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The Metabolomic Approach Identifies a Biological Signature of Low-dose Chronic Exposure to Cesium 137

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Cesium 137/Chronic ingestion; biomarker/Metabolomics/Low-doses.

Reports have described apparent biological effects of ^{137}Cs (the most persistent dispersed radionuclide) irradiation in people living in Chernobyl-contaminated territory. The sensitive analytical technology described here should now help assess the relation of this contamination to the observed effects. A rat model chronically exposed to ^{137}Cs through drinking water was developed to identify biomarkers of radiation-induced metabolic disorders, and the biological impact was evaluated by a metabolomic approach that allowed us to detect several hundred metabolites in biofluids and assess their association with disease states. After collection of plasma and urine from contaminated and non-contaminated rats at the end of the 9-months contamination period, analysis with a LC-MS system detected 742 features in urine and 1309 in plasma. Biostatistical discriminant analysis extracted a subset of 26 metabolite signals (2 urinary, 4 plasma non-polar, and 19 plasma polar metabolites) that in combination were able to predict from 68 up to 94% of the contaminated rats, depending on the prediction method used, with a misclassification rate as low as 5.3%. The difference in this metabolic score between the contaminated and non-contaminated rats was highly significant (P = 0.019 after ANOVA cross-validation). In conclusion, our proof-of-principle study demonstrated for the first time the usefulness of a metabolomic approach for addressing biological effects of chronic low-dose contamination. We can conclude that a metabolomic signature discriminated ^{137}Cs-contaminated from control animals in our model. Further validation is nevertheless required together with full annotation of the metabolic indicators.

INRODUCTION

Since the ionizing radiation emitted during the Chernobyl nuclear accident, various disorders have been reported in humans living nearby, including thyroid cancer,[1)] leukemia[2)] and other non-cancerous diseases, such as cataracts,[3)] atherosclerosis,[4)] abnormal cardiac features[5)] and other metabolic disturbances.[6)] However, chronic low-dose contamination by local foodstuff did not clearly lead to observations of specific diseases with the exception of cardiovascular symptoms.[5)] Experimental studies in rats chronically contaminated by ^{137}Cs, which is the most persistent radionuclide dispersed in this area (half-life of 30 years), have showed molecular and physiological effects in different metabolic pathways without, however, any major homeostatic deregulation.[7–10)]

Currently, analytical sensitivity is increasing with the use of new noninvasive methods. One of these is metabolomics, which appears useful[11)] for finding specific biomarkers of chronic radiocesium exposure. Metabolomics is the global profiling and quantitative characterization of metabolic phenotypes, *i.e.*, small molecules (< 1000 g/mol), in biofluids, cells, tissues, organs and organisms under specific sets of conditions, such as environmental or radiation exposures.[12)] Already used for drug toxicity modeling,[13)] metabolomics is sufficiently sensitive to guide human health choices.[14,15)] Moreover, noninvasive quantitative or qualitative profiling could provide useful diagnoses in cases of radiological accidents, nuclear terrorism or radiotherapy oncology treatment.[16)]

The aim of the present study was to evaluate the effects

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of radiocesium exposure on rat health with multiple tests usually used in toxicology. To mimic the environmental contamination following the Chernobyl accident, especially among children, known to be a sensitive subgroup in toxicology[17,18] and radiobiology,[19] rats were exposed to ^{137}Cs through drinking water from conception.[20] Consumption of food and drinking water were measured during the contamination protocol to monitor dietary behavior. Body weight and that of the main organs[21] were checked to evaluate any morphological or other possible specific effects. Blood cells were counted[22] and plasma metabolites measured to establish a ^{137}Cs toxicological diagnosis. Moreover, a mass-spectrometry-based metabolomic profile of rat plasma and urine, followed by a multivariate analysis, identified a specific metabolomic signature of low-level ^{137}Cs contamination.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley rats, aged 12 weeks and weighing 274 ± 2 g, were obtained from Charles River Laboratories (L'Arbresle, France). They were housed in pairs (male and female separated) upon arrival and allowed to recover from transportation for 2 weeks before the first day of contamination. During the experiment, they were maintained in a 12-h light/12-h dark cycle (regular cycle) at 21 ± 2°C and 50 ± 10% humidity, with access to a standard pellet rodent diet and water ad libitum. For the experiment, the rats were divided into two groups per sex, control and ^{137}Cs-exposed. A week after contamination began; male rats were mated with females for 48 h (1 male with 2 females in each group). After weaning, male offspring were housed in pairs from different mothers (assigned by randomization). Female offspring and mothers were euthanized. All experimental procedures were approved by the Animal Care Committee of the Institute of Radioprotection and Nuclear Safety (IRSN) and complied with French regulations for animal experimentation (Ministry of Agriculture Act No. 87-848, October 19, 1987, modified May 20, 2001).

Contamination procedure

Rats were contaminated with ^{137}cesium chloride (^{137}CsCl, CAS number 20334-19-4) obtained from CERCA (Pierrelatte, France). Parents of the offspring experimental group were exposed to ^{137}CsCl added to drinking water at a concentration of 6500 Bq/L (ca. 170 Bq/rat/day) a week before mating until euthanasia. This ^{137}Cs concentration corresponded to the maximum concentrations measured in milk in Belarus immediately after the Chernobyl accident.[23] Other study performed on the daily ^{137}Cs intake indicated a similar value (around 100 Bq) for inhabitants from Christinovska (Ukraine) that is included in the greatly contaminated zone II (between 555 and 1,480 kBq m^{-2}).[24] The control rats received uncontaminated mineral water. The food and water intake of both groups was monitored weekly throughout the contamination period. During pregnancy, offspring were theoretically contaminated by mother's blood and subsequently by mother's milk until weaning (receiving approximately 20% of the mother's daily dose).[25] Weaned males were exposed to ^{137}CsCl in their drinking water at a concentration of 6500 Bq/L until they were 9 months old.

Measurement of ^{137}Cs organ contamination

Radiocesium activity was measured in organs with a gamma counter (Cobra Perkin Elmer, Cobra gamma counter with a NaI detector, Packard Instruments, Courtaboeuf, France) as described previously.[26] The detection limit (DL) was calculated with the following formula (CTHIR, French GTN5):[27]

$$DL = \frac{4\sqrt{(2.B.T)}}{T},$$

where B is the background, obtained by averaging counts from 10 empty tubes, in counts per minute (cpm), and T is the duration of the counting period. The detection limit ranged from 4.2 to 12 counts per minute per sample, depending on the mass of the organ tested and on the duration of the counting period.

Dose calculation

The number of nuclear transformations in organs was estimated from 137Cs tissue measurements at 1, 3, and 9 months after the experiment started. To obtain a maximizing approximation, the activity in each tissue was assumed to be constant during the previous contamination period. The fraction of energy emitted from source tissue and absorbed in target tissue (AF) is taken from the voxel-based rat model designed by Stabin and co-workers.[28] Specific absorbed fractions were obtained by dividing AF by the tissue mass measured in the experiment and were convolved with the 137Cs and 137mBa emission spectra (International Commission on Radiological Protection 2008 Nuclear Decay Data for Dosimetric Calculations[29]) to derive specific effective energies (SEEs). Multiplying the SEEs by the number of nuclear transformations in the source tissues yielded the absorbed doses in the target tissue.

Collection of organs and biofluids

When rats were 9 months old, their urine was collected for 48 h. They were placed in metabolic cages (one per cage) in the morning with access to a ground pellet rodent diet and water (contaminated or not) ad libitum. Urine was collected twice a day, with sodium azide (0.01%)[30] added to prevent bacterial growth, and refrigerated at +4°C. Each rat's fractions were pooled, mixed, and centrifuged; supernatants were frozen at –80°C. The rats were then moved to conventional cages (one per cage) with food and drink ad libitum

until the evening to reduce stress. To control the diet cycle, food was removed in the evening until the next morning. Five hours before euthanasia, around 12 g of standard pellet rodent food was added directly to each cage to stimulate the desire to eat before sleep. Rats were anesthetized by inhalation of 5% isoflurane (Abbot France, Rungis, France) and euthanized by intracardiac puncture to collect blood in heparinized tubes. Whole blood was centrifuged (5000 rpm) and plasma supernatants were immediately frozen at –80°C. Organs were dissected on ice, weighed, deep-frozen in liquid nitrogen and stored at –80°C.

Blood cell counts

Complete blood counts (CBC) were measured by a MS-9 vet automatic counter (Melet-Schlossing, Osny, France). Remaining blood was centrifuged at 400 g for 10 min, and plasma was frozen (–80°C) for later use.

Biochemical panel

Biochemical measurements were taken from defrosted plasma samples by an automated spectrometric system (Konelab 20 from Thermo Electron Corporation, Cergy-Pontoise, France), with the manufacturer's biological chemistry reagents. The markers measured in plasma included glucose, albumin, total protein, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, phospholipids, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), triglycerides, total bilirubin, transferrin, iron, phosphorus, calcium, chlorine, potassium, magnesium, urea, creatinine and creatine kinase.

Urine and plasma sample preparation for metabolomic analysis

Plasma was thawed before either methanolic protein precipitation (to extract polar compounds) or organic solvent extraction (non-polar compounds). To extract polar compounds, 200 µL of plasma was mixed for 10 seconds with a double volume of methanol. The mixture was centrifuged at 13 000 rpm for 10 min (at +4°C). An equal volume of methanolic supernatant was taken from all tubes, evaporated under a nitrogen flow, and finally stored at –80°C until analysis. Before analysis, dry extracts were resolubilized with 100 µL of H_2O containing 0.1% formic acid for positive electrospray ionization (ESI+). Urine samples were centrifuged at 13 000 rpm for 10 min and then were diluted with water 350/1050 µL. Mixtures were centrifuged at 3000 rpm for 5 min and then placed in the liquid chromatography coupled to mass spectrometry (LC-MS) autosampler (at +4°C). Non-polar compounds were extracted with Bligh and Dyer's method: 200 µL of plasma was added to 200 µL of saline and 400 µL of methanol. The mixture was vigorously shaken for 30 s; 400 µL of chloroform was then added and mixed. After centrifugation (2000 g for 10 min at 4°C), the lower phase was taken with a Pasteur pipette and filtered on Na_2SO_4. The supernatant was re-extracted with 400 µL of chloroform, and the lower phases were assembled and dried under a nitrogen stream. Extracts were stored under a nitrogen atmosphere at –80°C until analysis. Before LC-MS processing, the samples were reconstituted with 1 mL acetonitrile-isopropanol mixture (5:2, v/v), 0.1% of formic acid and 1% of ammonium acetate.

LC-MS analysis

The samples were analyzed on an Agilent 1200 RRLC coupled to a Brüker microTOF ESI-hybrid quadrupole-time of flight mass spectrometer (Wissembourg, France). For the non-polar compounds (non-polar plasma), the liquid chromatographic conditions were: autosampler set at 15°C; column EC 100/2 Nucleodur C18 Isis, particle size 1.8 µm (Isis1) (Macherey-Nagel, Les Ulis, France). Solvent A: water + 0.1% formic acid + 0.1% ammonium acetate 1 M; solvent B: acetonitrile-isopropanol (5:2, v/v) + 0.1% formic acid + 1% ammonium acetate 1 M; eq with 60% B (40% A), 0–5 min 60–100% B, 5–10 min 100% B, 10–12 min 100–60% B, 12–15 min 60% B; flow 0.4 mL/min at 50°C. For polar compounds (plasma methanol extracts and urine), the liquid chromatographic conditions were: autosampler set at 4°C; column, EC 100/2 Nucleodur C18 pyramid, particle size 1.8 µm (Macherey-Nagel, Les Ulis, France); started from solvent A (95% water, 5% acetonitrile, 0.1% formic acid) to solvent B (95% acetonitrile, 5% water, 0.1% formic acid) from 0–10 min at 0.4 mL/min at 40°C column oven; thereafter switched to 95% A to 5% B for 6 min, held 2 min, and then returned to 100% A for 5 min.

The mass spectrometry (MS) conditions were as follows: full scan mode from m/z 50 to m/z 1500; capillary kV, 4.5; capillary temperature, 200°C; cone voltage at 40 V; drying gas flow set at 10 L.min-1 and nebulizing gas pressure (nitrogen) at 2.4 bar; positive electrospray ionization mode (ESI+).

Statistics

This animal protocol of ^{137}Cs contamination used two groups each of 20 rats. Each treatment group (control and contaminated) was divided in two subgroups (10 rats for the metabolomic study and 10 rats for other studies). The means of some measurements could be established for 10 to 20 individuals. Health diagnostic values were reported as means ± standard errors of the means (SEM). The control and contaminated groups were compared with Student's t-test in normal populations or the rank sum test in non-normal populations. Statistical significance was set at a p value less than 0.05. Statistical analyses were performed with Sigmastat statistical software (SPSS, Paris, France). Simca-P12 software (Umetrics, Umeå, Sweden) was used for all multivariate data analyses and modeling. Unsupervised hierarchi-

cal clustering was performed with Permutmatrix (www.lirmm.fr/~caraux/PermutMatrix/). Figure 1 describes the workflow of the metabolomic data analysis. After deconvolution of the raw data with the LC/MS-based data analysis software (XCMS),[31] the data from the plasma polar and non-polar compounds and from urine compounds were merged, and several filtration methods were applied to ensure proper data quality (Fig. 1). This reduced the number of variables to be analyzed per rat from 4139 to 600 (404 polar plasma, 185 non-polar plasma and 11 urine). Before multivariate analysis, the peak intensity values of the filtered data were scaled into units of variance. A principal component analysis of the pooled data with the quality control samples revealed that the analytical variance was satisfactory and lower than the biological variance. All the models evaluated were tested for overfitting, as described previously, with methods including permutation tests, cross-validation and cross validation-ANOVA.[15]

RESULTS

^{137}Cs activity in tissues and absorbed dose

^{137}Cs was measured in different organs (striated muscle, liver, kidney, brain, testicle, and biofluids) to check both the contamination level and homogeneity between the animals in each group (exposed versus control) (Table 1). Measurements showed that ^{137}Cs was incorporated into muscles at twice the rate as into other organs, which confirmed previous results.[32] Nonetheless, the high level of ^{137}Cs in urine demonstrated substantial intestinal absorption and urinary elimination of ingested radiocesium. The mean whole-body absorbed dose after 9 months of contamination was estimated at a maximum value of 4 mGy, under conservative assumptions for intermediate activity concentrations. Accordingly, this value can be considered to be the highest estimated dose received during the experiment.

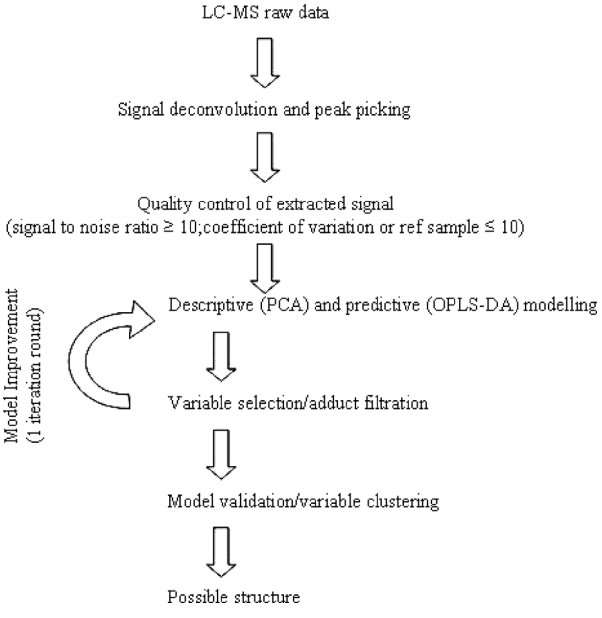

Fig. 1. Workflow of the metabolomic data analysis.

In order to assess the magnitude of the overestimate induced by the conservative assumptions, a simple exponential model of ^{137}Cs whole body retention was fitted to the observed activity data. If the retention after an acute intake is

$$R(t) = R(0) \times e^{-\lambda \cdot t}$$

where λ is the clearance rate and t the time after intake. The retention after a period t of chronic intake at a rate i may be expressed as

$$R(t) = R(0) + \frac{i}{\lambda} \times \left(1 - e^{-\lambda \cdot t}\right)$$

The number of nuclear transformations over this period is then the integral

$$A(t) = \left[\frac{i}{\lambda} + R(0)\right] \times t - \frac{i}{\lambda^2} \times \left(1 - e^{-\lambda \cdot t}\right)$$

While, under the conservative assumptions, the retention is supposed to stay constant between two measurements at times t_1 and t_2: for $t_1 < t < t_2$

$$R(t) = R(t_2) \quad \text{and} \quad A(t_2) = R(t_2) \times (t_2 - t_1)$$

The ingestion rate is approximately 170 Bq.d^{-1}. From the activities measured in organs, clearance rates of 0.8, 0.2 and 0.05 d^{-1} were estimated for rats of ages less than 3 weeks, from 3 weeks to 3 months and older than 3 months respectively. Comparing the number of nuclear transformations with such parameter values and the conservative assumptions yielded differences up to 8% over the duration of the experiment. It is therefore reasonable to assume the magnitude of the overestimate of the dose to be less than 10%.

Table 1. Mean ^{137}Cs radioactivity in different organs (Bq/g) in the rat after 9 months of chronic ingestion through drinking water (6500 Bq/L). DL: detection limit. Number of animals for each organ is indicated in parentheses.

	Striated muscle (4)	Urine (5)	Kidneys (4)	Liver (4)	Testicle (4)	Heart (4)	Brain (4)	Plasma (4)
Control	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
^{137}Cs	10,8 ± 0,8	9,8 ± 0,6	5,7 ± 0,6	5,5 ± 0,6	5 ± 0,5	4,6 ± 0,4	2,5 ± 0,2	< DL

Dietary behavior

We monitored drinking water and food consumption for three weeks before the end of contamination (once a week) and found no difference in the diets of the control and treated groups (Table 2).

Animals and organ weights

To identify any toxicological effect manifested morphologically, the whole body and principal organs were weighed, and the weights compared between the two groups. Calculation of absolute (Table 3) and relative weights (Table 4) showed no statistically significant effects.

Blood and plasma biochemistry panels

No imbalance in complete blood counts was observed after contamination (Table 5).

To examine the animal's health, we tested a number of plasma biochemical levels, including proteins, minerals, liver, cardiovascular and kidney markers (Tables 6&7). Except for slightly elevated creatinine levels in the contaminated rats ($P \leq 0.05$), within the physiological range, no differences were observed between groups.

Metabolic effects in plasma and urine

Overall, we analyzed 2053 metabolic features per rat, including 742 in urine, 368 non-polar and 941 polar in plasma.

Table 2. Mean dietary consumption was calculated for 3 weeks before euthanasia for each cage of two rats (mL/day/rat for water and g/day/rat for food). The number of animals for water and food is indicated in brackets.

	Water intake (20)	Food consumption (20)
Control	26,8 ± 1,4	25,3 ± 0,6
^{137}Cs	26,4 ± 0,8	25,0 ± 0,6

Discriminant analysis was performed to examine whether the ^{137}Cs intake produced a metabolic shift that could distinguish the contaminated from the non-contaminated rats (Fig. 2). The model including all the variables described the group variance with acceptable accuracy (R2Y = 0.46), but was not

Table 5. Mean blood results for rats. Number of animals for each measurement is indicated in parentheses.

	Control (20)	^{137}Cs (20)
Red blood cells (count.10^{+12}/L)	5,2 ± 0,4	5,9 ± 0,5
White blood cells (count.10^{+9}/L)	8,6 ± 0,1	8,3 ± 0,4
Hemoglobin (g/L)	13,71 ± 0,4	13,78 ± 0,53
Hematocrit (%)	45,92 ± 0,56	45,55 ± 2,23
Platelets (count.10^{+9}/L)	874 ± 50	869 ± 60
Lymphocytes (count.10^{+9}/L)	3,7± 0,3	4,12 ± 0,4
Monocytes (count.10^{+9}/L)	0,15 ± 0,01	0,19 ± 0,02
Granulocytes (count.10^{+9}/L)	1,31 ± 0,12	1,60 ± 0,15

Table 6. Plasma enzymes. Number of animals for each measurement is indicated in parentheses.

	Control (10)	^{137}Cs (10)
ALAT/GPT (U/L)	61 ± 10	49 ± 4
ASAT/GOT (U/L)	156 ± 21	147 ± 16
CK (U/L)	716 ± 182	599 ± 134
CK-MB (U/L)	1212 ± 326	1383 ± 303
Gamma-GT (U/L)	2,3 ± 0,4	2,9 ± 0,3
LDH (U/L)	1456 ± 313	1459 ± 338
Alkaline phosphatase (U/L)	128 ± 15	153 ± 7

Table 3. Mean ± SEM of whole-body and organ weight (g) in each group. Number of animals for each organ is indicated in parentheses.

	Total body (18)	Liver (10)	Heart (10)	Spleen (10)	Kidney (10)	Epididymis (10)	Testis (10)
Control (g)	536 ± 16	22 ± 1	1,54 ± 0,04	0,9 ± 0,1	1,72 ± 0,05	0,69 ± 0,01	1,96 ± 0,04
^{137}Cs (g)	548 ± 12	19,9 ± 0,2	1,49 ± 0,03	0,9 ± 0,1	1,67 ± 0,05	0,71 ± 0,02	1,99 ± 0,05

Table 4. Mean ratios of organ to whole body weight (%) for each group. Number of animals for each organ is indicated in parentheses.

	Liver (18)	Heart (10)	Spleen (10)	Kidney (10)	Epididymis (10)	Testis (10)
Control (%)	4,1 ± 0,3	0,28 ± 0,01	0,16 ± 0,02	0.31 ± 0,01	0,13 ± 0,01	0,36 ± 0,02
^{137}Cs (%)	3,7 ± 0,2	0,26 ± 0,01	0,14 ± 0,01	0,29 ± 0,01	0,12 ± 0,01	0,35 ± 0,01

Table 7. Proteins, carbohydrates, lipids, ions and other metabolites. Number of animals for each measurement is indicated in parentheses.

	Control (10)	^{137}Cs (10)
Albumin (g/L)	32,3 ± 0,5	32,8 ± 0,5
Total proteins (g/L)	69 ± 2	70 ± 1
Transferrin (g/L)	1,8 ± 0,1	1,7 ± 0,1
Glucose (g/L)	11,5 ± 0,6	11,4 ± 0,5
Triglycerides (mM)	1,5 ± 0,3	1,7 ± 0,2
Cholesterol (mM)	2,7 ± 0,2	2,7 ± 0,2
HDL-cholesterol (mM)	2,2 ± 0,2	2,1 ± 0,1
LDL-cholesterol (mM)	0,40 ± 0,05	0,36 ± 0,04
Phospholipids B (g/L)	2,0 ± 0,1	2,0 ± 0,1
Calcium (mM)	2,72 ± 0,03	2,68 ± 0,03
Iron (µM)	36 ± 0,2	39 ± 2
Chlorine (mM)	95,8 ± 0,8	96,3 ± 0,3
Potassium (mM)	4,2 ± 0,1	4,4 ± 0,1
Magnesium (mM)	0,73 ± 0,01	0,72 ± 0,01
Phosphorus (mM)	1,28 ± 0,04	1,28 ± 0,06
Creatinine (µM)	50 ± 2	53 ± 2*
Direct bilirubin (µM)	4,9 ± 0,3	4,7 ± 0,3
Total bilirubin (µM)	5,2 ± 0,5	5,2 ± 0,4
Urea (mM)	6,0 ± 0,2	6,2 ± 0,2

predictive (negative Q2Y = 0.36). To improve our model and find more accurate descriptors of ^{137}Cs ingestion, we selected variables according to the PLS score contribution index (variable importance in projection, VIP). This procedure filtered out the variables that made no important contribution to class discrimination. After this selection, applying several thresholds of variable-importance-in-projection (VIP) with SIMCA software, the discriminant analysis model obtained

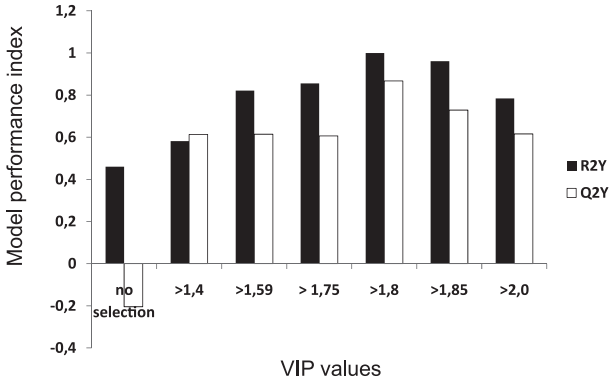

Fig. 2. Prediction of model performance after variable selection based on various VIP threshold values. 0 < R2Y < 1 and –1 < or Q2Y < 1. The best model was that with the highest R2Y and Q2Y values.

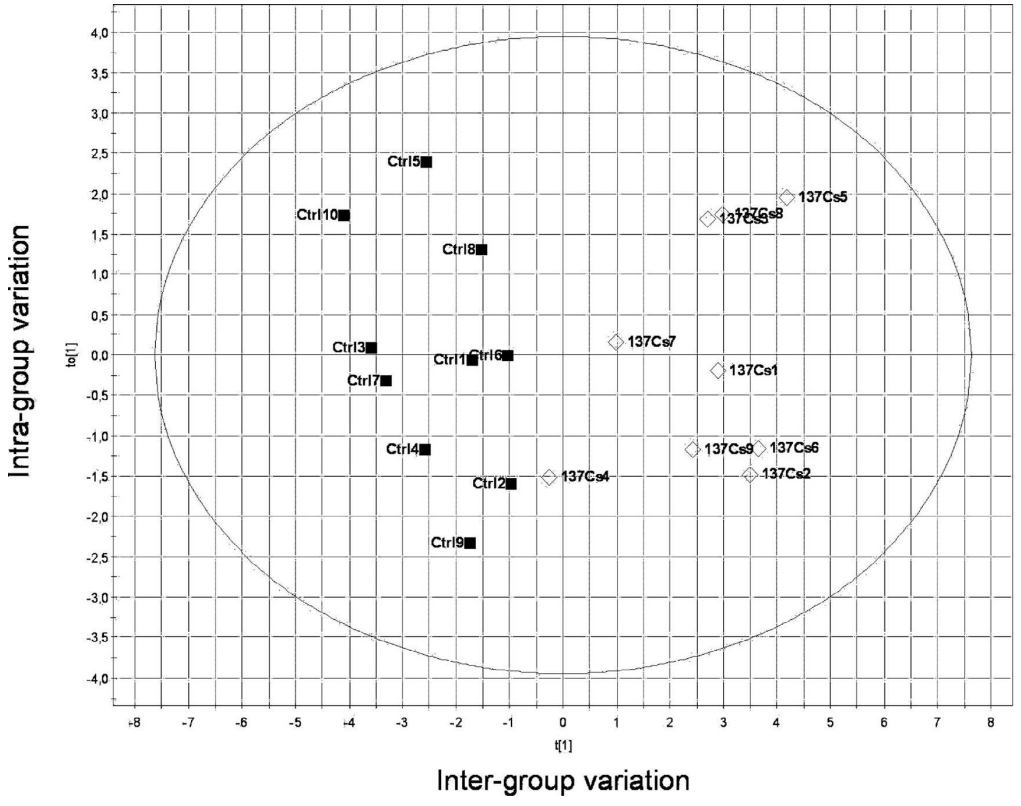

Fig. 3. Discriminant analysis (OPLS-DA) of the control and ^{137}Cs-treated rats, based on 26 metabolic variables sensitive to ^{137}Cs. Control rats: from Ctrl1 to 10, ^{137}Cs treated rats, from ^{137}Cs1 to 9.

described 99% of the group variance, with a prediction index (Q2Y) of 0.867 (Fig. 2). The model included only 41 variables: 35 polar plasma, 4 non-polar plasma, and 2 urine. A supplementary variable selection based on hierarchical clustering and linear regression analysis removed redundancy by discarding 16 more variables that were considered to be likely additional adducts of the same molecule. The final model constructed was fitted with 26 variables that discriminated the rats treated with ^{137}Cs from the control non-treated rats (Fig. 3). This discriminant analysis modeled 45.3% of the variation of the metabolic variables (R^2X), but most importantly 81.1% of the treatment group's metabolic characteristics (R^2Y) (prediction values Q^2 = 58.6% after cross-validation). With this combination of variables, the model assigned all the control rats to the non-contaminated group, and 88.9% of the ^{137}Cs-treated rats to the contaminated group (Fig. 4). The statistical discrimination level obtained between the groups was significant (P = 0.01951 after cross-validation ANOVA). We then challenged our class membership prediction model by the leave-one-out (LOO) technique, to predict class membership of the unassigned — left out — observation (an individual rat). For this validation, we took out one animal per group from the original dataset, thus leaving a training dataset. The rat taken out was then rein-

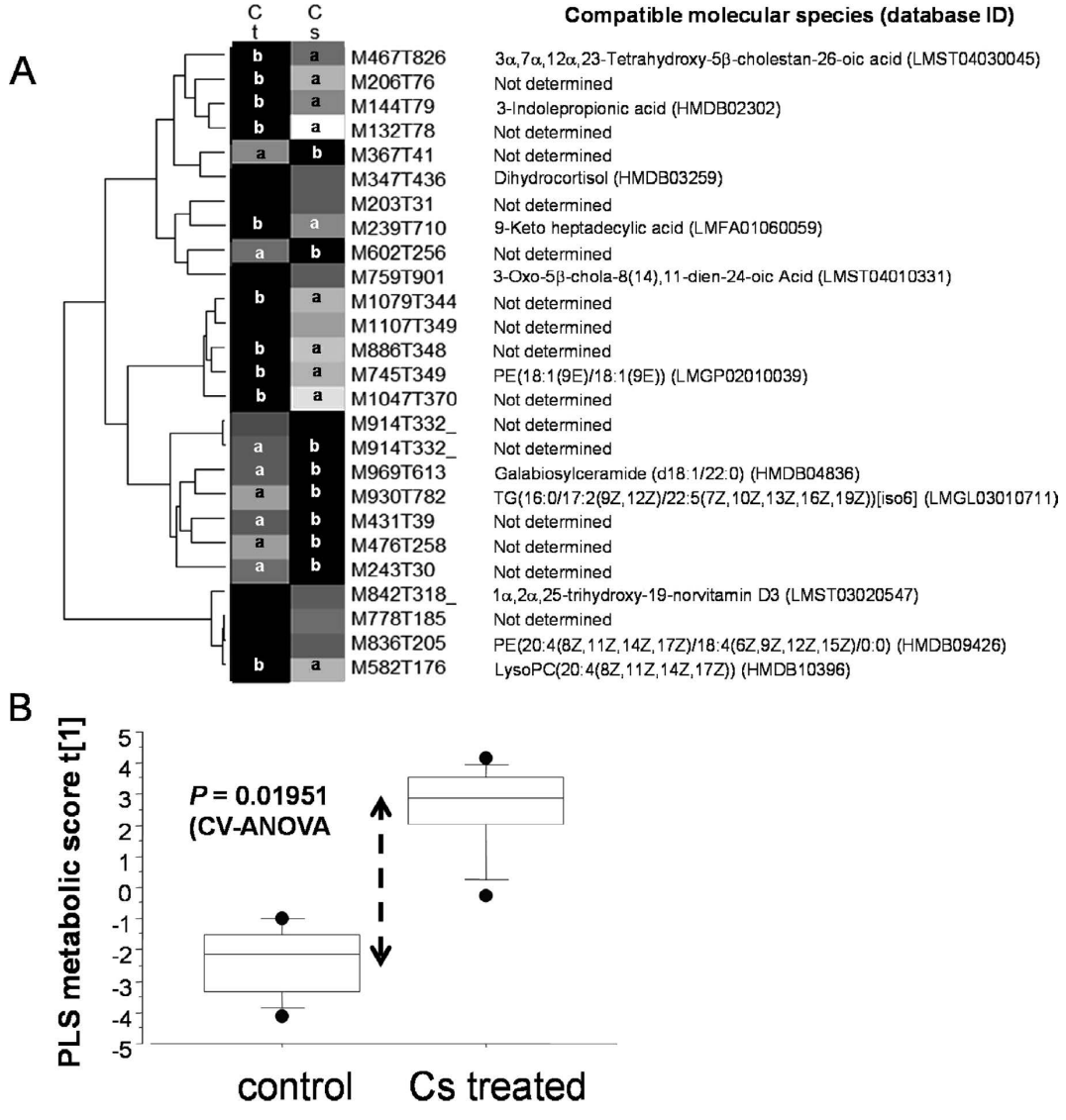

Fig. 4. A- Hierarchical clustering analysis and compatible structure to determine exact mass of the cesium sensitive metabolic dataset; the heatmap represent the relative intensity of the ions detected either in control (Ct) or ^{137}Cs-exposed (Cs) rats (dark box, increase; light box, decrease); a different letter in each row indicates a statistically significant difference in the univariate analysis (unpaired t-test, with $\alpha < 0.05$); B- Comparison of the ^{137}Cs sensitive composite metabolic score for ^{137}Cs contaminated and control rats.

Table 8. Prediction error rate for identifying rats as contaminated by ^{137}Cs or not, with the leave-one-out procedure.

%	Classification list	Misclassification table	z-score
Undefined	26.3	–	15.8
Correct	68.4	94.7	78.9
misclassified	5.3	5.3	5.3

troduced into this training set to assess whether the model could predict its class membership correctly. This test was performed for each rat. The error rate was determined by three different calculations: the misclassification table and the classification list of the SIMCA software, and a z-score calculated from the PLS *t*[1] scores obtained for each rat, from the intensity values of the metabolic variables (Table 8). The error rate was very low and identical for each assessment method. Depending on the test used, the metabolic signature correctly assigned from 68 up to 94% of the rats to their treatment group (control or ^{137}Cs contaminated). One false positive (*i.e.*, control rat positive for the metabolic response measured) was found, and one out of range (ultrasensitive to ^{137}Cs-treatment).

Finally, to obtain more insights into the metabolic response observed in ^{137}Cs-exposed rats, the metabolic component values were analyzed by hierarchical clustering. This analysis produced 5 clusters according to the statistical proximity of the variables (Fig. 4A). It is generally considered that this statistical proximity overlaps with co-regulated biological functions. Our attempt at structural elucidation identified some lipids (phospholipids, bile acids and sterol derivatives) and amino-acid breakdown derivatives (3-indolpropionic acid).

The results in Fig. 4A also indicated that the values for each variable did not always differ between the control and the ^{137}Cs-contaminated rats in univariate analyses, but the (multivariate) combined PLS metabolic scores, displayed as box-plots, differed significantly between the groups (Fig. 4B).

DISCUSSION

Metabolomics is a new analytical technique that measures all of the small organic molecules (excluding proteins and nucleic acids) detectable in biological fluids.[33] Its particularity is its high detection sensitivity for metabolites when coupled with mass spectrometry, its relatively noninvasive nature, and the fast data processing, directly from plasma or urine samples. For these reasons, metabolomics could be particularly interesting as a new analytical approach that might enrich more standard methods commonly used in human clinical chemistry. Metabolomics is already applied in pharmaceutical applications for finding biomarkers, notably in drug toxicology to diagnose drug-induced effects or for therapeutic monitoring applications.[34]

More recently, the use of high irradiation doses (> 10 Gy) in humans has created metabolomics applications in the domain of radiotherapy: the metabolic profile helps monitor response to treatment and simultaneously increases our knowledge of tumor pathophysiology.[35] Other experimental studies with more moderate gamma irradiation doses (between 3 to 8 Gy) found a metabolic dose-response relation in mouse urine 24 hours after irradiation. This analytical method may be adapted to noninvasive radiation biodosimetry in humans.[36] Moreover, urine has also been used to describe the course of a metabolic phenotype in rats after irradiation; it has showed effects on kidney functions following oxidative stress.[12] Other biological fluids, such as sebum, have also been explored as biomarker sources[37] in experiments demonstrating the diversity of potentially usable biofluids.

Nothing in the literature thus far describes the pertinence of metabolomic techniques in cases of radiotoxicity following chronic low doses of contamination by radionuclides. Nonetheless, the possibility of obtaining a specific metabolic fingerprint rapidly from accessible biofluids such as plasma or urine makes metabolomics a potentially original and relevant tool to diagnose and estimate the impact of environmental contamination, estimates that will be necessary for the medical management of populations in such cases[38,39] or for developing appropriate public health recommendations.

The aim of this study was to find a new and more sensitive tool to evaluate the possibility of physiological changes caused by low-dose ingestion of ^{137}Cs. A few studies have described clearly non-cancerous health consequences to populations living in territory contaminated by the Chernobyl fallout.[3,40,41] The issue of direct health consequences of low levels of internal ^{137}Cs exposure is far from resolved. The high exposure effects of irradiation observed in the liquidators and the possible role of other pollutants, such as the strontium 90 dispersed in the area during the accident,[42] as well as mental health effects due to the nuclear trauma[43] and the degradation of living conditions in a disaster area[44] must all be considered. For that reason, beginning research in experimentally standardized conditions is likely to be a good initial approach. To mimic the radiological exposure in territories around Chernobyl, rats were chronically exposed to radiocesium in their drinking water. During the 9 months of exposure, animals received a maximal dose of irradiation (less than 4 mGy) lower than the annual limit recommended for radiation-exposed workers by the International Commission on Radiological Protection (ICRP n°103)[45] and slightly higher than the maximum public annual limit dose of 1 mSv/y, which is itself higher than the residual radiological dose that persists around Chernobyl.[46] The irradiation dose to these rats thus ranged between the environmental doses around Chernobyl and the acceptable limit for workers. Ingestion through drinking water in our experiment was estimated at about 0.7 ng cesium a day per rat. At this level, the

quantity of dietary cesium metal intake is insignificant compared with the gamma-radiation activity. The weak organ irradiation is thus almost certainly the principal cause for possible biological effects.

To determine those potential effects, we measured the standard clinical biomarkers and biometric variables during and after treatment in each experimental group to check their health status. Problems such as anorexia, weight loss[47] and visceral tissue damage were not found, although they have been described with stronger external gamma irradiation.[48] Also, our study did not show a body weight increase as described in case of higher cumulated irradiation (6 Gy) at low-dose rate in rodents exposed during the same duration (9 months).[49] Moreover, these results confirmed the very low-dose range (0.004 Gy) of our study, consistent with results of similar experimental protocols carried out earlier in rats.[7–10,50,51] In addition, there was no imbalance in the proportions of blood cell types, such as that described in cases of high whole-body external irradiation (with a decrease white blood cells),[48,52] nor did we observe a change between the groups in hepatic, heart or kidney enzyme activity in plasma. Similarly, plasma lipid, protein and mineral concentrations did not change; such a change might have revealed a dysfunction or rupture in the organ or tissue integrity.

Nonetheless, previous experimental studies in rats chronically exposed to ^{137}Cs showed some modifications of genes and molecules, particularly in liver cholesterol metabolism.[9,51] Molecular and hormonal modifications have been described in testicular steroidogenesis and adrenal steroidogenic metabolism.[7] Exposure appears to modify vitamin D metabolism and cause mineral imbalances.[10] Finally, studies of the cardiovascular system have shown few genic, plasmatic biomarker or physiological modifications.[8] Accordingly, in this dose range (up to less than 4 mGy), the lack of observable major health effects is not surprising because these experimental results have indicated no major disturbances of genes or molecules. On the other hand, biological responses in different metabolisms indicate the possibility of a physiological response at low levels of ^{137}Cs exposure. Overall, the metabolomic approach, which provides a simultaneous analysis of all detectable metabolites in urine and plasma, may also offer a systemic metabolic fingerprint that reflects physiological adjustments and describes the specific metabolic regulation pathways in cases of low exposure.

The results we obtained in this first application were very encouraging. Our untargeted metabolomic approach found a combination of 26 robust metabolic markers that together were sensitive to ^{137}Cs contamination. In this specific combination, most of the metabolites were polar plasma metabolites, while a few of them were non-polar lipids and urinary metabolites. Although not all the rats could be correctly assigned according to the treatment, most were distinguished as contaminated or not contaminated with an accuracy that ranged from 68% up to 94% according to the statistical evaluation. Thus, our proof of principle study, which examined the potential of the metabolomic approach for characterizing low-dose ^{137}Cs contamination, provided satisfactory and promising results. It was possible to discriminate controls from contaminated animals and to confirm the interest of this new global technique compared with standard univariate analytical methods. The next step is to confirm and validate our results with more information on the rat treatment conditions, such as time of treatment, dose gradient, and determination of the correct molecular structure of each metabolite.

Later on, after complementary investigations, it will be necessary to assess the concentration of each ^{137}Cs sensitive metabolite in a subset of well-defined contaminated and control individuals in sufficient numbers to serve as a reference population and yield optimal delineation. This assessment could be performed by quantitative mass-spectrometry, such as GC-MS or triple-quad LC-MS instruments, which might allow the assessment of a selection of discriminating metabolites based on multiple reaction monitoring (MRM). A composite score from the concentration values could then be calculated according to the algorithm used here. Suspicious unknown samples analyzed in the same way could then be assigned — or not — to the ^{137}Cs contamination group, based on comparison of their composite scores with those of reference samples. To increase our knowledge about metabolic interactions with ^{137}Cs, another further objective should be the molecular identification of variables that can be quantitatively modified in case of contamination. This identification requires comprehensive LC-MS-based data. Unfortunately the mass databases are not yet fully optimized and it is often necessary to use time-consuming methods to elucidate the full structure of metabolites. At this state of our study, a first search in mass databases has shown a strong presumption for steroid molecules (Fig. 4), which is in accord with previous experimental results.[7,9,10,50,51]

In conclusion, metabolomics, applied to chronic low-dose exposure to ^{137}Cs, has for the first time provided a significant and specific metabolic signature in a case in which standard clinic analysis yielded no information. Hence metabolomics appears to be a sufficiently sensitive analytical tool for use in cases of chronic low-dose radiological exposure. Metabolic signature profiles for any pollutant could be used to sort and follow populations living or working in exposed areas. Moreover, determination of the metabolites most affected will make it possible to evaluate biological changes associated with long-term health effects on populations exposed to accidents or chronic industrial dumping and will improve our knowledge of metabolism networks. It is now necessary to confirm the interest of metabolomics with other radionuclides, under other experimental conditions such as time of exposure and dose ranges, other biological conditions such as age, sex, stress, and other animal models, and in multi-pollution approaches. Such studies will show the

relevance and specify the limitations of this new analytical technique in understanding the effects induced by chronic low-dose environmental exposures. Validation by methodological optimization remains necessary before its adoption for routine use.

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REFERENCES

1. Papadopoulou F and Efthimiou E (2009) Thyroid cancer after external or internal ionizing irradiation. Hell J Nucl Med **12**: 266–270.
2. Noshchenko AG, Bondar OY and Drozdova VD (2010) Radiation-induced leukemia among children aged 0–5 years at the time of the Chernobyl accident. Int J Cancer **127**: 412–426.
3. Sumner D (2007) Health effects resulting from the Chernobyl accident. Med Confl Surviv **23**: 31–45.
4. Trivedi A and Hannan MA (2004) Radiation and cardiovascular diseases. J Environ Pathol Toxicol Oncol **23**: 99–106.
5. Bandazhevskaya GS, *et al* (2004) Relationship between caesium (137Cs) load, cardiovascular symptoms, and source of food in 'Chernobyl' children – preliminary observations after intake of oral apple pectin. Swiss Med Wkly **134**: 725–9.
6. Sychik SI and Stozharov AN (1999) [Delayed effect of prenatal irradiation on the critical organs functional state in children after Chernobyl accident]. Radiats Biol Radioecol **39**: 500–504.
7. Grignard E, *et al* (2008) In vivo effects of chronic contamination with 137 cesium on testicular and adrenal steroidogenesis. Arch Toxicol **82**: 583–589.
8. Gueguen Y, *et al* (2008) Chronic contamination of rats with 137 cesium radionuclide: impact on the cardiovascular system. Cardiovasc Toxicol **8**: 33–40.
9. Souidi M, *et al* (2006) Chronic contamination with 137cesium in rat: effect on liver cholesterol metabolism. Int J Toxicol **25**: 493–497.
10. Tissandie E, *et al* (2009) Vitamin D metabolism impairment in the rat's offspring following maternal exposure to 137cesium. Arch Toxicol **83**: 357–362.
11. Robertson DG, Watkins P and Reily MD (2011) Metabolomics in Toxicology: Preclinical and Clinical Applications. Toxicol Sci **120**: S146–S70.
12. Lanz C, *et al* (2009) Radiation metabolomics. 3. Biomarker discovery in the urine of gamma-irradiated rats using a simplified metabolomics protocol of gas chromatography-mass spectrometry combined with random forests machine learning algorithm. Radiat Res **172**: 198–212.
13. Keun HC (2006) Metabonomic modeling of drug toxicity. Pharmacol Ther **109**: 92–106.
14. German JB, Hammock BD and Watkins SM (2005) Metabolomics: building on a century of biochemistry to guide human health. Metabolomics **1**: 3–9.
15. Martin JC, *et al* (2009) 1H NMR metabonomics can differentiate the early atherogenic effect of dairy products in hyperlipidemic hamsters. Atherosclerosis **206**: 127–133.
16. Patterson AD, Lanz C, Gonzalez FJ and Idle JR (2010) The role of mass spectrometry-based metabolomics in medical countermeasures against radiation. Mass Spectrom Rev **29**: 503–521.
17. Schwenk M, *et al* (2003) Children as a sensitive subgroup and their role in regulatory toxicology: DGPT workshop report. Arch Toxicol **77**: 2–6.
18. Wang L and Pinkerton KE (2007) Air pollutant effects on fetal and early postnatal development. Birth Defects Res C Embryo Today **81**: 144–154.
19. De Santis M, *et al* (2007) Radiation effects on development. Birth Defects Res C Embryo Today **81**: 177–182.
20. von Zallinger C and Tempel K (1998) Transplacental transfer of radionuclides. A review. Zentralbl Veterinarmed A **45**: 581–590.
21. Sellers RS, *et al* (2007) Society of Toxicologic Pathology position paper: organ weight recommendations for toxicology studies. Toxicol Pathol **35**: 751–755.
22. George-Gay B and Parker K (2003) Understanding the complete blood count with differential. J Perianesth Nurs **18**: 96–114; quiz 15–17.
23. Coulon R (1993) Le 137Cs et l'accident de Tchernobyl, projet international AIEA. In Actualités sur le césium. Jouve, Paris 10–16.
24. Handl J, *et al* (2003) Evaluation of radioactive exposure from 137Cs in contaminated areas of Northern Ukraine. Health Phys **84**: 502–517.
25. (2004) Annals of the ICRP. A report of: doses to infants from ingestion of radionuclides in mothers' milk. Ann ICRP **34**: iii, 15–267, 69–80.
26. Tourlonias E, *et al* (2010) Distribution of 137Cs in rat tissues after various schedules of chronic ingestion. Health Phys **99**: 39–48.
27. Millies-Lacroix JC (1994) Seuil de Décision et Limite de Détection. Applications aux Mesures de Radioactivité. CTHIR, Rapport technique GTN 5.
28. Stabin MG, *et al* (2006) Voxel-based mouse and rat models for internal dose calculations. J Nucl Med **47**: 655–659.
29. (2008) Nuclear decay data for dosimetric calculations. A report of ICRP Committee 2. Ann ICRP **38**: 7–96.
30. Griffin JL, *et al* (2001) Metabolic profiling of chronic cadmium exposure in the rat. Chem Res Toxicol **14**: 1428–1434.
31. Smith CA, *et al* (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem **78**: 779–787.
32. Tourlonias E, *et al* Distribution of 137Cs in rat tissues after various schedules of chronic ingestion. Health Phys **99**: 39–48.
33. Oldiges M, *et al* (2007) Metabolomics: current state and evolving methodologies and tools. Appl Microbiol Biotechnol **76**: 495–511.
34. Roux A, *et al* (2011) Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical

chemistry and toxicology: A review. Clin Biochem **44:** 119–135.
35. Wibom C, *et al* (2010) Metabolomic patterns in glioblastoma and changes during radiotherapy: a clinical microdialysis study. J Proteome Res **9**: 2909–2919.
36. Tyburski JB, *et al* (2008) Radiation metabolomics. 1. Identification of minimally invasive urine biomarkers for gamma-radiation exposure in mice. Radiat Res **170**: 1–14.
37. Lanz C, *et al* (2011) The production and composition of rat sebum is unaffected by 3 Gy gamma radiation. Int J Radiat Biol **87**: 360–371.
38. Blakely WF, Salter CA and Prasanna PG (2005) Early-response biological dosimetry–recommended countermeasure enhancements for mass-casualty radiological incidents and terrorism. Health Phys **89**: 494–504.
39. Coleman CN, *et al* (2003) Molecular and cellular biology of moderate-dose (1–10 Gy) radiation and potential mechanisms of radiation protection: report of a workshop at Bethesda, Maryland, December 17–18, 2001. Radiat Res **159**: 812–834.
40. Lazjuk GI, Nikolaev DL and Novikova IV (1997) Changes in registered congenital anomalies in the Republic of Belarus after the Chernobyl accident. Stem Cells **15**(Suppl 2): 255–260.
41. DeVita R, *et al* (2000) Health status and internal radiocontamination assessment in children exposed to the fallout of the Chernobyl accident. Arch Environ Health **55**: 181–186.
42. Izrael YA (2007) Chernobyl radionuclide distribution and migration. Health Phys **93**: 410–417.
43. Cwikel J, *et al* (1997) Two-year follow up study of stress-related disorders among immigrants to Israel from the Chernobyl area. Environ Health Perspect **105**(Suppl 6): 1545–1550.
44. Beehler GP, *et al* (2008) A multilevel analysis of long-term psychological distress among Belarusians affected by the Chernobyl disaster. Public Health **122**: 1239–1249.
45. (2007) The 2007 Recommendations of the International Commission on Radiological Protection. ICRP publication 103. Ann ICRP **37**: 1–332.
46. Taira Y, *et al* (2011) Current concentration of artificial radionuclides and estimated radiation doses from 137Cs around the Chernobyl Nuclear Power Plant, the Semipalatinsk Nuclear Testing Site, and in Nagasaki. J Radiat Res (Tokyo) **52**: 88–95.
47. Scanff P, *et al* (2004) Alteration of the enterohepatic recirculation of bile acids in rats after exposure to ionizing radiation. Can J Physiol Pharmacol **82**: 114–124.
48. Anno GH, *et al* (1989) Symptomatology of acute radiation effects in humans after exposure to doses of 0.5–30 Gy. Health Phys **56**: 821–838.
49. Nakamura S, *et al* (2010) Adiposity in female B6C3F1 mice continuously irradiated with low-dose-rate gamma rays. Radiat Res **173**: 333–341.
50. Racine R, *et al* (2010) Hepatic cholesterol metabolism following a chronic ingestion of cesium-137 starting at fetal stage in rats. J Radiat Res (Tokyo) **51**: 37–45.
51. Racine R, *et al* (2009) Molecular modifications of cholesterol metabolism in the liver and the brain after chronic contamination with cesium 137. Food Chem Toxicol **47**: 1642–1647.
52. Prat M, *et al* (2006) Use of flt3 ligand to evaluate residual hematopoiesis after heterogeneous irradiation in mice. Radiat Res **166**: 504–511.

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