low dermal absorption of glyphosate active ingredient for concentrated products, of less than 0.2%. Test material recovery in each of the four reported dermal absorption studies was very good, close to 100%. Most of the glyphosate was removed during skin surface washing at either eight or twenty four hours of in vitro human skin exposure. This also suggests significant potential for accumulation of glyphosate on the surface of the mice skin in George et al. (2010).

Proteomics is an emerging science, not yet yielding validated test methods for establishing human health endpoints. The up-regulation / down-regulation of protein expression reported after a single dermal dose of a glyphosate formulated product (proteomics experiment, group II), while interesting, does not demonstrate any toxicological endpoint. Rather, perturbations may well represent normal homeostatic fluctuations and be a natural response to insult. Further research and validation in this field will be necessary before such studies may prove useful in human health risk assessment.

4. Literature Review of Genotoxicity Publications

The following genotoxicity literature review was conducted by an expert in the field of genotoxicology. Relevant OECD Tier II-like summaries and Klimisch ratings (Klimisch, 1997), as described in introduction of the overall literature review, follow this genotoxicity literature review.

Review of Genotoxicity of Glyphosate and Glyphosate Based Formulations,
Larry D. Kier, PhD, Genotoxicology Consultant, Buena Vista, CO

Abbreviations AMPA, aminomethylphosphonic acid ; CB MN, cytokinesis block micronucleus; GBF, glyphosate based formulation; i.p., intraperitoneal ; NCE, normochromatic erythrocyte; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocyte; POEA, polyethoxylated tallow amine, tallowamine ethoxylate; SCE, sister chromatid exchange; SCGE, single cell gel electrophoresis (comet).

Abstract
An earlier review of the toxicity of glyphosate and the original Roundup™ formulation concluded that neither glyphosate nor the formulation pose a risk for the production of heritable/somatic mutations in humans (Williams et al., 2000). This review of subsequent glyphosate genotoxicity publications includes analysis of study methodology and incorporation of all the findings into a weight of evidence for genotoxicity. Two publications provided limited additional support for the conclusion that glyphosate and glyphosate based formulations (GBFs) are not active in the gene mutation assay category. The weight of evidence from *in vitro* and *in vivo* mammalian chromosome effects studies supports the earlier conclusion that glyphosate and GBFs are predominantly negative for this endpoint category. Exceptions are mostly for unusual test systems but there are also some unexplained discordant positive results in mammalian systems. Several reports of positive results for the SCE and comet DNA damage endpoints have been published for glyphosate and GBFs. The data suggest that these DNA damage effects are likely due to cytotoxic effects rather than DNA reactivity. This weight of evidence review concludes that there is no significant *in vivo* genotoxicity and mutagenicity potential of glyphosate or GBFs that would be expected under normal exposure scenarios.

1. Introduction
Glyphosate is the active ingredient in very widely used herbicide formulations in crop production, industrial turf, ornamental plants, forestry, roadsides, home lawns and gardens. Accordingly, the toxicity of glyphosate and its formulated products has been extensively studied. An earlier thorough review of glyphosate and glyphosate formulation safety and risk assessment included descriptions and analyses of genetic toxicology studies of glyphosate, the original Roundup™ formulation and other glyphosate based formulations (GBFs) (Williams et al., 2000). Subsequently, a fairly large number of genetic toxicology studies of glyphosate and GBFs have been published. These studies include a wide variety of test systems and endpoints. The number and diversity of the studies warrant careful examination and integration of their findings with the previous results to produce an updated assessment of the overall genotoxicity profile of glyphosate and GBFs.

2. General Review and Analysis Considerations
The published studies for review consideration were identified by literature searches for published reports containing references to glyphosate or GBFs that also contained searchable terms which indicated that genotoxicity studies were performed. Literature search utilized Chemical Abstracts (provided by Chemical Abstracts Service, a division of the American Chemical Society) and Web of Knowledge (Thompson Reuters), using the following modules: Web of Science℠, BIOSIS Previews®, MEDLINE®, and CAB Abstracts® (CABI) abstracting services. Search criteria were as follows (glyphosate acid and the various salts): glyphosat* OR glifosat* OR glyfosat* OR 1071-83-6 OR 38641-94-0 OR 70901-12-1 OR 39600-42-5 OR 69200-57-3 OR 34494-04-7 OR 114370-14-8 OR 40465-66-5 OR 69254-40-6 OR (aminomethyl w phosphonic*) OR 1066-51-9. Each identified publication was evaluated to verify that it contained original results of one or more genotoxicity studies on glyphosate or GBFs. Emphasis was placed on publications in peer-reviewed journals and abstracts or other sources with incomplete

information were not considered. Reviews without original data were not considered for evaluation; however, these reviews were examined to determine if there were any cited publications that had not been detected in the literature searches.

Each relevant publication was examined using several criteria to characterize the scientific quality of the reported genetic toxicology studies. Useful, objective criteria for this purpose were international guidelines for genetic toxicology studies developed by expert groups. These include principles for conducting studies, reporting results and analyzing and interpreting data. Some of the principles of the guidelines are generally applicable to categories of studies or all studies while others are specific for a particular type of test system and endpoint. Some of the specific types of studies encountered in the review do not yet have international guidelines; however, some of the guideline elements should be generically applicable to these studies. The guidelines for genetic toxicology tests developed for the Organization for Economic Cooperation and Development (OECD) are a pre-eminent source of internationally agreed and expert guidelines. Other regulatory international and national regulatory genetic toxicology testing guidance are usually concordant with the OECD guidelines. Table 1 presents some key OECD guideline criteria that were found to be relevant to analysis of the studies considered in this review.

Comparison of the published studies to the criteria in guidelines used for regulatory purposes does not represent an absolute judgment standard but it does serve to provide one means of characterization of the various published studies. Some of the criteria are rarely met in scientific publications. For example, data for individual cultures and individual animals are not commonly included in publications in scientific journals. These data are presumably collected but are usually summarized as means with a measure of variance for the treatment and control groups. This is not considered to be a significant omission in a scientific publication. However, other guideline features are more essential in demonstrating scientific quality standards and should be considered as having greater weight in evaluating a study. For example, there are consistent recommendations that assays involving visual scoring (e.g. chromosome aberration, micronucleus and sister chromatid exchange) should use slides that are independently coded so that scoring is performed without knowledge of the treatment or control group being scored. This guidance is good scientific practice and studies that do not include a description of coding or "blind" scoring in the methodology would appear to have a deficiency either in the methodology or the description of the methodology used. Other examples of guideline features that have clear experimental scientific value are the use of concurrent negative and positive controls and concurrent measurement and reporting of toxicity endpoints in main experiments, especially in *in vitro* mammalian cell assays.

Test materials, as described in the publications, were reviewed by industry experts to identify any publicly available and useful information on composition for the reported formulations to assist in interpreting the relevance of findings to glyphosate and/or formulation components. It should be noted that a common problem encountered in the published literature is the use of the terms "glyphosate", "glyphosate salt" or "Roundup" to indicate what may be any GBF that contains additional components such as surfactants. Published results from studies with different formulations have sometimes been incorrectly or inappropriately attributed to the active ingredient. The original Roundup formulation (containing 41% isopropyl amine glyphosate salt and 15.4% MON 0818 (a polyethoxylated tallowamine based surfactant blend), is no longer sold in many markets. However, other glyphosate based formulations are sold under the Roundup brand name with varying glyphosate forms, concentrations and surfactant systems. Clear identification of the test material is very important in toxicology studies because toxicity of formulations can be dramatically different than the active ingredient. The fact that test materials identified as Roundup formulations may actually have different compositions should be considered when comparing results of different studies. A major consideration, especially for DNA damage endpoints and for *in vitro* mammalian cell assays, is an assessment of whether observed effects might be due to toxicity or extreme culture conditions rather than indicating DNA-reactive mediated processes (Dearfield et al., 2011; Muller and Kasper, 2000; Scott et al., 1991; Thybaud et al., 2007b; Thybaud et al., 2007a). Relevant considerations include control of medium pH and osmolality for *in vitro* mammalian cell studies and whether effects are observed only at cytotoxic doses or in association with severe toxicity to the test system. Other important generic considerations in evaluating experimental results of each published study are evidence of experimental reproducibility and whether a biologically plausible dose response has been demonstrated.

Table 1. Genetic Toxicology Test Guideline Criteria

Area	Guidance	Reference
All studies	Test material purity and stability should be reported	OECD 471 (1997) OECD 473 (1997)
	Concurrent negative and positive controls should be included with each assay	
Assays with visual scoring	All slides should be independently coded before analysis (i.e. scored without knowledge of the treatment or control group)	OECD 473 (1997) OECD 479 (1986)
In vitro mammalian cell assays	Assay should be usually be conducted in the presence and absence of an appropriate exogenous metabolic activation system	OECD 473 (1997)
	Cytotoxicity should be determined in the main experiment	
	At least three analyzable concentrations should be used	
	Maximum dose determined by toxicity or 5 µg/ml, 5 mg/ml or 10 mM for soluble non-toxic test materials	
	Individual culture data should be provided	
In vivo mammalian assays	Five analyzable animals per group. Single sex may be used if there are no substantial difference in toxicity between sexes	OECD 475 (1997) OECD 474 (1997)
	Limit dose for non-toxic substances of 2000 mg/kg for treatments up to 14 days and 1000 mg/kg for treatments longer than 14 days	
In vitro chromosome aberration	Treatment for 3-6 hours in one experiment and harvest at 1.5 cell cycles. If negative a second experiment with continuous treatment for 1.5 cell cycles	OECD 473 (1997)
	Scoring of at least 200 metaphases ideally divided between duplicate cultures	
In vitro sister chromatid exchange	Treatment for 1-2 hours up to two cell cycles with harvest after two cell cycles in the presence of bromodeoxyuridine	OECD 479 (1986)
	Scoring of 25 metaphases per culture (50 per treatment group)	
In vitro micronucleus	Most active agents detected by treatment for 3-6 hours with harvest at 1.5-2 cell cycles after treatment. An extended treatment for 1.5-2 cycles in the absence of metabolic activation is also used	OECD 487 (2010)
	Scoring of at least 2000 binucleated cells or cells for micronuclei for each treatment or control group	
In vivo bone marrow chromosome aberration	Single treatment with first harvest at 1.5 cell cycles after treatment and second harvest 24 hour later or single harvest 1.5 cycles after last treatment for multiple daily treatments	OECD 475 (1997)
	Three dose levels usually recommended except when limit dose produces no toxicity	
	Concurrent measures of animal toxicity and toxicity to target cells	
	At least 100 cells analyzed per animal	
	Individual animal data should be reported	
In vivo erythrocyte micronucleus	Three dose levels for first sampling time	OECD 474 (1997)
	Treatment once with at least 2 harvests usually at 24 and 48 h after treatment or one harvest 18-24 h after final treatment if two or more daily treatments are used	
	Scoring of 2000 immature erythrocytes per animal or 2000 mature erythrocytes for treatments of 4 weeks or longer	

Table 2 presents a summary of genotoxicity test results for glyphosate and GBFs published subsequent to Williams et al. (2000). Test results are organized by the major genotoxicity assay categories of gene mutation, chromosome effects and DNA damage and other endpoints. Major features presented for each publication are the assay endpoint, the test system, the test material, the maximum dose tested and comments relevant to the reported conduct and results of the assay. For brevity, earlier reviewed individual publications of genotoxicity study results are referred to by citation of (Williams et al., 2000) rather than the original references reviewed in (Williams et al., 2000).

Table 2. Genetic Toxicology Studies of Glyphosate and Glyphosate Formulations Published On or After 2000

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro Gene Mutation						
Point mutation	Ames strains	Perzocyd 10 SL formulation	2 µg/plate (toxic)	Negative	TA1535 not used	Chruscielska et al., 2000
Wing spot test	Drosophila	glyphosate (96%)	10 mM in larval stage	Negative/ inconclusive[c]	Negative or inconclusive in crosses not sensitive to recombination events	Kaya et al., 2000
In Vitro Chromosome Effects—Mammalian Systems						
Cytokinesis block micronucleus	Bovine lymphocytes	Glyphosate formulation (62% glyphosate Monsanto source)	560 µM 48 h –S9	Positive?	PH, MA, SC, TO	Piesova, 2004
Cytokinesis block micronucleus	Bovine lymphocytes	Glyphosate formulation (62% glyphosate Monsanto source)	560 µM 48 h –S9 70 µM –S9 2 h +S9	Positive? Negative Negative	PH, SC, TO	Piesova, 2005
Chromosome aberration	Mouse spleen cells	herbazed formulation	50 µM?	Positive	Concentrations used not clear. PH, MA, SC, TO, RE	Amer et al., 2006
Chromosome aberration	Bovine lymphocytes	Glyphosate formulation (62% glyphosate) Monsanto source	1.12 mM (toxic) (24 h)	Negative	Chromosome 1 FISH analysis. PH, MA, PC, SC, TO, RE	Holeckova, 2006
Chromosome aberration	Bovine lymphocytes	Glyphosate formulation (62% glyphosate) Monsanto source	1.12 mM (toxic) (24 h)	Negative	PH, MA, SC, RE	Sivikova and Dianovsky, 2006
Chromosome aberration	Human lymphocytes	Glyphosate (96%)	6 mM (not toxic)	Negative	MA, IC, RE	Manas et al., 2009b
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009a
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009b

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro Chromosome Effects— Non Mammalian Systems						
Chromosome aberration	Onion root tip meristem	Roundup formulation (Bulgaria)	1% active ingredient (estimated 4.4-5.9 mM)	Negative	TO, IC, RE	Dimitrov et al., 2006
Micronucleus	Onion root tip meristem	Roundup formulation (Bulgaria)	1% active ingredient (estimated 4.4-5.9 mM)	Negative	TO, RE	Dimitrov et al., 2006
In Vivo Chromosome Effects—Mammalian Systems						
Bone marrow erythrocyte micronucleus	Mouse	Glyphosate	300 mg/kg i.p. Perzocyd 10 SL formulation	Negative Negative	DL, TO, SC, IM, RE DL, TO, SC, IM, RE	Ghuscielsk o et al., 2000
Bone marrow erythrocyte micronucleus	Mouse	Roundup 69 formulation	2 x 200 mg/kg i.p.	Negative	TO, SC, IE, RE	Coutinho do Nascimento and Grisolia, 2000
Bone marrow erythrocyte micronucleus	Mouse	Roundup™ formulation (Monsanto)	2 x 200 mg/kg i.p.	Negative	TO, SC, IE, RE	Grisolia, 2002
Bone marrow Chromosome aberration	Rabbit	Roundup™ formulation	750 ppm in drinking water	Positive?	DL, PC, TO, SC, IC	Helal and Moussa, 2005
Bone marrow Chromosome aberration	Mouse	Herbazed formulation (84% glyphosate)	50 mg/kg i.p. (1,3, 5 days) 100 mg/kg oral (1,7, 14, and 21 days)	Negative Positive	TO, SC, RE	Amer et al., 2006
Spermatocyte Chromosome aberration	Mouse	Herbazed formulation (84% glyphosate)	50 mg/kg i.p. (1,3, 5 days) 100 mg/kg oral (1,7, 14, and 21 days)	Negative Positive	TO, SC, RE	Amer et al., 2006
Bone marrow Chromosome aberration	Mouse	Roundup formulation (Bulgaria)	1080 mg/kg p.o. (1/2 LD_{50})	Negative	DL, TO, IC, RE	Dimitrov et al., 2006
Bone marrow erythrocyte micronucleus	Mouse	Analytical glyphosate (96%)	2 x 200 mg/kg i.p.	Positive	Erythrocytes scored? TO, SC, IC, RE	Manas et al., 2009b
Bone marrow Chromosome aberration	Mouse	Roundup™ formulation (Monsanto)	50 mg/kg i.p.	Positive	DL, SC, IC, RE	Prasad et al., 2009

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vivo Chromosome Effects—Non-Mammalian Systems						
Erythrocyte micronucleus	Oreochromis niloticus (Tilapia)	Roundup 69	170 mg/kg i.p. (maximum tolerated)	Negative?[c]	TO, RE	Coutinho do Nascimento and Grisolia, 2000
Wing spot test	Drosophila	Glyphosate (96%)	10 mM in larval stage	Positive/inconclusive[b]		Kaya et al., 2000
Erythrocyte micronucleus	Tilapia	Roundup™ formulation (Monsanto)	170 mg/kg (abdominal injection)	Positive	TO, RE	Grisolia, 2002
Erythrocyte micronucleus	*Crasseus auratus* (goldfish)	Roundup formulation	15 ppm glyphosate in water (2, 4 and 6 days)	Positive	TO, IE, RE	Cavas and Konen, 2007
	Prochilodus lineatus (tropical fish)	Roundup™ formulation (75% of 96 h LC50)	10 mg/L (6, 12 and 24 h) in water	Negative	DL, TO, SC, RE	Cavalcante et al., 2008
Erythrocyte micronucleus	Caiman eggs	Roundup® Full II formulation	1750 ue/egg	Positive	RE	Poetta et al., 2009
Erythrocyte micronucleus	Caiman eggs	Roundup® Full II formulation	Sprayed 2x with 100 litres of 3% in 30 days apart	Positive	DL, TO, RE	Poetta et al., 2010
In Vitro DNA Damage Mammalian Systems						
Alkaline SCGE	GM38 human fibroblasts and HT1090 human fibrosarcoma	Glyphosate (technical grade)	6.5 mM	Positive	MA, PH, TO, SC, RE	Monroy et al., 2005
Sister chromatid exchange	mouse spleen cells	herbazed formulation	50 µM?	Positive	Concentrations used not clear MA, PH, TO, SC, RE	Amer et al., 2006
Sister chromatid exchange	bovine lymphocytes	Glyphosate formulation (62% glyphosate, Monsanto)	1.12 mM (toxic)	Positive	PH, SC, RE	Sivikova and Dianovsky, 2006
Alkaline single cell gel electrophoresis (SCGE, comet)	Hep-2 cells	Glyphosate (analytical, 96%)	7.5 mM (limited by toxicity)	Positive	MA, PH, RE	Manas et al., 2009b
Alkaline SCGE	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/ml (toxic) (est. 3.43 mM)	Positive (-S9) Positive (+S9)		Mladinic et al., 2009a

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro DNA Damage Non-Mammalian Systems						
SOS	*E. coli*	Roundup BIO formulation	2.5 ug/sample	Positive		Raipulis J, 2009
Alkaline SCGE	Tradescantia flowers and nuclei	Glyphosate(technical, 96%)	700 µM	Positive	PH, SC	Alvarez-Moya et al., 2011
Bone marrow SCE	Mouse	herbazed formulation (84% glyphosate)	200 mg/kg p.o.	Positive	TO, SC, RE	Amer et al., 2006
Sperm abnormality	Mouse	herbazed formulation (84% glyphosate)	200 mg/kg p.o. (5 days)	Positive	TO, SC, RE	Amer et al., 2006
In Vivo DNA Damage Non-Mammalian Systems						
Erythrocyte Alkaline SCGE	Freshwater mussel larvae	Roundup formulation	5 mg/liter	Negative	TO, SC	Conners and Black, 2004
Erythrocyte alkaline SCGE	*Crasseus auratus* (goldfish)	Roundup formulation	15 ppm glyphosate in water (3, 4 and 6 days)	Positive	TO, RE	Cavas and Konen, 2007
Erythrocyte and gill cell alkaline SCGE	*Prochilodus lineatus* (tropical fish)	Roundup™ formulation (75% of 96 h LC50)	10 mg/l (6, 12 and 24 h) in water	Positive	DL, TO, RE	Cavalcante et al., 2008
Erythrocyte alkaline SCGE	Caiman eggs/hatchlings	Roundup® Full II formulation	1750 µg/egg	Positive	RE	Poletta et al., 2009
Erythrocyte alkaline SCGE	European eel	Roundup formulation	166 µg/liter	Positive	DL, SC, RE	Guilherme et al., 2010
Erythrocyte alkaline SCGE	Caiman eggs/hatchlings	Roundup® Full II formulation	Sprayed 2x with 100 l of 3%/ha 30 days apart	Positive	DL, RE	Poletta et al., 2010

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.

[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.

[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

3. Structure Activity Analysis

Glyphosate was evaluated using Derek for Windows (Llhasa Ltd., Leeds, UK, Version 11.0.0, October 24, 2009). No structural alerts were identified for chromosome damage, genotoxicity, mutagenicity or carcinogenicity. This small molecule consists of the amino acid, glycine, joined with a phosphonomethyl group. These moieties are not known to be genotoxic; therefore, the lack of structure activity alerts for glyphosate is expected.

4. Gene Mutation

As reviewed by Williams et al., (2000), most gene mutation studies for glyphosate and GBFs were negative. Gene mutation assays included numerous Ames/*Salmonella* and *E. coli* WP2 bacterial reversion assays, *Drosophila* sex-linked recessive lethal assays and a CHO/HGPRT *in vitro* mammalian cell assay. Of fifteen gene mutation assays reported, there were only two positive observations. A reported positive Ames/*Salmonella* result for Roundup formulation was not replicated in numerous other studies. There was one report of a positive result for a GBF in the *Drosophila* sex-linked recessive lethal assay but this was contradicted by a negative result for the same GBF in this assay reported by another laboratory. Further, the positive study had some features that hampered interpretation, including the lack of concurrent negative controls (Williams et al., 2000).

Subsequent to the Williams et al. (2000) review only two gene mutation studies have been reported (Table 2). One negative Ames/*Salmonella* assay result was reported for a GBF of undefined composition, Percozyd 10 SL (Chruscielska et al., 2000). Although this result is consistent with a large number of negative Ames/*Salmonella* results for glyphosate and GBFs, the reported study results have significant limitations. One of the recommended test strains, TA1535, was not used and results were only presented as "-" without presentation of revertant/plate data. A positive result for glyphosate was reported in the *Drosophila* wing spot assay which can indicate both gene mutation and mitotic recombination endpoints (Kaya et al., 2004). Small increases in small wing spot frequencies were observed in one of four crosses of larvae treated with up to 10 mM glyphosate. The lack of a positive response in the balancer-heterozygous cross offspring, which are insensitive to mitotic recombination events, suggests that there is no evidence for effects on gene mutation endpoint events such as intragenic mutations or deletions in this publication.

These gene-mutation publications add very limited data to the weight of evidence conclusion that glyphosate and GBFs do not pose significant risk for gene mutation.

5. Chromosome Effects

Assays to detect chromosome effects such as structural chromosome aberrations and micronucleus incidence constitute a second major genotoxicity endpoint category. A large number of publications with chromosome effects endpoints have been reported since the Williams et al. (2000) review. These are described in Table 2 and are separated into various test system categories which include *in vitro* cultured mammalian cell assays, *in vitro* tests in non-mammalian systems, *in vivo* mammalian assays and *in vivo* assays in non-mammalian systems. A *Drosophila* wing spot test (discussed previously) is also included in this category because results are relevant to somatic recombination.

5.1 In vitro Chromosome Effects

Two human and one bovine *in vitro* peripheral lymphocyte chromosome aberration studies of glyphosate were considered in the earlier review (Williams et al., 2000). One human lymphocyte *in vitro* study had negative results for glyphosate tested up to approximately 2-3 mM (calculated from reported mg/ml) in the absence and presence of an exogenous mammalian activation system. The other two studies with human and bovine lymphocytes and no metabolic activation system reported positive results at concentrations more than two orders of magnitude lower. The earlier review noted several other unusual features about the positive result studies including an unusual exposure protocol and discordant positive results for another chemical found negative in other laboratories.

As indicated in Table 2 both positive and negative results have been reported for glyphosate and GBFs in the nine *in vitro* chromosome effects assays published after the Williams et al. (2000) review. It is noteworthy that many of these studies have various deficiencies in conduct or reporting compared to internationally accepted guidelines for conduct of *in vitro* chromosome aberration or micronucleus studies (see Table 1). Perhaps the most significant deficiency was that coding and scoring of slides without knowledge of the treatment or control group was not indicated in seven of nine publications. This could be a deficiency in conducting the studies or perhaps a deficiency in describing methodology in the publications. Other common deficiencies included failure to indicate control of exposure medium pH, no use of exogenous metabolic activation and no reporting of concurrent measures of toxicity.

5.1.2 Results for glyphosate active ingredient

Three publications reported testing of technical glyphosate for micronucleus or chromosome aberration endpoints in cultured human lymphocytes (Manas et al., 2009b; Mladinic et al., 2009a; Mladinic et al., 2009b). Negative results for the micronucleus or chromosome aberration endpoints were observed in the absence of exogenous metabolic activation (S9) in all three publications. The maximum exposure concentration in the absence of S9 was in the range of 3-6 mM in these studies.

Two publications by one author reported cytokinesis block micronucleus results for cultured bovine lymphocytes treated with what was reported as 62% by weight isopropyl amine salt of glyphosate from a Monsanto Belgium source (Piesova, 2004; Piesova, 2005). This test material appears to be a manufacturing batch of the isopropylamine salt of glyphosate in water without surfactants, which is not sold as a GBF. In one publication no statistically significant increases in binucleated cell micronucleus frequency were observed with 24 hours of treatment (Piesova, 2004). For 48 hours of treatment a statistically significant increase in micronucleus frequency was observed in one donor at 280 µM but not at 560 µM and in a second donor at 560 µM but not 280 µM. The second publication reported negative results for the cytokinesis block micronucleus assay in bovine lymphocytes incubated with glyphosate formulation up to 560 µM for two hours in the absence and presence of a mammalian metabolic activation system (Piesova, 2005). This publication also reported positive results for 48 hours of treatment without S9. Curiously, in this second publication the same inconsistent dose response pattern was observed in which a statistically significant increase in micronucleus frequency was observed in one donor at 280 µM but not at 560 µM and in a second donor at 560 µM but not 280 µM. The lack of a consistent dose response pattern between donors suggests that the results with 48 hours of treatment are questionably positive.

Two other publications found negative results for the chromosome aberration endpoint in cultured bovine lymphocytes treated with what appears to be the same test material of 62% by weight isopropylamine salt of glyphosate from a Monsanto Belgium source, (Holeckova, 2006; Stivikova and Dianovsky, 2006). Both the studies used a maximum concentration of 1.12 mM which was reported to cause a decrease in mitotic inhibition of >50%. These two studies have several limitations including that an exogenous mammalian metabolic activation system was not used for chromosome aberration and scoring was not reported to be on coded slides. In addition, Holeckova (2006) only examined effects detectable by staining of chromosome 1 and did not report positive control results (Holeckova, 2006). Despite these limitations and the variable donor results, the results from these two studies are generally consistent with a lack of chromosome aberration effects of the isopropylamine salt of glyphosate on *in vitro* cultured mammalian cells in several experiments using high, toxic dose levels and exposures of 2-24 hours in the absence of S9.

One laboratory reported increases in cytokinesis-blocked micronucleus frequency in cultured human lymphocytes exposed to glyphosate for 4 hours in the presence of an exogenous human liver metabolic activation system (S9) in two publications (Mladinic et al., 2009a; Mladinic et al., 2009b). In both publications a statistically significant increase in micronuclei was observed with S9 at the highest dose level of glyphosate tested (580 µg/ml, ≈ 3.4 mM). Increased proportions of centromere- and DAPI-positive micronuclei were observed for the high dose with S9 suggesting that the induced micronuclei were derived from chromosomes rather than chromosome fragments. Statistically significant increases in the frequency of nuclear abnormalities (buds and bridges) and DNA strand breakage were also observed at the highest dose tested in both publications. In parallel experiments cytotoxic effects such as early apoptosis, late apoptosis and necrosis were observed and these effects were uniquely or preferentially observed in the presence of S9 and at the highest dose level tested (Mladinic et al., 2009a). Also, the negative control level of such endpoints as necrosis and alkaline SCGE tail moment was significantly increased in the presence of S9 (Mladinic et al., 2009a). It should be noted that glyphosate is mostly excreted unmetabolized *in vivo* in mammals with only very small levels of aminomethylphosphonic acid (AMPA) or an AMPA-related structure observed (Anadon et al., 2009; Brewster et al., 1991). These observations suggest that the observations of S9 mediated effects by Mladinic et al. are not likely to be due to *in vivo* relevant metabolites. It is possible that such effects might be generated by *in vitro* S9-mediated processes that are not relevant to *in vivo* processes such as genotoxic effects of low pH observed in the presence of S9 in *in vitro* assays (Cifone et al., 1987). The preponderance of *in vitro* genotoxicity studies conducted with exogenous mammalian metabolic activation systems has been negative, including a previously reviewed chromosome aberration study in human lymphocytes conducted up to a similar dose

level (Williams et al., 2000) and a bovine lymphocyte cytokinesis block micronucleus study (Piesova, 2005). Overall these results suggest the possibility of a weak aneugenic rather than clastogenic (chromosome breaking) effect occurring in the presence of S9 at high dose levels of glyphosate. The pattern of activity as well as the failure to observe activity in several other *in vitro* genotoxicity assays conducted with S9 suggests that the activity observed in the Mladinic et al. studies does not have a significant weight of evidence for *in vitro* genotoxicity and is not likely to be relevant to *in vivo* genotoxicity.

The recently published results for mammalian *in vitro* chromosome aberration and micronucleus assays demonstrate a weight of evidence that technical glyphosate and glyphosate salt concentrates are negative for these endpoints in cultured mammalian cells in the absence of an exogenous mammalian metabolic activation system. Five publications from four laboratories report negative *in vitro* mammalian cell chromosome or micronucleus results in the absence of exogenous activation while three publications from two laboratories report positive results. These results reinforce the Williams et al. (2000) conclusion that positive chromosome aberration results reported for glyphosate in cultured human lymphocytes in the absence of an exogenous metabolic activation system are aberrant.

Recent reports of positive chromosome aberration and micronucleus results for glyphosate in the presence of an exogenous mammalian activation system in cultured human lymphocytes in one laboratory (Mladinic et al., 2009a; Mladinic et al., 2009b) have no substantial reproducibility verification from other laboratories in the recent *in vitro* chromosome effects studies considered in this review because most of the studies performed by other laboratories (Table 2) did not employ an exogenous mammalian activation system. These results are discordant with one previously reviewed result demonstrating a negative result for glyphosate in cultured human lymphocytes with mammalian metabolic activation using the chromosome aberration endpoint (Williams et al 2000) and a negative result in the presence of S9 for the micronucleus endpoint in bovine lymphocytes (Piesova, 2005). The numerous consistent negative results for glyphosate and GBFs in gene mutation studies which employed exogenous mammalian metabolic activation and careful examination of the data suggests that the positive results indicate a possible threshold aneugenic effect associated with cytotoxicity rather than a DNA-reactive mechanism resulting in chromosome breakage. Thus, the weight evidence for the *in vitro* chromosome effect assays indicates a lack of DNA-reactive clastogenic chromosome effects.

5.1.3 Results for GBFs

Amer et al. (2006) reported positive *in vitro* chromosome aberration effects in mouse spleen cells for a formulation described as herbazed, which was reported to contain 84% glyphosate and 16% solvent, an unusually high glyphosate concentration for a formulation. The test material is not further characterized, lacking description of the glyphosate salt form and inert ingredients. The glyphosate concentrations used in the study are not clear because there are different descriptions of the concentration units (M or M glyphosate/ml medium) in the publication. Thus, the maximum concentration might have been 5 x 10^{-5} M (50 µM) or 5 x 10^{-5} M glyphosate/ml medium (50 mM). The former concentration, which was reported as toxic, would indicate effects at concentrations well below those typically found toxic for GBFs in cultured mammalian cells. The latter level of 50 mM would be well in excess of the limit level of 10 mM recommended in OECD guidelines (OECD 473, 1997). In addition to a question about the concentration used there are several other limitations to the reported study including no indication that pH of treatment solutions was controlled, no use of a mammalian metabolic activation system, no reported concurrent toxicity measurements and no reported use of coded slides for scoring. Given these limitations, the uncertainty about the concentrations used and the nature of the test material, these results should not be considered to have significant relevance or reliability with respect to glyphosate or GBFs.

In addition to *in vitro* mammalian cell studies there is also a report of negative results for the chromosome aberration and micronucleus endpoints in onion root tips incubated with a Roundup formulation (Dimitrov et al., 2006). The maximum exposure concentration (stated as 1% active ingredient) is estimated to be on the order of 4-6 mM. This study did not employ an exogenous mammalian metabolic activation system; however, it does provide evidence for a lack of chromosome effects for glyphosate and a GBF in a non-mammalian *in vitro* system. The result agrees with earlier reported negative onion root tip chromosome aberration results for glyphosate but is discordant with earlier reported positive results for a Roundup GBF in this system (Williams et al., 2000).

5.2 In vivo Chromosome Effects—Mammalian Systems

The Williams et al. (2000) glyphosate toxicity review presented results from *in vivo* mammalian chromosome effect assays. Results from several mouse bone marrow erythrocyte micronucleus studies of glyphosate and GBFs (e.g. Roundup, Rodeo and Direct) were negative for micronucleus induction. These included studies from different laboratories mostly following modern guidelines. The intraperitoneal (i.p.) route was used for most of the negative studies and maximum doses for many of the studies were toxic or appropriately close to LD50 values. In addition to i.p. studies a 13 week mouse feeding study was also negative for the micronucleus endpoint with an estimated maximum daily glyphosate dose of over 11,000 mg/kg/day. There was one published report of a weak positive mouse bone marrow micronucleus response observed for glyphosate and Roundup GBF. This study, which employed a smaller number of animals per group than other negative studies, was clearly aberrant from the numerous other negative studies not only in micronucleated cell frequency finding but also the finding of altered polychromatic erythrocyte to normochromatic erythrocyte (PCE/NCE) ratios. The overall weight of evidence from the earlier reviewed studies was that glyphosate and GBFs were negative in the mouse bone marrow erythrocyte micronucleus assay. The earlier review also noted a negative mouse dominant lethal result for glyphosate administered by gavage at a maximum dose level of 2000 mg/kg.

As indicated in Table 2, there are numerous subsequent publications of *in vivo* mammalian chromosome effects assays. With one exception, all of the *in vivo* mammalian studies were conducted in the mouse using either the bone marrow chromosome aberration or micronucleus endpoints. It should be noted that there are some fairly consistent limitations in the reported conduct of these studies compared to OECD guidelines. In most studies concurrent indications of toxicity (other than effects on the bone marrow) are not reported, coding of slides for scoring is not reported, individual animal data are not reported and fewer than recommended cells or metaphases per animal were scored. Other limitations encountered include use of only a single or two dose levels rather than three dose levels.

5.2.1 Results for glyphosate active ingredient

Two publications reported results for glyphosate in the mouse bone marrow erythrocyte micronucleus assay. Negative results were reported in one study which used a dose of 300 mg/kg of glyphosate administered once i.p. with sacrifices at 24, 48 and 74 hours after dosing (Chruscielska et al., 2000). This study had some limitations including the use of only one dose level, no reporting of toxicity other than PCE/NCE ratio, no reported coding of slides for scoring and scoring of 1000 PCE's per animal (scoring of 2000 PCE's per animal is recommended by OECD guidelines). A second publication reported positive results for glyphosate administered at 50, 100 and 200 mg/kg via i.p. injections repeated at 24 hours apart with sacrifice 24 hours after the second dose (Manas et al., 2009b). A statistically significant increase in micronucleated erythrocytes was observed in the high dose group. This study had limitations comparable to the negative study. A more significant potential difficulty with this second publication is that "erythrocytes" rather than polychromatic erythrocytes were indicated as scored for micronuclei. This does not appear to be a case of using "erythrocytes" to mean polychromatic erythrocytes because the term "polychromatic erythrocytes" is used elsewhere in the publication describing measurements of PCE/NCE ratios. Scoring of total erythrocytes instead of immature polychromatic erythrocytes for micronuclei would be inappropriate in an assay with the stated treatment and harvest times because of the transient nature of micronucleated PCE's in bone marrow (OECD474, 1997).

There is no definitive explanation for the discrepancy between the two publications. Although one study used a single dose with multiple harvest times and the second used two doses and a single harvest time, both are acceptable protocols and would not be expected to lead to such discordant results (OECD474, 1997). The negative result reported for the 13 week feeding study in the earlier review (Williams et al., 2000) confirms that positive results are not simply due to repeat dosing. The reported negative result (Chruscielska et al., 2000) seems to be in accord with a majority of earlier reviewed mouse bone marrow micronucleus studies of glyphosate using similar doses and the i.p. or feeding routes (Williams et al., 2000). Also, the apparent scoring of micronuclei in erythrocytes rather than just polychromatic erythrocytes raises a significant methodological question for the reported positive study.

5.2.2 Results for GBFs

There are several publications reporting *in vivo* mammalian bone marrow chromosome aberration and micronucleus endpoint results for Roundup GBFs. Three publications report negative results for Roundup

branded GBF in mouse chromosome aberration or micronucleus assays. Negative results were reported for two different Roundup branded GBFs administered at 2 x 200 mg/kg i.p. in mouse bone marrow erythrocyte micronucleus assays (Coutinho do Nascimento and Grisolia, 2000; Grisolia, 2002). The second study did not report coding of slides for scoring. Another publication reported negative results in mouse bone marrow studies for both the chromosome aberration and erythrocyte micronucleus endpoints (Dimitrov et al., 2006) using a dose of 1080 mg/kg administered orally (p.o.). In contrast, one publication reported positive results for Roundup GBF in mouse bone marrow for the chromosome aberration and erythrocyte micronucleus endpoints using a single maximum dose of 50 mg/kg i.p. (Prasad et al., 2009). Both the positive results and the magnitude of the increases in the chromosome aberration and micronucleus endpoint reported in this study are remarkably discordant with other reported results for Roundup and other GBFs in mouse bone marrow chromosome aberration and erythrocyte studies in a number of laboratories and publications (Table 2 and Williams et al., 2000). The reasons for this discordance are not clear. One unusual feature of the positive study is that the Roundup GBF was administered in dimethylsulfoxide. This is an unusual vehicle to use in *in vivo* genotoxicity studies, particularly for glyphosate which is water soluble and especially so in a formulated product. A published toxicity study found that use of a dimethylsulfoxide/olive oil vehicle by the i.p. route produced dramatically enhanced toxicity of glyphosate formulation or the formulation without glyphosate compared to saline vehicle and that the enhanced toxicity observed with this vehicle was not observed when the oral route was used (Heydens et al., 2008). These observations suggest that use of DMSO as a vehicle for administration of formulation components by the i.p. route might produce unusual toxic effects that are not relevant to normally encountered exposures. Regardless of the reasons for the discordant positive results it is clear that a large preponderance of evidence indicates that GBFs are typically negative in mouse bone marrow chromosome aberration and erythrocyte assays.

One publication reported positive results for bone marrow chromosome aberration in rabbits administered Roundup GBF in drinking water at 750 ppm for 60 days (Helal and Moussa, 2005). This study is relatively unique in terms of species and route of administration. The results do not report water intake in the test and control groups. Given the potential for water palatability issues with a formulated product, this is a significant shortcoming, as any effects noted may be attributable to dehydration (Saunders, 2005). This study had further limitations including the use of only a single dose level and not coding slides for scoring. Examination of the chromosome aberration scoring results showed that large increases for the treated group were observed for gaps and "centromeric attenuation" which were included in the summation and evaluation of structural chromosome aberration effects. Ordinarily gaps are scored but are not recommended for inclusion in total aberration frequency and centromeric attenuation is not included in ordinary structural aberrations (OECD 475, 1997, Savage, 1976). These unusual scoring and interpretive features raise significant questions about using this study to make conclusions about clastogenicity of the GBF tested.

Two other publications report *in vivo* mammalian chromosome aberration or micronucleus results for GBFs. An uncharacterized GBF, Perczyd 10L, was reported to be negative in a mouse bone marrow erythrocyte micronucleus assay (Chruscielska et al., 2000). The maximum dose level tested, 90 mg/kg i.p., was reported to be 70% of the i.p LD50 as determined experimentally by the authors. This study had several limitations including use of less than three dose levels and no reported coding of slides for scoring. Positive results were reported for another uncharacterized GBF, herbazed, in mouse bone marrow and spermatocyte chromosome aberration studies (Amer et al., 2006). No statistically significant increases in aberrant cells were observed in bone marrow cells for i.p. treatment of 50 mg/kg for 1, 3 or 5 days or in spermatocytes for 1 or 3 days treatment. Statistically significant increases in frequency of spermatocytes with aberrations were reported for 5 days of treatment with 50 mg/kg (i.p.). Oral treatment of 50 mg/kg and 100 mg/kg were reported to produce increases in aberrant cell frequency in bone marrow cells after extended treatments (14 and 21 days) but not after shorter 1 and 7 day treatments. Similarly, significant increases in aberrant cell frequencies of spermatocytes were reported at 14 and 21 days of 50 mg/kg oral treatment (negative for 1 and 7 days treatment) and at 7, 14 and 21 days of 100 mg/kg treatment (negative for 1 day treatment). Although not a genotoxic endpoint per se, it should be noted that statistically significant increases in frequency of sperm with abnormal morphology were also observed in mice treated with 100 and 200 mg/kg p.o. for 5 days. The positive results for the uncharacterized herbazed GBF were only observed after extended oral treatments (bone marrow and spermatocytes) and extended i.p. treatments (spermatocytes). The fact that positive results were not observed in an erythrocyte

micronucleus test of mice treated with glyphosate up to 50,000 ppm in feed for 13 weeks (Williams et al., 2000) provides direct evidence that extended glyphosate treatment by the oral route does not induce detectable chromosome effects. This treatment was longer and up to much higher glyphosate exposures than those used for the Amer et al. (2006) studies. Thus, it appears likely that these effects were due to some component(s) of the specific herbazed GBF tested rather than glyphosate.

In vivo mammalian assays for chromosome effects are an important category for characterizing genotoxicity that complements the gene mutation category. While some positive results have been reported the preponderance of evidence and published results are negative for glyphosate and GBFs.

5.3 In vivo Chromosome Effects—Non-Mammalian Systems

The Williams et al. (2000) review reported a few *in vivo* plant assays for chromosome effects in non-mammalian systems. These included negative results for glyphosate and positive results for Roundup GBFs for chromosome aberrations in an onion root tip assay and negative results for glyphosate with the micronucleus endpoint in a *Vicia faba* root tip assay.

Subsequent to the earlier review a number of publications reported results for erythrocyte micronucleus assays conducted on GBFs in several non-mammalian fish and reptile species with discordant results. One publication reported apparently negative results for the erythrocyte micronucleus test in *Oreochromis niloticus* (Nile tilapia) administered a test material described as Roundup 79 GBF, at an upper dose of 170 mg/kg i.p. (Coutinho do Nascimento and Grisolia, 2008). Although there was an increase in micronucleated erythrocyte frequency at the mid-dose level this was not observed at the high dose level and considerable variability in frequencies in different groups was noted. Negative results were also reported in another fish species (*Prochilodus lineatus*) exposed to 10 mg/liter Roundup branded GBF for 6, 24 and 96 hours (Cavalcante et al., 2008). This concentration was reported to be 96% of a 96 hour LC50. Positive results were reported for the erythrocyte micronucleus assay conducted in the fish *Tilapia rendalii* exposed to 170 mg/kg i.p. of another Roundup GBF (Grisolia, 2002). Examination of the micronucleus frequencies in this publication indicated that the negative control micronucleus frequency was considerably lower than the frequencies for all but one of 21 treatment groups for 7 different test materials. This suggests an unusually low control frequency and at least one treatment group was statistically significantly elevated for each of the 7 test materials, including many instances where the statistically significant increases were not consistent with a biologically plausible dose response. The possibility that the apparently significant increases were due to a low negative control value should be considered for this publication. Another publication reported positive erythrocyte micronucleus results in goldfish (*Carassius auratus*) exposed to 5 to 15 ppm of a Roundup GBF for 2 to 6 days (Cavas and Konen, 2007). The reasons for the discordant results are not clear for these fish erythrocyte micronucleus assays of Roundup GBFs. Although different species and GBF's were used in the different studies there were pairs of studies with positive and negative results that used similar treatment conditions (170 mg/kg i.p. or 10-15 mg/liter in water).

Results for an unusual test system of exposed caiman eggs are reported in two publications. In one study eggs were topically exposed in a laboratory setting to Roundup Full II GBF, and erythrocyte micronucleus formation was measured in hatchlings (Poletta et al., 2009). The GBF tested was reported to contain the potassium salt of glyphosate and alkoxylated alkylamine derivatives as surfactants. Statistically significant increases in micronucleated erythrocytes were observed in hatchlings from eggs treated with 500-1750 µg/egg. This system is quite unusual in the species tested and even more so in using an egg application with measurement of effects in hatchlings. Although there is some experience with a hen's egg erythrocyte micronucleus assay using *in ovo* exposure the erythrocytes are evaluated in embryos with only a few days between treatment and the erythrocyte micronucleus endpoint (Wolf et al., 2008). In the reported caiman egg assay there was presumably a single topical exposure followed by an egg incubation period of about 10 weeks before hatching. Biological plausibility raises questions whether genotoxic events *in ovo* can produce elevated micronucleated erythrocyte frequencies detectable after 10 weeks, given the number of cell divisions occurring in development of a hatchling.

A second publication by (Poletta et al., 2011) described two field experiments evaluating caiman hatched from eggs in artificial nests that were sprayed on incubation days 5 and 35. Experiment 1 dosed with two applications of Roundup Full II GBF and experiment 2, twelve months later, with the same dosing regimen except the second application at incubation day 35 included cotreatment with cypermethrin and endosulfan formulations. Increases in micronucleated erythrocyte frequency in hatchlings were reported

for both experiments. Additional measurements of growth showed small but statistically significant differences in total length and snout-vent length in 3 month old, but not 12 month old animals in both experiments. Alanine aminotransferase enzyme levels in serum of 3 month old animals in both experiments were significantly elevated (>2-fold control values). Alterations in these parameters suggest that the treated groups had some persistent biological differences from control group animals either as a result of treatment or some other factor. It is certainly possible that the micronucleus effects in both publications are associated with these persistent biological differences rather than from genotoxic effects induced in the embryos.

One published study reported a weak positive result in a *Drosophila* wing spot assay (Kaya et al., 2004). Statistically significant positive increases were only in one of four crosses for small twin spots and not for the two other wing spot categories (large wing spots and twin wing spots). As discussed above, only negative or inconclusive results were observed for crosses that were not subject to mitotic recombination effects. If the result was actually treatment related it only would indicate an increase in recombination events and not in somatic mutations.

The above *in vivo* chromosome effect assays in non-mammalian systems give discordant results for reasons that aren't precisely defined. Typically these results would be given lower weight than mammalian systems in being predictive of mammalian effects, especially since there is little or practically no assay experience with these systems in comparison with *in vivo* mammalian chromosome effects assays, such as the rat or mouse bone marrow chromosome aberration or erythrocyte micronucleus assays.

6. DNA Damage and Other Endpoints

A number of studies of glyphosate and GBFs have been published since 2000 which used various DNA damage endpoints in a variety of *in vitro* and *in vivo* systems. The DNA damage category includes endpoints such as sister chromatid exchange and DNA repair response in bacteria, but the most common DNA damage endpoint encountered was the alkaline single cell gel electrophoresis endpoint (alkaline SCGE) also commonly referred to as the "comet" assay. The alkaline SCGE endpoint has been applied to both *in vitro* and *in vivo* test systems.

In addition to DNA damage there are a few reports of other types of studies which can be associated with genotoxic effects even though the endpoints are not specific indicators of genotoxicity per se. These include sperm morphology and carcinogenicity studies.

6.1 In vitro DNA Damage Studies

Some positive results for glyphosate or GBFs in the SCE endpoint were reported in cultured human and bovine lymphocytes in the earlier review (Williams et al., 2000). These results tended to be weak, inconsistent and with limited evidence for dose response. A number of limitations were observed for the studies such as the failure to control pH, and abnormally low control values. Additional *in vitro* DNA damage endpoint results described in the earlier review included negative results for glyphosate in the *B. subtilis* rec-assay and in the primary hepatocyte/hepatocyte unscheduled DNA synthesis assay.

There are two subsequent publications using *in vitro* cultured mammalian cells and the SCE endpoint. Positive SCE results were reported for the uncharacterized herbazed GBF in mouse spleen cells (Amer et al., 2006). The dose response pattern for SCE response in this study was similar to the response for chromosome aberrations in this publication. Limitations of this study are in common to those described above for the chromosome aberration endpoint portion of the study; no indication that pH of treatment solutions was controlled, no use of a mammalian metabolic activation system, no reported concurrent toxicity measurements and no reported use of coded slides for scoring. Positive SCE results were also reported for cultured bovine lymphocytes treated with up to 1.12 mM glyphosate for 24 and 48 hours without exogenous mammalian metabolic activation (Sivikova and Dianovsky, 2006). The highest dose of 1.12 mM significantly delayed cell cycle progression with 48 hour treatment. These same concentrations for 24 h exposures did not induce statistically significant increases in chromosome aberrations which provides a clear example of a differential response of the SCE endpoint (Sivikova and Dianovsky, 2006). This is an important consideration in these publications, as chromosome effects are considered more relevant to genotoxicity than DNA damage.

Positive results for glyphosate are reported for the alkaline SCGE endpoint in three publications. Positive SCGE results were observed for two mammalian cell lines exposed to glyphosate for 4 hours at concentrations of 4.5-6.5 mM (GM39 cells) and 4.75-6.5 mM (HT1080 cells) (Monroy et al., 2005).

These concentrations are close to the upper limit dose of 10 mM generally recommended for *in vitro* mammalian cell assays and control of medium pH is not indicated. Characterization of nuclear damage was done by visual scoring without coding of slides being indicated. Positive alkaline SCGE results were also reported in Hep-2 cells exposed for 4 hours to 3.5-7.5 mM glyphosate (Manas et al., 2009b). Higher concentrations of glyphosate were reported to result in viability of <80% as determined by dye exclusion. As noted for the preceding publication, the concentrations employed were reasonably close to a limit dose of 10 mM and control of medium pH was not reported. This publication reported negative results for the chromosome aberration endpoint in cultured human lymphocytes exposed to up to 6 mM glyphosate for 48 hours and it should be noted that in this case an appropriate control of medium pH was reported for this human lymphocyte experiment. Positive alkaline SCGE results have also been reported for cultured human lymphocytes exposed to glyphosate at concentrations up to 580 µg/ml (estimated 3.4 mM) for 4 hours (Mladinic et al., 2009a). Effects were observed both in the presence and absence of S9 with statistically significant increases in tail intensity at 3.5, 92.8 and 580 µg/ml without S9 and at 580 µg/ml with S9. A modification of the alkaline SCGE assay employing human 8-hydroxyguanine DNA-glycosylase (hOGG1) to detect oxidative damage only indicated statistically significant effects on tail length for treatment with 580 µg/ml with S9. Increases in nuclear abnormalities (nuclear buds and/or nucleoplasmic bridges) were also observed at 580 µg/ml with and without S9 and in micronucleus frequency at 580 µg/ml with S9. Measurements of total antioxidant capacity and thiobarbituric acid reactive substances showed statistically significant increases at 580 µg/ml in the presence or absence of S9. Interpretation of the significance of metabolic activation effects is complicated by the observation that several of the endpoints (alkaline SCGE tail intensity and nuclear abnormalities) tended to show increases in the presence of S9 in negative controls or at the very lowest concentrations of glyphosate. A reasonable summation of the results in this publication is that alkaline SCGE effects and other effects such as nuclear abnormalities, early apoptosis, necrosis and oxidative damage were consistently observed at 580 µg/ml.

In addition to mammalian cell studies there are publications reporting positive alkaline SCGE effects for glyphosate in *Tradescantia* flowers and nuclei exposed to up to 700 µM glyphosate (Alvarez-Moya et al., 2011) and in the *E. coli* SOS chromotest for DNA damage conducted on a Roundup BIO GBF (Raipulis J, 2009). Observations of DNA damage in plants exposed to glyphosate are of questionable significance because of the herbicidal nature of glyphosate and the SOS chromotest provides only indirect evidence of DNA damage in a bacterial system.

Overall there appear to be a number of studies in which glyphosate or GBFs have been reported to produce positive responses in DNA damage endpoints of SCE or alkaline SCGE *in vitro* in mammalian cells. Most of these have occurred with exposures to mM concentrations of glyphosate. Although this dose level range is lower than the limit dose of 10 mM recommended for several *in vitro* mammalian cell culture assays (OECD473, 1997; OECD476, 1997; OECD487, 2010), an even lower limit dose of 1 mM was recently recommended for human pharmaceuticals, particularly because of concerns about relevance of positive *in vitro* findings observed at higher dose levels (ICHS2(R1), 2008; Parry et al., 2010). In addition, many of the studies have limitations such as not indicating control of medium pH and not coding slides for visual scoring.

Concerns over the possibility of effects induced by toxicity have led to several suggestions for experimental and interpretive criteria to distinguish between genotoxic DNA-reactive mechanisms for induction of alkaline SCGE effects and cytotoxic or apoptotic mechanisms. One recommendation for the *in vitro* alkaline SCGE assay is to limit toxicity to no more than a 30% reduction in viability compared to controls (Henderson et al. 1998; Storer et al. 1996; Tice et al. 2000). Importantly, dye exclusion measurements of cell membrane integrity, such as those reported in some of the above publications may significantly underestimate cytotoxicity that could lead to alkaline SCGE effects (Storer et al. 1996). Other recommendations include conducting experiments to measure DNA double strand breaks to determine if apoptotic process might be responsible for alkaline SCGE effects. Measurement of apoptotic and necrotic incidence were only performed in one publication (Mladinic et al., 2009a) and these measurements indicated both apoptotic and necrotic processes occurring in parallel with observations of alkaline SCGE effects. These direct observations as well as the reported dose responses, consistently suggest that biological effects and cytotoxicity accompany the observations of DNA damage *in vitro* in