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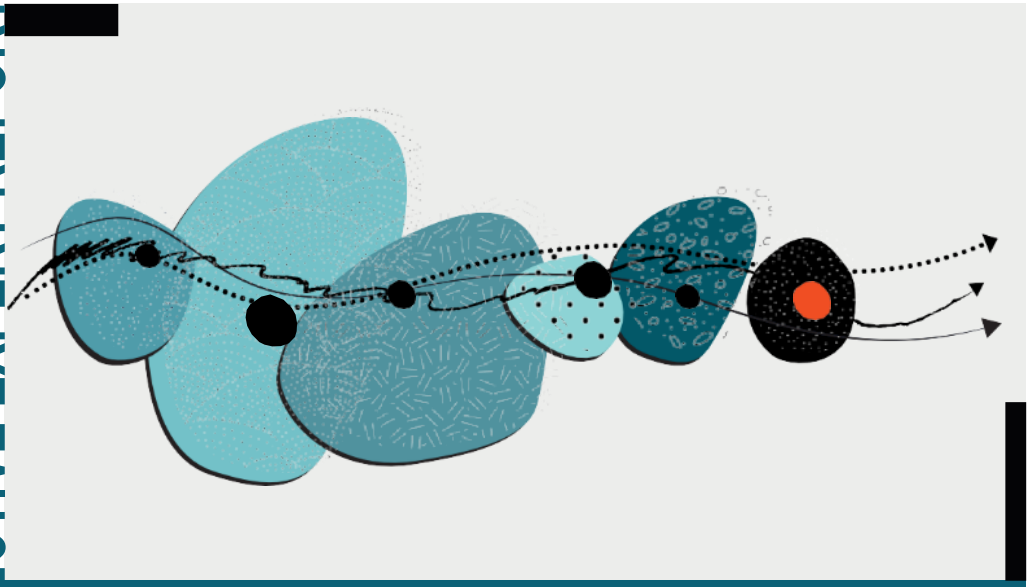

Glyphosate and glyphosate-based herbicides

Extract from “Pesticides and health effects: New data”

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This document on glyphosate was extracted and translated into English from the original version of the collective expert report published by Inserm in June 2021.

The complete report presents the work of a group of experts brought together by Inserm at the request of five Directorates General of the French government to update a collective expert report entitled "The effects of pesticides on health" published by the Institute in 2013. The procedure employed for this review is outlined in Annex 1.

This work on glyphosate is primarily based on data from the published scientific literature available as of the last quarter of 2019. Inserm compiled over 400 documents through searches of various bibliographic databases and through complementary literature searches carried out by the experts.

The group of experts chose to present an analysis of the literature on exposure and epidemiology data published since 2013 and on the toxicology and mechanisms of action of glyphosate.

This report was coordinated by Collective Expert Reports Unit of Inserm, which is part of its Public Health Thematic Institute.

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Glyphosate and glyphosate-based herbicides

Introduction

Glyphosate was first synthesized in 1950 as a potential pharmaceutical compound (IARC, 2017). The herbicidal properties of glyphosate were discovered by Monsanto in 1970 and the first commercial formulation containing glyphosate was marketed in 1974 under the name of Roundup (Farmer, 2010). This product received marketing authorization in France in 1975 under MA no. 7400057 (ANSES, 2019a). Patent protection expired outside the United States in 1991, and within the United States in 2000 (Székács and Darvas, 2012). Since then, glyphosate and glyphosate-based herbicides (GBH) have been marketed by many other companies.

Glyphosate is a total, foliar and systemic herbicide, which means: *i*) that it is effective on almost all annual or perennial weeds and is non-selective; *ii*) that it is absorbed by the leaves; and *iii*) that after absorption it is transported by the sap throughout the entire plant (the leaves and stem, through to the end of the roots and rhizomes (ACTA, 2019).

Glyphosate is an ingredient in several hundred commercial herbicides. These products are typically sold in the form of a soluble concentrate that is usually diluted to between 1 and 3% in an aqueous medium before application. Roundup Ultra, a "representative" formulation used by the health risk assessment agencies, has the following partial composition: glyphosate isopropylamine salt 485 g/L (equivalent to 360 g/L glyphosate as an active ingredient[1]) and the surfactant polyoxyethylene amine (POEA, 16%).

An analysis of the carcinogenic potential of glyphosate by the International Agency for Research on Cancer (IARC) reached a conclusion different from that of the European Food Safety Authority (EFSA). IARC classified glyphosate in Group 2A as "probably carcinogenic" (IARC, 2017), while

[1] To facilitate the interpretation of results, the concentrations of glyphosate in this report are shown in mg/L of glyphosate acid equivalent, with 5.9 µM corresponding to 1 mg/L.

evaluation by European agencies, led by the German Federal Institute for Risk Assessment (*Bundesinstitut fur Risikobewertung*; BfR) concluded that it should not be classified (BfR, 2015a), a conclusion adopted by EFSA and the European Chemicals Agency (ECHA). EFSA's opinion was shared by agencies outside the European Union: the Joint Meeting on Pesticide Residues (JMPR) of the Food and Agriculture Organization of the United Nations and the World Health Organization, the Australian Pesticides and Veterinary Medicines Authority, the New Zealand Environmental Protection Agency, the Health Canada Pest Management Regulatory Agency, and the US Environmental Protection Agency (EPA). In France, the *Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail* [French Agency for Food, Environmental and Occupational Health & Safety] (ANSES) concluded in 2016 that:

- the level of evidence for carcinogenicity in animals and humans was considered to be relatively limited and did not support classifying glyphosate in category 1A or 1B (known or presumed human carcinogens) under the criteria set out in Regulation (EC) No. 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP);
- in view of the limited level of evidence, the substance could arguably be classified in Category 2 but the Agency cannot decide on this point in the absence of a detailed analysis of all the studies.

In July 2019, ANSES thus drew up specifications for further studies of the genotoxic potential of glyphosate using the *in vivo* comet assay.

The difference in the assessment of the carcinogenic potential of glyphosate between the health risk assessment agencies and IARC arises from the following aspects in particular:

- a disparity between the databases used, with a body of regulatory data unknown to IARC and conversely some scientific articles not included in the BfR database;
- different criteria for interpreting the results of toxicology studies *(in vivo* and *in vitro)* and IARC's inclusion of certain animal models (oyster, mussel, fish, reptile, and earthworm) generally not included by the agencies;
- different objectives (strength of evidence and mechanisms of action for IARC, compared to risk assessment for the BfR). For example, IARC took into account the induction of oxidative stress and positive carcinogenesis tests in rodents in reaching its Group 2A classification.

The purpose of this collective expert report is not to analyze the various factors involved in this controversy, which has generated numerous

publications in both the scientific literature (Portier *et al.*, 2016; Mesnage and Antoniou, 2017; Tarazona *et al.*, 2017; Benbrook, 2018; Clausing *et al.*, 2018; Douwes *et al.*, 2018; Tarone, 2018) and in the media.

The purpose of this chapter is to present and analyze the available exposure science data on glyphosate (on its use, and environmental and population contamination), epidemiology data published since 2013, and the toxic mechanisms of action of glyphosate and GBH based on the recent literature.

Use, environmental contamination, and biomonitoring

Glyphosate is probably the most widely used herbicide in the world (Benbrook, 2016). In 2010, it was registered in over 130 countries, and estimated global production of the active ingredient totaled approximately 600,000 tons in 2008, 650,000 in 2011, and 720,000 in 2012 (IARC, 2017). The development in the 1990s of genetically modified organisms (GMOs) resistant to this herbicide ("Roundup Ready" plants, not grown in France), primarily corn, soy, and cotton, was accompanied by a significant increase in global use of glyphosate (Beckert *et al.*, 2011). Its worldwide consumption thus increased from 56,000 tons in 1994 to over 820,000 tons in 2014 (Table I), primarily in the agricultural sector (76% of the volume in 1994 and 90% in 2014).

Table I : Global use of glyphosate in agricultural and non-agricultural settings between 1994 and 2014

	1994	1995	2000	2005	2010	2012	2014
Glyphosate (1,000 kg)	56,296	67,078	193,485	402,350	652,486	718,600	825,804
Agricultural	42,868	51,078	155,367	339,790	578,124	648,638	746,580
Non-agricultural	13,428	16,000	38,118	62,560	74,362	69,962	79,224
Agricultural share (%)	76	76	80	84	89	90	90
Non-agricultural share (%)	24	24	20	16	11	10	10

Quantities of active ingredient, data from Benbrook, 2016.

Use in France

Like all synthetic phytopharmaceutical products, glyphosate has been banned in public spaces in France since January 1, 2017, and since January 1, 2019 it has been banned from sale to individuals. In October 2020, ANSES announced that agricultural use of glyphosate would be prohibited in situations where a non-chemical alternative (such as plowing, or manual or mechanical weeding) can be used instead, and also that the approved dosage rates would be reduced for certain uses (viticulture, fruit farming, field crops, and forestry). These decisions are designed to limit the amount of glyphosate used in agriculture in 2021, as the French government has announced its intention to phase out the pesticide by 2023.

In March 2018, 75 commercial products containing glyphosate were authorized in France as phytopharmaceutical products (ANSES, 2019b). In December 2019, ANSES announced the withdrawal of authorizations for 36 glyphosate-based products in France and the refusal of authorizations for 4 new products. In 2018, these products accounted for almost three-quarters of the tonnage of glyphosate-based products sold in the country for agricultural and non-agricultural uses.[2]

In France, as in most European countries, glyphosate is not used directly on growing crops, but to prepare the ground before they are planted. It therefore tends to be used between two agricultural crops, either to destroy regrowth of the previous crop or to clear the part of the field that remains unplanted and is invaded by weeds. To a lesser extent, glyphosate is also used in agriculture to control weeds around the edges of fields, roads and buildings. The French National Institute for Agricultural Research (INRA) has conducted research with a view to proposing alternatives to using glyphosate (Reboud *et al.*, 2017). Dependency on glyphosate varies across different agricultural systems. The following systems have, for example, been shown to have a high dependency on glyphosate: monoculture crops over a large area, field fruit and vegetables (for example, beet). Similarly, some systems have been shown to be highly if not totally dependent on glyphosate. These include conservation agriculture (i.e. no-till practices where mechanical weed control is avoided and that represents 3 to 4% of agricultural land), hazelnut production (around 200 producers, with 10

[2]. Published on 12/9/2019 on the ANSES website: https://www.anses.fr/fr/content/l%E2%80%99anses-annonce-le-retrait-de-36-produits-%C3%A0-base-de-glyphosate [accessed January 6, 2020].

tons of glyphosate used for this purpose in 2017), flax retting (in which flax stalks are left to break down in the field by the microorganisms present in order to harvest solely the fiber, which means the ground cannot be plowed; France is the world's leading producer of fiber flax), vineyards on sloping or stony terrain that makes mechanical weeding difficult (e.g. Banyuls, Maury, Côte-Rôtie, Condrieu, representing approximately 3,000 ha), and sugar cane.

As the INRA report highlights (Reboud *et al.*, 2017), glyphosate is a herbicide favored by farmers due to its broad spectrum of efficacy, low cost and ease of use. Its use has had several consequences: by enabling higher output, it has extended growing areas, the reduced need for plowing has changed the agricultural machinery sector, and it has made it possible for farmers to plant some crops in poorer soil.

Between 2009 and 2017, glyphosate was the most heavily used herbicide in France. Depending on the year, it was the most used (or second most used) phytopharmaceutical active substance (ANSES, 2019b). Figure 1 shows the change in sales of glyphosate between 2009 and 2017. Over this period, the annual quantity sold increased from 6,421 tons (in 2009) to 10,070 tons (in 2014) (Reboud *et al.*, 2017; ANSES, 2019b). A share of this quantity was sold in the form of commercial products authorized for use by the general public (non-professionals): between 13.9% (2017) and 23.7% (2013).

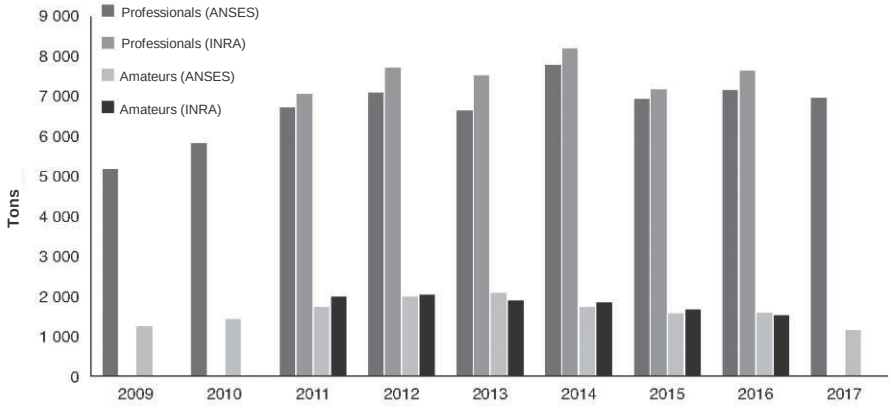


Figure 1: Quantities of glyphosate sold (in tons of active ingredient) for professional or amateur (non-professional and general public) use between 2009 and 2017 (according to ANSES, 2019b and Reboud *et al.*, 2017)

While the quantities of glyphosate sold annually at the national level varied only modestly between 2009 and 2017, sales at the departmental level differed substantially. According to INRA (Reboud *et al.*, 2017), between 2012 and 2016 national sales increased by 1.4% (8,980 to 9,110 tons), but in 10 departments the quantity of glyphosate sold per hectare of utilized agricultural land increased by more than 75%. The study authors explain these variations as a result of the destruction of permanent pastures in northern France, and the planting of new orchards and vineyards in the south.

The French Ministry of Agriculture regularly conducts surveys of farmers' crop practices.[3] These consist of sample surveys of agricultural plots for certain crops of interest, at various time intervals. ANSES has analyzed the data derived from these surveys (ANSES, 2019b). It found that, for field crops in 2014, the proportion of land treated at least once with glyphosate was 55.1% for sugar cane, 29% for sunflowers, 21.8% for protein peas, 18.9% for oilseed rape, 16.8% for barley, 13.3% for soft wheat, 12.9% for grain corn, 11.9% for triticale, 10.7% for durum wheat, 10.6% for forage corn, 10.3% for sugar beet, and 5.9% for potatoes. For viticulture in 2013-2014, the proportion of land treated at least once was estimated to be 72.3%. For arboriculture in 2012, the proportion was 60.6% for plums, 49.4% for apples, and 44.9% for cherries. Finally, for market gardening in 2013, the proportion was 10.1% for carrots and 9.2% for melons, with the other market garden crops studied being less affected (less than 3.6% of land treated). These results demonstrate that glyphosate is heavily used for perennial crops. For field crops, glyphosate is generally used in the intercropping period, i.e. between two successive crops. The INRA report (Reboud *et al.*, 2017) highlights that the use of glyphosate is significantly more common on plots receiving successive oilseed rape and straw cereals, or sunflower and straw cereals, and that, given the variations in rotation, the use of glyphosate varies from region to region. The crop systems used in French agriculture require significant weed control on fields. This is typically done chemically (limiting the need for machinery and labor) and using glyphosate because it is an effective and relatively low-cost herbicide.

[3] . Agreste - La statistique, l'évaluation et la prospective agricole. http://agreste.agriculture.gouv.fr/enquetes/pratiques-culturales/ [accessed January 6, 2020].

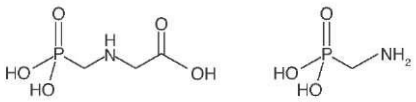
Environmental and food contamination

Glyphosate is a highly polar molecule that is insoluble in organic solvents and is amphoteric (both a base and an acid) (Figure 2). It is an aminophosphonate and is included variously as glyphosate-isopropyl ammonium, glyphosate-potassium, glyphosate-monoammonium, and glyphosate-dimethylammonium salts in the many different commercial formulations available.

As with most pesticides, glyphosate residues are present in the various compartments of the environment (air, soil, water, and food) that may be sources of exposure for the general population.

Glyphosate is rapidly and highly adsorbed in soil (especially soils that have an acid pH, are rich in minerals or low in phosphate), where it is metabolized and rendered inactive by microbial organisms (Mamy, 2004). Degradation of glyphosate results in the formation of several metabolites. The main degradation pathway (99%) involves the activity of the enzyme glyphosate oxidoreductase, which breaks down glyphosate to aminomethylphosphonic acid (AMPA, Figure 2) and glyoxylic acid, which are ultimately mineralized into CO_2 and inorganic phosphate. AMPA is also produced by glyphosate-resistant plants that express this enzyme, and can also result from the degradation of phosphonates, which are notably present in detergents. A lower proportion of glyphosate is degraded by other bacteria to N-methylglycine (sarcosine), glycine, and finally CO_2.

A recent study of arable land in Europe found that glyphosate and AMPA were present at concentrations greater than 0.05 mg/kg in almost half of the over 300 samples tested (Silva *et al.*, 2018). In France, the median concentrations of glyphosate and AMPA in soil from ten agricultural regions were 0.08 and 0.13 mg/kg respectively. AMPA is more persistent in soil than its parent compound, with a half-life that varies considerably depending on the degree of adsorption and degradation (from a few days to a few years) (EFSA, 2015a; ATSDR, 2019).



Glyphosate AMPA

Figure 2: Chemical formulae of glyphosate and its metabolite, aminomethylphosphonic acid (AMPA)

Glyphosate is shown in its acid form; the phosphate group can be anionic and form salts with cations (such as potassium and monoammonium).

Analytical methods

The physicochemical properties of glyphosate present a challenge for quantitative analytical techniques and demand rigorous validated methodologies, particularly since other secondary metabolites may form (following N-acetylation). Robust extraction and advanced purification methods are necessary to avoid matrix effects (the influence of the components of a sample other than the analyte), among others.

Various methodologies have been reported for quantification of glyphosate in water, cereals and other foods, soil, and other matrices. Most of these methods use mass spectrometry (MS) detection with an initial sample processing step adapted to each of the various matrices (water, food, etc.). To provide data on exposure, the analytical method must be sufficiently sensitive to monitor changes in glyphosate concentration based on dose or time. Assay characteristics consist of accuracy (absence of bias), precision (repeatability, intra- and inter-laboratory reproducibility) and quantification (the limit of detection, limit of quantification, and linearity of the model) (ANSES, 2015). The values for the limits of detection (LOD) and quantification (LOQ) are systematically reported in validation dossiers. Methods for estimating the LOD are set out in various regulations, guidelines, and standards. The purpose of these methods is to determine the limit below which the analyte is considered "not detected." In general, the LOQ represents the lowest concentration in a sample that can be quantified with acceptable precision and accuracy under the specified experimental conditions.

Quantification of glyphosate in urine is commonly performed to measure exposure in various populations; it uses either analytical chemistry or immunology methods, although these were initially developed for monitoring water, soil and food contamination. EFSA has proposed using total glyphosate, AMPA, N-acetyl AMPA and N-acetyl glyphosate concentrations for risk assessment, which favors analytical chemistry methods since antibody-based detection may be more challenging due to the diverse range of metabolites formed (EFSA, 2019a). For analytical chemistry-based glyphosate measurement, the detection techniques include capillary electrophoresis (Lanaro *et al.*, 2015), liquid chromatography and fluorescence detection (Wang *et al.*, 2016) and liquid chromatography and tandem mass spectrometry (LC-MS/MS), which is currently the method most commonly used (Bernal *et al.*, 2012; Liao *et al.*, 2018). As glyphosate is highly hydrophilic, non-volatile, has low molar mass and no chromophore

group,[4] it is difficult to analyze using HPLC (high performance liquid chromatography) with UV detection or fluorescence or GC (gas chromatography)-MS without a prior derivatization step (Yoshioka *et al.*, 2011). This involves addition of a chromophore agent to facilitate separation either by gas chromatography or by liquid chromatography, bearing in mind that each of these methods has specific constraints that must be taken into consideration. The derivatization step is generally performed using 9-Fluorenylmethyl chloroformate to obtain acceptable retention times in the chromatography columns (Botero-Coy *et al.*, 2013). Analysts have recently developed mass spectrometry (MS or MS/MS) with electrospray methods and have proposed several LC-MS/MS measurement methods adapted to different matrices (Martins-Junior *et al.*, 2009). Numerous articles report the use of MS detection assays on water and food samples but only a few articles report them in biological fluids such as urine. Urine samples are usually treated with an aqueous acid solution (0.1% formic acid). Glyphosate and AMPA have been measured in urine using LC-MS/MS with the addition of labeled internal standards (0.05 mg/mL $D_2{}^{13}C^{15}N$-AMPA and $^{13}C3^{15}N$-glyphosate). This method provides an assay linearity of 0.05 to 1,000 µg/L with an LOQ value of 0.1 µg/L (Jensen *et al.*, 2016). The LOD for glyphosate was set at 0.05 µg/L based on a signal-to-noise ratio greater than 3:1. It should be noted that assay linearity can be obtained over a larger concentration range, between 0.1 and 10 µg/L (Conrad *et al.*, 2017).

The enzyme-linked immunosorbent assay (ELISA) method is routinely used to measure pesticide levels in various matrices with low cross-reactivity for other substances (Byer *et al.*, 2011). One ELISA kit widely used to measure glyphosate, marketed by Eurofins Abraxis, is based on a competitive reaction between the glyphosate present in the sample and a glyphosate-enzyme complex for binding to an anti-glyphosate polyclonal antibody that is detected using a colored derivative. The quantification scale of a sample in water is 0.075 to 4 µg/L. Cross-reactivity for AMPA or glufosinate is low, around <0.1%.

The LC-MS/MS technique can be used to quantify glyphosate and AMPA and has higher sensitivity than the ELISA assay (LOD = 0.025 µg/L compared to 0.1 µg/L) (Byer *et al.*, 2008). There is however a strong

4. A chromophore is a group of atoms with one or more double bonds, forming a sequence of conjugated double bonds with the rest of the molecule, which usually gives it an easily analyzable color.

correlation between the results produced by these two methods (R^2 = 0.88), although the ELISA method tends to overestimate the concentration of glyphosate. This overestimation may be due to interference from ions, salts and/or phosphates (Clegg *et al.*, 1999). The ELISA method has been compared to the GC-MS assay and a correlation coefficient of 0.87 was estimated for urine (Krüger *et al.*, 2014). The measurement of glyphosate in water using HPLC was compared with a first-generation ELISA (Rubio *et al.*, 2003). For the immunoassay, the LOD was 1 µg/L or 0.1 µg/L if a prior derivatization step was performed. Interference with glufosinate or AMPA was very low, with cross-reactivity <0.01%. For the HPLC method, the LOD was 50 µg/L. More recently, the Eurofins Abraxis ELISA kit was used to monitor the contamination of groundwater, drinking water, and urine of farmers exposed to glyphosate (Rendon-von Osten and Dzul-Caamal, 2017). For measuring glyphosate in water, the LOQ was 0.13 µg/L and the maximum detectable concentration was 4 µg/L.

In summary, the two methodologies have similar sensitivity for glyphosate, with a low cross-reactivity effect from the matrix. However, analytical chemistry methods (MS) allow linearity across a greater range and can also be used to measure AMPA, which is not the case for ELISA assays.

Monitoring in ambient air

Due to its negligible vapor pressure[5] of around 9.8×10^{-8} to 1.94×10^{-7} mmHg, glyphosate is not prone to volatilizing from treated surfaces, and its presence in the atmosphere is related to the drift of spray droplets. ANSES has compiled all the measurements taken by the *Associations agréées de surveillance de la qualité de l'air* [Approved Air Quality Monitoring Associations] (AASQA) available between 2012 and 2015 (ANSES, 2019b). None of the AASQA analyzed glyphosate in ambient air. However, according to a study conducted in the PACA region, in the 83 analyses carried out between 2015 and 2016, glyphosate was detected 5 times, equating to a detection rate of 7%. Of the 4 sampling sites, 3 detected glyphosate in the air at least once. Samples were taken for 24 h with a sampling flow of 30 m^3/h and the glyphosate concentrations ranged from 0.18 to 1.04 ng/m^3 (Ravier *et al.*, 2019). Monitoring of pesticides in the air is not regulated and these concentrations are difficult to interpret at present.

5. https://pubchem.ncbi.nlm.nih.gov/compound/Glyphosate [accessed January 6, 2020].

Monitoring in the aquatic environment

Water quality regulations are derived from Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000, which establishes a framework for Community water policy.[6] In France, a water quality monitoring program has been put in place for the different categories of water (surface freshwater, groundwater, coastal and transitional waters). For rivers and canals, the monitoring system consists of 1,500 permanent "watercourse" sites across the country (mainland France), with further canal sites, international sites, and overseas departments sites.[7] Groundwater is monitored by 2,356 stations (in 2015).[8] The sites have been selected to provide a consistent and comprehensive picture of groundwater chemical status and provide representative control data.[9] The same applies to surface water. The pesticides that are measured are not the same across all stations. While there is a requirement for routine monitoring of certain pesticides (including atrazine and diuron), others, such as glyphosate and its metabolite AMPA, are only tested at some of the stations (at least 25% of them). Testing for different pesticides is mainly linked to the local context and the potential risk of environmental contamination. The earliest available data for glyphosate[10] are from July 2000. Over the year 2000 as a whole, 117 stations analyzed glyphosate. The respective station numbers were 2,038 in 2010 and 3,272 in 2018.

The acid form of glyphosate, N-(phosphonomethyl)glycine, is relatively insoluble in water (10.5 g/L at 20°C, pH = 1.9). It is typically used in salt form in order to significantly increase its solubility (<1 kg/L for the isopropylamine salt), with consequent potential for contamination of aquatic environments. The aquatic environment is the most monitored compartment of the environment in relation to pesticide contamination. It is covered by regulatory standards and numerous, regular monitoring

[6] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

[7] Circular of 13 July 2006 on the establishment and implementation of the surface freshwater monitoring program pursuant to Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

[8] Système d'information sur l'eau, SIE [French Water Information System]. Available from www.eaufrance.fr

[9] Directive 2006/118/EC of 12 December 2006 on the protection of groundwater against pollution and deterioration.

[10] Système d'information sur l'eau, SIE. Available from www.naiades.eaufrance.fr

plans.[11] The Order of 01/11/2007 (concerning the maximum limits and quality standards for raw water and water intended for human consumption referred to in Articles R. 1321-2, R. 1321-3, R. 1321-7 and R. 1321-38 of the French Public Health Code), establishes the following: *i*) for pesticides in raw waters from all sources, used to produce water intended for human consumption, a standard of 0.5 µg/L for each pesticide considered individually and of 2 µg/L for the sum of all individual pesticides detected and quantified (and their metabolites); and *ii*) for pesticides in water intended for human consumption, a standard of 0.1 µg/L for each pesticide considered individually and 0.5 µg/L for the sum of all individual pesticides detected and quantified (and their metabolites). These values apply to glyphosate. Only 4 pesticides are subject to the more stringent standards of 0.03 µg/L: aldrin, dieldrin, heptachlor, and heptachlor epoxide. These standards, which apply across the European Union,[12] were set on the basis of the analytical limits of detection at the time they were established and do not, under any circumstances, correspond to reference values for public health. As a result, they cannot be compared - from a public health risk point of view - to concentrations observed in other biological media or matrices (such as urine). The maximum values for glyphosate in drinking water in Canada[13] and the United States[14] are higher, at 280 and 700 µg/L respectively.

With regard to surface water, ANSES (ANSES, 2019b) has compared the data derived from monitoring plans to the environmental quality standard[15] which is set at 28 µg/L for glyphosate and 452 µg/L for its metabolite

[11] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community water policy.

[12] Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption.

[13] https://www.canada.ca/en/health-canada/semces/publications/healthy-living/guidelines-canadian-drinking-water-quality-guideline-technical-document-glyphosate.html [accessed January 6, 2020].

[14] https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations [accessed January 6, 2020].

[15] Environmental quality standards (EQS) are used to determine annual average maximum concentrations in order to protect organisms from possible prolonged exposure to pollutants in aquatic environments. The EQS for freshwater not intended for the production of drinking water is determined by applying a safety factor to the lowest dose with an acute toxic effect. The *Institut national de l'environnement industriel et des risque*s [French National Institute for Industrial Environment and Risks] (INERIS) set an EQS of 28 µg/L for glyphosate, calculated based on the NOEC (No Observed Effect Concentration) for a diatom (https://substances.ineris.fr/fr/ substance/cas/1071-83-6/3 [accessed January 10, 2020]). For AMPA, the EQS is 452 µg/L, calculated based on the EC_{50} in green algae. https://substances.ineris.fr/fr/substance/cas/1066-51-9/3 [accessed January 10, 2020].

AMPA. In mainland France, between 2007 and 2017, glyphosate testing was performed at 36.7% (2007) to 94.7% (2017) of the measurement sites where testing was performed for at least one pesticide. Glyphosate was quantified at 22.2% (2007) to 49.7% (2017) of these sites. Just one site, in 2014, had an annual average glyphosate concentration (70.2 µg/L) higher than the quality standard for surface water. We note that the quantification rate[16] increased regularly between 2007 and 2017, but that the limits of quantification also improved in parallel during this period. It is therefore not possible to directly link the increase in the quantification rate to an increase in environmental contamination by glyphosate without knowing the individual measurement values. For AMPA, the testing rate across the measurement sites was similar. It was quantified at 43.1% (2007) to 74.4% (2017) of these sites and was not recorded as exceeding the standards at any site. In the French overseas departments and regions (DROM), glyphosate testing was carried out at 44.6% (2007) to 100% (2011) of measurement sites. It was quantified in 2.1% (2008) to 16% (2007) of cases and none of the sites had an annual average above the standard. For AMPA, the testing rate ranged from 41.9% (2007) to 100% (2011), and the quantification rate from 9.2% to 28% (2013), and no nonconformities were observed.

For groundwater, the data presented by ANSES (ANSES, 2019b) were compared to the standard of 0.1 µg/L applying to water intended for human consumption. In mainland France between 2007 and 2017, glyphosate was quantified in 0.5% (2009) to 3.4% (2017) of the analyses carried out and the annual average concentration was higher than the maximum limit of 0.1 µg/L at 0.4% (2012) to 1.5% (2007) of measurement sites. AMPA was quantified in 0.8% (2009) to 4.7% (2012) of the analyses carried out and the standard was exceeded at 0.5% (2013/2014/2016) to 1.4% (2012) of the measurement sites. In the DROM, glyphosate was quantified in 0% (2010 to 2014) to 4.2% (2007) of analyses and the standard was exceeded at 0% (2010 to 2014) to 7.4% (2007) of measurement sites.

For water intended for human consumption, the non-compliance rate (>0.1 µg/L) ranged from 0.05% (2017) to 0.26% (2007) for glyphosate and from 0.04% (2017) to 0.29% (2011) for AMPA. None of the measurements exceeded the reference value derived from toxicology values (a health

[16].The number of analyses in which the pesticide in question was quantified as a percentage of all analyses of this parameter.

threshold) which is set at 900 μg/L for total glyphosate and AMPA concentrations (AFSSA, 2007).

In summary, glyphosate and AMPA are found in all aquatic compartments, to a greater extent in surface water and to a lesser extent in groundwater and water intended for human consumption. In surface water, the *Commissariat général au développement durable* (French General Commission for Sustainable Development) (2015) reported that between 2009 and 2013 the products with the highest total concentrations were, for herbicides, glyphosate and AMPA, followed by atrazine, metolachlor, S-metolachlor, chlortoluron, isoproturon, bentazone and their degradation products; for fungicides, boscalid; and for insecticides, imidacloprid. No cases in which the ecotoxicity threshold were exceeded were observed for glyphosate and AMPA. In groundwater, the most common substances, some of which have been banned for at least a decade, in their original or partially degraded form, were: atrazine (ethyl atrazine present at over 51% of the measurement sites in 2017), dimethachlor, metolachlor, metazachlor, simazine, bentazone, oxadixyl, alachlor, chlortoluron and glyphosate (*Ministère de la Transition écologique et solidaire*, 2019).

Monitoring in foodstuffs

Glyphosate and its metabolite AMPA are contaminants found in food products, raw agricultural products and processed products. Maximum residue limits (MRLs) for these compounds are set by law, and vary depending on the foodstuff. For human foodstuffs, the MRLs are 0.05 mg/kg for milk, eggs and meat, 0.1 mg/kg for most plant foods, and 10 to 20 mg/kg for seeds, while for animal feeds, they are around 500 mg/kg. These values are derived from good agricultural practices and have not been set as limits beyond which a toxic effect is observed in humans. They are generally conservative and much lower than those corresponding to an exposure level susceptible to induce toxicity.

Glyphosate was found, in trace amounts, as a contaminant of a glyphosate-tolerant genetically modified corn (the NK603 variant grown in Canada) treated or not treated with the herbicide (16 μg/kg for treated corn) (Chereau *et al.*, 2018). However, for genetically modified soybean, contamination with glyphosate and AMPA was found with a value of around 10 mg/kg while it was not detectable in organic soybean (Bohn *et al.*, 2014).

The 2017 European Union report on pesticide residues in food presents findings from all Member States along with Iceland and Norway, across a total of 88,247 samples (EFSA, 2019b). Glyphosate testing was performed in 2017 by 25 countries on 8,672 samples, of which 71 consisted of baby food samples and 306 samples of food of animal origin (including honey). In 97.5% of the samples, glyphosate was not quantified. In 2.2% of the samples (191 samples), glyphosate was quantified at levels above the limit of quantification, but below the MRL, and residue concentrations exceeded the MRL in only 21 samples (0.2%). Glyphosate residues were not detected in any of the baby food samples. Exceeded MRLs were identified in samples from Germany (7 honey samples), Italy (1 asparagus sample and 1 rye sample), Poland (1 buckwheat sample and 1 pear sample), Austria (1 honey sample), France (1 rice sample) and 8 samples of unknown origin (5 rye and 3 buckwheat).

Toxicokinetics in humans

There are few studies providing accurate toxicokinetic data for glyphosate in humans. Dermal absorption is considered to be the primary route of pesticide exposure in occupational settings (Lavy *et al.*, 1992; Connolly *et al.*, 2019a). Studies in monkeys found that dermal absorption, for both concentrated Roundup and a diluted dose equivalent to agricultural use, was 2.2% for the highest concentration tested (270 $\mu g/cm^2$). In *in vitro* studies carried out on human skin samples, a level of less than 2% was observed after 16 h of contact (Wester *et al.*, 1991). Inhalation is considered to be a minor route of exposure (Jauhiainen *et al.*, 1991; Johnson *et al.*, 2005; IARC, 2017).

In its toxicological profile for glyphosate, the US Agency for Toxic Substances and Disease Registry (ATSDR) reports being unable to locate any human or animal data concerning distribution of glyphosate after contamination via inhalation (ATSDR, 2019). Limited human data were found regarding distribution of glyphosate after oral intake. The detection of glyphosate in serum and/or urine samples from individuals who had ingested products containing glyphosate - intentionally or unintentionally - confirms absorption by the gastrointestinal tract (Hori *et al.*, 2003; Sribanditmongkol *et al.*, 2012; Zouaoui *et al.*, 2013). The absorption rate of glyphosate in cases of oral ingestion, estimated from data in rats, is approximately 20-30% (ATSDR, 2019). Recently, studies have been conducted in male volunteers who consumed a meal with a known glyphosate content (Zoller *et al.*, 2020) or ingested glyphosate dissolved in

water at a known concentration (Faniband *et al.*, 2021). Both studies found that the amounts of glyphosate excreted in the urine represented between 1 and 6% of the ingested dose. The AMPA metabolite represents only 0.01 to 0.3% of the ingested dose of glyphosate on a molar basis. These findings make it reasonable to assume that the level of absorption of glyphosate in the gastrointestinal tract in humans is much lower than that observed in rats. After absorption, glyphosate is readily distributed by the blood without significant accumulation in any particular tissue.

Glyphosate does not undergo significant metabolism in humans or rodents and only a small fraction (<1%) is metabolized to AMPA (ATSDR, 2019). Recent work by Zoller *et al.* (2020) and Faniband *et al.* (2021) confirms the very low metabolism of glyphosate in humans. Animal studies have identified the feces and urine as the main routes of elimination of glyphosate; the quantities excreted in urine reflect the quantity of glyphosate absorbed, and the quantities in the feces reflect the amount of glyphosate unabsorbed after oral exposure (ATSDR, 2019). IARC has estimated the serum half-life of glyphosate in rats exposed orally as 33 h (IARC, 2017), while the half-life calculated by Roberts *et al.* was 3 to 4 h for the elimination of glyphosate from the blood of patients who had intentionally ingested large amounts of products containing glyphosate (Roberts *et al.*, 2010). In other cases of poisoning, plasma concentrations of glyphosate decreased rapidly (within 2-3 days) from the start of observation (Talbot *et al.*, 1991). Connolly *et al.* recently reported a mean half-life of glyphosate between 5 h 30 and 10 h, depending on whether or not the concentrations were creatinine-adjusted or took into account the urinary excretion rate (Connolly *et al.*, 2019b).

Biomonitoring

The estimation of glyphosate exposure levels in the general population and among agricultural professionals falls under the scope of biomonitoring. Biomonitoring studies involve quantification of a chemical or biomarker in a biological compartment (such as blood, urine, sweat, hair, breast milk, or the umbilical cord) with the objective of assessing exposure to this agent. The development of biomonitoring methods requires a strategy for collecting and storing samples, consideration of the toxicokinetics of the product with its interspecies variations, and matrix selection (blood, urine, etc.) (Barr *et al.*, 2006).

Glyphosate absorbed after oral intake is primarily excreted unchanged in the urine, which validates quantification of this pesticide in the urine as a way to assess recent exposure compared to other matrices (blood, saliva, etc.) (Curwin *et al.*, 2006). Urine is therefore considered to be the best matrix for biomonitoring studies (Acquavella *et al.*, 2004; Curwin *et al.*, 2006; Krüger *et al.*, 2014; Niemann *et al.*, 2015). The guidelines advise adjusting the urine concentration of chemicals to creatinine when conducting environmental exposure studies (Barr *et al.*, 2005). Ideally, a 24-hour urine collection should be used for biomonitoring studies, but the elimination half-life of glyphosate [17] can be used to estimate recent exposure to glyphosate using spot urine samples collected over over the course of a day (Connolly *et al.*, 2018; Connolly *et al.*, 2019b).Few biomonitoring studies in France have provided data on exposure to glyphosate. The most commonly measured pesticides are from the organochlorine, organophosphate, pyrethroid and triazine families (Chevrier *et al.*, 2011; Fréry *et al.*, 2013; Dereumeaux *et al.*, 2016).

The PELAGIE cohort was set up in Brittany between 2002 and 2006 to study the impact of environmental contaminants on intrauterine and child development (Chevrier *et al.*, 2009). An exploratory study of prenatal measurements of herbicides in urine (using liquid chromatography coupled with triple quadrupole mass spectrometry; LOQ = 50 ng/L) was conducted in 47 women from the cohort in early pregnancy who reported using herbicides on their home lawns or pathways. Glyphosate was quantified in 43% of the women with a median level of 0.20 µg/L, and AMPA in 36% of women with a median level of 0.31 µg/L (Vandentorren and Guldner, 2013; ANSES, 2019b). The authors point out that no factors related to characteristics of weedkiller use (such as the frequency, or form of the product) appear to determine the detection rate or urinary level of this exposure.

Santé Publique France (the French Public Health Agency) runs a national biomonitoring program consisting of two complementary components, one of which has a perinatal focus (Dereumeaux *et al.*, 2016). This component studied 4,145 pregnant women included in the ELFE cohort who gave birth in France in 2011 (Vandentorren *et al.*, 2009; Pirus *et al.*, 2010) and it

[17] The time it takes for the blood concentration of glyphosate in the body to halve during its elimination phase.

measured 130 exposure biomarkers (including pesticides) in urine samples. Glyphosate was measured using LC-MS/MS (LOD = 15 ng/L; LOQ = 50 ng/L) in the urine of 1,036 women collected at the time of their admission to the maternity ward. Glyphosate and its metabolite were quantified in only a few cases: 0.3% of the samples had a glyphosate level above the LOQ; 0.1% for AMPA.

At the European level, since the debate over glyphosate emerged, measurement of this substance has been incorporated into urine testing for xenobiotics in biomonitoring programs, notably the European biomonitoring program HBM4EU.[18]

At the international level, a recent literature review identified studies documenting exposure levels in occupational settings (8 studies) and in the general population (14 studies) (Gillezeau *et al.*, 2019). The subjects in the occupational population consisted of either forestry workers (Jauhiainen *et al.*, 1991), horticultural workers (Connolly *et al.*, 2017; Connolly *et al.*, 2018), or farmers and their families (Acquavella *et al.*, 2004; Curwin *et al.*, 2006; Mesnage *et al.*, 2012; Jayasumana *et al.*, 2015b; Rendon-von Osten and Dzul-Caamal, 2017). An additional study on occupational exposure was published subsequently (Perry *et al.*, 2019) bringing the total to 9 studies conducted in the United States (n = 3), Ireland (n=2), Finland (n=1), France (n=1), Mexico (n=1), and Sri Lanka (n=1). Measurements were performed on samples from a total of 423 subjects, including 73 spouses and 148 children, and urinary levels of glyphosate ranged from 0.26 µg/L to 73.5 µg/L (data reported by Gillezeau *et al.*, 2019). The findings are difficult to compare as the occupational work and sampling times are fairly heterogeneous. These results provide only limited information on changes in levels of exposure over time. With regard to the French study (Mesnage *et al.*, 2012), the results concerned 5 people: a farmer, his wife and their 3 children. The concentrations of glyphosate measured in the worker were 9.5 µg/L after spraying glyphosate and 2 µg/L two days later. The literature review identified 14 studies conducted in the general population, covering a total of 3,298 subjects. The biological matrices were urine (n=11), breast milk and urine (n=1), and umbilical cord and maternal blood (n = 2). Pregnant women were the subject of four studies. The urinary levels ranged from 0.16 µg/L to 7.6 µg/L.

[18] https://www.hbm4eu.eu [accessed January 6, 2020].

Another literature review dating from 2015 reported the results of 7 studies in Europe and the United States documenting the level of exposure to glyphosate in urine (Niemann *et al.*, 2015). In the studies conducted in the United States, the average urine concentration was 2 to 3 µg/L, with major variations, including a maximum value of 233 µg/L, compared to an average of 1 µg/L with a maximum value of 5 g/L in the studies conducted in Europe. The authors compared the levels of contamination to a number of reference values and concluded firstly that exposure estimates were lower than the acceptable daily intake (ADI, the amount that can theoretically be ingested on a daily basis without risk to health) as well as the acceptable operator exposure level (AOEL), and secondly that workers or consumers were not exposed to levels likely to result in health risks.

Could urinary concentrations of glyphosate in the µg/L range be associated with a potential toxic effect? To answer this question, it is necessary to calculate the concentration of glyphosate in the urine for an individual exposed to the maximum dose with no toxic effect. In humans, the ADI following oral exposure is currently set at 0.5 mg/kg bw per day (EFSA, 2015a; upheld in 2018). This value is calculated based on the *no-observed-adverse-effect level (NOAEL)* set at 50 mg/kg bw per day on the basis of studies in rats and rabbits. The ADI is obtained by dividing the NOAEL by a safety factor of 100, corresponding to the inter- (10) and intra-species uncertainties (10) (which are therefore multiplied, 10x10=100). Therefore, for a person weighing 60 kg, with an absorption rate of 20% for glyphosate (Niemann *et al.*, 2015) and rapid urinary elimination with a urine volume of 1.5 to 2.0 L/day, the calculation is: 0.5 x 60 x 0.2/1.5 to 2 = 3.0 to 4.0 mg/L glyphosate in 24 h urine (or 3,000 to 4,000 µg/L). This value is much higher (3,000 to 4,000 times) than that found in urine, which is in the order of µg/L for the general population in Europe. Even if the ADI value is underestimated by a factor of 10, the safety factor would be 300-400, which given the current state of knowledge and experimental methods used would support no risk to health. In addition, in urine tests carried out in exposed workers, the average exposure corresponded to 0.4% of the ADI with a maximum of 2%, or an average of 2 µg/kg/day (Niemann *et al.*, 2015). Recent studies showing that intestinal absorption of glyphosate is less (1 to 6%, Zoller *et al.*, 2020; Faniband *et al.*, 2021) than initially estimated (20-30%, ATSDR, 2019) do not alter the ADI, as it is calculated by studying the link between the external dose and the occurrence of a toxic effect.

The biomonitoring studies described above provide information on levels of exposure to glyphosate and its metabolites. Interpretation of the

experimental data is based on practical and theoretical considerations (interindividual variability, whether or not the exposure is chronic, cocktail effects, intraspecies variation), and a risk to humans from exposure to glyphosate cannot be excluded on the basis of these studies. This highlights the importance of assessing the potential health risks of exposure to glyphosate through epidemiological studies, which are examined below.

Epidemiological data

Epidemiological studies of exposure to glyphosate have looked at several cancers, including solid tumors and hematological cancers (notably non-Hodgkin lymphoma; NHL), as well as non-cancer diseases such as respiratory diseases, Parkinson's disease, endocrine disorders, anxiety and depression, and developmental disorders.

Regulatory agencies assess the toxicity of pesticides such as glyphosate to humans and to the environment, yet living organisms are exposed not only to the active substance but also to co-formulants. This is also true for the vast majority of pesticides, which are formulations containing adjuvants of various kinds. Although few epidemiological studies on glyphosate have taken formulations into account, they nevertheless provide evidence regarding the public health risks potentially associated with occupational or environmental exposure to glyphosate.

Non-Hodgkin lymphoma

Data from the 2013 collective expert report

In the collective expert report of pesticides carried out by Inserm in 2013 (Inserm, 2013), glyphosate was associated with a significant excess risk of NHL, with a low or moderate presumption of a link depending on the populations exposed (farmers, occupational exposure encompassing any category of use: applicators, livestock farmers). This was also the case for the combination of glyphosate and malathion (Table II). For details on how the presumption of a link was categorized by the expert group, see Annex 2.

The review of the literature in 2013 found a heterogeneity of results between the cohort and case-control studies: there was no association between exposure to glyphosate and the risk of NHL in the Agricultural Health Study (AHS) cohort in the US, in contrast to the results of the case-control studies, notably those conducted in the US, Canada and Sweden.

In these studies, significant increases in the risk of NHL were observed and persisted after adjusting for exposure to other pesticides, but the findings were based on limited population sizes.

Table II: Exposure to glyphosate and occurrence of NHL: 2013 collective expert report

	Population	Presumption of a link
Glyphosate	Farmers	±
	Occupational exposure	+
Glyphosate + malathion	Occupational exposure	±

+ based on four case-control studies

± based on the results of a cohort study (farmers) and case-control study (glyphosate + malathion)

One of the case-control studies (Eriksson *et al.*, 2008) reported a statistically significant association with certain subtypes of B-cell lymphoma such as lymphocytic lymphoma/chronic lymphocytic leukemia. A case-control study conducted in Canada found that exposure to glyphosate alone did not increase the risk of NHL, while its use in combination with malathion resulted in a significant increase in risk (OR = 2.10; 95% CI [1.31-3.37]) (Hohenadel *et al.*, 2011).

New data

- ***Meta-analyses***

A systematic literature review accompanied by a meta-analysis based on 44 original research articles evaluated the associations between NHL and exposure to 21 pesticide chemical groups and 80 active ingredients, including glyphosate (Schinasi and Leon, 2014). The risk of NHL was positively associated with occupational exposure to glyphosate (mRR = 1.5; 95% CI [1.1-2.0]), a result observed from previously published studies already taken into account in the previous collective expert report (five case-control studies and the AHS cohort via the analysis published in 2005). The association was stronger when the link between exposure to glyphosate and B-cell lymphoma was considered (mRR = 2.0; 95% CI [1.1-3.6]), with no between-study heterogeneity.

A meta-analysis of agricultural cohorts, recently published by the AGRICOH consortium (Leon *et al.*, 2019), examined the role of exposure (in a binary manner: yes/no) to 14 pesticide chemical groups and 33 active ingredients, including glyphosate, and the risk of NHL and its major

subtypes. This work was based on data from three cohorts of agricultural workers: AHS (United States), AGRICAN (France) and CNAP (Norway), combining over 300,000 subjects, 2,430 cases and 3.5 million person-years under risk. Few of the associations investigated appeared to be significant but a statistically significant positive association was observed between glyphosate exposure and the risk of diffuse large B-cell lymphoma (DLBCL, one of the most common subtypes of NHL), after adjustment for the other pesticides (mRR = 1.4; 95% CI [1.0-1.9]). No between-study heterogeneity was found across the results of the three cohorts. This statistically significant increased risk was primarily based on data from the Norwegian cohort (CNAP) with a risk of DLBCL associated with exposure to glyphosate of RR = 1.7; 95% CI [1.1-2.7] (100 exposed cases), while the risks were slightly elevated but not significant in the US (AHS) and French (AGRICAN) cohorts, with the following respective results: RR = 1.2; 95% CI [0.7-2.0] (93 exposed cases) and RR = 1.1; 95% CI [0.5-2.2] (28 exposed cases). Glyphosate was used by 38%, 83% and 36% of farmers and agricultural workers in the CNAP, AHS and AGRICAN cohorts respectively.

Between 2013 and 2019, two other meta-analyses (Chang and Delzell, 2016; Zhang *et al.*, 2019) and a pooled analysis (Pahwa *et al.*, 2019) examined the relationship between occupational exposure to glyphosate and the risk of NHL by combining the same studies as Schinasi and Leon (2014). The meta-analyses were based on the same five previously published case-control studies (between 2001 and 2009) and on data from the AHS cohort. Zhang *et al.* included the most recent analysis of the AHS cohort, published in 2018 (Andreotti *et al.*, 2018), while Chang and Delzell used an analysis of the AHS published in 2005 (de Roos *et al.*, 2005).

The first meta-analysis (Chang and Delzell, 2016) found a statistically significant increased risk of NHL in farmers exposed to glyphosate, with mRR = 1.3; 95% CI [1.0-1.6] with little between-study heterogeneity (I^2 = 0%) and no publication bias detected. This association was robust in several secondary analyses using different statistical models, when the results of one study were substituted with those from a more recent analysis of the same population, and when both of these factors were tested together.

The second meta-analysis (Zhang *et al.*, 2019) focused on the subjects most exposed to glyphosate (in duration and intensity) and reported a slightly higher risk: mOR = 1.4; 95% CI [1.1-1.8]. An additional analysis based on the 2005 AHS data and the most highly exposed groups found a comparable risk of NHL: mRR = 1.5; 95% CI [1.1-1.9]. Several sensitivity analyses using different exposure selection criteria, based on the subtypes of NHL included,

the geographic location of the study, exposure to other pesticides, the type of statistical analysis, and the exclusion of one of the six studies in turn, found comparable risks. Overall, this latter study reported a slightly higher risk than the other two meta-analyses (Schinasi and Leon, 2014; Chang and Delzell, 2016). This study is the only one to tackle analysis of the groups most exposed to glyphosate. Another notable difference is the inclusion of multiple myeloma cases in the NHL group in the latest version of the AHS analysis (in line with the WHO classification and a publication from the InterLymph Consortium classifying this malignant blood disorder in the mature B-cell NHL group as it derives from monoclonal proliferation of plasma cells). However, a sensitivity analysis found that the results were not explained by the inclusion of myeloma. An association between multiple myeloma and glyphosate, if it existed, might have caused this increased risk, but this was not shown in the most recent analysis of the AHS cohort and is now based on a low level of evidence. No analysis by NHL subtype was included in the meta-analysis by Zhang *et al.* The only two results in a similar vein and specific to a subtype of lymphoma (DLBCL and more broadly B-cell lymphoma) were therefore those published by Leon *et al.* in 2019 and Schinasi and Leon in 2014, discussed above.

Finally, a pooled analysis (meta-analysis of individual data) published in 2019 examined the specific role of glyphosate exposure in the risk of NHL and its major subtypes (Pahwa *et al.*, 2019). It used case-control studies conducted in the US and Canada (North American Pooled Project), the majority of which were included in the meta-analyses described above. The authors found that subjects who used glyphosate were at increased risk of NHL (OR = 1.4; 95% CI [1.1-1.8]) but, after adjusting for exposure to the pesticides 2,4-D, dicamba and malathion, the risk was reduced and non-significant (OR = 1.1; 95% CI [0.8-1.5]). The authors reported the same findings for the analysis by NHL subtype. A statistically significant positive association with the risk of NHL was however observed in subjects in the 3rd tertile, consisting of those who had used glyphosate more than two days per year (OR = 1.7; 95% CI [1.0-2.9]; P-trend = 0.2) as well as in DLBCL subjects (OR = 2.1 [1.1-4.3]; P-trend = 0.2) which is consistent with the meta-analyses described above, particularly those by Zhang *et al.* These increased risks were not however found with the duration of exposure in years. The lifetime trend was significant only for lymphocytic lymphoma when the continuous exposure variable was analyzed, while the risk was increased but not significant when exposure was studied in a binary manner (exposed yes/no).

- ***Cohort studies***

The most recent analysis of the AHS cohort was published separately (Andreotti *et al.*, 2018) and focused specifically on exposure to glyphosate using the most up-to-date follow-up data (cancer incidence follow-up extended through 2012 in North Carolina and through 2013 in Iowa with 7,290 incident cancer cases, including additional information on exposure from a follow-up questionnaire). This analysis found no increased risk of NHL or its major subtypes. Data from the AHS cohort were analyzed in 2019 in conjunction with data from two other cohorts in an analysis conducted by the AGRICOH consortium (see above).

In summary, new data strengthen the presumption of a link between glyphosate and the risk of NHL in farmer populations (moderate presumption; Table III). This conclusion is based partly on the meta-analysis recently published by the consortium of agricultural cohort studies, AGRICOH (Leon *et al.*, 2019), which, based on data from the French and Norwegian cohorts (but not those from the US cohort (AHS)) found an association between diffuse large B-cell lymphoma and exposure to glyphosate, and partly on the three recent meta-analyses and pooled analyses conducted on older studies which systematically show an increased risk (Chang and Delzell, 2016; Pahwa *et al.*, 2019; Zhang *et al.*, 2019), particularly among the most exposed farmers. However, this summary must also take into account the fact that several studies, including a cohort study (AHS, in its most recent analysis conducted in 2018 and published separately), have not found any association and no dose-response relationship has been demonstrated. To our knowledge, no additional data have been published since the 2013 collective expert report to further support an increased risk of NHL from combined exposure to glyphosate and malathion.

Table III: Exposure to glyphosate and occurrence of NHL: new data

	Exposed populations	Presumption of a link
Glyphosate	Farmers, professionals	+

based on the results of three meta-analyses (new analyses but studies already known), a meta-analysis combining data from three cohorts (AGRICOH) and the four case-control studies included in 2013.

Multiple myeloma

Historically, multiple myeloma has been studied separately because it is a long-standing recognized entity considered to be distinct, and a separate section for this disease has therefore been maintained in this update of the collective expert report. A number of recent studies focusing on the effect of a specific pesticide active ingredient have however included cases of multiple myeloma in the NHL group, as in the most recent analysis of the AHS cohort, focusing on exposure to glyphosate (Andreotti *et al.*, 2018).

Data from the 2013 collective expert report

Few cohorts of workers are exposed primarily to a single class of pesticides or a specific pesticide. Cohort studies generally include a very limited number of cases, which makes it extremely difficult to study any potential relationship between this type of exposure and multiple myeloma. Data on glyphosate from the AHS cohort, available in 2013, did however show a trend for increased risk for certain intensity exposure categories but this was not statistically significant. This was also the case for three case-control studies (in Canada, the US, and France), which found a non-significant increase in risk for the most exposed subjects without adjustment for exposure to other pesticides.

New data

Since the Inserm collective expert report published in 2013, a meta-analysis has been published on exposure to glyphosate (Chang and Delzell, 2016) along with a recent update of the AHS cohort focusing specifically on glyphosate exposure (Andreotti *et al.*, 2018).

The 2016 meta-analysis on glyphosate by Chang and Delzell is based on three case-control studies published between 1993 and 2013 and two different analyses of the same data from the AHS cohort published in 2005 (de Roos *et al.*, 2005) and in 2015 (Sorahan, 2015). A statistically significant increased risk of multiple myeloma was observed in glyphosate-exposed farmers (mRR = 1.54; 95% CI [1.0-2.19]) based on the 2005 analysis of the AHS cohort and a risk of comparable magnitude (mRR = 1.4; 95% CI [1.0-1.9]) was found based on the Sorahan analysis published in 2015. This meta-analysis found no between-study heterogeneity of effects (I^2 = 0%) and no identifiable publication bias. This association was robust in several other secondary analyses.

More recently, a re-analysis of the AHS cohort was published. It was based on the data with the longest follow-up (Andreotti *et al.*, 2018) and specifically addressed exposure to glyphosate. It found no association between the latter and multiple myeloma.

Based on these results, the presumption of a link between the risk of multiple myeloma and exposure to glyphosate is low (±). It is based on a low level of evidence: the risk is elevated but at the limit of statistical significance in a meta-analysis of three case-control studies and one cohort study.

Hodgkin lymphoma

The 2013 Inserm collective expert report did not include any specific information on the link between glyphosate and Hodgkin lymphoma. This has been examined in two articles published since that time including a meta-analysis as well as an updated analysis of the AHS cohort, both specifically focusing on exposure to glyphosate.

New data

A meta-analysis looking specifically at glyphosate, published in 2016 (Chang and Delzell, 2016), jointly studied two case-control studies published between 2009 and 2012, and found no association between the risk of Hodgkin lymphoma and glyphosate exposure in farmers (mRR = 1.1; 95% CI [0.7-1.6]). However, the available data are based on limited population sizes.

The most recent re-analysis of the AHS cohort (Andreotti *et al.*, 2018) specifically addressed exposure to glyphosate and found no association with Hodgkin lymphoma. The number of exposed cases was low, reflecting the relative rarity of the disease (10% of lymphomas).

The scientific literature published since 2013 is thus relatively limited, consisting of three studies, including one meta-analysis (based on two case-control studies) and an updated analysis of the AHS cohort, both focused on exposure to glyphosate. These studies, which are based on a small number of cases, did not find an excess risk of developing Hodgkin lymphoma. In light of these findings, no presumption of a link between the risk of Hodgkin lymphoma and exposure to glyphosate can be established.

Leukemia

A study of the AHS cohort, analyzed in the 2013 collective expert report, reported that glyphosate was associated with an increased but not statistically-significant risk of leukemia, a relationship that was found only for the middle tertile of exposure. The authors of the study concluded that there was no association between exposure to glyphosate and the risk of leukemia.

Three meta-analyses have been published since 2013; two of these are based on data from three agricultural cohorts (AHS, AGRICAN and CNAP) and evaluate multiple pesticide families (El-Zaemey *et al.*, 2019; Leon *et al.*, 2019), while the third focuses specifically on exposure to glyphosate and is based on three case-control studies (Chang and Delzell, 2016). An updated analysis of the AHS cohort has also been published specifically looking at occupational exposure to glyphosate.

In this expertise a distinction is made between lymphoid leukemia (often studied with lymphoma and represented by the most common entity, chronic lymphocytic leukemia; CLL) and myeloid leukemia (the entity most commonly studied is acute myeloid leukemia; AML).

- ***Meta-analyses***

Meta-analyses of agricultural cohorts from the AGRICOH consortium found no association between exposure to glyphosate and CLL (it should be noted that none of the analyses looked at the risk of myeloid leukemia in these studies) (El-Zaemey *et al.*, 2019; Leon *et al.*, 2019). A meta-analysis published in 2016 focused specifically on glyphosate (Chang and Delzell, 2016). It analyzed data from three case-control studies published between 1990 and 2009 and did not find an association between the risk of CLL and glyphosate exposure in farmers (mRR = 1.0; 95% CI [0.6-1.5]). However, a meta-analysis of two studies evaluating the association between the use of glyphosate and the risk of hairy cell leukemia found an increased, but non-significant, risk (mRR = 2.5; 95% CI [0.9-7.3]).

- ***Cohort studies***

A recent re-analysis of the AHS cohort was published separately (Andreotti *et al.*, 2018) and focused specifically on exposure to glyphosate using the most up-to-date follow-up data (cancer incidence follow-up extended through 2012 in North Carolina and through 2013 in Iowa with 7,290 incident cancer cases, including additional information on exposure from a follow-up questionnaire). This analysis did not show an increased risk of CLL. However, the risk of AML was increased among the applicators in the

highest exposure quartile (exposure defined by intensity-weighted lifetime days of glyphosate use) compared to non-users (RR = 2.4; 95% CI [0.9-6.3], P-trend = 0.1), although this association was not statistically significant. The results were comparable when a five-year exposure lag (4th quartile RR = 2.3; 95% CI [1.0-5.5], P-trend = 0.07) and 20-year exposure lag (3rd tertile RR = 2.0; 95% CI [1.0-4.0], P-trend = 0.04) were used.

The presumption of a link between exposure to glyphosate and leukemia is weak (±), and is currently based only on the latest results from the AHS cohort, which require confirmation.

Genitourinary cancers

In an initial follow-up of the AHS cohort in 2001 (de Roos *et al*, 2005), no association was found between exposure to glyphosate and the risk of developing prostate cancer, based on 2,088 incident cases. Exposure to glyphosate was analyzed in an initial follow-up analysis of the AHS cohort in 2001 including 2,088 incident prostate cancer cases, and no association was observed (de Roos *et al.*, 2005). In a subsequent follow-up of the cohort, conducted through 2012 or 2013 (depending on the participants' state of residence) and including 2,844 incident cases, no association was observed with the risk of developing this cancer (Andreotti *et al.*, 2018).

Similarly, as part of the AHS, incident bladder cancers diagnosed between enrolment in the cohort (between 1993 and 1997) and 2011 were studied in connection with the use of around fifty substances - including glyphosate - with information gathered via a questionnaire completed during enrolment (Koutros *et al.*, 2016). The analysis included 321 cases of bladder cancer in men (women were excluded from the analysis as only one case was identified). When the cumulative number of lifetime days of use of glyphosate was taken into account, a non-significant increase in risk was observed (RR = 1.93; 95% CI [0.95-3.91]).

Respiratory disorders

In the AHS, the largest study to have investigated the link between exposure to specific active ingredients and various pulmonary function parameters, glyphosate was found to be associated with increased risk of both allergic wheeze (OR = 1.56; 95% CI [1.19-2.03]) and non-allergic wheeze (OR = 1.24; 95% CI [1.07-1.44]) after adjusting for body mass index (BMI), current asthma, age, smoking status, state, frequency of pesticide use and frequency

of diesel tractor use in over 22,000 farmers, with an exposure-response relationship observed for allergic wheeze (Hoppin *et al.*, 2017). Glyphosate was also associated with wheeze in 2,255 industrial applicators in a model adjusted for age, smoking status, asthma/allergy and BMI (OR = 1.38; 95% CI [1.03-1.86]), but this association was attenuated and no longer statistically significant after controlling for exposure to chlorimuron-ethyl in the model (Hoppin *et al.*, 2006). Finally, glyphosate was associated with allergic asthma in female farmers in a model adjusted for age, state, smoking status, and BMI and "growing up on a farm" (OR = 1.31; 95% CI [1.02-1.67]) (Hoppin *et al.*, 2008).

Regarding the impact of exposure to pesticides on asthma exacerbation, a cross-sectional study of 926 farmers with active asthma in the AHS cohort (Henneberger *et al.*, 2014) found a decreased risk of asthma exacerbation with the use of two herbicides including glyphosate (OR = 0.5; 95% CI [0.3-0.8] in a model adjusted for age, state, smoking status, allergy, and age of asthma onset (childhood compared to adulthood), suggesting a possible 'healthy worker effect' (people with asthma prone to exacerbations might avoid forms of exposure that trigger symptoms).

A study by Patel *et al.* of a population of over 11,000 farm operators found an increased risk of current asthma with the use of pesticides, herbicides or insecticides "without distinction" (meaning the association is observed for exposure to these broad categories of products, without identifying specific pesticide families or active substances involved). No association was found with the active ingredients considered separately, with the exception of a positive association close to the threshold for statistical significance for glyphosate (OR = 1.3; 95% CI [0.97-1.8]), after adjusting for sex and region (Patel *et al.*, 2018).

Colombia has a government program designed to combat the illegal cultivation of coca by aerial herbicide spraying (mainly using glyphosate). Based on herbicide spraying data and health care administrative data, a study found a statistically significant increase in the number of medical consultations for respiratory problems within 15 days of spraying pesticides. This study suggests a short-term effect of this exposure on respiratory health in a context of massive use (Camacho and Mejía, 2017).

In summary, the presumption of a link between glyphosate and respiratory health is weak, with a limited number of studies (most from the AHS cohort) showing an increased risk of wheeze (allergic or non-allergic) and asthma.

Parkinson's disease

Two studies, conducted in the US states of Washington (Caballero *et al.*, 2018) and Nebraska (Wan and Lin, 2016), reported a significant increased risk of Parkinson's disease associated with environmental exposure to glyphosate. The first estimated the relationship between exposure to pesticides at home and premature mortality from the disease (defined as <75 years) between 2011 and 2015. The authors combined geospatial data on land use with pesticide application data obtained from a survey of farmers during this period to define a crop-exposure matrix (Caballero *et al.*, 2018). Individuals living within 1,000 meters of pesticide-treated land at the time of their death were considered to be exposed. The authors selected 4 pesticides: glyphosate, diazinon, atrazine, and paraquat. The analyses were adjusted for sex, ethnicity, marital status and level of education. Exposure in the premature death group (≤74 years, n=659) was compared to that of the non-premature group (≥75 years, n = 3,932). No significant association was found between exposure to pesticides (all types) and premature mortality due to Parkinson's disease (OR = 1.19; 95% CI [0.98-1.44]). The association was significant for residential exposure to glyphosate (RR = 1.33; 95% CI [1.06-1.67]) but not for the other pesticides evaluated. Multivariate analyses taking into account exposure to several pesticides were not performed. The main limitation of this study is the use of death certificates to identify cases of Parkinson's disease, as this diagnosis is typically only included on death certificates in about 50% of cases (Paulson and Gill, 1995; Goldacre *et al.*, 2010; Benito-León *et al.*, 2014). Furthermore, comparison of death rates by age would require consideration of confounding factors (smoking and physical activity) whose frequency has changed considerably over time.

A population registry of new cases of Parkinson's disease in Nebraska identified 6,557 incident cases between 1997 and 2008 and was used to perform an ecological study (Wan and Lin, 2016). Land use for 19 agricultural crops was determined from satellite images (2005) and 5 of these were selected that together accounted for nearly all crops planted in the state. The use of pesticides for each crop was determined from several types of sources, including public data (US Geological Survey, National Center for Food and Agricultural Policy) and farmer surveys. Twenty pesticides (15 herbicides and 5 insecticides including 2 carbamates and 3 organophosphates), for which the authors considered the data to be sufficiently complete, were included in the analyses. A geographic information system was then used to determine the spatial distribution of

exposure to these pesticides. After adjusting for age, the incidence of Parkinson's disease in the counties of the state was not associated with exposure to pesticides. However, after excluding the reference group of counties with the highest incidence of Parkinson's disease, which were characterized by low exposure to pesticides, the incidence was higher in the counties with a high level of exposure to atrazine, bromoxynil, alachlor, metribuzin and glyphosate. In a multi-adjusted model incorporating the five pesticides and crop density, only alachlor was significantly associated with the incidence of Parkinson's disease. The limitations of the study include its ecological design at the county level (a relatively large geographical unit), and the lack of adjustment for confounding factors other than age. The relationships identified were only apparent after retrospective exclusion of the counties that had the highest incidence and which, according to the authors, were characterized by a high level of livestock but not other crops. However, the high incidence in these counties remains unexplained.

In a study published in 2007 based on data from the AHS (Kamel *et al.*, 2007), the authors identified 83 prevalent cases of Parkinson's disease at inclusion (1993-1997) and 78 incident cases by the end of follow-up (1999-2003). Diagnosis was based on self-reported disease and was not validated by a standardized neurological examination. The occupational exposure of the cases was compared to that of participants without Parkinson's disease (79,557 at baseline and 55,931 by the end of follow-up). The authors studied exposure to 43 specific pesticides including glyphosate; there was no association between this substance and prevalent (OR = 1.0; 95% CI [0.6-1.7]) or incident (OR = 1.1; 95% CI [0.6-2.0]) Parkinson's disease.

In France, a case-control study (224 cases including 118 men, and 557 controls including 291 men), was conducted using data on members of the *Mutualité Sociale Agricole* agricultural health insurance program (Elbaz *et al.*, 2009). Occupational exposure to pesticides was assessed using a two-phase procedure: interviews with occupational physicians and a case-by-case expert evaluation of exposure questionnaires. The association between 29 pesticide families and Parkinson's disease was evaluated, including for glyphosate, which was the only pesticide included in the organophosphate herbicide group; there was no significant association in men (OR = 1.5; 95% CI [0.8-2.7]; the number of exposed women was insufficient for detailed analyses).

In summary, two ecological studies have been conducted looking at the association of Parkinson's disease with residential exposure to glyphosate. One found an increased risk whereas the other, after adjusting for other pesticides, found no effect. The ecological design of these studies means

that it is not possible to infer a causal relationship. Two studies of occupational exposure to glyphosate reported no significant association.

Anxiety and depression

An association was found between depressive symptoms and pesticide exposure among 220 agricultural workers in a major coffee-growing region in Brazil (Conti *et al.*, 2018). Exposure to pesticides in general (without distinction of the chemical groups or active ingredients involved) was associated with a 5-fold increased risk of depressive symptoms (OR = 5.5; 95% CI [1.2-25.9]). However, specific exposure to glyphosate (38.6% of workers) was not associated with the risk of developing depressive symptoms.

A case-control study in France carried out a cross-sectional evaluation of the link between pesticides and self-reported treatment or hospitalization for depression (Weisskopf *et al.*, 2013). Only individuals working in agriculture were included (n = 567). Analyses were performed for pesticide groups identified in more than 5% of non-depressed individuals. Thirteen groups of herbicides were evaluated, among which were organophosphates including glyphosate and related herbicides. The analysis of 83 patients found an increased risk of depression associated with the use of herbicides in general (HR = 1.93; 95% CI [0.95-3.91]) that was more marked and became significant for individuals who had used them for longer than 19 years (HR = 2.31; 95% CI [1.05-5.10]) or when the number of hours of exposure was taken into account. The herbicide group-specific analyses found significant associations only for carbamates, dinitrophenols and picolinic acid. There was therefore no evidence of a specific link with glyphosate and related substances.

Thyroid disorders

In the AHS cohort (Shrestha *et al.*, 2018a, self-reporting of hypothyroidism at inclusion (n = 829) was studied in relation to lifetime use of a list of 50 pesticides, along with an indicator of cumulative exposure (intensity-weighted lifetime days of use; classified into three or four categories based on the numbers). This questionnaire-based measurement of exposure was performed at baseline for 35,150 professional applicators (98% men) with follow-up of 20 years. Sensitivity analyses showed that patient loss to follow-up had no impact on the measurement of the associations. In this study,

after adjusting for sex, age, region and smoking, the risk of hypothyroidism was increased in participants who had used four organochlorine insecticides (aldrin, heptachlor and lindane among participants over 62 years of age; chlordane among all participants), four organophosphate insecticides (coumaphos in the older group; diazinon, dichlorvos, and malathion for all participants) and three herbicides (dicamba, glyphosate, and 2,4-D across the sample). The HR (hazard ratio) ranged from 1.21; 95% CI [1.04-1.41] for chlordane and 1.54; 95% CI [1.23-19.4] for lindane in those aged over 62 years; for glyphosate it was 1.28; 95% CI [1.07-1.52]. When the use of glyphosate was modulated by duration and intensity of exposure, only individuals exposed in the first and second tertile (compared to the non-user reference group) had a significantly increased risk (HR = 1.27; 95% CI [1.03-1.69] and HR = 1.38; 95% CI [1.12-1.69] respectively). The risk was no longer significant in the most exposed individuals (HR = 1.17; 95% CI [0.94-1.45]) and the dose-response relationship was non-significant (p = 0.95).

In another study of the AHS cohort (Lerro *et al.*, 2018), a moderate, non-significant association was found between glyphosate exposure and circulating thyroid-stimulating hormone (TSH) for the 4th quartile of exposure compared to the non-exposed reference group (OR = 1.14; 95% CI [0.99-1.33]). All the associations were adjusted for age, region, BMI, smoking and other pesticides correlated with the pesticide studied.

A longitudinal analysis of the spouses of applicators from the same cohort was conducted after a 20-year follow-up period (Shrestha *et al.*, 2018b). It included 1,627 reported cases of hypothyroidism and 531 of hyperthyroidism. The use of glyphosate specifically was not associated with the risk of hypothyroidism (HR = 1.07; 95% CI [0.95-1.20]).

In summary, the data on exposure to glyphosate all come from the AHS cohort. An excess risk of hypothyroidism in male pesticide applicators has been observed but this was not found for their pesticide-applying spouses.

Birth defects and childhood diseases

A cohort study conducted on 71 women in India reported a negative correlation between urinary concentrations of glyphosate measured during pregnancy and the duration of pregnancy (Parvez *et al.*, 2018). No correlation was observed with birth weight or head circumference.

A systematic literature review exploring the link between exposure to glyphosate and birth defects, childhood diseases and altered sex ratios was

recently conducted (De Araujo *et al.*, 2016). No association between glyphosate and birth defects and childhood diseases was found except in relation to attention deficit hyperactivity disorder (OR = 3.6; 95% CI [1.3-9.6] in children born to glyphosate applicators. The study reporting this association was conducted in children aged one year and older whose parents were farmers in the Red River Valley (Minnesota) exposed to herbicides, including GBH (Garry *et al.*, 2002).

A case-control study conducted in the Central Valley region of California in the United States (von Ehrenstein *et al.*, 2019) found an increased risk of autism spectrum disorders in children associated with prenatal exposure to certain pesticides estimated by the presence of agricultural pesticide use within 2,000 m of the maternal residence. The pesticides associated with this increased risk included glyphosate (OR = 1.16; 95% CI [1.06-1.27]).

A case-control study conducted in North Carolina in the United States investigated the links between prenatal exposure to pesticides, estimated from their use within 500 m from the place of maternal residence, and the risk of birth defects (Rappazzo *et al.*, 2019). An increased risk of septal heart defects was observed in association with proximity to farms using glyphosate but also cyhalothrin, S-metolachlor, mepiquat, and pendimethalin.

In an ecological study conducted in Colombia, the authors reported an increase in consultations for miscarriages associated with herbicide-spraying campaigns as part of the fight against illegal crops (Camacho and Mejía, 2017).

Chronic kidney disease of unknown etiology

The hypothesis that glyphosate plays a role in the development of chronic kidney disease was first raised in the 1990s, specifically in relation to tropical areas (Sri Lanka, India, Egypt, and Central America), following epidemics of this disease in agricultural workers. These cases of chronic kidney disease with no known cause occur in young people, generally men, working in agriculture, and are thought to have resulted in the premature deaths of tens if not hundreds of thousands of these individuals. In some of these tropical areas, the prevalence of the disease is thought to be around 15% in adults under 70 years of age (Jayasumana *et al.*, 2015a), and is not explained by the presence of diabetes, hypertension or glomerulonephritis. Biochemical investigations have found an increase in plasma creatinine in a preclinical phase, suggesting impaired glomerular filtration, and the pathology evidence from patients points to a toxic nephropathy. Various

hypotheses have been suggested to explain the occurrence of this kidney injury: dehydration linked to physical labor in hot regions, exposure to metals (such as cadmium and lead), pesticides, and mycotoxins (such as ochratoxin A and citrinin).

A case-control study was conducted in Sri Lanka (125 cases and 180 hospital controls), which after adjusting for age, sex, level of education and family history of the disease, identified a link to well water consumption (OR = 2.52; 95% CI [1.12-5.70]) with up to a five-fold increase in risk when the water came from an abandoned well (glyphosate was usually detected; OR = 5.43; 95% CI [2.88-10.26]), and with the use of glyphosate (OR = 5.12; 95% CI [2.33-11.26]). Increased risks were observed for other pesticides (MCPA, bispyribac, and mancozeb) but the levels of risk appeared to be lower than with glyphosate (Jayasumana *et al.*, 2015a).

In 2014, a prospective study including 350 men and women working in agriculture (sugar cane and banana) aged 18-30 years was set up in Nicaragua to monitor their kidney function every 6 months and measure their exposure. Urine measurements were taken at baseline and at 6 months for the following substances: aluminum, arsenic, cadmium, chromium, cobalt, copper, lead, manganese, mercury, selenium, silicone and strontium along with ochratoxin A and citrinin. For pesticides, the urine tests included: 2,4-D, MCPA, glyphosate, metabolites of insecticides including pyrethroids, and metabolites of triazole, pyrimidine, and dithiocarbamate fungicides. Glyphosate was detected in approximately 30% of urine samples (limit of detection 0.1 µg/L). Although approximately 10% of men and 3% of women experienced a rapid decrease in kidney function after 2 years of follow-up, no differences were found between the levels of the various contaminants measured in their urine compared to those of individuals with stable kidney function (Smpokou *et al.*, 2019).

In summary, it is not currently possible to draw definitive conclusions regarding the etiology of this kidney disease, which has become a major public health issue in several tropical countries, although factors associated with agricultural activities or rural living have been identified in most locations. The involvement of pesticides remains an area of interest, but there is too little evidence to draw a conclusion about the specific role of glyphosate.

Toxicology data

The mode of action of glyphosate as a herbicide consists of inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which participates in the synthesis of aromatic amino acids in plants, fungi and certain bacteria (shikimate pathway) along with inhibition of phosphoenolpyruvate carboxylase (C4 and Crassulaceae plants) and the activity of nitrogenase (which enables certain bacteria to form NH_3 from atmospheric nitrogen). Unlike plants, bacteria and fungi, animals do not have a gene coding for EPSPS.

One of the objectives of this report is to describe the various mechanisms of glyphosate toxicity in animals. The extensive literature on *in vitro* and *in vivo* models includes studies where high treatment or exposure levels are often used. This practice is used by regulatory toxicologists who test a range of concentrations (elevated to less elevated) to demonstrate a critical effect. Accordingly, we have not taken into consideration numerous publications arising from *in vitro* experiments carried out using high doses. For example, *in vitro* genotoxicity tests are difficult to interpret if there is a high percentage of cell death.

Animal carcinogenicity studies

Carcinogenicity studies conducted in rats (at 2 years) and in mice (at 18 or 24 months) were analyzed by various agencies as part of the previous renewal of the authorization for glyphosate. European authorities appointed an expert committee coordinated by the German Federal Institute for Risk Assessment (BfR) that selected 6 studies in mice, and 10 studies in rats exposed to glyphosate orally, or in one case via the dermal route. IARC evaluated 3 and 7 of the mouse and rat studies, respectively, from among those included in the BfR report (Table IV). The IARC expert committee concluded that there was an increase in the incidence of tumors for certain studies in both mice and rats, whereas the BfR considered the carcinogenesis results in rodents to be negative. Both authorities agreed that 1 of the mouse studies and 3 of the rat studies were poorly conducted, and that 2 of the rat studies had negative results. The scientific arguments presented by EFSA and ECHA to repudiate the findings considered to be positive by IARC are set out below.

In mice

- Excess hemangiosarcomas in males (Atkinson *et al.*, 1993a) at high doses but with no significant difference when comparing the paired values (Fisher's test). This result was considered negative by ECHA and EFSA with an observed incidence thought to be indicative of a spontaneous effect not related to the treatment. The trend test was significant in males, so it remains difficult to interpret this result and to entirely overlook this increase.
- Excess renal adenomas in males of the CD-1 strain observed at the highest dose of exposure (4,841 mg/kg bw per day) (Knezevich and Hogan, 1983), not found in females. This result was considered negative for renal tumors by ECHA and EFSA given that the increase was found only at high doses and only affected one sex. In addition, another pathologist (EPA, 1985b) re-evaluated the slides and concluded that a tumor was present in the control group and that the nature of the tumors (adenoma or carcinoma) was difficult to assess.
- Excess malignant lymphomas in males and females in an oral carcinogenicity study in Swiss mice (Kumar, 2001). The evidence for a carcinogenic effect was judged to be limited. In addition, EFSA questioned the validity of the study due to a viral infection across all the mouse groups, arguing that this infection could have an impact on survival and the incidence of tumors, particularly lymphomas (EFSA, 2015a). EFSA's conclusion was however called into question by ECHA, which could not identify a viral infection (ECHA, 2016). In addition, as the results varied depending on the statistical analysis, it is difficult to rule out a positive result.
- The study by Wood *et al.*, not included by IARC, was reported as negative by ECHA and EFSA (Wood *et al.*, 2009b). However, the analysis of the incidence of lymphoma gave a positive result only in males, a result not taken into account by the BfR. A positive result for males, difficult to interpret, is noted.

In rats

- Excess pancreatic islet cell adenomas (significant difference for one or more groups without progression to malignancy) in males in an oral carcinogenicity study (Stout and Ruecker, 1990). An excess combined incidence of adenomas and carcinomas at the lowest dose was also observed. The results were considered positive for adenoma by ECHA and EFSA at high doses.

- Excess thyroid C-cell adenomas in females, without progression to carcinoma (Stout and Ruecker, 1990). This result was considered positive for adenomas and negative for adenocarcinomas. However, the spontaneous incidence of this benign tumor was relatively high (approximately 4-10%) in Sprague-Dawley rats with very few progressing to adenocarcinoma (ATSDR, 2019).

The BfR's argument was based on a debate about the statistical test used, the consideration of historical controls and the development of certain benign tumors with no progression to cancer. It should be noted that the values observed for historical controls depend on the location of the experiment, the strain, the date, and the number of animals and consequently are not generalizable (Clausing *et al.*, 2018).

In summary, experimental carcinogenesis data on exposure to glyphosate and the occurrence of some tumor types establish a link, but this is generally limited to a single sex and in some cases to sensitive strains or very high doses of exposure. Looking at these results as a whole, the level of evidence for carcinogenicity in rodents is not null, but relatively limited.

Genotoxicity studies

A battery of *in vitro* and *in vivo* tests is used by the agencies in accordance with OECD guidelines to characterize a genotoxic effect, i.e. one that results in one or more types of DNA damage such as breaks (assessed by the comet assay), chromosomal aberrations (micronucleus test, sister chromatid exchange assay, and chromosomal aberration test) and formation of DNA adducts (unscheduled DNA synthesis test; UDS). These DNA changes may lead to mutations in the form of point mutations or chromosomal rearrangements.

One of the tests currently widely used, particularly in the studies described below, to characterize DNA breaks (i.e. clastogenic effects) induced by a genotoxic agent (or during processes such as apoptosis or cell suicide) is the comet assay. This is an agarose gel electrophoresis technique, often conducted in alkaline conditions, performed on various cell types in culture *(in vitro)* or from animals *(in vivo)* exposed for example to glyphosate or GBH. The cells are suspended in a low-melting-point agarose gel and then undergo lysis by incubation with an alkaline detergent. The nuclei released then undergo electrophoresis and the DNA is visualized after neutralization using a fluorescent intercalating agent. If the DNA is undamaged, it forms a compact sphere. If it is damaged, atypical comets are produced at cytotoxic

concentrations and the test must be carried out in a range of concentrations *in vitro*, the highest of which corresponds to approximately 20% cell mortality (LD_{20}). When performed under alkaline conditions, the comet assay quantifies not only the presence of single- or double-strand DNA breaks but also alkali-labile sites. Finally, endonucleases recognizing oxidized bases such as Fpg (DNA-formamidopyrimidine glycosylase) and endo III (endonuclease III) produced by *E. coli*, or hOGG1 (8-oxoguanine DNA N-glycosylase 1) produced by human cells can be used to quantify oxidized DNA bases, one of the signs of oxidative stress. This method is used by research laboratories and in regulatory toxicology because it is non-invasive, sensitive, rapid and quantitative. However, it is particularly difficult to compare results if the electrophoresis is not carried out in parallel (for example, across different studies).

Positive and negative results have been obtained from *in vitro* and *in vivo* tests for exposure to glyphosate and GBH and are presented below. As a reminder, we have not included the numerous publications derived from high-dose *in vitro* experiments, as genotoxicity tests of this type are difficult to interpret if there is a high percentage of cell death.

Studies demonstrating a genotoxic effect

- ***In vitro tests on leukocyte blood cells***

Numerous publications report clastogenic events in animal or plant systems. A few recent examples demonstrating induction of DNA breaks are described below.

Exposure of peripheral blood mononuclear cells (PBMCs, consisting of lymphocytes and monocytes) to glyphosate at a concentration of 42.3 mg/L (0.25 mM) caused DNA damage detected by the comet assay under alkaline conditions, not all of which was repairable (Kwiatkowska *et al.*, 2017). A second study using the same model with a more extensive range of products (glyphosate, AMPA and Roundup 360 PLUS) was conducted more recently (Wozniak *et al.*, 2018) and found that Roundup 360 PLUS induced comets in alkaline media from 5 µM with a concentration of glyphosate 50 to 100 times lower than those obtained with pure glyphosate (0.25 mm, equivalent to 42.3 mg/L) and AMPA (0.5 mM, equivalent to 55.5 mg/L), respectively. These results cannot be explained by general cytotoxicity, as the viability of cells exposed to glyphosate at 169 mg/L (1 mM) is 98.8% (Wozniak *et al.*, 2018). The nature of the breaks was characterized in this study: double-strand breaks were observed at 1.69 mg/L (10 µM) of glyphosate in the form

of Roundup 360 PLUS and 169 mg/L (1 mM) for pure glyphosate. Among the mechanisms explored to explain these breaks, the authors reported an increase in the production of reactive oxygen species (ROS) following exposure to Roundup 360 PLUS and glyphosate (Wozniak *et al.*, 2018), the potential effects of which will be discussed below. Comparison of these two studies shows that the formulation produces a much more pronounced genotoxic effect than glyphosate alone.

Other similar blood cell models have been used. Cytotoxic and genotoxic effects were evaluated using the MTT assay (which measures intracellular dehydrogenase activity) and the comet assay in Burkitt lymphoma cells (Raji cell line) exposed to glyphosate for 20 to 120 min at concentrations ranging from 0.017 to 2,535 mg/L (0.1 µM and 15 mM). Cytotoxicity was observed at 1,690 mg/L (10 mM) while breaks were quantified at 169 and 845 mg/L (1 and 5 mM) at early measurement points, and were absent at later measurement points, indicating the cells' ability to repair these breaks. The authors concluded that for concentrations of up to 16.9 mg/L (100 µM), glyphosate is not cytotoxic and does not produce detectable DNA breaks (Townsend *et al.*, 2017), likely due to an effective single-strand (dependent on XRCC1/PARP/Lig3) and/or double-strand (dependent on Ku70-80/DNA-PK/Lig4) break repair mechanism. This result differs from that obtained with PBMCs and can be explained by the fact that the latter do not replicate their DNA and therefore exhibit little to no break repair activity, while Raji cells rapidly divide and have effective mechanisms for repairing DNA damage (replication being a potentially genotoxic process). These results thus demonstrate that the choice of cell model is key to assessing genotoxicity (and cytotoxicity).

The choice of species may also be important. For example, exposing armadillo *(Chaetophractus villosus)* lymphocytes to glyphosate at concentrations of 65, 97.5, 130 and 260 mg/L (in the form of Roundup Full II) induced a genotoxic effect with chromosomal aberrations (130 mg/L), and sister chromatid exchanges (65 mg/L) (Luaces *et al.*, 2017). These concentrations of glyphosate are much higher than those identified with PBMCs (but the product used was also different).

Finally, DNA breaks were induced in human lymphocytes exposed to 200 mg/L (1.8 mM) AMPA (Mañas *et al.*, 2009a), a concentration with no effect on the mitotic index value. This genotoxic activity of AMPA may be important to consider, as plants and soil microorganisms are able to metabolize glyphosate into AMPA and it is found in soil, plant residues and water (Mamy *et al.*, 2016; Tong *et al.*, 2017).

- ***In vitro tests in other cell models***

Models other than the white blood cells described above have been studied, as the distribution of glyphosate in the body can affect both blood cells and all organs.

Human oral epithelial cells (TR146 cell line) were exposed to either glyphosate or Roundup Ultramax (Koller *et al.*, 2012). Significant genotoxic effects were observed using the comet assay from 20 mg/L pure glyphosate and for the micronucleus test (another genotoxicity test) from 20 mg/L glyphosate in the form of the GBH Roundup Ultramax (with no damage to cell integrity) with markers of cell death activated from 20 mg/L for these formulations (necrosis for both, apoptosis with pure glyphosate).

Human liver cells from the HepG2 cell line were exposed to 0.5, 2.91 and 3.5 mg/L glyphosate for 4 or 24 h. The dose of 0.5 mg/L induced oxidative damage and DNA breaks after 24 h of exposure (Kauba *et al.*, 2017).

In a comet assay using Hep-2 cells, a positive result was obtained after treatment with glyphosate at concentrations from 3 mM (500 mg/L) (Mañas *et al.*, 2009b), while treatment of the same cell line with AMPA caused DNA breaks from 2.5 mM (277 mg/L) (Mañas *et al.*, 2009a).

Exposing hamster ovary cells (CHO-K1) to AMPA resulted in production of micronuclei at a dose 1,000 times lower than the dose of glyphosate required to achieve the same effect (Roustan *et al.*, 2014). However, the low mutagenic effect observed with glyphosate is explained by the very low level of hepatic metabolism (<1%) (BfR, 2015a).

- ***In vivo tests***

Various *in vivo* models can be used to assess genotoxic effects (including experiments in mice, fish, and crustaceans).

Genotoxic effects were observed in the kidney and liver cells of mice treated with two intraperitoneal injections of 300 mg/kg glyphosate (high concentration) after 4 h of exposure (but not after 24 h), along with formation of micronuclei in the bone marrow (Bolognesi *et al.*, 1997). This effect was also observed by Mañas *et al.*, who found a genotoxic effect at 200 mg/kg glyphosate (two intraperitoneal injections 24 h apart) in mice (Mañas *et al.*, 2009b). At lower doses (25 and 50 mg/kg), chromosomal aberrations and micronuclei were observed at 24, 48, and 72 h in the bone marrow of mice exposed intraperitoneally (Prasad *et al.*, 2009). The results were positive for both doses and across the different time points.

Exposure of *Channa punctatus* fish to Roundup (41% glyphosate) at very high sublethal doses (1/10, 1/8 and 1/5 of the LD_{50}) caused DNA breaks observed in red blood cells and branchial cells from 3.25 mg/L glyphosate (1/10 of the LD_{50}) (Nwani *et al.*, 2013). CAT and SOD antioxidant enzyme activity was reduced in the blood and gills from the lowest dose of exposure. Induction of DNA breaks was also detected by the comet assay in the erythrocytes of another type of fish *(Oreochromis niloticus)* exposed to glyphosate at the environmentally-relevant dose of 0.12 mg/L. Similar results were obtained in eel *(Anguilla anguilla)* gill cells exposed to glyphosate or Roundup Ultra (Guilherme *et al.*, 2010; Guilherme *et al.*, 2012; Guilherme *et al.*, 2014) with observation of the production of oxidative damage (8-oxoG) and reduction of antioxidant defenses in the gills and liver.

Two crustacean models have also been studied in genotoxic studies: exposing crabs *(Eriocheir sinensis)* to glyphosate for 96 h at concentrations of 4.4, 9.8, 44 and 98 mg/L (very high doses since the LD_{50} = 98 mg/L) caused DNA breaks revealed by the comet assay from 4.4 mg/L (Hong *et al.*, 2017). Oxidative stress, with the presence of genotoxic damage, was also observed in the red blood cells of shrimp exposed to Roundup (41% glyphosate) for 96 h at sublethal doses (0.35, 0.70, 1.40, 2.80 and 5.60 mg/L; LD_{50} = 11.2 mg/L) (Hong *et al.*, 2018). The comet assay was positive from the lowest concentration and micronuclei were induced from 1.4 mg/L. These effects were partly due to a decrease in antioxidant defenses (SOD and CAT) from 0.35 mg/L, as in the *Channa punctatus* fish model. Clastogenic events such as induction of DNA breaks and chromosomal aberrations have also been reported in plants (Lioi *et al.*, 1998; Alvarez-Moya *et al.*, 2011; Frescura *et al.*, 2013).

In summary, in various experimental models, numerous positive results have been obtained using *in vitro* and *in vivo* comet assays. However, the protocols vary substantially in terms of the models, exposure times, types of products used (glyphosate or formulations) and doses. For the studies conducted in parallel, genotoxic effects were obtained at lower concentrations of glyphosate used in the form of GBH compared to the active ingredient alone. In comparison to exposure levels, several *in vitro* tests have found genotoxic effects at concentrations similar to those that can be detected in the environment. In France, for example, glyphosate concentrations do not exceed 0.07 mg/L in surface water, a value similar to that inducing genotoxic effects in *Oreochromis niloticus* (0.12 mg/L) (Alvarez-Moya *et al.*, 2014).

Studies not demonstrating a genotoxic effect

- ***In vitro tests***

Two studies reported that rat liver cells exposed to glyphosate *in vitro* showed no unscheduled DNA synthesis (UDS test) (Li and Long, 1988; Rossberger, 1994). However, this method is now considered insufficiently sensitive and the corresponding OECD guideline (OECD test no. 486) has not been updated, with other methods such as the comet assay recommended instead (OECD, 2015). This test also consists of staining replication following DNA excision repair, and glyphosate has not been shown to be a direct or indirect producer of DNA adducts. These results do not therefore provide any information that can be used to assess genotoxic potential.

Negative results were reported by the BfR from manufacturer-produced studies which were not available to the experts, as they have not been made public.

- ***In vivo tests***

In plants, no induction of chromosome aberrations was observed following exposure of onion bulbs (*Allium cepa*) to concentrations of glyphosate isopropylamine up to 2.88 mg/L, (IC_{50} = 5.5 mg/L), but positive results were obtained following exposure to 1.44 and 2.88 mg/L of the active ingredient in the form of Roundup (IC_{50} =1.2 mg/L) but with no dose-response relationship potentially due to the toxic effect (Rank *et al.*, 1993). No induction of micronuclei was observed when beans (*Vicia faba*) were exposed to glyphosate for 4 days (De Marco *et al.*, 1992).

In rodents, no effect was observed in mice in the dominant lethal assay (EPA, 1980), no induction of micronuclei was seen in the bone marrow in mice following a single intraperitoneal exposure of either glyphosate isopropylamine salt (100-200 mg/kg) or glyphosate isopropylamine salt in the form of Roundup (133 and 200 mg/kg) (Rank *et al.*, 1993) and no induction of chromosome aberrations was found following a single intraperitoneal exposure in rats (Li and Long, 1988).

In summary, the studies showing a lack of genotoxicity from glyphosate appear to be less substantial in both qualitative and quantitative terms than those suggesting a positive effect. If we compare two fairly similar studies with opposite results, such as in mice (intraperitoneal exposure to 200 mg/kg glyphosate) (Rank *et al.*, 1993; Mañas *et al.*, 2009b), it is very interesting to note that the study by Mañas *et al.* includes a second injection 24 h after the first, suggesting that repeat exposure (a plausible phenomenon) is an important consideration. This would make the short- or long-term nature of exposure to glyphosate or GBH an important parameter. In addition, in the studies that were conducted in parallel (and are therefore comparable), the positive results were more pronounced with the commercial preparations than the active ingredient. This increase in the effects induced by the commercial products is due to the added surfactants.

With the micronucleus test in vertebrate models, different from the comet assay typically used in a large number of studies, a meta-analysis of the literature also concludes that there is a genotoxic effect (Ghisi Nde *et al.*, 2016). Finally, sexual dimorphism is observed with a more marked effect in male rodents than females.

Among the studies with positive results, the doses used are sometimes consistent (although higher) with environmental exposure. Genotoxic effects have been demonstrated by some authors (Alvarez-Moya *et al.*, 2014; Hong *et al.*, 2017) in less conventional experimental models (fish and crustaceans).

Mutagenic effects

As reported by ECHA, negative results were obtained for glyphosate in mutagenesis tests on bacteria (the Ames test) and in mutagenesis tests on eukaryotic cells including those evaluating the *Hprt* site of CHO cells and the *TK* locus of mouse lymphoma cells (ECHA, 2016). However, positive results have been reported from a sister chromatid exchange assay performed on bovine lymphocytes from 2.87 mg/L (17 µM) (Lioi *et al.*, 1998).

In conclusion, looking at the results obtained from genotoxicity and mutagenicity tests as a whole, numerous studies have been published with the results generally supporting a genotoxic effect. On the other hand, almost all of the results reported from *in vitro* mutagenesis tests in bacteria or eukaryotic cells have been negative. Direct or indirect induction of oxidative stress, which is potentially involved in a process of genetic

instability (and may explain genotoxic observations) has however been seen in various species and cell systems. This result was found, for example, in fish at exposure levels comparable to those found in the environment (Nwani *et al.*, 2013).

Mechanisms of toxicity

Although glyphosate is selective for plants, it can disrupt processes other than the shikimate metabolic pathway that could potentially lead to chronic diseases in animals. For example, many publications report potential carcinogenic or non-carcinogenic effects, including endocrine disruption (ED). In addition, many diseases in vertebrates, including in humans, are associated with changes in the microbiota, and some bacteria are known to express EPSPS, which is the enzyme targeted by glyphosate in its mode of action as an herbicide.

A joint literature search for the terms "cancer" and "glyphosate" identified a number of mechanistic studies that sometimes appear contradictory but were often carried out under different experimental conditions. Despite sometimes relatively high doses, commercial GBHs also contain a varying percentage of glyphosate. For example, "Roundup Ready" (360 g/L glyphosate) is diluted to between 1 and 3% for an effective concentration of use between 3.6 and 10.8 g/L (21-64 mM).

Below we consider various mechanisms of glyphosate toxicity by looking at different aspects (acute/chronic, organelles/cell nucleus, multi-generation, etc.) in turn, beginning with cytotoxicity.

Cytotoxicity

In an early study, treatment of rat hepatoma cells (HTC cell line) with high doses of Roundup between 1 and 10 mM (169 to 1,690 mg/L) for 4 to 48 h (Malatesta *et al.*, 2008) had no effect on cell viability but was associated with changes in certain nuclear, cytoplasmic and mitochondrial characteristics (size and number of cytoplasmic vacuoles, number of lysosomes, structural chromatin changes, decreased inner/outer mitochondrial membrane length ratio).

More recently, Koller *et al.*, showed that treatment of human buccal epithelial cells (TR146 cell line) with glyphosate at a concentration greater than 10 mg/L when in the form of Roundup, or 200 mg/L for the active substance alone, resulted in significant extracellular release of lactate

dehydrogenase (LDH, a marker of cell viability normally found only in the cytoplasm). These concentrations are much lower than those of sprayed solutions that may come into contact with this type of cell orally or on the skin more generally. Impaired mitochondrial function (as assessed by the XTT test) and neutral red uptake, were observed with glyphosate in the form of Roundup from 60 mg/L and 100 mg/L respectively (but not with the active substance alone) (Koller *et al.*, 2012).

The use of atomic force microscopy (and Peak Force Tapping) on human epidermal cells (HaCaT cell line) treated with 2,535 or 5,070 mg/L (15-30 mM) glyphosate (i.e. at doses that are high but also consistent with sprayed solutions) for short periods (<24 h) resulted in membrane changes including flattening, and changes in the nature of cell protuberances (Heu *et al.*, 2012).

A difference in cellular toxicity between glyphosate and GBH was expected due to the low toxicity of glyphosate compared to surfactants, which are sometimes present in high concentrations (Mesnage *et al.*, 2013; Mesnage *et al.*, 2015; Vanlaeys *et al.*, 2018). A transcriptome analysis of HepaRG cells exposed to 600 µM glyphosate found no modifications compared to control cells, while the effect of the GBH on transcriptional response may be 1,000 times greater (Mesnage *et al.*, 2018).

In Sertoli cells (TM4 cell line), Roundup Bioforce was more toxic than glyphosate with deleterious effects on mitochondrial function attributed to the co-formulant POEA (Vanlaeys *et al.*, 2018). The co-formulant POEA is reported to be genotoxic at concentrations that are non-toxic for cells, as well as potentially reprotoxic (EFSA, 2015a). Despite a lack of data from (short- and long-term) toxicology tests, this agent has been banned.[19]

Looking beyond 2D models, new 3D models are yielding results that, while *in vitro*, more closely approach cell biology. The effects of glyphosate have thus been tested in liver organoids, showing potential cytotoxicity from 42.3 mg/L (0.25 mM). This threshold was 423 mg/L (2.5 mM) for cardiac organoids with an IC_{50} for production of ATP (a marker of cell viability) of around 1,690 mg/L (10 mM) and disturbances in the beating activity of cardiac organoids from 42.3 mg/L (0.25 mM) (Forsythe *et al.*, 2018). These results are relatively consistent with those obtained *ex vivo* by Gress *et al.*,

[19]. European Commission 2016/1313, Implementing Regulation published in the Official Journal of the European Union on August 2.

who investigated the effects of Roundup, at concentrations equivalent to those measured in the blood following acute poisoning, and of glyphosate, on cardiac function (in male rats and female rabbits). Cardiac dysfunction (arrhythmias and conduction blocks) were reported in this study (Gress *et al.*, 2015).

Mitochondrial toxicity

Mitochondrial toxicity - disturbances in mitochondrial function, leading, for example, to apoptosis or generation of reactive oxygen species (ROS) - is associated with many diseases as an initiating or contributing event. Various tests evaluating mitochondrial toxicity *in vitro* and *in vivo* are typically used by research laboratories, but none have been validated by regulatory authorities. The results of these studies are presented below.

Exposure of the nematode *Caenorhabditis elegans,* a small transparent nonparasitic worm, for 24 h to the GBH TouchDown (52.3% glyphosate) caused inhibition of mitochondrial respiration associated with disruption of the proton gradient and decreased production of ATP (Bailey *et al.*, 2018). This inhibition of respiration was accompanied by the production of H_2O_2 (a reactive oxygen species) but at glyphosate concentration of 9.8%, which is higher than that used for field spraying (0.4 to 2.2%) (Bailey *et al.*, 2018) but in the order of the LC_{50} value for acute exposure (Negga *et al.*, 2011).

Exposure of zebrafish (*Danio rerio*) for 7 days to 0.065 mg/L glyphosate (as GBH Scout; 720 g/kg glyphosate) impaired the functioning of the mitochondrial respiratory chain by inhibiting complexes I and IV and inducing membrane hyperpolarization of central nervous system mitochondria (Pereira *et al.*, 2018). Inhibition of complexes I and IV results in the formation of ROS responsible for the neurotoxic effect (Abramov *et al.*, 2010). Thus, from 0.065 mg/L (i.e. a dose lower than 0.12 mg/L or 0.3 µM, a genotoxic concentration in *Oreochromis niloticus*), the fish displayed behavioral changes and at 1 mg/L they remained at the bottom of the aquarium. This change in the behavior of fish was also observed previously (Bridi *et al.*, 2017).

A reduction in mitochondrial activity and in several glutathione S-transferases was observed in a zebrafish hepatocyte cell line (ZF-L) exposed to to Roundup and glyphosate at 0.65 and 3.25 mg/L, respectively (Lopes *et al.*, 2018).

In summary, mitochondrial toxicity can be observed at environmental doses (the value of 0.065 mg/L is lower than the highest concentration detected in surface water: 0.070 mg/L). There is a time lag between the fundamental biological questions regarding the mechanisms of toxicity, particularly regarding mitochondrial toxicity, and the tools validated by the regulatory agencies, that may partly explain some controversies, particularly regarding glyphosate.

Carcinogenesis

When evaluating carcinogenesis, it is necessary firstly to determine whether a substance can affect tumor initiation and/or promotion (if it affects both, it is referred to as a complete carcinogen). Second, analysis of carcinogenesis is not limited to genotoxicity (associated with initiation) and must be evaluated on the basis of non-genotoxic effects (as is the case with cell proliferation of a cancer clone or tumor promotion step).

A seminal mechanistic study on the genotoxicity of glyphosate was conducted using two human cell lines (BEAS-2B, human bronchial epithelium; and SHSY-5Y neuronal) that demonstrated inhibition of DNA repair activity after exposure to the substance for 24 h at concentrations between 10-100 µM (Alleva *et al.*, 2016). A honey extract rich in polyphenols (supporting antioxidant activity) restored mitochondrial activity and inhibited ROS formation in both cell lines.

In mice, a proteomic study conducted in a skin carcinogenesis model over a period of 32 weeks (with different treatment conditions, initiating agents, promoting agents, etc.) showed that glyphosate has no initiating or complete carcinogen effects of its own. However, in combination with an initiating agent, it showed a promoter effect at doses much lower than those previously tested (25 mg/kg bw; twice weekly), particularly as the glyphosate-modified proteomic profile resembles that of 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a model phorbol ester used as the promoting agent (George *et al.*, 2010). Despite the previously reported genotoxic effect related to oxidative stress, a tumor promoter effect based on other mechanisms may also be possible.

Glyphosate as a potential estrogen mimetic

In the early 2000s, Lin *et al.* studied various cell parameters after treating the human cell line MCF-7 with Roundup and glyphosate. They observed that glyphosate stimulates cell proliferation from 0.228 mg/L and Roundup

from 1 mg/L, independently of a classic estrogen stimulation effect (the concentrations resulting in maximum proliferation were 2.28 mg/L and 10 mg/L respectively) (Lin and Garry, 2000). The question of whether glyphosate has estrogenic properties has been addressed by several studies, such as that by Hokanson *et al.*, in which a microarray analysis also conducted in MCF-7 found a modification in the expression of estrogen-sensitive genes after exposure to glyphosate 15 mg/L[20] for 18 h in the presence or absence of estradiol (3 x 10^{-10} M) (Hokanson *et al.*, 2007). These findings are in agreement with those of Thongprakaisang *et al.* in 2013 who showed that glyphosate at concentrations as low as 0.169 ng/L (10^{-12} M) induces a proliferative effect in the human breast cell line T47D (a hormone-dependent breast cancer cell line), likely associated with stimulation of the estrogen pathway (as it is not observed in the ER-negative cell line MDA-MB-231, a non-hormone-dependent breast cancer cell line) (Thongprakaisang *et al.*, 2013). A recent study reported that glyphosate at concentrations as low as 10^{-11} M (1.69 ng/L) effects markers of proliferation from in cholangiocarcinoma cell lines expressing or not expressing the estrogen receptor ERα. The proliferative effect (increase in S-phase cells) was observed in ERα-expressing cells with cell proliferation blocked by an ERα antagonist (Sritana *et al.*, 2018). MDA-MB-468 (ER-negative) and MCF-7 (ER-positive) cells were exposed to 186 mg/L (1.1 mM) of glyphosate in the form of Roundup Original or 1,110 mg/L (10 mM) of AMPA for 48 h for the purposes of transcriptome analysis (Stur *et al.*, 2019). Roundup and AMPA caused dysregulation of estrogen-dependent genes and eleven pathways, including the cell cycle and DNA damage repair pathways, in triple-negative cells.

Given the importance of the dose in toxicology, it may be useful to compare the results obtained at much higher doses. Glyphosate, but not the components used for GBH (some of which are known, such as POEA, and others are unknown due to the trade secret regarding formulations), can activate ERα at high concentrations (Mesnage *et al.*, 2017). Li *et al.* tested the influence of glyphosate at concentrations between 15 and 50 mM (2,535 and 8,450 mg/L) on the growth of 8 cell lines and identified growth inhibition in some from 15 mM, a phenomenon not observed in two normal but immortalized prostate epithelial cell lines (Li *et al.*, 2013). This

[20]. The dose was calculated using a 15% glyphosate solution.

was also observed with AMPA, with the same concentration interval (Parajuli *et al.*, 2015). Apoptotic processes were also stimulated by AMPA from 50 mM (5,552 mg/L) (Li *et al.*, 2013). These studies were conducted using different cell models and suggest that glyphosate activates estrogen receptors, an activation pathway associated with cell proliferation. The "dose-effect relationship" also appears to play a leading role here, with activation of ERα observed at high doses (Mesnage *et al.*, 2017).

In addition to effects via estrogen receptors, several studies have reported an inhibitory effect from glyphosate on aromatase. Roundup Bioforce was thus tested on embryonic 293 cells and placental (choriocarcinoma) JEG-3 cells to assess potential hormonal activity (Benachour *et al.*, 2007). At a dilution of 0.01% (equivalent to 35.5 mg/L or 210 µM glyphosate) for 24 h, Bioforce Roundup had an inhibitory effect on the activity of aromatase, an enzyme responsible for transforming certain androgen precursors into estrogens. An inhibitory effect on aromatase was also reported in JEG3 cells following exposure *in vitro* to two different GBHs (Glyphos and Médallon) and ingredients: glyphosate and surfactants. The concentrations used were 1.2 to 3 times lower than the NOEC *(No Observed Effect Concentration)* of Glyphos (75 mg/L) and Médallon (500 mg/L). Glyphosate (NOEC = 3,100 mg/L) alone had no effect while the co-formulants and GBHs inhibited aromatase by 25 to 40%, which points to an effect from the co-formulants rather than glyphosate (Defarge *et al.*, 2016). The half maximal inhibitory concentration (IC_{50}) of glyphosate on aromatase was observed in JEG3 cells and in an *in vitro* microsomal preparation using a Roundup concentration of 0.04% (equivalent to 144 mg/L glyphosate) and 0.6% (equivalent to 2.16 g/L glyphosate), respectively. Roundup tested on the microsomal preparations was 4 times more active than the active ingredient (with an equivalent concentration of glyphosate, likely due to the presence of co-formulants) (Richard *et al.*, 2005).

These results are interesting as they suggest pro-estrogen effects via estrogen receptors observed at high but also at low doses. The inhibition of aromatase adds a complex element to data interpretation on which the following section may shed some light.

The biochemical mechanism of action and question regarding direct binding to ERα

A possible interaction between glyphosate and sex hormone regulatory pathways was evaluated by the EPA using the EDSP *(Endocrine Disruptor*

Screening Program) Tier I series of tests, producing a negative result for estrogen, androgen and thyroid receptors (EPA, 2015). A recent study reported the same result by excluding binding of glyphosate to the ERα receptor. However, it suggested activation of this receptor through non-genomic mechanisms (Mesnage *et al.*, 2017). In this study, conducted using high-throughput approaches and tools (omics), the authors show that glyphosate is probably not an effective ligand of ERα but nevertheless promotes a non-estrogenic transactivation pathway (protein kinase A). Specifically, glyphosate promotes proliferation of MCF-7 and T47D cells at 59 µM (>10 mg/L).

Taking into account all of these findings, glyphosate and GBH could activate the ERα signaling pathway, through mechanisms that do not involve binding to the receptor (estrogen-independent, ERα-dependent), which would be consistent with the inhibition of aromatase also observed. These effects are also consistent with possible tumor promoter properties.

Glyphosate as a potential endocrine disruptor

The carcinogenic potential of glyphosate remains an open question, and the results of the small number of recent epidemiology and genotoxicity studies do not provide any definitive answers. Focusing the controversy on a potential carcinogenic effect could obscure other possible mechanisms of toxicity, in particular an endocrine disruptor effect.

Endocrine disruptors (EDs) are substances that can interfere with the hormonal system. Since 2017 (EU regulations 2017/2100 and 2018/605), a phytopharmaceutical substance has been identified as an ED if: *i*) the substance or mixture of substances has a mode of action that alters the function of the endocrine system; *ii*) it produces an adverse effect in an intact organism or its progeny; and *iii*) the adverse effect is a consequence of this mode of action.

Although data from the literature are contradictory, a 2017 publication from EFSA (EFSA, 2017) stated that glyphosate was probably not an ED, but left the debate open. This analysis was partly based on the following negative results:

- the EPA *EDSP Tier 1* test found a negative result (EPA, 2015);
- no effect on steroidogenesis in the MA-10 mouse line (Forgacs *et al.*, 2012);
- no effect on synthesis of vitellogenin (Xie *et al.*, 2005);

- no effect on regulation of PXR in COS-7 cells (Kojima *et al.*, 2011) or the Ah receptor in Hepa1c1c7 cells (Takeuchi *et al.*, 2008).

These various tests do not allow for the identification of other hormonal signaling pathways that could be disrupted. Indeed, as described above that glyphosate appears to have estrogen activation properties without direct binding to the ERα receptor. The recent literature suggests an ED mode of action that could act at the level of developmental or reproductive function.

Impaired development associated with hormonal dysregulation

Glyphosate and GBH affect various bodily functions, including metabolism, reproductive function, and the nervous system. In 2010, it was reported that a GBH (Roundup Classic, containing 48% glyphosate salt) affected the development of frogs and chickens (Paganelli *et al.*, 2010). The effects appear to be related to disruption of retinoic acid signaling. Essentially, the formulation impaired development of the neural crest, which may be mediated by an increase in endogenous retinoic acid since retinoic acid antagonists were able to reverse the teratogenic effect of glyphosate. Other experiments have reported embryotoxicity in rats exposed to GBH during pregnancy (Dallegrave *et al.*, 2003; Dallegrave *et al.*, 2007).

Impaired reproductive function

A number of studies have investigated the potential effect of glyphosate, or more frequently GBH, on reproduction, mainly in rodents.

- ***The effect of glyphosate and GBH in males***

Changes to sperm have been found in several studies using various cell models (rats and humans) and *in vivo* models (mice and rats).

A recent meta-analysis of studies in rodents concluded that exposure to glyphosate is associated with a reduction in sperm concentration (-2.774 x 10^6/sperm/g/testis; 95% CI [-0.969 to -4.579]) (Cai *et al.*, 2017). This finding is supported by recent studies involving glyphosate or various formulations of GBH and the three species described above; impaired reproductive function was observed in rats exposed orally for 12 weeks to Roundup containing glyphosate in the form of a potassium salt at doses of 3.6, 50.4 and 248.4 mg/kg bw per day (Owagboriaye *et al.*, 2017). A decrease in testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and increased prolactin levels were reported, along with reduced sperm concentration and motility, and an increase in sperm morphology

abnormalities. These results are consistent with those from an earlier study involving exposure of pregnant rats to GBH at doses of 50, 150 or 450 mg/kg glyphosate during gestation (21-23 days) and lactation (21 days). Treatment did not induce maternal toxicity, however, adverse effects on reproductive function in male offspring were observed, with a decreased sperm count, increased abnormal sperm count, and decreased serum testosterone levels (Dallegrave *et al.*, 2007). Some of these effects may be related to the modification not of sperm function, but of cells important to their development. Indeed, acute exposure to Roundup Original (360 g/L) for 30 min induced oxidative stress and activated multiple responses including Sertoli cell mortality in prepubertal rat testes (De Liz Oliveira Cavalli *et al.*, 2013). This effect was observed with glyphosate under the same exposure conditions and was counteracted by pretreatment with antioxidants. More recently, pregnant mice were exposed to Roundup 3 Plus or glyphosate in drinking water at doses of 0.5 (corresponding to the ADI), 5 or 50 mg/kg/day from embryonic day 10.5 to 20 days postpartum. The results showed that in male offspring sacrificed at 5, 20, 35 days and 8 months of age, the sperm count decreased sharply at doses of 0.5 and 5 mg/kg/day (GBH and glyphosate respectively). The number of undifferentiated spermatogonia decreased 60% at the dose of 5 mg/kg/day in the glyphosate group (Pham *et al.*, 2019). Furthermore, mice exposed to Roundup (360 g/L glyphosate and 18% (mass/volume) of the surfactant POEA) by gavage (glyphosate concentrations of 60, 180 and 540 mg/kg bw per day) showed a decrease in sperm motility and count and an increase in sperm morphology abnormalities. Excessive germ cell apoptosis was accompanied by an overexpression of XAF1 [21] at high doses of 180 and 540 mg/kg (Jiang *et al.*, 2018). Finally, human sperm motility and DNA fragmentation were tested *in vitro* following exposure to 0.36 mg/L glyphosate (Anifandis *et al.*, 2018a). Motility was reduced after one hour of incubation but with no effect on DNA fragmentation, an effect whose mechanism may be dependent on mitochondrial dysfunction (Anifandis *et al.*, 2018b).

Alteration of the mammary gland (consistent with impaired estrogen function) was also observed in two studies: in male rats exposed at birth to Roundup FULL II at 2 mg/kg glyphosate administered subcutaneously every two days from postnatal day 1 to 7 (PND1 to PND7), and sacrificed

[21]. X-linked inhibitor of apoptosis-associated factor 1.

on PND21 or PND60, development of the mammary gland was observed with an increase in stroma and terminal end buds at 21 days and mast cell infiltration at 60 days (feminization) (Altamirano *et al.*, 2018). When pregnant rats were exposed to a GBH (3.5 or 350 mg/kg bw per day) from the 9th day of gestation until weaning, atrophy of the development of the mammary gland was observed in male offspring on PND60 (Gomez *et al.*, 2019). These effects differ obviously, but the exposure protocols also varied as do the role of the hormonal systems at the various stages.

Indeed, further evidence of disruption of the masculinization process was reported in a study of rats exposed to GBH during gestation that reported hypersecretion of androgens and increased gonadal activity in male offspring during adulthood (Romano *et al.*, 2012).

- ***The effect of GBH in females***

Exposure of rats to GBH (Roundup FULL II; 2 mg/kg bw per day of glyphosate) administered subcutaneously from postnatal day 1 to 7 (PND1 to 7) increased uterine cell proliferation on PND8 but not on PND21 (Guerrero Schimpf *et al.*, 2017). The ERα pathway was activated on PND8 in the stromal compartment and repressed on PND21 in the luminal compartment. Furthermore, when rats were ovariectomized on PND21 and treated with 17β-estradiol until PND60, uterine epithelium hyperplasia was observed in rats exposed to GBH during the first week after birth, suggesting that early exposure to GBH increases the sensitivity of the uterus to estradiol (Guerrero Schimpf *et al.*, 2018). These results are consistent with the mechanistic hypothesis of estrogen stimulation (though independent of binding to ERα, see above).

Among the mechanisms that could potentially explain these findings include modification of steroidogenesis (or the hormones that regulate it), which is supported by experimental data reported by several studies.

As previously described, a decrease in testosterone, LH and FSH, and an increase in prolactin levels (associated with reduced sperm concentration and motility) were observed in male rats exposed orally for 12 weeks to Roundup (Owagboriaye *et al.*, 2017). In a more recent study that reported effects involving both GBH and glyphosate, rats were exposed either to 1.75 mg/kg bw per day of glyphosate (purity >99.5%) or to the same dose of the active ingredient in the form of Roundup Bioflow (41.5% glyphosate isopropylamine salt) in drinking water from the 6th day of gestation to PND120 (Manservisi *et al.*, 2019). This exposure resulted in impairment of

the endocrine system in offspring, accompanied by: *i*) an increase in anogenital distance on PND4 in males and females treated with Roundup and in males treated with glyphosate; *ii*) delayed age at first estrus and increased testosterone concentration in females (consistent with the aromatase inhibition effects described above); and *iii*) increased plasma TSH concentration in males treated with glyphosate as well as decreased dihydrotestosterone and increased brain-derived neurotrophic factor (BDNF) in males treated with Roundup. In the study by Pham *et al.*, exposure of pregnant mice to Roundup 3 Plus or glyphosate in drinking water at doses of 0.5 (corresponding to the ADI), 5 and 50 mg/kg/day from embryonic day 10.5 up to 20 days after birth, found that in male offspring (sacrificed on day 5, 20, 35 and at 8 months of age) exposure to glyphosate but not to GBH affected both the weight of the testes (reduction) and the morphology of the seminiferous tubule epithelium on day 20, and also decreased serum testosterone concentration on day 35. In the 8-month-old mice, a decrease in testosterone was observed in the GBH group (Pham *et al.*, 2019). Finally, treatment of mouse MA-10 Leydig cells with Roundup was shown to inhibit steroidogenesis at a non-cytotoxic concentration, while glyphosate had no effect. This result again illustrates that formulations are more toxic that glyphosate and indicates that they can have ED effects (Walsh *et al.*, 2000).

- ***The potential effect of glyphosate or GBH on reproductive function***

GBHs were used in the majority of these studies, raising the question of the contribution of glyphosate to the observed toxic effects. The formulations can also exhibit toxic properties unrelated to the concentration of glyphosate, as reported in invertebrate systems (Niemeyer *et al.*, 2018). Is there a specific effect from certain co-formulants, or a cocktail effect between compounds including glyphosate? This is to be compared with earlier research indicating that the ED mode of action might not be linked solely to the active ingredient but rather to the co-formulants (Mesnage *et al.*, 2013; Defarge *et al.*, 2016). In fact, several studies have reported effects of both glyphosate and GBH. The study by Manservisi *et al.* in which rats were exposed to either 1.75 mg/kg bw per day of glyphosate (purity >99.5%) or the formulation Roundup Bioflow (41.5% glyphosate) in drinking water from the 6th day of gestation through to 120 days after birth, found that both treatments resulted in endocrine system disruption (with differences

between them, but also commonalities such as a change in anogenital distance in males) (Manservisi *et al.*, 2019).

Similarly, the study by Pham *et al.* reported decreased sperm counts in the offspring of pregnant mice exposed to Roundup 3 Plus or glyphosate in drinking water at doses of 0.5 (corresponding to the ADI), 5 and 50 mg/kg/day from E10.5 to 20 days postpartum (Pham *et al.*, 2019).

In summary, an ED mode of action has been observed following exposure to GBH but also to glyphosate at low doses, i.e. less than 50 mg/kg/day (a value corresponding to the NOAEL in rats). Phenotypic ED effects from GBH and glyphosate are strongly suspected following numerous studies of androgen and estrogen function, but with complex effects that could be explained, for example, for estrogens through both their action on aromatase (anti-estrogen) and through hypersensitization to the action of estradiol (pro-estrogen).

Intergenerational effects and epigenetic changes

Epigenetic studies are used to identify mechanisms of action that modify gene expression in a reversible, transmissible and adaptive manner, without changing the nucleotide sequence (i.e. the DNA). This type of change in the promoter region of genes, for example, can extinguish or conversely stimulate gene expression. Although widely debated, the idea of this type of change being passed down through generations is increasingly accepted. It is becoming more common in experimental research for studies to explore the effects of treating pregnant animals (so called generation F0 ; and only this generation) with a contaminant and then evaluating the effects through to the 3rd generation (F3) that has never been exposed to the contaminant, unlike the F1 (exposed as fetuses) and F2 (exposure of the F1 gametes) generations. The effects of glyphosate and GBH are beginning to be explored in such studies.

In a 2018 study, rats (F0) were exposed orally to 2 or 200 mg/kg bw per day of glyphosate in the form of GBH (Magnum Super II containing 66.2% glyphosate potassium salt) between the 9th day of gestation and the day of weaning (21 days after birth). No toxic effects were found for either dose on reproductive characteristics, including length of gestation, litter size and birth weight. However, exposure of F0 animals to 2 mg/kg bw per day of glyphosate led to reproductive function abnormalities (decreased implantation sites, miscarriages) in the F1 generation. In the F2 generation, fetal growth delays and developmental abnormalities (fetal malformations)

were observed (Milesi *et al.*, 2018). This heritability suggests an intergenerational effect that could be explained by exposure to GBH *in utero* of the F1 generation or their germ cells (for the F2 generation) (Figure 3). It would however have been interesting for this study to examine the F3 generation in order to identify potential effects in the absence of exposure to GBH (so-called "transgenerational" effects).

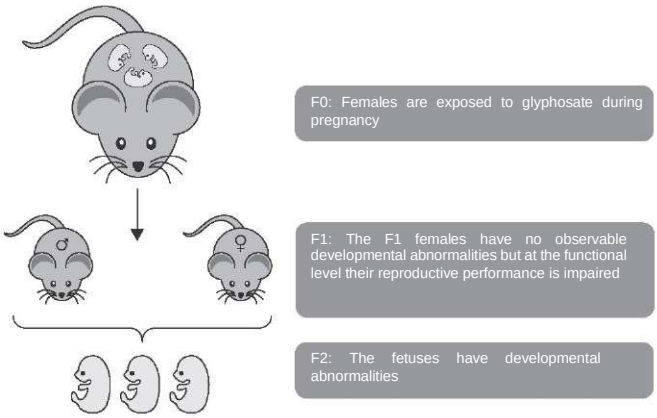


Figure 3: Intergenerational effects of an environmental pollutant: the example of glyphosate

The intergenerational effects noted suggests transmission of epigenetic information (i.e. not involving alteration of the DNA sequence) linked to exposure to GBH. The processes involved in epigenetic regulation include post-translational modifications of histones, chromatin structure, DNA methylation, non-coding RNA, and RNA methylation. Three recent studies provide evidence for some of these mechanisms.

To better understand the biological mechanisms underlying the results described above, the same authors exposed pregnant rats (F0) to 350 mg/kg bw per day of glyphosate (in the form of the GBH Magnum Super II) orally during gestation and lactation. Their female offspring (F1) were mated and sacrificed on the 5th day of gestation (preimplantation period). The uteruses were collected and a decrease in methylation of the ERα promoter and post-translational modifications of histones were observed in parallel with an increase in ERα messenger levels (Lorenz *et al.*, 2019). This decrease in methylation of the ERα promoter may lead to an increase in expression of the receptor, which would explain the increase in sensitivity to estrogens, a phenomenon described above, albeit in a different context.

In other work, rats (F0) were exposed intraperitoneally to glyphosate from days 8 to 14 of gestation at a dose of 25 mg/kg bw per day. F1 animals (fetal exposure) produced F2 offspring (germ cell exposure), that produced an F3 generation (no exposure to glyphosate). The various generations were euthanized at the age of one year and an analysis of sperm epigenetic markers was conducted. DNA methylation profiles (differentially methylated regions; DMR) were different for F1, F2 and F3 in comparison to control F0 animals. The majority of DMRs were not associated with genes, but with intergenic regions. Most of the genes associated with DMRs are involved in signaling, metabolism, receptors and cytokines (Kubsad *et al.*, 2019; Ben Maamar *et al.*, 2020). Treatment did not impact the health of F0 and F1 animals, but an increase in disease frequency was observed in the F2 and F3 generations. This result suggests transgenerational epigenetic heritability.

Finally, an overall decrease in DNA methylation was reported in peripheral lymphocytes exposed *in vitro* to glyphosate at a concentration of 42.3 mg/L (0.25 mM) (Kwiatkowska *et al.*, 2017). At concentrations of 0.25 and 0.5 mM, glyphosate increased methylation of the p53 promoter with no change to the p16 promoter.

In summary, an epigenetic mode of action by glyphosate and GBH has been observed in several studies, including one at values of exposure lower than the NOAEL for a short exposure period.

Neurotoxic effects

Exposure to GBH has been reported to potentially affect the development and physiology of vertebrates (Dallegrave *et al.*, 2007; Paganelli *et al.*, 2010; Zhang *et al.*, 2017). Among the disturbances induced by glyphosate include alterations to central nervous system (CNS) homeostasis and behavior, described below.

Recent studies show that GBH induce a change in the concentration of several neurotransmitters including dopamine, norepinephrine, serotonin and glutamate in various regions of the brain such as the hippocampus, dark matter and prefrontal cortex in rodents (Hernández-Plata *et al.*, 2015; Cattani *et al.*, 2017; Gallegos *et al.*, 2018; Martinez *et al.*, 2018[22]; Yu *et al.*,

[22] Erratum : unfortunately, a reference had been omitted in the French version and has been added here.

2018). However, they often use high doses of exposure (>50 mg/kg/day) and the effects of glyphosate versus GBH are rarely compared within the same study. For example, Cattani *et al.* reported oxidative stress in rats exposed continuously to GBH starting during prenatal life (by exposure of their mothers to 1% GBH in drinking water beginning on the 5th day of gestation) until postnatal day 15 or 60 (exposed indirectly via lactation until weaning, and then directly via drinking water). The changes included depletion of the glutathione pool (GSH), decreased expression of GST and SOD in hippocampal cells, and abnormal cholinergic and glutamatergic neurotransmission (Cattani *et al.*, 2017). For reference to other species, 1% GBH corresponds to 0.36% glyphosate (equivalent to 3,600 mg/L) ; a 35 g mouse drinks around 5 mL a day, and thus, the dose in this experiment would correspond to approximately 18 mg/day or 514 mg/kg/bw per day. The neurotoxic effects of GBH were accompanied by a depressive state and reduced mobility. The reduced neurotransmitter concentrations could explain the locomotor deficits or symptoms of anxiety and depressive-like behavior also observed in other recent studies, in rodents exposed to glyphosate or GBH (Bridges, 2016; Ait Bali *et al.*, 2017; Cattani *et al.*, 2017; Ait Bali *et al.*, 2018; Gallegos *et al.*, 2018).

Other models are also now being used to investigate the potential neurotoxic effects of GBH and glyphosate. In zebrafish, Pereira *et al.* have shown the induction of alteration of the mitochondrial respiratory chain (complexes I and IV) in certain neurons by exposure to glyphosate or GBH (doses of 0.065, 1.0 and 10.0 mg/L) (Pereira *et al.*, 2018). It is widely accepted that oxidative stress and/or disruption of mitochondrial physiology contribute to the development of neurodegenerative diseases (Rugarli and Langer, 2012). Consistent with this observation, a neurobehavioral effect was observed in zebrafish after 7 days at the lowest dose of exposure to GBH (0.065 mg/L), corresponding to exploratory activity solely at the top of the aquarium compared to the control group (Pereira *et al.*, 2018). In addition, in larvae (after 3 days post-fertilization) and in adults exposed to glyphosate or Roundup for 96 h, the authors observed reduced locomotion in adults (0.5 mg/L glyphosate and 0.065 or 0.5 mg/L Roundup), impaired memory in adults (0,.5 mg/L Roundup) and decreased interocular distance in larvae (0.5 mg/L glyphosate). Exposure to glyphosate and Roundup also reduced aggressive behavior in adults (Bridi *et al.*, 2017).

The studies above focused on the effects of glyphosate and GBH on brain development in offspring following perinatal exposure, which is logical

given the neuroplasticity of the CNS during development. One recent animal study, however, examined potential neurotoxic effects in exposed mothers. Dechartres *et al* exposed pregnant rats to glyphosate or Roundup 3 Plus at a low dose of glyphosate (5 mg/kg bw per day; i.e. 1/10 of the NOAEL) from the 10th day of gestation through to the 21st day postpartum (PND21) (Dechartres *et al.*, 2019). This low dose of exposure affected maternal licking behavior and neurogenesis in the postpartum period, as illustrated by an increase in the population of postmitotic immature neurons in the dorsal dentate gyrus. Although no direct neurotoxic effect was demonstrated, it is interesting to note that glyphosate or the formulation altered the *Bacteroidetes* and *Firmicutes* ratio in the mother's gut microbiota.

With regard to the mechanisms inducing neurotoxic effects, the metal chelating property of glyphosate has been studied very little and may contribute to the neurotoxicity observed in various experimental models (Mertens *et al.*, 2018).

These more marked neurotoxic effects with GBH compared to glyphosate are associated with dysbiosis of the gut microbiota. The link between dysbiosis of the gut microbiota (with these EPSPS-expressing bacteria) and numerous diseases including cancer and psychiatric disorders (Galland, 2014; Kosumi *et al.*, 2018) should prompt further investigation of the effect of glyphosate on microbial populations, as discussed below.

Glyphosate and dysbiosis of the gut microbiota

Glyphosate is used on plants for its inhibitory effect on the shikimate pathway, a biosynthetic pathway found in many bacteria, and it consequently has the potential to effect animal and environmental microbiota. Here we will limit our analysis to symbiotic microbiota in animals and, in particular, to the gut microbiota.

An *in vitro* toxicity test of glyphosate on the microbiota of chicken was performed to assess its impact on bacteria that may provide protection from pathogenic bacteria (Shehata *et al.*, 2013). Pathogenic bacteria such as *Salmonella enteritidis, S. gallinarum, S. typhimurium, Clostridium perfringens* and *C. botulinum* are resistant to glyphosate. Conversely, protective bacteria such as *Enterococcus faecalis, E. faecium, Bacillus badius, Bifidobacterium adolescentis* and *Lactobacillus spp.* are moderately or highly susceptible to glyphosate. We can therefore hypothesize that by disrupting the balance of the microbiota, glyphosate predisposes organisms to diseases related to bacterial dysbiosis.

When bovine rumen microbiota were fermented *in vitro*, the induction of dysbiosis by glyphosate, which promotes the development of *Clostridium botulinum* (pathogenic glyphosate-resistant bacteria, see above) and production of botulinum neurotoxin were observed in the rumen fluid (Ackermann *et al.*, 2015).

In rodent models, exposure to Roundup Grand Travaux Plus (450 g/L glyphosate) present in drinking water at doses of 50 ng/L (0.1 ppb), 100 mg/L (400 ppm) and 2,250 mg/L (5,000 ppm) induced dysbiosis of the rat gut microbiota with an increase in the *Bacteroidetes* family and a decrease in the *Lactobacillaceae* family (Lozano *et al.*, 2018). Oral exposure of rats to 1.75 mg/kg/day glyphosate from the 6th day of gestation up to 13 weeks did not affect animal survival, weight or dietary behavior (Panzacchi *et al.*, 2018). The same results were obtained for an equivalent dose of glyphosate in the form of Roundup Bioflow (containing 41.5% glyphosate isopropylamine salt), which at the time of study publication was defined as the chronic reference dose set by the US Environmental Protection Agency. However, when rats (F0) were exposed using the same experimental design from day 6 of gestation to postnatal day 125 (PND125), a significant change was seen in the composition of gut microbiota in F1 generation animals on PND31 (corresponding to prepuberty in humans): the *Bacteroidetes/Firmicutes* ratio was increased with glyphosate and Roundup Bioflow (Mao *et al.*, 2018). This increase in the proportion of *Bacteroidetes* is consistent with the study by Lozano *et al.* In mice, two studies have found an disruptive effect from Roundup on gut microbiota associated with symptoms of anxiety or depression after exposure at high doses (250 and 500 mg/kg bw per day) (Ait Bali *et al.*, 2017; Ait Bali *et al.*, 2018).

These reported effects in animal models, used to study mechanisms related to human health, must not cause us to overlook glyphosate toxicity on ecosystems. A toxic effect on insects, and in particular bees, has been reported. Bees were exposed orally (syrup) to glyphosate at doses of 5 and 10 mg/L (Motta *et al.*, 2018), corresponding to environmental exposure levels (Herbert *et al.*, 2014). Exposure to glyphosate during early gut colonization increased the mortality of adult bees when exposed to opportunistic pathogens (Motta *et al.*, 2018). Exposure of nurse bees to a sublethal dose of Roundup Original (glyphosate/artificial feed in a ratio of 2.16 µg/g) resulted in ultrastructural changes in the hypopharyngeal glands, degeneration of the endoplasmic reticulum and structural changes in mitochondria in these cells. This impact on the hypopharyngeal glands

results in impaired royal jelly production, with an effect on the development and survival of bee colonies (Faita *et al.*, 2018).

Finally, the hypothesis that GBH might promote the generation of co-resistance of coliform bacteria to glyphosate and antibiotics has recently been suggested (Kurenbach *et al.*, 2015; van Bruggen *et al.*, 2018). This hypothesis also raises the question of a selection effect for glyphosate-resistant weeds, along with changes to symbiotic microbiota in plants and animals.

Conclusion

Glyphosate is the most widely used herbicide in the world, and the most used in France. Due to the methods and circumstances of application, dermal absorption is considered to be the main route of exposure among both professional and non-professional users. In addition, contamination of foodstuffs may result in oral exposure of consumers. Glyphosate undergoes very little metabolism in the body (less than 1%) and is excreted in the urine unchanged. It does not have a high bioaccumulation potential, and its half-life in humans is estimated to be between 5 and 10 h.

Quantification of glyphosate in the urine is the most appropriate method for estimating and monitoring population exposure over time. However, this requires rigorous analytical methods (extraction, separation and detection techniques). The urinary concentrations frequently found in occupationally exposed populations or in the general population are in the µg/L range. These values are 100 to 1,000 times lower than those expected for chronic exposure corresponding to the ADI currently set by EFSA, that is 0.5 mg/kg/day (EFSA, 2015a). This reference value is, however, based on experimental data in laboratory animals and it does not enable one to rule out all risk to humans, particularly in cases of repeated and long-term exposure. Many epidemiological studies have therefore considered the public health impact of occupational exposure (and to a lesser extent residential exposure) to glyphosate-based formulations. These studies have investigated the occurrence of both cancer and non-cancer diseases.

In 2013, the Inserm collective expert report concluded that exposure to glyphosate was associated with an excess risk of non-Hodgkin lymphoma (NHL), with a moderate presumption of a link for occupationally exposed populations (considered as a whole without distinction of the specific employment categories), and a weak presumption of a link specifically with respect to farmers. New data have been acquired since 2013. A follow-up

analysis of the AHS cohort in the United States found no increased risk of developing NHL or its major subtypes among licensed pesticide applicators exposed to glyphosate. However, a meta-analysis published by the AGRICOH consortium, combining the AHS cohort with two other agricultural worker cohorts (AGRICAN in France and CNAP in Norway) and including over 300,000 subjects, with 2,430 cases of NHL, found a statistically significant association between the risk of developing diffuse large B-cell lymphoma and exposure to glyphosate. The presumption of a link between exposure to glyphosate and the risk of developing NHL is therefore considered by the Inserm expert group to be moderate regardless of occupational category (farmer or other).

Regarding multiple myeloma, the 2013 Inserm collective expert report did not conclude on the existence of a link between exposure to glyphosate and the risk of developing this disease, as the few French and North American case-control studies and the AHS cohort were based on a limited number of cases. Recently, a new follow-up analysis of the AHS cohort found no association with multiple myeloma. However, a meta-analysis of earlier case-control studies, as well as some data from the AHS cohort, found an increased risk among farmers exposed to glyphosate that was at the limit of statistical significance. Taking these new data into account, the presumption of a link with respect to the risk of multiple myeloma is considered to be weak (±). It is based on a low level of evidence: a risk that is elevated, but at the limit of statistical significance in a meta-analysis of three case-control studies and one cohort study.

Very little information was available on exposure to glyphosate and the occurrence of Hodgkin lymphoma at the time of the 2013 Inserm collective expert report. Little has been published in the scientific literature since then: three studies, including a meta-analysis (based on two previous case-control studies) and a follow-up analysis of the AHS cohort. No association was found between glyphosate exposure and the occurrence of Hodgkin lymphoma. In view of these results, no presumption of a link can be established.

Only a single study looking at exposure to glyphosate and the risk of leukemia was available at the time of the 2013 Inserm collective expert report. This study, that focused on occupational exposure in the AHS cohort and did not include acute myeloid leukemia, suggested an increased risk in the second tertile of exposure to glyphosate. The evidence was thus insufficient to establish a link.

Since 2013, two meta-analyses have been published based on data from the AGRICOH consortium, concluding that there is no association with the risk of developing chronic lymphocytic leukemia (CLL). A third analysis of three case-control studies also concluded that there was no association with CLL. Two of the three case-control studies evaluating the risk of developing hairy cell leukemia (a subtype of CLL) found an increased, though not significant, risk associated with glyphosate exposure. A recent follow-up analysis of the AHS cohort did not confirm the trend for increased risk of CLL reported previously. Finally, this new follow-up of the AHS study, based on a limited number of cases, identified for the first time a more than two-fold increase in the risk of acute myeloid leukemia among the most exposed subjects, which did not reach statistical significance. Taking into account in particular the latest results from the AHS cohort, the presumption of a link between exposure to glyphosate and the risk of developing leukemia is considered to be weak. This presumption is based on a single study (AHS cohort) that was well conducted but requires confirmation.

Prostate cancer and bladder cancer have been studied in relation to exposure to glyphosate in the AHS cohort. With regard to prostate cancer, the various follow-up analyses of the cohort over time have not shown any excess risk. For bladder cancer, researchers observed an excess risk but this was not statistically significant. At present, based on the available studies, it is not possible to establish a presumption of a link between exposure to glyphosate and the occurrence of prostate and bladder cancer.

Concerning non-cancer diseases, some studies, primarily within the AHS cohort, point to occupational exposure in the agricultural sector to multiple pesticides, including glyphosate, being associated with an increased risk of wheeze (with or without an allergic component) in male farmers and industrial applicators and of allergic asthma in their pesticide-applying spouses. In view of the limited number of studies and the fact that the results are primarily based on a single cohort study (AHS), the presumption of a link to respiratory health is considered to be weak.

Other studies, all based on the AHS cohort, have reported an excess risk of hypothyroidism in male glyphosate applicators, without evidence of a dose-response relationship. Among their glyphosate-applying spouses, no association was found with the risk of hypothyroidism.

Finally, various studies have explored whether occupational or residential exposure to glyphosate might be linked to the occurrence of anxiety and

depressive disorders, Parkinson's disease, and chronic kidney disease of unknown etiology in adults, with birth defects and neurobehavioral disorders in young children, and with growth indicators in newborns (gestational length, birthweight). However, at present the ecological nature of some studies, imprecision in measuring exposure, low subject numbers and the inconsistency of the conclusions, make it impossible to reach definitive conclusions and thus establish a presumption of link.

And what about the biological plausibility of the epidemiological associations described above? Numerous experimental studies have been carried out on both cancer and non-cancer diseases in relation to this issue.

In recent years, glyphosate has been thrust into the spotlight by a debate regarding its carcinogenicity. This stemmed from the divergence between the conclusions of IARC and the other national and international agencies responsible for the classification and regulation of chemicals. These divergent conclusions are largely due to the different approaches and criteria used.

For carcinogenicity testing in laboratory animals as well as mutagenicity studies, the level of evidence is relatively limited. However, many studies show genotoxic damage (DNA breaks or structural changes). These changes, if not faithfully repaired, can lead to the development of mutations and trigger carcinogenesis. Such effects are consistent with the direct or indirect induction of oxidative stress by glyphosate, observed in various species and cell systems, sometimes at exposure levels compatible with those that may be experienced by human populations.

In addition to glyphosate's ability to induce production of reactive oxygen species, other toxicological characteristics have been described. Glyphosate is used to block amino acid synthesis in plants. Its principal mode of action involves blocking an essential enzyme that is expressed by plants, but also by fungi and certain bacteria. Unlike these organisms, animals and humans do not have the gene coding this enzyme. However, experimental studies suggest deleterious effects related to mitochondrial toxicity (associated with behavioral disturbances in models such as zebrafish) and endocrine disruption, including impaired steroidogenesis and activation of estrogen pathways without binding to estradiol receptors. The results of studies published since 2013, that must be confirmed, also indicate dysregulation of the concentration of neurotransmitters (consistent with behavioral alterations), but also of the gut microbiota in several animal species including humans, that are a logical target of glyphosate as certain bacteria also express the enzyme targeted in plants. Such mechanisms merit further

investigation and greater consideration during regulatory evaluation procedures.

Finally, although beyond the scope of this review, the expert group notes that the potential effects of glyphosate use on ecosystems and their regulation raises important questions surrounding the environment and its indirect impact on human health. These deserve to be addressed using an integrated and systemic (i.e. "One Health") approach and incorporated by policy makers in the decision making process on equal standing with social and economic considerations.

Table IV: Comparison of the IARC and BfR/EFSA/ECHA analyses of carcinogenesis testing in rodents[1]

References Strain, sex, duration	Substance and doses[2]	Results[3]	Analysis of data by IARC	Analysis of data by BfR/EFSA/ECHA
Mouse studies				
Knezevich and Hogan, 1983 (EPA, 1985a; EPA, 1985b; EPA, 1986; EPA, 1991d) CD-1; (M/F); 24 months; TOX9552381 (Monsanto)	Glyphosate (99.7%) at 0, 1,000, 5,000, 30,000 ppm (equivalent to 0, 157/190, 814/955, 4,841/5,874 mg/kg bw per day); oral exposure (pellets)	Males: renal tubule adenoma (0/49, 0/49, 1/50, 3/50 animals); no kidney data for females Re-examination of slides (EPA, 1986): renal tubule adenoma (1/49, 0/49, 0/50, 1/50 animals) [NS]; renal carcinoma (0/49, 0/49, 1/50, 2/50 animals) [p = 0.037]; tubule adenoma or renal carcinoma combined (1/49, 0/49, 1/50,3/50 animals) [p = 0.034]	The 1986 pathologists' report confirms no increase in incidence by comparing the treated groups to the control; however, the trend test gives a p value = 0.016	Paired analysis result negative. Historical controls for adenoma or renal carcinoma value up to 6%. Toxicity cannot be ruled out at high doses. Renal tumor result considered negative
Atkinson *et al.*, 1993a (JMPR, 2006) CD-1; (M/F); 24 months; TOX9552382 (Cheminova)	Glyphosate (98.6%) at 0, 100, 300, 1,000 ppm; oral exposure (pellets)	Males: hemangiosarcoma (0/50, 0/50, 0/50, 4/50); histiocytic sarcoma in hematopoietic tissue (0/50, 2/50, 0/50, 2/50); lymphoma (4/50, 2/50, 1/50, 6/50) Females: hemangiosarcoma (0/50, 2/50, 0/50, 1/50); histiocytic sarcoma in hematopoietic tissue (0/50, 3/50, 3/50, 1/50); lymphoma (14/50, 12/50, 9/50, 13/50)	Trend test p <0.001 for hemangiosarcoma in males (non-significant for females and other tumors in both sexes)	Paired analysis result negative. The base incidence for hemangiosarcoma is 12% if all organs are considered. The observed incidence is indicative of a spontaneous effect unrelated to treatment
George *et al.*, 2010; Swiss; (M); 32 weeks; ASB2012-11829, initiation-promotion study	GBH (Roundup Original: 41% glyphosate, 15% POEA); 25 mg/kg bw; skin exposure	Skin tumors (papilloma): controls: 0/20; GBH (3 times a week): 0/20; GBH (1 or 9 doses) then TPA: 0/20; DMBA then GBH (3 times a week): 8/20	Short duration of treatment, no control for solvents, no histopathology assessment, no information on age at start of experiment. Conclusion: study not relevant	Study inadequate to test the carcinogenic effect of glyphosate

Table IV: (cont.)

References Strain, sex, duration	Substance and doses[2]	Results[3]	Analysis of data by IARC	Analysis of data by BfR/EFSA/ECHA
Wood *et al.*, 2009b; CD-1; (M/F); 18 months; ASB2012-11492 (Nufarm)	Glyphosate (95.7%): 0, 500, 1,500, 5,000 ppm (equivalent to 71/98; 234/299; 810/1,081 mg/kg bw per day); oral exposure	No increase in tumor incidence, but in males: lymphoma: (0/51, 1/51, 2/51, 5/51); females: lymphoma: (11/51, 8/51, 10/51, 11/51)	An analysis performed for IARC by an external expert concluded that there was a significant association for males (p = 0.004); study not considered	Negative result
Kumar, 2001; Swiss albino; (M/F); 18 months; ASB2012-11491 (ADAMA)	Glyphosate (95.14%): 0, 100, 1,000, 10,000 ppm (equivalent to 15; 151; 1,460 mg/kg bw per day for males and females)	Increased incidence of lymphoma at high doses in males, histological change in the stomach; males: lymphoma (10/50, 15/50, 16/50, 19/50); females: lymphoma (18/50, 20/50, 19/50, 25/50)	An analysis performed for IARC by an external expert concluded that there was a significant association for males (p = 0.05); study not considered	Taking into account historical controls (18 to 27% lymphoma in males, or up to 50%, but the study puts this value down to a viral infection of the "CFW Swiss" strain), some indications of carcinogenesis but not sufficient to be taken into account for classification
Sugimoto, 1997; CD-1; (M/F); 18 months; ASB2012-11493 (Arysta)	Glyphosate (97.56% and 94.61%): 0, 1,600, 8,000, 40,000 ppm (equivalent to 165/153; 838/787; 4,348/4,116 mg/kg bw per day)	No positive response for carcinogenicity; males: lymphoma (2/50, 2/50, 0/50, 6/50); females: lymphoma (6/50, 4/50, 8/50, 7/50)	Study not considered	Negative result
Studies in rats				
Séralini *et al.*, 2014; Sprague-Dawley; (M/F); 24 months	GBH: 0, 1.1 x 10^{-8}%, 0.09%, 0.5% (equivalent to 0, 50 ng/L, 400 mg/L, 2.25 g/L glyphosate); exposure through drinking water	Males: no increase in tumor incidence; females: mammary tumors (5/10, 9/10, 10/10, 9/10), pituitary abnormalities (6/10, 8/10, 7/10, 7/10)	No information on the composition of the formulation, little information on and no discussion of histopathology, number of animals too small. Conclusion: study not relevant	Group size too small (10 M and 10 F), not taken into account in CLP assessment
Chruscielska *et al.*, 2000; Wistar; (M/F); 24 months	Glyphosate (ammonium salt): 0, 300, 900, 2,700 mg/L; exposure through drinking water	No increase in tumor incidence (55 M and 55 F)	Little information on diet, histopathology and tumor incidence	Salt tested rather than the acid, debatable protocol, not taken into account in the CLP assessment

Table IV: (cont.)

References Strain, sex, duration	Substance and doses[2]	Results[3]	Analysis of data by IARC	Analysis of data by BfR/EFSA/ECHA
Milburn, 1996 (JMPR, 2006); Wistar; (M/F); 12 months	Glyphosate (95.6%): 0, 2,000, 8,000, 20,000 ppm; oral exposure (pellets)	No increase in tumor incidence (24 M and 24 F)	Limited duration of exposure	Not mentioned
Atkinson *et al.*, 1993b (JMPR, 2006); Sprague-Dawley; (M/F); 24 months; TOX9750499 (Cheminova)	Glyphosate (98.7% and 98.9%): 0, 10, 100, 300, 1,000 mg/kg bw per day; oral exposure (pellets)	No increase in tumor incidence	Negative result	Negative result
Brammer, 2001 (JMPR, 2006) Wistar; (M/F); 24 months; ASB2012-11488 (Syngenta)	Glyphosate (97.6%): 0, 2,000, 6,000, 20,000 ppm; oral exposure (pellets)	No increase in tumor incidence	Negative result	Negative result
Stout and Ruecker, 1990 (EPA a,b, c et d)); Sprague-Dawley; (M/F); 24 months; TOX9300244 (Monsanto)	Glyphosate (96.5%): 0, 2,000, 8,000, 20,000 ppm; oral exposure (pellets)	Males: pancreatic adenoma (1/58, 8/57, 5/60, 7/59), pancreatic carcinoma (1/58, 0/57, 0/60, 0/59), combined adenoma and carcinoma (2/58, 8/57, 5/60, 7/59), hepatic adenoma (2/60, 2/60, 3/60, 7/60), hepatic carcinoma (3/60, 2/60, 1/60, 2/60) Females: pancreatic adenoma (5/60, 1/60, 4/60, 0/59), pancreatic carcinoma (0/60, 0/60, 0/60, 0/59), combined adenoma and carcinoma (5/60, 1/60, 4/60, 0/60), thyroid adenoma (2/60, 2/60, 6/60, 7/60) and thyroid carcinoma (0/60, 0/60, 1/60, 0/60)	Pancreatic adenoma in historical controls 1.8 to 8.5% Additional statistical analyses (EPA, 1991a): males: pancreatic adenoma p <0.05, trend test p = 0.016, no progression to carcinoma; hepatic adenoma trend test p = 0.016, no progression to carcinoma; females: thyroid adenoma trend test p = 0.031, no progression to carcinoma	Positive result for pancreatic tumor at low dose considered to be incidental. Hepatic adenoma in rats and thyroid adenoma in females but with no progression to carcinoma

Table IV: (cont.)

References Strain, sex, duration	Substance and doses[2]	Results[3]	Analysis of data by IARC	Analysis of data by BfR/EFSA/ECHA
Lankas, 1981 (EPA a,b, c et d) Sprague-Dawley; (M/F); 26 months; TOX2000-595 and TOX2000-1997 (Monsanto)	Glyphosate (98.7%): 0, 30, 100, 300 ppm; oral exposure (pellets)	Males: pancreatic adenoma (0/50, 5/49, 2/50, 2/50), pancreatic carcinoma (0/50, 0/49, 0/50, 1/50), combined adenoma and carcinoma (0/50, 5/49, 2/50, 3/50) Females: pancreatic adenoma (2/50, 1/50, 1/50, 0/50), pancreatic carcinoma (0/50, 1/50, 1/50, 1/50), combined adenoma and carcinoma (2/50, 2/50, 2/50, 1/50)	No positive trend in incidence of pancreatic tumors and no progression to carcinoma but increase in one of the male groups (30 ppm, Fisher's exact test $p < 0.05$)	No increase in tumor incidence
Wood *et al.*, 2009a Wistar; (M/F); 24 months; ASB2012-11490 (Nufarm)	Glyphosate (95.7%): 0, 1,500, 5,000, 15,000 ppm increasing up to 24,000 ppm (equivalent to 86/105, 285/349, and 1,077/1,382 mg/kg bw per day); oral exposure (pellets)	No increase in tumor incidence	Study not considered	Negative result
Enomoto, 1997 Sprague-Dawley; (M/F); 24 months; ASB2012-11484 (Arysta)	Glyphosate (94.61% and 97.56%): 0, 3,000, 10,000, 30,000 ppm (equivalent to 104/115, 354/393, 1,127/1,247 mg/kg); oral exposure (pellets)	No increase in tumor incidence	Study not considered	Negative result
Suresh, 1996 Wistar; (M/F); 24 months;TOX9651587 (ADAMA)	Glyphosate (96.0% and 96.8%): 0, 100, 1,000, 10,000 ppm (equivalent to 6.3/8.6, 59.4/88.5, 595.2/886 mg/kg); oral exposure (pellets)	No increase in tumor incidence	Study not considered	Negative result

[1]: the data in this summary table are derived from an analysis of regulatory approaches (Clausing *et al.*, 2018) and reports on glyphosate published by European and other agencies (BfR, 2015a; BfR, 2015b; EFSA, 2015b; EPA, 2017; IARC, 2017). The references for unpublished industry studies are provided for clarity.

[2]: the doses separated by a slash are the data for males and females.

[3]: the numbers separated by a slash correspond, for each exposure group, to the number of animals with the disease compared to the total.

Abbreviations: CLP: Classification, Labelling and Packaging of Substances and Mixtures; DMBA: 7,12-Dimethylbenz[a]anthracene; GBH: Glyphosate-based herbicide; JMPR: Joint Meeting on Pesticide Residues of the Food and Agriculture Organization of the United Nations and the World Health Organization; POEA: polyoxyethylene amine; TPA: 12-0-Tetradecanoylphorbol-13-acetate.

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• • Study financed by a pesticide company

• • • One or more authors affiliated with a pesticide company

• • • • Study where a conflict of interest was identified

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Annex 1. Inserm Collective Expert Reviews : principles and methods

The aim of Inserm Collective Expert Reviews[23] is to summarize scientific knowledge on topics in the health sector through a critical analysis of the international literature. They are conducted at the request of institutions (ministries, health agencies or insurance bodies, etc.) who want up-to-date research data relevant to their public policy decisions.

Since their inception in 1994, over 80 collective expert reviews have been conducted on numerous subjects. Inserm is responsible for the conditions under which the expert reviews are performed (selection of source documents, constitution of expert groups based on the qualifications and independence of its members, transparency of the process, etc.) in accordance with its Charter of Expertise, which sets out its ethical principles.[24]

The Collective Expert Reports Unit of Inserm, which is part of its Public Health Thematic Institute, manages the scientific and technical coordination of the expert reviews following a defined procedure consisting of six main stages.

Commission of the collective expert review

The first stage involves *i*) working with the commissioning body to ensure the request is clearly defined, *ii*) verifying that sufficient scientific literature exists on the topic in question, and *iii*) drawing up an agreement outlining the scope and principal topics of the expert review, as well as the project duration and budget. During this stage the commissioning body's request is translated into scientific questions that will be addressed by the experts.

Literature searching

A document base is assembled from articles identified through searches of international bibliographic databases, along with other documents identified from the gray literature (institutional reports, etc.) relevant to the scientific questions posed. This document base is updated during the expert

[23] Brand name registered by Inserm.

[24] Inserm Charter of Expertise available from: https://www.inserm.fr/wp-content/uploads/media/entity_documents/inserm-charteexpertise.pdf

review and is complemented by the experts according to their area of expertise.

Constitution of a multidisciplinary expert group

For each expert review, a group of 10 to 15 experts is formed whose composition is determined by the scientific fields required to analyze the literature and answer the questions asked, while also ensuring the complementarity of approaches and disciplines.

Experts are selected from across the French and international scientific community. The selection is based on scientific expertise as demonstrated by their publications in peer-reviewed journals and by peer recognition. The experts must be independent of the commissioning body and of recognized lobbying groups. Prior to the start of the expert review, all the experts are required to complete and sign a declaration of interests that is kept on file by Inserm. The composition of the expert group is approved by the executive management of the Inserm Public Health Thematic Institute.

The experts' work lasts from 12 to 18 months depending on the amount of literature analyzed and the complexity of the topic.

Critical analysis of the literature by the experts

Over a series of expert group meetings, each member presents their critical analysis of the literature on a given topic, which is then discussed by the group. This analysis leads to the drafting of the various chapters of the expert report, that is constructed through collective discussion to ensure its coherence and articulation.

Individuals outside the group of experts may be invited to participate in these meetings to provide a complementary approach or viewpoint. Depending on the topic, meetings with civil society organizations may also be arranged by the Collective Expert Report Unit in order to learn about their issues of concern and to identify other sources of data.

Summary and recommendations

A summary is produced that brings together the key points of the literature analysis and outlines the principal findings and overarching themes. Most collective expert reports include recommendations for action or research intended for decision-makers. These recommendations, formulated by the group of experts, are based on scientific arguments resulting from the analysis. The collective expert review procedure does not generally include

an assessment of their feasibility and social acceptability. Such assessment may be subject to other types of expert review.

Publication of the collective expert report

After submission to the commissioning body, the collective expert report, consisting of the analysis, summary and recommendations, is published by Inserm. In agreement with the commissioning body, various communications strategies may be used, such as press releases, press conferences, or seminars open to various stakeholders including patient organizations, professionals, researchers, and institutions.

The expert reports are available from bookstores and can be accessed from the Inserm website.[25] The full collection is also available from iPubli,[26] which provides free access to Inserm publications.

[25] https://www.inserm.fr/information-en-sante/expertises-collectives

[26] http://www.ipubli.inserm.fr/handle/10608/1

Annex 2. Qualification of the links between glyphosate exposure and disease

The presumption of a link between glyphosate exposure and the occurrence of each disease or health event examined was categorized according to the scale used in the 2013 and 2021 Inserm collective expert reports on pesticides, that is : strong (++), moderate (+), or weak (±).

The degrees of presumption are evaluated in accordance with the following general principles adapted from Wigle *et al.* (2008) and a report from the US National Academy of Medicine (formerly US Institute of Medicine) published in 2000 :

- (++) the presumption of the link is strong if there is a high-quality meta-analysis showing a statistically significant association, or several high-quality studies from different groups showing statistically significant associations ;
- (+) the presumption of the link is moderate if there is at least one high-quality study showing a statistically significant association ;
- (±) the presumption of the link is weak if the studies are of insufficient quality or have inconsistent findings, or do not have sufficient statistical power to conclusively support the existence of an association.

These degrees of presumption represent a framework of reasoning that provides for some flexibility. For example, if a meta-analysis exists but it is based on studies that are limited in number or heterogeneous, the group of experts may assign it limited weight when assessing the presumption of the link. Conversely, a rigorous case-control study based on very good characterization of exposure may be considered more informative and more robust than several cohort studies of moderate quality in which characterization of exposure is based on a questionnaire.

Toxicology data from the academic literature was also analyzed to assess the biologic plausibility of a link between exposure to glyphosate and the development of disease, and also to flag effects that have not been observed or not investigated in the epidemiologic studies. These data are derived from the fields of biochemistry, cell biology, genetics, histology, and physiology on the basis of *in silico*, *in vitro*, and *in vivo* models (molecular modeling, cell and tissue lines, and animal testing). The following aspects were taken into consideration : *i*) the relevance of the experimental models; *ii*) the level of exposure ; and *iii*) the coherence of the findings relating to the mechanism(s) of toxicity.