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Radiation-Induced Lens Opacity and Cataractogenesis: A Lifetime Study Using Mice of Varying Genetic Backgrounds

R. A. McCarron,[a] S. G. R. Barnard,[a,b,1] G. Babini,[c,d] C. Dalke,[e] J. Graw,[e] S. Leonardi,[f] M. Mancuso,[f] J. E. Moquet,[a] D. Pawliczek,[e] S. Pazzaglia,[f] I. De Stefano,[f] E. A. Ainsbury[a] for the LDLensRad Consortium[2]

[a] *Public Health England, Centre for Radiation, Chemical and Environmental Hazards, Chilton, United Kingdom;* [b] *Durham University, School of Biosciences, Durham, United Kingdom;* [c] *Department of Physics, University of Pavia, Pavia, Italy;* [d] *Department of Woman and Child Health, Fondazione Policlinico A. Gemelli, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy;* [e] *Helmholtz Zentrum München GmbH, German Research Centre for Environmental Health, Neuherberg, Germany; and* [f] *Laboratory of Biomedical Technologies, Agenzia Nazionale per le Nuove Tecnologie, l'Energia e lo Sviluppo Economico Sostenibile (ENEA), Rome, Italy*

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Recent epidemiological findings and reanalysis of historical data suggest lens opacities resulting from ionizing radiation exposures are likely induced at lower doses than previously thought. These observations have led to ICRP recommendations for a reduction in the occupational dose limits for the eye lens, as well as subsequent implementation in EU member states. The EU CONCERT LDLensRad project was initiated to further understand the effects of ionizing radiation on the lens and identify the mechanism(s) involved in radiation-induced cataract, as well as the impact of dose and dose-rate. Here, we present the results of a long-term study of changes to lens opacity in male and female adult mice from a variety of different genetic (radiosensitive or radioresistant) backgrounds, including mutant strains *Ercc*2 and *Ptch*1, which were assumed to be susceptible to radiation-induced lens opacities. Mice received 0.5, 1 and 2 Gy ^{60}Co gamma-ray irradiation at dose rates of 0.063 and 0.3 Gy min^{-1}. Scheimpflug imaging was used to quantify lens opacification as an early indicator of cataract, with monthly observations taken postirradiation for an 18-month period in all strains apart from 129S2, which were observed for 12 months. Opacification of the lens was found to increase with time postirradiation (with age) for most mouse models, with ionizing radiation exposure increasing opacities further. Sex, dose, dose rate and genetic background were all found to be significant contributors to opacification; however, significant interactions were identified, which meant that the impact of these factors was strain dependent. Mean lens density increased with higher dose and dose rate in the presence of *Ercc*2 and *Ptch*1 mutations. This project was the first to focus on low (<1 Gy) dose, multiple dose rate, sex and strain effects in lens opacification, and clearly demonstrates the importance of these experimental factors in radiobiological investigations on the lens. The results provide insight into the effects of ionizing radiation on the lens as well as the need for further work in this area to underpin appropriate radiation protection legislation and guidance.

INTRODUCTION

Lens opacities and radiation-induced cataracts are known to occur after exposure to relatively high doses of 1 Gy or above of ionizing radiation. This process was, until recently, assumed to be a tissue reaction/deterministic effect (*1*). However, the combined results from the most recent epidemiological studies and the small number of mechanistic, animal studies suggest that radiation cataractogenesis may have a lower threshold and may be described by a linear, non-threshold model (*2*). Recently published epidemiological studies and reanalyses involving Chernobyl clean-up workers (*3*), Japanese atomic bomb survivors (*4*) as well as astronauts, residents of contaminated buildings and radiological technicians (*5*) have suggested an increased incidence of lens opacities at lower doses. As summarized by Ainsbury *et al*. (*2*), the weight of evidence indicated that the threshold for cataract development is less than was previously recommended. This supported a further recommendation by the International Commission for Radiological Protection (ICRP) to reduce the practical threshold for radiation cataract to 0.5 Gy, together with a reduction in

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[1] Address for correspondence: Public Health England, PHE CRCE, Chilton, Didcot, Oxford, Oxfordshire OX11 0RQ, United Kingdom; email: stephen.barnard@phe.gov.uk.

[2] LDLensRad Consortium members: E. Ainsbury, M. Ahmadi, T. Azizova, A. Antonelli, G. Babini, S. Barnard, C. Dalke, L. Dauer, I. De Stefano, J. Dynlacht, L. Garrett, J. Graw, N. Hamada, S. M. Hölter, M. Jarrin, M. Kadhim, A. Kalligeraki, S. Kunze, S. Leonardi, M. Mancuso, R. McCarron, J. Moquet, D. Pawliczek, S. Pazzaglia, R. Quinlan, A. Saran, R. Tanner, B. Tanno, M. Ung and A. Uwineza.

dose limit of occupational radiation exposure in the eye lens to 20 mSv per year, averaged over 5 years, with no one year exceeding 50 mSv (*2*). In the U.S., the NCRP recommended a reduction in annual occupational lens dose limit to 50 mGy absorbed dose (*6*). However, the ICRP and NCRP were explicit regarding the limitations of these recommendations, not least of which was that they were based almost solely on available epidemiological data, with limited mechanistic understanding, and acknowledging the lack of sufficient evidence on the impact of dose rate and evidence for acute, compared to protracted, dose (*7*). The current work highlights the need for relevant *in vitro* and *in vivo* mechanistic data at low dose.

Mice are the ideal *in vivo* model for use in cataract research; they have very comparable pathologic changes to humans and are genetically well categorized (*8*). Due to the eye size and severity of effects, cataractous lenses are relatively easy to detect and quantify in mice without the need of major technical equipment (*9*). The *Ercc*2 gene, also known as DNA helicase, produces the protein XPD (xeroderma pigmentosum, complementation group D) which is involved in gene repair (*10*). A mutation in the *Ercc*2 gene in mice leads to recessive cataracts, with heterozygotes also demonstrating a higher sensitivity to radiation (*11*). These mice are observed to be radiosensitive when quantifying the DNA damage repair response in peripheral lymphocytes using γ-H2AX. In epidemiological studies, polymorphisms in *Ercc*2 have been observed, leading to an increase in susceptibility to age-related cataract (*12*). *Patched 1* mice (*Ptch1*) are another example of a mutant strain that has been used during radiation-induced cataracts investigations (*13*). *Ptch1* is often termed a tumor suppressor gene due to its role in preventing cells from uncontrolled proliferation (*14*). Mice heterozygous for *Ptch1* are reportedly highly susceptible to cataract induction by radiation exposure at postnatal age (*13*), making them useful for investigating the mechanisms of radiation-induced cataracts (*15*).

In a recently published study, the lifetime effects of radiation were investigated in mouse lenses after a single dose of 0.063, 0.125 and 0.5 Gy of γ-ray radiation, delivered at 0.063 Gy min^{-1} (*16*), alongside sham-irradiated controls. The study was the first to make use of Scheimpflug imaging to investigate radiation-induced lens opacity in mice, and it was found to be a highly efficient and sensitive method compared to traditional slit lamp (*17*). Dalke *et al*. (*16*) investigated the effect of dose and gender in wild-type B6C3F1 hybrid mice and corresponding heterozygous *Ercc*2 mice. In that study, at over 24 months postirradiation, there was no evidence of a significant effect of sex or genotype. The dose effect was highly statistically significant, particularly after 0.125 and 0.5 Gy doses, with less of an increase of lens opacity over a lifetime after the lowest dose of 0.063 Gy. However, the increase in opacification associated with this effect was on the order of 1% and thus was not likely to have a significant effect on vision.

The EU CONCERT-funded LDLensRad project aims to further understand and clarify the biological mechanism(s) associated with radiation-induced cataract (https://bit.ly/3mRaT7p). The findings presented herein represent data collated from three project partners, investigating the effect of both dose and dose rate of radiation, and the influence of genetic predisposition and sex, on lens opacity assessed within *in vivo* lenses using Scheimpflug imaging over a period of 12–18 months, as an early marker of cataract development postirradiation.

MATERIALS AND METHODS

Irradiation and Scheimpflug image analysis were performed at three different facilities: Public Health England (PHE, UK), Helmholtz Zentrum Munchen (HMGU, Germany) and the Agenzia Nazionale per le nuove Tecnologie, l'Energia e lo Sviluppo Economico Sostenibile (ENEA, Italy). In all instances, post hoc power calculations were performed to ensure that sufficient numbers of mice were used within each experimental group.

Mice

PHE. 120 inbred female C57BL/6 (C57BL/6Jola/Hsd) mice obtained from Envigo, UK (Blackthorn, UK) received ^{60}Co γ-ray irradiation at 0 (control) 0.5, 1 or 2 Gy, at dose rates of 0.063 and 0.3 Gy min^{-1}. This strain is reported to be radioresistant based on postirradiation survival rate and reduced incidence of radiogenic neoplasms, compared to BALB/c mice, as well as upregulation of their anti-tumor cytotoxic function demonstrated in NK cell-enriched splenocytes after X-ray irradiations (*18*). Thirty inbred female 129S2 (129S2/SvHsd) mice, also obtained from Envigo, UK, received 0 and 2 Gy irradiation at dose rates 0.063 and 0.3 Gy min^{-1}. This strain was used for its reported radiosensitive nature (*19*). At PHE, only female mice were used, due to housing constraints, meaning mice could be housed more efficiently at the facility. Groups of 4 mice were housed together with food (RM3I, LBS Biotechnology, Hookwood, UK) and water available at all times, with the health status of the mice checked daily throughout the study. To carry out the relevant procedures, approval from the Home Office was granted for a project license and personal license for each individual involved with mouse work. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986, with approval from the local AWERB (Animal Welfare and Ethical Review Body) at PHE.

HMGU. Wild-type F1 hybrids from C57BL/6JGxC3HeB/FeJ matings (B6C3F1) and B6C3F1 mice with a heterozygous point mutation in the *Ercc*2 gene (*Ercc*$2^{+/S737P}$) were used to compare radiation responses in the wild-types to the *Ercc*2 mutation, with higher sensitivity to radiation, and supposed susceptibility to cataracts (*16*). A total of 324 animals were used, with 162 male and 162 female (81 *Ercc*$2^{+/-}$ and 81 B6C3F1 each) receiving 0, 0.5, 1 or 2 Gy irradiation at dose rate 0.3 Gy min^{-1}.

ENEA. Mice lacking one *Ptch1* allele (n = 303) maintained on two different genetic backgrounds, CD1 (named *Ptch*$1^{+/-}$/CD1; n = 153) and C57BL/6 (named *Ptch*$1^{+/-}$/C57BL/6; n = 150) were enrolled in the study as well as their wild-type counterparts (CD1 and C57BL/6 mice, n = 78 and n = 81, respectively) (*13*). Mice of both sexes were equally distributed among irradiated groups (2 Gy, 1 Gy and 0.5 Gy; dose rates 0.063 Gy min^{-1} and 0.3 Gy min^{-1}). A group of mice of each genotype comprised the nonirradiated control group (n = 65).

Irradiation

All irradiations were performed at room temperature, with a horizontal geometry using whole-body *in vivo* exposure. Control mice

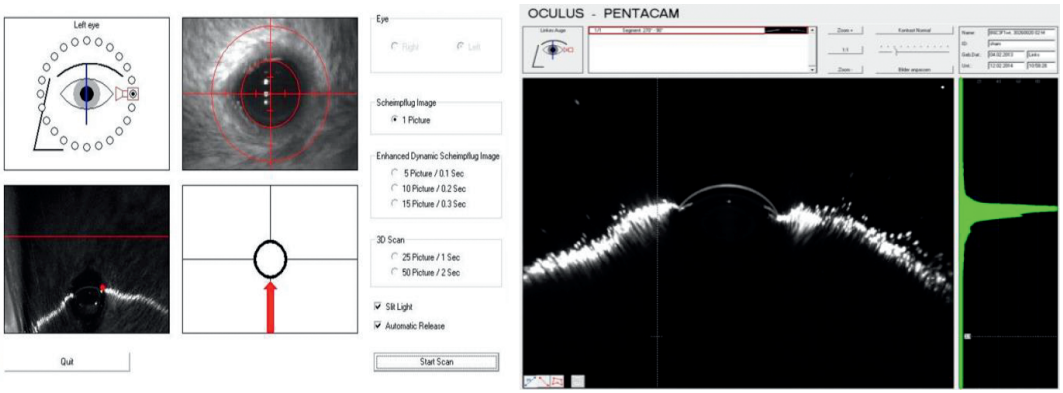

FIG. 1. Setup of the Scheimpflug imaging system demonstrating eye and camera orientation using the Pentacam Oculus software displaying ''densitometry across a line'' analyzing tool.

were sham irradiated, i.e., they were taken to the exposure facility exactly per the procedure for irradiated mice, but the source was not turned on. Mice were irradiated at approximately 8–12 weeks of age, as this ensured that lens growth and changes to cell densities involved in myopia had occurred, with emmetropia shown to be established at 6 weeks of age (*20*).

PHE irradiations were performed at a custom-built ^{60}Co-gamma irradiation facility at the Medical Research Council (MRC, Harwell Campus, Didcot, UK). The irradiation system is calibrated with traceability to national standards, with all doses delivered to within 5% accuracy, with a horizontal geometry. The exposure chamber allows for up to four cobalt-60 sources to be used at any one time. Given the decay of the sources, either two or four sources were used to achieve the desired dose rates of 0.063 and 0.3 Gy min^{-1}, respectively. Irradiations were performed at room temperature with whole-body *in vivo* exposure. This method demonstrated no adverse effects.

HMGU irradiations were performed in the irradiation facility under a ^{60}Co source (Eldorado 78 teletherapy irradiator; Atomic Energy of Canada Limited, Chalk River, Canada). Dosimetry was conducted using the UNIDOS II dosimeter (secondary electrometer, calibration was based on the primary standards of the Physikalische-Technische Bundesanstalt-Braunschweig, Germany). Mice were irradiated at room temperature with the animals seated in a plexiglass cylinder with a lid to prevent rearing of the mice. Doses of 0, 0.5, 1 and 2 Gy were administered at a dose rate of 0.3 Gy min^{-1}. Mice of the same genetic background were irradiated at a dose rate of 0.063 Gy min^{-1} in a previously reported study (*16*).

ENEA irradiations were performed using a horizontal ^{60}Co beam with a field size of 10×10 cm at 100 cm distance from the source and a dose rate of 0.16 Gy min^{-1}, as determined using a reference ionization chamber calibrated in terms of absorbed dose to water with traceability to the Italian primary standard of absorbed dose. Two different dose rates (0.063 Gy min^{-1} and 0.3 Gy min^{-1}) were employed, varying the source distance at 161.7 cm and 74.1 cm, respectively. Mice were irradiated in a PMMA holder with 4-mm-thick walls, ensuring electronic equilibrium conditions. The midpoint of the PMMA holder was located at a distance from the source required to receive the correct dose-rate within $\pm 2\%$, and the source distance was changed every four months. The irradiation time (t_{irr}) to deliver the required absorbed dose was calculated daily, accounting for the ^{60}Co source decay and according to the formula: $t_{irr} = D/\dot{D} - t_{err}$, where D is the delivered dose, $\dot{D}$ is the actual dose rate during irradiation, t_{err} is the timer error. The number of mice simultaneously irradiated was established to ensure a beam uniformity within 1%, resulting in $n = 3$ and $n = 1$ for the lower and higher dose rates, respectively. This animal study was performed according to the European Community Council Directive 2010/63/EU, approved by the local Ethical Committee for Animal Experiments of the ENEA, and authorized by the Italian Ministry of Health (no. 1233/2015-PR).

Scheimpflug Imaging

The mouse lenses were imaged monthly as described elsewhere (*21*). All three institutes used the same calibration settings, refined by HMGU from a previously published study (*16*). Atropine eye drops were used to dilate pupils at all three facilities; however, short-term general anesthetic (isoflurane) was used in mice only at PHE to reduce stress and enable accurate imaging on the Scheimpflug camera. This slight variation in methodology was not expected to affect the results. Indeed, there is a lack of evidence in the literature to suggest an influence of anesthetic on lens opacification. All mice were restrained by hand during the Scheimpflug imaging process using the scruffing technique, which helps to reduce mobility and keep eyelids open during the imaging procedure and lasts only a few seconds. All strains, except for the 129S2 mice, were followed to 18 months; the 129S2 mice were followed to 12 months due to time constraints related to the local animal facilities.

All three facilities used the same imaging technique, equipment and software via a Scheimpflug OCULUS Pentacam (Fig. 1). Mean, maximum and minimum lens density was recorded as a measure of opacity across the lens (Fig. 2).

Statistical Analysis

Statistical analyses were performed using Minitab® 18. Two-sample *t* test-based power analysis was applied to calculated group numbers. Kaplan-Meier survival analysis was performed to confirm that radiation did not significantly reduce group numbers over the lifetime of the study. Anderson-Darling normality testing was followed by assessment of the power to detect significance of the end points based on the sample sizes at the end of the assessment period. General linear model analysis of variance (ANOVA) was then applied to analyze lens mean and maximum opacification as a function of factors (with levels in parentheses) for each of the individual strains: Month postirradiation (1–18); sex (male or female, except for the 129S2 mice, which were female only); dose (0, 0.5, 1 or 2 Gy); dose rate (0.063 or 0.3 Gy min^{-1} for all, except the B6C3F1, *Ercc2*$^{+/S737P}$ mice which were administered only the 0.3 Gy min^{-1} dose rate), with interaction effects between the factors assessed where indicated in the model fits. For C57BL/6 mice only, two laboratories housed these mice, i.e., PHE and ENEA; thus, laboratory was added as a factor in this case. Tukey's post hoc testing was then applied to further investigate the influence of the factor levels for significant factors. The analysis was then repeated for the combined data, with the additional factor of strain: CD1, *Ptch1*$^{+/-}$/CD1, C57BL/6, *Ptch1*$^{+/-}$/C57BL/6, B6C3F1, *Ercc2*$^{+/S737P}$ or 129S2. A significance level of $P = 0.05$ was assumed throughout.

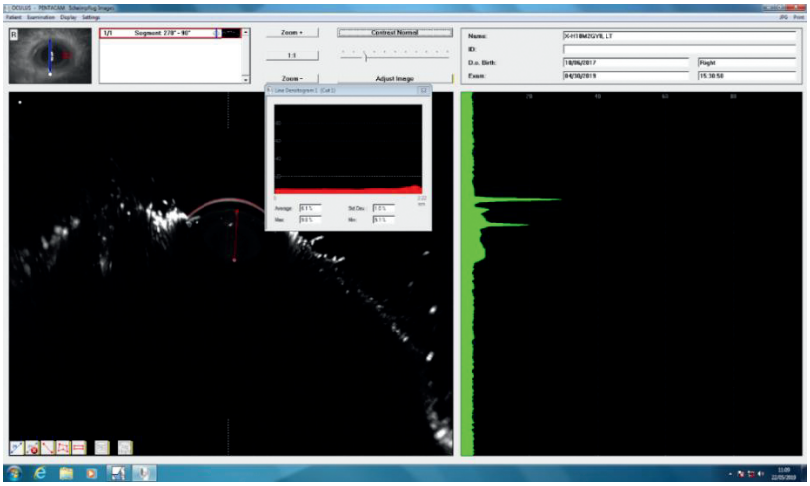

FIG. 2. Lens densitometry analyses using Pentacam Oculus imaging software of the Scheimpflug imaging system.

RESULTS

All the raw data for the results presented in this article are available to view on STORE[DB] website. See also Supplementary Figs. S1–S6 (https://doi.org/10.1667/RADE-20-00266.1.S1) for survival data for all Kaplan-Meier plots from each institute.

In terms of survival, 93% of female C57BL/6 mice from PHE survived to 18 months postirradiation (data not shown), with a total of eight premature deaths out of 120 mice over an 18-month period (approximately 7%). Five of these premature deaths occurred among the nonirradiated mice. One hundred percent of 129S2 mice survived to their end point of 12 months (data not shown). Power calculations confirmed that for these strains, all group sizes remained large enough to draw conclusions regarding the significance of the experimental factors at 12–18 months postirradiation. For the pooled B6C3F1 and *Ercc2*$^{+/S737P}$ mice, survival probabilities differed. Overall log-rank test of the Kaplan-Meier plot revealed a significant difference of $P = 2.6E{-}04$. The hazard for early mortality within the observation for 2 Gy irradiated mice was 3 times higher than for controls ($P = 0.002$). There was no significantly increased hazard for the 0.5 or 1 Gy irradiated animals. Information can be extrapolated from the survival data of mouse colonies enrolled at ENEA. No significant differences were found in survival of nonirradiated wild-type mice, with 70% of CD1 and 77.7% of C57Bl/6 mice surviving to 18 months. Survival of all wild-type mice, irradiated at 0.5, 1 or 2 Gy, was not significantly different from those of their nonirradiated counterparts. In mutant mice (*Ptch1*$^{+/-}$ maintained on both genetic backgrounds), survivals declined in all groups, confirming that Shh-dependent pathologies occurred in this mouse model (*22*). However, no significant differences were found after irradiation at any of the delivered doses with respect to the nonirradiated group.

Figures 3 and 4 illustrate the clear difference of lens opacification in radiation responses between the strains and sexes, in addition to the strain-dependent variation in dose and dose-rate response.

Normality testing revealed that in almost all cases, the data were normally distributed, which indicated that ANOVA was appropriate.

ANOVA was initially applied for each strain individually, with interactions in the model of the order of 2. For the CD1 mice, for maximum lens opacity, month, dose, dose*sex, and dose rate*sex were all found to be highly significant factors ($P < 0.001$). There was no indication of independent significance of dose rate or sex as for the CD1 mice ($P = 0.186$ and 0.680, respectively). These results were closely mirrored for mean lens opacity, with the addition of month*dose as significant ($P = 0.005$). For the *Ptch1*$^{+/-}$/CD1 mice, for maximum opacity, month, dose and dose rate were all highly significant factors ($P < 0.001$), as were dose*sex and dose rate*sex ($P = 0.023$ and 0.002, respectively). For mean opacity, month, dose rate and dose rate*sex were significant ($P < 0.001$). Dose was not found to be significant ($P = 0.078$) and neither was sex in this group ($P = 0.421$ for maximum opacity and $P = 0.291$ for mean opacity). For the C57BL/6 mice, significant effects on maximum opacification were detected with factors month, sex and laboratory (for all, $P < 0.001$). No significant effects of dose or dose rate, or any of the interactions, were apparent (for all, $P > 0.050$). The reasons for this are not clear, although, the most likely explanation is that this is due to the confounding effect of laboratory, as discussed further below. However, for mean opacification, lab, month, dose, dose rate, sex, month*dose and dose*sex were all statistically significant factors ($P < 0.001$). For the *Ptch1*$^{+/-}$/C57BL/6 mice, for maximum opacity, month, sex ($P = 0.017$), month*dose, month*dose rate and dose*dose rate were all statistically significant (for factors other than sex, $P < 0.001$). These results were mirrored for mean

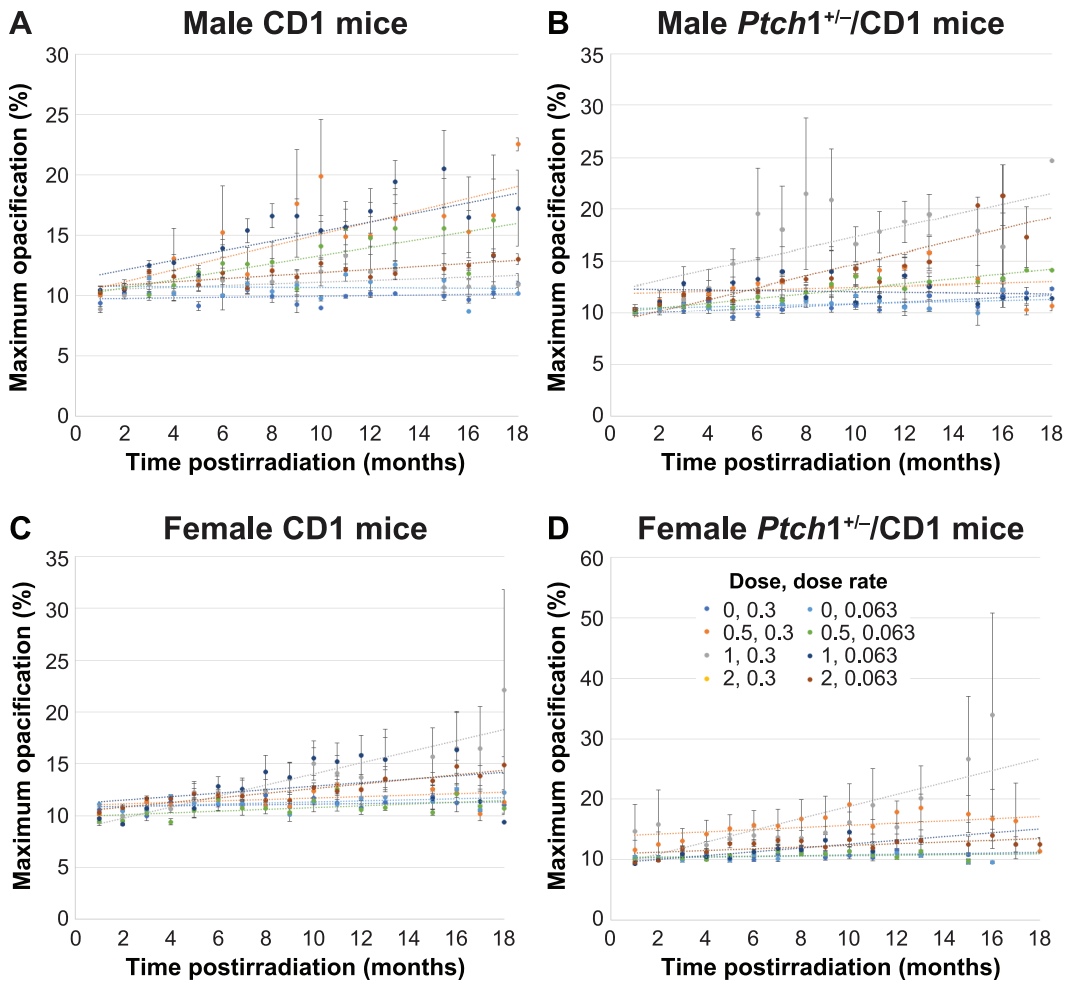

FIG. 3. Maximum lens opacification measured by Scheimpflug imaging at months 1–18 postirradiation in Ptch1$^{+/-}$ and wild-type counterpart mice. Radiation doses are in Gy and dose rates in Gy min^{-1}. Error bars are based on group standard error.

opacity, with the exception of the additional significant factor: month*sex, with $P = 0.037$. Dose and dose rate were not independently significant factors for either mean or maximum opacity. For the B6C3F1 mice, month, month*dose (both $P < 0.001$) and month*sex ($P = 0.002$) were significant factors for maximum opacity; dose was not significant, with $P = 0.064$. For mean opacity, month, dose, month*dose and month*sex were all significant ($P < 0.019$). For the *Ercc2*$^{+/S737P}$ mice, month, dose, month*dose and month*sex were all significant predictive factors of maximum opacity (for all, $P < 0.001$), and for mean opacity, month, dose, sex, month*dose and month*sex were all significant factors (for all, $P < 0.001$). Finally, for the 129S2 mice, only month and dose were significant predictors of maximum opacity (for both, $P < 0.001$); only month and dose*dose rate were significant predictors of mean opacity (for both, $P < 0.001$), with dose at borderline significance, with $P = 0.054$.

For the combined data across all strains, applied to assess the global impact of the experimental factors on lens opacification in this study, ANOVA revealed that each of the individual experimental factors (strain, dose, sex, dose rate and month) were all highly significant ($P < 0.001$). In addition, the following interactions were detected: Month*dose ($P = 0.003$); dose*dose rate ($P < 0.001$); dose*sex ($P < 0.001$); dose rate*sex ($P < 0.001$). Month*sex was not significant ($P > 0.975$), and there was insufficient data to investigate further, higher-order interactions.

As shown in Fig. 3, CD1 background mice had significantly higher maximum lens opacities than other strains. Post hoc testing revealed the strain responses were also all significantly different from each other: those with *Ptch*1$^{+/-}$ mutations, the wild-type CD1, C57BL/6, *Ercc*2$^{+/S737P}$, B6C3F1 and 129S2 mice. Nevertheless, as expected, opacification increased with time for most strains. Tukey's post hoc testing revealed that the increases were significantly different in blocks of 5 months on average, so, for example, the average response in months 1–5 was significantly different from the average response in months 5–10, and so on ($P < 0.05$).

Each of the doses, except for 0 and 2 Gy, were also significantly different from each other, with Tukey's test *P*

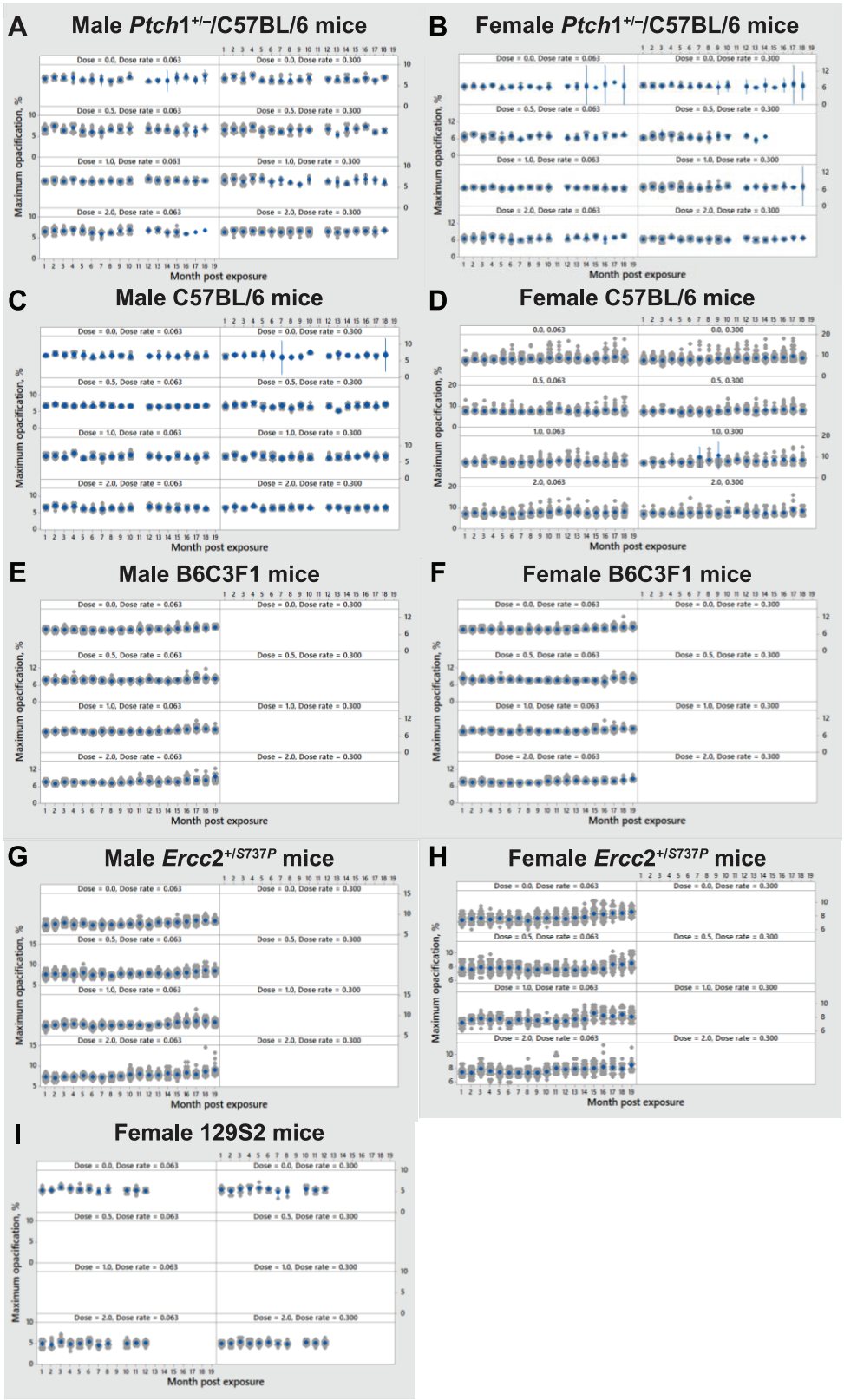

FIG. 4. Maximum lens opacification measured by Scheimpflug imaging at months 1–18 postirradiation in all strains of mice. Radiation doses are in Gy and dose rates in Gy min^{-1}. Uncertainties are individual point standard deviations.

≤ 0.002 for all pairwise differences. For 0 and 2 Gy, $P = 0.842$. However, this lack of significance was chiefly driven by the slightly unexpected lower overall values of opacification at 2 Gy compared to 1 Gy for female (but not male) mice irradiated at a dose rate of 0.3 Gy min^{-1}. Also of note is the lower opacification at 1 Gy compared to 0.5 Gy for the female mice irradiated at 0.063 Gy min.

DISCUSSION

To date, there have been very few studies investigating the effects of low-dose radiation on cataracts in mouse models. Indeed, to our knowledge, this study was the first to focus on low (<1 Gy) dose, multiple dose rate, sex and strain effects in lens opacification. The unique findings within this study, which were only made possible using combined data from the three institutes, was the highly significant effect of these experimental factors, and in many cases the significant interactions of these. Statistical interaction effects occur when the effect of one variable depends on the value or response of another variable. Here, the results clearly indicate that not only are the effects of dose, dose rate, strain and sex inter-related, but that studies involving these factors need to control for or co-investigate all of these factors to give valid results.

As shown in Figs. 3 and 4, and confirmed by ANOVA with post hoc analyses, on average, lens opacification increased over time, as the animals aged after irradiation. The association of cataract and age is well documented in both animal and human studies, with the most recent hypotheses suggesting that radiation adds to the ''cataractogenic load'' by accelerating lens aging, possibly by an amount proportional to dose (*23*). The data from this study support this hypothesis: Increased opacification with age (postirradiation, in this case) happens anyway, with radiation exposure increasing opacification further, with higher dose and dose rates having a greater effect. It should be noted that while age of exposure is known to be an important factor in lens opacification in mouse models (*15, 24*), age was not a factor during this study; all mice were irradiated at approximately 10 weeks of age. This has been investigated in the wider LDLensRad project and the results will be reported separately. The differences in whether sex, for example, has an influence on age- and dose-related response across the different strains, is evident in the comparison of Fig. 4E and F and Fig. 4G and H. To date, there have been few human epidemiological studies of radiation-induced cataract reporting a significant effect of gender, despite females being known to have a higher background risk of cataracts in general (*25*). Azizova *et al.* (*26–28*) did demonstrate a significantly higher ERR/Sv for posterior subcapsular cataract in females compared to males ($P < 0.0001$). There is, however, clear evidence that estrogen accelerates progression of radiation-induced opacification (*29–31*). Further, after low-LET irradiation, female rats show a lower incidence of cataract than males, although at high LET, it is males that demonstrate a higher incidence of cataract (*32*). From the pooled mean lens density data across all strains presented in this study, female mice demonstrated a statistically significant higher mean lens density compared to males.

Statistical analyses also indicated significant differences in radiation-induced lens density between each of the strains, with CD1, 129S2 and C57BL/6 mice, both with and without *Ercc*2 and *Ptch1* mutations, all significantly different from each other. This suggests that genetic factors have a significant influence upon the susceptibility to radiation-induced lens opacities in mouse models at lower doses (*19*). This is as has been reported in previous studies, albeit using higher doses (*33, 34*), with heterozygosity of the Atm and/or Rad9 genes resulting in earlier development of posterior subcapsular cataracts. This finding is not unexpected; $Ptch1^{+/-}$ mice have demonstrated increased incidence of spontaneous and radiation-induced cataract (*13, 15*). In humans, $Ptch1^{+/-}$ often results in Gorlin syndrome, also characterized by radiosensitive fibroblasts (*35*). Similarly, $Ercc2^{+/-}$ mice demonstrate a higher incidence of nuclear and cortex cataract, and also a DNA repair deficiency (*11, 36*). The significance of strain, and in particular these mutations, suggest that genetic predisposition to cataract is likely to be an important confounder during radiation-induced cataractogenesis.

Similarly to the findings from Dalke *et al.* (*16*), in this study, radiation dose had a significant effect on increased lens opacity, with increasing dose further increasing opacity. While it is possible that the combined results from Dalke *et al.* and this study may indicate a threshold of exposure, it is important to note that the long latency period for radiation cataract, and the limitations of investigating this mouse model of short lifetimes, mean that further conclusions regarding the existence or not of a threshold cannot be drawn. In addition, this study incorporates a wider range of doses (0.5–2 Gy), delivered at both 0.063 Gy min^{-1} and an additional higher dose rate of 0.3 Gy min^{-1}. A number of strains, both mutated and inbred, were investigated concurrently. The data show that while for some strains the increases in opacification associated with radiation exposure were minimal, for others, radiation had, both statistically and visually, highly significant effects.

In this work, opacification was measured in terms of percentage maximum or mean lens density using the Scheimpflug imaging technique and densitometry analysis. In most cases, the mean and maximum lens opacities at 12–18 months were well below the threshold of ~14% for vision-impairing cataracts (*37*) in humans. However, the CD1 mice, both wild-types and $Ptch1^{+/-}$, demonstrated opacities higher than this level, indicating mice may have experienced some visual impairment. Figure 3 also demonstrates that these groups had a large amount of variability, and in many cases, this is due to smaller group sizes as the mice aged and had to be sacrificed.

The Scheimpflug principle describes an optimal imaging condition using an obliquely tilted object, which when applied to the human eye allows documentation of the anterior eye segment with a depth of focus, and minimal distortion (*38*). Therefore, slit image photography is reported to be the most precise and versatile method of documenting light scattering and biometry of the anterior eye segment (*38*). The Scheimpflug camera was originally comprised of a slit lamp Newvicon tube camera and an online computer, and was designed to photograph, store and analyze lens opacities (*39*). During optimal conditions, the camera can create a 3D calculation of lens density by rotating 180 degrees around the central axis of the lens, capturing up to 100 images at different points in approximately 2 s. Using Scheimpflug densitometry, the camera can then detect lens opacification, or loss of transparency, using a calculation from the measure of reflected light (*40*). However, regarding cataract detection and measurement, it is important to note that Scheimpflug may not be the ideal method for tracking early radiation-induced opacities, particularly PSC, as the method has been reported to lack the sensitivity to detect this in mice (*41*).

While the use of data from the three institutes (PHE, HMGU and ENEA) facilitated the statistical power necessary to investigate the large number of experimental factors included in these experiments, in terms of radiation exposure, it was only feasible to investigate four doses (only two for the 129S2 mice) and at only two dose rates (just one for the *Ercc*$2^{+/-}$ mice) using only one quality of radiation. 129S2 mice were only observed up to 12 months postirradiation, due to time constraints associated with the local facilities and project.

Only one strain, inbred C57BL/6, was investigated at more than one laboratory, and ANOVA revealed statistically significant differences for both maximum and mean opacity in this case. Differences in animal husbandry between the three institutes were to be expected and are certainly a consideration during this study, and this might explain the significant observed difference between the laboratories that investigated these mice. Indeed, for the study overall, confounders that may influence the results, but were not controlled for, included animal diet and specific housing conditions, as this was institution specific.

Mice were irradiated at approximately 8–12 weeks of age while fully conscious, for comparison to previously reported studies of lens opacity development in mice (*16, 17*) The slight variation in ages of irradiation was not predicted to have a considerable impact as a cofounding factor due to lens development already having been established by this time. Emmetropia was recently reported to occur at 4–6 weeks post-natal development, although further investigation is needed to establish what effect this may have on future lens exposures to radiation, to establish optimal murine age (*20*). The slight variation in ages of irradiation is not predicted to have a considerable effect as a cofounding factor.

Likewise, the slight variation in methods with the use of anesthetic only at PHE facilities are considered. However, this was not expected to effect results, due to the lack of evidence found in the literature to suggest that anesthetic effects lens opacification. Background lens opacity in the control lenses of anesthetized C57BL/6 mice was similar to that reported by Dalke *et al*. (*16*), where no anesthetic was administered. Similarly, during this study, sham-irradiated mice from PHE and HMGU showed no significant difference in opacification with and without anesthetic use, respectively. However, the fact that the laboratory was a significant factor for the irradiated mice remains, and as such, this requires further investigation.

Total-body irradiations were performed during this study, without shielding to the rest of the body; therefore the effects of radiation were not restricted to the eye lens, and may affect other tissues and organs within the body (*16*). However, dosimetry was carefully calculated and calibrated using a common method across all three institutes to ensure that doses to the lenses varied by no more than 5%.

Current radiation protection regulations (ICRP 118; BSS 2015) consider only exposure dose, not the rate in which it is delivered. Although more work is clearly needed, the need to include consideration of dose rate and perhaps even sex [as has already been considered by, e.g., NASA (*42*)] may become apparent as further data emerge. The minimum threshold dose estimated to increase the incidence of radiation cataracts varies depending on study design and methodology (*2*). Likewise with epidemiological data, despite the suggestion of most investigators that the threshold dose should be between 100 mGy–1 Gy for lens opacities (*43*), other findings (*44*) have shown that cataract risk remains statistically significant at doses $<$100 mGy cumulative occupational exposure to the lens (*44*). These findings indicated that a revision of the allowable radiation exposure to the eye should be considered (*3*). In addition, despite early results from the LDLensRad study reporting an inverse dose-rate effect of DNA repair in the lens (*45*), this phenomenon was not supported within the pooled lens opacification data across all strains presented here, with the higher dose rate having a significantly greater mean lens opacity than those of lenses exposed to the lower dose rate. Following this work, further research is needed to consider the implications for radiation workers exposed periodically, or on a daily basis, over their working lifetimes. For example, if ionizing radiation does contribute to the ''cataractogenic load'' (*23*), i.e., if those exposed to radiation experience opacification at earlier time points than would otherwise be expected, this would indicate that radiation protection require consideration of other factors contributing to predisposition and to cataract development, and thus, a complete shift in the way radiation protection is applied.

CONCLUSIONS

The EU CONCERT LDLensRad study was the first to look at the effects of low-dose ionizing radiation on opacification in the lens, taking into account dose rate and sex, while also comparing responses from mice of different genetic backgrounds. The results of this study clearly demonstrate the importance of each of these experimental factors in terms of lens opacification in response to ionizing radiation and, although this work has by necessity been carried out in mouse models, the results clearly suggest that the risk of cataract development depends not only on dose and time after exposure, but also on dose rate. It is crucial that such future studies into the effects of radiation in humans or animal models also include further investigation into the impact of dose rate and protraction, and should ensure all these factors are appropriately tested or controlled. Moving forward, it is entirely possible that dose rate in particular, and perhaps also sex, will need to be further considered for the radiation protection regulations.

SUPPLEMENTARY INFORMATION

Fig. S1. Kaplan-Meier survival plots from the CD1 wild-type mice at ENEA.

Fig. S2. Kaplan-Meier survival plots from the C57BL/6 wild-type mice at ENEA.

Fig. S3. Kaplan-Meier survival plots from the Ptch1$^{+/-}$/CD1 heterozygous mice at ENEA.

Fig. S4. Kaplan-Meier survival plot from the Ptch1$^{+/-}$/C57BL/6 heterozygous mice at ENEA.

Fig. S5. Kaplan-Meier survival plot of mouse survival under all exposure conditions for C57BL/6 mice at PHE.

Fig. S6. Eighteen-month Kaplan Meier survival plots for *Ercc*2 mutated and wild-type mice from HMGU.

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