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Lead Author: DH-PHE

With contributions from: All project partners (HMGU, ENEA, DU, OBU) and Advisory Board members

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Abstract

The lens of the eye is known to be more radiosensitive than previously thought but, despite a substantial reduction in occupational dose limits based on recent epidemiological information and reanalyses, the mechanisms of low dose radiation cataract induction are still unclear. This is an important current public health issue, for instance for medical radiation workers, many of whom will need to amend their working practices despite a clear understanding of the effects of chronic, low dose, ionising radiation exposure.

The LDLensRad project aims to bring together experts from across Europe to answer a number of key research questions on this topic, including: how does low dose radiation cause cataracts; is there a dose rate effect, and how does genetic background influence cataract development after radiation exposure. CONCERT Deliverable 9.45 describes progress in year 3, from January – December 2019, in the format of minutes of the final scientific meeting which took place in Rome in December 2019.

Content

Progress summary – minutes of LDLensRad 2019 Final Meeting

The meeting was attended by representatives of all partners and the Advisory Board members[1].

Introduction

The LDLensRad coordinator kicked off the meeting with a brief welcome and details of the project background, outlines and aims, deliverables and milestones which have to date all been successfully completed on time. LDLensRad was a successful EJP CONCERT first call project, focused on the 'Improvement of health risk assessment associated with low dose/dose rate radiation.' The overall objectives were: "To advance knowledge to solve the question of how radiation causes and/or promotes cataracts."

The initial aims were:

- How does low dose radiation cause cataract?
- Is there a dose rate effect?
- How does genetic background influence cataract development after radiation exposure?

In addition, we intended to look at the shape of the dose response (in time), advancing the debate regarding the nature of cataract (deterministic, stochastic or both), biomarkers or bioindicators of global radiosensitivity, and also education of early career scientists.

WP1: Scheimpflug imaging

Discussion of progress in WP1 started with Task 1.1.1: Irradiations and long term Scheimpflug imaging at HMGU with wildtype (B6C3F1) and heterozygous mutant *Ercc2*$^{+/S737P}$ mice. CD presented 19 mth Scheimpflug data demonstrating no significant radiation-induced lens opacifications in 10 wk irradiated mice given doses of 0, 0.5, 1 and 2 Gy at 0.3 Gy/min. However, the influence of strain, and the interaction of sex and strain, strain and dose were statistically significant. The issue of lack of clarity of the back of the lens was discussed briefly, with reference to the OCT and histology results of DP (discussed below). Anterior changes are, however, clear. As expected opacification was much more prevalent in the mice irradiated at 2 days old (P2) with a clear, highly significant, dose effect. Differential survival and wider pathology was observed in comparison to the INSTRA (0.063 Gy/min irradiated) mice, which needs further investigation.

RM presented PHE's data on C57BL/6J (C57) female mice. Again, the age-related increase in opacification was clear, with significant differences from background evident around 6 mths post irradiation. There was a significant effect of dose and dose rate (0.3 vs 0.063 Gy/min) at 2 Gy and a significant interaction of dose and dose rate but, as with the HMGU 10 wk data, no opacification which might be considered 'clinically significant' (i.e. > 14.1%, which is considered to be vision impairing in humans). Only female mice were used at PHE so it is not possible to assess the impact of sex, or the impact of interaction of sex with other factors, on opacification in this model. The vast majority of the

[1] No names only initials are used

mice survived 18 months so the impact of even 2 Gy on survival in this model was minimal. Imaging on the 129S mice is now complete but analysis is still in progress.

Data from the *Ptch1*$^{+/-}$ mice on C57 and CD1 backgrounds and wt mice at 10 wk and 2 day from ENEA were then presented by MM. CD1 mice had higher opacities than the C57s and, in contrast to the PHE data, the C57 mice demonstrated no increase in opacification with age over the 18 month follow up period. There was a strain dependent dose rate effect, but in 10 wk mice no 'clinically significant' opacification was observed. In the P2 mice, there were significant strain dependent dose and dose rate effects resulting in opacification of the lens up to ~ 40% (likely vision impairing), which also markedly impacted on survival. It was concluded that background strain had a greater influence on opacity than the presence or absence of the *Ptch1* mutation. It is hypothesised that *Ptch1* may compound genetic predisposition.

LA then presented information on the initial combined 10 wk ANOVA results. The data can be summarised as follows:

Significant effects of dose and dose rate have been identified in some models. However, for all models apart from the P2 *Ptch1*$^{+/-}$, P2 B6C3F1 wild type and *Ercc2*$^{+/-}$, all measured lens densities (19 mths post exposure) < 14.1% (which is 'clinically significant' in humans).

Age and genetic background significantly contribute to cataract risk. In most mouse models: the effects of aging and strain clearly outweigh the effect of ionising radiation dose or dose rate.

For *Ptch1*$^{+/-}$ P2 and *Ercc2*$^{+/-}$ irradiated mice, the effect of age at irradiation is strongly influenced by genetic background.

There are clear interaction effects (in dose, dose rate, age, strain and sex) so the impact of dose and dose rate is dependent on the status of these other factors.

Further investigation of the husbandry conditions is to be carried out in order to ensure the 'strain' differences are fully inclusive of differences in animal housing facilities too.

Short and long term in vitro and in vivo endpoints

DP then presented details of the OCT, histology and immunohistochemistry.

The data are clear in that OCT is much more efficient in terms of detection of PSC, so OCT and/or histology are preferable over Scheimpflug images. New ways of measuring and new phenotypes of cataract have thus been defined and there is a clear effect of radiation in terms of cataract induction for both posterior and anterior cataracts.

In the 10 wk mice, at 12 months post irradiation, there appears to be a threshold of approximately 2 Gy for alterations in lens structure, however, by 20 months the response is linear in dose – so a key message of this work is that, as with the epidemiological observations, the 'threshold' appears to be time dependent. There was also no evidence for 'static' cataracts, though this was not the main focus of the study.

In the P2 mice, the dominant phenotype is posterior cortical cataract and retinal atrophy beginning ~6 months post irradiation.

Visual acuity was impacted by radiation with sex and genotype influence, but the relationship between visual acuity and opacification was unclear, leading to further questions regarding the impact of radiation outside the lens on vision – and, possibly, on the question of the ICRP definition of detriment. Effects in female mice were greater than those in male throughout.

These results will be published in a manuscript to be submitted before end December 2019.

In the C57 mice from PHE, there was a similar dose response but no dose rate effect.

SB then provided an update of the DNA damage and proliferation work on samples from PHE and HMGU mice. The inverse dose rate effect is consistent across strains, with ENEA samples still to be analysed. To our knowledge an inverse dose rate effect has been reported only once elsewhere, in Hela cells (Mitchell, J. B.; Bedford, J. S.; Bailey, S. M. Dose-rate ef- fects in plateau-phase cultures of S3 HeLa and V79 cells. Radiat. Res. 79:520-536; 1979.), but this relates only to clonogenic cell survival – this reference should be reported with these data to prompt further discussion.

The strains showed differential repair in terms of detection of 53PB1 foci at 4 and 24 hours post-irradiation, indicating *Ercc2*$^{+/-}$ mice are either more efficient in DNA damage repair or there is less damage. The influence of single strand breaks was discussed together with a hypothesis around the potential role of growth factors as discussed in Barnard et al., 2019 and transcription factors for the XPD DNA damage repair protein, in which the *Ercc2*$^{+/-}$ mice are deficient. In lymphocytes, *in vitro*, repair was slower in *Ercc2*$^{+/-}$ samples, potentially indicating differential repair mechanisms in the lens as compared to other tissues. Further investigation will be carried out when the ENEA data analysis is complete.

The proliferation analyses completed to date in the PHE and HMGU mice indicate that ionising radiation reduces proliferation, with region of the lens epithelium (nucleus or cortex), dose and dose rate all significantly involved. There were significant strain differences but no significant effect of time post exposure (4 or 24 hours) or sex in this case.

RQ then presented details of the lens morphology work at DU, including the novel method of assessing cell density via 3D mounting and image processing. The data indicate slow but dynamic development of opacities associated with radiation exposure at 0.5 - 2 Gy 12 months post exposure, with clear evidence of lens repair too, and gender and strain effects. The hypothesis of acceleration of aging due to a combination of effects was explored, together with the link to the 'compensatory response' of the lens indicated by the inverse DNA damage dose rate response presented by SB. Further attention should be paid to alpha beta crystallin and proteomic work would be desirable. Protective and/or mitigating agents should also be further explored.

BT then presented details of the NGS carried out at ENEA. Initial analysis of miRNome identified miRNA indicative of radiation responses. Pathway analysis then revealed the role of Toll-like receptor 'danger' signalling which alerts the immune system with a pro-oxidant response. The differential responses between strains are interpreted to indicate a potential switching off of the DNA damage response and "Danger signals pathways" in the *Ptch1*$^{+/-}$ CD1 mice due to early events in the chain of DNA damage response. Focused NGS was then carried out on DNA repair mechanisms, including network visualisation. It was concluded that *Ptch1*$^{+/-}$ C57 lens cells have slower proliferation rates mainly due to p53 pathway upregulation. Further consideration of the p53 pathway leads to the conclusions that irradiated CD1 Ptch1$^{+/-}$ mice show changes related to posterior/ anterior cataracts caused by EMT (via

the TGFb pathway). It is thus hypothesised that in *Ptch1*$^{+/-}$ C57, p53 could prevent the formation of cataracts by triggering the death of aberrantly proliferating cells and inhibiting EMT.

The results thus far in terms of in vitro experiments regarding intracellular communication and ROS carried out at OBU were then presented by MA. Cell viability, DNA damage assessed via the comet and gamma—H2AX immunofluorescence assays, ROS, senescence and telomeric effects have all been assessed and indicate dose responses (0 – 0.5 Gy Cs 137 gamma irradiation) at 1 h but not 24 hr post exposure in immortalised human lens epithelial (B3) cells. Exceptions are cell viability and senescence studies where the dose response can be seen at 24 h and 15 days respectively.

Dose rate (0.3 vs 0.063 Gy) effects were also observed in most endpoints. Telomere length and telomerase activity demonstrated no significant differences with dose, dose rate or time post exposure. Further work is ongoing with primary human lens epithelial cells, together with assessment of cellular signalling and genetic predisposition.

AU then presented the lipidomic and proteomic results to date, testing the hypothesis that IR causes oxidative stress, leading to cholesterol oxidation into various oxysterols which gradually lead to cataractogenesis. 50 Gy X-irradiation of bovine lenses did not change cholesterol levels, however, oxysterols were impacted with a trend of dose dependent increase, and changes persisted to 18 days post irradiation. In vivo irradiation of mouse lenses led to transient dose dependent changes in oxysterol levels with differential responses in the nucleus and cortex of the lens.

The effect of IR on advance glycation end products (AGEs) was also investigated, as post translational modifications (PTM) are known to lead to opacification. Ionising radiation was found to alter the PTM in the cortex and nucleus, with differential dose dependence in terms of AGE formation and amino acid oxidation. With assistance from the University of Milan, VC1-pull down was applied (Degani et al., 2017), to identify a number of AGE bound proteins related to lens development and morphology. It was concluded that IR induces changes in the lipids and proteins and that the cortex is more sensitive than the nucleus, but also, it should be noted that acute damage can clearly be repaired in vivo.

WP3

Irradiation effects on behaviour of the HMGU mice were presented by S H-K. An overview of the current understanding of irradiation effects on neuroinflammation and Parkinson's disease was presented as context. Behaviour and social interaction were assessed by open field, prepulse inhibition, social discrimination and y-maze following IR of the HMGU mice at 10 wks to 0 – 2 Gy (0.3 Gy/min) at 4, 12 and 18 months post irradiation. Age was the strongest indicator overall but the responses were clearly also dependent on dose, sex, genotype, with various factor interactions identified for the different endpoints. The fact that at 4 months, visual acuity was not affected was discussed, indicating that it is not the lens which is driving these observations. The relationship with neurogenesis was also discussed.

SP then presented the final details of the irradiation effects on the mouse brain in the ENEA mouse models. A brief introduction regarding what is known about radiation in the brain and a reiteration of the role of the sonic hedgehog pathway for wide regulation of cell growth and differentiation (and which is deficient in *Ptch1*$^{+/-}$ mice) was followed by a report of the observations that ionising radiation

> 0.5 Gy significantly reduced survival of the mice due to development of medulloblastoma (MB) in CD1 background mice, which was not observed in any unirradiated mice. There were no significant effects of dose rate on survival or MB induction in the CD1 mice. In the C57 mice, ionising radiation reduced survival in a less marked manner, and there was no MB induction above background in the P2 irradiated C57 mice. Genetic background was thus the dominating factor in MB development in this study.

In terms of neurogenesis, the results show a significantly slower rate of basal neurogenesis in C57 (-50%) compared to CD1 mice pointing to important genetic background related-differences between the two mouse strains. No difference in the long-term response (4 months post-IRR) of the neuronal population of the DG to adult irradiation with 2Gy were observed at any of the two dose-rates in WT CD1 and C57 mice, although the genetic background-dependent differences were maintained. At 6 weeks post exposure, impairment of Sox2 and Dcx populations was observed in the C57-*Ptch1*$^{+/-}$ mice only. It is thus concluded that sensitivity of *Ptch1*$^{+/-}$ mice to irradiation was strongly exacerbated on a C57 background both after neonatal or adult irradiation.

The link between these observations and the NGS data (BT) was discussed. Efforts will now continue to attempt to understand the molecular mechanisms accounting for the different response to irradiation in *Ptch1*$^{+/-}$/*CD1* and *Ptch1*$^{+/-}$/C57 mice.

WP4

The statistical analysis of data, under WP4, was then discussed. The original plans were for individual tasks, that each lab/individual was responsible for the provision of high quality uncertainty estimates on each and every measurement made – to ensure the analysis methods are driven by the data. Analysis would nominally be with variance-based approaches for multiple experimental factors – 'ANOVA' or non-parametric equivalents where necessary, together with multivariate approaches where appropriate.

It was then intended to focus on development of methodological models where possible to investigate whether data could be combined with 'systems' style modelling. Finally, whether it is possible to apply pathway-based and network-based analysis methods, for example to propose 'Adverse Outcome Pathways,' would be investigated. In practice, although individual endpoint analysis is almost complete, as we are only now at the stage where the data are being finally collated and thus methodological modelling is still underway. However, as an example, MANOVA has been applied to cell density and proliferation data from HMGU, and demonstrates a borderline statistical link between the endpoints in terms of mutation status (wildtype (B6C3F1) and heterozygous mutant *Ercc2*$^{+/S737P}$ mice).

Advisory Board (AB) report

The final day of the meeting started with the AB report presented by NH. In general, the AB reported that the project is very well represented on the ResearchGate page (https://www.researchgate.net/project/LDLensRad-the-European-CONCERT-project-starting-in-2017-Towards-a-full-mechanistic-understanding-of-low-dose-radiation-induced-cataracts) – people are paying attention to the project. At the time of the 2019 AM we have posted 33 updates, have 63 followers, and have had 1419 'reads.' The project is well designed as a whole, but some improvements may further strengthen the merit of the project.

For WP1 and WP2: The major assumptions underlying the latest ICRP recommendations include no dose rate effects and progression of minor opacities into VIC. ICRP recognizes the importance of mechanistic research to further inform adequate RP.

There is growing epidemiological evidence for radiation cataracts, and the LDLensRad data suggest endpoint dependent impact of dose rates. Various biological changes could be detected including histological data from the eye, biochemical data from bovine lenses, molecular data from next generation sequencing (NGS), cellular data from in vitro cultured human cells. The relevance to manifestation of lens opacities for radiation protection and relevance needs to be carefully considered.

The epidemiological evidence for the progressive nature of cataract is unclear, so further attention needs to be paid to this issue, particular in interpretation of the Scheimpflug imaging results.

One of the strengths of the LDLensRad is that the three institutes (PHE, ENEA, HMGU) used very consistent irradiation conditions for studies with mouse models, which makes inter-lab comparison feasible. However, further description of the similarities and differences in the animal facilities and husbandry (bedding, feed, water, etc) will help identify the potential causes for the 'differences' in the results and make clear the need for consistency in future research.

Discussion of age and time since exposure effects was a running theme throughout presentation of the results. This poses the question of which population is more sensitive: young or old individuals? At younger ages of exposure, background levels are lower, but onset times can be longer, and vice versa.

For the OCT/Histological work: A at 12 mths there/ appeared to be a threshold, however, linear doses were observed at 20 mths post irradiation. This resembles the data seen in the A-bomb survivors; careful interpretation is needed for the radiation protection implications.

Regarding tumour related factors: Evidence is limited but increasing for the possible involvement of various tumour related factors in radiation cataract. This has implications for the stochastic/deterministic (tissue reaction) classification of the nature of cataracts.

Regarding WP3: Integration of the wider systemic effects has been very useful. The biological developments in mice are supported epidemiologically by the Mayak worker and INWORKS studies, and may likely be supported by other studies.

For WP4: A clear strength of the project is that the statistical significance can (generally) be tested in a consistent way. AOP is a hot topic, this should be investigated further.

For WP5: The presence of LDLensRad consortium members at a large number of international scientific meetings has succeeded in disseminating the work of LDLensRad. Upcoming meetings that we might consider include COSPAR 2020.

Regarding the editorial for the special issue: The introductory paper should clearly mention the objectives of the project including the strong and more hypothetical motivations, also highlights of the main findings, introduction to the key issues in the field of RP and the impact of the main findings. The papers presenting scientific results should clearly reflect the strengths and limitations including the Scheimpflug imaging not always detecting PSC.

In summary, each of these aims outlined at the start of the project has been met or exceeded very satisfactorily. For final reporting, we need to stress the education and training aspects including travel and poster awards – the early career scientists have done a great job. We should also be clear that there are no 'negative' data – all the results are valid whether positive or 'negative' in terms of radiation response and ultimately establishing whether there is a true threshold dose for radiation cataractogenesis. The field of eye lens dosimetry is advancing, with additional information at low doses, which should in future years yield much more detailed epidemiological data thus forthcoming

on this issue. The final paper of the special issue should highlight all these relevant issues regarding the landscape of lens research going forward.

The AB also provided some thoughts regarding future work. In order to investigate further the most interesting effects, e.g. the inverse dose rate effects, we should collect additional dose and dose rate points. The validation we did prior to starting the experiments is important, including the physics of calibration and comparison between the different labs' dosimetry systems, and thus needs to be clearly defined in the relevant publications. We must keep in mind that while animal models are useful for mechanistic understanding, humans are not the same as mice! For mechanistic modelling, we should rely on the data which are really consistent in order to ensure that the results are high impact and of key relevance for radiation protection. We should also keep in mind the developing epidemiological and other mechanistic data which are emerging, and further work with epidemiologists going forward. LET and RBE will also be hugely important going forward – this should be high priority for the next project proposals.

Dissemination plan

In terms of scientific dissemination, the project has been represented at a large number of international scientific meetings with approximately 30 poster and/or oral presentations to date. Further dissemination is expected to take the form of additional conference presentations as well as a proposed special issue of Radiation Research focused on LDLensRad.

The journal has accepted the proposal and discussion took place regarding how to collate and present the joint project findings. It was agreed for each task leader to collate data into a rough draft paper to be ready for circulation amongst the partners by July 2019. Further discussion will take place regarding how to best organise the data for publication, with ~10 manuscripts to be ready for submission by end November 2020. Publication of the special issue should then take place ~ March 2021.

It was also agreed that the project PhD students and others who have a more pressing need to publish as soon as possible are welcome to submit to the special issue as soon as the papers are ready as online publication will, as usual, occur as soon as manuscripts are accepted.

Finally, it was agreed to approach a separate journal, likely Mutation Research Reviews, with a list of related reviews, including: the review of 'What is a cataract?' to consider definition of lens opacities and cataracts, suitability of cataract surgery as a surrogate for vision impairing cataracts remain obscure, the lens opacification classification system suitable for radiation cataracts.

For public and wider stakeholder dissemination, following the successful LDLensRad stakeholder engagement and involvement workshop which took place in May 2018, LA and partners from other CONCERT projects are now organising a Stakeholder Dissemination Workshop which will take place in Newcastle on the 30th March 2020. The aim is for early career scientists to give short presentations focusing on impact of outputs to invite members of the public, patients, medical professionals, CONCERT WP5 scientific stakeholders and reps from NGOs and any other. This will be followed by group and one-to-one discussions and feedback from Stakeholders together with live and retrospective artistic interpretation of public impressions of the research outputs and implications. A permanent home will be found for the resulting artwork to further promote public discourse and understanding on the topic of ionising radiation research to support radiation protection. It is also intended to write a manuscript reporting the event for a public engagement journal.

The first announcement has already gone out the CONCERT project coordinators and invitations to stakeholders will be send in January 2020.

STORE: Data collation and sharing

LDLensRad data collation and sharing was discussed, with the plans as per the AM 2018 reiterated – in brief, to put all the raw data on the STORE file sharing facility for project partners as soon as possible, then for the data to be made public when the publications are accepted and made available online.

Conclusions and Future research

It was agreed by all partners that the project has been highly successful in terms of generation of data that advance knowledge regarding the low dose and dose rate effects of ionising radiation on the lens.

The implications of the work for radiation protection will be assessed in the special issue, but will include the key points that dose rate matters, and that it is the interaction between dose, dose rate, genetic background, sex and age, that is important.

However, further work is needed and, as discussed in the AB report section above, the priorities are for understanding the relationship between our data and human cataract; understanding the dose rate and sex differences in responses, and for widening the exposure profiles to include higher LET radiation which is of key relevance for space research. Further work on the wider systemic effects of radiation in relation to the lens/brain and on radiation mitigators is also a high priority.