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Analysis of chromosome damage by sister chromatid exchange (SCE) and redox homeostasis characterization on sheep flocks from Sardinian pasturelands

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UNIVERSITÀ DEGLI STUDI DI TORINO

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1 **ANALYSIS OF CHROMOSOME DAMAGE BY SISTER CHROMATID EXCHANGE (SCE)**
2 **AND REDOX HOMEOSTASIS TESTS ON SHEEP FLOCKS FROM SARDINIAN**
3 **PASTURELANDS**

4

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21

22

23 **Abbreviations**

24 Sister chromatid exchange, SCE; 5-Bromodiuxyuridine, 5-BrdU; reactive oxygen species, ROS;
25 Retinol, Ret; alpha-Tocopherol, Toc; Ascorbic Acid, Asc; Nitro-tyrosine, N-Tyr; protein-bound
26 carbonyls, PC; lipid hydroperoxides, LPO; superoxide dismutase, SOD; glutathione peroxidase, GPx;
27 Total Antioxidant Capacity, TAC; Enzyme linked immunosorbent assay, ELISA; high performance
28 liquid chromatography, HPLC.

29

Abstract

Over the last decades, an increase of pollutants of diverse origin (industrial, military, mining, etc.) was recorded in several areas of Sardinia Island. We report the results of a multidisciplinary and complementary study based on cytogenetic and physiological analyses. The data obtained show the effects of the environmental impact on six sheep flocks (Sardinian breed) grazing on natural pasturelands next to possible polluted areas and compared to three herds grazing in different areas far from those potentially contaminated and used as control. Sister chromatid exchange (SCE) test was used as cytogenetic test to analyze chromosomal damages and it was performed on peripheral blood samples collected from 129 adult sheep (age >4 years) randomly selected from polluted (92 animals) and control (37 animals) areas. Two types of cell cultures were performed: without (normal cultures) and with addition of 5-Bromodiuxyuridine (5-BrdU). SCE-mean values estimated over 35 cells counted for each animal were 8.65±3.40, 8.10±3.50, 8.05±3.08, 7.42±3.34, 9.28±3.56 and 8.38±3.29 in the exposed areas, whereas the average values were 7.86±3.31 in the control group. Significant increasing ($P<0.01$) of SCEs were found in three investigated areas of Southern Sardinia. Furthermore, sheep of the same flocks were characterized for blood redox homeostasis in order to define the potential targets of oxidative damage and to identify biomarkers of the extent of animal exposure to environmental contaminants. The plasma level of Asc, Toc and Ret were found significantly lower ($P<0.001$) in exposed sheep (I, II, IV and V) than in control group. TAC, as well as GPx and SOD activities were higher in control than in the exposed groups ($P<0.001$). Finally, plasma levels of N-Tyr, PC, and LPO were significantly lower ($P<0.001$) in the control group than in the exposed groups.

Keywords: Sister Chromatid Exchanges, Sardinian sheep, Environmental pollution, Chromosome fragility, Redox homeostasis, Bio-monitoring

55 **1. Introduction**

56 Sardinia is the second largest island in the Mediterranean Sea, well known for its natural landscapes
57 and for its endemic species. The island is considered as major center of plant diversity. Despite that,
58 various areas of this region are at environmental risk due to the contamination and/or pollution
59 generated by civil and industrial activities.

60 The most polluted areas are localized in the southwest of the Island (Boni et al. 1999; Sanna et al.
61 2003; Beccaloni et al. 2013) where high concentration of volatile organic compounds (benzene,
62 formaldehyde, xylene), polycyclic aromatic hydrocarbons and heavy metals have been found as result
63 of metallurgic industrial activities (Peluso et al. 2013) and mining actions (Madeddu et al. 2013;
64 Varrica et al. 2014; Cidu et al. 2014). In the same areas, residues of chemical emission due to military
65 tests have been also found (Cristaldi et al. 2013).

66 A total of 18 areas assessed as industrial, mining, military and urban zones have been considered at
67 environmental risk in Sardinia Island (Biggeri et al. 2006).

68 In this context it is worth mentioning that noxious pollutants, such as sulfur oxides, nitrogen oxides,
69 hydrocarbons, carbon monoxide, transition metals, may induce DNA damage and genome mutation,
70 thus exerting carcinogenic effects (Goulart et al. 2005; Mateuca et al. 2005; Cavallo et al. 2008).
71 These pollutants also promotes highly reactive oxygen species (ROS) production (Matès et al. 2010),
72 and depression of several ROS quenching systems (Ishida et al. 2009), with subsequent accumulation
73 of toxic compounds in blood and tissues (Knerr et al. 2006). In physiological conditions, the
74 antioxidant defence system, provided by enzymes and antioxidants, scavenges ROS thus limiting or
75 preventing oxidative damage (Halliwell, 2012). The imbalance between ROS production and
76 neutralizing capacity of antioxidant mechanisms may lead to oxidative stress (Cadenas and Davies,
77 2000; Halliwell and Gutteridge, 2000), which is associated with modifications of physiological and
78 metabolic functions (Halliwell and Gutteridge, 2000).

79 In the last decades, the epidemiological data on the incidence of human pathologies analyzed in
80 Sardinia, showed a general increasing trend as well as rising mortality rates have been reported for
81 the most prevalent types of cancer in both sexes (Budroni et al. 2013).
82 A recent investigation of the National Association of Italian Veterinarians (FNOVI) on sheep farms
83 located in potential polluted areas reported a correlation between the incidence of cancers in sheep
84 farmers and the emergence of genetic malformations in newborn lambs (Mellis and Lorrai, 2013). In
85 this respect, the farm animals represent good environmental sentinels (especially those naturally
86 pastured) to facilitate the assessment of human exposure to environmental contaminants. For instance,
87 they can be used as a monitoring system to reveal early environmental contamination, to monitor
88 contamination of the food web, to investigate the presence of contaminants in environmental media
89 (van der Schalie et al. 1999).
90 Among the farm animals, sheep are particularly suitable to be used as environmental sentinels. The
91 reason for that lies in their feeding characteristics. In fact, differently from the other ruminants, about
92 12% of the daily feed for sheep raised on pasture is represented by soil, where usually pollutants
93 accumulate and their permitted values are several times higher than those admitted in plants (i.e.
94 dioxin's values are 0.75ng/Kg and 10ng/Kg in plants and soil, respectively).
95 Cytogenetic tests represent direct and sensitive methods that have been used for detecting DNA
96 damages at the chromosomal level in the biomonitoring of different species (Penders et al. 2012;
97 Wójcik et al. 2013; Yang et al. 2014). In particular, Sister Chromatid Exchange (SCE) is a short-term
98 test for the detection of reciprocal exchanges of DNA between two sister chromatids, involving DNA
99 breakage and subsequent re-junction. The increased frequencies of SCE as consequence of negative
100 conditions, like pollutants exposition, leads to DNA single strands breaks as it has been reported in
101 several studies on livestock populations (Rubes et al. 1997; Di Meo et al. 2000; Iannuzzi et al. 2004;
102 Perucatti et al. 2006; Di Meo et al. 2011; Genualdo et al., 2012; Wójcik et al. 2013). The goal of this
103 study is to evaluate the impact of environmental pollutants in some areas of Sardina Island on the
104 genome damage of sheep flocks living in this region by using the SCE test. Furthermore, as the

105 analysis of blood redox homeostasis has become an important complementary tool for the evaluation
106 of health and metabolic status of dairy cows (Bernabucci et al. 2005; Castillo et al. 2003, 2005, 2006),
107 and feedlot calves (Castillo et al. 2012), we also characterized blood redox homeostasis of sheep in
108 order to define the potential targets of oxidative damage in blood, and to describe physiological
109 changes associated with exposure to pollutants.
110
111 **2. Materials and Methods**
112 *2.1. Farm selection*
113 The different areas were selected according to the chemical emissions of the different activities as
114 reported in former investigations (table 1).
115 The cytogenetic study was performed in Sardinia on 129 adult sheep (age >4 years) of Sardinian
116 breed, 92 grazing on natural pasturelands near to possible polluted areas selected as follows: two
117 herds (~12 sheep for each herd) located in the Northern area (industrial and military zones) and four
118 flocks (~15 sheep for each flock) located in the Southern area (military, industrial and mine zones)
119 of the island. For comparison, 37 sheep, reared in three different herds, were randomly selected in
120 areas far from possible polluted zones and used as control (Fig. 1).
121 Furthermore, physiological investigations were performed on 80 exposed sheep (20 per each group)
122 and 20 sheep used as control group belonging to the same farms.
123
124 *2.2. Cell cultures*
125 Whole blood samples were collected from the jugular vein using sterile vacutainer tubes
126 containing sodium heparin as anticoagulant. About 1 ml of whole blood sample was added to the
127 culture mix composed of 7 ml of RPMI medium, enriched with fetal calf serum (20%), L-glutamine
128 (0.25%), antibiotic-antimycotic mixture (0.5%) and concanavalin A (20 μg/ml) as mitogen. Culture
129 flasks were incubated at 37.8°C for 72 h. Cell cultures from the investigated animals were treated for
130 conventional (normal cultures) and 5-bromodeoxyuridine (BrdU) incorporation, the latter added 28

131 h before harvesting at final concentration of 10µg/ml to obtain preparations to be treated for SCE-
132 test. Both cell cultures were gently shaken once a day and subjected to 1.5 h of colcemid (0.5 µg/ml)
133 treatment, followed by centrifugation steps, hypotonic (KCl 75mM) and fixative treatments according
134 to Iannuzzi and Di Berardino (2008). Slides obtained from both normal and BrdU-treated cultures
135 were stained for 10 min with acridine orange (0.01 % in buffer phosphate), washed with distilled
136 water, and mounted in P-buffer. Slides obtained from normal cultures were used to detect structural
137 chromosomal abnormalities (in particular Robertsonian translocation or sex chromosome
138 aneuploidy), slides obtained from BrdU-treated cells were used for SCE-test. In particular, slides used
139 for normal cultures were used to get CBA-banding following the protocol reported by Iannuzzi and
140 Di Berardino (2008). At least 20 and 35 complete metaphases (2n=54) were studied to get CBA-
141 banding and SCE-test, respectively, for each animal. Slides were observed with a NIKON E-1000
142 fluorescence microscope (Nikon Instruments Europe B.V) equipped with FITC specific filter (ex 450-
143 490) and provided with image-analysis software system (RS Image image-acquisition software,
144 Photometrics Company). Digital images were captured at 100x magnification in gray-scale. All
145 metaphases were carefully examined by at least two expert cytogeneticists.

146

147 *2.3. Blood redox homeostasis characterization*

148 Plasma concentrations of non-enzymatic antioxidants (Ascorbic Acid, Retinol, and alpha-
149 Tocopherol), and the activities of enzymatic antioxidants (superoxide dismutase and glutathione
150 peroxidase) were measured, and used as markers of the antioxidant defense system. The total
151 antioxidant capacity was also assessed, as well as the oxidative damage to protein and lipid
152 peroxidation induced by the interaction of free radicals with polyunsaturated fatty acids. Therefore,
153 blood samples were collected into heparinised tubes, early in the morning, in the same day and under
154 the same environmental conditions. Plasma was obtained by centrifugation (500 g for 15 min at 4°C)
155 and processed, by the same operator, for titration of antioxidants, SOD and GPx activity, total

156 antioxidant capacity (TAC), nitro-tyrosine (N-Tyr), protein-bound carbonyls (PC), and
157 hydroperoxides (LPO).
158

159 *2.3.1. Determination of antioxidants and lipid hydroperoxides (LPO)*

160 Plasma samples were processed for determination of Asc concentration as previously described
161 (Spagnuolo et al. 2011), and analysed by high performance liquid chromatography (HPLC) using an
162 anion exchange column (Nucleosil 100-NH_2, 5 µm, 250 x 4.6 mm i.d). Ret and Toc levels were
163 measured according to a published procedure (Spagnuolo et al. 2003), and analysed by HPLC, using
164 a reverse phase C18 column (Nova-PAK C18, 4µm, 125 x 2 mm i.d.). The Total Antioxidant Capacity
165 (TAC) was measured by the Trolox Equivalent Antioxidant Capacity Assay, according to Miller et
166 al. (1993a), and expressed as µM concentration of Trolox equivalents (Miller et al. 1993a; Spagnuolo
167 et al. 2001).

168 LPO concentration was measured by a colorimetric quantitative assay, using the Lipid Hydroperoxide
169 Assay Kit of Cayman Chemical, according to the manufacturer's instructions.
170

171 *2.3.2. Determination of nitro-tyrosine (N-Tyr)*

172 Nitrated protein levels in plasma samples were measured by ELISA, as previously reported
173 (Spagnuolo et al. 2001). Plasma samples were diluted (1:500, 1:2000, 1:5000, and 1:10000) with
174 coating buffer (7 mM Na2CO3, 17 mM NaHCO3, 1.5 mM NaN3, pH 9.6), and incubated in the wells
175 of a microtitre plate overnight at 4°C. Standard curves were obtained with serial dilutions of nitrated
176 bovine serum albumin (BSA). N-Tyr was detected by incubation with Rabbit anti-N-Tyr antibody
177 (Covalab; 1: 800 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween 20, pH 7.3,
178 supplemented with 0.25 % BSA; 1 h, 37°C), followed by Goat anti Rabbit IgG-horseradish peroxidase
179 linked (GAR-HRP) diluted 1:2000 as the primary antibody. Colour development was monitored at
180 492 nm, as previously described (Spagnuolo et al., 2003). Data were reported as nmol of N-Tyr per
181 mg of protein.

182

183 *2.3.3. Determination of protein-bound carbonyls (PC)*

184 PC concentration in plasma samples was titrated by ELISA according to Buss et al. (1997).
185 Protein derivatization was carried out with a dinitrophenylhydrazine (DNP) solution (10 mM in 6 M
186 guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5), to a final protein concentration
187 of 3 mg/mL. Samples were incubated at room temperature for 45 min vortexing every 10-15 min.
188 Each sample was then diluted (1: 800-1: 15000) with10 mM sodium phosphate buffer, pH 7.0,
189 containing 140 mM NaCl, and incubated (overnight at 4°C) in the wells of a microtitre plate. PC were
190 detected by incubation (1 h at 37°C) with Rabbit anti-DNP antibody diluted 1:1000 with PBS
191 supplemented with 0.2 % gelatine and 0.05% Tween 20, followed by GAR-HRP antibody (diluted
192 1:2500 as the primary antibody). Colour development was monitored at 492 nm, as previously
193 described (Spagnuolo et al. 2003). A six-point standard curve of oxidized BSA was included in each
194 plate. A blank for DNP reagent in PBS without protein was subtracted from each absorbance. Data
195 were reported as nmol of carbonyls per mg of proteins.

196

197 *2.3.4. Evaluation of plasma activity of glutathione peroxidase (GPx) and superoxide*
198 *dismutase (SOD)*

199 GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR),
200 using the glutathione peroxidase assay kit of Cayman Chemical, according to the manufacturer's
201 instructions. GPx activity was expressed as nmol of NADPH oxidized per minute per mL of sample.
202 SOD activity was measured with the superoxide dismutase assay kit of Cayman Chemical, according
203 to the manufacturer's instructions. SOD activity was expressed Unit/mL. One unit of SOD is defined
204 as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

205

206 *2.4. Statistical analysis*

207 Summary statistics were calculated for both single animals and animal groups. The distribution
208 of data within each group was tested for normality according to Shapiro-Wilk (1965). Significant
209 departures from symmetry ($P<0.05$) was observed in all groups with the exception of the exposed VI,
210 whose kurtosis was 0.19. The Log transformation of the data showed that groups with higher SCE
211 means tend also to have more-variable data, indicating that ANOVA on the raw data may be
212 problematic. As consequence, alternative procedures like non parametric tests are more appropriate.
213 In order to ensure the strictness of the result, both parametric (ANOVA on raw data) and
214 nonparametric tests (Kruskal-Wallis) were performed to point up the differences between and within
215 the analysed groups. Tukey's and Dwass-Steel-Critchlow-Fligner's pairwise were used to make all
216 possible comparisons between the groups. Bonferroni correction was applied as default restriction
217 and differences were considered significant if $P\leq0.01$.
218 The samples for measurement of SOD and GPx activities, PC, N-Tyr, or LPO concentration were
219 processed in triplicate. The titration of Toc, Ret, and Asc was carried out on duplicates. Values were
220 expressed as mean ± SD. Significance of statistical differences was evaluated by one-way ANOVA,
221 followed by Bonferroni's test for multiple comparisons, using the Graph Pad Prism 5.01 program
222 (Graph Pad Software, San Diego, CA, USA).
223
224 ## 3. Results and discussion
225 The CBA-banding, very useful to detect sex chromosome abnormalities, did not reveal any numerical
226 and structural abnormality in all studied animals.
227 The SCE-test was applied to six groups of sheep reared on natural pasturelands near to possible
228 polluted areas, as well as on three groups located far from polluted areas and used as control. Analysis
229 of variance using both parametric (ANOVA) and non-parametric (Kruskal-Wallis) approaches
230 revealed significant differences among the groups (table 3). SCE-mean values per cell were higher in
231 three exposed sheep groups (Esp. I, V and VI) compared to the control. The remaining exposed groups
232 (II, III and IV) did not show significant differences compared to the control (table 2 and 4). The mean

233 values of II, III, IV exposed groups and control were comparable to previously published data on SCE
234 test for sheep reared in Campania region (Perucatti et al. 2006). No inter-individual differences were
235 detected among the samples belonging to the same group, whereas inter-group differences were
236 clearly evidenced from multiple comparison tests. In fact, both Tukey's and Dwass-Steel-Critchlow-
237 Fligner's pairwise highlighted the same significant differences (table 4). In particular, the groups I
238 and V showed SCE mean values higher than the number of exchanges counted in the other exposed
239 groups. Interesting seems to be the data of the exposed group III, whose mean SCE value is not
240 different from the control, but also closer to the exposed group VI (table 2).
241 Blood redox status of sheep living in different geographic areas of Sardinia, exposed to different
242 environmental contamination, was characterized, and the results were compared with those obtained
243 from animals bred in the unpolluted area. Plasma level of Ret, Toc, and Asc, the total antioxidant
244 capacity (TAC), and GPx and SOD activities, here used as markers of the antioxidant defence system,
245 are shown in table 5. The concentrations of Asc, Toc and Ret were found significantly lower
246 ($P<0.001$) in plasma collected from sheep reared in polluted areas (I, II, IV and V) than in control
247 group (K). TAC, as well as GPx and SOD activities were higher in control than in the exposed groups
248 ($P<0.001$). These findings demonstrate that the exposure to environmental pollutants severely impairs
249 the blood antioxidant defence system. By comparing the groups from the polluted areas, we found
250 that plasma concentration of Ret and Toc was significantly lower in sheep of group V than in the
251 other exposed groups (I, II and IV; $P<0.001$), but did not differ among groups from polluted areas (I,
252 II, IV and V). Conversely, the other markers of the antioxidant defence system did not differ among
253 the two exposed groups. These findings suggest that liposoluble antioxidants play a key role in the
254 protection from environmental pollutants, and that the lipophilic compartment represents a specific
255 target of damage in exposed animals, in agreement with data previously obtained from bovine
256 exposed to steel plant-derived contaminants (Spagnuolo et al. 2012).
257 The extent of oxidative damage to proteins and lipids was evaluated by measuring plasma
258 concentrations of PC, N-Tyr, and LPO, and a significantly higher extent of oxidative modifications

259 of protein and lipid was also observed in exposed sheep. As shown in Table 6, plasma levels of N-
260 Tyr, PC, and LPO were significantly lower ($P<0.001$) in the control group than in the exposed groups.
261 In addition, by comparing the four groups from polluted areas, significant differences in the extent of
262 oxidative modifications to plasma proteins and lipids were also found. Indeed, the concentration of
263 N-Tyr, PC and LPO was higher in the plasma from animals of group V ($P<0.01$ and $P<0.05$
264 respectively) than from the other exposed groups. It is worth mentioning that N-Tyr level represents
265 the footprint of protein oxidative damage induced by peroxynitrite (Halliwell, 1997), PC may be
266 introduced into proteins by direct oxidative attack to proteins themselves (Kristal and Yu, 1992), or
267 by reactions with aldehydes originated during lipid peroxidation processes (Uchida and Stadtman,
268 1993), and LPO is an index of the extent of lipid peroxidation induced by the interaction of free
269 radicals with polyunsaturated fatty acids. Therefore our results strongly suggest that lipid
270 peroxidation and its intermediates, as well as peroxynitrite production, are crucial in determining
271 oxidative modifications to protein and lipid in sheep reared in polluted areas.
272 On the basis of the location of the samples collection and the results of data analysis, it is clearly
273 shown that differences exist between the groups collected in the northern and southern areas of the
274 island.
275 Such a result might be connected to the territorial distribution of industrial, military and mining areas.
276 In fact, for its strategic position in the middle-west of Mediterranean Sea, Sardinia Island has
277 important oil-refineries (for instance Porto Torres) and one of the biggest petrochemical park in
278 Europe (Sarroch industrial estate). Moreover, there is a military training area (Quirra zone) and
279 location of active and disused mines as listed by the Italian Agency for the territory (MATT, 2006).
280 With the exception of the mining areas spread on the territory, most of the other centres of activity
281 are located in the southern part of the island in a triangle of about 250 km where the groups with
282 higher SCE-means (I, V and VI), lower concentration of Ret and Toc and higher level of N-Tyr, PC
283 and LPO in the plasma (especially V) were sampled. The location of samples collection also explains
284 the trend of the exposed group III which shows intermediate values of SCE. In fact, it is located near

285 a large industrial area for steel production. However, differently from other polluted areas, here the
286 values of the contaminants increase when the distance from the industrial pole decrease. Such a
287 condition might be the result of the wind direction, always reported as E-NE by the Environmental
288 Protection Agency of Sardinia (ARPAS, 2012).
289 The higher DNA breakages of these samples agrees with a former study carried out in Sarroch
290 industrial estate on DNA damages caused by malondialdehyde–deoxyguanosine adducts (Peluso et
291 al. 2013). The data, drawn from this research, show the problematic situation of this region. Indeed,
292 a higher incidence of teratogenesis in animals and a higher cancer incidence for both sheep and sheep
293 farmers of the area V have been reported (Mellis and Lorrai, 2013).
294 The level of pollution present in these areas was already reported by several studies (Forte et al. 2011;
295 Peluso et al. 2013; Cidu et al. 2013). In particular, among the most represented pollutants there are
296 sulphates, As, Cd, Cr, Hg, Ni, Pb, Zn, Sb, Mn, Tl, in different distribution in the soil–water–plant
297 system in the south-western part and Rb, Tl, W, Al, Ti in the PISQ area as reported by ARPAS (2012,
298 2013). Furthermore, the disused mine sites in the proximity of the samples collection sites still release
299 various metals and metalloids into environment, contributing negatively to such condition as reported
300 by Varrica et al. (2014).
301 This mixture of air pollutants has a large impact on health of people living in this region. In fact, for
302 some combined or mixed exposures the health effects may increase more than the expected effects of
303 the individual components (Silins and Högberf, 2011). It is well established that several
304 environmental mutagens may induce chromatin damages (Bryant et al., 2004) and overwhelm
305 antioxidant defences. Since the chromatin is the main component of chromosomes, damage at the
306 chromosomal level (especially when double DNA breakages occur) may denote chromosome
307 fragility. The higher is this chromosome fragility, the higher is the risk of mutations in animals, and,
308 indirectly humans (Iannuzzi et al. 2004). This enhanced chromosome fragility can result in gene
309 mutations which are often the first step for the onset of cancer, immune-enzymatic defects and
310 reproductive problems. In previous studies, adverse effects of environment air pollution on human

311 and animals reproduction have been demonstrated, including increased risk of foetal growth,
312 restriction for pregnant woman (Dejmek et al. 1999, 2000), decreased sperm quality in young men
313 (Selevan et al. 2000), that included sperm DNA damage, and reduced fertility for the animals
314 (Gustavsson, 1980; Ducos et al. 2008).
315 Furthermore, since the oxidative stress impairs health, fertility and zootechnical performance of dairy
316 cows (Miller et al. 1993b), and is involved in the etiology of several diseases and metabolic disorders
317 (Smith et al. 1984 Bernabucci et al. 2002, 2005), the exposure to environmental pollutants, as
318 affecting blood redox homeostasis of sheep, might be associated with modifications of physiological
319 and metabolic functions.
320 Therefore, our study is a further indication of a potential risk of adverse effect on the health of the
321 exposed population, which suggests to implement the surveillance activity in this region, especially
322 in the southern part.
323

324 ## 4. Conclusion

325 To our knowledge, this is the first report of chromosome fragility by cytogenetic test in livestock
326 reared in Sardinia. The data obtained in this study confirm that animal biomonitoring is a powerful
327 tool for risk assessment from natural and anthropogenic exposure to environmental pollutants. In
328 particular, monitoring the livestock population by cytogenetic tests might be a good tool to control
329 indirectly the food chain, to preserve health problems, and to avoid management problems and
330 income losses at farm level. In this respect the herbivores, consuming large volumes of fodder, are
331 the most suitable mammals to be used as environmental sentinel. This is particular important in
332 Sardinia region where the larger Italian sheep population is reared. The SCE data obtained in the
333 present study represent a baseline level for the Sardinian sheep and it represents an essential step for
334 future assessment of health risks in relation to environmental hazards. We also propose that the
335 characterization of blood redox status might represent an useful tool for identifying animals exposed
336 to environmental pollutants. In addition, as plasma concentrations of Ret, Toc, PC, N-Tyr and LPO

337 significantly differ among groups from different geographic areas, with different types and degree of
338 environmental contamination, they could represent selective and specific markers for bio-monitoring
339 the extent of exposure to specific pollutants.

340

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344

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523

524 **Table 1**. Chemical emissions due to the various activities of selected areas of Sardinia Island

Areas	Activity	Emissions	References
Control	Agriculture	n.a.	
Exposed I	Mining	Ag,Ba,Cd,Cr,Ni,Pb,Rb,Sb,U,V,Zn	Pirastu et al. 2011
			Safronova et al. 2012
			Madeddu et al. 2013
			Varrica et al. 2014
Exposed II	Military	former nuclear submarine base	Aumento et al. 2004
			Hernandez et al. 2011
Exposed III	Industrial	Hg, Cd, Pb, Zn	Schintu and Degetto 1999
			Sanna et al. 2003
Exposed IV	Industrial	IPA,	De Luca et al. 2003
			Pirastu et al. 2011
Exposed V	Military	Rb, Tl, W, Ti and Al, Cd, Pb	Cristaldi et al. 2013
			Gatti et al. 2013
Exposed VI	Military/Industrial	PCDD, PCDF, PCB	Storelli et al. 2012

525

526 **Table 2**. Number of animals, examined cells, SCE mean values and standard deviations in sheep
527 reared in polluted and control areas of Sardinia Island (Italy).
528

Animals		Examined cells	SCEs		
Group	N	N	N	Mean	SD
Exposed I	19	630	5754	8.65[c]	3.40
Exposed II	19	630	5388	8.10[a]	3.50
Exposed III	12	420	3383	8.05[a,b]	3.08
Exposed IV	13	420	3377	7.42[a]	3.34
Exposed V	11	315	3575	9.28[c]	3.56
Exposed VI	18	560	5280	8.38[b,c]	3.29
Control	37	1225	10185	7.86[a]	3.31

[a,b,c] Means within columns without a common superscript differ (P<0.01)

529

530 **Table 3**. Parametric (One-way ANOVA) and nonparametric (Kruskal-Wallis H-test) analysis and of damaged cells showing differences among

531 treatment groups.

ANOVA					Kruskal-Wallis		
Sources of variation	D. of freedom	Sum of squares	Mean sum of square	*F ratio*		Observed	D. of freedom
Between groups	6	1027,36	171,226	15,45*	H	90,73*	6
Within groups	4193	46477,4	11,0845				
Total:	4199	47504,8					

*p< 0.0001

532

Table 4. P-value obtained from multiple comparisons among the groups performed by Tukey pairwises test (above the diagonal) and Dwass-Steel-Critchlow-Fligner pairwises test (below the diagonal). Significant values after Bonferroni correction are indicated by asterisks.

	I	II	III	IV	V	VI	Control
I		0.002*	0.011	<0.0001*	0.146	0.991	<0.0001*
II	0.000*		0.999	0.521	<0.0001*	0.032	0.997
III	0.015	0.986		0.274	<0.0001*	0.099	0.956
IV	<0.0001*	0.564	0.195		<0.0001*	<0.0001*	0.875
V	0.204	<0.0001*	<0.0001*	<0.0001*		0.019	<0.0001*
VI	0.999	0.004*	0.074	<0.0001*	0.096		0.004*
Control	<0.0001*	1	0.934	0.579	<0.0001*	0.000*	

Table 5. Markers of the antioxidant defence system in plasma of sheep

	Control	I	II	IV	V
Ret (µg/ml)	0.65±0.05***	0.48±0.04^{aaa}	0.47±0.04^{ccc}	0.46±0.04	0.41±0.03^{e}
Toc (µg/ml)	2.18±0.08***	1.28±0.08^{aaa}	1.26±0.08^{ccc}	1.24±0.05	1.09±0.07^{eee}
Asc (µM)	6.93±0.67***	5.97±0.41^{bbb}	5.37±0.37^{cc}	6.24±0.28^{ddd}	5.85±0.40
GPx (nmol/min/ml)	149.8±46.9***	90.9±30.3	85.5±28.4	101.0±33.6	83.6±27.8
SOD (U/ml)	1.64±0.37***	0.91±0.23	0.86±0.21	1.02±0.25	0.84±0.21
TAC (µM)	106.5±12.3***	86.6±10.0	81.4±9.4	84.8±9.7	78.8±9.1
N	20	20	20	20	20

Ret: Retinol; Toc: α-Tocopherol; Asc: Ascorbate; TAC: Total Antioxidant Capacity (µM concentration of Trolox equivalents); GPx: Glutathione Peroxidase Activity; SOD: superoxide dismutase.

Sheep reared in not polluted area were regarded as control group.

***Control vs I, II, IV and V ($P < 0.001$)

^{aaa}I vs V ($P < 0.001$)

^{bbb}I vs II ($P < 0.001$)

^{ccc}II vs V ($P < 0.001$)

^{ddd}II vs IV ($P < 0.001$)

^{eee}IV vs V ($P < 0.001$)

^{e}IV vs V ($P < 0.05$)

Table 6. Markers of oxidative stress in plasma of sheep

	Control	I	II	IV	V
PC (nmol/mgP)	18.81±2.87***	47.47±6.68[aa]	42.72±6.01[ccc]	38.95±7.38	55.54±7.81[eee]
N-Tyr (nmol/mgP)	12.58±0.94***	21.93±2.29[aaa]	19.73±2.06[bb, ccc]	15.92±0.92[ddd]	24.12±2.52[eee]
LPO (µM)	13.37±2.46***	20.46±4.58[a]	18.55±1.94[ccc]	17.00±1.89	23.94±5.36[eee]
N	30	20	20	20	20

PC: protein-bound carbonyls (nmol per mg of protein); N-Tyr: nitro-tyrosine (nmol per mg of protein); LPO: lipid hydroperoxides (µM).

Sheep reared in not polluted area were regarded as control group.

***Control vs I, II, IV and V ($P < 0.001$)

[aaa]I vs V ($P < 0.001$)

[aa]I vs V ($P < 0.01$)

[a]I vs V ($P < 0.05$)

[bb]I vs II ($P < 0.01$)

[ccc]II vs V ($P < 0.001$)

[ddd]II vs IV ($P < 0.001$)

[eee]IV vs V ($P < 0.001$)

Fig. 1. Localization of the farms investigated: A-Control; B-Exposed I; C-Exposed II; D-Exposed III; E-Exposed IV; F-Exposed V; G-Exposed VI.