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Thomas Jaylet, Roel Quintens, Mohamed Abderrafi Benotmane, Jukka Luukkonen, Ignacia Braga Tanaka, Chrystelle Ibanez, Christelle Durand, Magdalini Sachana, Omid Azimzadeh, Christelle Adam-Guillermin, et al.

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1 **Development of an Adverse Outcome Pathway for radiation-induced**  
2 **microcephaly via expert consultation and machine learning**

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97 understanding of ionizing radiation exposure through the development of AOPs and integrative  
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## 115 **Development of an Adverse Outcome Pathway for radiation-induced** 116 **microcephaly via expert consultation and machine learning**

117 **Background:** Brain development during embryogenesis and in early postnatal  
118 life is particularly complex and involves the interplay of many cellular processes  
119 and molecular mechanisms, making it extremely vulnerable to exogenous insults,  
120 including ionizing radiation (IR). Microcephaly is one of the most frequent  
121 neurodevelopmental abnormalities that is characterized by small brain size, and is  
122 often associated with intellectual deficiency. Decades of research span from  
123 epidemiological data on *in utero* exposure of the A-bomb survivors, to studies on  
124 animal and cellular models that allowed deciphering the most prominent  
125 molecular mechanisms leading to microcephaly. The Adverse Outcome Pathway  
126 (AOP) framework is used to organize, evaluate and portray the scientific  
127 knowledge of toxicological effects spanning different biological levels of  
128 organizations, from the initial interaction with molecular targets to the occurrence  
129 of a disease or adversity. In the present study, the framework was used in an  
130 attempt to organize the current scientific knowledge on microcephaly progression  
131 in the context of ionizing radiation (IR) exposure. This work was performed by a  
132 group of experts formed during a recent workshop organized jointly by the  
133 Multidisciplinary European Low Dose Initiative (MELODI) and the European  
134 Radioecology Alliance (ALLIANCE) associations to present the AOP approach  
135 and tools. Here we report on the development of a putative AOP for congenital  
136 microcephaly resulting from IR exposure based on discussions of the working  
137 group and we emphasize the use of a novel machine-learning approach to assist  
138 in the screening of the available literature to develop AOPs.

139 **Conclusion:** The expert consultation led to the identification of crucial biological  
140 events for the progression of microcephaly upon exposure to IR, and highlighted  
141 current knowledge gaps. The machine learning approach was successfully used to  
142 screen the existing knowledge and helped to rapidly screen the body of evidence  
143 and in particular the epidemiological data. This systematic review approach also  
144 ensured that the analysis was sufficiently comprehensive to identify the most  
145 relevant data and facilitate rapid and consistent AOP development. We anticipate  
146 that as machine learning approaches become more user-friendly through easy-to-  
147 use web interface, this would allow AOP development to become more efficient  
148 and less time consuming.

149            Keywords: Adverse Outcome Pathway; AOP; microcephaly; brain; development;  
150            ionizing radiation

151    **Abbreviation list**

152	AOP(s)	Adverse Outcome Pathway(s)
153	AO	Adverse outcome
154	BH	Bradford Hill criteria
155	DDR	DNA damage response
156	DSB(s)	DNA double-strand break(s)
157	KE(s)	Key event(s)
158	KER(s)	Key event relationship(s)
159	Gy	Gray
160	HR	Homologous recombination
161	IR	Ionizing radiation
162	LET	Linear energy transfer
163	MIE	Molecular initiating event
164	NHEJ	Nonhomologous end-joining
165	NPP	Nuclear power plant
166	qAOP(s)	Quantitative Adverse Outcome Pathway(s)
167	RGCs	Radial glia cell(s)
168	Sv	Sievert
169	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
170	WoE	Weight of evidence
171	WG	Working group
172	ZIKV	Zika virus

173

174    **Epidemiological studies on IR-induced microcephaly**

175            Microcephaly is a medical condition characterized by a small head size less than  
176            the expected average for age, gender and considered population. Based on the literature,  
177            its definition may vary qualitatively (e.g. exclusion of other syndromes) or  
178            quantitatively (2 or 3 standard deviations below the mean, which may also reflect

179 severity, with dependence on the chosen reference population) (Passemaid et al. 2013;  
180 Becerra-Solano et al. 2021). Microcephaly is classified either as primary when brain  
181 development is altered during embryogenesis, or acquired when the brain is normal at  
182 birth but suffer damages postnatally resulting in impaired growth (Passemaid et al.  
183 2013). Both cases result in reduced brain size/volume and is frequently associated with  
184 intellectual disability (Woods 2004). Several risk factors have been identified for  
185 microcephaly, including infections during pregnancy (e.g. syphilis, herpes simplex,  
186 HIV, Zika), maternal exposure to ionizing radiation or chemicals (e.g. mercury, arsenic,  
187 alcohol), pre- and perinatal injuries to the developing brain (trauma, hypoxia-ischemia)  
188 and genetic abnormalities. This wide range of risk factors can partly explain the  
189 variations in incidence rates observed in normal populations (from 1 to 150 cases per  
190 100,000 birth) (Passemaid et al. 2013; Becerra-Solano et al. 2021). In addition, the  
191 consideration of additional criteria (e.g. mental disability) as indicators of microcephaly,  
192 can create even more disparities in the number of cases accounted in the  
193 epidemiological cohorts from different populations. Recent studies dealing with  
194 microcephaly are related to Zika virus (ZIKV) infection during gestation, where the  
195 estimated prevalence rate is of 2.3% (95% CI = 1.0-5.3%) among all pregnancies  
196 (Coelho and Crovella 2017).

197         Associations between microcephaly and ionizing radiation (IR) have first been  
198 suspected from a series of early clinical observations of newborns with small head size  
199 and ophthalmic defects with or without mental disability, when pregnant women were  
200 exposed to pelvic irradiation (more than 10 mSv of X-rays) (Goldstein and Murphy  
201 1929; Goldstein 1930) . Data from Atomic-bomb survivors of Hiroshima and Nagasaki  
202 exposed *in utero* between 8-25 weeks of gestation showed statistically significant  
203 associations between IR exposure and small head sizes (defined here as head

204 circumference inferior to two standard deviations below the sex and age specific mean  
205 of the studied population of 1473 children) (Plummer 1952; Miller and Blot 1972;  
206 Otake and Schull 1993; Otake and Schull 1998). Some craniostenosis cases were  
207 potentially included which raised some controversy (Otake and Schull 1998). The most  
208 radiosensitive period of embryonic development of the human brain was established as  
209 between 8-15 weeks of gestation (Miller and Blot 1972; Otake and Schull 1998). More  
210 recently, three papers reported increased prevalence rates of microcephaly (defined as  
211 occipito-frontal circumferences of at least three standard deviations below the standard  
212 for age and sex proposed by the World Health Organization,  
213 <https://www.who.int/tools/child-growth-standards>) in children of women residing in  
214 areas impacted by the Chernobyl fall-out of the Rivne Province of Ukraine at the time  
215 of pregnancy. However, as these papers considered geographically aggregated data  
216 rather than individual data (Wertelecki et al. 2014; Wertelecki et al. 2017; Wertelecki et  
217 al. 2018), they currently provide a lower weight of evidence compared to the atomic  
218 bomb survivor studies in Hiroshima and Nagasaki.

### 219 **Aims of the AOP working group on IR induced microcephaly**

220 The Adverse Outcome Pathway (AOP) framework allows the description of  
221 events from the molecular initiation of the toxic effects to the adverse effects observed  
222 in the organism at levels relevant for human and biota populations. It describes the  
223 fundamental biological events from a Molecular Initiating Event (MIE) and propagation  
224 through essential Key Events (KEs) across the different biological scales of  
225 organization to result in an Adverse Outcome (AO). The Key Event Relationships  
226 (KERs) represent links between the KEs and are supported by experimental data  
227 evaluated as empirical evidence using modified (tailored) Bradford Hill (BH) criteria  
228 (Becker et al. 2015). Thus, the AOP describes in a simple way the scientific knowledge

229 of the most critical toxic effects at different biological levels of organization from  
230 molecular mechanisms to the occurrence of the disease or adverse effects of regulatory  
231 or research focus (Villeneuve et al. 2014; OECD 2018). This concept can also be used  
232 to model the combined effects caused by single- or mixtures of different stressors to  
233 identify and quantify the links between the biological events and the health outcomes  
234 (Beyer et al. 2014; Salbu et al. 2019).

235         The Organization for Economic Cooperation and Development (OECD)  
236 launched in 2012 a program on the development and assessment of AOPs to support  
237 chemical risk assessment. Through this program, guidance and a dedicated web-based  
238 platform ([www.aopwiki.org](http://www.aopwiki.org)) emerged to facilitate AOP development and uptake from  
239 the scientific and regulatory communities. As the development of AOP are relatively  
240 new to the field of low dose radiation research (Chauhan et al. 2022), a workshop  
241 dedicated to AOP of IR effects was organized jointly by the Multidisciplinary European  
242 Low Dose Initiative (MELODI) and the European Radioecology Alliance (ALLIANCE)  
243 on April 12-16, 2021. The aim was to foster interactions between radiobiologists,  
244 radioecotoxicologists and other communities, including chemical toxicologists, in order  
245 to define the particularities of the AOP when applied to the radiation field and prioritize  
246 the development of AOP relevant for health and environmental effects (Chauhan et al.  
247 2021). Here we report on the discussion of a working group (WG) dedicated to the  
248 development and proposing of an AOP for fetal neurotoxicity resulting from IR  
249 exposure. The WG is composed of scientists knowledgeable in the field of brain  
250 development, low dose radiation research (including epidemiology), chemical toxicity  
251 research and computational science. Developmental toxicity encompasses many  
252 pathologies, and it was rapidly recognized that brain development is particularly  
253 vulnerable to IR. Microcephaly provided a favorable case study as : i) the outcome

254 (decrease in brain size) is clearly defined and measurable, ii) epidemiological data is  
255 available to demonstrate an increased risk of microcephaly following *in utero* exposure  
256 to IR, iii) mechanistic understanding of the effects is well documented, and iv)  
257 microcephaly was not documented in AOP-Wiki (<https://aopwiki.org>).

258         Discussions centered first on the exposure situation to IR. It was acknowledged  
259 that most of the evidence, primarily epidemiological data, were obtained for acute  
260 exposure at a relative low dose of IR (> 0.1 Gy) during embryonic development and that  
261 most effects were studied using low LET IR, including both gamma and X-rays.  
262 Virtually all data were obtained from human and rodent studies (mouse and rat),  
263 although the existence of two studies on wild animals (monkey and birds in Fukushima  
264 and Chernobyl, respectively (Møller et al. 2011; Hayama et al. 2017)) was  
265 acknowledged, but not considered sufficiently supportive for expanding the taxonomic  
266 application domains of the AOP. During the review of the available human data, the  
267 group considered accidental *in utero* exposures to the dose > 0.1 Gy as the preferential  
268 scenario, since occupational and environmental exposures are unlikely (Applegate et al.  
269 2021). This dose is in the range of the threshold value for which therapeutic abortion  
270 should be considered in human (0.1 Gy) (Nakagawa et al. 1997). The frequency and  
271 severity of microcephaly is dose-dependent in epidemiological data and animal  
272 experiments, but the dose-threshold of occurrence of microcephaly is different in human  
273 and mouse (above 0.3 Gy in the mouse (Verreet, Verslegers, et al. 2016) and above 0.1  
274 Gy for the human (Otake and Schull 1998)).

275         Data at a lower dose (< 0.1 Gy) are scarce, and microcephaly was proposed to be  
276 an endpoint without dose threshold. To perform a comprehensive review, the group  
277 decided to consider all available data irrespective of the dose, in order to detect potential  
278 early molecular or cellular key events of microcephaly. It was also discussed that dose

279 and dose rates would be important for the development of quantitative AOPs (qAOP),  
280 the quantitative characterization of stressor-response and response-response  
281 relationships along an AOP (Conolly et al. 2017; Song et al. 2020; Moe et al. 2021),  
282 although such effort was not undertaken in the present work. In this context, dose  
283 dependencies were considered as potential strategy to assess the weight of evidence  
284 (WoE) between adjacent KE events according to the modified Bradford-Hill criteria  
285 (Becker et al. 2015).

286 The group considered different possible MIEs for the microcephaly AOP.  
287 ‘Deposition of energy’ (found as MIE 1686 in AOP-Wiki) was proposed as a possible  
288 consensus between the different WGs during the workshop (Chauhan et al. 2021). In the  
289 context of microcephaly, such MIE can be linked directly to the first KE ‘increased  
290 DNA damages’ for which compelling evidence of essentiality exist. A coherent parallel  
291 can be made with an already existing MIE, ‘deposition of particles’, as ‘Deposition of  
292 energy’ conveys the notion of interactions between photons (or high LET particles) and  
293 living matter at the origin of the effects observed at the molecular level. However, this  
294 MIE does not distinguish non-ionizing from ionizing radiation exposures, while the  
295 body of evidence collected for microcephaly comprises only IR. Alternative proposed  
296 MIE were ‘deposition of ionizing energy’ (MIE 1550) or ‘increase, DNA damages’  
297 itself (KE 1194). However, for the sake of coherence and transposability with the  
298 MELODI/Alliance WGs it was decided to keep ‘deposition of energy’ as the MIE for  
299 microcephaly and highlighted that further discussion and harmonization of potential  
300 MIEs could be relevant within the radiation community.

301 The integration of the effects observed at different levels of biological  
302 organization, central for the AOP framework, was identified as closely linked to the  
303 concept of systems biology. The existence of a high-throughput dataset describing the

304 effects of IR on gene expression leading to microcephaly in the mouse embryonic brain  
305 (Mfossa et al. 2020) was discussed as an interesting approach to identify KEs and  
306 characterize the KERs, but such analysis was beyond the reach of the group. Instead, the  
307 screening of additional databases useful for AOP development, such as the Comparative  
308 Toxicogenomics Database (CTD), GeneCards, AOP-Wiki and PubMed databases were  
309 highlighted as useful approaches to capture and assess evidence systematically. The  
310 integration of these data to the results of the scoping review, also performed by the WG,  
311 was thus proposed as an innovative way to incorporate the existing knowledge to the  
312 microcephaly AOP and ensure its comprehensive assembly.

### 313 **Identification of key events through weight of evidence considerations**

314 AOP development is based on the available knowledge from studies that support  
315 each KE and KER. The analysis of the evidence collected in the scientific literature is a  
316 tedious and challenging process made by experts that must understand, organize and  
317 evaluate the WoE along the AOP. This process is at the basis of the mapping of the  
318 knowledge made during the construction of AOPs, and is made through the effective  
319 assessment of biological plausibility, causality and consistency of the empirical  
320 evidence according to the modified Bradford-Hill criteria, as described by Becker *et al.*  
321 (Becker et al. 2015). The WG performed the scoping review of the literature in several  
322 steps: i) the constitution of a preliminary AOP (or AOP ‘skeleton’) based on experts’  
323 knowledge, ii) consolidation of the AOP by evaluation of the scientific evidence, and  
324 iii) proposition of a hypothetical AOP based on consensus among experts. The second  
325 step is the heart of the compilation of evidence to develop AOPs. The WG decided to  
326 prioritize the evaluation of scientific articles focusing on microcephaly induced by IR,  
327 but, as these articles are limited in number, functional studies from genetic mutations  
328 leading to microcephaly were also included. In addition, genetic studies on

329 microcephaly were considered important to provide evidence of essentiality and  
330 causality between KEs. The group decided to focus pragmatically on the effects of IR  
331 on neurogenesis, as a first step towards a more comprehensive and integrated evaluation  
332 of the effects on other type of cells in the brain like oligodendrocyte, astrocyte or  
333 endothelial cells implicated in the vascularization of the brain and transport across the  
334 blood-brain barrier.

335 ***Increased DNA damage leads to TP53 activation and apoptosis of progenitors***  
336 ***in the developing brain***

337 Controlling the number and differentiation of progenitor cells during brain  
338 development is critical to generate the appropriate number of neurons (and other type of  
339 cells) and to achieve normal expansion of the cerebral cortex (Noctor et al. 2004;  
340 Kriegstein and Alvarez-Buylla 2009; Xing et al. 2021)(Pinson and Huttner 2021). The  
341 best documented cause of primary microcephaly is the loss of radial glial cells (RGCs)  
342 and intermediate progenitors by apoptosis (Baala et al. 2007; Sessa et al. 2008). Such  
343 losses occur by the induction of genomic instability (Marthiens et al. 2013) and the  
344 accumulation of unreparable DNA damages (through cell division) via mutation in  
345 critical genes involved in the DNA damage response (DDR) (Buck et al. 2006;  
346 O'Driscoll and Jeggo 2008; Zhou et al. 2013; Bianchi et al. 2017), or defective  
347 chromosomal segregation during mitosis that leads to prolonged cell arrest and TP53-  
348 mediated apoptosis (Chen et al. 2014; Insolera et al. 2014; Phan et al. 2021). In  
349 addition, other mutations can lead to excessive and inappropriate TP53 activation in the  
350 developing brain, ultimately leading to microcephaly (or microcephaly-associated  
351 syndromes) because of increased apoptosis of neural progenitors (Bowen and Attardi  
352 2019).

353 As a well-known mutagen, IR induces microcephaly. Compelling evidence from human

354 epidemiological studies and laboratory animals exposed *in utero* demonstrated the link  
355 between radiation-induced DNA damage and apoptosis of progenitors in the mouse  
356 cerebral cortex as the origin of microcephaly (Nowak et al. 2006; Saha et al. 2014;  
357 Barazzuol and Jeggo 2016; Kashiwagi et al. 2018; Mfossa et al. 2020). Microcephaly is  
358 observed after exposure throughout the neurogenesis period, from E11 to E17 in the  
359 mice and between weeks 8 and 25 of gestation in humans (Verreet, Verslegers, et al.  
360 2016), and is apparent in the domain of the low dose radiation (near 0.2 Gy for humans  
361 and 0.3 Gy for the mouse). In both species, the decreased brain size was strongly  
362 correlated to altered cognitive performance (Verreet, Rangarajan, et al. 2016; Verreet,  
363 Verslegers, et al. 2016). Importantly, the central role of TP53-induced apoptosis was  
364 demonstrated by transcriptome-wide expression studies of TP53 early transcriptional  
365 response to radiation exposure in mouse (Quintens et al. 2015; Mfossa et al. 2020) and  
366 zebrafish (Jaafar et al. 2013). Moreover, the partial rescue of the microcephaly  
367 phenotype when conditional *Trp53* knock out mice are exposed to IR, provides a direct  
368 causal link between radiation-induced apoptosis and microcephaly (Mfossa et al. 2020).

369 The evidence describing the effects of IR on DNA is already present in two different  
370 AOP (<https://aopwiki.org>, AOP#272, AOP#293) and it is out of the scope of this work  
371 to present the detailed mechanisms of DNA damages and repair (see for instance (Lu et  
372 al. 2019; Tang et al. 2022)). However, it remain important to consider that during  
373 neurogenesis, the repair of DNA double-strand breaks (DSBs) via nonhomologous end-  
374 joining (NHEJ) or homologous recombination (HR) depend on the developmental stage,  
375 as DSB in early progenitors (E13 in the mouse) are mainly repaired by HR, while  
376 progenitors present at later stage requires NHEJ (Orii et al. 2006; Li et al. 2020). The  
377 causality between the KE increased DSB – TP53 activation – apoptosis, is well  
378 described and the biological plausibility is considered strong. Increased DSB and

379 apoptosis are two KEs already present in AOP-Wiki (respectively KE 1194 and KE  
380 1262 or KE 1365), but TP53 activation was not yet proposed as a KE (except KE 1923  
381 ‘Altered gene expression, TP53 dependent apoptosis pathway). Many other functional  
382 studies evidenced a role of DDR and TP53-dependent responses similar to those elicited  
383 by IR, suggesting the activation of identical underlying mechanisms (Pilaz et al. 2016;  
384 Mao et al. 2016; Baumgartner et al. 2018; Shi et al. 2019; Keil et al. 2020).

385 Beside apoptosis, mitotic spindle defects (Shimada et al. 2016) and endoplasmic  
386 reticulum stress (Passemar et al. 2019) were also implicated in microcephaly in the  
387 context of IR exposure. Finally, data showed that TP53 have also a role in cell-fate  
388 determination during neurogenesis, independent of its classical function in the induction  
389 of apoptosis. Indeed, *in vivo* study using *Trp53* mutant mice demonstrated that Trp53  
390 hyperactivation led to a precocious differentiation of RGC into neurons during  
391 embryonic development (Liu et al. 2013; Mfossa et al. 2020). In this case, the pool of  
392 RGC is depleted too early (Gruber et al. 2011), limiting the production of neurons  
393 throughout neurogenesis and thereby contributing to a decreased brain size. Given all  
394 these data, the WG considered TP53 activation as a genuine KE essential for the  
395 appearance of microcephaly via two independent KE, namely apoptosis and premature  
396 neuronal differentiation.

### 397 ***Oxidative stress***

398 Deposition of ionizing energy may induce oxidative stress, an imbalance between  
399 oxidants and antioxidants in favor of the former. In addition, IR can directly damage  
400 DNA but also indirectly through the production of reactive oxygen species (Kim et al.  
401 2019). Oxidative stress produces mainly single-strand DNA breaks, whereas more  
402 detrimental effects of radiation are thought to be contributed by the radicals formed

403 along the radiation track where they induce clustered DNA-damage (Sharma et al.  
404 2016). Although oxidative stress has been associated with a variety of diseases, such as  
405 cancer and cardiac diseases, there is currently only limited evidence of its involvement  
406 in radiation-induced microcephaly. This limited evidence includes the occurrence of  
407 oxidative stress and cell cycle arrest in hippocampal neuronal progenitor cells (Oh et al.  
408 2013) and increased neuroinflammation in the brain of adult Wistar albino rats (Kale et  
409 al. 2018). Although it is challenging to directly link a measure of oxidative stress to  
410 DNA damage, some studies have demonstrated quite clearly a key role played by  
411 oxidative stress in radiosensitivity and IR-induced DDR, which remain to be  
412 investigated in the case of microcephaly (Hong et al. 2013; Yazlovitskaya et al. 2015)  
413 (Nikitaki et al. 2015). In addition to acute DNA damage by radiation-induced oxidants,  
414 several studies have found radiation to cause persistent oxidative stress (Datta et al.  
415 2012; Cosar et al. 2012), which in turn might result in a prolonged increase in DNA  
416 damage. Taken together, we acknowledge that oxidative stress might be involved in  
417 parallel or as a complementary pathway that can be described by an AOP network, but  
418 we did not include it as a KE in the AOP due to the low weight of evidence of this  
419 process during microcephaly progression.

#### 420 *Neuroinflammation*

421 Neuroinflammation is considered as a key component of the stress response in the brain  
422 after exposure to various stressors in adults and during embryogenesis. It has therefore  
423 been considered as a possible KE during the construction of the preliminary AOP for  
424 microcephaly. Recent studies associating neuroinflammation and microcephaly are  
425 related to ZIKV infection during gestation. Indeed, many studies highlighted that  
426 infections by ZIKV during pregnancy is characterized by polyfunctional immune  
427 activation associated with increased levels of inflammatory cytokines in the amniotic

428 fluid of ZIKV-positive pregnant women, including the presence of interferon-gamma-  
429 inducible protein 10, interleukin-6 (IL-6) and IL-8, vascular endothelial growth factor  
430 (VEGF), monocyte chemoattractive protein 1 (MCP-1) and granulocyte colony-  
431 stimulating factor (G-CSF) (Ghouzzi et al. 2017). This inflammatory response is  
432 associated with neuronal damages during neurogenesis, leading to microcephaly (Tappe  
433 et al. 2016; Ornelas et al. 2017; Lima et al. 2019). In the context of IR, several studies  
434 reported a microglial activation after exposure of embryonic, young or adult rodents to  
435 IR, which elicited neuroinflammation associated with neuronal loss and cognitive  
436 deficit (Quintens et al. 2015; Verreet, Verslegers, et al. 2016; Osman et al. 2020;  
437 Constanzo et al. 2020). Gene expression changes in irradiated microglia of juvenile  
438 mice highly overlap with those seen in the developing mouse brain and converge on the  
439 TP53 pathway and inflammation (Osman et al. 2020; Mfossa et al. 2020). Importantly,  
440 pharmacological inhibition of several cytokines can confer neuroprotection depending  
441 on the stage of development and type of injury, highlighting the contribution of  
442 neuroinflammation in the appearance of neurological disorders (Hagberg et al. 2015). In  
443 addition there is a strong relationship between chronic inflammation and oxidative  
444 damage after exposure to IR (Najafi et al. 2017; Yahyapour et al. 2018). Indeed, after  
445 exposure to very high dose of IR (> 1Gy), necrosis is more common than apoptosis,  
446 which leads to the release of damage-associated molecular patterns by necrotic cells  
447 which stimulate more T-cells and dendritic cells, amplifying the production of reactive  
448 oxygen species (ROS) and inflammatory mediators (Multhoff et al. 2011; Yahyapour et  
449 al. 2018). In contrast, at lower doses of IR, macrophages detect apoptotic cells and can  
450 release anti-inflammatory cytokines such as transforming growth factor beta (TGF- $\beta$ )  
451 that keep inflammation under control. Disruption of non-cell autonomous signaling like  
452 TGF- $\beta$  can have a role in the progression of microcephaly as they are also essential for

453 cortical migration during development (Zhang et al. 2016). The role of these factors in  
454 the sense of bystander effects of radiation has so far not been demonstrated. The  
455 interaction between inflammation and oxidative stress is thus complex. It is noteworthy  
456 that infection of human neural progenitor cells by Zika virus (Ghouzzi et al. 2017),  
457 irradiation of the mouse embryonic brain (Quintens et al. 2015) and mutation in the  
458 DDR gene citron kinase (Di Cunto et al. 2000), all results in TP53-mediated apoptosis  
459 of the developing neuronal progenitors (Quintens 2017). As radiation-induced apoptosis  
460 triggers inflammation (Boyd et al. 2021), it is possible that neuroinflammation  
461 contributes to microcephaly, but this process remains poorly documented and could also  
462 be considered as secondary compared to apoptosis. The WG decided thus to exclude  
463 neuroinflammation from the list of possible KE until a causative role has been  
464 established.

#### 465 **Radiation-induced microcephaly, test-case application of a machine learning** 466 **method**

467 Given the enormous amount of existing knowledge in the scientific literature and other  
468 data sources, the identification of relevant biological information to build AOP is a  
469 complex and time-consuming task, especially in deciphering sparse data. Thus, to assist  
470 in the AOP development process, the WG discussed that the use of artificial intelligence  
471 (AI) and automated data mining methods can represent a complementary approach to  
472 the expert manual review of the available data. From this perspective, an innovative AI  
473 tool, called AOP-helpFinder, that combines graph theory and text mining, was  
474 developed to automatically explore the scientific abstracts from the PubMed database  
475 (Carvaillo et al. 2019; Jornod et al. 2021). This tool was previously successfully applied  
476 in several studies to identify and extract associations between stressors (bisphenol S,  
477 bisphenol F and pesticides) and KEs of interest (Carvaillo et al. 2019; Rugard et al.

478 2020; Jornod et al. 2021). Epidemiological studies linking microcephaly and IR  
479 exposures being sparse in the literature, and then difficult to identify, we used the AOP-  
480 helpFinder tool to quickly identify and extract, in a systematic manner, all published  
481 existing information that could help AOP development. To do so, the user-friendly  
482 AOP-helpFinder webserver was applied using the key words ‘ionizing radiation’ and  
483 biological events ‘Microcephaly’ and ‘Small head size’ (<http://aop-helpfinder.u-paris->  
484 [sciences.fr/](http://aop-helpfinder.u-paris-sciences.fr/), accessed on October, 19), with the options “Reduced search: 20%” and  
485 “Refinement filter: yes”, in order to extract as much information as possible. Doing so  
486 the co-occurrence of ‘ionizing radiation’ with ‘Microcephaly’ or ‘Small head size’ in  
487 the abstract of PubMed database was scanned. Other phenotypical terminology such as  
488 brain atrophy, gliosis, or reduced neurogenesis were considered less precise. After  
489 automatic computational exploration of 125,226 abstracts related to IR from PubMed  
490 database, the AOP-helpFinder tool allowed to gather 47 articles associating IR and  
491 microcephaly (or small head size). Among these 47 links, a manual curation of the  
492 abstracts allowed to keep only the 9 epidemiological articles dealing with exposure to  
493 IR in humans. All these 9 publications were fully evaluated and discussed in the WG.  
494 Two types of epidemiological studies were discriminated, either related to A-bomb  
495 survivors in Hiroshima and Nagasaki (Plummer 1952; Miller and Blot 1972; Otake and  
496 Schull 1998) or exposures after the Chernobyl NPP accident (Wertelecki et al. 2014;  
497 Wertelecki et al. 2017; Wertelecki et al. 2018). After removing potential redundancy  
498 within these studies (including metanalysis and confirmational studies), the WG  
499 proposed to incorporate this knowledge via non-adjacent KER between the MIE ‘energy  
500 deposition’ and microcephaly, as these studies demonstrated a clear link between the  
501 two events, but without intermediate KEs.

502 **Preliminary AOP based on expert consultation**

503 Based on the discussion in the WG, it was possible to propose a preliminary AOP  
504 related to radiation-induced microcephaly (Figure1).

505 The MIE (MIE1686, Deposition of Energy) leads to the increased DNA damage at the  
506 molecular scale (KE 1194, Increase, DNA damage), which is considered in this AOP as  
507 the main route by which IR exerts their effects at higher levels of biological  
508 organization. The large amount of data describing the link between IR and DNA  
509 damages already present in AOP-Wiki, and the additional evidences gathered in the  
510 context of neurogenesis (Table1), led to WG to propose a ‘High’ WoE for this KE.  
511 ‘Activation of TP53’ (KE 1974) is proposed as a novel essential KE. The existing KE  
512 1923 ‘altered gene expression, P53 dependent apoptosis pathway’ was not used as it is  
513 restricted to apoptosis. This KE is first supported by a time course analysis by  
514 immunoblot of phosphorylated TP53 on (E14) primary mouse striatal stem irradiated  
515 acutely to 1 Gy and 2 Gy of X-rays (Kashiwagi et al. 2018). The authors also quantified  
516 TP53BP1 by immunofluorescence and describe that the peak of DNA damages (10 min  
517 after irradiation) precedes, as expected, the peak of TP53 activation (3h to 5h after  
518 irradiation). Similar data were obtained by Limoli *et al.* (Limoli et al. 2004), but with  
519 higher dose (5 Gy). In the context of microcephaly, the link between increased DNA-  
520 damages and TP53 activation was demonstrated by immunofluorescence using  $\gamma$ H2Ax  
521 and TP53BP1 on brain cryosections and immunoblotting against phosphorylated TP53  
522 in *Citk*<sup>-/-</sup> E14.5 mouse embryos (Bianchi et al. 2017; Mfossa et al. 2020). Together, this  
523 WoE for ‘Activation of TP53’ (KE 1974) was considered high. TP53 activation is  
524 necessary for the induction of two possible cellular outcomes in the developing brain,  
525 increased apoptosis (KE1365, ‘Increase apoptosis’) or ‘Premature cell differentiation’  
526 (KE 1979), which could both contribute to ‘microcephaly’ (AO 1978). The WG

527 identified the KE1365, 'Increase apoptosis' (already present in AOP-Wiki), with high  
528 level of confidence in the context of brain development, as described by analysis using  
529 cleaved Caspase-3 (CC3) (Shimada et al. 2016; Mfossa et al. 2020), TUNEL assay  
530 (Saha et al. 2014; Barazzuol and Jeggo 2016; Bianchi et al. 2017; Mfossa et al. 2020) on  
531 mouse brain cryosections, western blotting (Kashiwagi et al. 2018) and microscopic  
532 analysis of pyknotic nuclei (Nowak et al. 2006; Etienne et al. 2012). The KE 'Premature  
533 cell differentiation' (KE 1979) was supported by immunostaining of the neuronal  
534 markers on mouse brain cryosections (Liu et al. 2013; Mfossa et al. 2020). We noted the  
535 existence of the KE 1560 'altered differentiation', but considered that this KE was not  
536 adequate as altered differentiation can also lead to defective cell function and this term  
537 does not convey the notion of temporality during development. In addition, both IR-  
538 induced apoptosis and premature differentiation were ablated in conditional *Trp53*  
539 knock out mouse, which adds to the WoE (Mfossa et al. 2020). But, together, as few  
540 studies were identified, this WoE was considered moderate. Finally, the abundant  
541 epidemiological data in the field of radiation is incorporated as a non-adjacent KER  
542 between energy deposition and the adverse outcome (AOP 441, <https://aopwiki.org>).

543 The proposed AOP represents the foundation on which more dedicated work will be  
544 needed. Indeed, despite the willingness of the WG to follow the modified Bradford-Hill  
545 criteria to assess the WoE for the KER, this process was only initiated, and it was  
546 evident that additional effort will be needed to compile the evidence in a systematic and  
547 transparent manner. Additional potential KE were considered in the initial phase of the  
548 AOP construction and might be considered for future development of the AOP towards  
549 an AOP network. For transparency the complete list of the unused events is provided in  
550 Supplementary Table 1.

## 551 **Conclusion**

552 In this work, we proposed an AOP for radiation-induced microcephaly and highlighted  
553 the important molecular and cellular processes leading to this adverse outcome. The  
554 WG considered accidental exposure to IR ( $> 0.1$  Gy) of low LET energy (gamma and  
555 X-rays) during pregnancy (between 8 and 25 weeks post-fertilization in human), as a  
556 well-known risk factor for microcephaly. The effects of IR on RGs and intermediate  
557 progenitors in the developing brain were considered as the main KEs leading to  
558 microcephaly. The AOP proposed here is a simplistic representation of the known  
559 mechanisms leading to microcephaly after exposure to IR. It relies on epidemiological  
560 data and is completed by mechanistic evidences that support the relationship between  
561 IR and microcephaly. As such, this AOP can facilitate the predictive extrapolation to  
562 other animal species, given that the processes of neurogenesis are to a certain extent  
563 similar and the KERs conserved, and can thus help understand the effects on brain size  
564 observed on wild animals after the Fukushima and Chernobyl nuclear accidents (Møller  
565 et al. 2011; Hayama et al. 2017).

566 Besides a classical knowledge gap in the field of low-dose ( $< 0.1$  Gy) and low-dose rate  
567 ( $< 5$  mGy/h), the development of the AOP also emphasized the need for more studies on  
568 cell fate after irradiation. Indeed, if apoptosis is well documented, the effects of IR on  
569 premature neuronal differentiation, cell migration, cytokinesis defects and their link  
570 with cellular senescence, oxidative stress and neuroinflammation remain scarce, as well  
571 as their contribution to microcephaly. The WG also identified the effect on non-  
572 neuronal tissues as a major gap, especially for the vascular system and microglia. More  
573 functional analysis (i.e. mutants, transgenesis and other mechanistic data) are thus  
574 required to delineate causes and consequences in the context of DNA damage induced  
575 by IR. It is also noteworthy that most AOP related to DNA damage in the AOP-Wiki

576 database relate to cancer effects. If cancer is indeed a major outcome following  
577 exposure to a relative low to moderate dose of IR, non-cancer effects (including  
578 developmental effects but also cardiovascular or cognitive effects in adults) remain less  
579 studied and should not be neglected. The final proposed AOP is relatively simple and  
580 more dedicated work is required to assess WoE in a systematic and transparent manner  
581 as well as identifying additional MIEs and KEs relevant for the same AO. This effort  
582 would entail developing additional AOPs that collectively can form an AOP network  
583 where the relevance of each AOP can be assessed in terms of developing quantitative  
584 stressor-response and response-response relationships. In this regard, automated  
585 analysis by machine learning approaches will help to screen the huge literature rapidly,  
586 and permits consolidation of KEs, but limits still exists for this automated process as  
587 different AO and KE terminology can affect the search. More generally, system biology  
588 approaches including omics data will be useful to consolidate the molecular events of  
589 the AOP, and will be of use by bringing a quantitative aspect to the AOP.

590

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597

598 Disclosure statement

599 All authors declare that they have no competing interests. The opinions expressed and  
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602

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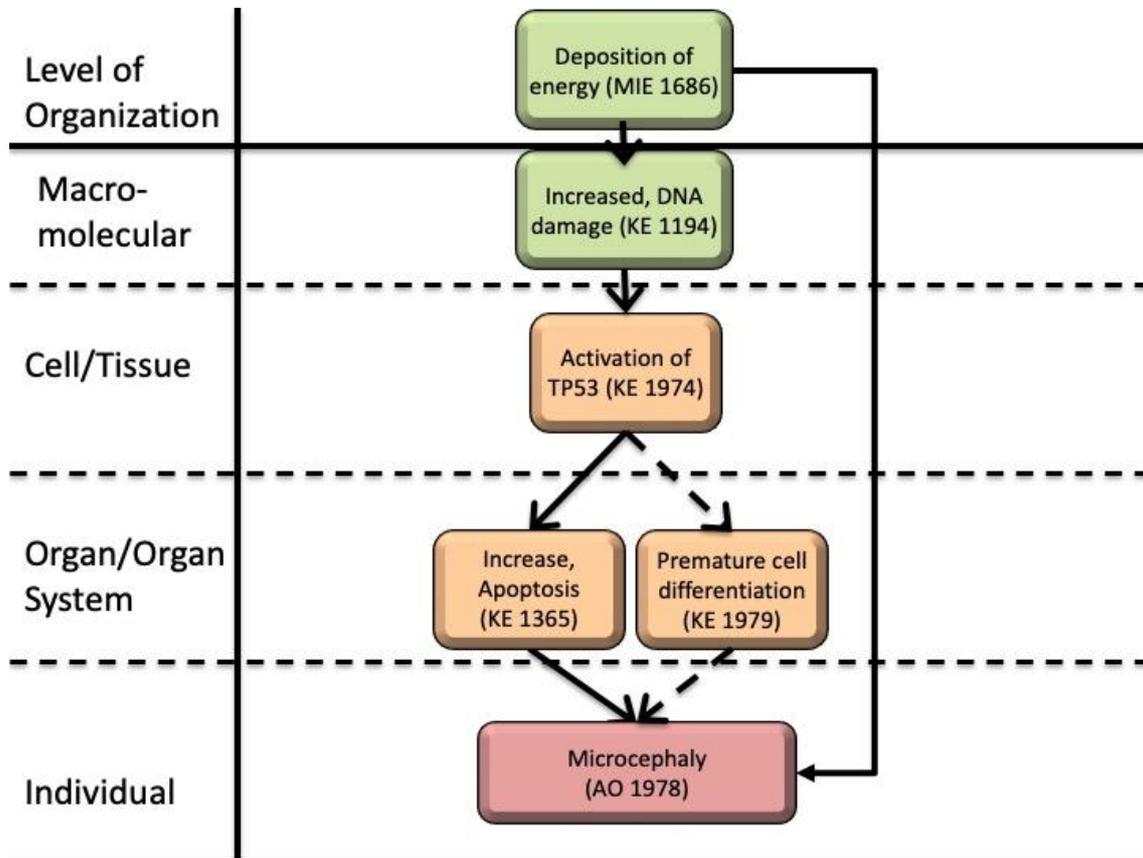
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928 Figure 1. Description of the preliminary AOP: Ionizing radiation-induced DNA damage  
929 leads to microcephaly via apoptosis and premature cell differentiation (AOP 441,  
930 <https://aopwiki.org/aops/441>). MIE (Molecular Initiating Event), Key Events (KE),  
931 Adverse Outcome (AO). Key event relationship with high and moderate confidence are  
932 depicted respectively by solid and dashed lines.



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938 Table 1. Description of the Weight of Evidence (WoE) used for the preliminary AOP .  
 939 MIE (Molecular Initiating Event), Key Events (KE), Adverse Outcome (AO). The  
 940 event-identities (Event ID) in AOP-Wiki are indicated, as well as the bibliographical  
 941 references used.

Type	Event ID	Title	Detection method	References	WoE
MIE	1686	Direct Deposition of Energy	Dosimetry	Chauhan V et al. 2021	High
KE	1194	Increase, DNA damage	Immunostaining Western Blot	Barazzuol and Jeggo 2016 Saha et al. 2014 Kashiwagi et al. 2018 Nowak et al. 2006 Mfossa et al. 2020 Bianchi et al. 2017	High
KE	1974	Activation of TP53	Immunostaining Western Blot	Kashiwagi et al. 2018 Limoli et al. 2004 Bianchi et al. 2017 Mfossa et al. 2020	High
KE	1365	Increase, Apoptosis	Immunostaining Western Blot TUNNEL assay Pycnotic nucleus	Shimada et al. 2016 Saha et al. 2014 Barazzuol and Jeggo 2016 Kashiwagi et al. 2018 Nowak et al. 2006 Mfossa et al. 2020 Bianchi et al. 2017 Etienne et al. 2012	High
KE	1979	Premature cell differentiation	Immunostaining	Mfossa et al. 2020 Liu et al 2013	Moderate
AO	1978	Microcephaly	Head size	Plummer 1952 Miller and Blot 1972 Otake and Schull 1998 Otake and Schull 1993 Shimada et al. 2016 Bianchi et al. 2017 Mfossa et al. 2020	High

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944 Supplementary Table 1. Description of the potential KE explored in the initial phase of  
 945 AOP construction. The comment indicates how the potential KE was considered in the  
 946 preliminary AOP after expert consultation.

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Term	KE in AOP wiki	Comment
Increased, oxydative stress	1088	Insufficient direct evidence
Oxidative Stress	1392	Insufficient direct evidence
Increase in reactive oxygen and nitrogen species (RONS)	1632	Insufficient direct evidence
Neuroinflammation	188	Insufficient direct evidence
Increase in inflammation	1633	Insufficient direct evidence
Increased DNA double breaks	1635	Related to DNA damage
Cell cycle arrest	None	Insufficient direct evidence
Cell cycle, disrupted	1505	Insufficient direct evidence
Defective cell division	None	Insufficient direct evidence
Defective spindle assembly	None	Insufficient direct evidence
Senescence	None	Insufficient direct evidence
Lipid peroxidation	1511	Insufficient direct evidence
Increase, Lipid peroxidation	1445	Insufficient direct evidence
Protein carbonylation	None	Insufficient direct evidence
Peptide Oxidation	209	Insufficient direct evidence
Mitochondria dysfunction	1816	Insufficient direct evidence
Altered gene expression, P53 dependent apoptosis pathway	1923	Difficulty to assess essentiality
Change in gene expression levels (omics data)	None	Difficulty to assess essentiality
Increased gliosis	None	Insufficient direct evidence
Brain atrophy	None	Related to microcephaly
Reduced brain size	None	Related to microcephaly
Reduced neurogenesis	None	Related to premature neuronal differentiation
General Apoptosis	1513	Related to Increase apoptosis
Neuronal death by apoptosis	None	Related to Increase apoptosis
Premature Neuronal differentiation	None	Related to Premature cell differentiation
Altered vascularization	None	Insufficient direct evidence
948 Impaired blood-brain transport	None	Insufficient direct evidence

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