ATTACHMENT

to the comment related to the public consultation on glyphosate on behalf of Pesticide Action Network (PAN Germany), Hamburg, and Health and Environment Alliance (HEAL), Brussels.

Detailed comments concerning:

Volume 1, 2.6.5.1. Short summary and overall relevance of the provided information on long-term toxicity and carcinogenicity, part 2.6.5.1.1 Animal studies (pp. 250-298)

Note: all page references made here refer to RAR Volume 1 (2021/06 version)

Part 1
Carcinogenicity studies in mice (pp. 234-237; 254-258; 280-298; 310-311 of RAR, Volume 1)

For background information on the critique below concerning "limit" dose, two-sided testing, historical control data, and quantitative dose-response comparisons across studies etc., please refer also to Clausing et al. (2018)[1]

Malignant Lymphoma

<u>#1: Top doses of two mouse studies (1997, 1983) were too high</u>

<u>Background</u>
1997 Study (RAR, p. 235):
40,000 ppm (corresponding to 4,348 mg/kg bw in males; 4,116 mg/kg in females)
Reduced body weight gain in top dose (males "up to -7%; up to -18% in females)

1983 Study (RAR, p. 236):
30,000 ppm (corresponding to 4,841 mg/kg bw in males; 5,874 mg/kg in females)

1983 Study (RAR, p. 236)
Reduced body weight (gain not presented in RAR): "*Mean body weights for the high-dose males were generally lower than in controls; differences from control were as great as -11 % (at Week 102)*"

<u>Claim</u>
"*The increase in renal tumour incidences in CD-1 mice was thus only observed in two studies with extremely high dose levels (4841 mg/kg bw/day and 4348 mg/kg bw/day), which is far* ***above the recommended maximum dose of 1000 mg/kg bw/day*** *according to OECD TG 453*", (RAR, p.293).

[1] Clausing, P. et al. (2018): Pesticides and public health: an analysis of the regulatory approach to assessing the carcinogenicity of glyphosate in the European Union. Journal of Epidemiology and Comminty Health **72**:668–672. doi:10.1136/jech-2017-209776; open access.

Assessment:
This alleged "recommended maximum dose" does not exist for carcinogenicity studies. OECD TG 453 describes this "limit dose" explicitly for chronic toxicity studies. Instead, for carcinogenicity studies, the OECD Guidance 116 (p. 54) recommends *a maximum concentration* of the test substance in dietary studies: 50,000 ppm. The studies discussed here are at 30,000 and 40,000 ppm. In addition, the top doses used in the 1997 and 1983 studies *do not exceed the maximum tolerated dose (MTD)*, and, thus, *are compliant* with the recommendations of OECD Guidance 116 for selecting the top dose. OECD Guidance 116 (p. 53): *"… the top dose should ideally provide some signs of toxicity such as slight depression of body weight gain (not more than 10%)*". However, the concern with regard to a higher depression of body weight gain is that this may obscure carcinogenic effects (see also next paragraph). According to OECD Guidance 116 (p. 53, emphasis added): "*The MTD is often used in the assessment of a chronic toxicity or a carcinogenicity study to* ***decide whether the top dose tested was adequate to give confidence in a negative result***." The concept of using the MTD to select the top dose is also applied by ECHA (2017, p.385). MTD-considerations are not limited to a decrease in body weight gain. An exceedance of the MTD includes tissue necrosis, metabolic saturation and a reduced life span due to effects other than tumours, (OECD Guidance 116, p. 53). Such effects, however, were not observed in the 1983 and 1997 study. As for the MTD, the only concern remaining would be that the top doses of these two studies were **not adequate to give confidence in a negative result**. In fact, the 1983 Study – the study with the strongest depression of body weight gain (28% reduction over all) – is the only study with a negative result concerning malignant lymphoma (ML). In other words, there is reason (recommended by OECD) to disregard this study in the overall evaluation of ML. Taking this approach there would be three positive studies concerning ML and two studies invalid for different reasons which did not show an effect.

Detailed explanation for the consideration of lower body weight gain: The concern regarding false negative results, because of a decreased body weight gain of more than 10% is explained in detail in the "ILSI Principles for Dose Selection in Chronic Rodent Bioassays" (OECD Guidance 116, p. 64): "*Historically, scientists have adopted a 10% decreased body-weight gain at the end of pre-chronic studies (typically 90 days duration) as the target that should not be exceeded in chronic (carcinogenicity) studies. It is now recognised that there is a positive correlation between body weight and the occurrence of certain tumours in rodent species and strains used in safety assessment or for hazard identification; i.e., the higher the body weight between 6 and 18 months on test, the higher the probability that the animal will develop some tumours. Moreover, the lower the body weight, the less sensitive the animal may be to agent-induced toxicity, including cancer. A significant decrease in body-weight gain therefore could reduce the animal's ability to respond to compound-induced toxicities*."

#2: Two-sided testing is (more) appropriate

Claim
"*In the AGG analysis on the relevance of malignant lymphomas in mice, two-sided testing is applied as this is in-line with how the statistical analysis was established in the study protocols*.", (RAR, p.289).

Assessment:
Common sense and OECD Guidance 116 calls for the use of one-sided tests.

"In a carcinogenicity study, the expectation is often that the change will be an increase in tumours in the treated group, so a one-sided test may be considered more appropriate, although this can be controversial. If the treatment could also be protective (i.e., reduce tumour incidence or delay it) then a two-sided comparison may be more appropriate", (OECD 116, p.133). In case of pesticides, a therapeutic action is not the research question and, therefore, it is irrelevant for the assessment of glyphosate.

According OECD TG 451 (p.2), the "*statistical methods* ***most appropriate*** *for the analysis of results, given the experimental design and objectives, should be established before commencing the study.*" When a statistical analysis is "established" in the study protocol, it is still unclear, whether the most appropriate statistical method was selected. For the sake of transparency the authorities should:

- disclose the respective parts of the study protocols, e.g. by quoting them.
- describe their judgment whether the method used in the study report is considered the "most appropriate" and why
- apply the most appropriate method themselves (re-calculate), if it was not used in the study report.

Besides, a one-sided error can be applied to any statistical method and does not depend on the statistical method.

#3: Statistical Significance vs. Biological relevance

In the RAR (p. 290, first paragraph), referring to OECD Guidance 116, emphasizes that "*statistical significance is not the only criteria to decide if an effect is treatment-related*". What is neither mentioned nor applied here, is that biological relevance – as pointed out in OECD Guidance 116 – can also be recognized without statistical significance, if indicated (see the Summary at the end of this – malignant lymphoma - section).

#4: The 2009 Study

The statement on p. 289, last paragraph: "*… in the case that effects occur only at the highest dose, … This is the case for the studies by [blacked-out] (2009) and [blacked-out] (1997) …*" **is a wrong statement that doesn't correspond to the actual findings**. The 2009 Study shows a clear dose-response with ML incidences of 0, 1, 2, 5 in Control, Low, Mid and High Dose groups. This is even acknowledged in the RAR on the same page further above.
This study shows ML incidents in all exposure groups in a dose response pattern and should not be dismissed.

#5: Quantitative Comparisons

The RAR relies heavily on quantitative comparisons, representing the major argument to dismiss the augmented incidence of ML in the mouse studies. However, in order to make such quantitative comparisons, stringent requirements for comparability should be applied, similar to the use of historical control data (HCD).

The recommendations made by OECD Guidance 116 for the use of HCD need to be taken into account. More specifically, comparisons should only be made

- between the same strain

- within a limited window of time (5 years according to OECD Guidance 116)
- at least under similar housing conditions (according to OECD Guidance 116 even in the same laboratory)

OECD Guidance 116 (p. 135) emphasizes "It is widely recognized that large differences can result from disparities in factors such as pathology nomenclature, strain, husbandry, pathologists."

Applying the criteria mentioned above the following is revealed:

- Only three studies (1983, 1993, 2009) have been performed in the same strain (Crl:CD-1). It should be noted that the strain used in the 1997 Study (Crj:CD-1) is a different strain according to text book knowledge / general standards of laboratory animal science. The indifferent use of "CD-1" suggesting that all four studies were conducted with the same strain is incorrect.
- Of the three studies performed in the same strain (a precondition for comparability) all were more than 10 years apart from the closest other study.
- Housing conditions are not taken into consideration in the RAR. Stress effects (immunosuppression) induced by particular housing conditions (including, but not limited to individual versus group housing and housing on wire bottom cages versus shoe box cages) can have a major influence on tumor incidences.

In conclusion, while <u>qualitative</u> comparisons between different studies are reasonable in the framework of Weight of Evidence (WoE) considerations, <u>quantitative</u> comparisons as made in the RAR are scientifically flawed.

<u>#6: Malignant Lymphoma WoE considerations</u>

The WoE considerations summarized in the RAR on p. 292 need to be re-considered taking into account the following facts:

1. **Increased incidence** of MLs in male mice of the top dose in 3 of 5 studies in three different mouse strains – indicating a robust effect (according to RAR all 3 with statistical significance, depending on the statistical method used)
2. **Dose-dependent** ML response in 2 of the 3 studies with effects: in males of the 2001 Study using Swiss mice (incidences: 10, 15, 16, 19) and in the 2009 Study using Crl:CD-1 mice (incidences: 0-1-2-5)
3. **Findings supported by HCD** in male mice for the 1997 and 2001 Study (no HCD available for the 2009 Study). RAR (p. 281) for the 2001 Study: "*maximum HCD exceeded in the intermediate and the high dose groups*". For the 1997 Study appropriate HCD from 7 studies were available, one of the was an outlier, according to the RAR (p.282): "*However, it is noted that 6 studies out of the 7 are all below 6% and only one has a much higher incidence, with a value of 19.2%. The latter study might be an outlier and if excluded the min—max range would be 3.8%—6% …*". The ML-incidence of the high dose group was two times higher than the upper limit of the respective HCD (outlier excluded).

Concerning the summary presented on RAR p. 292, the following should be noted:

- "*Swiss mice, overall no significant trend was observed and together with the high variability in the background incidence, the apparent increase in ML is not considered treatment related*";
  Comment: The "apparent increase in ML" was higher than the upper limit of the HCD range. Therefore, "the high variability in the background incidence" is not relevant. Thus, this remark is obsolete. In addition, a slight dose-response is exhibited by the ML incidences of this study. Furthermore, the trend test was described as "borderline significant" and the Peto-test commissioned by the authorities was significant, based on one-sided error.
  The conclusion that "the apparent increase in ML is not considered treatment related" is flawed and contradicted by the existing facts.
- "[blacked-out] and [blacked-out] CD-1 mice (remark: obviously reference is made to the 1997 and 2009 Study): an increase in MLs. Only trend sign, but not the pairwise comparison;"
  Comment: The flowchart in OECD guidance 116 (p. 123), showing a trend test for analyzing tumour incidences implies that this method is preferred. Trend test results should not be played off against those from pairwise comparisons. OECD Guidance 116 (p. 116) explains: "*Significance in either kind of test is sufficient to reject the hypothesis that chance accounts for the result. A statistically significant response may or may not be biologically significant* ***and vice versa***" (emphasis added).
- "*Only for one of the three studies in CD-1 mice HCD is available, showing that the incidence at the top dose level was within HCD range ([blacked-out], 1997);*"
  Comment: This contradicts the assessments made on p. 282 of the RAR (for a summary of the details see "3. Findings supported by HCD" in the beginning of this section #6). Using flawed HCD containing an outlier is deceptive and wrong.
- "*Variability in background incidence was shown based on the (limited) historical control data*;"
  Comment: Because in the two studies with valid HCD the incidence of the top dose is above the upper HCD limit in both cases, this statement is irrelevant for WoE considerations and should be deleted
- "*The increases in ML incidences appeared to be confined to the high dose groups in the CD-1 mice which were around or above the OECD limit dose of 1000 mg/kg bw/day*;"
  Comment: Reference to "the OECD limit dose of 1000 mg/kg bw/day" is deceptive. As explained in details above (item #1), this limit dose does not apply to carcinogenicity studies and evaluated by the OECD-recommended MTD concept the doses were appropriate.
- "*No clear dose-effect concordance between studies was observed*;"
  Comment: As explained in item #5 (quantitative comparisons), the studies are not comparable with regard to the mouse strain used and/or the time when the studies were conducted. Therefore, it is futile to perform an analysis of dose-effect concordance between studies.

#7 Obsolete considerations on oncogenic viruses for which no evidence exists

The RAR (p. 290, 4th paragraph) admits that "no information is available on the possible abundance of oncogenic viruses in the mice colony that was used for carcinogenicity testing in the study"[2] and that "there are no indications that the mice in this study had a suspected

[2] It concerns the 2001 study in Swiss mice

viral infection" and "it is unclear whether or not this could have introduced any uncertainty in the findings related to malignant lymphomas."
In other words, the text above is purely speculative, trying to weaken the evidence for the observed increased incidence of ML in this study. This is made by an extensive quote from CLH report of 2016, which itself contains flawed citations and biased use of literature. The CLH report, cited on page 290 of RAR, states: "*According to older articles, control incidences in male mice of Swiss or Swiss-derived strains may reach 18–27.5% and exceed 36% in females (Sher, 1974, Z22020; Roe and Tucker, 1974, ASB2015-2534; Tucker, 1979, Z83266). In a more recent publication, Tadesse-Heath et al. (2000, ASB2015-2535) even mentioned a nearly 50% lymphoma (mostly of B cell origin) incidence in a colony of CFW Swiss mice but also emphasised the contribution of* ***widespread*** *infections with murine oncogenic viruses to the high but remarkably variable incidence of tumours of the lymphoreticular system in this species"* (emphasis added).

Keeping in mind the improvements of the microbiological quality of laboratory rodents during the last 30 years it is dishonest to refer to publications from the 1970ies to sow doubt about a study conducted more than 20 years later, in particular when no evidence exists concerning the speculated infection. In addition, the word "**widespread**" was a pure invention by the authors of the CLH report – no such word is contained in the publication by Tadesse-Heath. In addition, Wogan and Pattengale (1984), cited in the CLH report, state: "*Virtually all spontaneous or induced lymphomas which have been studied in mice contain oncogenic viruses."* This leads to the conclusion that there is no particular "strain" vulnerability of Swiss mice.

Th entire speculation about this virus infection should be deleted from the current RAR.

Taken together:
For male mice ML as a result of treatment with glyphosate are obvious (see table below). In addition, according to the RAR, some support is also provided by the findings in female mice of the 2001 Study and of a 1999 study (not fully included in the RAR considerations, because the study report was not available).

Study of	Mouse strain	Statistically significant	Borderline significance	Dose-dependent	HCD available	Supported by HCD
1983	Crl:CD-1					
1993	Crl:CD-1					
1997	Crj:Cd-1	+			+	+
2001	Swiss		+	+	+	+
2009	Crl:CD-1	+		+		

Taken together, "the database as a whole in the species and the respective strains" (RAR, p. 209, first paragraph) demonstrates that malignant lymphoma is a statistically significant and biologically relevant tumor in male mice. The concordance of this finding and reports about an increased risk of tumors of lymphoid tissue in epidemiological studies should be noted. Taking into consideration the evidence that glyphosate is capable of eliciting genotoxic effects and oxidative stress, it is futile to call this chemical an "otherwise more or less inert substance" (RAR, p. 296, first paragraph).

It should be noted that tumours in lymphoid tissues have been reported in epidemiological studies and several meta-analyses confirmed a significantly increased risk.

Kidney tumors

Technical Remark: Erroneously in RAR reference is made to a "1993" study (p. 292, last paragraph, p. 293, legend to the table). The correct year is 1983. Likewise (p. 293 last paragraph) the year of the study performed in Swiss mice (report number Toxi 1559.CARCI-M) should be 2001, instead of 1997.

#1: Top doses of two mouse studies (1997, 1983) were too high

For Background and Assessment – see item #1 in the section on Malignant Lymphoma section.
The arguments presented there, also apply

- to the claim made on p.295:

  The two studies in CD-1 mice in which an increase in renal tubule tumours were seen ([redacted], 1983, study report no. 77-2061 and [redacted], 1997, study report no. 94-0151) used very high dose levels (>4000 mg/kg bw/day), which is well in excess of the limit dose for carcinogenicity testing as recommended by OECD guidance document 116. Since the other CD-1 mice studies were conducted at lower dose levels (up to 1000 mg/kg bw/day)

- to the statement on p. 297 ("The incidence at the top dose was outside HCD range in the study, however the relevance of this finding is limited as this dose was **above the maximum recommended dose** (according to OECD TG 453 (2009))", emphasis added), and
- to the quote from Risk Assessment Committee (RAC, 2017) opinion (RAR p. 297, 3rd paragraph) that the RAC "gives less weight to the findings at these very high doses". Discussion around the decrease in body weight "by up to 11% and 15%" ignores the fact that OECD Guidance 116 (p. 53) points out that a too strong reduction in body weight (gain) would be an obstacle for having "confidence in a **negative** result" (emphasis added). In the RAC (2017) opinion, however, this perspective is turned upside down.

#2: Historical Control Data

In the RAR, referring to the 1983 study (p. 292, last paragraph), it is written: "*The applicant has stated that historical control data is no longer available.*" This statement is wrong. HDC from at least five different laboratories are available for the 1983 study and they all strengthen the statistical significance of the tumour finding. These HDC are documented in detail in the following US EPA-documents :
https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-170.pdf
https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-249.pdf
This includes, in particular, data from 16 long-term carcinogenicity studies that had been performed in the same laboratory (bio/dynamics) as the Monsanto study and had been completed in the relevant time window between 1978 and 1982. In these 16 studies, 3 out of 815 male control mice developed kidney tumors. According to the US-EPA memorandum of February 1985, these HCD demonstrate that the probabilty of seeing 4 or more male CD-1 mice with kidney tumours (this is the outcome of the Monsanto study) is about p = 0,0064. It is stated: "*Another way of saying this is that if Glyphosate were truly unrelated to kidney*

production[3] *we would expect to see 4 or more tumors in less than 1 out of 100 experiments of the type sponsored by Monsanto*."
HCDs from four other laboratories that had been submitted to the US EPA by Monsanto also show that kidney tumours are rare in male CD-1 mice. The tumour incidence of 6% in the high dose group of the 1983 study is above the range of all HCD.

Besides it should be noted that OECD Guidance 116 emphasizes that the concurrent control is always the most important consideration

According to the RAR referring to the 2001 Study (p. 293, last paragraph) "*The increase at the mid (3.8%; 1/26) and high dose (4.0%; 2/50) was above HCD mean, but within HCD range (mean 2.0%; range 0-6%, based on 8 studies using the same strain of mice, from the same lab, years 1996-2002)*."
Because kidney tumors are rare, a spontaneous incidence of 6% appears very high. The control group incidences of all 8 studies should be disclosed– it could be possible that the upper limit of 6% is due to an outlier.

<u>#3: Contradictory terminology concerning kidney pathology</u>

On p. 293, second paragraph from bottom, it is stated: "*In the study by (1983), nonneoplastic kidney pathology in the form of chronic interstitial* ***nephritis*** *was reported to be increased, but is* ***not considered to be a precursor*** *for renal tubular cell adenoma*" (emphasis added).
On p. 295, second paragraph from bottom citing from the CLH report (2016) it says: "*There was a positive trend for chronic interstitial* ***necrosis*** *...*" (emphasis added). However, this is wrong. According to the original study report (Pathology Annex, Table 19C) it is interstitial **nephritis.** This clarification is important in the context of the "excessive" dose discussion, because "necrosis" could be an indication that the MTD was exceeded. However, interstitial nephritis has nothing to do with exceeding the MTD.

The "high dose" discussion in the last paragraph on p. 295 is misleading with regard to the 1997 Study, because the pathological changes in gastrointestinal tract have nothing to do with kidney tumors, and the discussion is purely speculative with regard to the 1983 Study, because the paragraph ends saying "there is not sufficient evidence to support this".

<u>#4: Glyphosate – an inert substance?</u>

Even the RAR itself (p. 227) admits that "… some of the *in vitro* and *in vivo* studies suggest that glyphosate may induce oxidative stress". Therefore, it is futile to call this chemical an "otherwise more or less inert substance" (RAR, p. 296, first paragraph).

More detailed information on oxidative stress elicited by glyphosate in the kidney of mice (Gao et al. 2019) can be found in Part 2 (Oxidative Stress – the neglected mode of action for carcinogenicity assessment of glyphosate)

In summary:
In males a **statistically significant** increase in kidney tumors (adenoma and/or carcinoma) was seen **in three of the five** mouse studies. **In two** studies, **dose-dependence** was observed with incidences (Control – Low – Mid – High dose) of 0 – 0 – 1 – 2 in the 1983 Study for renal carcinoma and 0 – 0 – 1 – 2 in the 1997 Study for renal tubular adenoma. In addition,

[3] Should read "kidney <u>tumor</u> production"

the results of both studies are supported, though by valid HCD. Detailed incidences of all 8 studies are needed to clarify whether an "outlier value" of one study is the reason for the upper limit of the range of 6% which is very high for spontaneous kidney tumors.
A recent publication provides an organ and species-specific potential mode of action for the occurrence of kidney tumours in mice.

Haemangisosarcoma

#1: Historical Control Data

Historical control data was provided for the study by [redacted], 1993 (6 studies terminated between September 1988 and September 1991) which showed a mean incidence of 3.3% with a range of 0-8%. Therefore, the reported incidence at the top dose was within the historical control range. No historical control data was available for the

- With 4/50 the incidence (8%) at the top dose of the 1993 Study is not "within", but at the upper edge of the (valid) HCD. Besides, detailed information on these HCD needs to be disclosed to convince stakeholders that the upper limit of 8% is not an outlier. Besides, OECD Guideline 112 recommends the use of the interquartile range instead of the "simple" range to avoid wrong conclusions.
- Reference to HCD from 52 studies (Giknis and Clifford 2005) from 11 different laboratories over a time span of 13 years (RAR p. 298, 1st paragraph) has been rejected in the RAR itself saying that such an argument is "no longer considered appropriate as only HCD should be considered which is obtained from studies using the same strain of mice from the same test facility gathered from a period of 5 year as closely matching the period that the study was performed" (RAR, p. 297, bottom of page). Therefore, it is obsolete and misleading to refer to this type of HCD
- It would be more appropriate to mention that OECD Guidance 116 (p. 135) emphasizes "that the concurrent control group is always the most important consideration in the testing for increased tumour rates".

In summary:
With the precautionary principle in mind ("Member States shall not be prevented from **applying the precautionary principle where there is scientific uncertainty** as to the risks … posed by the plant protection products …", EU Regulation 1107/2009, emphasis added), it is unacceptable to dismiss the two studies with statistically significant increases in haemangiosarcoma.

Part 2
Oxidative Stress – the neglected mode of action for carcinogenicity assessment of glyphosate

Oxidative stress is a recognized mechanism of carcinogenicity[4], and, therefore needs to be part of weight-of-evidence analysis.

[4] IARC (2015): Some organophosphate insecticides and herbicides. IARC Monographs 112, p.388-390. RMS Germany (2015): Final addendum to the Renewal Assessment Report - public version – GLYPHOSATE; Addendum 1 to RAR: Assessment of IARC Monographs Volume 112 (2015): Glyphosate, p.73-78.

On page 256 it is stated: "*A 'weight of evidence' approach should and may be applied, therefore, as a general principle*." However, this approach is not followed. Existing evidence concerning oxidative stress as a possible mode of action (MoA) has been ignored. This is astonishing, because Table 56 (page 307) explicitly mentions MoA as one of the factors to be considered during hazard assessment. It should be noted that IARC recognized oxidative stress elicited by glyphosate as a MoA.

Remarkably, this mechanism is completely ignored during the current RAR although it was addressed in the last assessment. More specifically, the previous assessment has the following statement (RMS Germany, 2015, p. 78): "*In general the documentation of the majority of studies on oxidative stress in section 4.2.3 of IARC Monographs Volume 112 (2015, ASB2015-8421) can be confirmed. It is noted that here is a lack of positive controls for oxidative stress in all in vitro and in vivo studies described in section 4.2.3 (ii) Nonhuman mammalian experimental systems of the IARC monograph. From the available data on glyphosate, there is some indication of induction of oxidative stress from testing in human cell cultures and in mammalian (in vivo) experimental systems*."

While not spelled out, the remark that "here is a lack of positive controls for oxidative stress" seems to serve the purpose of inherently dismiss these studies even though no guideline exists making positive controls for oxidative stress markers mandatory.

Since 2015 a considerable amount of additional studies have been published. A simplified strategy of searching "glyphosate" AND "oxidative stress" in the PubMed database, performed in September 2021, provided 214 hits. The results are summarized in the table below. An initial review of titles and abstracts, followed by selected full text analysis revealed 32 papers using glyphosate active ingredient and/or AMPA in vertebrate *in vivo* or *in vitro* test systems (studies using intraperitoneal injection were discounted, but will be mentioned separately below as "proof-of-concept"-studies). Of these 32 studies, 18 were in vivo studies, 13 of which were performed in rats or mice. Ten studies were published prior to 2020, eight of them were mentioned/assessed in the RAR, but none of them in Section 2.6.5 of Volume 1 or Volume 3 B.6.5.

Criterion	**Number of studies**
PubMed "glyphosate" AND "oxidative stress"	214
Glyphosate (a.i.) or AMPA vertebrate in vivo/in vitro systems	32
Vertebrate in vivo studies	17
Studies in rats and mice	12
Published prior to 2020	10
Considered in RAR	8
Considered in Carc-Section	0

It should be clear that studies/parameters on oxidative stress are not part of guidelines of regulatory study. Therefore, some of the criteria[5] to evaluate reliability are not applicable, i.e. "Study in accordance to valid internationally accepted testing guidelines" and "Study performed according to GLP". In other words, dismissing publications because of this would not be appropriate

[5] Volume 3 – B.6.7-B.6.10 (A), p. 779

One of the most important studies was published by Gao et al. (2019). ICR mice were treated by oral gavage for 28 days with 400 mg/kg body weight. In Section 2.6.3.1 (*Specific target organ toxicity-repeated exposure [STOT RE]*) the study was evaluated as follows: "*The in vivo part of the study is considered as reliable with restrictions, as this was an investigative study which only included observation of body weight, liver and kidney weight, kidney histology and several other kidney parameters*" (Volume 1, p. 162). Body weight gain was recorded and was unaffected. However, the glyphosate-treated group showed statistically significant reductions in the levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and a significant increase in the malondialdehyde (MDA) level in the kidney. Furthermore, an upregulation of the NMDA Receptor1 (NMDAR1) was seen. It was suggested that the pro-oxidant/antioxidant imbalance was related to NMDAR1-upregulation. Glyphosate, *N*-(Phosphonomethyl)-glycin, is structurally related to glycine – a known NMDA-receptor agonist which provides a molecular explanation or at least hypothesis for the effects seen in this study. Therefore, the authors conclude "*that the increase in ROS levels and apoptosis induced by glyphosate was a result of NMDAR1 activation in renal tubular epithelial cells*". These findings are in stark contrast to the claim in Volume 1, p. 296, that glyphosate would be unable to cause renal tumours, because it is a "*more or less inert substance*". Importantly, the involvement of the NMDA-receptor was validated by Gao et al. (2019) by blocking the NMDA-receptor which "*not only ameliorated glyphosate-induced increases in [Ca2+] and ROS levels, but also attenuated apoptosis*".

In contrast, Dai et al. (2016) did not find significant changes between groups concerning SOD, CAT, and MDA after treating Sprague Dawley rats by oral gavage with 0, 5, 50 or 500 mg glyphosate/ kg body weight for 5 weeks. While this study was assessed as a Category A study ("reliable with restrictions") in Vol. 3 section B.6.6.3.1. it should be noted that this applies to reproductive toxicity. Weight-of-evidence considerations need to account for confounding factors relevant for the endpoints under consideration. Reduced food consumption is well-known to be negatively correlated with lipid peroxidation.[6] Therefore, the significantly reduced consumption of food and the dose-dependent reduction of body weight (as an indirect indication of reduced food consumption) in the glyphosate groups of Dai et al. (2016) suggest that this study cannot be considered as "reliable with restrictions". Rather these decreases may have masked the oxidative stress elicited by the test substance. Therefore, the study should only be considered supportive.

All <u>four</u> arguments summarized on p.296 of Volume 1 are obsolete:

- *The effects occurred at very high dose levels above the OECD-recommended limit of 1000 mg/kg bw/day and exceeding the MTD.*
  <u>Comment:</u> This limit does not exist for carcinogenicity studies – please, refer to Clausing et al. (2018, footnote 1) or consult the guidelines/guidance themselves
- *If the whole database is taken into consideration it is clear that the top dose incidences are comparable to those observed in controls and low dose groups in the other studies or are only slightly higher (RMS: argument no longer considered appropriate as only HCD should be considered which is obtained from studies using the same strain of mice from the same test facility gathered from a period of 5 year as*

[6] Cf. Rao et al. (1990): J. Nutr. 120:602-609; Xia et al. (1995): J. Nutr. 125 195-201; Kim et al. (1996): Aging 8:123-129.

closely matching the period that the study was performed. Refer to RMS comment below)
Comment: As RMS itself confirms, this argument is no longer valid. To the contrary, the "RMS comment below", points out that a valid HCD exist for the 1997 mouse carcinogenicity study and that the incidence of kidney tumours at the top dose of this study was outside the HCD range. Then it is attempted to dismiss these facts with the wrong reference to an alleged limit dose (see above)

- *No pre-neoplastic kidney lesions were observed.*
  Comment: Portier (2020, page 6/7) lists a statistical evaluation of pathologic kidney changes in these studies, some of which can be considered pre-neoplastic
- *There is no plausible mechanism.*
  There is a plausible mechanism, see above -paper of Gao et al. (2019)

In conclusion the claim made on page 297 of Volume 1("The current RMS did not find any new information which would change the outcome of this conclusion") is not supported by the evidence.

In addition to the two publications reviewed above (Dai et al. 2016 in rats; Gao et al. 2019 in mice), further *in vivo* studies have been published and are briefly summarized in the two tables below.

Summary of in vivo studies with rats and mice

Of the seven rat studies, five (including Dai et al. 2016 - see above) appeared – for different reasons – unreliable. Of the remaining two, Owagboriaye et al. (2019) did not observe changes in oxidative stress markers, and Tang et al. (2020) saw dose-dependent effects.

Of the five mouse studies, one was considered unreliable. Of the remaining four (ncluding Gao et al. 2019 - see above), Manas et al. (2013) did not see effects on oxidative stress markers although relatively high doses were used, whereas effects were noted in the studies of Ren et al. (2018) and Zhang et al. (2021).

Studies in Rats

Reference	*Strain* Route Doses (mg/kg bw) Duration	Glyphosate Source/Purity	Comments
Dedeke et al. (2018)	*"Albino rats"* Oral gavage 3.6, 50.4, 248.4 12 weeks	Sigma-Aldrich	SOD, CAT, GPx, MDA and GSH in kidney Analysis of renal tissue for glyphosate, no residues in low dose, residues in mid-dose higher than in top-dose. The explanation offered for this paradox (more re-absorption of glyphosate at the lower dose) remains speculative. Observed changes inconsistent. In general, significant changes of endpoints after 3.6 mg/kg, but not at mid- and top-dose, GPx increased at 3.6, decreased at 50.4, unchanged at 248.4, etc. Considered unreliable.
Larsen et al. (2012)	*Wistar* Oral (drinking water) Approx. 0.9, 9 30 d, 90 d	Sigma-Aldrich	TBARS*(liver, kidney, small intestine), GSH (liver), GPx (liver, kidney, small intestine) Authors claim indications of adaptive/protective reactions. Reliability is questionable: Fig. 2 and 3 show one and the same control data for 30 d and 90 d

			treatment duration, likewise for the data in Table 1 and 2). Considered unreliable.
Milic et al. (2018)	*Wistar* Oral gavage 0.1, 0.5, 1.75, 10 28 d	Sigma-Aldrich	Reduced body weight gain at end of study (2-3 times lower at end of treatment) in all glyphosate groups (more pronounced in lower doses). No significant disturbances of oxidative stress markers were detected. Comment: Note the low doses and the lower body weight gain in glyphosate treated groups (which is surprising at these doses and at the same time may indirectly indicated reduced food consumption as a confounding factor)
Nozdrenko et al. (2021)	*Wistar* Oral gavage 0.01 30 d	Not declared	CAT, TBARS, GSH, H_2O_2 in blood Markers of oxidative stress 2-3 times higher in glyphosate group. Unclear how such a marked effect can happen. Considered unreliable.
Owagboriaye et al. (2019)	*"Albino rats"* Oral gavage 3.6, 50.4, 248.4 12 weeks	Sigma-Aldrich	CAT, MDA, GSH in blood No changes as compared to control group (significant changes for the same doses when treated with Roundup in the same experiment)
Tang et al. (2020)	*Sprague-Dawley* Oral gavage 5, 50, 500 35 d	Shanghai Ryon Biological Technology Co., Ltd.	MDA, SOD, H_2O_2, GSH, GPx in intestinal tissue Some dose-dependent decreases of SOD, GSH and GPx observed in several tissues (duodenum, jejunum, ileum) statistically sign. at 50 mg/kg and 500 mg/kg

*thiobarbituric acid reactive substance

Studies in Mice

Reference	*Strain* Route Doses Duration	Glyphosate Purity	Comments
Ganesan et al. (2020)	*C57BL/6* Orally 0.25,0.5,1,1.5,2 20 w	Sigma-Aldrich	Dosed "with a pipette tip" does not sound like a reliable dosing procedure, in particular not with such a narrow dose spacing. Results concerning oxidative stress were inconclusive. Considered unreliable.
Manas et al. (2013)	*Balb c* Drinking water at concentrations adjusted for doses of 40 and 400 glyphosate or 100 of AMPA 14 d	Sigma-Aldrich 96%	TBARS, SOD, CAT in liver, kidney, lung, heart. Authors' conclusion: minor effects seen on oxidative stress parameters (significant decrease of SOD in heart, and significant increase of CAT in kidney). Note: strong effects for DNA damage as measured by comet assay in blood and liver
Ren et al. (2018)	*ICR* Drinking water 5 g / L Gestational day 1-19	Shanghai Ryon Biological Technology Co., Ltd.	MDA, CAT, SOD and total antioxidant capacity (T-AOC) in ovary; MDA, CAT, GPx and (T-AOC) in serum Feed intake unaffected; In ovary: CAT significantly reduced In serum: GPx significantly reduced
Zhang et al. (2021)	*ICR* Drinking water 0.7 mg/L 12 w	Ehrenstorfer (Germany) 98%	Study designed to investigate combined effects of glyphosate and hard (Ca^{2+}) water. Third experiment with glyphosate (active ingredient) alone, hard water alone, and hard water + glyphosate combined. Endpoints: DHE (fluorescent probe to measure intracellular reactive oxygen species), MDA, SOD. DHE significantly increased for glyphosate (about twice as high as control) combined with hard water about four times higher than control MDA, SOD slightly, but significantly increased in combined group

Additional considerations

Studies in rats (Astiz et al. 2009) and mice (Bolognesi et al. 1997) using intraperitoneal injection demonstrated strong effects on oxidative stress markers. While not being a representative route of exposure, intraperitoneal injection ensures high bioavailability. Thus, these studies can be considered as a "proof of concept" for this type of effects.

IARC (2015) assessed a large number of studies published prior to 2015 performed *in vivo* in non-mammalian species or in *in vitro* test systems, including human cells. Based on this review IARC (2015, p. 399) concluded: *"There is strong evidence that glyphosate, glyphosate-based formulations, and aminomethylphosphonic acid (AMPA) can act to induce oxidative stress based on studies in experimental animals, and in studies in humans in vitro."*

As noted above, RMS Germany (2015, p. 78) partially agreed with the assessment of IARC by stating: "*From the available data on glyphosate, there is some indication of induction of oxidative stress from testing in human cell cultures and in mammalian (in vivo) experimental systems.*"

In addition to the *in vivo* mammalian studies reviewed above, IARC's conclusion is reinforced by more recent *in-vitro* studies and studies performed in non-mammalian species.

More specifically,

- Abdel-Halim et al. (2020) oxidative stress in WPM-Y.1 cells at 25 µM concentration of glyphosate (95% purity);
- Barbasz et al. (2020) analyzed the effect of analytical grade glyphosate on human immune cells (HL-60 and U-937) on MDA concentration and total antioxidant activity at concentrations between 0.225 and 3.6 mg/mL;
- Fathi et al. (2019) injected 10 mg glyphosate (analytical grade)/kg egg mass on Day 6 of incubation and investigated MDA, and SOD in liver and kidneys of newly hatched chicks;
- Fathi et al. (2020) injected 10 mg glyphosate (analytical grade)/kg egg mass on Day 6 of incubation and investigated MDA, SOD and GPx in liver, duodenum, jejunum and ileum of embryos on Day 15 of incubation;
- Kasuba et al. (2017) exposed HepG2 cells for 4 or 24 hours with analytical grade glyphosate at concentrations of 0.5 µg/mL, 2.91 µg/mL and 3.5 µg/mL. Subsequently, TBARS, GPx, formation of reactive oxygen species, and total antioxidant capacity were measured;
- Le Du-Careé et al. (2021a) analyzed the effects on TBARS and CAT in the F2 generation of rainbow trout after multi-, inter- and transgenerational exposure to a mean concentration of 123 ng/L of analytical grade glyphosate;
- Le Du-Careé et al. (2021b), in contrast, did not observe changes in oxidative stress markers after exposure to 1 µg/L glyphosate (analytical grade) for 10 months.

Overall Conclusion

In addition to the strong evidence for oxidative stress caused by glyphosate already identified by IARC (2015), further evidence is now available. It should be noted that this was partially agreed upon by RMS Germany (2015). To some degree, the results of the recent studies are variable which – from a general perspective – is expected for biological test systems, in particular for a highly dynamic process as the oxidative/antioxidative equilibrium. But the majority of the valid studies published between 2015 and 2021 support the conclusion drawn by IARC in 2015. Importantly, the study of Gao et al. (2019) provides an "indirect" positive control, earlier claimed as lacking by RMS Germany (2015, p.78) by showing that the glyphosate-induced increase of reactive oxygen species could be ameliorated by blocking the NMDA-receptor. The demonstration of the involvement of the NMDA-receptor also establishes a plausible molecular mechanism, which is further supported by the structural similarities of (Phosphonomethyl)-glycine, i.e. glyphosate, and the known NMDA-receptor agonist glycine. Remarkably, these effects were observed in the same organ (kidney) and the same species (mouse) where a significant increase of renal tumour incidences was seen based on Cochran Armitage trend tests in the carcinogenicity studies of 1983, 1997 and 2001 (RMS Germany, 2015).

References

Abdel-Halim, K.Y. and Osman, S.R. (2020) 'Cytotoxicity and Oxidative Stress Responses of Imidacloprid and Glyphosate in Human Prostate Epithelial WPM-Y.1 Cell Line', *Journal of Toxicology*. Edited by Y.-C. Hseu, 2020, pp. 1–12. doi:10.1155/2020/4364650.

Astiz, M., de Alaniz, M.J.T. and Marra, C.A. (2009) 'Antioxidant defense system in rats simultaneously intoxicated with agrochemicals', *Environmental Toxicology and Pharmacology*, 28(3), pp. 465–473. doi:10.1016/j.etap.2009.07.009.

Barbasz, A. *et al.* (2020) 'Toxicity of pesticides toward human immune cells U-937 and HL-60', *Journal of Environmental Science and Health, Part B*, 55(8), pp. 719–725. doi:10.1080/03601234.2020.1777059.

Bolognesi, C. *et al.* (1997) 'Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup', *Journal of Agricultural and Food Chemistry*, 45(5), pp. 1957–1962. doi:10.1021/jf9606518.

Dedeke, G.A. *et al.* (2018) 'Comparative Assessment on Mechanism Underlying Renal Toxicity of Commercial Formulation of Roundup Herbicide and Glyphosate Alone in Male Albino Rat', *International Journal of Toxicology*, 37(4), pp. 285–295. doi:10.1177/1091581818779553.

Fathi, M.A. *et al.* (2019) 'Effect of in ovo glyphosate injection on embryonic development, serum biochemistry, antioxidant status and histopathological changes in newly hatched chicks', *Journal of Animal Physiology and Animal Nutrition*, 103(6), pp. 1776–1784. doi:10.1111/jpn.13181.

Fathi, M.A. *et al.* (2020) 'Disruption of cytochrome P450 enzymes in the liver and small intestine in chicken embryos in ovo exposed to glyphosate', *Environmental Science and Pollution Research*, 27(14), pp. 16865–16875. doi:10.1007/s11356-020-08269-3.

Ganesan, S. and Keating, A.F. (2020) 'Ovarian mitochondrial and oxidative stress proteins are altered by glyphosate exposure in mice', *Toxicology and Applied Pharmacology*, 402, p. 115116. doi:10.1016/j.taap.2020.115116.

IARC (2015): Some organophosphate insecticides and herbicides. IARC Monographs, Volume 112, Lyon, France.

Kašuba, V. *et al.* (2017) 'Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line', *Environmental Science and Pollution Research*, 24(23), pp. 19267–19281. doi:10.1007/s11356-017-9438-y.

Larsen, K. *et al.* (2012) 'Effects of sub-lethal exposure of rats to the herbicide glyphosate in drinking water: Glutathione transferase enzyme activities, levels of reduced glutathione and lipid peroxidation in liver, kidneys and small intestine', *Environmental Toxicology and Pharmacology*, 34(3), pp. 811–818. doi:10.1016/j.etap.2012.09.005.

Le Du-Carrée, J. *et al.* (2021) 'Developmental effect of parental or direct chronic exposure to environmental concentration of glyphosate on the larvae of rainbow trout, Oncorhynchus mykiss', *Aquatic Toxicology*, 237, p. 105894. doi:10.1016/j.aquatox.2021.105894.

Le Du-Carrée, J., Morin, T. and Danion, M. (2021) 'Impact of chronic exposure of rainbow

trout, Oncorhynchus mykiss, to low doses of glyphosate or glyphosate-based herbicides', *Aquatic Toxicology*, 230, p. 105687. doi:10.1016/j.aquatox.2020.105687.

Mañas, F., Peralta, L. and et al. (2013) 'Oxidative stress and comet assay in tissues of mice administered glyphosate and AMPA in drinking water for 14 days', *Journal of basic and applied genetics* [Preprint].

Milić, M. *et al.* (2018) 'Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate', *Archives of Industrial Hygiene and Toxicology*, 69(2), pp. 154–168. doi:10.2478/aiht-2018-69-3114.

Nozdrenko, D. *et al.* (2021) 'Analysis of Biomechanical Parameters of Muscle Soleus Contraction and Blood Biochemical Parameters in Rat with Chronic Glyphosate Intoxication and Therapeutic Use of C60 Fullerene', *International Journal of Molecular Sciences*, 22(9), p. 4977. doi:10.3390/ijms22094977.

Owagboriaye, F. *et al.* (2019) 'Comparative studies on endogenic stress hormones, antioxidant, biochemical and hematological status of metabolic disturbance in albino rat exposed to roundup herbicide and its active ingredient glyphosate', *Environmental Science and Pollution Research*, 26(14), pp. 14502–14512. doi:10.1007/s11356-019-04759-1.

Ren, X. *et al.* (2018) 'Effects of glyphosate on the ovarian function of pregnant mice, the secretion of hormones and the sex ratio of their fetuses', *Environmental Pollution*, 243, pp. 833–841. doi:10.1016/j.envpol.2018.09.049.

RMS Germany (2015): Renewal Assessment Report, Glyphosate, Addendum 1 to RAR, Assessment of IARC Monographs Volume 112 (2015): Glyphosate; https://www.efsa.europa.eu/en/news/glyphosate-background-documents-published

Tang, Q. *et al.* (2020) 'Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats', *Environmental Pollution*, 261, p. 114129. doi:10.1016/j.envpol.2020.114129.

Zhang, L. *et al.* (2021) 'Involvement of mitochondrial fission in renal tubular pyroptosis in mice exposed to high and environmental levels of glyphosate combined with hard water', *Environmental Pollution*, 283, p. 117082. doi:10.1016/j.envpol.2021.117082.