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Name, title and organisation of the scientific representative of the project's coordinator:

Coordinator:	*Univ.-Prof. Dr. med. Michael Molls, Technische Universität München, Germany*
Scientific Manager:	*PD Dr. med. Nicolaus Andratschke; Technische Universität München, Germany*
Tel:	*+ 49 (0) 89 4140 4501*
Fax:	*+ 49 (0) 89 4140 4882*
E-mail:	*Klinik-fuer-Strahlentherapie@lrz.tu-muenchen.de*
Project website:	*www.cardiorisk.eu*

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CARDIORISK

Logo:

Project title: The mechanism of cardiovascular risks after low radiation doses

Website: ***www.cardiorisk.eu***

Contractors involved (CARDIORISK consortium):

The project is coordinated by Univ.-Prof. Dr. med. Michael Molls, Klinik und Poliklinik für Strahlentherapie und Radiologische Onkologie, Technische Universität München, Germany;

Scientific Manager: PD Dr. med. Nicolaus Andratschke, Klinik und Poliklinik für Strahlentherapie und Radiologische Onkologie, Technische Universität München, Germany;

Address: Ismaninger Str. 22, 81675 München, Germany

Other partners and team leaders:

02 TUD: Prof. Wolfgang Doerr, Technical University of Dresden, Germany

03 NKI: Dr. Fiona Stewart, Netherland Cancer Institute, Netherlands

04 IRSN: Dr. Marie-Catherine Vozenin, Institut de Radioprotection et de Sureté nucléaire Villejuif, France

05 QUB: Prof. Dr. Kevin Prise, University of Belfast, United Kingdom

06 USFD: Dr. Chryso Kanthou, University of Sheffield, United Kingdom

07 MSCCI: Prof. Piotr Widlak, Maria Sklodowska-Curie Cancer Institute, Poland

08 HELMUC: Dr. Soile Tapio, Helmholtz Centre Munich, Germany

09 UL: Dr. Annegret Glasow, University of Leipzig, Germany

10 STUK: Dariusz Leszczynski, STUK Helsinki, Finland

11 CARIM: Prof. Mat Daemen, Cardiovascular Research Institute Maastricht, Netherlands

12 GABO:mi: Dr. Claudia Speiser, Gesellschaft für Ablauforganisation :milliarium mbH & Co KG, Germany

13 UROS: Prof. Dr. Guido Hildebrandt, University of Rostock, Germany

1.1 Executive summary

The CARDIORISK project addressed both macrovascular and cardiac microvascular radiation damage after local irradiation with low, intermediate and high doses.

Various experimental in vitro and in vivo methods and models were developed to describe and quantify the effects of ionising radiation occurring at low doses. Small animal high precision irradiation set-ups were deployed to reproducibly irradiate either the heart, the arteria carotis with the aortic arch or the peripheral arteria saphena. Irradiation consisted of doses ranging from 0.2 to 16 Gy. High radiation doses have been explicitly included to serve as a reference at which structural and clinical damage can be expected and to investigate whether different mechanism of action are involved at low versus high radiation doses.

As the animals, heart tissue or cells have been centrally prepared and provided to all members of the research consortium, results obtained in this research programme apply to identical biological material, treated identically with radiation. Molecular and cellular responses at different times can thus be related directly to histopathological and functional changes of the irradiated cardiac microvasculature or the irradiated arteries.

Radiation-induced cardiovascular disease represents a late occurring event. Therefore, animals were followed up to 60 weeks and investigated at different time points after irradiation (for late effects at 20, 40 and 60 weeks).

Even though changes at the molecular level induced by low doses were observed in the locally irradiated murine hearts weeks after the exposure, a persistent functional cardiac impairment could not be observed during a follow-up of up to 60 weeks. As inflammatory, prothrombotic and adhesive markers did not exhibit a consistent pattern, inflammation as a causative factor does not seem to play a relevant role.

Long-term persistent stress responses are observed even at intermediate and low doses. With the exception of the oxidative stress response in cardiac mitochondria, they are rather a consequence than a cause of radiation induced cardiovascular disease. The findings of mitochondrial disturbances are intriguing as they develop early and persistent after very low radiation doses. Further investigation on long-term persistence and correlation with other late structural and functional changes is necessary to clarify the true relevance.

The angiogenic capacity of cardiac endothelial cells is strongly inhibited even at low to intermediate radiation doses as demonstrated in 2 newly developed ex vivo in vitro angiogenesis assays which points to a decreased capacity to repair/ regenerate in the case a second clinically relevant insult to the heart occurs. The most relevant and consistent finding was a linear dose and time dependent reduction of the microvascular density in the mid part of the heart. Though not statistically significant, a decrease in MVD is seen even at a low dose of 2 Gy. This decrease is preceded by a time and dose dependent loss of alkaline phosphatase expression in endothelial cell.

The results of the CARDIORISK project permit the following conclusions, which may impact profoundly future research in the area of low-dose radiation risk assessment in the cardiovascular system:

1) Radiation at low doses does not per se induce atherosclerotic changes in medium to large arteries.

2) Inflammation does not play a major role in the development of radiation induced CVD; stress responses can be regarded as a consequence of the effects of radiation induced CVD.

3) The reduction in microvessel density and the inhibition of neoangiogenesis even at low to intermediate doses does not translate into apparent functional and structural effects leading to the clinical picture of CVD.

4) These major conclusions support the hypothesis that low-dose radiation exposure and related subtle subclinical changes are permissive factors for the development of CVD which reduce the capacity of the heart to recover if a clinically relevant event/ insult secondary to irradiation occurs.

The CARDIORISK identified the progressive reduction of the capillary network at intermediate and low radiation doses (<2 to 8 Gy) as the key mechanism leading to radiation-induced heart disease. This microvascular effect does not lead to measurable ischemic cardiomyopathy but is likely to reduce the compensatory capacity of the heart to insults from other sources such as coronary heart disease etc. In addition, there are also strong suggestions of direct myocardial injury by low radiation doses after similar latencies. Despite the wide range of research methods and biological targets used in CARDIORISK, some key molecular and cellular processes of this microvascular effect are still largely unknown. Future research should focus on resolving these open questions, in particular:

For this research, an integrated programme bringing together cardiological and radiobiological scientists is obligatory. The experience of CARDIORSIK has demonstrated the great potential of this cooperation.

1.2 Summary description of project context and objectives:

The aim of this collaborative research project is to elucidate the pathogenesis of early and late alterations in the microcirculation of the heart and of atherosclerotic lesions in arteries after exposure to low radiation doses in comparison to high radiation doses. A major goal was investigation of early molecular, proinflammatory and prothrombotic changes as well as alterations of myocardial perfusion, cardiac cell integrity and immunological processes.

CARDIORISK to achieve the following objectives:

- Elucidation of the pathogenic mechanisms of radiation-induced heart disease and of radiation-induced vascular damage after low and moderate radiation doses at the tissue, cell and molecular level (the primary objective of this project). In the context of this experimental study, low radiation doses are defined as average heart doses between 0.01 and 0.5 Gy, while moderate doses as average heart doses between 0.5 and 5 Gy.
- Determination of the radiation dose dependence of the severity, latency, and rate of progression of cardiovascular radiation damage.
- Elucidation of the interaction between radiation-induced cardiovascular damage with other risk factors e.g. elevated cholesterol levels for atherosclerotic vascular damage and for microvascular damage.
- Clarification of the histopathological and biochemical (proteomic) development of cardiovascular radiation damage, in particular elucidation of differences in responses at high (≥2 Gy such as in radiotherapy) and low radiation doses (≤0.5 Gy as encountered in radiation protection).
- Development of a dose specification system for inhomogeneous dose distribution in the heart and the cardiovascular system, which will be based on the identification of critical subvolumes in the heart and their anatomical distribution in the organ.

1.3 Description of the main S&T results/foregrounds of CARDIORISK

The CARDIORISK project addressed both macrovascular and microvascular radiation damage after local irradiation. Different groups were developing and implementing various experimental methods and models making use of the same cells and tissues most of which were centrally prepared and provided to all members of the research consortium. This way, results obtained in this research programme apply to identical biological material, treated identically with radiation. In vitro assays and in vivo experiments were all based on cells, tissue or animals from two genetically different mouse strains: ApoE-/- mice deficient in Apo-Lipoprotein E and prone to the development of atherosclerosis and C57Bl6 mice as normal counterpart. Molecular and cellular responses at different times can thus be related directly to histopathological and functional changes of the irradiated cardiac microvasculature or the irradiated arteries.

Development of experimental methods

The major tasks of the CARDIORISK consortium within the first two years were the development and establishment of experimental systems to study the radiation biology of cardiovascular effects. Both in vivo and in vitro model systems were deployed to describe and quantify the effects of ionizing radiation occurring at low doses.

Local irradiation of the heart, the a. carotis and the A. saphena (partners TUD, NKI)

Small animal irradiation setups were developed to reproducibly irradiate either the heart (TUD, NKI), the a. carotis with the aortic arch (NKI) or the peripheral A. saphena (TUD). Irradiation consisted of doses ranging from 0.2 to 16 Gy. Animals or heart tissue were then distributed to all consortium members for further analysis on comparable biological material. As radiation-induced cardiovascular disease represents a late occurring event, animals were followed up to 60 weeks and investigated at different time points after irradiation (20, 40 and 60 weeks).

In Dresden, a total of 1648 animals were irradiated locally to the heart at doses between 0 (sham irradiation) and 16 Gy. Before irradiation, the correct position of the hearts was verified by digital radiographs, resulting in a total body dose of 4 mGy. Subsequently, single local heart doses of 0.2, 2, 8 or 16 Gy were applied. A control group received sham irradiation with 0 Gy. Live animals or heart preparations were then distributed between the partners for the respective experiments. The animal numbers are summarized in table 1. An additional 291 animals were irradiated locally to the heart at the NKI (doses of 0, 2, 8 and 16 Gy). These animals were used for assessment of cardiac function, prior to evaluation of morphological damage and changes in gene and protein expression (NKI and CARIM).

Table 1: Distribution of the animals/hearts irradiated at TUD between the partners

Partner	***C57BL/6***	***ApoE-/-***	***Total***
HELMUC	*148*	*100*	*248*
TUM	*39*	*27*	*66*
UL	*120*	*138*	*258*
MSCCI	*212*	*72*	*284*
IRSN	*114*	*84*	*198*
USFD	*125*	*110*	*235*
TUD	*160*	*160*	*320*
Additional animals	*17*	*22*	*39*
Total: 1648	***935***	***713***	***1648***

Preparation of cardiovascular endothelial cells, cardiomyocytes and tissue for functional assays (partners USFD, MSCCI, TUM)

As endothelial cells (EC) are among the putative target cells, methods were developed to extract primary EC from non-irradiated and irradiated hearts after different radiation doses (0.2-16 Gy) and different follow-up times.

Cardiac endothelial cell extraction methods were developed and optimized by three partners (USFD, MSCCI, TUM). They consistently yielded about 100.000 cells/ heart after different radiation doses and at different time points after irradiation up to 68 weeks.

Development of ex vivo angiogenesis assays (partner USFD)

Cardiac injury triggers repair processes in the myocardium that are dependent on neo-vascularisation. Angiogenesis is therefore central to cardiac repair. To determine whether radiation damage modulates the capacity of cardiac tissues to repair and re-vascularise, novel assays/approaches to determine 'angiogenic' activity of cells derived from the mouse heart at predetermined time points after in vivo irradiation were developed by USFD:

In the first approach, explants of both atrial tissue and ventricular myocardium were established in three-dimensional fibrin gels and outgrowth of endothelial sprouts from these explants were used to assess endothelial sprouting after irradiation Each heart section was dissected and ~50 tissue explants about 1 mm^3 in size were embedded in a fibrin-gel and overlaid with growth medium, incubated for 10 days and then assessed for extent of sprouting using a semi-quantitative approach. Explants with no sprouts were scored as 0. Explants with a few sprouts were scored as 1 or 2, while explants with extensive sprouting were assigned scores of 3 or 4.

In a second approach, a 'novel' fibroblast-endothelial self-assembling angiogenesis assay was developed and used to monitor cardiac endothelial cell migration, and remodeling into capillary-like structures among fibroblasts/ myofibroblasts/ pericytes. Hearts of irradiated animals were enzymatically digested into a single cell suspension, counted and equal numbers of viable cells plated in 24-well cluster plates. After 7 to 10 days, cultures were fixed and stained with lectin to visualise endothelial cells forming capillary like structures surrounded by fibroblasts/pericytes.

In a third approach, a co-culture model for CEC and cardiac fibroblast extracted from (sham-)irradiated mouse hearts was developed to investigate the influence of fibroblast on the angiogenic properties of CEC.

Fibroblasts were plated, allowed to grow for 7 - 10 days to establish post-confluent conditions. Fibroblasts were then irradiated. Endothelial cells were plated on these irradiated fibroblast beds and allowed to grow for 10 days in cultures after which cultures were fixed and stained with lectin to visualise endothelial cells forming capillary like structures.

In vivo imaging of cardiovascular function (partners TUD, NKI, CARIM)

To study cardiovascular changes in vivo over the whole follow-up period of 60 weeks within the living animals, non-invasive imaging methods were implemented (partners TUD, NKI, CARIM).

For imaging of the peripheral A. saphena, a new imaging technology was validated by TUD called optical coherence tomography (OCT) which allows structural as well as functional vessel imaging in vivo at specific time points. With OCT imaging, arterial diameter of A. saphena under normal conditions, after vasoconstriction and vasodilatation at day 1 and 3 months after irradiation with 0-16 Gy were investigated. The left A. saphena was locally irradiated with 2 to 10 Gy and investigated 3, 6, 9, and 12 months after irradiation to validate the suitability of the method for investigations of late functional changes in arteries exposed to different radiation doses. The arteria of the right leg served as an individual control. OCT after local application of saline served as a control as well. Vasodilation and vasoconstriction was pharmacologically induced. The resulting 2D-OCT-cross sections of the A. saphena are used to define the respective inner arterial diameters under the different conditions.

For imaging of the heart, SPECT-CT was validated and used as well as ultrasound imaging. With these imaging modalities cardiac blood volume, end diastolic and systolic volumes, ejection fraction and fractional shortening were measured, as relevant cardiac function parameters.

Development of Dynamic Adhesion Assay (partner QUB)

The dynamic adhesion assay was further developed by creating a sealed system that allows to carry out the whole experiment under sterile conditions. A preconditioning approach is now used under flow conditions, which leads to re-organization of the cellular cytoskeleton and pre-orientation of the cells in the direction of the flow. Significant challenges still remain with non-uniform adherence of monocytes and currently work is continuing to improve this.

Investigation of macrovascular effects

Structural changes (partners TUD, NKI)

A. carotis: Atherosclerotic changes are observed at high doses only and are mouse strain dependent

Wild type C57Bl6 mice did not develop atherosclerotic lesions within a 30-week follow-up period after carotid artery irradiation (below, at and above the bifurcation) irrespective of dose. 20% of mice developed early fatty streaks at 30 weeks after 14 Gy, but no mature atherosclerosis was observed.

Local high dose (8 and 14 Gy) irradiation to the carotid region of ApoE-/- mice resulted in more atherosclerosis and an inflammatory lesion phenotype in the long term (~30 wks follow-up). At 22-30 weeks after carotid artery irradiation of ApoE-/- mice there was a two-fold increase in the number of atherosclerotic lesions and total plaque burden after 8-14 Gy, compared with age and sex-matched controls. The majority of lesions in the irradiated carotid arteries of ApoE-/- mice irradiated with 14 Gy were granulocyte rich, with thrombotic features (iron containing macrophages, thrombin deposits, endothelial cell bands indicative of stimulated angiogenesis), whereas these features were much less common in age-matched controls or after irradiation with 8 Gy. In the short term (1-4 wks), 14 Gy also resulted in a higher incidence of fatty streaks. On the other hand, a lower dose of 2 Gy did not affect lesion size or phenotype.

Heart

There were no obvious atherosclerotic changes in after irradiation of hearts of C57Bl6 mice. However, at 20 weeks after 16 Gy to hearts of ApoE-/- mice there was foam cell accumulation in the endocardium of 17/20 hearts examined, versus 1/14 in age matched controls. There were also atherosclerotic lesions in the coronary arteries of 3/15 of the hearts irradiated with 16 Gy, compared with 1/14 in controls. Atherosclerotic changes at longer follow-up times are currently being evaluated.

A. saphena: No atherosclerotic lesions regardless of radiation dose and time of follow-up

In contrast, no atherosclerotic lesions in A. saphena of C57Bl6 or ApoE-/- mice were observed irrespective of dose and interval after irradiation. For detection of fatty streaks, fat uptake and foam cells oil red and CD45 stainings were conducted in all 10 weeks animals. Some fat vacuoles were detected in Tunica intima in four animals (two of the 2 Gy and the 16 Gy group). CD45 staining showed no monocytes in any layer of the A. saphena. Hematoxylin – Eosin staining showed no pathological changes after irradiation (e.g. edema, plaques). Circumference of A. saphena was measured in 18 animals and varied by 10 % without effect of irradiation. CD45 stainings and circumference measurements will therefore not be followed up in the main cohort.

In vivo optical imaging of vascular function (TUD)

For imaging of the peripheral A. saphena , the imaging technology called optical coherence tomography (OCT) was validated which allows structural as well as functional vessel imaging in vivo at specific time points.

Radiation does not alter the functional compliance of the arteria saphena

Independent of post-irradiation interval and dose, the diameter at maximum vasoconstriciton was about 50% of the initial control diameter. After vasodilation, the diameter increased to 1.5x the control diameter, again independent of time-interval and dose. Therefore, no radiation induced changes in functional compliance of the A. saphena were observed. Clearly higher vasodilatatory values are found for $ApoE^{-/-}$-mice at 3 months, compared to C57BL/6-mice. No leg contracture was observed.

The role of inflammatory, adhesive and thrombogenic responses (partners TUD, NKI, UL, UROS)

Investigation of A. carotis (NKI):

To investigate whether the increased incidence of fatty streaks (short term) and inflammatory lesion phenotype (long term) is caused by upregulation of inflammatory molecules directly after irradiation, expression of several inflammatory and thrombotic molecules were analyzed up to 60 weeks after irradiation using immunohistochemistry and micro-array analysis.

The following inflammatory markers were examined: P-selectin, ICAM1, VCAM1, TM, eNOS, MCP1, TF, endoglin, vWF, PAI-1. The amount (percentage area positive for signal) and intensity of the inflammatory proteins in the endothelium of the carotid arteries were quantitatively analyzed at three positions: above, around and below the bifurcation (1, 0 and -1) on the carotid arteries.

Expression pattern of inflammatory and prothrombotic factors is independent of structural changes

There was significantly less VCAM1 expression in unirradiated carotid arteries of C57Bl6 mice than in ApoE-/- mice. For the other markers, there were no base line differences between the strains.

1 day after 14 Gy irradiation CD31 expression was significantly decreased in the ApoE-/- mice. There were no significant differences in the percentage of EC expressing ICAM1, VCAM1 or TM, and no differences in TF expression in the arterial wall. 1 week after irradiation with 14 Gy, but not lower doses, the percentage of EC expressing ICAM1 was reduced in both strains of mice and VCAM1 was reduced in ApoE-/- mice only. 4 weeks after irradiation with 14 Gy the expression of TF and TM was increased in ApoE-/- mice but was reduced in C57Bl6 mice. Irradiation did not cause significant changes in expression of CD31, eNOS, MCP1 or endoglin in the carotid artery at 1 or 4 weeks. 8 Gy irradiation did not result in changes in expression of ICAM1, VCAM1, TM, TF, 1 and 4 weeks after irradiation in the C57BL6 mice.

mRNA analyses:
The first analyses have been performed on the ApoE-/- carotid arteries at 1 and 4 weeks after irradiation with 2, 8 and 14 Gy. TM and MCP1 were the only genes that were significantly upregulated at 4 weeks after 8 Gy.

Investigations of A. saphena (TUD, UL, UROS):

Sections of irradiated A. saphena were prepared for immunohistochemical analysis of thrombotic and inflammatory changes in C57/Bl6 and ApoE-/- mice 3, 6, 9, 12, 18 months after irradiation. Twelve different markers were tested for suitability and six (VCAM, E-Selectin, MCP-1, Thrombomodullin, CD31 and iCAM) were finally chosen for evaluation. Six markers were not included in the final studies (CD45, Thy-1, Alkaline phospahtase), as the Tunica intima is negative for these markers, von Willebrand factor, KC and Tissue factor stained too weak for quantitative evaluation despite of testing different antibodies and protocols.

Strain specific expression of inflammatory and prothrombotic factors without structural atherosclerotic changes

Local increases of CD31 mean fluorescence in C57/Bl6 mice 6 months after irradiation were observed. CD31 showed only local significances and a tendency to decrease 3 months after irradiation in Bl6.

ICAM1- mean fluorescence intensity showed increases at early time points (3 and 6 months Bl6; 3 months ApoE) already at intermediate doses > 2Gy, whereas after 9 months no changes were found.

In ApoE-/- mice VCAM showed a partial increase at 3 months post radiation mainly at 16 Gy. C57/Bl6 mice showed a higher and dose- dependent increase also at 3 months and additionally at 6 months. The total area of VCAM-expression showed a tendency to expand at later time points and higher doses.

In ApoE deficient mice local increases in total area of Thrombomodulin 6 and 12 months after radiation exposure were found. In C57/Bl6 fluorescence intensity partially increased 6 months post irradiation. In ApoE-/- a dose-dependent 3 months post radiation could be revealed. Irradiation with 2 Gy increased the fluorescence intensity whereas 8 and 10 Gy resulted in decreased fluorescence intensities.

MCP-1 positive area showed high variation but no significant and consistent changes. No significant effects in E-Selectin expression in both ApoE and Bl6 were detected.

Investigation of microvascular effects in irradiated heart

In vivo functional imaging of microvascular perfusion (partners NKI, CARIM)

Novel in vivo imaging methods (microSPECT/CT, ultrasound) were used for evaluation of cardiac blood volume and function. One of the tracers used (myoview) can be used to measure changes in cardiac microvascular perfusion in humans undergoing SPECT/CT. We had hoped to use this tracer quantify focal and/or overall reduction of blood perfusion in the irradiated mouse heart. However, this turned out not to be possible due to the extremely high heart beat rate in the mouse. We therefore had to rely on invasive methods (immunohiostochemistry after injection of FITC/lectin)) for evaluation of perfusion in the mouse heart .

SPECT/CT using the blood pool tracer Tc^{99m}-HSA, performed at NKI, showed that cardiac blood volume of C57Bl6 mice decreased significantly at 20 weeks after 2- 16 Gy (18-22%), with a further decrease (20-36%) at 60 weeks after 2-8 Gy.

Gated SPECT/CT using the Tc^{99m}-myoview (at NKI) demonstrated a significant reduction in end diastolic volume (EDV) and end systolic volume (ESV) and increased ejection fraction (EF) at 20 and 40 weeks after irradiation.

Ultrasound measurements (at CARIMI) on C57Bl6 mice also showed significantly decreased EDV and ESV volumes and increased EF at 20 weeks after 16 Gy, but these parameters, as measured by US, had largely normalized by 40 weeks. Cardiac function of ApoE-/- mice was measured at 20 weeks after 0 and 16 Gy, by both ultrasound and gated SPECT (myoview). Both analyses indicated very similar reductions in EDV and ESV and increased EF in ApoE-/- mice as in C57Bl6 mice at 20 weeks after irradiation.

Similar reductions in EDV and ESV were seen in mice irradiated in Dresden and assessed by ultrasound investigations (at TUD). In these studies there were no consistent changes in EF in irradiated mice, however, there was a trend for increased EF at 20 weeks after irradiation in all dose groups.

Morphometry of microvascular density (partners TUD, NKI, UL)

Three groups investigated capillary density of irradiated hearts using different methods and criteria of quantification looking at different parts of the left ventricle.

NKI:
Microvascular density (MVD) was determined in transverse frozen sections of mouse hearts (male C57Bl6 and ApoE-/-) at 20, 40, and 60 weeks after 0, 2, 8 and 16 Gy (uniform, whole heart irradiation). An anti-CD31 antibody with DAB staining was used to visualize cardiac vasculature of the central or apical part of the heart. To quantify the percentage of perfused microvessels, FITC-lectin was injected i.v. 5 minutes before tissue harvesting. For quantification of microvessels, five random fields (40x objective) were photographed and a computerized morphometry system was used to quantify MVD. Vessels beneath a size of 1.5 or above 200 μm^2 were automatically excluded from the measurements.

UL:
Apical parts of transverse frozen sections from male ApoE-/- and C57/Bl6 mice were evaluated at 20, 40 and 60 weeks after 0, 0.2, 2, 8, and 16 Gy. A fluorescent CD31 antibody was used to detect the vasculature and results are expressed as CD31+ events normalized per DAPI positive nuclei.

TUD:
Formalin fixed, paraffin-embedded sections were used to evaluate the number of microvessels per cardiomyocyte in the papillary muscle. This was based on a staining of adjacent sections with CD31/DAB, to detect endothelial cells, and laminin, to distinguish the cell boundaries of the cardiomyocytes. The number of microvessels per cardiomyocyte was counted manually, at 20 and 40 weeks after 0, 8 and 16 Gy (male ApoE-/- and C57/Bl6 mice).

Dose and time dependent decrease in MVD is accompanied by decrease in perfusion, but not hypoxia
In C57Bl6 hearts of mice irradiated at the NKI (figure 1), there was a transient increase in microvascular density in the central part of the left ventricle (16-20%) at 20 weeks after 2-8 Gy, and a trend for a decrease after 16 Gy (no significant changes in apical regions). A significant decrease in microvascular density was observed at 40 weeks after 16 Gy and 60 weeks after 8 Gy, indicative of progressive loss of microvessels after mid to high doses. In ApoE-/- mice, capillary loss occurred earlier and at lower doses (from 20 weeks after 8 Gy).

Analysis of CD31 and FITC-lectin stained sections showed that 92% of microvessels in hearts of control C57Bl6 mice were perfused at 20 weeks after treatment. There was no significant change in the number of perfused vessels after irradiation with 2 to 16 Gy. At 40 weeks after 16 Gy, the reduction in MVD was accompanied by a very small, but significant, decrease in perfusion of those vessels (84% of the remaining microvessels were perfused after 16 Gy, versus 87% in controls). In ApoE-/- mice, the reduction in MVD at 40 weeks after 8-16 Gy was also accompanied by a small reduction in the perfusion of remaining vessels (86% versus 91% in age matched controls). However, these small reductions in perfusion did not lead to any detectable hypoxia (EF5 staining).

In C57/Bl6 mice irradiated at TUD (figure 2) and analyzed at UL, there were no changes in CD31+ events, normalized per DAPI nuclei, in the apical part of hearts at 20 weeks after irradiation, but at 40-60 weeks after 8-16 Gy there were decreases in MVD (only significant at 40 weeks after 16 Gy). In ApoE-/- mice, there were no consistent changes in MVD.

The morphometric analysis of the microvascular density in the papillary musvle by TUD, however did not reveal any consistent dose or time dependent changes.

C57Bl6

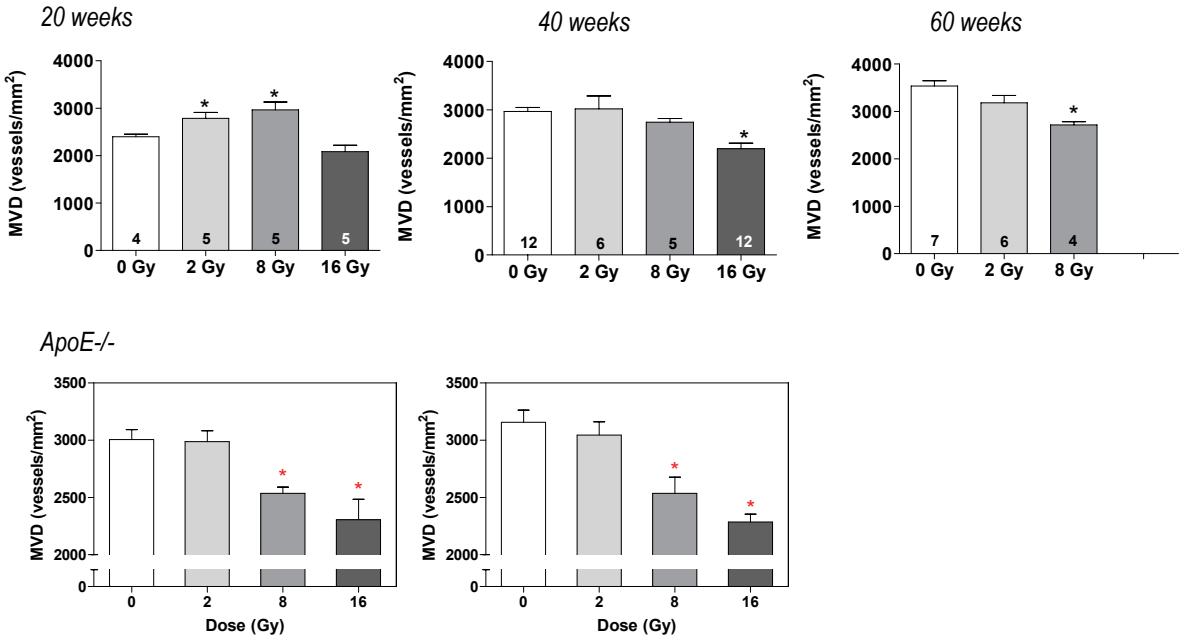

Figure 1: *Capillary density measured in central part of the left ventricle of C57Bl6 and ApoE-/- mice (irradiated at NKI). Values represent mean ± SEM, * p<0.05 compared to age-matched controls.*

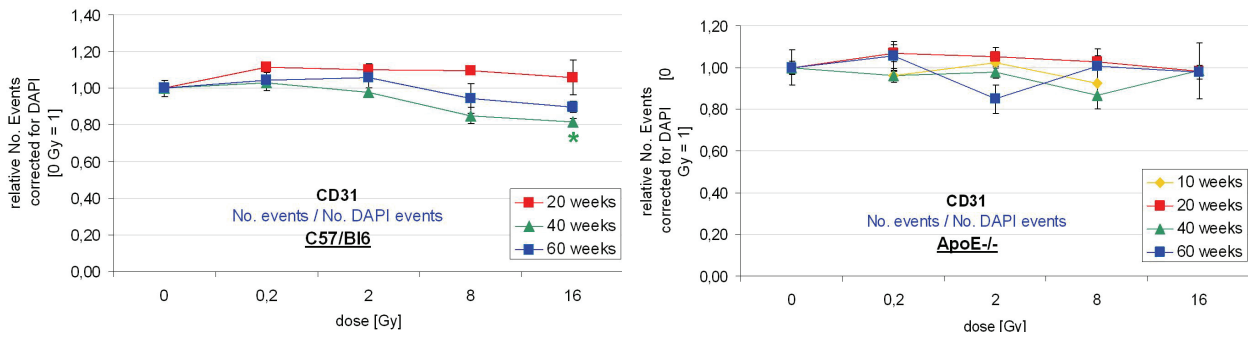

Figure 2: *Capillary density measured in apical part of the left ventricle of C57Bl6 and ApoE-/- mice (irradiated at TUD and evaluated at UL). Values represent mean ± SEM, * p<0.05 compared to age-matched controls.*

Loss of alkaline phosphatase already occurs at low radiation doses and precedes reduction in micro vessel density (partner NKI)

To investigate whether structural changes in microvascular density were associated with functional changes, changes in expression of alkaline phosphatase (ALP) von Willebrand factor (vWF) in microvessels, evidence of vascular leakage (staining for albumin) and collagen deposition in interstitial areas was investigated by NKI.

At 20-60 weeks after irradiation with 2-16 Gy, the amount of capillary staining for ALP was reduced by up to 50 % in both strains of mice, with more pronounced response in ApoE-/- mice (figure 3 and 4, mid panel).

In C57Bl6 mice (figure 3), there was a significant decrease (30-44%) in percentage tissue stained for ALP at 20 weeks after irradiation with 8-16 Gy. By 40 weeks, the 2 Gy dose group also had significantly less ALP expression, indicative of further progression of endothelial damage in small blood vessel. At 60 weeks the ALP expression in irradiated groups (2 and 8 Gy) was 50% of the mean control value, but these differences were borderline significant (p=0.05).

In ApoE-/- mice (figure 4), there was an earlier reduction in microvascular density than in C57Bl6 mice. At 20 weeks after 8-16 Gy MVD was already significantly less than in the controls and this persisted until at least 40 weeks after 8-16 Gy (longer times were not evaluated). There was also an earlier, and more pronounced, decrease in ALP expression in microvessels of irradiated ApoE-/- hearts than in C57Bl6 mice. At 20 weeks there were significant, dose related, reductions in ALP after all doses and by 40 weeks the ALP levels in vessels of irradiated hearts were only 55-36% of controls.

There was a significant increase in capillary vWF expression (prothrombotic) at 20-40 weeks after high doses (8-16 Gy) in both strains (figure 3 and 4, lower panel). This response was slightly less marked than was seen in C57Bl6 mice.

C57Bl6 mice

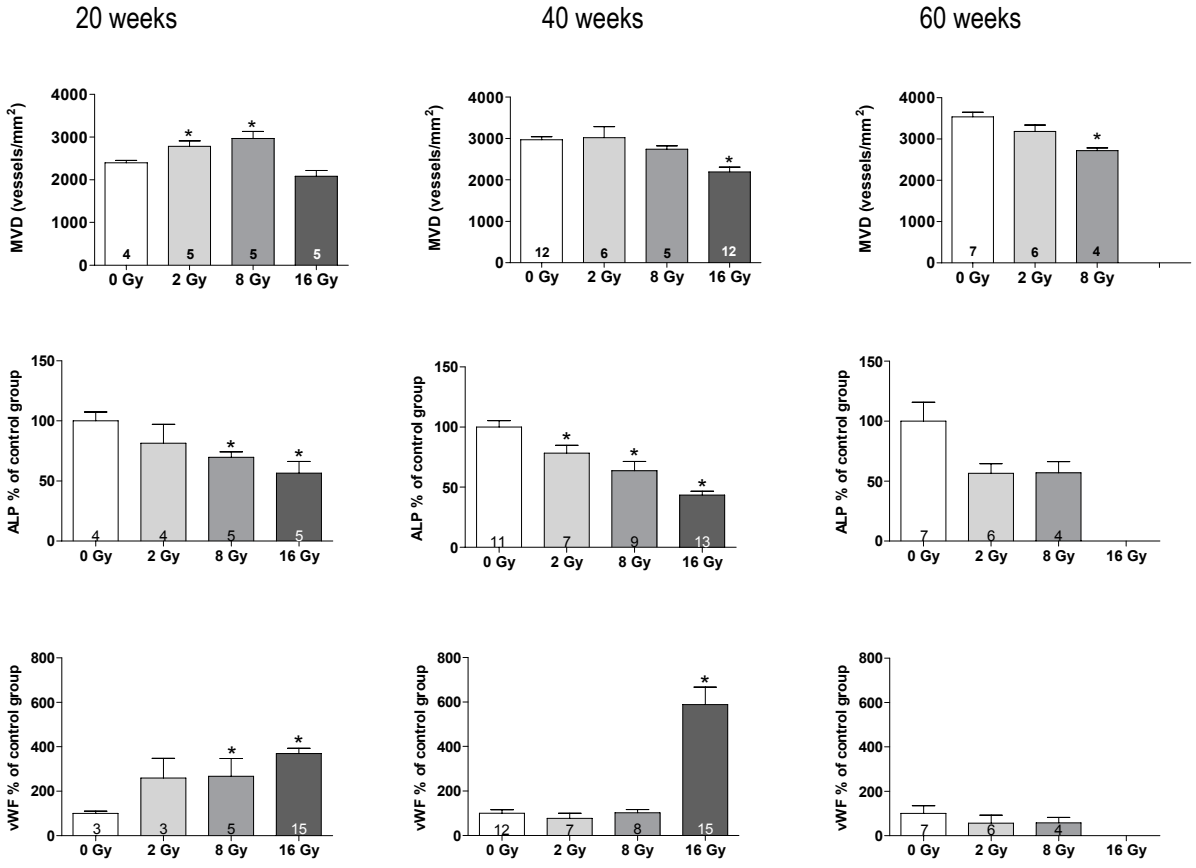

Figure 3: *Microvascular alterations in hearts of C57Bl6 mice at 20-60 weeks after irradiation. Mean ± SEM, * p<0.05 compared to age-matched control;* (upper panel) Quantification of MVD per unit area. (mid panel) ALP positive tissue areas as % of LV tissue area. (lower panel) vWF positive tissue areas as % of LV tissue area.

ApoE-/- mice

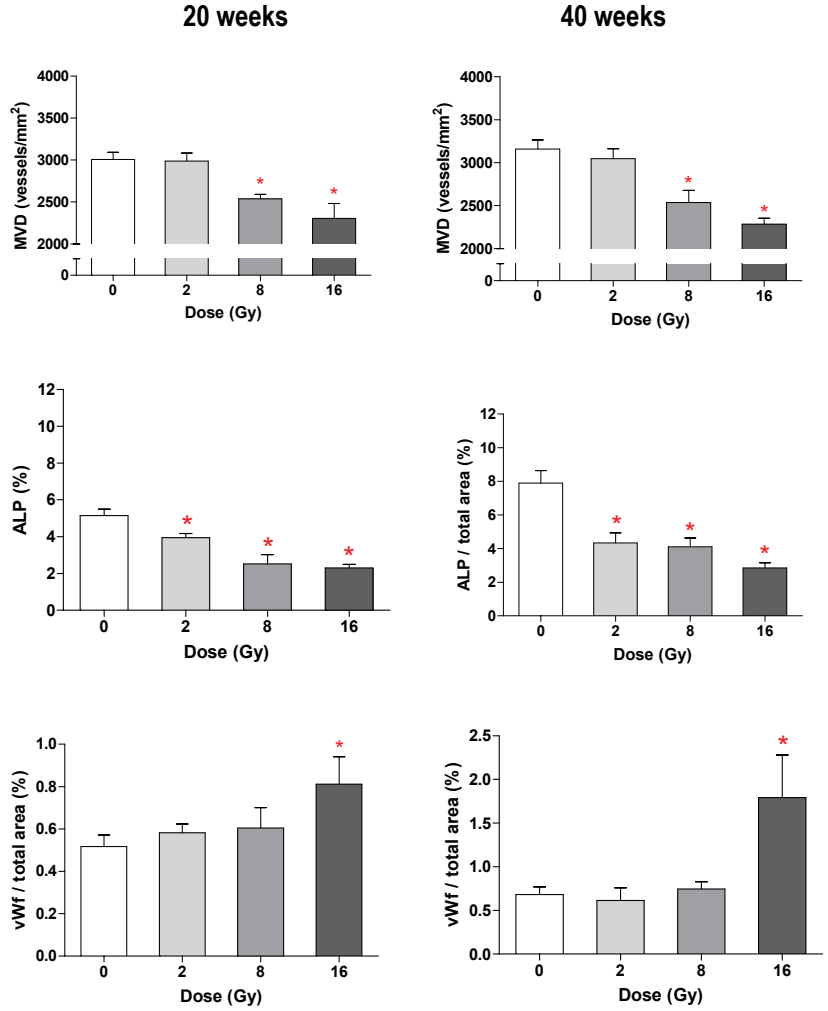

Figure 4: *Microvascular alterations in hearts of ApoE-/- mice at 20-40 weeks after irradiation. Mean ± SEM, * p<0.05 compared to age-matched control;* (upper panel) Quantification of MVD per unit area. (mid panel) ALP positive tissue areas as % of LV tissue area. (lower panel) vWF positive tissue areas as % of LV tissue area.

To investigate whether the structural and functional changes in the microvasculature were associated with vascular leakage, albumin deposition in the myocardium was examined. At 40 weeks, half of the C57Bl6 hearts irradiated with 2 Gy and almost all hearts irradiated with 8-16 Gy showed albumin in the myocardium; this was absent or very mild in controls. After 16 Gy, myocardial albumin was extensive in 5 of 11 hearts and all these animals also had diffuse amyloidosis, which was confirmed with a Congo red staining. Amyloidosis was not seen in the irradiated ApoE-/- hearts.

Structural effects:

Epicardial thickness more than doubled at 20 weeks after 8-16 Gy and remained increased at 40 weeks. This was associated with the presence of iron containing macrophages (indicative of previous haemorrhage).

Increased interstitial collagen deposition (indicative of fibrosis) was present in the myocardium at 40 weeks after 8-16 Gy and at 60 weeks after 2-8 Gy. In ApoE-/- hearts, these changes were seen earlier and at lower doses (20 weeks after 8-16 Gy, 40 weeks after 0.2-2 Gy.

Electron microscopy studies showed dilated mitochondria with lysis of the cristae in cardiomyocytes at 10 weeks after 8 Gy. Myofibrils showed focal lysis and disrupted structure and endothelial cells were dilated.

No relevant structural disturbance were seen at 2 Gy or lower doses.

At 40 weeks, half of the hearts irradiated with 2 Gy and almost all hearts irradiated with 8-16 Gy showed extravascular albumin in the myocardium (Table 2); this was absent or mild in controls. After 16 Gy, myocardial albumin was extensive in 5 of 11 hearts and all these animals also had diffuse amyloidosis. The remaining 6 animals from this group exhibited a more focal amyloidosis.

Table 2: *Incidence albumin deposition in myocardium at 40 weeks*

	Mild	*Strong*	*Any deposition*
0 Gv	*1/5*	*0/5*	*1/5*
2 Gv	*4/8*	*0/8*	*4/8*
8 Gyy	*8/8**	*0/8*	*8/8**
16 Gv	*5/11*	*5/11*	*10/11*#*

** p<0.05 compared to age-matched controls. # hearts with strong albumin deposition also had diffuse amyloidosis.*

There was an increase in interstitial collagen deposition from 40 weeks after 8-16 Gy and 60 weeks after 2-8 Gy in C57Bl6 mice (figure 5). In ApoE-/- mice this collagen deposition occurred earlier (from 20 weeks).

C57Bl6

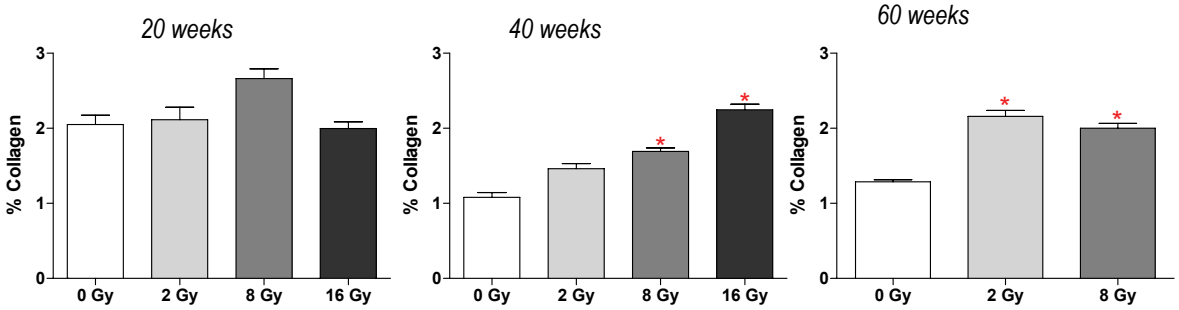

*Figure 5: Quantification of interstitial collagen of left ventricle of C57Bl6 at 20, 40 and 60 weeks after irradiation. Values represent mean ± SEM, * p<0.05 compared to age-matched controls.*

Stress response and immune function in irradiated hearts (partners TUM, MSCCI, HELMUC)

Membrane Hsp70 expression only predicts short term radiation-induced stress response (TUM)

The cell surface expression and secretion of hsp70 on mouse heart ECs was analyzed by determining membrane Hsp70 expression density on viable mouse heart ECs. A maximum membrane expression of Hsp70 was observed already at 8 weeks after irradiation which then normalised at 10 weeks. Reduced cell surface expression levels were observed thereafter until 60 weeks after irradiation.

Serum Hsp70 expression indicates long-term persistence of radiation-induced stress response (TUM)

Hsp70 protein levels were determined in the serum of mice after punction of the heart. Serum analysis of Hsp70 protein levels after irradiation revealed a significant increase only 60 weeks after irradiation (figure 6).

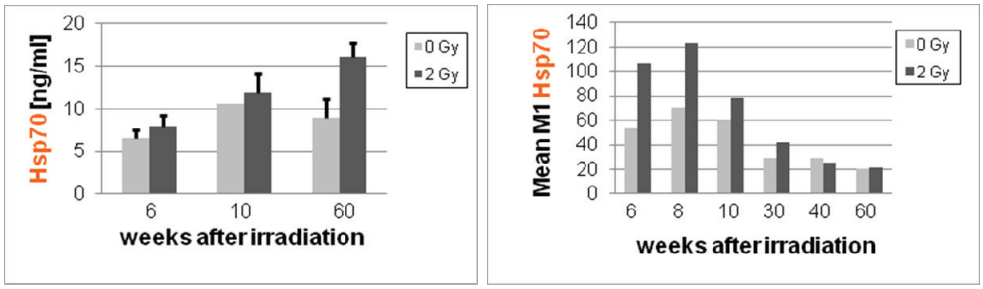

Figure 6: *Hsp70 expression in serum (left panel) and endothelial cells (right panel) after irradiation*

Hsp70 expression in whole heart preparations (MSCCI)

Changes in the expression of the major stress-responsive gene *Hsp70i* were assessed in both cardiac endothelial cells and in a whole heart tissue up to 40 weeks after irradiation with 0.2, 2, 8 and 16 Gy doses. Mice treated with heat shock were treated as a positive control.

Gene expression analysis (MSCCI)

Cardiac endothelial cells (CEC) isolated from juvenile mice were irradiated in vitro with 2 and 8 Gy, and then 24 hours after the exposure cells were collected and RNA isolated. In addition, animals were irradiated in vivo with 2 and 8 Gy and CECs were isolated 12 and 36 hours, 6 days, 20 and 40 weeks after the exposure, and then RNAs were purified directly after the cell isolation.

Only at 8 Gy up-regulation of VCAM1 gene was observed early after radiation exposure.

At 20 and 40 weeks of follow-up no significant difference between irradiated and sham-irradiated animals for VCAM1, E-Selectin, Bnip and Glut1 (hypoxia-related genes) expression was observed.

Radiation caused up-regulation of Hsp70i gene expression, which was visible in a dose-dependent manner within 24 hours after irradiation in vitro and in vivo in CEC isolated from irradiated animals at 20 and 40 weeks after the exposure.

Proteom analysis in in endothelial cells in vitro (HELMUC)

The human endothelial cell line EA.hy926 and the human coronary artery endothelial cell line (HCAEC) were irradiated in vitro with 0.2 Gy gamma (Co-60) and analysed for proteomic changes 4h and 24h later. In Ea.hy926, 15 significantly differentially expressed proteins were identified, of which 10 were up-regulated and 5 down-regulated, with more than ± 1.5-fold difference compared to unexposed cells. Pathways influenced by the low-dose exposure included the Ran and RhoA pathways, fatty acid metabolism, and stress response. Radiation of 0.2 Gy and 1 Gy did not cause phosphorylation changes in the proteome of EA.hy926 cells when examined 10 min, 30 min, or 4 h later using the total cell lysate or cytosolic fraction. In HCAEC, the total number of deregulated spots after 4 h and 24 h was 29 and 26, respectively. The identification of the proteins represented as deregulated spots by ESI LC-MS/MS elucidated several pathways affected by radiation including cell death, cell morphology, RhoA signalling and actin-based motility by Rho.

Inflammatory response and thrombotic changes in irradiated hearts (partners CARIM, NKI, UL, UROS, IRSN)

ApoE-/- and C57/Bl6 mice sacrificed 20, 40 and 60 weeks after irradiation with 0, 0.2, 2, 8, and 16 Gy (8 animals per dose) were analyzed for CD31, thrombomodulin, VCAM-endocard and CD45.

Increased mean area of CD31 staining 20 and 40 weeks post irradiation may indicate endothelial swelling at high doses (8 and 16 Gy) and was found in ApoE-/- mice only. Similarly CD31 fluorescence intensity increased in ApoE-/- mice only, indicating an increased extravasation potential already after irradiation at 2 Gy. At high doses (8, 16 Gy) a time depend immigration of leukocytes was detected, by increase of CD45 positive cells, in both endocardium and myocardium at 40 weeks post irradiation. This behaviour is not due to the onset of spontaneous atherosclerosis in the ApoE-/- genotype because it was found a similar reaction in the C57/Bl6 wild type model. Also VCAM expression on the endocard showed a trend to increase after irradiation at 16 Gy at all time points in both mice strains. Changes in all four markers (CD31, Thrombomodulin, VCAM-endocard and CD45) indicate a proinflammatory response mostly at high doses (8 and 16 Gy). At low dose (0.2 Gy) only small changes are seen at a few time points with no significances.

A reduction of Thrombomodulin (TM) positive events was found 20 and 40 weeks (C57/Bl6), and 60 weeks (ApoE-/-) after irradiation at 16 Gy. TM exhibits anti-inflammatory, antithrombotic properties, partly by being a sink for thrombin but also by reducing the expression of adhesion molecules. Reduction of TM positive capillaries might indicate a proinflammatory response in the background of unchanged overall capillary count (see CD31 results).

The ICAM-1 staining was weak and did not appear to be influenced by irradiation.

Analyses of inflammatory gene expression

Microrarray data are only available for C57 Bl6 mice at present. There were no large changes in inflammatory gene expression at 20 weeks, but VCAM1, ICAM 1 and KFL2 were slightly increased at 20 weeks after 2 Gy. Pathway analysis scored high for cell-to-cell signaling pathway including CXCL1, S100A8 and S100A9. Gene expression data at 20 weeks after 16 Gy showed modest increases in endoglin and decreases in VCAM1. Pathway analysis scored high in cell death/development disorder-pathways, including MYL4, myosin, CTGF, PAI-1, TIMP3 and MMP.

By 40 weeks after 2 Gy, the expression levels of PAI-1, TM, ICAM1 and KLF2 were increased and pathway analysis scored high for cellular movement-immune cell trafficking pathway and molecular transport/cell cycle – pathway. These pathways include upregulation of LYZ, CCL2 and CCL4. Gene expression data from 16 Gy showed increases in Ki67, PAI-1, P-selectin and endothelin and significant down-regulation of Smad5 and PDGFb. Pathway analysis indicate high scores for cell movement/immune cell trafficking pathway including SPP1, CXCL13 and ESM1.

Using real-time PCR expression of the inflammatory genes (VCAM-1, MCP-1, E-Selectin) was investigated. Neither samples of ApoE-/- mice sacrificed 20 weeks after irradiation nor mice sacrificed 60 weeks after irradiation showed any change in expression of inflammatory marker gene VCAM or E-selectin normalized to reference genes GAPDH and ß-actin. Due to very few native material MCP-1 could not be amplified for any of the samples.

Functional integrity

Studies in Endothelial cells (partners USFD, MSCCI, QUB)

Experiments at USFD focused on analyzing angiogenic responses in the heart tissue following irradiation with the aim to determine whether radiation damage modulates the capacity of cardiac tissues to repair and re-vascularise. Since isolation and subculture of enough endothelial cells from older mice proved challenging, alternative assays/approaches to determine 'angiogenic' activity of cells derived from the mouse heart were developed described in page 6.

The results demonstrate that radiation decreases the capacity of reparative angiogenesis in heart tissues. Severe impairment of this process was statistically significant at high radiation doses while a trend for a reduced angiogenic activity is evident at moderate to low doses (C57Bl6 mice: figure 7; ApoE-/- mice: figure 8). Radiation at high doses (8 and 16 Gy) inhibited capillary-like tube formation in ApoE-/- mouse heart cultures at 20 weeks post-irradiation. Capillary-like structures were affected both qualitatively and quantitatively. In particular, in the irradiated groups these were less prominent than the controls and were characteristically shorter and narrower, fewer in numbers and had fewer branching points. There were no significant differences in the total number of viable cells initially extracted from irradiated and non-irradiated hearts. However, once in culture, cells from the 8 and 16 Gy irradiated groups showed a reduction in growth and viability correlating with the profound inhibition of capillary-like network formation seen at these radiation doses. A similar response was seen in C57BL/6 hearts at 20 and 40 weeks post-irradiation.

A dose dependent reduction in 'angiogenic index' was observed 20 weeks post-irradiation. A similar pattern was observed at 40 and 60 weeks post irradiation. Statistical significance was achieved only for the 8 and 16 Gy dose groups.

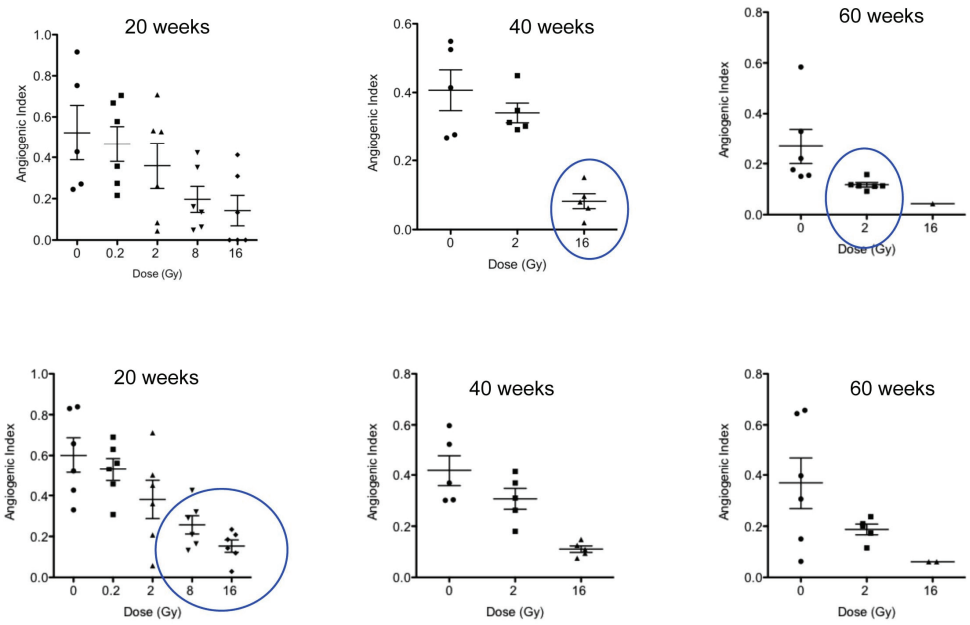

Figure 7: *Semi-quantitative analysis of sprouting of C57BL/6 mouse heart explants. Top row, ventricular explants; lower row, atrial explants. Data that are statistically significant are denoted by a blue circle (partner USFD).*

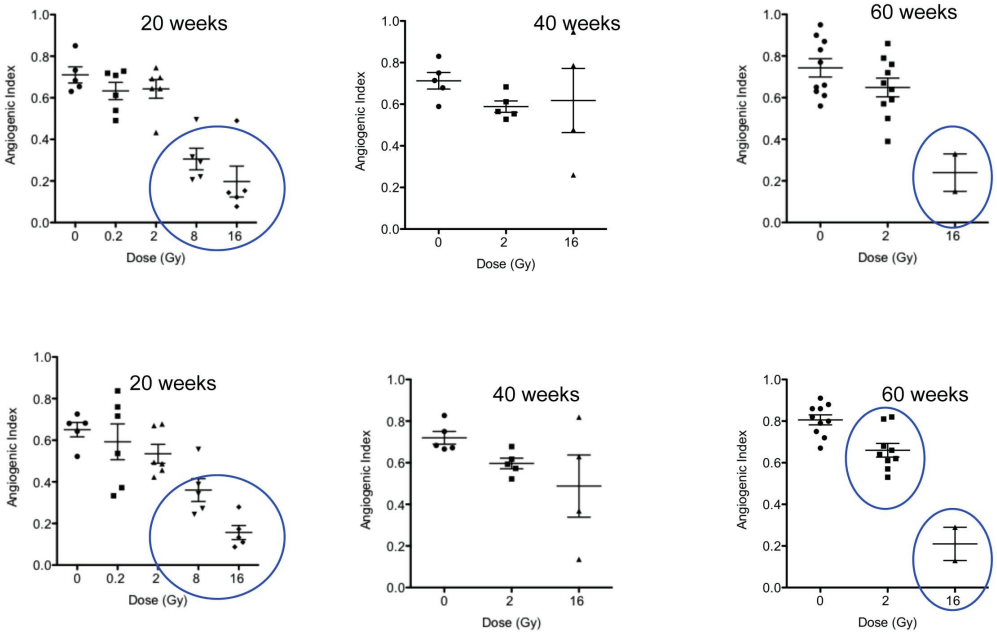

Figure 8: *Semi-quantitative analysis of sprouting of ApoE-/- mouse heart explants. Top row, ventricular explants; lower row, atrial explants. Data that are statistically significant are denoted by a blue circle (partner USFD).*

Work at MSCCI focused on analyzing irradiation-induced stress responses in cardiac endothelial cells (CEC). These included analysis of expression of stress-response genes described above as well the analysis of cytosceletal structures (figure 9).

Analysis of cytoskeletal structures (actin stress fibres) was analysed in CEC isolated from animals irradiated with 2 and 8 Gy, 20 weeks after irradiation (cells isolated 40 weeks after irradiation were not suitable for analysis). Stress fibre analysis was also performed on CECs isolated from animals irradiated in Gliwice at shorter times after irradiation and on CECs from juvenile animals irradiated in vitro. Filamentous structures of actin were detected upon staining with fluorescently-labeled phalloidin.

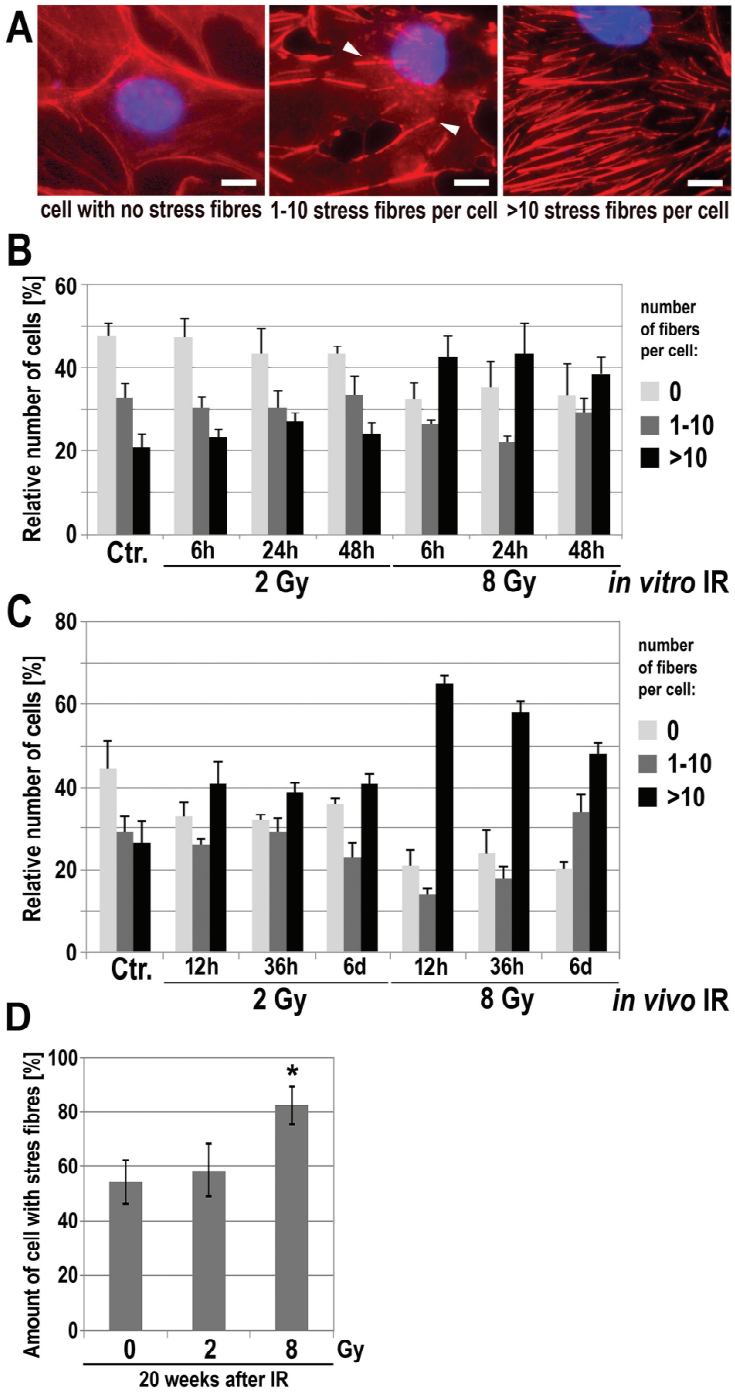

Figure 9: *Radiation-induced stress fibers in cardiac endothelial cells (partner MSCCI). (A) – Examples of cells with different number of actin stress fibers (marked with arrowheads). B) – CECs isolated from juvenile mice, exposed in vitro to 2 or 8 Gy and analysed at 6, 24 and 48 hours after irradiation. (C) – CECs isolated from 8-weeks-old mice 14 and 36 hours, and 6 days after animal irradiation with 2 and 8 Gy. (D) – CECs isolated from irradiated mice 20 weeks after the exposure. Asterisks refers to* $p<0.05$.

Significant changes were observed in CECs irradiated in vitro with 8 Gy. Mice were irradiated with 2 Gy and 8 Gy, and at 12 hours, 36 hours, 6 days and 20 weeks after irradiation endothelial cells were extracted. Irradiation with 2 Gy increased the number of cells with high number of stress fibres (to about 40%); such changes persisted for 6 days after exposure but were not seen after 20 weeks. Irradiation with 8 Gy resulted in a highly significant increase in numbers of stress fibres. We conclude that radiation induced dose-dependent changes in actin cytoskeleton (i.e., formation of contractile stress fibres) in cardiac endothelial cells irradiated in vivo and in vitro.

Irradiation with 16Gy did not increased permeability of monolayers formed by CECs to dextran (MW 40,000 Da), neither 3 nor 24 hours after exposure. Therefore, this part of the experimental program was not pursued further.

Work at QUB focused on studying intercellular communication in endothelial cell cultures. Originally, freshly isolated endothelial cells were to be used, but this proved difficult, therefore QUB used established mouse endothelial cell lines for these studies. Previously, the characterisation of various endothelial cell lines was completed in collaboration with UL and the decision was made to consolidate work using a mouse cardiac endothelial cell line H5V thereafter shared by all members of the consortium working on endothelial cell lines. Other mouse endothelial lines used included mlEDN1 (Lymph node large vessel); bEND3 (brain microvascular); H5V (Heart microvascular) and previously MHEC5-T (heart microvascular) and SVEC4-10 (Lymph node large vessel). In particular, QUB focused on the characterisation of the cytokine profile of endothelial cell lines after irradiation at 0.1 and 2 Gy both in the presence and absence of TNFα. The aim was to understand mechanisms related to the focal nature of cardiovascular disease associated with response of endothelial cells to low and targeted doses of radiation.

In addition, the impact of irradiated monocytes on adhesion properties was determined and changes in adhesion properties, both static and dynamic, were evaluated along with cytokine changes in endothelial cell monolayers. This was extended to situations where cells are irradiated both directly and under bystander conditions.

The brain derived bEND.3 cells responded to irradiation with doses between 0.05 Gy and 5 Gy with a significant increase in adhesion properties under both static and dynamic conditions. Statistically significant changes in adhesion properties were not observed in the other two cells lines While pro-inflammatory effects of radiation were observed only in bEND.3 cells, in all three cell lines evidence for radiation-induced anti-inflammatory effect 24h after irradiation of pre-activated with TNFα cells was seen. Analysis of inflammatory cytokine expression profile of H5V, bEND.3 and mlEND1 cells suggests that radiation induced pro-inflammatory response can occur in all three endothelial cell lines as early as 30 minutes after irradiation in both microvascular and lymph node derived endothelial cells. Elevated levels of MCP-1 and decreased expression of TGFbeta persisted for more than 24 hours in pre-activated cells. An anti-inflammatory effect of radiation on pre-activated endothelial cells was also observed as early as 30 min after irradiation.

X-ray exposure with partially shielded cultures indicates a role for bystander signalling for both cytokine expression and monocyte adhesion. This is observed at 0.1 and 2 Gy and suggests intercellular communication plays an important role.

Antibody blocking cell surface adhesion molecules (ICAM-1, VCAM-1 and E-sel), led to significant decrease in monocyte adhesion in mlEND1 and bEND.3 cells with the effects being more pronounced in pre-stimulated cells.

The important role of the Nf$\kappa\beta$ pathway in radiation-induced inflammation was confirmed by adding to the media Bay11-7085 prior to treatment with radiation or TNFα, which led to a significant decrease in adhesion (results not shown).

Irradiation of the monocyte population with low and moderate doses (0.1 Gy and 2 Gy) causes decrease in the ability of monocytes to adhere to endothelial cells, suggesting potential radiation-induced anti-inflammatory effect. The effect was observed in all three cell lines – bEND.3, mlEND1 and H5V.

Studies in heart tissue and cardiomyocytes (partners HELMUC, IRSN)

Mitochondrial proteom and function (HELMUC)

The radiation-induced in vivo effects on cardiac mitochondrial proteome and function were investigated at 20 and 40 weeks after local irradiation of the heart. Myocardial mitochondria were isolated from whole heart tissue and tested for proteomic and functional alterations. The proteomic analysis was done using peptide (ICPL) and protein quantification (2D-DIGE).

Altogether 18 proteins were found deregulated 20 weeks after 2 Gy-irradiation, of which 12 were up-regulated and 6 downregulated. Deregulated proteins belonged to OXPHOS complexes, carbohydrate metabolism or mitochondria-associated cytoskeleton.

After 40 weeks, 49 proteins were deregulated in C57BL/6 cardiac mitochondria. Of these 12 were upregulated and 37 were downregulated. At 40 weeks, ApoE-/- cardiac mitochondria showed deregulation of 23 proteins of which 8 were upregulated and 15 downregulated. In both strains, the majority of the deregulated proteins belong to OXPHOS complexes or structural proteins. 12 deregulated proteins were shared between C57BL/6 and ApoE-/-

Generally, protein expression changes in C57BL/6 were most severe in the energy producing respiratory chain and structural proteins, whereas most changes in ApoE-/- were seen in lipid metabolism.

Mitochondrial respiration was measured using succinate as the substrate to analyse the intactness of mitochondria and to determine the efficiency of oxygen consumption. Succinate–stimulated respiration decreased significantly in mitochondria from mouse hearts irradiated with 2 Gy after 20 w and 40 w, respectively. No significant alteration was found in the succinate-driven respiration after irradiation with 0.2 Gy of C57BL/6 mice or with any dose or time point in ApoE-/- mutant. No changes in mitochondrial membrane potential were registered in mitochondria isolated from either C57BL/6 or ApoE-/- at 40 weeks using different stimuli.

RhoROCK pathway (IRSN)

Acute radiation effects on primary cardiomyocytes isolated from 12-week old C57BL6 and irradiated in vitro (0; 0.2; 8; 16 Gy) and long-term radiation effects in primary CM isolated from sham-irradiated and irradiated C57BL6 and ApoE-/- mice at 0.2, 2 and 16 Gy; 20, 40 and 60 weeks post-irradiation were performed.

Investigation of acute and delayed radiation-induced Rho/ROCK and Smad pathway activation along with remodelling of the actin cytoskeleton were performed in isolated CM using biochemical assays and immunohistochemistry. The activation of the Rho/ROCK pathway by monitoring Rho protein isoprenylation by immunoblotting. ROCK kinase was assessed by monitoring phosphorylation of its target myosin-light chain (MLC) and upregulation of CTGF, a known downstream target protein of the pathway. CTGF is also a target of the TGF-b pathway together with PAI-1. Activation of the following members of the TGF-b cascade was studied: TGF-b1, TGF-b RII, Smad2/3/4, PAI-1. Immunostaining was performed to assess radiation-induced remodelling of the alpha-actin-sarcomeric that constitutes the main cytoskeletal component of cardiomyocytes. The pharmacological inhibitor of ROCK, Y-27632, was used to modulate the phenotypical characteristics of CM isolated from animals 20 and 40 weeks post-irradiation. Pharmacological experiments (with Y-27632) could not be performed 60 weeks post-irradiation due to the fragility of the CM isolated at this time-point. In addition, planned knock down siRNA approaches were withdrawn due to the small yield of CM isolated from irradiated animals and to their fragility.

Radiation-induced sequential activation of Smad and Rho pathways and cytoskeletal remodelling in primary cardiomyocytes (IRSN)

The balance between TGF-b1/Smad and Rho/ROCK signalling in response to low and high doses of irradiation was investigated in CM, 4 h and 24 h post-irradiation. Irradiation at 0.2, 2 and 16 Gy induced early phosphorylation of Smad2/3 and a dose dependent increased expression of Smad4, indicative of Smad pathway activation 4 h post-irradiation. This was followed by radiation-induced increase of the isoprenylated Rho 24 h after irradiation, indicative of Rho activation. A trend for Rho activation was observed after exposure at 0.2 Gy but only reached significance for doses above 2 Gy. Consistently, a subsequent production of CTGF occurred 24h after exposure of CM to 16 Gy. Immunofluorescence studies showed a radiation-induced remodelling of the central network of α-sarcomeric actin, which is the main structural protein of cardiac muscle. Actin remodelling was observed after low dose (0.2 Gy), a dose at which Rho proteins were not significantly activated suggesting involvement of additional mechanisms. Such alteration of actin network was also visible in irradiated CM at high dose as early as 4 h after irradiation and persisted at 24h, consistent with the alteration of Rho isoprenylation observed after exposure to high doses.

Rho/ROCK pathway is involved in control of actin remodelling in primary cardiomyocytes (IRSN)

Actin remodelling is thought to depend upon modulation of the Rho/ROCK pathway. To confirm this assessment primary CM were exposed to the pharmacological inhibitor of ROCK, Y-27632. First, the inhibition of ROCK kinase activity upon Y-27632 incubation was monitored by studying myosin light chain phosphorylation (MLC). MLC phosphorylation decreased significantly 1 h after exposure to Y-27632 and was sustained at, 4, 6 and 1h after exposure. Consistently, alteration of the actin network associated with striation disappearance (Small Square) was obvious 4 h after exposure to Y-27632 and persisted at 6 and 16 h.

Primary cardiomyocytes can be isolated from irradiated mice (IRSN)

In order to investigate the TGF-beta1/Smad and Rho/ROCK signalling pathway as a late effect in irradiated cardiomyocytes in vivo, the isolation of CM from irradiated mice was optimized. CM was successfully isolated from non-irradiated and irradiated C57Bl6 and ApoE -/- mice 20, 40 and 60 weeks post-irradiation. 20 weeks post-irradiation, the yield, quality and shape of CM was good with good adhesion capability indicative of cell's quality. It allowed sub-culturing and pharmacological modulation. 40 and 60 weeks post-irradiation, quality and shape of CM

was insufficient to provide good adhesion and perform long-term culture, therefore cells were lysed immediately after isolation.

Modulation of the TGF-ß cascade (IRSN)

In CM no alteration of the TGF-ß cascade was observed 20 weeks post-irradiation, regulations occurred at later time point. Immunoblot analysis showed a global activation of the TGF-ß cascade in CM isolated from C57BL6 that mainly occurred at late time points. TGF-ß increased 40 and 60 weeks post-irradiation in animals irradiated with 0.2 Gy, 2Gy and 16 Gy (40 weeks only available). Interestingly, Smad 2/3 and smad4 inductions were dose dependant 40 weeks post-irradiation. 60 week post-irradiation Smad 2/3 level remained high whereas Smad 4 dropped. Finally, the two TGF-ß targets studied (CTGF and PAI-1) were only moderately stimulated, mostly 40 weeks post-irradiation. Similarly to what was observed in cardiac tissue, Smad7 expression dropped 40 and 60 weeks post-irradiation. Western-blot analysis showed increased TGF-ß1 levels in CM isolated from ApoE -/- irradiated at low and intermediate doses. However, the protein level of canonical members of the TGF-ß cascade, Smad 2/3 remained stable. A trend to induction was observed for Smad4. CTGF expression was not modulated at 0,2 Gy whereas PAI-1 significantly picked at this dose and doses above. At higher doses, CTGF protein level moderately but significantly increased. Smad7 expression dropped 60 weeks post-irradiation.

In conclusion, the results show that cardiomyocyte physiology and molecular responses are altered even by low radiation doses. Differences were found between CM responses between irradiation in vitro or after cell isolation from the irradiated tissue. PAI-1 activation was observed in the both mouse strains suggesting that the TGF-ß/Smad/PAI-1 cascade operated in fibrogenesis. Yet amyloidosis development which was observed in C57Bl6 but not in ApoE deficient mice, was dependent of ApoE-/-, and the activation of another mechanism must be considered. The current results show that the molecular imprint at low dose is different from that obtained at moderate/high dose. Activation of the Smad2/3 pathway occurs immediately after irradiation but neither Rho pathway nor CTGF were activated after exposure at 0.2Gy. The TGF-ß cascade is differentially activated in both mouse strains. Smad 2/3/4 seemed to operate in C57Bl6 whereas other protein seemed involved in ApoE. This difference may explain the differential pathogenic picture observed in histology. However, the Rho pathway was not found activated in CM despite immunohistochemical pattern.

Overall summary and conclusion

The CARDIORISK project addressed both macrovascular and cardiac microvascular radiation damage after local irradiation with low, intermediate and high doses.

Various experimental in vitro and in vivo methods and models were developed to describe and quantify the effects of ionising radiation occurring at low doses. Small animal high precision irradiation set-ups were deployed to reproducibly irradiate either the heart, the arteria carotis with the aortic arch or the peripheral arteria saphena. Irradiation consisted of doses ranging from 0.2 to 16 Gy. High radiation doses have been explicitly included to serve as a reference at which structural and clinical damage can be expected and to investigate whether different mechanism of action are involved at low vs. high radiation doses.

As the animals, heart tissue or cells have been centrally prepared and provided to all members of the research consortium, results obtained in this research programme apply to identical biological material, treated identically with radiation. Molecular and cellular responses at different times can thus be related directly to histopathological and functional changes of the irradiated cardiac microvasculature or the irradiated arteries.

As radiation-induced cardiovascular disease represents a late occurring event, animals were followed up to 60 weeks and investigated at different time points after irradiation (for late effects at 20, 40 and 60 weeks).

Macrovascular effects

Arteria carotis (partners NKI, CARIM)

In C57Bl6 mice even high doses of radiation did not result in mature atherosclerotic lesions up to 30 weeks after treatment, although small fatty streaks were seen in the carotid arteries of a few mice. High doses of irradiation (cardiorisk defined moderate doses as 0.5 to 5 Gy) (8-14 Gy) accelerated the development of atherosclerosis in

ApoE-/- mice (atherosclerosis prone). There is therefore an interaction between high cholesterol levels (ApoE-/- mice) and irradiation with respect to the accelerated development of atherosclerosis. Low and moderate dose irradiation (2 Gy) did not accelerate the development of atherosclerosis in either C57Bl6 and ApoE-/- strain.

There does not appear to be a direct link between early changes in expression of the inflammatory/thrombotic markers examined so far and the development of radiatio- induced atherosclerosis in the carotid artery. High-dose irradiation led to a reduction in inflammatory markers ICAM1 and VCAM1, whereas these markers were associated with the initiation of age-related atherosclerosis. High-dose irradiation caused an increase in prothrombotic tissue factor (TF) in atherosclerosis-prone ApoE-/- mice, but this was counterbalanced by an increase in expression of anti-thrombotic Thrombomodulin (TM). In the irradiated C57Bl6 mice (which do not develop atherosclerosis) the reverse pattern was seen: decreased expression of both TF and TM.

Arteria saphena (TUD)

No atherosclerotic lesions in A. saphena of C57Bl6 or ApoE-/- mice were observed irrespective of dose and interval after irradiation. The analyses of proinflammatory/ prothombotic marker expression in A. saphena of C57/Bl6 and ApoE-/- mice showed strain specific differences. C57/Bl6 wild type mice tended to have lower basal levels in expression of CD31, ICAM, VCAM and MCP1. ApoE-/- mice instead showed a higher basal level of inflammatory/adhesion molecules. Surprisingly the C57BL6 mice showed stronger changes in expression after irradiation, i.e. VCAM, MCP-1 and iCAM. It was expected that the inflammation-prone ApoE-/-mice would react stronger to the irradiation challenge.

On the functional level as investigated by OCT imaging, irradiation did not affect compliance (vasoconstriction or vasodilatation) of the A. saphena (at all irradiation levels or timepoints).

These results point to a site-specific response to irradiation. Thus, in the larger conduit arteries only the high dose accelerated atherosclerosis, which could not be explained by changes in a panel of well-known pro-inflammatory or pro-thrombotic factors. In the A. saphena however, irradiation did increase expression of pro-inflammatory markers yet did not result in vascular disease or functional changes. The results suggest that either there are other inflammatory/ thrombotic mediators of radiation-induced atherosclerosis, or that other mechanisms are involved in the conversion of age-related atherosclerosis in ApoE-/- mice towards an inflammatory phenotype.

With regard to the site specifity, different flow and arterial blood pressure conditions in the a. carotis compared to the laminar flow in the a .saphena may significantly influence the development of atherosclerotic plaques, as these plaques predominantely develop at and around the biufurcation of the A. carotis. This is a radiation independent phenomenom and stresses the notion that radiationi per se does not induce atherosclerosis, especially at low radiation doses, unless another permissive factor/ event is present.

Microvascular effects (partners, NKI, CARIM, TUD, UL, MSCCI, HELMUC, TU, UROS, IRSN)

Functional effects (NKI, CARIM):

A dose- and time-dependent significant reduction in end diastolic volume and end systolic volume and increased ejection fraction could be demonstrated using two independent imaging methods: SPECT-CT and ultrasound imaging, Despite the consistent changes in the geometry of the heart after irradiation, and the less consistent changes in ejection fraction, the cardiac function of irradiated mice remained within acceptable normal limits during the follow-up of 60 weeks.

Microvessel density and perfusion (partners NKI, UL, TUD):

A dose dependent decrease in MVD from 40 weeks after irradiation with 8-16 Gy in C57Bl6 mice was observed in the mid and apical parts of the heart but not in the papillary muscles. In ApoE-/- mice, decreases in MVD in the mid section of the heart occurred earlier, and at lower doses, than in C57Bl6 mice. The results for the C57Bl6 at 60 weeks and the ApoE-/- mice at 40 weeks are compatible with a linear dose dependence, even though the effects at the 0.2 Gy and 2 Gy dose points are not significantly different from the controls. Decreased MVD after high doses was accompanied by a very small reduction in the percentage of remaining vessels that were perfused, but this was never less than 84% and did not lead to tissue hypoxia.

NKI observed, in both mouse strains, reductions in alkaline phosphatase expression in endothelial cells of irradiated hearts occurring earlier and at lower doses than reductions in microvascular density

Reduced levels of alkaline phosphatise (ALP) seem to be an early indicator of EC damage, preceding EC death. These microvascular changes (MVD and ALP decreases) were more severe in ApoE-/- mice than C57Bl6 mice, in that they occurred earlier and after lower doses. At 20 weeks after low doses in C57Bl6 mice, there was a transient increase in MVD, despite falling levels of alkaline phosphatase. This was interpreted as indicating angiogenesis in damaged hearts at early times after low to moderate doses. After higher doses the angiogenic response is unable to keep pace with EC loss. An increased expression of pro-thrombotic vWF and pro-inflammatory VCAM-1 was observed in remaining microvessels after cardiac irradiation. Other markers of inflammation or thrombosis were not markedly changed at the protein level.

Atherosclerosis in large vessels effects (partners CARIM, NKI, UL, UROS, IRSN , TUD):

Contrary to expectations, there was no clear picture on a dose and time dependant change in inflammatory and prothrombotic markers in large vessels, with respect to the development of atherosclerosis. However, high doses of irradiation (8-16 Gy) in combination wit elevated cholesterol (ApoE-/- mice) stimulated the formation of fatty streak lesions (foam cells) and atherosclerosis in carotid arteries, and in the endocardium and coronary arteries.

Stress response (partners TUM, MSCCI, HELMUC):

Serum analysis of Hsp70 protein levels after irradiation revealed a significant increase 60 weeks after irradiation. Up-regulation of major stress-induced factor Hsp70i (both at the level of transcript and protein) was observed in the heart several weeks after irradiation of animals but not after long follow-up times.

Functional integrity (partners USFD, MSCCI, QUB, IRSN, HELMUC):

Radiation inhibited angiogenic sprouting in a dose-dependent manner with the most pronounced effect in the high dose groups. Endothelial morphogenesis into 3D capillary-like structures was significantly inhibited by 8 and 16 Gy. In these high dose groups, capillary-like structures were both quantitatively and qualitatively different from controls.

CM physiology and molecular responses are altered by ionizing radiation even after low doses yet CM responses to ionizing radiation differed between in vitro irradiation and after cell isolation from the irradiated tissue. PAI-1 activation was observed in the two strains suggesting that the TGF-ß/Smad/PAI-1 cascade was involved in fibrogenesis. Yet amyloidosis development was observed only in C57Bl6 but not in ApoE deficient mice. The molecular imprint of low radiation doses is different from that after moderate or high radiation doses. Activation of the Smad2/3 pathway occurred immediately after irradiation but neither the Rho pathway nor CTGF were activated after low doses. The TGF-ß cascade is differentially activated in both mouse strains. Smad 2/3/4 seemed to operate in C57Bl6 whereas other proteins seemed to be involved in ApoE. This difference may explain the differential pathogenic picture observed in histology.

In C57Bl6 mice, ionising radiation caused non-transient mitochondrial alterations in three major biological categories: the pyruvate metabolism, the oxidative phophorylation and the mitochondria-associated cytoskeleton. Radiation-induced impairment of the respiratory chain was tightly coupled to increased reactive oxygen species (ROS) levels in the heart and was reflected as increased protein oxidation already at 4 weeks after exposure. The succinate-driven respiration stayed impaired at least until 40 weeks after exposure. The proteomic changes increased with time from 24 (4 w) to 46 (40 w) deregulated proteins in C57BL/6 mice. With 0.2 Gy dose, much less proteomic changes were seen (6 proteins after 4 weeks) compared to 2 Gy. In accordance with this, no functional changes (membrane potential, ROS production, respiration) were observed with the 0.2 Gy dose at any time point tested (4 w, 20 w, 40 w).

ApoE deficient mice showed less mitochondrial proteome alterations than C57Bl/6 at 40 weeks (26 vs. 46) but there was a big overlap in the deregulated proteins (12) between the two strains. The direction of deregulation was the same in 10 out of 12 overlapping proteins. The deregulation of the mitochondrial complexes was less severe in ApoE than in C57Bl/6; this was confirmed by the functional measurements where no effect in the succinate-driven respiration was seen with $ApoE^{-/-}$, in contrast to C57Bl/6. However, the proteins involved in lipid metabolism were more affected in $ApoE^{-/-}$; they were more in number and the deregulation was more severe. There was almost no radiation-effect on the structural proteins in $ApoE^{-/-}$.

Final summary and conclusions

No clinically manifest cardiovascular disease was observed neither in C57Bl6 nor ApoE-/- mice even at 60 weeks after high doses of irradiation. The high rate of sudden death in the high dose group at 60 weeks could not be unequivocally attributed to changes in cardiovascular function or structure.

The most relevant and consistent finding is the linear dose and time dependent reduction of the microvessel density in the mid part of the heart. which was preceded by a time and dose-dependent loss of alkaline phosphatase expression in endothelial cells. There was also evidence of vascular leakage at doses of 2 Gy and above, with inflammatory cell invasion and a modest increase in collagen deposition after higher doses.

The angiogenic capacity of cardiac endothelial cells is strongly inhibited even at low to intermediate radiation doses as demonstrated in 2 newly developed ex vivo in vitro angiogenesis assays.

Long-term persistent stress responses were observed even at intermediate and low doses. With the exception of the oxidative stress response in cardiac mitochondria, they seem to be a consequence rather than a cause of radiation induced cardiovascular disease. The biological relevance of the mitochondrial impairment is not clear at the moment, but deserves further investigation.

The results of the CARDIORISK project permit the following conclusions which may impact profoundly future research in the area of low-dose radiation risk assessment in the cardiovascular system:

1) Radiation at low doses does not per se induce atherosclerotic changes in medium to large arteries.

2) Inflammation does not play a major role in the development of radiation induced CVD; stress responses can be regarded as a consequence of the effects of radiation induced CVD.

3) The reduction in microvessel density and the inhibition of neoangiogenesis even at low to intermediate doses does not directly translate into the clinical picture of CVD, but it may well compromise the ability of the heart to respond to subsequent stress.

4) These major conclusions support the hypothesis that low-dose radiation exposure and related subtle subclinical changes are permissive factors for the development of CVD which reduce the capacity of the heart to recover if a clinically relevant event/ insult occurs.

Outlook and future research

The CARDIORISK identified the progressive reduction of the capillary network at intermediate and low radiation doses (<2 to 8 Gy) as the key mechanism leading to radiation-induced heart disease. This microvascular effect does not lead to measurable ischemic cardiomyopathy but is likely to reduce the compensatory capacity of the heart to insults from other sources such as coronary heart disease etc. In addition, there are also strong suggestions of direct myocardial injury by low radiation doses after similar latencies. Despite the wide range of research methods and biological targets used in CARDIORISK, some key molecular and cellular processes of this microvascular effect are still largely unknown. Future research should focus on resolving these open questions, in particular:

1. The molecular targets in capillary endothelial cells which lead to functional damage, e.g. loss of alkaline phosphatase. Since CARDIORISK provided evidence that endothelial cells isolated from different organs and different endothelial cell lines show very different responses to low dose radiation exposure (not just “radiosensitivity” but the nature of response) those studies should use predominantly endothelial cells isolated directly from the hearts of the investigated animals.
2. The role of radiation-induced reduction of alkaline phosphatase in capillary endothelial cells and their role for capillary function,
3. The molecular mechanisms of low and intermediate dose radiation damage to cardiomyocytes (in particular mitochondria and energy metabolism,
4. The mechanisms of progression of general and focal loss of alkaline phosphatase and possible consequential random destruction of entire capillarie.
5. Elucidation of the apparent intercellular communication processes between endothelial cells within a capillary for the development of capillary damage.
6. The dependence of reduction of the capillary network on dose (<2Gy) and time (>1year) in longer living animals.

7. The relationship of radiation-induced microvascular radiation damage on the topographical dose distribution in the hearts, on age at exposure with regard to different clinical manifestations of radiation-induced heart disease, in particular ischaemic heart disease, valvular disease and conduction defects.

For this research, an integrated programme bringing together cardiological and radiobiological scientists is obligatory. The experience of CARDIORSIK has demonstrated the great potential of this cooperation.

1.4 Deliverables and Milestones Tables

LIST OF DELIVERABLES									
Del. no.	***Deliverable name***	***WP no.***	***Lead beneficiary***	***Nature 1)***	***Dissemi-nation Level 2)***	***Delivery date from Annex I***	***Delivered yes/no***	***Actual/ Forecast delivery date***	***Comments***
D1	Draft criteria and performance/research indicators	1	01	R	RE	2008-04-30	yes	2008-04-30	
D2	Employment report	1	01	R	RE	2008-03-30	yes	2008-03-30	
D3	Quality Management Report	1	01	R	RE	2008-04-30	yes	2008-04-30	
D4	Draft Implementation plan	1	01	R	CO	2008-07-31	yes	2008-07-31	
D5	Gender equality plan	1	01	R	RE	2008-03-31	yes	2008-03-31	
D6	Dissemination plan	1	01	R	CO	2008-07-31	yes	2008-07-31	
D7	Training plan	1	01	R	RE	2008-04-30	yes	2008-04-30	
D8	Mid term progress report	1	01	R	RE	2009-07-31	yes	2009-07-31	Annual Report year2
D9	Document detailing experimental evidence defining risk of CV effects at low	1	01	R	Re	2010-06-30	yes	2011-06-30	Final report
D10	Final plan for the use and dissemination of Foreground	1	01	R	Re	2010-06-30	yes	2010-06-30	Final report
D11	Report on awareness and wider social implications	1	01	R	Re	2010-06-30	yes	2010-06-30	Final report
D12	Report on isolation + proliferative capacity	2	02	R	PU	2009-01-31	yes	2009-01-31	
D13	Report on dose dependence of yield	2	02	R	PU	2010-01-31	yes	20010-01-31	
D14	Report on dose dependence (a, saphena)	3	11	R	PU	2010-01-31	yes	2010-01-31	
D15	Report on dose dependence of functional and structural changes in irradiated a. carotis	3	03	R	PU	2011-01-30	yes	2011-01-30	
D16	Report on microvascular density	4	03	R	PU	2010-01-31	yes	2010-01-31	
D17	Report on dose dependence of microvascular density in homogenously irradiated hearts	4	03	R	PU	2011-01-30	yes	2011-01-30	

LIST OF DELIVERABLES									
Del. no.	***Deliverable name***	***WP no.***	***Lead beneficiary***	***Nature 1)***	***Dissemination Level 2)***	***Delivery date from Annex I***	***Delivered yes/no***	***Actual/ Forecast delivery date***	***Comments***
D18	Report on the effects of low radiation doses	5	09	R	PU	2010-01-31	yes	2010-01-31	
D19	Report on dose dependence of inflammatory markers	5	09	R	PU	2010-01-31	yes	2010-01-31	
D20	Report on morphogenetic response of micr. EC	6	06	R	PU	2010-01-31	yes	2010-01-31	
D21	Report on specific protein changes (in vitro)	7	08	R	PU	2010-01-31	yes	2010-01-31	
D22	Report on specific protein changes in microvascular endothelial cells irradiated in vivo 1 – 12 months before analysis	7	08	R	PU	2011-01-30	yes	2010-01-31	
D23	Workshop on experimental techniques	8	01	O	RE	2009-01-31	yes	2009-01-31	No report
D24	Workshop on experimental techniques	8	01	O	RE	2010-01-31	yes	2010-02-08	No report
D25	Symposium on radiation-induced cardiovascular disease	8	01	O	PU	2009-07-30	yes	2010-06-07	No report
D26	Conference with stake-holders on mechanisms and prevention of cardiovascular radiation risks	8	01	O	PU	2011-01-30	yes	2010-06-07	No report

Footnotes:

1) R = Report, P = Prototype, D = Demonstrator, O = Other
2) PU = Public, PP = Restricted to other programme participants (including the Commission Services), RE = Restricted to a group specified by the consortium (including the Commission Services). CO = Confidential, only for members of the consortium (including the Commission Services).

LIST OF MILESTONES

MS. no.	*Milestone name*	*Work-package no*	*Lead beneficiary*	*Delivery date from Annex I*	*Achieved yes/no*	*Actual/ Forecast achievement date*	*Comments*
MS1	Establishment of SAB	1	01	2008-03-31	yes	2008-03-31	
MS2	Establishment of memorandum	1	01	2008-04-30	yes	2008-04-30	
MS3	External technical review by the European Commission	1	01	2009-08-30	yes	2009-08-30	
MS4	Delivery of irradiated hearts (9m, go/ no go)	2	02	2008-10-31	yes	2008-10-31	
MS5	Ability to propagate EC from irradiated hearts	2	02	2009-01-31	yes	2009-01-31	
MS6	Preperation of CM from irradiated + non-irradiated heart	2	02	2009-01-31	yes	2009-01-31	
MS7	Delivery of tissue from A. saphena	2	02	2009-01-31	yes	2009-01-31	
MS8	Validaton of OCT Imaging methodology	3	11	2009-01-31	yes	2009-01-31	
MS9	Validation of SPECT imaging sensitivity	4	03	2009-07-31	yes	2009-07-31	
MS10	Establishing of morphometry technique (go/ no)	4	03	2008-10-31	yes	2008-10-31	
MS11	Selection of appropriate in vivo stress parameters	5	01	2010-01-31	yes	2010-01-31	
MS12	Selection of appr. Assays for inflamm. Response	5	09	2010-01-31	yes	2010-01-31	
MS13a	Selection of appropriate method of co-culture techniques – fibroblasts	6	06	2009-07-31	yes	2010-01-31	
MS13b	Selection of appropriate method of co-culture techniques	6	06	2009-07-31	yes	2010-01-31	

LIST OF MILESTONES							
MS. no.	***Milestone name***	***Work-package no***	***Lead beneficiary***	***Delivery date from Annex I***	***Achieved yes/no***	***Actual/ Forecast achievement date***	***Comments***
MS14	Feasibility of morphogenic and migration assays	6	06	2010-01-31	yes	2010-01-31	
MS15	Selection of appropriate methods to study effects on cytoskeleton and barrier properties in irradiated EC	6	06	2009-07-30	yes	2009-07-30	
MS17	Quantitative analysis of protein changes	7	08	2010-01-31	yes	2010-01-31	
MS 18	Identification and validation of differentially expressed proteins	7	07	20010-07-30	yes	2010-07-30	
MS 19	Validation of protein changes in vivo	7	07	2010-09-30	yes	2010-09-30	
MS 20	Website management and content established and online.	8	08	2008-03-30	yes	2008-03-30	

1.5 The potential impact

Socio-economic impact and the wider societal implications of the project

The CARDIORISK consortium has developped a series of experimental systems to study the radiation biology of cardiovascular effects at low doses in vivo and in vitro. The major goal was to provide the impetus necessary to place radiation protection considerations of late cardiovascular effects in a biological context.

The estimation of radiation risk is primarily based on the assumption that, except for heritable, genetic radiation damage, the only somatic radiation damage to be considered in radiation protection is radiation-induced cancer. This dogma, which went unchallenged for several decades, has been challenged by the epidemiological evidence showing radiation-induced mortality from cardiovascular and cerebrovascular diseases of similar magnitude as from radiation-induced cancer. These epidemiological data demands a reassessment of the concepts, quantities and methods of how risk is defined in radiation protection. In order to incorporate cardiovascular and cerebrovascular radiation risks into the overall system of radiation protection, knowledge is required on pathogenic mechanisms, which only radiobiological experiments can provide.

The main problems for the incorporation of the new epidemiological evidence of radiation-induced late cardiovascular and cerebrovascular damage arise in the definition of dose and the shape of the dose response relationship. In the present system of radiation protection, the effective dose is defined as the sum of the mean radiation doses in the respective organs at risk, which is multiplied with the tissue weighting factor. While the tissue weighting factor may be relatively straightforward to define from epidemiological studies – and it is obvious that the tissue weighting factor for the heart and the brain will have to be increased significantly from their present value of 0.025, the question of dose definition cannot be resolved by epidemiological data. The answer to this problem has to be based on scientific evidence which requires full knowledge on the pathogenesis of the respective radiation-induced fatal diseases including in particular information on the critical structures which trigger the pathogenic pathways and their anatomical distribution in the organ. Only radiobiological experiments such as those performed in the CARDIORISK project were seen to put the methods of how to define radiation dose in the heart in situations of inhomogeneous radiation exposure -which are the rule in particular in diagnostic radiology - on a sound basis of scientific evidence. The present system of radiation protection assumes proportionality between mean organ dose and the risk of radiation-induced cancer since cancer is likely to be a clonal disease, arising from a single stem cell that has been transformed and clonally expanded through many additional steps of progressing malignancy. In contrast to this, cardiovascular diseases and cerebrovascular diseases are examples of obvious multicellular origin. All scientific bodies (such as ICRP, BEIR and USCEAR) have consistently argued that the distinction between stochastic and deterministic radiation effects can be based on the distinction of single cell clonal effects (such as germ cell mutations for heritable diseases and somatic cell mutations for radiation induced cancer), justifying a linear dose risk relationship, and multicellular radiation effects (such as late organ damage), which is observed only after radiation exposure has exceeded a threshold. This distinction can no longer be upheld in view of the epidemiological evidence.on cardiovascular radiation mortality of the A-bomb survivors. The resolution of this problem is of great importance for radiation protection since the entire concept of no-threshold linear dose risk relationships and the concept of stochastic radiation effects has been put into the limbo.

The CARDIORISK project has produced an enormous amount of biological data which relate to the pathogenesis of macrovascular and microvascular radiation damage. There is evidence in the mouse cardiovascular system that that large vessels such as carotid artery, arteria saphena and probably coronary arteries follow different pathogenic pathways but which are all associated with inflammatory processes which, however, are only triggered by high radiation doses which are characteristic of radiotherapy. In contrast, microvascular radiation damage is elicited already by lower radiation doses and develops independent of pro-inflammatory changes which lead to structural changes in the capillary network in the myocardium. This damage starts with functional changes in the capillary endothelial cells causing progressive disappearance of entire capillaries. There is some evidence for a simultaneous stress response in the irradiated myocardial cells although it remains open whether both effects develop independently or one is the consequence of the other.

For macrovascular radiation effects in the heart the results of the CARDIORISK project suggest a threshold-type dose response relationship which depends on the radiosensitivity of several well investigated processes involved in the activation of the inflammatory conversion of age-related atherosclerosis which occur after shorter latencies than the microvascular changes. The target structure in the heart where these processes take place and for which,

therefore, the specification of local dose has to be defined are the coronary arteries, and in particular the left anterior descending coronary artery as has also been suggested by the findings of the RACE project.

The CARDIORISK project produced evidence that microvascular radiation damage is different following different pathogenic kinetics in different parts of the ventricular myocardium. These topographical differences may be due to pathophysiological factors or to the different response criteria used by the different CARDIORISK partners. More studies need to be performed on the mechanisms and modifying factors which influence the reduction of the microvascular network. The data produced by CARDIORISK suggest that the reduction and rarefication of the capillary network is progressing over much of the life-span and a linear dose response relationship cannot be excluded. The linear dose dependence, though only validated in the dose range of 2 to 8 Gy, is difficult to reconcile with the observation that functional alterations in endothelial cells and rarefication occur throughout much of the myocardium of the ventricles, and that severity of this effect progresses with dose and time. This puts a big question mark on the concept of linear dose response relationships in general and clonal origin of "stochastic" radiation damage and may have implications for the entire system of radiation protection in all areas of low dose radiation exposure including the out-of-field exposures in radiation oncology. These findings also suggest that the whole myocardium should be delineated as the sub-volume for which dose should be defined and reported which would be, for microvascular damage, the mean myocardial dose.

In conclusion, different doses to the heart may have to be considered in radiation protection, i.e. the mean dose to the myocardium, and in radiation oncology where in addition, the local doses to the coronary arteries have to be determined and minimised as well.

Impact on other EURATOM projects

Close cooperation with the integrated FP7 project NOTE which includes studies of cardiovascular damage after low dose and protracted total body irradiation had been agreed. The leader of WP2 of NOTE (UROS) was a member of our consortium. He used irradiated hearts, arteries and endothelial cells from the CARDIORISK project and investigate their response with the same methods he used in the hearts of the NOTE project in order to compare the cardiac effects of total body irradiation with those of localised heart irradiation. Moreover, the leader of WP4 of NOTE, who aimed at using the data produced in the NOTE project to develop a bio mathematical model of radiation-induced cardiovascular risk, was invited to all CARDIORISK meetings and had direct access to all results produced by the CARDIORISK project to incorporate them into the bio mathematical model of radiation-induced cardiovascular.

Main dissemination activities and exploitation of results

All results will finally be published in the scientific open literature. However, equally important is the direct input of the results of the described studies and our conclusions from the new data into the discussions of the international committees responsible to adjust the radiation protection concepts, rules and regulations to the progress of knowledge.

Dissemination to specialist audiences

The primary dissemination vehicle is the presentation of scientific results to the international community. The target audiences are mainly the radiation biology and radiation therapy communities, besides the cardiology and general oncology communities.

So far, results from the CARDIORISK project have been presented at 16 national and 55 international meetings and published in 30 scientific article in Journals with an median Impact factor of 3,56 (range: 1,649 – 6,401).

As this project addressed long-term cardiovascular effects, final pivotal publication can only now be prepared after all follow-up times have been analysed. From every partner at least 1 seminal publication is planned for publication.

To specifically address the radiation biology and therapy community, a dedicated issue of Radiotherapy & Oncology ("Green Journal", IF 4.3) about the CARDIORISK project, the results and conclusions is planned. In a peer-review process 1 paper from each partner is being considered for submission together with an introductory editorial.

A review paper which described the overall design of the CARDIORISK project was written by the Scientific Secretary and published in 2010 in Radiotherapy & Oncology.

By far the most important dissemination activities during the lifetime of the project were:

(1) The DoReMi exploratory workshop on Radiation-Induced Cardiovascular Disease from low dose exposure in November 2010 in Bombon , France

The Bombon meeting in November 2010, provided a unique opportunity to inform those radiation protection experts who do research on Cardiovascular radiation risks about the activities of CARDIORISK. Of the about 30 participants who discussed the issues relevant to cardiovascular radiation risks were seven who were associated with the CARDIORISK project who, in lectures and in the very extensive discussion period which lasted four days, had ample opportunity to present the state of the research concepts, methods and findings to date. The final conclusions and recommendations for further research which were passed on the last day were apparently strongly influenced by the CARDIORISK project. Since also key scientists in the field from the USA and Japan participated in the workshop the work of CARDIORISK was spread world-wide.

(2) The CARDIORISK symposium in June 2011 which heralded the successful conclusion of the project

The CARDIORISK symposium in June 2011 followed a similar approach. In total 53 scientists participated, among them 5 invited experts outside the project.

Of the 9 key-note lectures of the symposium, four were given by work package leaders of CARDIORISK who presented summaries of the results described in this report.

The other five talks were given by the leading experts in the field of cardiovascular research in the context of radiation exposure, radiation therapy and chemotherapy coming from specialties like radiation oncology, radiation biology, oncology, cardiology and epidemiology presenting complimentary information from experimental and in particular clinical data:

- David Cutter, University of Cambridge, Clinical Trial Service Unit, United Kingdom
- Peter van Luijk, University Medical Center of Groningen; The Netherlands
- Steve Lipshultz, Professor and Chairman of Department of Pediatrics, University of Miami Miller School of MedicineUniversity of Miami, USA
- Larry Marks Department of Radiation Oncology, University of North Carolina, USA
- Günther Schellong, KInderklinik Münster, Gemany (represented by Prof. K.R.-Trott)

All talks highlighted the role of the CARDIORISK project for planning future research in radiation-induced cardiovascular diseases. Especially the presentations and vivid participations of the invited international experts in this field during the whole symposium was seminal for the discussion of the CARDIORISK results and their possible impact as well as for the definition of a possible roadmap for future research.

Dissemination and training to graduate and undergraduate audiences

The members of CARDIORISK are all involved in academic teaching, and the new knowledge on basic radiation biology as well as experimental models, techniques and design arising form CARDIORISK were immediately included in lecture and course material at the graduate and undergraduate level.

Additionally, workshops on experimental methods were organised as satellite symposia to the annual meeting of CARDIORISK as a platform to teach epidemiological evidence and research concepts as well as discuss and exchange knowledge on specific topics or method and techniques involved in the CARDIORISK project. Target audience for the workshops were students at the graduate and undergraduate level as well as scientific staff. Work group leader were chosen as expert teacher for these workshops.

Senior CARDIORISK members participated as expert speakers in the European MSc course in radiation biology as another means of dissemination of new knowledge in understanding radiation biology.

Dissemination to other EURATOM contracts

CARDIORISK has signed a memorandum of understanding with the NOTE project to signal close cooperation between two medium to large scale projects involved in the research of effects after low radiation dose. Close interaction between the two projects was already immanent, as three partners (QUB, UL, UROS) were involved in both, CARDIORISK and NOTE, respectively. This facilitated communication and discussion of results tremendously.

An informal cooperation has been established to the ALLEGRO project, as one of the members of the Scientific Advisory Board of CARDIORISK was a member of the management board of the ALLEGRO project. Discussion of

preclinical findings and possible impact on clinical decision making in radiation oncology as addressed by ALLEGRO has been intensively discussed.

Dissemination to the public and stakeholders

The CARDIORISK web site was developed to present the aims and key results of the research as well as public events like symposia and workshops to the public in an engaging and informative manner.

The project presented itself from the beginning on with press releases in the lay press or with short communications to individual university newsletters targeted at a wider audience interested in general scientific activities.