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Biochemical approach to prediction of multiple organ dysfunction syndrome

L ROY, PhD, J M BERTHO, PhD, M SOUIDI, PhD, M C VOZENIN, PhD, P VOISIN, PhD and M BENDERITTER, PhD

Institut de Radioprotection et de Sûreté Nucléaire, BP 17, F-92262 Fontenay-aux-Roses Cedex, France

Abstract. Variation in many biological parameters can be directly measured in different tissues following radiation exposure. These biological parameters are a useful complement to the sometimes non-existent clinical symptomatology and to the often incomplete physical reconstruction of the accidental radiation exposure situation. For many years, the scoring of unstable chromosome aberrations in peripheral blood lymphocytes was stated as an accurate reference in cases of acute, whole body and homogeneous irradiation. However, most radiation overexposures are heterogeneous, fractionated or their evaluation delayed. New methods were thus added to the conventional cytogenetic ones to set up a multiparametric panel of biological dosimetry. These methods have been developed in the cytogenetic area (translocations, micronuclei, prematurely condensed chromosomes, Qdr, Dolphin) on circulating lymphocytes as well as on resident cells, in order to assess the dose received locally. The dose itself is not sufficient to predict multiple organ dysfunction syndrome (MODS). In fact, at the level of the organ, biological dosimetric indicators should be complemented by bioindicators of prognosis and diagnosis. Some of these indicators appear promising and are presented here in this paper (Flt-3, citrulline, oxysterols); they refer to either structural or functional alterations. The final purpose should be to establish a cartography of the dose received by various organs and to assess damage as soon as possible to predict MODS leading to eventual multiple organ failure.

Introduction

Multiple organ failure was first described in the 1970s when it was pointed out that patients treated in Intensive Care Units following burns or trauma did not die from simple or isolated organ failure but from multiple organ failure (MOF), which is defined as a total or partial loss of two or more organs with vital functions [1]. It is not clear from the clinical signs of highly irradiated people whether the patients really die of radio-induced MOF. However, patients first suffer from multiple organ dysfunction syndrome (MODS). The object of this presentation is to predict these dysfunctions at a cellular level as a complement to clinical signs.

Cases of MODS combined with ionising radiation are linked to high dose exposure. The probability of developing such a syndrome is related to the dose received. It is therefore important to determine this dose as accurately as possible. Today, the most sensitive technique to estimate a biological dose is using the conventional cytogenetic technique based on scoring of dicentrics [2]. An equivalent whole body dose can then be evaluated. It is also possible to quantify dose heterogeneity using mathematical models. These models are used to calculate the dose received by exposed lymphocytes together with the percentage of exposed lymphocytes. However, they do not provide information on the dose received locally by individual organs. Because exposed organs are involved in MODS, one way to predict MODS would be to know the dose received by the organs themselves. This can only be achieved by estimating the dose directly on resident cells, and in this paper this possibility will be presented for the case of skin fibroblasts.

Dose assessment contributes to the diagnosis of radiation exposure. However, in this respect, biological dosemeters are not sufficient in defining functional and physiological consequences of the dose received by the patient. Other biological indicators have to be assessed to improve the diagnosis and to give some indication on the injury prognosis. Indeed, the effects of radiation exposure depend both on intrinsic factors such as radiosensitivity and on external factors such as clinical pathologies. Furthermore, one particularity of MODS is that some organs not directly exposed can be defective and therefore an estimate of dose is not always sufficient. Two main requirements are necessary to improve MODS diagnosis. The assay must be applicable in daily clinical practice and it must be highly correlated with the intensity of organ damage. Our strategy is to develop a multiparametric approach for MODS diagnosis based on the selection of highly specific physiological markers. Plasma FMS-like tyrosine kinase 3 (Flt-3) ligand level is proposed as a biomarker of bone marrow damage, and plasma citrulline level as a biomarker of intestinal mucosa damage. Finally, plasma oxysterols can be considered as specific physiological markers of different organs involved in MODS.

Address correspondence to Laurence Roy, IRSN/DRPH/SRBE, BP 17, 92262 Fontenay-aux roses Cedex, France. E-mail: Laurence.roy@irsn.fr.

Indicators of dose

Whole body dose estimate

In the 1960s, Bender et al [3] proposed the use of dicentrics and centric rings scoring in peripheral blood lymphocytes to estimate the exposure dose. Today this technique is still regarded as the most specific method.

Many studies on animal models and patients treated by radiotherapy indicated a close correlation between the results obtained *in vitro* and *in vivo*. Therefore, dose–effect relationships can be easily established by *in vitro* blood sample irradiation (Figure 1). The slope and shape of the dicentric curves depend on the radiation quality and dose rate, which makes it possible to increase the accuracy of the dose estimate when the physical characteristics of the accident are known. Biological dosimetry by conventional cytogenetics provides its best estimates when the irradiation is whole body and homogeneous [4]. All blood peripheral lymphocytes and lymph nodes are irradiated in a homogeneous way so that the dose found, integrated over the whole body, is regarded as representative of the radiation dose to the bone marrow. The specificity of this technique is secured by the fact that less than 1 dicentric for 1000 lymphocytes is generally found in the normal population [5]. Moreover, this frequency is practically independent of age and sex. Thus, the precision is a function of the number of cells studied, inducing a possible lower limit dose of approximately 0.01 Gy. The yield of dicentrics is limited, as a saturation effect is observed for high doses of ionising radiation (above 5 Gy). In this case, the scoring of centric rings should be more appropriate. This technique was applied at the Tokai-mura accident where the whole body dose of the most injured patient was approximately 15–20 Gy [6]. This required that laboratories involved in biological dosimetry also built a centric ring calibration curve in addition to dicentric dose–effect curves. At this level of dose, the yield of lymphocytes is usually very low, so a chemical agent can be added to culture (calyculine A; VWR, France) to increase DNA condensation and therefore increase the number of analysable lymphocytes [7].

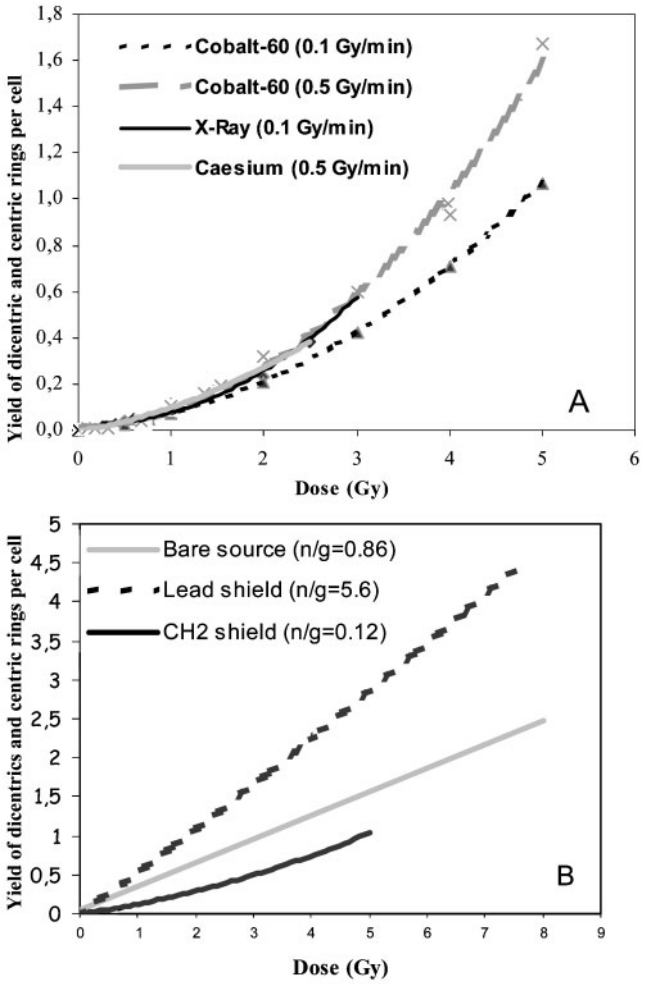

Figure 1. Dose–effect relationships established after *in vitro* exposure of blood to various radiation qualities and dose rates. Dicentrics and centric rings were scored among peripheral blood lymphocytes after Fluorescence Plus Giemsa staining. Calibration curves were calculated by iteratively re-weighted maximum likelihood method. (a) Low-LET (linear energy transfer) exposures: cobalt-60, dose rate 0.5 Gy min^{-1} and 0.1 Gy min^{-1}; caesium-137, dose rate 0.5 Gy min^{-1}; and X-rays, dose rate 0.1 Gy min^{-1}. (b) High-LET exposures at three different fission neutron/γ ratios: 0.86; 5.6; and 0.12.

Heterogeneous dose estimate

When the dose received is distributed heterogeneously throughout the body, the conventional cytogenetic approach is less precise, as the dose is estimated for a mixture of exposed and non-exposed lymphocytes. To get an idea of the dose received by the exposed lymphocytes, mathematical models have been set up [2, 8]. These so-called Qdr [9] and Dolphin [10] models are based on the distribution of dicentrics among cells. Indeed, in the case of a homogeneous exposure, the distribution is expected to follow a Poisson law, which is not true when exposure is heterogeneous. Therefore the aim of this approach is to identify cells that were exposed homogeneously and where aberrations are distributed according to a Poisson law. The result obtained is the dose received by the exposed lymphocytes. It also allows an estimation of the fraction of exposed lymphocytes. This can have an influence as it is related to haematological consequences following exposure. Such a model was applied to the accident at Lia in Georgia in 2001. A strontium source of very strong activity was found by three persons, who carried it on their back for minutes, resulting in severe skin burns. Conventional biological dosimetry was estimated and the corrected results are presented in Table 1. A good correlation was observed between the heterogeneous dose estimate and other indictors of dose such as electron spin resonance (ESR) measurements. One main limitation to this approach is the impossibility of estimating the dose received locally. For this purpose, a cytogenetic dose estimate was developed for resident cells and particularly for skin cells.

Table 1. Results of the dose estimate of two patients of the Lia accident (Georgia) exposed to a strontium source of strong activity. Whole body dose estimate was performed according to standard conventional cytogenetics. In addition, dose heterogeneity was taken into account together with the percentage of estimated exposed lymphocytes according either to the Qdr model or to the Dolphin model

Patient	Whole body dose (Gy)	Dolphin dose (Gy)	Qdr dose (Gy)	% of exposed lymphocytes
DM	3.1	5.4	4.9	72
MG	4.4	5.7	5.7	84

Localised dose estimate

Skin is the first targeted organ in all cases of radiological exposure. We have developed a new method of biodosimetry in the case of localised irradiation based on analysis of skin fibroblast chromosomal changes. Stable translocations were measured with the fluorescent *in situ* hybridisation (FISH) technique. As fibroblast cells are quiescent, a chemical inducer of chromosome condensation, *i.e*. calyculine A, was used to increase significantly the number of analysable cells. A reference curve was built after exposure of human skin explants (excess surgical tissue from resected abdominal skin from healthy patient) to a γ-source of caesium-137.

Our approach was tested on cells isolated from the skin of the same irradiated victim from Lia, Georgia described above. The victim was treated in France at the Percy Hospital (Centre de Traitement des Brûlés) on day 79 post irradiation for a moist desquamation. The lesion was surgically removed at day 88 post irradiation for skin graft. Skin excision of the irradiated area was quickly collected from the surgical unit and was available for cell extraction and cytogenetic analysis. A complete dose cartography (11 different areas) of the irradiated area was thus obtained (Figure 2). The dose range obtained correlated highly with the clinical symptoms and also with the physical dose as obtained by ESR on bone measurements.

Biological indicators of radiation-induced damage

Plasma Flt-3 ligand concentration as a physiological marker of bone marrow damage

Bone marrow syndrome is induced in the range 3–8 Gy and requires several weeks to be expressed as the haematopoietic cells are lost. The challenge here is to propose a simple and sensitive marker of radio-induced bone marrow damage.

Bertho et al [11] proposed the level of Flt-3 ligand in the plasma as a biomarker of radiation-induced aplasia. The Flt-3 ligand is a cytokine that acts mainly on haematopoietic and lymphoid stem and progenitor cell proliferation and differentiation [12]. It has been shown that Flt-3 ligand concentration is increased in the blood of patients with aplastic anaemia or undergoing chemotherapy [13]. Moreover, an inverse correlation between the number of colony-forming cells and the serum concentration of Flt-3 was reported in these patients. These data suggest that the Flt-3 ligand concentration in the peripheral blood might serve as a biomarker for bone marrow damage. According to Bertho et al [11], experiments on different animal models clearly showed that the increase in plasma Flt-3 ligand concentration is directly linked to the radiation-induced damage to the bone marrow, even if the irradiated bone marrow volume was not sufficient to observe severe aplasia. Dose effect, time effect, volume effect and high correlation of this parameter to the intensity of bone marrow damage, as observed on different animal models, make the plasma Flt-3 ligand a useful potential physiological marker for radio-induced bone marrow damage monitoring during the medical management of the irradiated patient. The same group validated this hypothesis by follow-up of 27 patients undergoing radiotherapy [14]. A negative correlation between plasma Flt-3 ligand concentration and the number of circulating white blood cells and platelets during radiotherapy was observed. Moreover, the overall amount of Flt-3 ligand in the blood of these patients correlated directly with both the cumulated radiation dose and the proportion of irradiated bone marrow (Figure 3). These results need to be extended by monitoring the Flt-3 ligand concentration in patients undergoing large field, high-risk radiotherapy or high-risk combined radiochemotherapy. However, these results together are sufficient to include the Flt-3 ligand in the panel of MODS biomarkers as a biomarker of bone marrow damage. Other molecules,

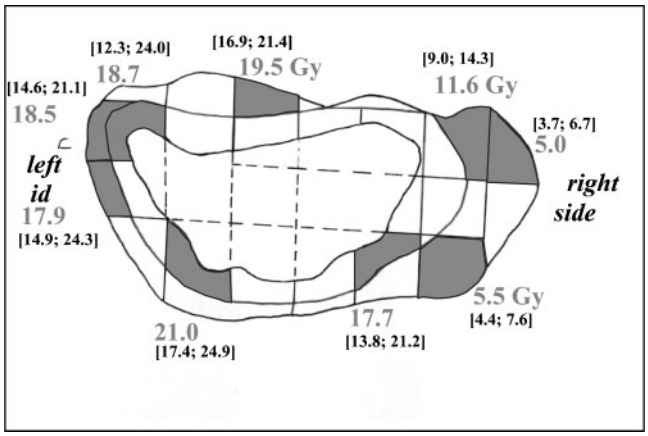

Figure 2. Dose estimates at different positions of a skin biopsy resulting from surgical excision from a patient accidentally exposed to a strontium source of very strong activity (Lia, Georgia, 2001). Values in parentheses are 95% confidence intervals.

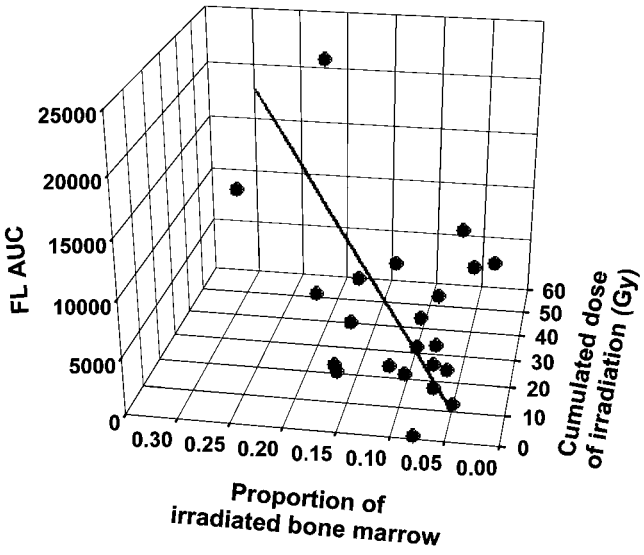

Figure 3. Three-dimensional representation of the multiple linear regression analysis for 22 patients receiving localised radiation therapy. For each patient, the area under the curve (AUC) of Flt-3 ligand concentration (FL) according to the time post irradiation was calculated. The multiple linear regression analysis showed a correlation of AUC for FL with both the cumulated irradiation dose ($p=0.012$) and the proportion of irradiated bone marrow ($p=0.017$).

such as cell adhesion molecules, CD95 or CD95 ligand, were also shown to be increased in the blood of irradiated animals or patients. However, none of these molecules is specific to an organ or a physiological function, whilst variations in the concentration of serum Flt-3 ligand remain specific for damage to the haematopoietic system.

Plasma citrulline concentration as a physiological marker of intestinal mucosa damage

Gastrointestinal syndrome appears approximately 4–10 days after exposure to whole body doses in the range 5–12 Gy and is associated with bloody diarrhoea and breakdown of the gastrointestinal mucosa. The challenge here is to propose simple and sensitive markers of the radio-induced intestinal mucosa damage. Lutgens et al [15] proposed the plasma level of citrulline as a physiological marker enabling quantification and monitoring of epithelial radiation-induced small bowel damage. The small intestinal enterocyte contains specific enzymes, *i.e.* pyrroline-5-carboxylate synthase, involved in citrulline production, making the small bowel the principal source of circulating citrulline. Consequently, according to these authors, the plasma citrulline level is highly dependent upon the intestinal cell mass. The authors clearly demonstrate that plasma level kinetics are dose-dependent and in accordance with radiation injury to the intestinal mucosa. All these experiments were performed after whole body irradiation of Naval Medical Research Institute mice. The data obtained here challenge the use of plasma citrulline as an assay for monitoring epithelial radiation-induced intestinal damage in clinical practice.

Plasma oxysterol concentrations as physiological markers of MODS

We propose plasma concentration of oxysterols 24S-hydroxycholesterol (24S-OH), 27-hydroxycholesterol (27-OH) and 7α-hydroxycholesterol (7-OH) [16] as specific indicators of, respectively, brain, lung and liver damage after radiation exposure. Oxysterols result from tissue-specific enzymatic (cytochrome P450) degradation of cholesterol: CYP46A1 for the brain [17], CYP27A1 for the lung [18, 19] and CYP7A1 for liver [20] (Figure 4). Variations of plasma oxysterol levels were recently proposed as physiological indicators of different pathologies such as neurodegenerative disease [21] or hepatic pathologies [22].

A new clinically practical technique was developed by the Institut de Radioprotection et de Sûreté Nucléaire group to detect plasma concentrations of these three oxysterols simultaneously. This technique is based on high performance liquid chromatography separation after treatment of the hexane-extracted samples by cholesterol oxidase. In a first experiment, plasma oxysterol concentration was measured 3 days after *in vivo* total body exposure of rats (10 Gy, ^{60}Co), at which time metabolism alterations were described (hepatic). A significant decrease in the level of 7-OH is observed together with an increase of 24S-OH when exposed rats are compared with controls (Figure 5). On the other hand, no change in the plasma level of 27-OH after irradiation was observed 3 days post radiation, perhaps because other undamaged tissues (endothelium, macrophages, etc.) also produce 27-OH. We established that variation in plasma 7-OH concentration was highly correlated with CYP7A enzyme activity in the liver, reflecting physiological modifications of liver following radiation exposure.

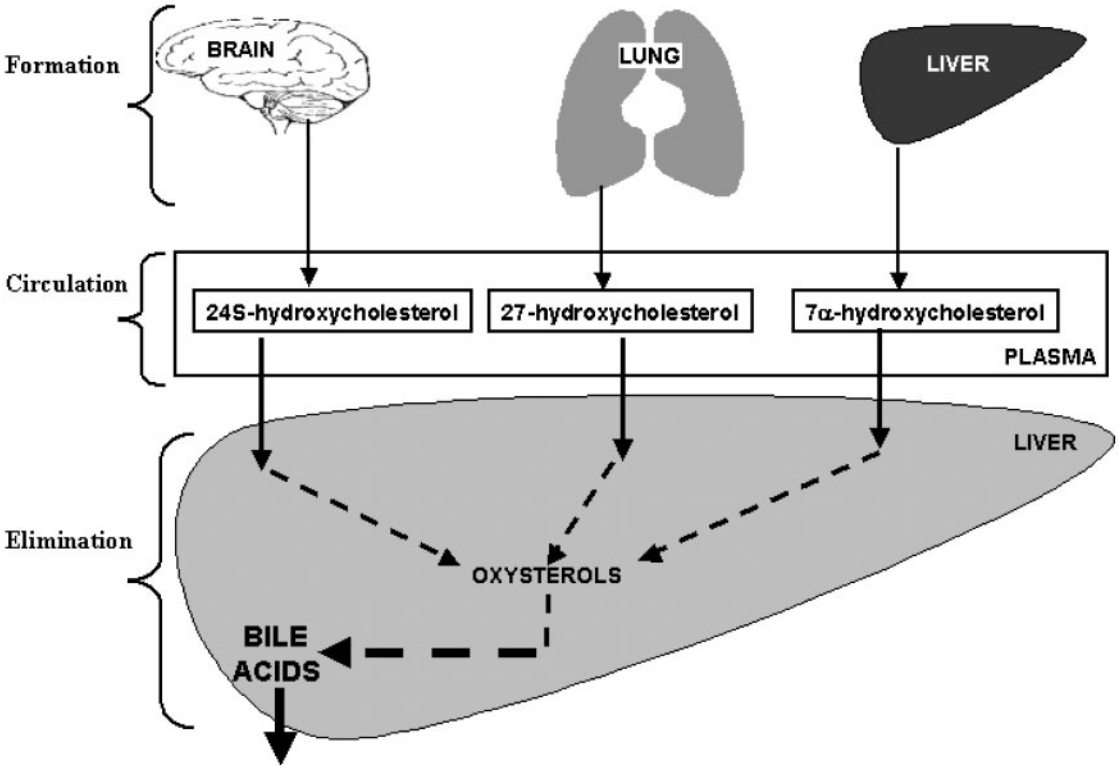

Figure 4. Major plasma oxysterols: origin and elimination.

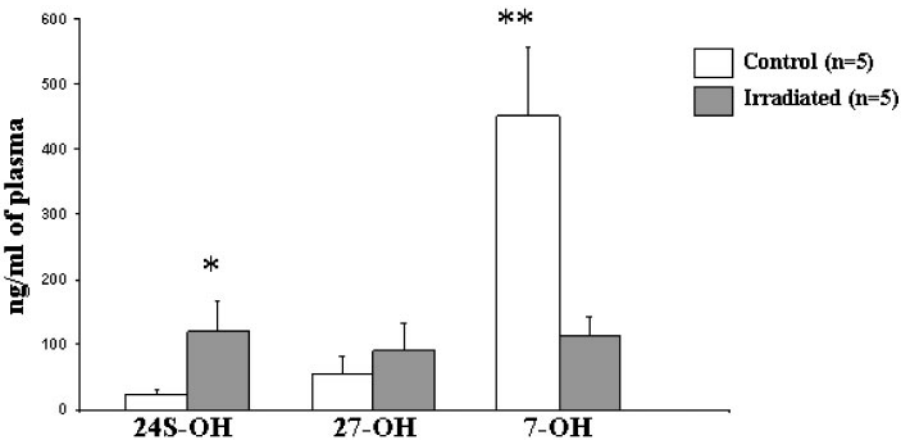

Figure 5. Plasma oxysterol concentrations in control rats and in rats 3 days after a whole body dose of 10 Gy γ ^{60}Co irradiation. 24S-OH, 24S-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; 7-OH, 7α-hydroxycholesterol. *Significantly different from control (Student's *t*-test, $p<0.05$); **significantly different from control (Student's *t*-test, $p<0.01$).

These preliminary data are in favour of changes in plasma levels of oxysterols post exposure. The variations observed both for 7-OH and 24S-OH could be indicators of, respectively, brain and liver damage, reflecting some modifications of cholesterol metabolism in these organs. Our data obtained here encourage the use of oxysterols as an assay of multi-organ radio-induced damage in clinical practice. However, new experiments need to be performed to verify these results, *e.g.* establishment of a dose–effect curve, a kinetic curve, and determination of the sensitivity of each oxysterol.

Conclusion

Biological dosimetry based on the scoring of dicentrics whatever the accidental overexposure conditions remains the reference technique at the cellular level. However, at the organ and organism level, this approach must be enhanced by the use of biological indicators to improve diagnosis and prognosis of radio-induced injuries. Our experimental and clinical data indicated plasma Flt-3 ligand level as a physiological marker of bone marrow damage, plasma citrulline level as a physiological marker of intestinal mucosa damage, and plasma oxysterols as physiological markers of MODS. The kinetics observed for these parameters allow the monitoring of radiation-induced organ damage in clinical practice and may be useful for guiding treatment of MODS. Other specific markers of organ damage are now under investigation in our laboratory. For this purpose, proteomics could be a technology of choice to determine new bioindicators of radiation exposure. Nevertheless, the panel of techniques proposed in this paper could be used to quantify radiation exposure as well as to optimise the treatment of patients following either radiotherapy or accidental overexposure.

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