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Review

# Ionizing radiation biomarkers for potential use in epidemiological studies

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ABSTRACT

Ionizing radiation is a known human carcinogen that can induce a variety of biological effects depending on the physical nature, duration, doses and dose-rates of exposure. However, the magnitude of health risks at low doses and dose-rates (below 100 mSv and/or 0.1 mSv $min^{-1}$) remains controversial due to a lack of direct human evidence. It is anticipated that significant insights will emerge from the integration of epidemiological and biological research, made possible by molecular epidemiology studies incorporating biomarkers and bioassays. A number of these have been used to investigate exposure, effects and susceptibility to ionizing radiation, albeit often at higher doses and dose rates, with each reflecting time-limited cellular or physiological alterations. This review summarises the multidisciplinary work undertaken in the framework of the European project DoReMi (Low Dose Research towards Multidisciplinary Integration) to identify the most appropriate biomarkers for use in population studies. In addition to logistical and ethical considerations for conducting large-scale epidemiological studies, we discuss the relevance of their use for assessing the effects of low dose ionizing radiation exposure at the cellular and physiological level. We also propose a temporal classification of biomarkers that may be relevant for molecular epidemiology studies which need to take into account the time elapsed since exposure. Finally, the integration of biology with epidemiology requires careful planning and enhanced discussions between the epidemiology, biology and dosimetry communities in order to determine the most important questions to be addressed in light of pragmatic considerations including the appropriate population to be investigated (occupationally, environmentally or medically exposed), and study design. The consideration of the logistics of biological sample collection, processing and storing and the choice of biomarker or bioassay, as well as awareness of potential confounding factors, are also essential.



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**Contents**



## 1. Introduction

Ionizing radiation (IR) is a known carcinogen but the magnitude of risk at low doses and dose-rates (i.e. below 100 mSv and/or 0.1 mSv $min^{-1}$) remains controversial due to a lack of direct human evidence [1]. Epidemiological studies of radiation exposed populations can provide evidence of risk. The most important of these epidemiological studies for risk assessment is the Life Span Study of the survivors of the atomic bombings of Hiroshima and Nagasaki [2,3]. Studies of those occupationally exposed to radiation are becoming an increasingly important source of information on low dose/low dose-rate exposures [4,5]. However, it is recognised that epidemiological studies can be limited in statistical power to detect excess risk under these conditions. This is because the population sizes required to detect excessive risk become enormous when very small increases in risk are being investigated. In general this dose limit is at the 50–100 mSv level for acute radiation exposures, though increased risks have been observed at much lower doses for *in utero* exposures [6].

There is therefore a need to improve the evidence available on which to base low dose/low dose-rate radiation risk assessment. This is widely recognised throughout the world and several research programmes address this issue. In Europe, the DoReMi (Low Dose Research towards Multidisciplinary Integration) Network of Excellence (http://www.doremi-noe.net) aims to encourage and develop multidisciplinary approaches to low dose risk research. DoReMi is funded by the European Commission's EURATOM programme for a 6 year period. In the longer term it is envisaged that coordination and integration of European research will be facilitated by MELODI (Multidisciplinary European Low Dose Radiation Risk Research Initiative; http://www.melodi-online.eu/).

Improved evidence on the magnitude of health risk of low radiation doses is anticipated to emerge from the integration of

epidemiological and biological research through molecular/biomarker epidemiology and mechanistic studies. Such an integration is by no means new but it is complex [7–10]. It will require coordinated action and careful planning in terms of: definition of study questions, choice of appropriate and sufficiently large populations, validation of logistical and ethical aspects of sample collection and processing, and analysis of appropriate, sensitive and specific biomarkers over a wide range of doses.

The objective of the DoReMi network is to promote multidisciplinary integration and therefore offer a particularly suitable vehicle to help develop and plan large scale molecular/biomarker epidemiological studies. This review summarises the multidisciplinary work undertaken to date that has provided insight into suitable biomarkers for use in population studies. It highlights the outstanding needs in radiation biomarker development for molecular epidemiological studies and the research areas, and in particular in the -omics fields, that might provide biomarkers of high sensitivity and specificity for radiation research. It will not consider radiation biomarkers for clinical diagnosis, treatment assessment and prognosis of individual cases and emphasis will be given to research areas where potential biomarkers are already identified. Clearly the biomarker field is vast and this review is based on discussions in a workshop of epidemiologists and biologists involved in biomarker studies (most of the authors of this review) held under the auspices of DoReMi, contact with additional experts and the systematic review of the published literature on the topic using Medline and ISI Web of Knowledge. It is hoped that this review will help refine and focus research needs for improved low dose risk estimation, a topic that is of public and occupational health significance.

## 2. General considerations on biomarkers for use in epidemiological studies

### 2.1. Definitions

A biomarker has been defined as "any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological" [11]. Biomarkers can be used for multiple purposes in epidemiological investigation [12], including (1) estimation or validation of received dose, thus improving the validity of a correlation between exposure and biological responses; (2) investigation of individual susceptibility and (3) early detection of a radiation induced health effect. Multimarker approaches should be particularly useful in epidemiological studies, both for assessing exposure–response relationships and how these vary with individual susceptibility, and to understand better disease mechanisms and the interplay of different possible pathways. Conversely, carefully planned molecular epidemiological studies are essential for the validation and

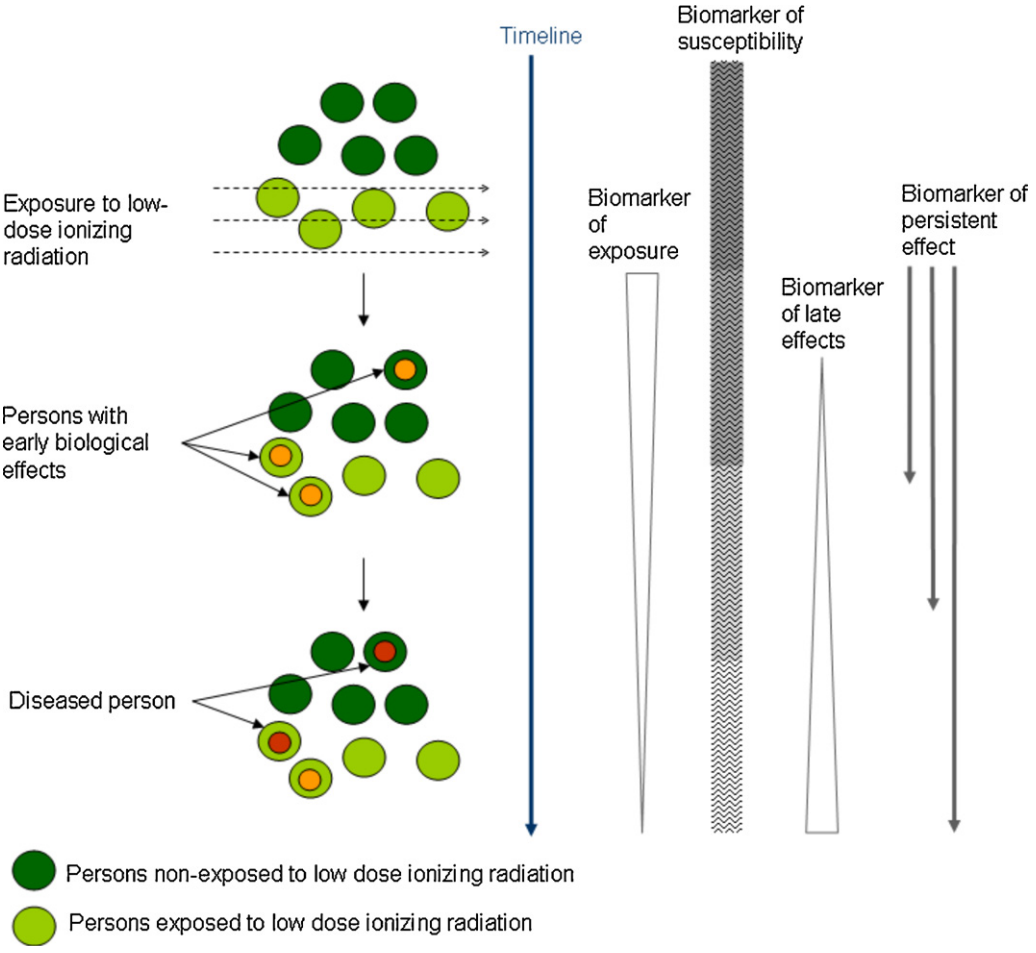


**Fig. 1.** Timing of radiation induced disease processes and relation with the different types of biomarkers.
*Biomarkers of exposure* are available at some point after exposure and are suitable for estimating the dose received. *Biomarkers of susceptibility* can be available before, during or after exposure and can predict an increased risk of radiation effects. Biomarkers of susceptibility would be expected to remain constant throughout the lifetime of an individual. However, certain gene and protein expression profiles could vary with age. *Biomarkers of late effects* can be used to assess health effects that are present a long time after exposure, before clinical detection of the radiation induced disease or death. *Biomarkers of persistent effects* allow the assessment of radiation effects present a long period of time after exposure.

verification of biomarkers, to determine their specificity and sensitivity as well as factors that might influence them (such as age, smoking status or individual sensitivity).

Over the past decades, the definition and classification of the different types of biomarkers have varied slightly, depending on the biomedical field considered [11–22]. For the purpose of the current review, we have chosen to classify biomarkers into four categories, related to temporal parameters (Fig. 1):

- *Biomarkers of exposure*: these are available at some point after exposure and are suitable for estimating the dose received. Examples include chromosomal aberrations as discussed in Section 4.1. They may be short-lived (e.g. dicentrics) or long-lived (e.g. the stable translocations measured using Fluorescence in situ hybridization [FISH] have been measured decades after exposure in Mayak workers [23]).
- *Biomarkers of susceptibility* can be available before, during or after exposure and can predict an increased risk of radiation induced health effects. Biomarkers of susceptibility would be expected to remain constant throughout the lifetime of an individual. However, certain gene and protein expression profiles could vary with age.
- *Biomarkers of late effects* can be used to assess health effects that are present a long time after exposure, before clinical detection of the radiation induced disease or death (the usual endpoints in classical epidemiological studies).
- *Biomarkers of persistent effects* allow the assessment of radiation effects present a long period of time after exposure.

This classification is justified not only with respect to the timing of processes that can be measured with these biomarkers, but also in considering the most adequate designs and sampling procedures in molecular epidemiological studies, as discussed in Section 3. It should be noted, however, that overlap exists between these different types of biomarkers.

### 2.2. Characteristics of a good biomarker

Although the definitions, nature and uses of biomarkers are multiple and rapidly evolving with the -omics technologies, some characteristics of an ideal biomarker can be listed, including its sensitivity and specificity, reproducibility and known variability in the general population [11]. For use in large scale molecular epidemiological studies, particularly in young people, an additional desirable characteristic is the possibility of using non-invasive procedures for collection of biological samples [22].

Determining if a biomarker is a good biomarker for molecular epidemiological studies is complex because this encompasses different concepts and will very much depend on the biological samples that can be collected [13,14,18,24–26]. This includes establishing the:

- validity of the assay measuring the biomarker (lack of systematic error and minimal random error of the measurement, compared to "the truth");
- validity of the marker (sensitivity, specificity, reproducibility, and biological plausibility). In many senses this is the critical issue with regard to the selection and/or development of biomarkers for use in studies of exposure related health risk. There is a need for a good understanding of what the marker is actually measuring and to evaluate factors which may modulate the biomarker yield such as the level of dose received and exposure to other agents which may provoke similar biological responses.
- suitability of the marker and assay for use in an epidemiological study. As described in Section 3, this is related to the study design – retrospective or prospective –, logistics and timing of sample collection and to the characteristics of the population studied, including the dose distribution. For instance, prospective cohort studies in which periodic contact with the study subjects is possible would be suitable for establishing the relationship between valid biomarkers and risk of disease because the resulting correlations would not be affected by reverse causality bias (e.g. disease, instead of exposure, modulating biomarker responses).
- invasiveness and acceptability of the sample collection. Sampling methods together with the nature and quantity of the biological samples (urine, saliva, blood, etc.) will have practical implications for the selection of a biomarker in epidemiological studies, particularly in children.

Those important characteristics are discussed in Section 4 for each biomarker considered in this review.

### 2.3. Biomarkers following in vitro exposure

While a biomarker can be measured in human subjects or directly in material obtained from human subjects exposed to radiation, an important tool for measuring a subject's biological response to radiation exposure is via the in vitro irradiation of biological material under defined experimental conditions (Fig. 2 and Fig. 3). Such a bioassay is the method of choice for assessing, for example, DNA repair capacity because the dose received can be tightly controlled and responses with respect to time can be more easily measured than in vivo. Bioassays assessing inducible damage, repair capacity and a cell's ability to survive can be useful as surrogate assays to test and predict individual radiation susceptibility. They are also particularly suitable in retrospective studies, such as case-control studies or retrospective cohort studies, in which it is not possible to measure biological responses immediately after an exposure that has occurred years in the past.

Bioassays are usually performed on cell lines derived from human subjects but primary cells can also be collected and irradiated in vitro (e.g. rate of loss of γH2AX in lymphocytes or hair follicles following in vitro irradiation) (Fig. 3). It should be kept in mind, however, that the extrapolation of results from in vitro experiments to human tissues is limited by the inherent reduced complexity of in vitro, compared to in vivo experiments, which reflect interactions between multiple tissues and biological pathways.

## 3. General epidemiological considerations

Epidemiological studies in which individual information is collected and biological samples are analyzed can be either prospective or retrospective [27]:

- Prospective studies: In *prospective cohort studies* (Fig. 4) a population with a given characteristic – for example survivors of the atomic bombs in Japan – is identified at the time of exposure or shortly thereafter and is followed up prospectively over time until diagnosis of (or death from) a particular disease (cancer, cardiovascular disease, etc.).
- Retrospective studies: They include *retrospective cohort studies* defined on the basis of historical records many years after exposures (cancer survivors, miners, workers in the nuclear industry) and *case-control studies* (Fig. 4). In case-control studies, individuals with the disease of interest (cases) are identified and compared to controls (i.e. persons which do not have that disease and are matched to the cases with respect to age, sex and other potentially confounding factors) selected from the population from which the cases arise and historical

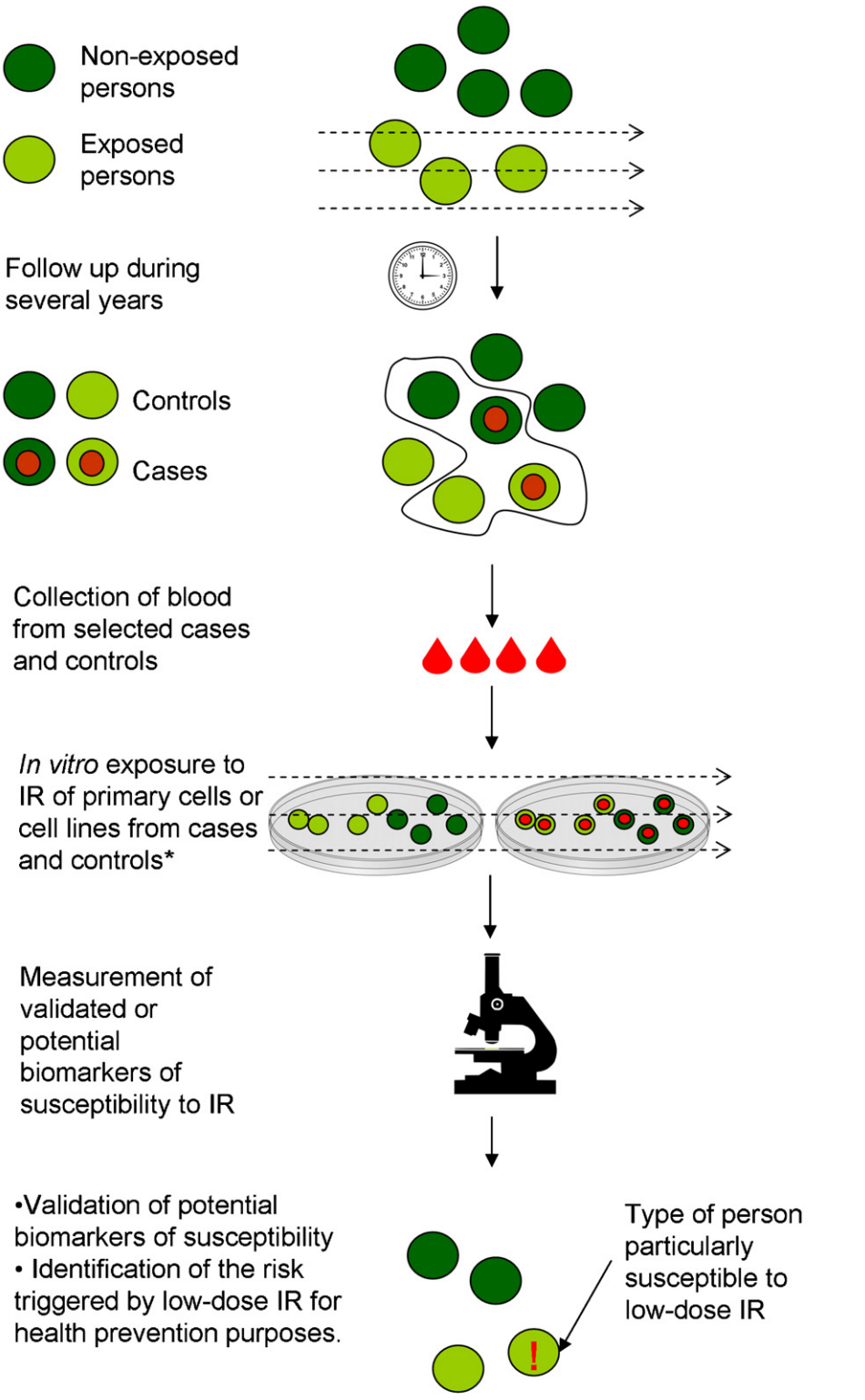


**Fig. 2.** Example of a retrospective study (here a case-control study nested in a cohort) to investigate susceptibility to low-dose ionizing radiation.

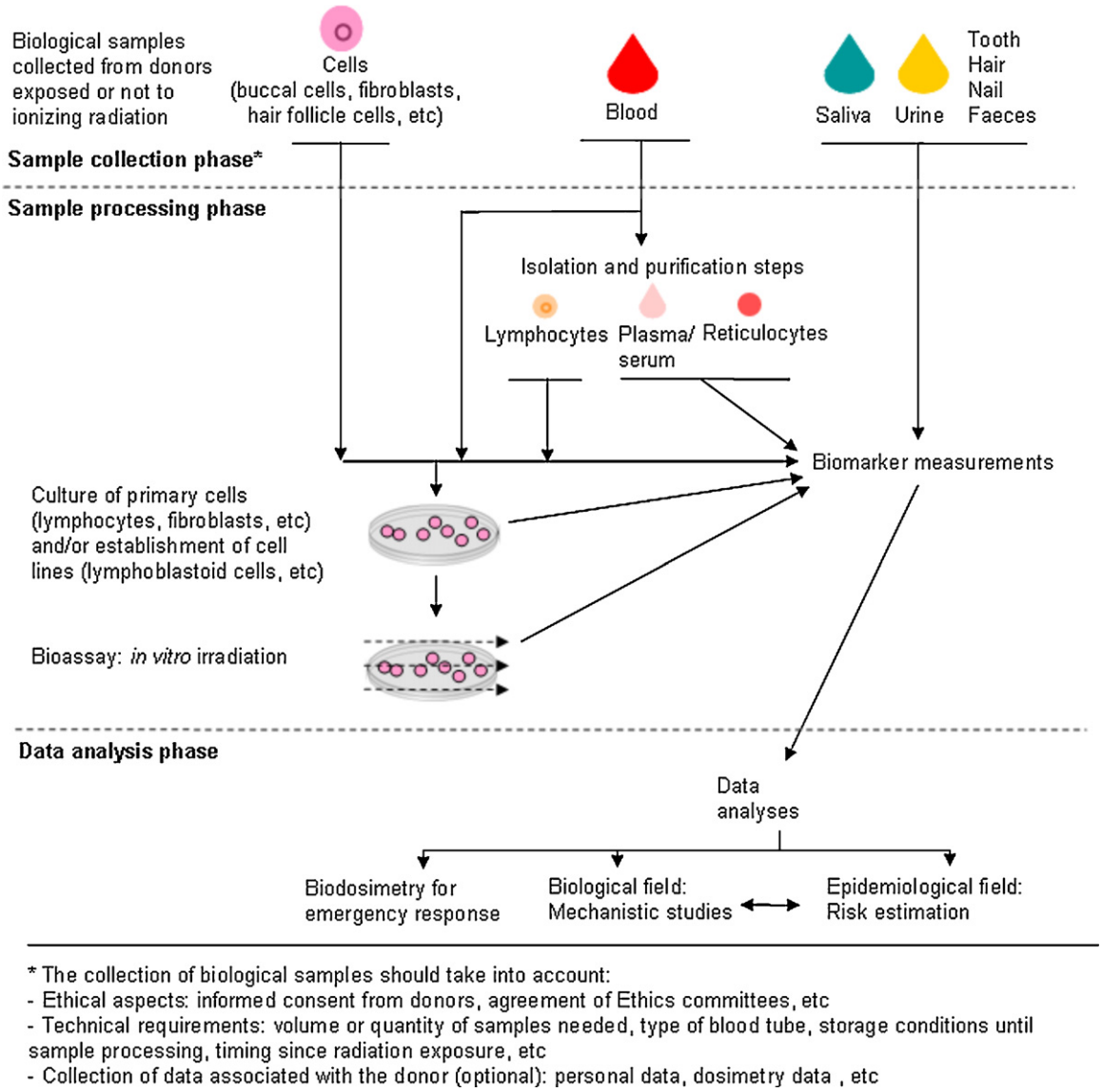


**Fig. 3.** Collection and use of biological samples to study the effects of ionizing radiation.

exposure is reconstructed (for example case-control studies of thyroid cancer in young people following the Chernobyl accident [28]). A special type of case-control study, which may be particularly suitable for molecular epidemiological studies in terms of logistics and costs, is a *nested case-control study* (Fig. 2 and Fig. 4). It is a case-control study in which the cases and the controls are identified from an exposed cohort under investigation. This is an efficient study design which allows the expensive and complex analyses of biological samples to be conducted on a restricted number of subjects/samples (cases of a specific disease and a subgroup of disease-free people; for example a case-control study of childhood leukaemia nested within a cohort of paediatric Computed Tomography [CT] patients would require analyses on several hundreds of subjects, rather than on hundreds of thousands).

These different types of studies imply different constraints for the collection and use of biological samples. While in prospective studies it may be possible to collect biological samples soon after exposure (e.g. early Chernobyl emergency recovery workers on whom biological dosimetry was conducted for triage, Fukushima plant workers) and repeat the sampling later for each individual, in retrospective studies exposure has occurred a long time in the past and in general it is not possible to measure early markers of exposure or markers of early effects unless biological samples have been banked before the start of the study. Retrospective studies may allow, however, on a limited number of subjects (for example through the conduct of nested case-control studies) the study of radiation sensitivity through the collection of samples which could be used in bioassays (Fig. 2).

A further important consideration in the planning of molecular epidemiological studies is the limited (and, in record linkage studies, non-existent) contact with study subjects which makes it logistically difficult in many studies to collect samples at different and relevant time points from the same individual. A notable exception are cohorts of cancer survivors who undergo active follow-up for many years and for whom it may be possible to collect samples which would allow the study of different types of biomarkers over time. Worker populations are also often followed-up at regular intervals by occupational physicians. Although biological samples collected under such circumstances are rarely kept, the results of standard biological analyses (cholesterol levels, and other potential effect modifiers or confounders for cardiovascular diseases and other diseases) and, in some countries, the

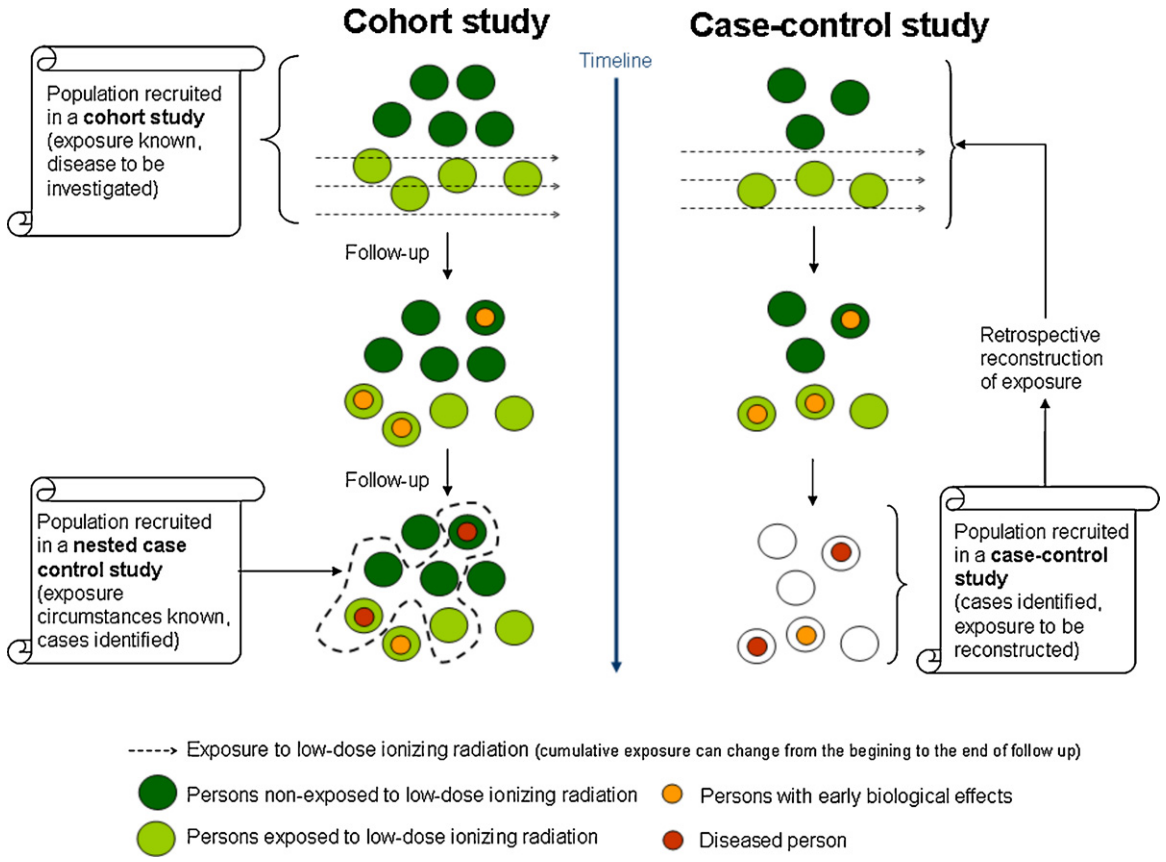


**Fig. 4.** Populations recruited in different types of study design.
In a *cohort study*, a population with a given characteristic (for example survivors of the atomic bombs in Japan) is identified at the time of exposure or shortly thereafter and followed up prospectively over time until diagnosis (or death) from a particular disease (cancer, cardiovascular disease, etc.). In a *case-control study*, cases of a disease of interest are identified and their exposure compared to that of controls (i.e. persons which do not have that disease and are as close as possible to the cases with respect to age, sex and other factors of interest) selected from the population from which the cases arise (for example case-control studies of thyroid cancer in young people following the Chernobyl accident). A *case-control study nested in a cohort* is a particular type of case-control study in which the cases and controls are drawn from a cohort under observation (for example a childhood leukaemia case-control study, conducted within a cohort of paediatric CT patients).

results of chromosomal aberration studies, may be available for molecular epidemiological studies.

### 3.1. Collection and use of biological samples in epidemiological studies

A variety of biological samples can be used for biomarker measurements in epidemiological studies, given appropriate ethics approvals and informed consent (see Section 3.2) and do depend on the nature of the exposure–internal vs. external. These include blood, saliva, buccal cells, skin fibroblasts, urine, faeces, hair, hair follicle cells, and nail clippings (Fig. 3). In vivo analysis of electron spin resonance (ESR) of tooth enamel is also possible (see Section 4.7.4). In cancer patients, tissue may also be available from biopsies and/or surgery (generally including healthy as well as tumour tissue). It is beyond the scope of this paper to discuss the technical requirements for sample collection and processing, but this is an important issue for consideration in molecular epidemiological studies (for example the type of anticoagulant to be used for blood collection may affect the usefulness of the sample for specific analyses). More technical details can be found in the 2011 cytogenetic dosimetry guide from the International Atomic Energy Agency [29].

Because of the burden imposed on study participants, collection of samples for the purpose of a molecular epidemiological study should be well justified, minimally invasive, pose no health risk and interfere minimally with the participant. Blood should be collected only when the added information cannot be obtained from elsewhere. DNA for genotyping can be extracted for example from saliva and from exfoliated cells collected from the mouth (buccal cells) or urine (urothelial cells) [30,31]. The collection of saliva or buccal cells with commercially available kits has been validated and used in many molecular studies for extraction of DNA and more recently, RNA [32–37]. This method presents important logistical advantages – in particular when the study population is geographically dispersed and also because it does not require trained personnel – and ethical and compliance advantages compared to blood, particularly in studies involving children and/or repeated sampling. It has to be recognised that the quantity of DNA that can be isolated from different biological samples will vary, although recent technological advances described below can be used to amplify DNA from many sources. Biopsy and other tissue collection outside the setting of surgery are rare with the exception of hair, hair follicle, toe nails and skin punch biopsies that can be used to isolate fibroblasts and are ethically acceptable in some countries.

The development and validation of biomarkers of exposure, susceptibility, persistent and late effects are therefore limited by access to appropriate biological samples collected and stored under appropriate conditions. In many situations there is only a small window of opportunity after which the study subject may no longer be available or conditions may have changed. In most cohorts, moreover, multiple or repeated sample collection is not feasible.

As discussed by Holland et al. [38], the main challenges of molecular epidemiology are: (a) obtaining a large amount of

information from limited samples; (b) making provisions for evaluation of future biomarkers; and, (c) maximizing the information that can be obtained from banked samples. Indeed the banking of samples is, in itself, a central issue especially for long-term studies and for future sample use in new studies. Storage can affect the quality of the samples and determine their future usefulness for the evaluation of different markers. For instance samples collected at biopsy and surgery for diagnostic purposes are usually fixed in formalin and embedded in paraffin before histological examination and long-term storage. This fixation process leads to structural changes in RNA, thus limiting the usefulness of such biological material for the assessment of RNA expression although shorter microRNAs (miRNAs) can be successfully extracted from such samples. In recent years techniques such as whole genome amplification (WGA) and multiple displacement amplification (MDA) have proven efficient for the amplification of small amounts of DNA, including DNA from single cells. The amplified DNA is suitable for multiple downstream applications, such as sequencing, short tandem repeat analysis, array comparative genomic hybridization and methylation analysis (see for instance [39,40]).

### 3.2. Ethical considerations

The use of biological samples raises a number of ethical issues which in most countries are governed by law. The study design, questionnaires and informed consent documents usually have to be approved by institutional and/or national ethical review boards before the start of the study. In addition, because data are collected and stored, many countries also require approval from the agencies that oversee data protection and freedom of information and from the ethical review committees of all participating hospitals. These approvals are frequently a prerequisite for applying for financial support of the study. In addition, if the collection of biological material can potentially create a risk for the subject participating in the study (e.g. the collection of blood samples), national ethical committees also require that this is covered by appropriate indemnity arrangements.

Informed consent has to be obtained from study participants prior to the collection and use of biological samples and/or their medical, social and occupational data [41]. Potential risks and harms for the participant must be clear, as well as the fact that a participant enters the study voluntarily with the right to withdraw. In recent years the nature of the informed consent has changed in many countries as it is now not just a question of the participant acknowledging that they are aware of the potential risks associated with giving a sample for a specific purpose at the time, but requires a broader consent of the participant because of the rapidly expanding biotechnological progress in sample analysis. In such circumstances it has to be understood by the participants that they agree to the use of their samples for unspecified assays at some time in the future and that the material together with the data may be stored for undefined periods of time, e.g. if cell lines are established out of subjectś primary cells. Guidelines exist to assure the proper and legal handling of specimen and health data [42–45]. Usually it is required that a subject's sample and data are completely anonymous. That means there is no link to the subject's name or address. If this is not possible, because a participant has the possibility to withdraw his consent to a later time point, the sample and the data have to be coded. For this purpose, data and samples are usually dissociated from the personal identification with an independent person such as the data protection officer of an organization, holding the codes so that a person can be eventually identified with their prior written informed consent. This guarantees that people who are analyzing samples and data are not able to link the results to a person. If a subject withdraws from the study all of the stored biological materials of this individual have to be destroyed and any analyzed data can only be used in the future in a totally anonymous way. Another example of when coding of data is needed is for longitudinal studies, where sequential sampling of biological material and data occurs. For instance the collection of health data over certain time periods may be required to follow up the appearance of biomarkers and their association with disease development. It is likely that such studies will be the preferred ones in the future and will require that participating populations accept the concept of repositories that combine medical, genealogical and lifestyle information with biological samples. Indeed to be effective to study the interaction of environmental exposures such as radiation and genetic factors, tens of thousands of people should be involved and it is likely that national cohorts will need to be combined into European or cross-continent cohorts (e.g. European Strategy Forum on Research Infrastructures: European Biobanking and Biomolecular Resources Research Infrastructure [BBMRI], launched in 2008 [www.bbmri.eu]). This possibility also means that in the informed consent the participants agree that their stored material and data can be given to other international researchers for analysis. National and international data sharing and material transfer are regulated by specific transfer agreements which govern their use and any intellectual property generated through such research. Because of the ethical problems arising with such an open and wide consent including questions of autonomy to decide how biological samples and personal data are used, several countries have already set up or are trying to set up legislation on "biobanking" [46].

One major issue arising from such molecular epidemiological research is how to inform subjects about the results of the biological analysis carried out. With the increasing information on genetic susceptibility to severe diseases and their corresponding biomarkers, biological analysis can reveal information about putative individual susceptibility to potential health risks. At the present time many studies do not give information on individual risks to study participants. Explaining a risk of developing perhaps an incurable or even untreatable disease to a supposed healthy subject could cause severe distress and even psychological damage. In addition if the subject has been given individual risk information, he/she might have to inform his/her life or health insurance which could lead to discrimination in healthcare availability and also in the workplace. In coming years these issues will evolve as the understanding of the risks associated with individual susceptibility and the specificity and sensitivity of different endpoints to assess individual risk become clearer.

## 4. Biological classification of ionizing radiation biomarkers

The diverse structures and functional entities in the cell are all targets for radiation induced damages as discussed in different sections in this review. However, which is the most relevant target in terms of identifying and validating different types of biomarkers after exposure to low dose and dose rate exposures still remains to be fully determined. For instance, whilst DNA damage repair mechanisms represent an important aspect in responses to high doses of IR, other cellular responses such as cytokine regulation and changes in the transcriptome, proteome, epigenome or metabolome implicated in the global response to irradiation may be more relevant, particularly following exposures to low doses of radiation. This can be illustrated by the fact that most severe cases of over response to IR amongst patients who underwent radiotherapy showed impaired DNA damage repair, whereas most moderate cases did not [47].

The classical approach to discover or measure biomarkers relevant to IR exposure and responses uses a candidate approach to

evaluate endpoints based on previous hypotheses and known biological mechanisms. The high-throughput technologies or -omics technologies, which often depend on the use of microarrays, are an alternative approach that tests the entire genome or the modulations of the transcriptome, proteome, epigenome or metabolome. Unlike hypothesis-driven research, it has the potential to identify biomarkers of IR with no prior assumptions about underlying biological mechanisms. This is a potent tool but the analysis and interpretation of the data obtained remains challenging [48].

In this review we consider a non-exhaustive list of potential biomarkers for IR exposure and response that are implicated in cellular or physiological mechanisms and rely on a hypothesis-driven or an -omics approach. Keeping in mind that many biomarkers could be classified in multiple categories (e.g. γH2AX represents a protein modification but is strongly associated with DNA damage, 8-oxo-dG represents a nucleotide damage but is also generated by reactive oxygen species (ROS), the phosphoproteome is influenced by epigenetic modifications but is also implicated in many signalling pathways), we have classified them as (a) cytogenetic biomarkers, (b) biomarkers related to nucleotide pool damage and DNA damage, (c) biomarkers related to germline inherited mutations and variants, (d) biomarkers related to induced mutations, (e) biomarkers related to transcriptional and translational changes, (f) biomarkers related to epigenomic modifications, and (g) other biomarkers, including biophysical markers of exposure (Fig. 5). For certain of these, little information is available on their usefulness for radiation molecular epidemiological studies but several show potential and the purpose of this section is to review the available information. Due to the nature

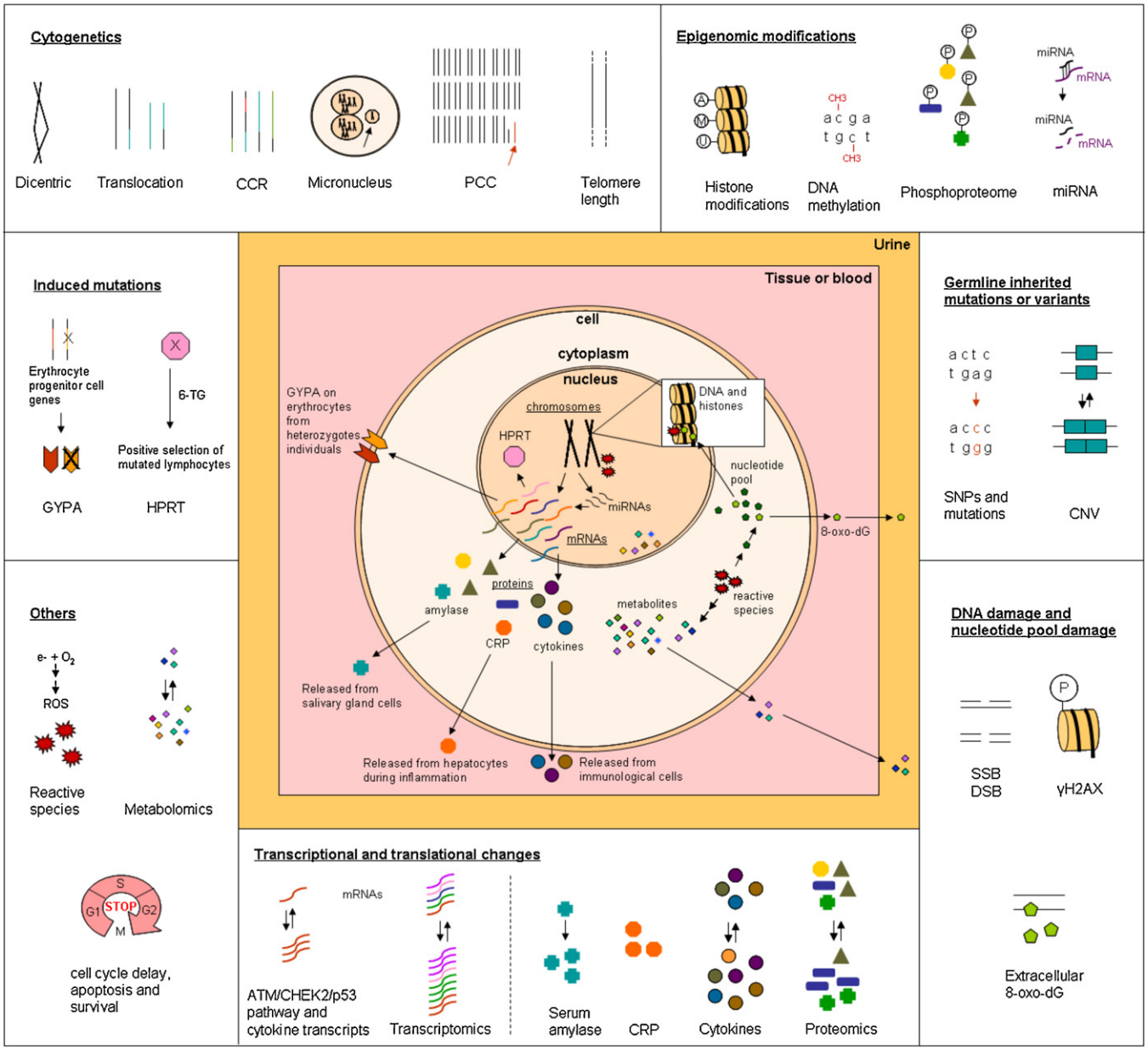


**Fig. 5.** Overview of the biomarkers of ionizing radiation covered in this review.
Vertical double lines represent pairs of chromosomes and horizontal double lines represent double strands of DNA.
A: acetyl group; CCR: complex chromosomal rearrangement; CNV: copy number variant; CRP: C-reactive protein; DSB: double strand break; GYPA: glycophorin A; HPRT: hypoxanthine-guanine phosphoribosyltransferase; M: methyl group; miRNA: microRNA; P: phosphate group; PCC: premature chromosome condensation; ROS: reactive oxygen species; SNP: single nucleotide polymorphism; SSB: single strand break; U: ubiquitin; 6-TG: 6-Thioguanine; 8-oxo-DG: 8-Oxo-deoxyguanosine.

**Table 1**
Cytogenetic biomarkers.

| Biomarkers | Assays/methodology | Sensitivity | Specificity to IR and confounders | Time window after exposure during which assays might be performed | Biological material needed to perform the assays[a] |
|---|---|---|---|---|---|
| Dicentrics | Dicentric chromosome assay | 0.1–5 Gy | Almost exclusively induced by IR | Before renewal of PBL | WB: fresh; PBMC: fresh and frozen[b] |
| Translocations | Single colour FISH G-banding | 0.25–4 Gy | Confounding factors: smoking; strong age effect | Years | WB: fresh; PBMC: fresh and frozen[b] |
| CCR | Multiple colour FISH | Unknown | High LET and heavy ion exposure | Before renewal of PBL | WB: fresh; PBMC: fresh and frozen |
| PCC | PCC assay combined or not to FISH chromosome painting or c-banding | PCC fragments: 0.2–20 Gy PCC rings: 1–20 Gy | IR specific to a large extent | PCC fragments: ideally immediately after exposure PCC rings: before renewal of PBL | PBMC: fresh and frozen |
| Telomere length | Flow cytometry Quantitative-FISH Southern blot qPCR | Not yet established | Not specific: modulated by viral infection Potential confounders: age, oxidative stress | Not yet established | WB, PBMC, cell lines: fresh and frozen |
| Micronuclei | Cytokinesis block micronucleus assay Micronucleus centromere FISH assay for low doses Flow cytometric detection of DNA in reticulocytes | 0.2–4 Gy[c] but limited sensitivity at doses <1 Gy. Selective scoring after centromere FISH: ~100 mGy | Not specific: modulated by genotoxins Confounding factors: age, gender | In lymphocytes: before renewal of PBL In reticulocytes: not yet established | WB, PBMC: fresh and frozen[b]; Reticulocytes: fresh |

CCR: complex chromosomal rearrangement; FISH: Fluorescence in situ hybridization; PBMC: Peripheral Blood Mononuclear Cell (lymphocytes, monocytes, etc.); PBL: Peripheral blood lymphocytes (T lymphocytes for assays requiring cycling cells); PCC: premature chromosome condensation; qPCR: quantitative polymerase chain reaction; WB: whole blood.

[a] Sample storage conditions listed when known.

[b] Frozen samples give lower yields of scorable cells.

[c] Dose range for photon equivalent acute whole-body exposure 24 h ago.

and biokinetics of the dose distribution, we have considered separately approaches for the biological estimation of dose for internal emitters.

### 4.1. Cytogenetic biomarkers

Cytogenetics focuses on the study of chromosomes, in particular chromosomal anomalies. Several cytogenetic endpoints are routinely used as biomarkers of exposure as they show a high degree of specificity and sensitivity. Other cytogenetic measurements might be useful as biomarkers of late effects but need to be validated against well defined outcomes/endpoints. Cytogenetic biomarkers are summarised in Table 1.

#### 4.1.1. Dicentrics

Dicentric chromosomes (i.e. chromosomes with two centromeres) are, with only very few exceptions, induced by IR. They are stable within non-dividing cells such as lymphocytes but as the half-life of blood lymphocytes is in the order of months/years depending on the sub-population [29], the dicentric is the biomarker of choice for investigating recent exposure to IR. In general, as there are no major confounders influencing the yield of dicentrics, its natural occurrence is very low (generally in the order of 0.5–1/1000 cells scored). Individual dose assessment can be achieved for homogeneous whole-body exposures to doses as low as 100 mGy for low-linear energy transfer (LET) IR if up to 1000 cells are analyzed. However after exposure to low doses, the calculated estimates often carry large uncertainties, mainly due to the insufficient number of cells scored. Since the dicentric assay is very laborious, counting sufficiently large numbers of cells will be a limiting factor and will limit the possibilities for adequate dose estimation in the low dose range on an individual level. Automated systems are under development and provide very reproducible results but their major limitation is the dicentric's detection efficiency that remains around 50–70% [29]. Nevertheless, automated dicentric assays are currently being investigated in the framework of the European Multibiodose project (http://www.multibiodose.eu) that is aimed at analysing and adapting biodosimetric tools to manage high scale radiological casualties.

In addition to acute whole-body exposures, dose estimation for protracted and partial-body exposure can also be achieved by scoring dicentrics in lymphocytes. It should be noted that in order to estimate dose, calibration curves are necessary. Although the scoring of dicentrics is most suitable as a biomarker for external exposures [49], it can also be informative after internal exposures to radionuclides that disperse fairly uniformly around the body. Isotopes of caesium and tritiated water are two such examples [29]. Dicentric aberrations are unstable because their frequency decreases with the turnover of peripheral blood lymphocytes. Thus, for reliable dose assessment, dicentric aberration assays should be performed within a few weeks of exposure. If performed later, the precision of the assay is diminished as the dose calculation requires the use of half-time estimates for the disappearance of dicentrics [29].

#### 4.1.2. Translocations

In contrast to dicentrics, translocations are chromosomal aberrations that have been shown to persist in peripheral blood lymphocytes for years and can thus be used as biomarkers of past exposures [29]. This persistence reflects the presence of translocations in the lymphatic stem cells and is affected by many factors. These include exposure conditions, such as dose rate and whole-body vs. partial-body exposure. As translocations may be parts of complex chromosomal rearrangements (CCRs) [50] that are unstable (see Section 4.1.3), it is essential to distinguish their origin within the cell. The cells that are scored also influence measurements and dose calibration for translocations, as it has been shown that the presence of unstable chromosomal aberrations in the same cell reduces the frequency of translocations with time [51]. Therefore, only cells free of any unstable chromosome damage should be scored.

A common feature of translocations among non-exposed subjects is the large inter-individual variation in their number, age being the most important confounding factor [52]. Therefore, natural occurrence and accumulation with lifespan can confound very low dose exposure estimations. Despite the strong age-effect for translocations, subjects of the same age may show large variation in translocation frequencies. The reasons for the age-dependent baseline frequency are not fully established, but some of them may be linked with cellular mechanisms changing with age such as DNA repair. From a number of confounders tested, smoking has been demonstrated to increase translocation frequencies in some studies, but not in others, possibly due to variations in cigarette types or numbers smoked [53]. Other sources of variation may include clastogenic agents in the diet or environment, gender, ethnicity and genetic polymorphisms in genes involved in cellular defence mechanisms. Individual dose assessment using translocations is strongly dependent on the personal baseline frequency of this aberration. As this information is almost never available, the alternative is to refer to a large study of non-exposed individuals and apply corresponding age-related baseline frequencies in individual dose calculations [52]. Using frequencies of unexposed subjects, it has been shown that the minimum detectable acute dose increases linearly with age at a rate of 1.8 mGy per year from the age of 20 up to 69 years. For chronic exposure, the corresponding value is 15.9 mGy per year [54].

As for dicentrics, the precision of using translocation analysis as an exposure biomarker at doses <1 Gy would be vastly improved by substantially increasing the number of translocations scored per subject (e.g. $n = 30$) and thus increasing the number of cells scored up to thousands of cell equivalents, in contrast to the prevalent practise of a few hundred per individual [53]. However, from a practical point of view, the time required for the analysis would severely limit the use of such an approach in large molecular epidemiological studies. FISH (or "chromosome painting") is commonly used for the detection of inter-exchanges, such as translocations and dicentrics. In studies assessing exposures to IR that are either chronic or have occurred in the past, single (or multiple) colour FISH together with a pancentromeric probe is commonly applied for identification and quantification of two-way or one-way translocations. Currently the assay could be semi-automated, for instance through the use of a metaphase finder system, but there is still a need for a fully automated image analyser that would reliably differentiate normal cells from cells with chromosomal aberrations [53,55].

#### 4.1.3. Complex chromosomal rearrangements

CCR involve a minimum of three or more breaks in two or more chromosomes [56] and can be described as a combination of several simple aberrations, such as translocations, dicentrics, ring chromosomes or acentric fragments. The introduction of multi-colour FISH into cytogenetics has widened the spectrum of aberrations amenable to detection (reciprocal translocation, insertion, and deletions) of radiation exposure, and it has made it possible to refine the detection and definition of CCRs that were only possible before with conventional staining (Giemsa staining or standard methodology). CCR's natural occurrence is very low and its presence without exposure was almost exclusively confined to some populations and may imply a DNA repair defect. The CCRs are unstable since they decrease with the turnover of peripheral blood lymphocytes [57]. The presence of CCRs is considered to be a signature for high LET and heavy ion exposure [58,59]. There have been several reports that past irradiation can leave a permanent signature in the genome even several years after the exposure, as evident in plutonium workers exposed in 1949 in the Mayak Production Association near Ozyorsk, Russia [23], New Zealand nuclear test veterans exposed in 1957–1958 [60], patients treated with X-ray irradiation for ankylosing spondylitis [61], Hodgkin lymphoma patients treated with chemotherapy associated to radiation therapy [62], Hiroshima atomic bomb survivors [63,64], and Chernobyl cleanup workers [65–67]. However, currently there is no study assessing the usefulness of this potential biomarker after low dose exposure and further studies are needed to investigate the effect of low-LET radiation exposure on its occurrence. In addition, as for translocation scoring, CCR scoring with FISH is technically demanding and without automation, it is unlikely that from a practical point of view this biomarker could be used in large scale epidemiological studies.

#### 4.1.4. Premature chromosome condensation (PCC)

Measurement of chromosomal aberrations requires that the cells of interest are in mitotic phase where chromosomes are condensed and visible. PCC techniques can induce a condensation of the chromosomes in quiescent and cycling cells whether by fusion with mitotic cells or by chemical treatment. In quiescent cells the number of excess PCC fragments (>46 chromosomes for human) are scored. In cycling cells, it is also possible to score ring chromosomes, dicentrics and translocations if the PCC assay is combined with FISH chromosome painting or c-banding [29,68–72].

PCC has been reported to be most useful for assessing high dose acute exposures to low LET radiation [73]. Dose–response data from 0.2 to 20 Gy have been achieved for the PCC fragment assay whereas with the PCC ring method, the sensitivity ranges from 1 to >20 Gy [73–75]. PCC has been successfully used in some cases of exposure, especially for problems of radioprotection and assessment of doses received after medical imaging (X-ray and nuclear medicine) [76]. When scoring excess PCC fragments, sampling and lymphocyte isolation after treatment should be performed without delay, otherwise repair kinetics should be taken into account [75].

#### 4.1.5. Telomere length

Telomeres are heterochromatic domains composed of repetitive DNA (TTAGGG repeats) bound to an array of specialized proteins situated at the very end of chromosomes. The length of the telomere repeats and the integrity of the telomere binding proteins are both important for telomere protection. Loss of telomere function can lead to genomic instability and cancer progression [77] and is associated with radiation-induced genetic instability, increased radiation sensitivity, loss of cellular viability, and senescence [78–81]. The association between telomere length and individual radiation sensitivity was initially based on the fact that accelerated telomere shortening was commonly observed in cells from patients with IR sensitive syndromes (reviewed in [82]). Later, it was proposed that short telomeres contribute to genomic instability in the aged progeny of irradiated cells [83–86]. It has also been shown that telomere length modulates chromosome in vitro radiosensitivity in healthy individuals as the group with short telomeres presented higher frequencies of IR-induced micronuclei (MN) when compared to the long telomeres group [87]. Moreover telomere alterations and genomic instability are described in long-term cultures of normal human fibroblasts irradiated with X-ray and protons [88]. The quantification of telomere lengths in cohorts of persons exposed to radiation shows potential as a biomarker of risk for secondary malignancies and late complications [62]. However, further studies are needed to validate the use of this biomarker in molecular epidemiological studies, in particular for low-dose IR. It should also be noted that age and oxidative stress are potential confounders and that viral infection can influence telomere length. From a practical point of view, the automation of the quantification of telomere lengths has resulted in the ability to evaluate large numbers of cells quickly and efficiently. Flow

cytometry and a slide-based image (Q-FISH) analysis have both been used with considerable success [89,90]. Shortened telomeres can also easily be detected by a PCR assay although problems of reproducibility have been reported [91].

#### 4.1.6. Micronuclei

MN form when intact chromosomes or fragments are not properly segregated into daughter cell nuclei at anaphase but instead remain in the cytoplasm after cell division. They can be visualised as small spherical objects using any conventional DNA dye. In comparison to most other cytogenetic techniques, MN are far easier to score both manually and using automated microscopy slide scanning and image analysis systems [29,92,93]. In fact, as for the dicentric assay, one of the workpackages of the European Multibiodose project focuses entirely on automated MN assay. As MN form only during cell division and, like dicentrics, are lost when cells continue to divide, the most reliable quantitative results are achieved by blocking the cell cycle progression of PHA-stimulated lymphocytes at the stage of cytokinesis after the first mitosis using cytochalasin B. Microscopic scoring of MN is then only performed in binucleated cells. Such measurements can be made on fresh whole blood or frozen peripheral blood lymphocytes; the subsequent sample preparation, scoring and analysis procedures have been largely harmonised among biodosimetry labs [29], and an ISO standard is in preparation.

In addition to MN scoring to assess exposure, the same samples can also be analyzed for the nuclear division index, different modes of cell death as well as other types of chromosomal aberrations such as nucleoplasmic bridges – which are correlated to dicentrics and centric rings – and nuclear buds which are associated with gene amplification in an approach known as the 'Cytome Assay' [94].

MN measured by cytokinesis-blocked micronucleus (CBMN) assay show promise as a biomarker for individual radiosensitivity and susceptibility to environmental carcinogens [95]. Consistent with this notion, a recent twin study provided evidence for the high heritability of baseline and induced MN frequencies [96].

Due to variable base levels in different individuals, the standard CBMN assay cannot detect acute whole body doses below 200 mGy for low-LET IR. In addition a wide range of clastogenic and aneugenic agents (i.e. agents causing chromosome breakages and abnormal number of chromosomes, respectively) can induce MN, and confounding factors include age and gender. Most of this background 'noise' of MN in non-exposed individuals can be attributed to the loss of one intact copy of the X-chromosome. Selective scoring of MN that are negative for centromere-specific FISH signals can significantly improve the sensitivity to a minimum detectable acute whole body gamma-ray dose of ~100 mGy for individuals [97]. First steps have been made towards the development of an automated analysis system for the micronucleus centromere assay [98] which would enable large-scale studies of cohorts exposed to low-to-moderate radiation doses. Other limitations of the CBMN assay include the minimum delay of 3 days between sampling and first results becoming available, loss of the signal with lymphocyte turnover (as discussed above for dicentrics) and its inability to detect non-uniform exposures [97].

As an alternative to the microscopic CBMN assay in lymphocytes, MN can also be detected by flow cytometric detection of DNA in reticulocytes [99]. Because micronucleus-carrying reticulocytes are normally trapped in the spleen, only a small fraction can be detected in the blood. However, this population of recently matured reticulocytes can be increased by immunomagnetic enrichment or flow sorting of transferrin receptor positive cells [100]. Initial results are encouraging but more studies are needed to assess the full potential and limitations of the reticulocyte micronucleus assay and its use as a biomarker for radiation exposure and individual sensitivity, in particular at low doses.

### 4.2. Biomarkers related to nucleotide pool damage and DNA damage

IR can induce a variety of DNA damage either directly or indirectly via ionization events produced by radiation-induced reactive ROS. For example exposure of mammalian cells to 1 Gy of gamma or photon radiation has been estimated to lead to 1000 single-strand breaks (SSB), 500 damaged bases, 40 double strand breaks (DSB) and 150 DNA-protein cross-links [101]. However the formation of these DNA lesions is not unique to IR and thus they can not be used per se as biomarkers of exposure unless a number of confounding factors are taken into account, including age, syndromes associated with oxidative stress and exposure to other genotoxins (including smoking, certain occupational settings and chemotherapy). DNA strand breaks can be measured directly or using surrogate endpoints such as the presence of γH2AX foci or assays such as the comet assay. A brief outline of these techniques is presented below but it should be noted that there is vast literature discussing the use and limitations of such measurements that is beyond the scope of this review. There are also a number of methods such as HPLC-ESI-MS/MS and to a lesser extent HPLC-ECD that are sensitive enough for measuring base lesions formed under conditions of severe oxidative stress including exposure to IR [102], that will also not be further discussed here. Finally, IR and ROS can produce damage to the nucleotide pool, the measurement of which has potential as a biomarker of exposure (see Table 5). Biomarkers related to nucleotide pool damage and DNA damage are summarised in Table 2.

#### 4.2.1. DNA single/double strand breaks

Since SSB and especially DSB are highly characteristic of the DNA lesions formed after exposure to IR, assays detecting their formation and persistence or the individual's ability to repair this type of damage can be used as biomarkers for exposure or individual radiation sensitivity.

To measure DNA SSB and/or DSB generated by IR several techniques with different sensitivities in terms of the lesions detected and the level of detectable DNA damage exist. These include techniques such as alkaline or neutral filter elution [103,104], alkaline unwinding [105,106], sucrose gradient centrifugation [107–109] or pulsed field gel electrophoresis [110–113]. However the utility of such techniques for molecular epidemiological studies investigating low doses effects is limited as none can be used to investigate DNA damage after exposures to doses under 2 Gy and at the single cell level.

The comet assay is a relatively easy, quick and automatable test to detect direct DNA-damage at the single cell level and with a higher sensitivity compared to methods described above [114]. Its main advantage is the requirement of only minimal numbers of cells (~10.000) or volumes of whole blood (10 μl) [115]. It has been widely used to measure both in vitro and in vivo DNA damage and repair following exposure of mammalian cells to various genotoxic agents including some chemicals, IR and non-IR [116–119]. The assay can be performed in neutral or in alkaline conditions. Both methods detect SSB and DSB however the alkaline assay is often used to detect SSB and alkali-labile sites whereas the neutral comet assay is often used to detect DSBs, although it has to be noted that this variant of the comet assay lacks sensitivity and specificity. With the alkaline comet assay an irradiation dose range from 100 mGy to 8 Gy can be investigated as well as DNA repair capacity. Because it can be automated, the assay is highly suitable for a screening assay in human populations although some important criteria need to be taken into consideration: (a) the specificity for radiation exposure is low, since oxidative stress in

general induces single strand breaks detected by the assay, and (b) inter-experimental variability is very high in the test system, so the test has to be performed under highly standardized conditions with an automated analyses and the inclusion of reference samples so that comparisons can be made over-time [120]. In addition to being used as an endpoint to assess DNA damage levels, the comet assay can be used as a bioassay for instance to evaluate DNA repair capacity. Using such an approach, biological samples are irradiated in vitro and the level of persisting DNA damage with time used as a biomarker of susceptibility [121]. The results from such assays need to be interpreted with caution as a number of confounding factors including age of the subject, smoking and diet have been reported.

#### 4.2.2. γH2AX

The phosphorylation of the histone variant H2AX at the site of DNA DSBs lead to the formation and accumulation of γH2AX foci in the cell nucleus within a few minutes of DNA damage. The maximum yield of foci is detected within 30–60 min after irradiation, depending on the dose, and after this, the number of foci usually decreases to baseline level within days [47,75]. The measurement of γH2AX foci could be used as a direct endpoint assessing the formation of damage and therefore as a biomarker of exposure, whereas its persistence with time in irradiated samples can be used to evaluate DNA repair capacity and thus as a biomarker of susceptibility (see also [47] for recent review).

Several studies on patients medically exposed to low dose radiation have shown that the γH2AX assay is very sensitive, and that foci after doses below 20–10 mGy can be detected. A study on patients with differentiated thyroid carcinoma who underwent $^{131}$I therapy showed that exposure to radionuclide incorporation can be detected by γH2AX assay in mononuclear peripheral blood leukocytes after absorbed doses to the blood below 20 mGy [122]. Another study on patients who underwent angiographic procedures and received from 2.2 to 99.9 mGy to blood has concluded on the reliability and sensitivity of γH2AX immunofluorescence microscopy [123]. Studies investigating γH2AX foci in blood samples of CT scanned patients before and after the CT scan, have shown that γH2AX focus induction and loss are sensitive enough to be used as biologic dosimeters [124,125]. However, extensive use of the assay as a biomarker of exposure directly in biological samples obtained from a study subject is regarded as limited due to the fast decline of the signal as well as variation of foci frequencies between individuals.

Despite these limitations, if samples can be obtained in appropriate time windows, the γH2AX assay has potential to reveal low dose hypersensitivity [126]. Löbrich et al. have investigated loss of γH2AX foci in lymphocytes of CT scanned patients and have demonstrated that the loss of γH2AX foci correlated with DSB repair [124]. Interestingly, one radiosensitive patient presented elevated residual foci after CT, suggesting an impaired DNA repair. The kinetics of γH2AX foci loss might thus be used as a biomarker of susceptibility in in vivo or in vitro studies. However, as discussed above, due to the fast decline of the signal after exposure, in vitro irradiation of subject's cells to detect a loss in γH2AX foci might be more feasible in a large scale epidemiological study.

When considering γH2AX foci frequency or foci loss as potential biomarkers of exposure or susceptibility, it should be kept in mind that previous studies have provided evidence that at very low doses (1.2–10 mGy), the loss of foci in irradiated mice or nondividing primary human fibroblasts is impaired over several days compared to higher dose exposure [127,128]. Therefore, the use of γH2AX foci loss as a biomarker of susceptibility in studies investigating the effects of less than 10 mGy might not be suitable because the kinetics of foci loss will be difficult to establish. It should be also noted that a similar level of persistent radiation induced γH2AX foci, depending on radiation quality (X rays vs. γ-rays) and types of sample (whole blood vs. isolated lymphocytes), was reported after exposure to 5 and 200 mGy [264]. Regarding the use of γH2AX foci frequency as a biomarker of very low dose exposure, the persistence of the foci might be an opportunity to increase the time frame of sampling and testing after irradiation and also to discriminate between irradiated and non irradiated cells.

Optimization of γH2AX foci analysis according to cell cycle phases and its limitations has been reviewed recently elsewhere [129]. Automation may improve the possibilities to apply the technique as a biomarker in large scale accidents and molecular epidemiological studies to investigate radiation sensitivity. There are two ways to automate the assay: (a) flow cytometric analysis, and (b) automated microscopic analysis. Flow cytometric analysis has the disadvantage that only total fluorescence instead of single foci are analyzed. As mentioned above for the dicentric assay and the MN assay, the automation of γH2AX assay for rapid triage in case of mass casualty scenario is one of the topics of interest in the European Multibiodose project. Although there already exist some publications that have tested or used automated assays [130–132], large scale molecular epidemiological studies have not been performed using the assay so far.

The possibility of using hair bulbs for γH2AX foci assays is highly interesting in the context of an epidemiological study because the collection of such biological samples is less invasive for the subjects than blood or tissue collection. Although such an assay has not yet been reported in humans for low doses, a study in non human-primates exposed to high doses (1–8.5 Gy) showed promising results [133]. The slower rate of γH2AX foci loss in hair bulbs compared to lymphocytes reported in this study could also be an opportunity to increase the time frame of sampling and testing after irradiation. Finally, it should be noted that the measurement of γH2AX foci in eyebrow hair follicles was used in a pharmacodynamics study assessing the activity of PARP Inhibitors [134].

#### 4.2.3. Extracellular 8-oxo-dG

In the last few years attention has been drawn to the nucleotide pool as a target of IR. It has been suggested that extracellular 8-oxo-dG primarily originates from the sanitization process of 8-oxo-dGTP, thus the yields of extracellular 8-oxo-dG may serve as a biomarker of the intracellular oxidative stress (see also Section 4.7.1 on ROS) [135–137]. The mechanism(s) of radiation induced nucleotide pool damage are not well understood, dose–response relations suggest that in the low dose region (1–100 mGy) radiation will trigger a stress response reaction leading to an endogenous formation of ROS that is the dominating cause of 8-oxo-dGTP production [135–137]. The dose–response relations are not linear and saturates for doses in the 0.1–1 Gy range. Regarding radiation-induced endogenous formation of ROS, mechanistic studies in cellular model systems have shown that it is transient with a time span over a few hours.

In parallel with the formation of 8-oxo-dGTP, the levels of the nucleotide pool sanitizing enzyme with 8-oxo-dGTPase activity (hMTH1) were increased following exposure to low dose radiation suggesting that subsequent ROS production will trigger the activation of cellular defence systems against oxidative stress [136]. Increased serum levels of 8-oxo-dG have also been suggested to correlate with inflammatory responses – that are known to generate ROS – in a group of haemodialysis patients [138]. Thus although extracellular 8-oxo-dG may be used as a biomarker of oxidative stress it lacks the specificity for a biomarker of exposure to IR.

**Table 2**
Biomarkers related to DNA damage and nucleotide pool damage.

| Biomarkers | Assays/methodology | Sensitivity | Specificity to IR and confounders | Time window after exposure during which assays might be performed | Biological material needed to perform the assays[a] |
|---|---|---|---|---|---|
| SSB/DSB | Comet assay (alkaline Single Cell Gel Electrophoresis assay) | 0.1–8 Gy | Not specific: modulated by several mutagens and oxidative stress<br>Confounding factors: age, smoking, diet | Minutes to days post-irradiation | WB: fresh<br>PBMC: fresh and frozen;<br>Fibroblasts: fresh and frozen;<br>Buccal cells: fresh |
| γ-H2AX | Immunofluorescence staining<br>Flow cytometry<br>High throughput electrochemiluminescent platform | 0.01–8 Gy | Not specific: also formed in response to UV and other genotoxins | Minutes to days post-irradiation | PBMC, fresh and frozen;<br>Fibroblasts: fresh and frozen<br>Buccal cells: fresh<br>Tissues: frozen and FFPE<br>Hair follicle bulb[b]: fresh;<br>Hair follicle bulge stem cells[c]: fresh |
| Extracellular 8-oxo-dG | HPLC-ECD Modified ELISA | 1–100 mGy<br>Saturation for doses between 0.1 and 1 Gy | Not specific: also formed by endogenous oxidative stress<br>Confounding factors: unknown | 1–2 h post irradiation | WB: fresh; PBMC: fresh;<br>Serum: fresh and frozen;<br>Cell lines, Saliva, Urine |

DSB: Double Strand Break; ELISA: Enzyme-linked Immunosorbent Assay; FFPE: Formalin-Fixed Paraffin-Embedded; HPLC-ECD: High Performance Liquid Chromatography coupled to Electrochemical Detection; PBMC: Peripheral Blood Mononuclear Cell (lymphocyte, monocytes, etc.); SSB: Single Strand Break; WB: whole blood.
[a] Sample storage conditions listed when known.
[b] Reported for non-human primates.
[c] Reported for mice [140].

The extracellular levels of 8-oxo-dG have also been studied in search for biomarkers of individual susceptibility. It has been shown that the urinary levels of 8-oxo-dG, produced during radiotherapy, differed significantly between a cohort of patients that developed severe side effects when treated for breast cancer compared to a group that showed no side effects [139]. This observation needs to be validated in a larger cohort before extra cellular 8-oxo-dG can be used as a biomarker of susceptibility. Further studies are also needed to investigate if such differences are seen under conditions of low acute and chronic exposures and to determine the impact of confounding factors such as age, smoking, diet and medical conditions, e.g. autoimmune disease and viral infections, that might alter levels of oxidative stress.

### 4.3. Biomarkers related to germline inherited mutations/variants

Biomarkers related to germline inherited mutations or variants are summarised in Table 3.

#### 4.3.1. Single Nucleotide Polymorphisms (SNP) and inherited gene mutations

It is well accepted that individual heterogeneity in responses to radiation exist yet the genetic determinants and the molecular mechanisms of altered sensitivity and even resistance remain poorly understood. The deleterious clinical consequences of inherited defects in DNA repair systems are apparent from several human syndromes. Examples are individuals carrying mutations in the Ataxia Telangiectasia Mutated (*ATM*) gene, which is essential for DNA DSB recognition and the following damage response cascade activation. These individuals have an increased risk to develop cancer and when treated during radiation therapy often develop severe side effects of the normal tissue. However such deleterious mutations are too rare in the general population to be of a major public health impact in terms of responses to radiation. Radiation sensitivity can be regarded as a quantitative complex phenotype or trait due to the combined effect of many more frequently found susceptibility sequence variants, such as SNPs – i.e. a variation at a single position in a DNA sequence present in

**Table 3**
Biomarkers related to inherited and induced mutations.

| Biomarkers | Assays/methodology | Sensitivity | Specificity to IR and confounders | Time window after exposure during which assays might be performed | Biological material needed to perform the assays[a] |
|---|---|---|---|---|---|
| SNPs and inherited gene mutations | Various candidate gene approaches<br>SNP arrays/GWAS<br>NGS (potentially) | Unknown | Not specific<br>Confounders unknown | Not time dependent | WB: fresh, frozen, dried;<br>PBMC: fresh and frozen;<br>Fibroblasts: fresh and frozen;<br>Tissue: fresh, frozen, FFPE |
| CNV and CNA | CGH, array CGH<br>FISH<br>NGS | Unknown | Not specific<br>Confounders:<br>CNA: age;<br>CNV: unknown | CNV: Not time dependent<br>CNA: Not yet established | WB: fresh, frozen, dried;<br>Tissue: fresh, frozen, FFPE |
| GYPA (in heterozygous MN blood group only) | Flow cytometer | >1 Gy | Not specific: formed after exposure to other genotoxins | Years | WB: fresh;<br>Erythrocytes |
| *HPRT* | Thioguanine selective cloning assay<br>PCR | >90 mGy | Not specific: formed after exposure to other genotoxins | Months | PBMC: fresh and frozen |

CGH: comparative genomic hybridization; CNA: copy number alteration; CNV: copy number variant; FFPE: formalin-fixed and paraffin-embedded; FISH: fluorescence in situ hybridization; GYPA: glycophorin A; GWAS: genome-wide association study; *HPRT*: hypoxanthine-guanine phosphoribosyl transferase; NGS: next generation sequencing; PBMC: peripheral blood mononuclear cell (lymphocyte, monocytes, etc.); PCR: polymerase chain reaction; SNP: single nucleotide polymorphism; WB: whole blood.
[a] Sample storage conditions listed when known.

more than 1% of a population – interacting with environmental factors. A comprehensive search for the genetic risk factors for radiation sensitivity would ideally examine all the genetic differences in a large number of affected individuals and controls. It may eventually become feasible to accomplish this by complete genome sequencing (using next generation sequencing [NGS] for instance) but to date attempts to identify genetic variants associated with radiation sensitivity have almost exclusively used a candidate gene approach assessing the frequency of common variants in cases and controls and more recently using genome wide association studies (GWAS). GWAS compare arrays of SNPs from cases and controls in an attempt to identify functional DNA-sequence variants that influence radiation sensitivity. One limitation of GWAS compared to NGS is the fact that only common genetic variants are investigated whereas with NGS, rare variants can also be identified. Such studies have often been carried out in a clinical setting where for instance early and late normal tissue reactions to radiotherapy toxicity can be well documented [141–143]. Many of the recent genetic association studies have focused on identifying effects of SNPs in candidate genes, among which DNA repair and cell cycle control genes have been extensively studied because of their critical role in maintaining genome integrity. SNPs in genes involved in profibrotic and inflammatory cytokines, endogenous antioxidants, general metabolism and homeostasis may be of particular relevance in situations where exposure is to low doses of radiation which may generate elevated levels of oxidative stress. A potentially interesting genome to characterise in terms of sequence variants is that of the mitochondria, as well as the genes encoding other proteins associated with mitochondrial function, which play a key role in the endogenous control of ROS in cells.

Many of the SNP association studies have often yielded conflicting results—see for instance a critical review of the association studies between SNPs in the Transforming Growth Factor β (TGFβ) gene and clinical radiation sensitivity [143]. As highlighted in this review many studies are very heterogeneous in terms of patient selection, tumour site, radiotherapy technique and normal tissue endpoints and are small in size so are severely underpowered from a statistical point of view to detect an association between the presence of a specific allele at a SNP and the risk of developing complications linked to treatment. In addition many studies investigated several SNPs and a number of different endpoints yet rarely have taken steps to correct for multiple testing which leads to a high risk of false positives by chance.

The need for large populations with well documented dosimetry and biological samples that can be used for the extraction of DNA is possible to establish in the medical setting and has led to the establishment of a Radiogenomics Consortium [144]. This Consortium aims to develop an assay capable of predicting which cancer patients are most likely to develop radiation injuries resulting from treatment with a standard radiotherapy protocol and to identify genes possessing SNPs associated with the development of radiation-induced adverse effects ranging from early to late and even second cancers. Such studies will be highly relevant for other radiation exposed cohorts which could be used to identify and validate genetic biomarkers using candidate gene approaches and whole genome association studies.

#### 4.3.2. Copy number variants (CNV) and alterations (CNA)

Advances in technologies that allow whole genomes to be screened for variations such as high-resolution SNP arrays have made it possible to identify CNVs. These structural variants that arise from deletions (loss) or duplications (gain), can include entire genes or regions of transcribed sequence, or, can comprise only nontranscribed sequences. The role of CNVs in cancer has only emerged in the last few years when constitutional CNVs was observed in the Li-Fraumeni cancer susceptibility syndrome and in neuroblastoma [145]. The impact of CNV on biological approaches and in particular after radiation exposures is also a research field that has been very little studied either in vivo or in vitro. A recent publication has shown that mouse ATM/CHK2/p53 activity was very dependent on *ATM*, *Trp53* (p53) and *Chek2* (CHK2) copy number before and after in vitro ionizing irradiation and that the relationship between gene copy number and transcriptional induction after radiation was linear for p21 and Puma and correlated well with cancer incidence in p53 variant mice [146] (see also Section 4.5.1). Clearly certain CNV show associations with radiation responses and by extrapolation may be exploitable as biomarkers of variation in responses. However, their usefulness in molecular epidemiological studies remains to be established.

Acquired CNVs (or copy number alterations [CNA]) have also been found in certain tumours. For example IQGAP1 CNA is associated with the invasiveness of thyroid cancer and may represent a novel prognostic marker and therapeutic target for this cancer [147]. In addition, a study conducted on young patients with papillary thyroid carcinoma exposed to an estimated average dose of 150 mGy after the Chernobyl accident showed an amplification of the region 7q11.22–11.23 in a subgroup of exposed patients, and could potentially represent a biomarker of exposure, although possibly limited to certain cases or dose ranges [148]. However whether the presence of specific CNAs reflects the tumour aetiology remains to be examined. Other biomarkers related to mutations induced by IR are discussed in the following section.

### 4.4. Biomarkers related to induced mutations

Two somatic mutation assays, the Glycophorin A (GYPA) and hypoxanthine-guanine-phosphoribosyl transferase gene (*HPRT*) mutation assays (described below and see also [75] for recent review), are frequently used in the biodosimetry field. However whether radiation induced tumours have a specific mutation profile remains to be established. As noted in Section 4.3.2, a recent study has identified a potential genetic signature of radiation-induced thyroid tumours [148]. In another study assessing the mutational pattern of a reporter gene in mouse fibroblasts upon fractionated IR exposure, a high proportion of T:A → G:C transversions was observed [149]. Some hints have also been obtained from medical settings. For instance in one study investigating the *TP53* mutation pattern in radiation-induced sarcomas, a high proportion (58%) of sarcomas showed a somatic inactivating mutation for one allele of TP53, systematically associated with a loss of the other allele, and a high frequency of short deletions (52% of the mutations) [150]. In addition, the lack of mutations in CpG dinucleotides together with the presence of recurrent mutation sites at codons 135 and 237 also seemed to be specific for radiation tumorigenesis. However these observations are limited in many respects and clearly need to be extended to examine more tumour types and other tumour sites.

Biomarkers related to induced mutations are summarised in Table 3.

#### 4.4.1. Glycophorin A in MN blood group heterozygotes

GYPA is a glycoprotein present on the surface of erythrocytes. The gene has two allelic forms, *gpaM* and *gpaN* that give rise to proteins that differ in only two amino acid residues. Alterations at the *GYPA* locus in erythrocyte progenitor cells produce variant red cells that have lost the expression of one of the alleles. This allele loss can be detected using fluorescent labelled antibodies against the M and N forms of GYPA in individuals heterozygous for the MN blood type (gpaM/N) (50% of the human population is heterozygous with respect to the MN blood group). Thus the GYPA assay

**Table 4**
Biomarkers related to transcriptional and translational changes.

| Biomarkers | Assays/methodology | Sensitivity | Specificity to IR and confounders | Time window after exposure during which assays might be performed | Biological material needed to perform the assays[a] |
|---|---|---|---|---|---|
| ATM/CHK2/p53 pathway | Various candidate gene approaches, e.g. TAQMAN, qPCR | Unknown | Not specific | Probably hours to days | PBMC, WB, cell lines |
| Changes in RNA levels identified by transcriptomics | Microarrays, qPCR, Nanostring™ NGS (potentially) | Unknown | Unknown at present time | 1–3 days | PBMC, WB, cell lines |
| Serum amylase | Serum amylase assays | >1 Gy | Not specific | 24 h after exposure | Serum |
| CRP | ELISA | >1 Gy | Not specific: modulated by levels of inflammation | Blood: Years<br>Saliva: up to 8 h | Serum and saliva with in vivo exposure only. |
| Cytokines | ELISA | >1.2 mGy | Not specific | Within 24 h | PBMC: fresh; serum |
| Proteins identified by proteomics | NanoHPLC-LC-MS/MS<br>SELDI-TOF-MS | Good to very good | Unknown at present time/remains to be fully established | 10 min–40 weeks[b] after exposure with doses varying from 0.2 to 16 Gy | Serum: frozen;<br>Primary cells and cell lines;<br>Tissue: frozen |
| | 2DE/2D-DIGE;<br>MALDI-TOF/TOF;<br>Western blot;<br>Enzymatic assays;<br>Tissue arrays | Moderate to very good | Unknown at present time/remains to be fully established | 10 min–40 weeks[b] after exposure with doses varying from 0.2 to 16 Gy | Serum/plasma: frozen;<br>Primary cells and cell lines;<br>Tissue: frozen |

CRP: C-reactive protein; ELISA: enzyme-linked immunosorbent assay; HPLC-LC-MS: high-performance liquid chromatography-mass spectrometry; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight; NGS: next generation sequencing; PBMC: peripheral blood mononuclear cell (lymphocyte, monocytes, etc.); qPCR: quantitative polymerase chain reaction; SELDI-TOF-MS: surface enhanced laser desorption/ionization time-of-flight mass spectrometry; WB: whole blood; 2DE/2D-DIGE: two-dimensional protein gel electrophoresis/two-dimensional difference gel electrophoresis.
[a] Sample storage conditions listed when known.
[b] For mice. For humans: possibly more than 60 years.

determines the frequency of erythrocytes lacking the M or the N allelic form, measuring the hemizygous (M/0 or N/0) and the homozygous (M/M or N/N generated by mitotic recombination, gene conversion, etc.) phenotype cells with the help of a flow cytometer [151]. The frequency of GYPA variants has been investigated in several studies on individuals exposed to IR. Dose-dependent increase in mutation frequency and long term persistence (years) has been observed in subjects exposed to high doses (>1 Gy), such as A-bomb survivors and radiation accident victims. However, the assay is not able to detect exposure to low doses [75]. In addition, control populations show considerable variability of the variant frequency. Although the GYPA assay has many advantageous features such as low cost and speed, and is relatively unaffected by environmental factors, it lacks the possibility of in vitro dose calibration and is applicable to only half of any study population [152].

#### 4.4.2. Hypoxanthine-guanine phosphoribosyl transferase gene

Hypoxanthine-guanine phosphoribosyl transferase (HPRTase) is a protein implicated in the salvage of adenines and guanines from degraded DNA. Mutations in the *HPRT* gene after irradiation can be easily detected in cultivated cells by adding the toxic nucleoside analog 6-thioguanine (6-TG) because, unlike wild type cells, mutated cells are able to survive in this otherwise toxic medium. The *HPRT* somatic mutation assay has been frequently used to investigate the mutagenic effects of IR in populations occupationally or environmentally exposed to low dose radiation [75]. A study on Russian Chernobyl Clean-up workers aimed at investigating *HPRT* mutation in lymphocytes nine years after exposure observed that *HPRT* mutant frequency was sensitive enough to assess past exposure to low doses [153]. However, because it declines over time, this biomarker was not considered as a suitable retrospective biodosimeter (stable for months) [75]. More recently, *HPRT* has been used to investigate in vitro the adaptive response of lymphoblastoid cell lines primed with low doses of X-rays or heavy-ion radiation [154,155]. The mutation frequency of cells primed with low doses of X-rays (20–100 mGy) was reported to be similar to the mutation frequency of the non exposed cells, whereas for cells primed with low doses of heavy ion radiation (10 mGy), the differences were more marked, depending on the cell line. In the framework of a large scale epidemiological study, the use of *HPRT* mutations as a biomarker would be strongly limited by the technical requirements and the time necessary to select the mutants.

### 4.5. Biomarkers related to transcriptional and translational changes

Biomarkers related to transcriptional and translational changes are summarised in Table 4.

#### 4.5.1. Biomarkers related to changes in RNA levels

While a wide variety of transcriptional responses to IR have been described, the relationship of these responses to health is rarely clear. In most cases, gene expression profiling has been performed after a short time following exposure. Several studies have been published that indicate that microarray based analysis of early transcriptional responses may predict early or late developing adverse normal tissue reactions to radiotherapy [156,157]. Furthermore, simple quantitative real-time PCR assays hold some promise as being predictive for normal tissue reactions [158].

With the advent of microarray technology during the late 1990s it soon became possible to analyze the mRNA expression of all known genes simultaneously. Later, microarrays that cover all known exons were developed, providing increased accuracy but also allowing analysis of transcript and splice variation. Nowadays, more and more high-throughput studies are using NGS technology to sequence entire transcriptomes (RNAseq). Since the costs of these technologies are dropping rapidly, it is expected that microarrays will become obsolete for gene expression analysis in the near future. In addition, some comparative studies suggest that quantitative PCR and direct molecule counting methods such as Nanostring™ hold advantages compared to microarrays [159]. Still, the use of microarray technology has provided significant advantages in the search for mRNA biomarkers of radiation exposure as well as for the understanding of the biological pathways that are involved in the radiation response.

*4.5.1.1. ATM/CHK2/p53 pathway.* Several examples of potential candidate approach biomarkers of IR can be found among the transcripts involved in the ATM/CHK2/p53 pathway (also discussed in Section 4.3.2). ATM is a kinase which is activated by DSBs and which phosphorylates and activates p53 directly or via the phosphorylation of the kinase CHK2. Many of the transcriptional responses to IR are p53 dependent and p53 is known to be a key tumour susceptibility gene. Assays using human blood and cells have suggested that transcriptional assessment of the ATM/CHK2/p53 pathway function in vitro could serve as a biomarker of cancer risk [146] but a larger validation study is required and there is a need to evaluate confounding factors [160]. Moreover, the ATM/CHK2/p53 pathway is an early responsive-genes mechanism able to predict exposure to radiation after few hours to few days, but not long time after exposure such as months or years.

One of the earliest microarrays studies investigating the effect of low to moderate doses of γ-rays (0.2–2 Gy) found a dose-dependent induction of a number of genes in isolated human peripheral blood lymphocytes up to three days after irradiation [161]. Most of these genes were involved in p53-regulated pathways such as DNA damage repair, cell cycle arrest and apoptosis. However, the effect of radiation was much less pronounced at the later time points, indicating a decay of the DNA damage signal. Since then, several other studies have confirmed these findings often using different methodologies (different microarray platforms, different doses, radiation quality, or cell types) and although they often found different genes to be differentially expressed after irradiation, all of them found a p53-regulated response which was attenuated with time [162–164]. This indicates that, at least at early time points (<24 h), the p53-regulated response is pivotal providing several possible biomarkers (e.g. CDKN1A, DDB2, CCNG1, PCNA, TNFRSF10B, GADD45A,...) for early exposure to a large range of radiation doses.

Over the past few years, gene expression profiles, obtained using microarray technology, have also been used for biodosimetry purposes. Most of these studies used blood cells (peripheral blood mononuclear cells, whole blood, cultured lymphocytes) for radiation doses between 0.5 and 10 Gy, over a time period between 4 and 24 h. Again, from these studies it is evident that genes involved in p53-regulated pathways are the best suited predictors of radiation exposure [160,164–169]. Whether this would also be the case for other cell types or tissues, remains to be investigated.

Thus, it seems that changes in gene expression of certain genes are very well suited to estimate radiation exposure, potentially providing several advantages over the more classical cytogenetic assays which are time consuming and labour intensive. First, the expression analysis of limited numbers of genes is easy to perform, quick, and cheap. Second, these assays need only a limited amount of material (blood, RNA) as shown by a recent study in which a biodosimetry device was used to analyze gene expression from small blood volumes (30 μl) [165]. However, in the context of an epidemiological study, an important disadvantage is that the effects on gene expression seem to be short-lived and whether changes that are specific to radiation will be identified remains to be established. Thus, whether changes in gene expression may be suited for dose estimation years after the exposure needs further investigation although samples stored appropriately soon after exposure could be investigated at later times.

Expression profiling has also been used to try and identify finger-prints of radiation induced cancers in particular as they often have no specific histological characteristics. Under such circumstance an expression profile that could be used to confirm the aetiology of a tumour would be an enormous benefit to epidemiological studies. Recent publications have suggested that gene expression profiles can discriminate sporadic from post-radiotherapy-induced thyroid carcinomas [170] and sarcomas [171]. The validation and the assessment of the robustness of these molecular markers that could represent a radiation-induction signature is essential and could help identify the specific molecular pathways deregulated in radiation-induced cancers and lead to the development of new biomarkers of exposure and susceptibility.

*4.5.2. Biomarkers related to changes in protein levels*

Different organs respond to radiation by altering the level of protein expression and their post-translational modification status. Therefore, it is reasonable to believe that protein expression profiling can be used to successfully find radiation-associated protein biomarkers in biological samples such as urine, blood/serum or even tissue. The usage of tissue samples has been limited mostly to radiation-induced cancers. The primary advantage of using tissue is that protein expression is tissue-specific and some of the biomarkers will be maximally expressed in the targeted tissue. The obvious primary disadvantage is the difficulty in obtaining tissue specimens by biopsy or autopsy. In contrast, protein-rich samples of biological fluids – such as serum, urine or saliva – can be collected in a non-invasive or semi-invasive manner, and quantification of radiation-induced protein expression can be automated using high-throughput proteomic technologies. In addition, both pre-separation methods and mass spectrometers have seen remarkable and consistent improvements over the past decade [172].

However, the discovery of radiation-associated protein biomarkers remains an enormous challenge within radiobiological research because of the time- and dose-dependent variation of protein expression. Animal and cellular studies have been used as a tool to identify potential biomarkers that then may be tested in molecular epidemiological studies [173–177]. Marchetti et al. conducted a literature review of candidate protein biomarkers for individual radiation biodosimetry of exposure to IR [178]. The study included human, animal and cellular studies using in vivo and in vitro irradiation. All identified radiation-responsive proteins, 261 in total, including 173 human proteins, were tabulated and assigned to nine priority groups. This method resulted in a proposed panel of 20 candidate protein biomarkers for different doses and time points after exposure. Since the study by Marchetti (2006) some new data concerning radiation-induced changes in different proteomes have been published. Concentrating only on studies with human material we summarised the recent studies, adding the human data from Marchetti et al.; the radiation-responsive proteins identified in these studies are shown in Supplementary Data A.

*4.5.2.1. Amylase, Flt3-ligand and C-reactive protein (CRP).* So far, the changes in the level of only two proteins have been used as bioindicators for radiation exposure: the amylase that indicates radiation-induced damage of the parotid gland [179,180], and Flt3-ligand, a hematopoietic cytokine indicating damage of the bone marrow [181]. Both are relatively easy to measure from serum/plasma using a clinical blood chemistry analyser or commercial sandwich enzyme-linked immunosorbent assay (ELISA), respectively. However, analyzed separately these biomarkers are neither specific nor sensitive enough to estimate the received dose, especially if the time from the exposure exceeds 48 h.

The variation in the level of CRP, a protein secreted by hepatocytes in response to inflammation, provides an accurate estimation of the level of inflammation, but it is not specific to radiation exposure or a particular disease; for instance, it is modulated in case of cancer, rheumatoid arthritis, cardiovascular diseases, or upon high doses of IR. An epidemiological study conducted in the A-bomb survivors cohort 50 years after the

original exposure, has shown a persistent increase of 28% in the level of CRP per Gy in persons exposed [182]. However in this study, the frequency of persons exposed to low dose radiation was not detailed (medium–low dose group: 0.005–1.5 Gy, mean ± SD: 0.66 Gy ± 0.45, $n$ = 164). In the context of an epidemiological study, it is interesting to note that CRP can be measured by high-sensitivity ELISA in saliva, a less invasive method than venipuncture [183]. However, this assay has still to be validated, in particular for low concentrations of CRP.

*4.5.2.2. Changes in protein levels identified by proteomics.* Proteomic tools combined with statistical and bioinformatics methods are able to discriminate several classes of samples, such as non-exposed and exposed, based on a few variables. In the classical gel-based proteomic analysis such as two-dimensional differential in-gel electrophoresis (2D-DIGE) protein quantification follows after a separation based on the charge and molecular mass. Proteins labelled with fluorescent dyes (Cy2, Cy3, Cy5) are analyzed in a laser-activated fluorescence scanner. Coupled with mass spectrometry this method is able to find radiation-induced alterations in protein expression levels and/or post-translational modifications. The gel-free tandem mass spectrometry (LC-MS/MS) method is based on the peptide quantification and is often combined with different labelling methods on a cellular level (Stable Isotope Labelling with Amino acids in Cell culture, SILAC), protein level (Isotope Coded Protein Labelling, ICPL) or peptide level (Isobaric Tag for Relative and Absolute Quantitation, iTRAQ)[184]. Although superior in sensitivity, gel-free methods rely almost exclusively on evaluating protein abundance as the criteria for finding biomarkers; it is likely that many biomarkers are post-translationally modified, and are therefore not being identified in LC-MS/MS studies. For high throughput studies the surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) technology remains a very promising approach with clinical applications [185], but exactly what is detected is not clear.

*4.5.3. Transcriptional and translational changes in cytokines*

In addition to the Flt3-ligand mentioned above, studies conducted during the last decade have provided additional evidence of the involvement of cytokines in response to IR. These low molecular weight glycoproteins are mainly produced by immune cells and play a central role in the mediation and regulation of immunity, through driving the development and activation of immune cells, inflammation, and hematopoiesis [186]. They are known to function in the form of networks and are central players in cell-cell communication [186,187]. Cytokine signalling pathways can activate a range of final cellular impacts including apoptosis, inhibitors of apoptosis, protein synthesis and cell growth, and the inflammatory response. They can also significantly impact upon ROS generation and activation of antioxidants (reviewed in Section 4.7.1). Interestingly, there is also evidence that these pathways can impact upon damage response signalling and/or DNA repair [188,189], processes described elsewhere in this review.

Cytokines produced due to radiation-induced DNA damage are thought to play a key role in the inflammatory reactions that are associated with ionizing or UV radiation [190]. In general, they cause a broad range of effects that are relatively tissue-specific. They can impact on the microenvironment around the damaged cell/tissue by affecting the surrounding stroma, epithelial composition and growth stimulation. TGFβ for example acts via autocrine, paracrine and endocrine mechanisms, and, depending on the environment, can exert either tumour suppressor or tumour promoter activity. Indeed TGFβ orchestrates a complex network of cellular response to radiation exposure functioning both as a sensor and signal transducer of radiation induced stress. TGFβ signalling is efficiently activated after radiation exposure and appears to be particularly important in regulating the normal tissue response after radiation therapy [191,192].

At low dose and low dose rates of IR, cytokines play an important role in the establishment of bystander effects (i.e. response of cells that were not directly irradiated but were near irradiated cells or in contact with irradiated cell medium). In a bystander cell model using normal human lung fibroblasts (HFL-1) and testing low doses of high LET radiation, TGFβ1 contributed to increase DSBs [193]. In a study conducted by Ojima et al. (2008) [194], a supra-linear dose–response was observed in normal human fibroblasts irradiated with doses from 1.2 to 200 mGy. This hypersensitivity was ascribed to bystander effects. The mechanisms behind them were attributed to ROS, nitric oxide and TGFβ1. These factors are thought to be released in the medium of irradiated cells and cause DSBs in non-irradiated cells cultured in the same medium. However the dose/response relationship of these changes is clearly complicated. IL6, IL8, MCP-1 and RANTES were identified in the growth medium of human dermal fibroblasts after exposure to 2 Gy and IL-6 was reported to promote γH2AX foci formation whilst MCP-1 caused a drop in spot occupancy (i.e. fraction of the nuclear area covered by γH2AX spots) [195]. Finally, microarray experiments using blood collected from donors exposed to chronic low dose radiation following the Chernobyl accident (range: 0.18–49 mSv during 11–13 years) showed a transcriptional modulation of several cytokines including TNF-α/β, IL-1β, IL-2, IL-10, IL-12β, chemokines (IL-8), macrophage colony-stimulating factor (M-CSF), and apoptosis-inducing receptors [196].

Overall, these findings suggest that alterations in the transcriptional profile of certain cytokines might be suitable as biomarkers of exposure to low dose radiation. However further validation, including the validation of statistical methods used to analyze the data from microarrays, is required to use them as such. The specificity of such changes as a marker of radiation exposure, and not just a marker of altered oxidative stress, needs to be fully evaluated as well as the impact of confounding factors such as viral infections, immune deficiency and age.

*4.6. Biomarkers related to epigenomic modifications*

Exposure to radiation has long been known to result in epigenomic modifications which will affect gene regulation after DNA damage induction. Through various check-points, epigenomic modifications allow a very tight control of the amount of mRNAs and proteins in the cell. First, the initiation of the transcription of genomic DNA can be affected by the remodelling of the chromatin structure via histone modifications. Second, given that transcription regulation requires access of transcription factors for activation or repression and splicing, DNA methylation may compromise the transcription by preventing the binding of transcription factors to the promoter region of the gene. Third, additional check-points exist via miRNA which can bind to transcribed mRNAs preventing their translation into proteins. Other check-points exist also at the protein level (e.g. protein phosphorylation), allowing a more specific functional modulation.

After DNA damage, post transcriptional regulation by miRNA occurs at later time points than the fast (sec/min) but often transient post-translational protein modifications, such as phosphorylation and ubiquitination, but before transcriptional regulation changes (hours/days)[197]. The time-scales of these changes and their stability will impact on their usefulness as biomarkers in molecular epidemiological studies.

*4.6.1. Histone modifications*

Histones are considered as key players in epigenetics because of their specific properties that can regulate gene expression primarily by influencing the structure of the chromatin. Like most

other proteins, histones are also subject to post-translational modifications, especially, but not exclusively, on their C-terminal tails. At least eight different types of covalent histone modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination and proline isomerization) can occur and they often occur on different residues simultaneously. Recently, no less than 51 distinct "chromatin states" with different biological roles were identified in human T lymphocytes based on the existence of specific combinations of histone modifications [198,199]. The changes in the profile of these modifications in response to IR are likely to be dose- and time-dependent and whether the various modifications are inter-related or mutually exclusive, remains to be fully established.

The function of histone modifications is exerted mainly through two distinct mechanisms: influencing the overall structure of the chromatin, which can occur over short or long distances, or by modulating the binding of effector molecules [200]. The effects of histone modifications have implications not only for gene transcription, but also for DNA repair, replication and recombination.

Probably the best known histone modification that occurs in response to radiation-induced DNA damage is the phosphorylation of the serine-139 residue of histone H2AX (γH2AX) near the site of the damage. The use of γH2AX as a biomarker of DNA DSBs has been discussed in Section 4.2.2. The main mechanisms by which histone modifications influence DNA repair are via the recruitment of DSB repair factors, as in the case of phosphorylation or sumoylation of histones, or by modifying the chromatin structure to render the DNA more or less accessible to the DNA repair machinery. In general, methylation of histones, which occurs mainly on lysine and arginine residues, leads to a more compact conformation of the chromatin, making the DNA less accessible to transcription factors but also less accessible to DNA repair factors. Recent studies have shown that methylation of several histone residues is induced by high doses of IR but the IR specificity of such changes is not well described [201,202]. Acetylation of histone tails, which is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), mainly renders the DNA more accessible to the transcription machinery and therefore promotes transcriptionally active euchromatin. However, changes in chromatin conformation also affect the accessibility to other proteins with a DNA-based function such as DNA repair proteins and several lines of evidence have demonstrated that histone acetylation of different histone residues (e.g. H4K16 and H3K56) plays an important role in modulating DNA damage repair mechanisms induced by moderate to high doses of IR [203,204].

Overall, the published literature on the effects of irradiation on methylation, acetylation, ubiquitination and other post-translational modifications of histones almost exclusively reports studies that have investigated the effects of high doses using in vitro models ([200,202,204–209] and Supplementary Data B) and clearly not all the players in these processes have yet been fully characterised and others probably remain to be identified. It is clear that further studies are needed before assessing whether these epigenomic changes can serve as possible biomarkers for epidemiological studies on the effects of low dose exposure to IR in humans. In particular, for use as biomarkers of exposure, the relationship between the doses received and the profile and extent of histone modification and its persistence need to be established, as well as the specificity of any changes in terms of the spectra of agents that will induce the modifications. It is possible that, as for γH2AX, a limited persistence of the signal after irradiation and the fact that several types of DNA damaging agents can produce the modification, will make them unsuitable for retrospective studies but they may prove to be useful biomarkers of susceptibility or persistent effects. Significant advances in understanding the effects of IR on epigenetic states are required before fully validated biomarkers become available.

#### 4.6.2. DNA methylation

DNA methylation consists of the covalent addition of a methyl group (–CH3) to a cytosine, mainly in a CpG dinucleotide. This heritable DNA modification induces a local change in the chromatin structure which ultimately leads to the modulation of transcription, most frequently repression. DNA methylation is a key component in the control of gene expression in specific tissues and the germline and its involvement in carcinogenesis is well known [210,211]. Several studies have shown that exposure to IR can lead to both hypo- and hypermethylation [210,212,213]. Low-dose radiation can regulate DNA methylation, which in turn can affect low-dose radiation responses such as bystander effects or genomic instability [214].

However, given the variability of the results of studies on the methylation status of DNA after irradiation, in particular at low-dose ranges, it is unlikely that currently DNA methylation would be a suitable biomarker of exposure, effect or susceptibility in an epidemiological molecular study addressing the effects of radiation exposure. Further studies are needed to confirm at least the changes induced by different doses and/or radiation quality of irradiation and the impact of this change (hypo- or hypermethylation) on gene expression, as well as the effect of confounding factors such as age and other exposures. Recent technological advances such as Methyl-CpG binding domain protein sequencing (MBD-seq) that is widely used to survey DNA methylation patterns should help resolve these issues [215]. In addition based on promising results in the cancer detection field, the possibility of measuring the methylation pattern of candidate genes in biological samples as a biomarker of cancer risk needs to be fully evaluated [216,217].

#### 4.6.3. miRNA

miRNA are small (around 22 nucleotides) highly conserved non-coding RNAs that bind to mRNA and promote mRNA degradation or altered translation. They are involved in post-transcriptional regulation of gene expression and play a role in numerous physiologic processes in normal and malignant cells. The efficiency of miRNA-mRNA pairing, and consequently gene expression levels, can be affected by miRNA levels and also by SNPs (see also Section 4.3.1) in miRNA genes as well as SNPs located in miRNA binding sites of mRNA 3′UTRs [218]. MiRNA expression profiles are very tissue specific and tumours show highly specific expression signatures which can be correlated with tumour state and tumour prognosis [219,220]. There are many recent studies that have shown that their expression profiles can be modulated following exposure to both low and high LET irradiations and it has been suggested that these miRNA expression signatures can be used as biomarkers of radiation exposure (see recent reviews [197,210,214]). Templin et al. reported that 4 h after in vivo exposure of radiotherapy patients to 1.25 Gy of IR an upregulation of the expression of a considerable proportion of the human miRNAome of peripheral blood cells was detected [221]. Interestingly, among all the genes controlled by miRNAs under these experimental conditions, they found that biological processes such as hemopoiesis and the immune response were targeted by miRNA control whereas metabolic processes were under-represented. In contrast Aypar et al. showed that in GM10115 cells only three miRNA involved in two major pathways were altered after high LET irradiations while six miRNA involved in five major pathways were altered after low LET irradiations [213]. A number of DNA damage responsive miRNAs that regulate many cellular processes and pathways have been described [197]. For instance the miRNA-138 modulates DNA damage responses by repressing histone H2AX

expression [222] (the phosphorylation of which has been proposed as a biomarker of the formation of DNA DSB; see Section 4.2.2). However as discussed by Woulters et al., it is hard to extract common miRNA responses from such studies since different microarray platforms, types of radiation, doses and dose-rates have been used, which may all influence outcome [197]. In addition, as miRNA levels are themselves modulated both by genetic and epigenetic alterations, a complex interplay exists between the miRNAs and their targets which appears to be cell type specific. The use of the expression profile of miRNAs as biomarkers is very much in the developmental stages and clearly more information on sensitivity, specificity to IR, the timing of expression changes after exposure and the biological material in which such changes can be measured is necessary.

#### 4.6.4. Phosphoproteome

Of the post-translational modifications, phosphorylation is essential in signal transduction, gene regulation, and metabolic control in cells, especially in response to intracellular and extracellular changes and stimuli. Therefore the identification of phosphoproteins, which phosphorylation sites regulate protein function, and the upstream signalling kinases involved in this modification will provide valuable insight into the molecular mechanisms that regulate the cellular responses to IR. The analysis of the phosphoproteome can be achieved by using immunohistochemical based techniques such as reverse phase protein arrays that typically characterise one phosphoprotein, and often only one specific phosphorylation site, at a time. Such techniques are hampered by the availability of validated antibodies, tissue handling variability, and intratumoral heterogeneity. However, in the clinical setting this technique has shown great promise by, for instance, identifying dysregulated signalling pathways in tumours, providing insight into patient-specific differences that may impact on clinical outcome [223,224]. Recent technological advances in LC-MS technology now also enable the broad proteome-wide study of phosphorylation (phosphoproteomics) and allow the identification of thousands of phosphorylation sites (and often multiple sites in an individual protein) in a particular proteome; such an approach has been used to examine the phosphoproteome of primary human skin fibroblasts 1 h after exposure to 20 and 500 mGy [225]. Phosphorylation sites on proteins such as TP53BP1 but also on previously unidentified radiation-responsive proteins such as the candidate tumor suppressor SASH1 were identified [225]. Those results show the potential of such an analysis to identify radiation-induced post-translational modification but in terms of validation for use of a biomarker of any category many questions remain and their applicability to biological samples collected in a molecular epidemiological setting will need validating.

### 4.7. Other biomarkers

#### 4.7.1. Reactive oxygen species

ROS are molecules or ions, such as superoxide, peroxide or hydroxyl radicals that result from the interaction of an electron with an oxygen molecule. IR can lead directly to their production, creating directly induced (or targeted) DNA damage or damages to the nucleotide pool as mentioned in Section 4.2.3. However, a plethora of evidence has shown that IR can also lead to the activation of ROS indirectly via a range of signalling processes, release of ROS from the mitochondria or changes to the cell's microenvironment, including the activation of an inflammatory response. Frequently, the antioxidant capacity of a cell adapts to ROS levels change, so that the steady state level of ROS and/or ROS damage is minimised. If we define oxidative stress as a situation where the generation of ROS exceeds the antioxidant capacity of a cell, then IR can also lead to oxidative stress. Furthermore, oxidative stress itself can lead to a situation where ROS levels increase, further confounding the situation. In addition to ROS, reactive nitrogen species such as peroxynitrite anions ($ONOO^-$) can form through interaction of nitric oxide with superoxide anions.

The precise signal that initiates stress response signalling is unclear. However, there is evidence that extranuclear targets can mediate genotoxic effects of radiation. The bystander response (also briefly discussed in Section 4.5.3) demonstrates that signalling can be relayed from a damaged cell to an undamaged cell, demonstrating that the target might be extracellular. Additionally, oxidants, as well as other free radicals, can react with lipids, proteins and DNA. For example, lipid peroxyl radicals and their decomposition products and intermediates of lipid peroxidation can lead to the generation of reactive aldehydes including 4-hydroxynonenal (4-HNE). In general, ROS are short lived and therefore not suitable as a biomarker but there are examples where elevated ROS production can be maintained for some considerable time after radiation exposure, and elevated levels of ROS have been reported after IR. Assays to monitor ROS levels are unlikely to be useful for biomonitoring purposes due to the short half life of ROS and the fact that the response is not specific to radiation exposure. The products of ROS damage, such as protein and lipid oxidation products have the potential to be exploited as biomarkers. Some possible products are listed in Supplementary Data C. However, as for monitoring ROS levels, there is likely to be a lack of specificity for IR. A recent review has considered the range of oxidatively generated base damage to cellular DNA and considered procedures to measure such damage [226]. Again, however, this is unlikely to have major application for biomonitoring purposes due to their short half life. The activation of antioxidant levels can represent a potential biomarker although again such activation can be transient. Further, the tentative biomarkers of oxidative stress listed in Supplementary Data C need with a few exceptions to be further characterized regarding dose–response relations, persistence in time and specificity. As reviewed above most of these biomarkers are likely to be induced by several types of stressors. A possible route to improve the situation is to combine a set of these biomarkers.

#### 4.7.2. Metabolites and metabolomics

Metabolomics is a recent "omics" technology that focuses on the study of metabolites, which are usually defined as small molecules with a mass less than 1 kDa. Although not exhaustive, many metabolite databases – e.g. Human Metabolome database (http://www.hmdb.ca/), Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/), METLIN Metabolite Database (http://metlin.scripps.edu/) – are available [227]. Metabolomics methodologies which mainly involve chromatography and mass spectrometry-based techniques, have been recently reviewed elsewhere [228]. Because metabolites result from the interactions between several complex cellular networks, metabolomics can provide a qualitative and quantitative overview of the global perturbations induced by IR in cells and biological fluids. In fact, many of the urinary metabolites observed specifically following radiation exposure represent products resulting from oxidative stress. Studies in rodents using metabolomics have shown an increase of purine and pyrimidine derivatives, such as 2′-deoxyuridine and thymidine, in urine after an exposure of 1–3 Gy of γ rays [229–231]. In humans, a study performed on patients who underwent a 131I-therapy observed an increase of salivary isoprostaglandins, whereas no increase in isoprostane levels in urine was observed in a study of patients treated for prostate cancer [232,233]. By testing the entire metabolome without focusing on a particular metabolite, metabolomics might detect

many other biomarkers of IR in urine or saliva of persons recruited in an epidemiological study. However, currently, there is no evidence that the metabolomics technology is sensitive enough to detect metabolite modulations after low dose radiation in humans.

#### 4.7.3. Biomarkers associated with cell cycle delay, apoptosis and cell survival

The cell cycle is composed of the G1 phase (cell growth), the S phase (replication of the DNA), the G2 phase (preparation to divide) and the mitotic phase (cell division). Exposure to radiation is known to lead to delays in the progression through this cycle and to impact on cell survival; a number of bioassays based on these endpoints have been used to assess radiosensitivity.

A modest radiosensitivity in heterozygous carriers of mutations in the *ATM* gene was identified using a radiation-induced G2 phase cell cycle delay assay in lymphoblastoid cells [234]. A similar assay performed on lymphoblastoid cells (3 Gy) identified a radiosensitive sub-group within breast cancer patients [235]. The association between abnormal radiation-induced cell cycle delay and breast cancer cases has also been confirmed in other studies, although opposite results regarding the direction of changes (reduced or prolonged) in cell cycle delay for cancer patients were reported [236,237]. Finally, several studies, including case-control studies, performed on lymphocytes or lymphoblastoid cells from lung cancer patients have showed a reduced cell cycle delay after irradiation with 1–2.5 Gy [238–240]. Inter-individual variation in radiation-induced cell cycle delay, radiation-induced apoptosis and human population variation in the expression of certain DNA repair, cell cycle and apoptosis genes has been shown to have a significant heritable component [241–243].

Regarding apoptosis, significantly reduced apoptotic responses increasing with age have been observed in breast cancer patient's lymphocytes irradiated with 4 Gy [244] although another study reported an increased radiation-induced apoptotic responses in breast cancer cases' lymphocytes after 5 Gy [237]. A very low induction of apoptosis in AT homozygotes together with a reduced level in AT heterozygotes and in breast cancer patients compared to normal individuals was also observed in a study where lymphocytes were exposed to 4 Gy [245].

Skin fibroblast clonogenic cell survival assays has been used to test in vitro radiosensitivity in recurrence-free breast cancer patients [246]. However the difficulty associated with obtaining fibroblasts from subjects and the time needed to complete the assay (2–3 months) would severely limit the use of such bioassay in a large-scale molecular epidemiological studies.

In summary, it is clear that there is significant inter-individual variation in cell cycle and apoptotic responses following radiation exposure. Bioassays based on these cellular endpoints hold promise as biomarkers of radiation sensitivity. However the data available are not entirely consistent, responses to low dose radiation exposure are poorly characterized and it is likely that some of the assays are very sensitive to experimental conditions. Therefore, further validation will be needed before considering cell cycle delay and apoptosis as biomarkers of IR in large scale epidemiological studies including the careful evaluation of potential confounding factors such as age, smoking status, and ethnicity.

#### 4.7.4. Biophysical markers

The concentration of radicals that are formed by IR and that persist for a sufficiently long time in solid biological materials such as bones, tooth enamel, finger nails and hair can be measured by electron paramagnetic resonance (EPR or alternatively ESR) spectroscopy. EPR dosimetry is a non-destructive spectroscopic technique based on the correlation between the intensity or amplitude of the radiation-induced signals with the dose absorbed for instance in the tooth enamel [247]. A number of potential confounding factors have been identified such as UV exposure and the health status of the teeth (see [247] for an extensive review). The invasive sampling necessary to obtain teeth and bones limits the application of this technique to retrospective dosimetry. However several groups are investigating alternative non-invasive methodology that measure non organic radicals. For instance Trompier F, et al. [248] have investigated the potential of carrying out EPR spectrometry on the plastics that can be found in personal effects such as glasses (CR-39, polycarbonate), mobile phones (PMMA, polycarbonate), watches and buttons, although signal fading would appear to be a limitation for the use of such an approach in epidemiological studies and these personal items should have been in use for many years to provide relevant information about past exposure. In addition transportable EPR spectrometers, developed to facilitate tooth dosimetry in an emergency response setting, have been developed which allow the upper incisors to be used as a dosimeter (see for instance [249]). However the lower limits of detection could be a limiting factor outside an emergency setting where EPR tooth dosimetry is likely to be a valuable resource for triage following potential radiation exposure of a large population.

### 4.8. Biological determination of exposure/intake of internal emitters

Due to the nature of emitted radiations and to the biokinetics of dose distribution from internally incorporated radionuclides, estimation of dose from internal emitters poses particular challenges and biological monitoring has long been used in occupational, medical and accidental settings to provide a qualitative indication of exposure or a quantitative assessment of absorbed dose to a specific organ or tissue over a specific time period.

The body content of radionuclides can be measured directly in vivo using detectors set outside the body. The energy spectrum indicates the isotopic composition, while the counting rate is converted into activity through calibration with anthropomorphic phantoms. Such techniques provide a quick and convenient estimate of activities in the body or in a specific organ such as the thyroid for iodine or the lung for insoluble aerosols. This, especially when carried out with dedicated mobile units, is the best measurement technique immediately after a major accident when multiple measurements have to be performed (for example cohorts of children with measured thyroid activity have been established and followed in Belarus and Ukraine [250,251] and extensive measurements have been made in the regions most affected by the Fukushima accident in Japan) in the same area over a short period of time, and for the monitoring of short lived radionuclides such as radiopharmaceuticals used in nuclear medicine. However this approach is feasible only for radionuclides emitting radiation that can "escape from" the body: X-rays, $\gamma$ or energetic $\beta$ particles but not pure $\alpha$ or low energy $\beta$ emitters.

In vitro or indirect measurement involves the analysis of excreta or other biological samples (nose blow, nasal smear, saliva, blood or biopsy). This is the only measurement approach for radionuclides which emit no penetrating radiation. Periodic excreta (in particular urine) measurements have been widely used since the 1950s to monitor intake of radionuclides in occupational settings because of their high sensitivity and applicability to any radionuclide. Retrospective individual dose reconstruction has been conducted from historical urine measurements in several epidemiological studies, including the case-control study of lung cancer and leukaemia risk in Belgium, French and UK uranium and plutonium workers in Alpha-Risk [252,253], studies of plutonium workers at the Mayak Production Association [254], of tritium exposure in CANDU reactors in Canada [255] and

**Table 5**
Temporal classification of IR biomarkers.

| Biological classification of IR biomarkers | | Temporal classification of IR biomarkers | | | |
|---|---|---|---|---|---|
| | | Exposure | Susceptibility | Late effects | Persistent effects |
| Cytogenetics | Dicentrics | √ | P | P[a] | P |
| | Translocations | √ | P | P[a] | √ |
| | CCR | √ (high LET IR) | P | P[a] | √ |
| | PCC rings and fragments | √ | | | |
| | Telomere length | P | P | P[a] | P |
| | Micronuclei | √ | P | P[a] | |
| Nucleotide pool damage and DNA damage | SSB/DSB | √ | P | | |
| | γ-H2AX | √ | P | P | P |
| | Extracellular 8-oxo-dG | (oxidative stress) | P | | |
| Germline inherited mutations/variants and induced mutations | SNP, CNV and inherited gene mutations | | √ | P (minisatellites in offspring) | P |
| | CNA | P | | | P |
| | GYPA | √ | | | √ |
| | *HPRT* | √ | | | √ |
| Transcriptional and translational changes | Changes in the mRNA levels of the ATM/CHK2/p53 pathway | √ | P | | |
| | Changes in RNAs identified by transcriptomics | √ | P | P | P |
| | Serum amylase | √ | | | |
| | CRP | √ | | | √ |
| | Proteins identified by proteomics | P | P | P | P |
| | Cytokines | P | P | P | P |
| Epigenomic modifications | Histone modifications | P | P | P | P |
| | DNA methylation | P | P | P | P |
| | miRNA | √ | P | P | P |
| | Phosphoproteomics | P | P | | |
| Other biomarkers | ROS | √ | P | P | P |
| | Metabolites and metabolomic | √ | P | P | P |
| | Cell cycle delay, apoptosis and survival | P | P | | |
| Direct dosimetry on samples | EPR/ESR | √ | | | √ |
| | Internal emitters | √ | | | |

√: direct evidence that this biomarker could be used as such; P: potential or theoretical use; CCR: complex chromosomal rearrangement; CNA: copy number alteration; CNV: copy number variant; CRP: c-reactive protein; DSB: double strand break; EPR/ESR: electron paramagnetic resonance/electron spin resonance; GYPA: glycophorin A; *HPRT*: hypoxanthine-guanine-phosphoribosyl transferase; IR: ionizing radiation; miRNA: microRNA; PCC: premature chromosome condensation; SNP: single nucleotide polymorphism; SSB: single strand break; ROS: reactive oxygen species.
[a] Chromosomal aberrations due to genomic instability.

at the Savannah River site in the US [256] and of radionuclide exposure among Rocketdyne/Atomics International workers in the USA [257].

Individual in vivo or in vitro measurement of radionuclide emissions provides direct evidence of exposure. However, measurement results only provide point information on the contamination at a given time. Models, combined with scenarios of exposure including the assumed rate of exposure and the physico-chemical nature of the incorporated material, have to be applied to evaluate the individual intake and dose and to quantitatively assess exposure. If the measurement is performed reasonably soon after intake and if the mode of intake, chemical form of the nuclide and solubility are known, the typical detection limit allows the assessment of doses down to 1 mSv and even below in some situations. Generally for all measurement techniques, the sensitivity in terms of dose assessment decreases with time after exposure due to radioactive decay and biological clearance. The precision of the assessed dose strongly relies, however, on the information available on the exposure (assessed doses can vary by an order of magnitude or more for Pu depending on solubility) and individual physiology as well as on the number and combination of measurement data on individuals. In the case of chronic exposure, the sensitivity and quality of the dose reconstruction will depend directly on the timing of measurements. The frequency of measurement should be consistent with the radionuclide involved: while a yearly measurement of long-lived and strongly retained plutonium would allow a reasonable estimate of dose, a dose assessment for short-lived radiopharmaceuticals would require daily to monthly measurement [258].

## 5. Discussion

### 5.1. Potential use of biomarkers for epidemiological studies

As reviewed above (Tables 1–4) and summarised in Table 5, there are a number of endpoints that can be assessed in an epidemiological setting using biological samples that could be obtained from a large proportion of a study population with relative ease, and that show potential in one or more of the four categories of biomarkers discussed above: biomarkers of exposure, of susceptibility, of persistent effects and/or of late effects. At this time, the best established and validated markers, at moderate and high doses, are biomarkers of exposure measured shortly after irradiation. There is a need to validate potential biomarkers of exposure, susceptibility, and late and persistent effects at low doses.

As shown in Table 5, some biomarkers may fit in more than one category, depending on the timing of biological sampling. As discussed above, most potential biomarkers (as is the case for biomarkers of other types of exogenous and endogenous exposures

such as UV light and certain chemical carcinogens) are subject to confounding. In addition, with the possible exception of dicentric chromosomal aberrations and of in vivo or in vitro measurement of radiation from internally incorporated radionuclides, it appears unlikely, at present, that biomarkers will be found in the near future that provide an indisputable signature of radiation effects: biomarkers with 100% specificity (only present in case of radiation exposure) and 100% sensitivity (always present in case of radiation exposure).

Nevertheless, what can be gained from the identification of biomarkers (the gain of molecular epidemiology compared to "classical" epidemiology) is an improvement in the quantification of the relationship between radiation exposure and late effects. Compared to already available dosimetric information, exposure biomarkers should allow a better classification of individuals by level of exposure or dose, whereas effect biomarkers should allow a better classification of potentially diseased and healthy individuals. The information provided by biomarkers should help in reducing biases due to misclassification, and therefore, should allow a more precise estimation of the exposure-risk relationship. Also, the identification of biomarkers of susceptibility should facilitate analyses on more homogeneous populations and provide more precise estimations of the exposure-risk relationship in different population subgroups, reducing selection biases and providing information on the variability of risks between individuals, which has important implications for radiation protection particularly in medical and occupational settings.

Serious consideration should therefore be given to the integration of epidemiology and biology through the exploitation of existing samples in epidemiological studies and the joint planning of future molecular epidemiological studies, with careful collection and processing of samples, and for future analyses of biomarkers that will provide answers to key questions in radiation protection research. The identification of predictive biomarkers suitable for large scale analysis requires the investigation of cellular mechanisms that control the cellular and tissue response to low doses of radiation and that thus confer sensitivity. Finding biomarkers of relevance to mechanism identification may require growth or analysis of cells, including cell lines, that may not be suitable for use as a predictive biomarker of susceptibility but could be of use for a limited subset of people, for example in the framework of nested case-control studies of specific diseases.

### 5.2. Possible limitations of molecular epidemiology studies

As in classical epidemiological studies, potential limitations of molecular epidemiological studies concern confounding, bias and random error [259]. Typical confounders or risk modifiers are age, gender and ethnicity. Cancer-related potential confounders include smoking status, exposure to occupational carcinogens, medical treatment, other sources of exposure to IR, and factors related to cardiovascular diseases (i.e. blood pressure, weight, cholesterol, etc.). If no or limited information on such factors is available or confounders are not adequately controlled for in the statistical analyses, confounding cannot be excluded. Uncertainty in the assessment of radiation exposure or dosimetry as well as inaccuracy in the determination of the disease or biomarker under investigation could also lead to bias. Particularly the use of validated biomarkers is an important issue in molecular epidemiological studies.

Most of the published molecular epidemiological studies of low dose radiation effects are small-scaled and suffer from low statistical power. Sufficient statistical power is an essential criterion for an informative study to detect an association of a biomarker with either radiation exposure, persistent or late radiation induced effects or genetic susceptibility, if present. Moreover possible interaction of these biomarkers with other biomarkers or risk modifiers may require even larger studies. The statistical power is determined by several factors such as the study size, the range of radiation exposure, the number of cases and duration of follow-up, but also the ratio of intra-individual variation to inter-individual variation of a biomarker. If the expected effect of a relationship or difference is small, as it may be in the field of radiation research focussing on the low dose range, very large studies are necessary to detect these effects if they exist.

Presently, a large number of "classical" epidemiological studies on IR and cancer exist [260]. Most of these studies deal with the relationship between radiation exposure and cancer risk. The number of studies is appreciably lower for studies on radiation induced non-cancer effects such as cardiovascular diseases or on the late effects of internal contaminations. Most studies are based on either medically (diagnostic or therapeutic), occupationally (e.g. nuclear worker, uranium miner, Mayak worker, Chernobyl liquidators) or environmentally (Mayak, Chernobyl residents, etc.) radiation exposed groups [260]. They differ with respect to type of radiation such as external radiation or internal exposure (plutonium, radon, uranium, etc.) and thus involve different pathways and different radiation related diseases.

A recent review of these studies by some of the co-authors (Pernot et al. in preparation), demonstrated that currently only a limited number of studies have collected and stored biological samples – generally only at one point in time – and that samples may be available only for relatively small proportions of the cohorts, thus limiting the statistical power of the studies. Further, validation of sample collection and storage is needed to ensure that stored samples can be used for analysis of specific biomarkers.

Generally prospective studies have advantages in comparison to retrospective studies because biological samples and dosimetric information are collected before disease of onset, and there is the possibility of getting repeated measurements and biological samples over time. In contrast to this, retrospective studies, in which exposure may have taken place a long time ago, would involve collecting biological samples many years after exposure, and in case-control studies, after the cases have developed the disease of interest—thus possibly affecting the results of the biomarker analyses. Prospective follow-up is useful to study biomarker of exposure, susceptibility and early and late effects as well as their evolution over time. However, starting now prospectively new large molecular epidemiological studies may involve a long duration before the occurrence of the disease under investigation and will involve considerable costs. A much less time and resource consuming approach would be to conduct nested case-control studies within large scale epidemiological cohorts and collect biological samples and reconstruct doses only on cases of the disease of interest and on a limited number of controls. This could reduce the number of subjects (and in parallel study costs) from hundreds of thousands to several hundreds. It would allow the investigation of biomarkers of susceptibility and biomarker of persistent effects and provide important opportunities for biomarker validation.

Another method to overcome the limitation of insufficient power in molecular epidemiological studies is pooling of these studies to increase the study size and the precision of risk estimates. In contrast to meta-analyses, pooled studies use the original individual data, which allow interaction tests and subgroup analyses including dose–response relationships to be performed [261]. However, pooled studies require standardized data on exposure, disease, potential confounders and effect modifiers; and standardized methods for collection and storage of biomarkers to avoid heterogeneity between studies. Next to that, different ethical and data safety rules across the laboratories and countries have to be considered. The legal requirements

needed for molecular epidemiological studies are generally much more complex than those for classical epidemiological studies and can be particularly complex for retrospective sample collection. Informed consent from all study participants should be available in order to use the biological data for future study questions and by other researchers.

Overall, the major limitations in conducting large molecular epidemiological studies are the costs, the accessibility to biological samples, the participation rate of subjects, and logistical difficulties in recruiting sufficiently large number of subjects. In addition, very strict ethical and data safety protection rules could hamper the establishment of molecular epidemiological studies and biobanks as discussed in Section 3.2.

### *5.3. Can biomarkers provide insight into the shape of the dose–response curve?*

One of the goals of molecular epidemiological studies considered in this manuscript is to define the shape of the dose response for radiation induced health effects (for example cancer or cardiovascular disease) following low doses of radiation exposure in order to optimise guidelines governing radiation protection. While the study of the dose-response of specific biomarkers is essential, it should be noted that biomarker yield may not be synonymous with health effect. Some biomarker responses appear to be linear with dose, while others are not. For example, it has been shown that G2/M checkpoint arrest has a defined threshold for its activation, that DSB repair may not function efficiently at low doses and that the bystander effect reaches a plateau after low doses [127,128,262]. For most biomarkers directly related to DNA damage, linear dose–response relationships apply at short times after exposure. In contrast, for biomarkers more dependent on cellular processing of direct damage, more complex linear-quadratic and other relationships may be observed. Thus, the dose-response for a particular health effect (for example cancer induction) is at present difficult to evaluate as it may be the result of a number of parallel processes (DNA damage, repair, epigenetic effects, tissue interaction effects, etc.) which might have different dose-responses. It is therefore essential to study not only the dose-response in the context of specific bioassays but to gain insights into the shape of the dose–response curve for health effects, by interfacing molecular analysis with epidemiological studies.

## 6. Conclusions

When considering suitable biomarkers of IR for use in large scale epidemiological studies, criteria such as sensitivity (in particular for low dose exposure), specificity to IR, persistence, availability of biological samples, technical applicability to large scale and cost should be taken into account. These requirements considerably reduce the number of possible candidates and explain why, currently, there is no ideal biomarker for assessing exposure, effect or susceptibility of low dose radiation exposure. There are some good validated biomarkers for acute radiation exposure to doses above 100 mGy (e.g. dicentrics) but not for lower doses, although some good candidates do exist (e.g. γH2AX) (Table 5). Likewise, there is currently no suitable biomarker of radiation susceptibility after low dose exposures. However, there is clearly the potential to identify suitable genetic biomarkers of susceptibility as large cohorts of exposed individuals do exist which could be used to for instance for GWAS. Technological developments and reduction in their costs will probably help the evaluation of genetic variations (SNPs and CNV) but extrapolating this information for the development and validation of any kind of biomarker is going to be for the future. More generally, the -omics fields are fast moving and such studies may identify endpoints that can be used for different classes of biomarkers. Many of the tests based on transcriptomics methods are criticised and challenged on their ability to predict disease risk over long periods of time. At least one study provides some reason for optimism that transcriptional responses bear a relationship to lifetime disease risk [146]. However further validation of -omics endpoints is required to ensure their specificity to IR and their usefulness as biomarkers of exposure and susceptibility.

Because multiple end points and tissues are involved in the responses to low dose radiation, a multi-marker approach will provide information about the interplay of different possible pathways and will be needed to evaluate an individual's risk. An unbiased multiparametric approach will be needed to identify novel biomarkers [263].

Ultimately, the integration of biology with epidemiology requires careful planning and enhanced discussion among the epidemiology, biology, and dosimetry communities. This will serve to determine the most important questions to be addressed, the appropriate study design and population to be investigated (either occupationally, environmentally or medically exposed). Also essential are the logistics of biological sample collection, processing and storing and the choice of biomarker or bioassay, as well as awareness of potential confounding factors.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrrev.2012.05.003.

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