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Mode of action of Cr(VI) in immunocytes of earthworms: implications for animal health

Susanna Sforzini [a], Michael N. Moore [a, b, c], Zhuofan Mou [c], Marta Boeri [a], Mohamed Banni [d], Aldo Viarengo [a]

[a] Department of Sciences and Technological Innovation (DiSIT), University of Piemonte Orientale "A. Avogadro", V.le T. Michel 11, 15121 Alessandria, Italy

[b] European Centre for Environment & Human Health (ECEHH), University of Exeter Medical School, Truro, TR1 3HD, UK

[c] Plymouth Marine Laboratory, Plymouth, PL1 3DH, UK

[d] Laboratory of Biochemistry and Environmental Toxicology, ISA, Chott-Mariem, Sousse, Tunisia

Corresponding author:

Aldo Viarengo

Department of Sciences and Technological Innovation (DiSIT), University of Piemonte Orientale "A. Avogadro", V.le T. Michel 11, 15121 Alessandria, Italy

Phone: + 390131360370

Fax: +390131360390

e-mail: viarengo@unipmn.it

Abstract

Chromium (Cr) is one of the major and most detrimental pollutant, widely present in the environment as a result of several anthropogenic activities. In mammalian cells, Cr(VI) is known to enhance reactive oxygen species (ROS) production and to cause toxic and genotoxic effects. Less commonly investigated are the effects and mode of action of this contaminant in invertebrates, particularly in soil organisms. In this work, earthworms of the species *Eisenia andrei* were exposed for 1 and 3 days to various sublethal concentrations of Cr(VI) (2, 15, 30 μg mL^{-1}) using the paper contact toxicity test. In amoeboid leukocytes we investigated intracellular ROS and lipoperoxide production, oxidative DNA damage, and the effects on different cell functions. The analysis of the results shows that Cr(VI) triggered severe adverse reactions; the first events were an increase of intracellular ROS levels, generating in the cells oxidative stress conditions leading to membrane lipid peroxidation and oxidative DNA damage. Lysosomes showed relevant changes such as a strong membrane destabilization, which was accompanied by an increased catabolism of cytoplasmic proteins and accumulation of lipofuscin. With an increase in the dose and/or time of exposure, the physiological status of intracellular organelles (such as lysosomes, nucleus and mitochondria) showed further impairment and amoebocyte immune functions were adversely affected, as shown by the decrease of the phagocytic activity. By mapping the responses of the different parameters evaluated, diagnostic of (oxidative) stress events, against lysosomal membrane stability, a "health status" indicator (able to describe the stress syndrome from its early phase to pathology), we have shown that this biomarker is suitable as a prognostic test for health of earthworms. This is viewed as a crucial step toward the derivation of explanatory frameworks for prediction of pollutant impact on animal health.

Keywords: chromium(VI); immunocytes; earthworms; oxidative stress; reactive oxygen species

47 # 1. Introduction

48 Chromium (Cr) is one of the major and most detrimental environmental pollutants (Singh and
49 Yadava, 2003; Peralta-Videa et al., 2009). Besides its natural occurrence, Cr is widely present in the
50 environment as a result of several anthropogenic activities such as electroplating, manufacturing
51 industries and ferrochrome production (Roca-Perez et al., 2010; Liao et al., 2013). Between the two
52 most stable and common oxidation states of Cr (i.e. the trivalent [Cr(III)] and the hexavalent
53 [Cr(VI)] forms), Cr(VI) is much more toxic to most living organisms (Levina and Lay, 2005;
54 Peralta-Videa et al., 2009). Several studies have indicated that in different biological systems
55 Cr(VI) can readily cross cell membranes (Arslan et al., 1987; Chatterjee et al., 2009). The reductive
56 metabolism of Cr(VI) results in the production of Cr(III), a process that can generate variable
57 amounts of reactive Cr(V/IV) intermediates and reactive oxygen species (ROS) (Salnikow and
58 Zhitkovich, 2008). Despite the abundance of data underlining the potential molecular mechanisms
59 of Cr(VI) cytotoxicity and genotoxicity in vertebrates (Bagchi et al., 2001; Shrivastava et al., 2002),
60 relatively less investigated are the effects and mode of action of this contaminant in invertebrates, in
61 particular in edaphic organisms notwithstanding the extensive Cr(VI) soil contamination (ATSDR,
62 2012).

63 Earthworms are very important organisms in terrestrial ecosystems. These invertebrates are often a
64 major component of soil fauna communities and their activity is essential for soil formation and
65 fertility (Lee, 1985; Blouin et al., 2013). Previous studies reported adverse effects of Cr(VI) on
66 worm survival and reproduction (Soni and Abbasi, 1981; Abbasi and Soni, 1983; Sivakumar and
67 Subbhuraam, 2005). However, only a few data exist for the Cr(VI)-induced responses of more
68 sensitive biomarkers at lower level of functional complexity: the evaluation of these parameters is
69 of great relevance to highlight early warning signals of detrimental changes before the further
70 impairment of the organism; and finally, before negative consequences at the population and
71 ecosystem level (Depledge et al., 1993; Viarengo et al., 2007). In particular, Manerikar et al. (2008)

and Bigorgne et al. (2010) showed that Cr(VI) provoke a sustained increase in the level of DNA damage in coelomocytes of exposed worms.

Coelomic amoebocytes (cells circulating in the coelomic fluid constituting the hydrostatic skeleton of earthworms) are immunocytes involved in a broad range of defence functions (Engelmann et al., 2005; Bilej et al., 2010). However, amoeboid leukocytes, non-invasively extruded from the coelomic cavity, have proved an appropriate target for assessing toxic and genotoxic effects of chemicals, allowing for the detection of sensitive physiological responses in exposed animals (Eyambe et al., 1991; Homa et al., 2005; Sforzini et al., 2012; Hayashi and Engelmann, 2013; Muangphra et al., 2015). Cr(VI) has been found to be involved in the alteration of the immune response in different cell types (Shrivastava et al., 2002; Ciacci et al., 2011).

One of the aims of this work was to investigate the alterations caused by Cr(VI) in amoebocytes of earthworms and the possible processes that lead to the effects observed: this could help to understand how chromium compromises the immune function of these organisms, rendering them potentially more susceptible to additional stress conditions. To this end, earthworms of the species *Eisenia andrei* (Bouché, 1972) (Oligochaeta, Lumbricidae), commonly used for standard toxicity tests and ecotoxicological studies (OECD, 2004; ISO, 2008; Lee et al., 2008; Irizar et al., 2014), were exposed for 1 and 3 days to different sublethal concentrations of Cr(VI) using the paper contact toxicity test (OECD, 1984). In amoebocytes we investigated intracellular ROS production and the alterations caused in different cellular compartments such as lysosomes and mitochondria; moreover, at the nuclear level, the genotoxic damage was determined by evaluating the oxidative DNA damage. The modifications of the membranes were also studied by evaluating the generation of lipoperoxides that may compromise the membrane functions. The phagocytic activity of the amoebocytes was finally evaluated to reveal if the cellular (oxidative) stress conditions can affect also the immune response of Cr(VI) exposed worms.

A second objective was the development of conceptual and predictive models of lysosomal and other biomarker reactions as both diagnostic and prognostic biomarkers for health status in the earthworms. Multivariate statistical analysis was used to develop an appropriate conceptual framework and statistical models for the role of a multi-biomarker assessment, as well as lysosomal function and responses to environmental variables, particularly chemical pollutants (Moore et al., 2004, 2006; Sforzini et al., 2015). Previous studies on different organisms have shown that there is a strong relationship between LMS, as an indicator of cellular health, and the responses of numerous stress biomarkers (Moore et al., 2006; Sforzini et al., 2015). Principal component analysis was used to integrate multi-biomarker data to test a predictive statistical model of lysosomal function (membrane stability) in the amoebocytes of earthworms. We propose that such models will provide the necessary basis for explanatory frameworks that will facilitate the development of a predictive capacity for estimating risk to the health of sentinel animals associated with the possibility of future environmental events.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated.

2.2. Animals

Earthworms of the species *E. andrei* were cultured essentially as described in the OECD guidelines (OECD, 1984, 2004). Organisms were selected from a synchronised culture with an homogeneous age structure. Adult worms with a clitellum of similar size and weight (of 400 to 500 mg) were utilised in the experiments.

121 *2.3. Paper contact toxicity test*

122 The filter paper test was performed as described in the OECD guideline for the testing of chemicals
123 (OECD, 1984). Worms were kept on clean moist filter paper for 3 h before being placed in test
124 dishes to allow them to void their gut contents. Animals were then washed with deionised water and
125 dried before use. $K_2Cr_2O_7$ was dissolved in Milli-Q water to give the range of Cr(VI) concentrations
126 used in the experiment i.e. 2, 15, 30 µg mL^{-1}. Then, 1 ml of each contaminant solution was spread
127 onto a filter paper (Whatman grade 1), evaporated to dryness and placed on the bottom of a Petri
128 dish. Control filter papers were treated with 1 ml of Milli-Q water. After drying, 1 ml of deionised
129 water was added to each dish to moisten the filter paper. The test was performed in the dark at 20 ±
130 1 °C and for a period of 1 and 3 d. At least ten replicates per treatment for each assay, consisting of
131 one worm per dish, were used.

132

133 *2.4. Coelomocytes harvesting*

134 Earthworm coelomocytes were obtained by a non-invasive ethanol extrusion method (Eyambe et
135 al., 1991; Fugère et al., 1996), with modifications, as previously described by Sforzini et al. (2012).
136 Cell viability Trypan-blue exclusion method was assessed immediately after the extrusion and
137 resulted >95% in all cases. The coelomic fluid contains two main types of coelomocytes i.e. i) the
138 chloragocytes (eleocytes), cells differentiating from the chloragogenous tissue which play nutritive
139 functions and contribute to homeostasis and humoral immunity; and ii) the amoebocytes (either
140 hyaline or granular), originating from the lining of the coelomic cavity which represent effectory
141 immunocytes involved in a broad range of defence functions including phagocytosis (Plytycz et al.,
142 2006; Bilej et al., 2010; Hayashi and Engelmann, 2013).

143

144 *2.5. Oxidative stress*

145 *2.5.1. ROS and lipoperoxides detection*

Coelomocytes were placed on polylysinated slides where they were allowed to adhere for 15 min in a humidity chamber at 20 ± 1 °C. Both amoebocytes and eleocytes firmly attached to the polylysinated slides; the analyses were performed only on amoebocytes. For the evaluation of ROS and lipoperoxides generation, the cells were incubated with the fluorescent probes hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO-9-AC, Invitrogen-Molecular Probes, Eugene, USA) (20 µM, 10 min), for the detection of hydroxyl and superoxide radicals; dihydrorhodamine (DHR) 123 (Invitrogen-Molecular Probes, Eugene, USA) (10 µM, 5 min), able to highlight the generation of hydrogen peroxide and peroxynitrite; and BODIPY® 581⁄591 C11 (Invitrogen-Molecular Probes, Eugene, USA) (5 µM, 10 min), specific for lipid peroxides. Then, excess dye was eliminated and the cells were washed and kept moist with Hanks' Balanced Salt Solution (HBSS). Slides were viewed under 630x magnification by an inverted photo-microscope (Zeiss Axiovert 100M) equipped for fluorescence microscopy using a FITC emission filter. Images were analysed using an image analysis system (Scion Image) that allowed for the quantification of radicals and lipoperoxides generation, that were expressed as fluorescence intensity.

2.5.2. Lipofuscin lysosomal content

Lipofuscin lysosomal content was determined using the Schmorl reaction (Moore, 1988; Sforzini et al., 2011), with modifications. After adhesion (as described above), cells were fixed for 10 min in formol-calcium at 4 °C and then immersed for 5 min in the reaction medium containing 1% ferric chloride and 1% potassium ferricyanide in a ratio of 3:1. Slides were rinsed in 1% acetic acid for 30 sec, followed by rinsing in distilled water and mounted in glycerol gelatin. The lysosomal accumulation of lipofuscin (dark blue granules due to the reduction of ferricyanide to ferrocyanide) was quantified by image analysis as described above and expressed as a percentage variation with respect to controls.

171 *2.6. DNA damage*

172 DNA damage was assessed by the alkaline Comet assay essentially as described by Sforzini et al.
173 (2012). To reveal also the oxidative damage to DNA, the formamidopyrimidine-DNA glycosylase
174 (Fpg) was introduced in the method, as described by Collins et al. (1993), with modifications.
175 Essentially, the method requires the use of the enzyme in an extra step in the standard alkaline
176 Comet assay: after lysis, slides were washes with the enzyme buffer (0.1 M KCl, 0.5 mM
177 Na_2EDTA, 40 mM HEPES, 0.2 mg/ml bovine serum albumin, pH 8.0) and then incubated for 15
178 min with Fpg (1/4000 in enzyme buffer). After electrophoresis, slides were washed with PBS, fixed
179 in ice-cold ethanol (70%) for 5 min and subsequently stained with DAPI (4',6-diamidino-2-
180 phenylindole) (ICN Biomedicals Inc., USA), a DNA-specific fluorescent probe. Slides were
181 observed with an inverted photo-microscope (Zeiss Axiovert 100 M) equipped for fluorescence
182 microscopy at 400× magnification. A total of 100 cells were scored for each sample and the
183 captured images were analyzed for the % DNA content in the tail, by use of the Comet Score
184 image-analysis software (TriTek CometScore^{TM}).

185

186 *2.7. Alterations of cell functions*

187 *2.7.1. Mitochondrial functionality*

188 Mitochondria were labelled using MitoTracker® Green FM (Invitrogen-Molecular Probes, Eugene,
189 USA). After adhesion (as described above), the cells were incubated with the probe (50 nM) for 30
190 min at 20 ± 1 °C. Then, excess probe was eliminated and the cells were washed and kept moist with
191 HBSS. Slides were viewed under 630x magnification by an inverted photo-microscope (Zeiss
192 Axiovert 100M) equipped for fluorescence microscopy using a FITC emission filter. Images were
193 analysed using an image analysis system as described above that allowed for the quantification of
194 the mitochondrial mass, that was expressed as a percentage change in fluorescence intensity with
195 respect to controls.

196

197 *2.7.2. Lysosomal membrane stability*

198 Lysosomal membrane stability was determined as described by Sforzini et al. (2011). After
199 adhesion (as described above), the cells were incubated in a working solution of neutral red (NR),
200 obtained by diluting 10 µL of a stock solution of NR (20 mg of NR in 1 mL of dimethyl sulfoxide -
201 DMSO) with 990 µL of Hanks' Balanced Salt Solution (HBSS) (Sigma product H8264). After 5
202 min, excess dye was eliminated and the cells were washed and kept moist with HBSS. The retention
203 time of NR dye within the lysosomes (NRRT) was monitored after 1 h. Slides were viewed under
204 630× magnification by an inverted photo-microscope (Zeiss Axiovert 100M) equipped for
205 fluorescence microscopy using a rhodamine emission filter. Images were analysed using an image
206 analysis system as described above that allowed for the quantification of the lysosomal NR leakage,
207 that was expressed as a percentage change in fluorescence intensity with respect to controls.

208

209 *2.7.3. Autophagy*

210 The autophagic process was assessed using fluorescein diacetate 6-isothiocyanate (Diacetyl-6-
211 FITC) as described by Moore et al. (2008), with slight modifications. After adhesion (as described
212 above), cells were incubated with the probe (30 µg/ml in HBSS) for 30 min at 20 ± 1 °C. Then,
213 excess probe was eliminated and the cells were washed and kept moist with HBSS. The autophagic
214 transfer and compartmentalization of the FITC-labeled cytoplasmic proteins in lysosomes was
215 monitored after 4 h. Slides were viewed under 630x magnification by an inverted photo-microscope
216 (Zeiss Axiovert 100M) equipped for fluorescence microscopy using a FITC emission filter. Images
217 were analysed using an image analysis system as described above that allowed for the quantification
218 of the autophagic rate, that was expressed as fluorescence intensity.

219

220 *2.7.4. Phagocytic activity*

221 Phagocytosis was evaluated quantifying cellular intake of fluorescent bioparticles (*Escherichia coli*
222 K-12 Strain; Abs/Em maximum=505/513 nm; Invitrogen-Molecular Probes, Eugene, USA). After
223 the extrusion (as described above), coelomocyte suspension was incubated with bacteria, at 1:100
224 ratio, for 3 h at 20 ± 1 °C. The suspension was maintained in gentle agitation to avoid the
225 sedimentation of the cells. After incubation, cells were washed, fixed in absolute methanol for 20
226 min and spread on the slides. Slides were viewed under 630x magnification by an inverted photo-
227 microscope (Zeiss Axiovert 100M) equipped for fluorescence microscopy using a FITC emission
228 filter. Images were analysed using an image analysis system as described above that allowed for the
229 quantification of fluorescence of the cells, which reflects the number of bioparticles engulfed, that
230 was expressed percentage change with respect to controls.
231
232 *2.8. Univariate statistical analysis*
233 The non-parametric Mann-Whitney *U*-test was used to compare the data from treated earthworms
234 with those of the controls ones; moreover, the same test was used to identify, for each
235 concentration, significant changes with increasing exposure time.
236
237 *2.9. Multivariate analysis*
238 Biomarker data for earthworms exposed to Cr(VI) were analysed using non-parametric multivariate
239 analysis software, PRIMER v 6 (PRIMER-E Ltd., Plymouth, UK; Clarke, 1999; Clarke &
240 Warwick, 2001). All data were log transformed [$\log_n(1+x)$] and standardised to the same scale.
241 Principal component analysis (PCA), hierarchical cluster analysis and non-metric multi-dimensional
242 scaling analysis (MDS), derived from Euclidean distance similarity matrices were used to visualise
243 dissimilarities between sample groups. The results were further tested for significance using
244 analysis of similarity (PRIMER v6 - ANOSIM), which is an approximate analogue of the univariate
245 ANOVA and reflects on differences between treatment groups in contrast to differences among

246 replicates within samples (the *R* statistic). Under the null hypothesis H_0 ("no difference between
247 samples"), $R = 0$ and this was tested by a non-parametric permutations approach; there should be
248 little or no effect on the average *R* value if the labels identifying which replicates belong to which
249 samples are randomly rearranged.
250 The PRIMER v6 - BIO-ENV routine (Spearman's Rank Correlations) linking multivariate
251 biomarker response patterns was used to identify "influential biomarkers" - small subsets of
252 biomarkers capturing the full MDS biomarker response pattern.
253 Finally, in order to map integrated biomarker data onto "health status space" by using lysosomal
254 membrane stability (LMS); first principal components (PC1) for the biomarker data were derived
255 using PRIMER v6 and then plotted against the LMS values (as a measure of cellular well-being) for
256 each treatment (Allen and Moore, 2004; Moore et al., 2006; Sforzini et al., 2015).
257

258 ## 3. Results

259 *3.1. Biomarker responses*
260 The results demonstrate that exposure of *E. andrei* for 1 and 3 d to different concentrations of
261 Cr(VI) by the filter paper test induced significant changes in the different parameters evaluated on
262 coelomocytes of treated earthworms (Figs.1-3), without resulting in mortality of animals or
263 affecting coelomocyte viability (data not shown).
264 Cr(VI) provoked a relevant increase of ROS levels in coelomocytes. In particular, the use of the
265 fluorescent probe TEMPO-9-AC, specific for the detection of hydroxyl radicals and superoxide,
266 revealed a significant increase of the fluorescence intensity of cells of worms exposed for 1 d at all
267 the concentrations, with greater effects at higher ones i.e. 15 and 30 μg mL^{-1}; after 3 d, the
268 fluorescence reached a plateau (Fig. 1A). Similar results were obtained utilizing the probe DHR
269 123, able to highlight the generation of hydrogen peroxide and peroxynitrite. Fluorescence intensity

showed a significant increase in coelomocytes of worms exposed for 1 d to 15 and 30 µg mL^{-1}; maximal effects were observed after 3 d (Fig. 1B).

The generation of high levels of ROS in cells of Cr(VI) exposed worms determined membrane lipid peroxidation. In particular, the use of the probe BODIPY (specific for lipoperoxides) highlighted a significant increase of the fluorescence intensity of coelomocytes of worms exposed for 1 days to higher concentrations i.e. 15 and 30 µg mL^{-1}; after 3 days, significant changes were observed all the concentrations utilised, with maximal effect at 30 µg mL^{-1} (Fig. 1C). In line with these results, lysosomal accumulation of lipofuscin (representing the end products of membrane lipid peroxidation) showed a relevant increase at all the concentrations after 1 day. It is interesting to note that at the higher concentrations strongest effects were observed after 1 day of exposure with a decrease in lipofuscin accumulation at 3 days. The possible explanations of this effect include an enhanced rate of secretion of lipofuscins into the coelomic fluid and/or a decreased lysosomal autophagic activity.

Cr(VI) also provoked genotoxic effects in terms of DNA damage that was evaluated by use of the alkaline Comet assay and introducing in the method the use of the enzyme formamido pyrimidin-glycosylase (Fpg), that allows to highlight also the oxidative DNA damage (Fig. 2). The results of the alkaline Comet assay indicated that after 1 day of exposure, there was a significant increase in DNA damage at 15 and 30 µg mL^{-1}. However, after 3 days, significant changes were observed at all the concentrations utilised. A similar trend was observed using the Fpg enzyme with significant variations in cells of worms exposed to all the different experimental conditions (doses and days of exposure). In particular, the oxidative DNA damage was greater than the damage observed by the standard alkaline method; this change was significant for all the concentrations after 1 day of exposure.

Significant alterations of various cellular physiological parameters were observed in amoebocytes of Cr(VI) treated worms as shown in Figure 3. In particular, mitochondrial functionality (evaluated

295 utilising the fluorescent probe MitoTracker®) showed, after 1 day, a decrease with respect to
296 controls at all the concentrations; greater significant changes were observed at 15 and 30 µg mL^{-1} (-
297 42% and -84% with respect to controls respectively). Stronger alterations were measured in cells of
298 worms exposed to all the Cr(VI) concentrations for 3 days: at 2 and 15 µg mL^{-1}, mitochondrial mass
299 showed a significant reduction with respect to 1 day, while at 30 µg mL^{-1} the value has been
300 confirmed to be extremely low (Fig. 3A). Even more dramatic changes were induced by Cr(VI) on
301 LMS, an indicator of cellular health in eukaryotic cells (Fig. 3B).A significant decrease in LMS was
302 induced in worms exposed for both 1 and 3 days to all the different concentrations, with maximal
303 effect at 30 µg mL^{-1} (-97% after 3 days, with respect to controls) (Fig. 3B). Pathological reactions
304 involving the lysosomal system are often linked to augmented autophagic sequestration of cellular
305 components (Moore and Viarengo, 1987; Moore et al., 2008); the results showed in amoebocytes of
306 Cr(VI) exposed worms a significant increase of FITC-protein adducts into the autophagic-
307 lysosomal compartment (Fig. 3C). The only exception was the highest concentration after 3 days of
308 exposure, in which the autophagy value was lower.
309 Finally, relevant changes were observed also in the phagocytic activity of amoebocytes. As shown
310 in Fig. 3E the effect was significant after 1 days at the highest concentration (-32% with respect to
311 controls); at 3 days, all the different doses caused a significant decrease in phagocytic activity
312 (about -40% with respect to controls).
313
314 *3.2. Multivariate analysis of biomarker reactions*
315 Principal component (PCA) and hierarchical cluster analysis of all the biomarker reactions showed
316 that Chromium (Cr VI) had a detrimental effect on the earthworm coelomocytes (Fig. 4A).
317 Analysis of similarity shows that these clusters were significantly different (ANOSIM, R Statistic:
318 $R = 0.925$, $P < 0.001$).

The Controls for day 1 and day 3 were clustered together, with Cr VI treatments clearly separated (Fig. 4A). The Cr VI (30 μg.ml-1 - 3 days) treatment group was separated from all of the other groups, with the remaining Cr VI treated groups in two intermediate clusters Fig. 1A). MDS analysis gave a very similar pattern (not shown). The lysosomally-related subset of biomarkers (LMS, FITC-diacetate - autophagy, and lipofuscin; Fig. 4B) showed a similar pattern for PCA and hierarchical cluster analysis, and the clusters were also significantly different (ANOSIM, R Statistic: $R = 0.83$, $P < 0.001$).

Figure S3 (see supplementary information) shows the pattern of lysosomal changes, with Cr VI induced increases in autophagy, lipofuscin and a corresponding decrease in lysosomal membrane stability. The highest concentration of Cr VI exposure for a period of 3 days showed a large increase in lysosomal lipofuscin, combined with decreased autophagy (FITC-diacetate) as compared with the other Cr VI treatments. This finding is indicative of failed autophagy (Moore et al., 2006, 2008)

Multiple regression analysis of the biomarker data indicated that most of the biological parameters were correlated (Fig. 5). Of all the biomarkers tested, lysosomal membrane stability was the most strongly correlated ($RS > 0.8$ or -0.8, $P < 0.001$; with the exception of FITC diacetate $RS = 0.644$, $P < 0.001$ & Phagocytosis $RS > 0.63$, $P < 0.001$) with the other parameters (inversely correlated for all biomarkers except mitochondrial function and phagocytosis which were directly correlated). FITC-diacetate and phagocytosis were less strongly correlated ($RS > 0.63$ or -0.63, $P < 0.001$; although these were still stronger that correlations with other biomarkers). The correlations between the three lysosomal biomarkers are shown separately in Figure S4 (see supplementary information).

As most of the biomarkers were strongly correlated with each other, the BIO-ENV routine for combinations of biomarkers (Table 1) indicated that various combinations of the lysosomal parameters (lipofuscin, lysosomal membrane stability and autophagy - FITC-diacetate) were influential biomarkers in the Cr VI treatments, as were all of the remaining biomarkers. LMS on its

344 own captured most of the variance in "health status space"(Table 1); while LMS + lysosomal
345 autophagy (FITC-diacetate) had a Spearman's rank correlation coefficient (RS) value of 0.877
346 (Table 1).
347
348 *3.3. Modelling lysosomal and biomarker reactions to Cr VI treatment*
349 By plotting lysosomal membrane stability against the first principal component (PC1) of all of the
350 remaining cellular biomarker data (Fig. 6), we effectively integrate the selected multi-biomarker
351 data and the graph reflects the gradient of toxicity between the samples ($R = 0.951$, $P < 0.001$). PC1
352 is a measure of the contaminant gradient with the left-hand side being the most impacted and the
353 right-hand side the least affected (Fig. 6).
354 Lysosomal membrane stability plotted against the PC1 for the other lysosomally-related biomarkers
355 showed a similar relationship, although it was not as strongly correlated ($R = 0.920$, $P < 0.001$; Fig.
356 6).
357 Cr VI exposure concentrations were also significantly inversely correlated with PC 1 for all the
358 biomarkers at 1 and 3 days respectively (Fig. S5 – see supplementary information).
359

360 ## 4. Discussion

361 In this study, we investigated the (oxidative) stress effects and the genotoxicity in *E. andrei*
362 amoeboid immunocytes by exposure of worms, for different times, to different sublethal
363 concentrations of Cr(VI). For this purpose, we used the paper contact toxicity test (OECD, 1984),
364 that is easy to perform and gives highly reproducible results, important aspects for the development
365 of novel biomarkers at cellular/molecular level. The selected Cr(VI) concentrations are sublethal;
366 and the amounts are related to the Italian legal limits for residential soils (2 mg/Kg), industrial soils
367 (15 mg/Kg) and higher (30 mg/Kg). In the analysis of the results, using multivariate statistics

368 (Clarke, 1999; Clarke and Warwick, 2001) is also used to help elucidate the potential mechanisms
369 of Cr(VI) action in worms.
370 Coelomic amoebocytes are immunocytes involved in a broad range of defence functions
371 (Engelmann et al., 2005; Bilej et al., 2010). The use of biomarkers suitable for highlighting
372 oxidative stress (e.g. reactive oxygen species -ROS- and lipoperoxide production, lipofuscin
373 accumulation, oxidative damage to DNA) and cell injury (such as lysosomal and mitochondrial
374 alterations) is essential to gain more information about how, and to what extent a chemical (in this
375 instance Cr) can alter the immune system of worms: an impairment that could reach compromise
376 their ability to survive in the environment.
377 The results demonstrated that Cr(VI) caused relevant alterations in coelomocytes of exposed
378 worms. A clear separation between controls and treated animals was found, as shown by
379 multivariate analysis of the biomarker data.
380 Even the lowest dose of 2 μg mL^{-1} Cr(VI) at 1 d of treatment caused an increase of intracellular
381 ROS level that generated in the cells oxidative stress conditions as highlighted by the accumulation
382 in lysosomes of lipofuscins (end-products of membrane lipid peroxidation -Viarengo and Nott,
383 1993; Terman and Brunk, 2004) and the increase in the level of oxidative DNA damage. Moreover,
384 a relevant peturbation in the activity of the lysosomal vacuolar system (in terms of reduction of
385 lysosomal membrane stability and enhanced autophagy) was observed. Studies on eukaryotic cells
386 indicated that the reductive metabolism of Cr(VI) results in the production of Cr(III), a process that
387 can generate variable amounts of reactive Cr(V/IV) intermediates and radicals (Salnikow and
388 Zhitkovich, 2008). In vertebrates, it has been demonstrated that Cr(VI) induces oxidative stress
389 through enhanced production of ROS leading to oxidative deterioration of lipids and proteins and
390 genomic DNA damage (Bagchi et al., 2001; Shrivastava et al., 2002; Caglieri et al., 2008). The
391 alkaline version of the comet assay is a sensitive method for the detection of DNA single- and
392 double-strand breaks and alkali-labile sites. Reactive (oxygen) species cause DNA breaks, and

393 breaks can also appear as intermediates in DNA repair. A more specific indicator of oxidative attack
394 is the presence of oxidised purines or pyrimidines. The basic comet assay was modified to detect
395 these, by introducing an incubation with the bacterial repair enzymes Endonuclease III, specific for
396 oxidised pyrimidines, and formamidopyrimidine DNA glycosylase (FPG), acting on 8-oxo-7,8-
397 dihydroguanine (8-oxoGua) (Collins et al., 1993; Dusinska and Collins, 1996). In vertebrates, the
398 generation of Cr(VI)-induced oxidative DNA damage using the FPG-modified version of the comet
399 assay has been demonstrated (Hodges et al. 2001; Lee et al., 2004). In invertebrate systems, few
400 studies have been done also utilising the DNA repair enzymes (Gielazyn et al., 2003; Hook and
401 Lee, 2004; Emmanouil et al., 2007; Dallas et al., 2013; Hertel-Aas et al., 2011); in earthworms, we
402 successfully employed this method to reveal oxidative DNA damage induced by Cr(VI). Lysosomes
403 appear to be a target for many pollutants, as lysosomes accumulate toxic metals and organic
404 xenobiotics, which may perturb normal function and damage the lysosomal membrane (Allison and
405 Mallucci, 1964; Viarengo et al., 1985; Sforzini et al., 2014). Pathological reactions involving the
406 lysosomal system are also often linked to augmented autophagic sequestration of cellular
407 components; in particular, there is evidence that autophagy may have a protective role in the context
408 of oxidative stress through the degradation and recycling of oxidized proteins and damaged
409 organelles (Bergamini et al., 2003; Cuervo, 2004; Moore, 2008). It is likely that reactive free
410 radicals produced during biotransformation contribute to the damaging effects on the lysosomal
411 membrane and build up of lipofuscin (Kirchin et al., 1992; Winston et al., 1996). Lipofuscin is an
412 end product of oxidative attack on lipids and proteins and is also an indicator of autophagy (Terman
413 and Brunk, 2004; Moore, 2008).
414 With the increase of the dose and/or time of exposure, the effects become more severe. Principal
415 component analysis revealed a greater distance of the different treatments from the controls
416 compared to the lowest dose at 1 d. In particular, the highest dose of Cr(VI) (30 µg mL^{-1}), 3 d
417 treatment group was the most distant, with the remaining Cr(VI) treated groups (2 µg mL^{-1} 3 d; 15

418 µg mL^{-1} 1 and 3 d; 30 µg mL^{-1} 1 d) in an intermediate cluster. In these latter exposure conditions,
419 the data revealed a further sustained increase of intracellular ROS generation and lipid peroxidation.
420 The level of DNA damage increased (as highlighted by the results of both the standard and the
421 "Fpg-modified" Comet assay); and the mitochondria showed a reduced mass. In the same cells,
422 which have shown such a wide range of severe subcellular alterations, an impairment of their
423 functions was also observed as demonstrated by the decrease of their phagocytic activity.
424 Metal-mediated formation of free radicals causes various effects; among these enhanced lipid
425 peroxidation. Lipid peroxides are known to be induced by Cr(VI) in vertebrates as well
426 invertebrates (Sridevi et al., 1998; Hojo et al., 2000; Barmo et al., 2011; Gao et al., 2016).
427 The chromium genotoxicity manifests as several types of DNA lesions along with oxidative damage
428 (Shrivastava et al., 2002) (Casadevall et al., 1999; Hodges et al., 2001; Levina and Lay, 2005;
429 Manerikar et al., 2008). There is evidence that the generation of DNA single strand breaks by Cr
430 involves oxidizing species (Salnikow and Zhitkovich, 2008). DNA single-strand breaks are also
431 introduced as intermediate of base excision-repair process of the oxidative DNA damage. The
432 increase of the level of the damage to DNA induced by Cr(VI), as highlighted by the standard
433 alkaline comet assay, may also reflect both alkali-labile sites and true single-strand breaks following
434 the repair of DNA-adducts.
435 Mitochondria are highly dynamic organelles responding to cellular stress through changes in overall
436 mass, interconnectedness, and sub-cellular localization. Change in overall mitochondrial mass may
437 reflect an altered balance between mitochondrial biogenesis and rates of mitophagy, two processes
438 that are tightly regulated in response to cellular stress, including oxidative damage and redox state
439 (Boland et al., 2013). Common mechanisms involving the Fenton reaction, generation of the
440 superoxide radical and the hydroxyl radical appear to be involved for chromium primarily
441 associated with mitochondria, microsomes and peroxisomes (Valko et al., 2005). Cr(VI) combines
442 with NADH/NADPH to form pentavalent chromium in mitochondria (Chiu et al., 2010). Previous

studies have demonstrated in mammals deleterious effects of Cr(VI) on mitochondrial physiology (Myers et al., 2010; Das et al., 2015) and of cellular respiration, resulting in aberrant oxygen metabolism and subsequent indirect formation of ROS (Cohen et al., 1993; Hodges et al., 2001). As regards to the negative effects of Cr on amoebocyte immune activity, these data are in line with previous results obtained in vertebrates as well in invertebrates. Khangarot et al., 1999 showed that chromium exposed fish exhibit higher susceptibility to bacterial infection; and the phagocytic activity of splenic and pronephros macrophages is significantly decreased. Johansson et al. (1986a, 1986b) reported a reduction of the phagocytic activity of lungs and pulmonary macrophages of chromium-exposed rabbit. Ciacci et al. (2011) demonstrated a decreased phagocytic activity of hemocytes in Cr(VI)-exposed *Mytilus galloprovincialis*.

When earthworms were exposed for 3 d to 30 μg mL^{-1} Cr(VI), the health status of the cells showed a further deterioration. The high levels of ROS determined a strong increase of lipoperoxides in cell membranes; moreover, the dramatic reduction of LMS combined with a failed autophagy (a strong decrease of the level of the *in situ* FITC-labeled intracellular proteins was observed when compared to other Cr(VI) treatments) are indicative of a extremely relevant damage to lysosomal vacuolar system.

Overall, a cascade of events appears to be verified in immune cells of Cr(VI) exposed worms with the increase of the dose and the time of exposure. Multiple regression analysis of the biomarker data indicated that most of the biological parameters were strongly correlated. In particular, of all the biomarkers utilised, LMS was the most strongly correlated with the other parameters.

Many pollutants may exert both toxicity and genotoxicity directly, as well as through oxidative stress. The resulting damage to membranes, proteins and DNA can contribute to decrease protein synthesis, and to enhance cell injury and physiological dysfunction (Viarengo, 1989; Kirchin et al., 1992; Winston et al., 1996; Lowe et al., 2006; Moore et al., 2006). Lysosomal membrane stability (LMS) in blue mussels is directly correlated with total oxyradical scavenging capacity (TOSC),

polyribosome formation; and is inversely proportional to DNA damage, lipofuscin formation, autophagy-related lysosomal swelling and autophagic accumulation of lipid (Krishnakumar et al., 1994; Regoli, 2000; Dailianis et al., 2003; Kalpaxis et al., 2004; Moore et al., 2006).

In this study, multiple regression analysis of the biomarker data indicated that LMS is significantly correlated to mitochondrial function and inversely correlated to intracellular ROS levels and the oxidative damage of membrane lipids and DNA (measured with TEMPO, DHR, BODIPY, lipofuscin and Comet-Fpg), as well as to DNA damage (COMET).

Lipofuscin content is a good indicator of oxidative stress, and showed a consistent pattern of correlations across the various treatments (Moore et al., 2006). The BIO-ENV routine indicated that experimental exposure of worms to Cr(VI) resulted in the functionally related sub-sets of biomarkers: LMS + lipofuscin, LMS + autophagy, and LMS + lipofuscin + autophagy emerging as effective combinations of lysosomal biomarkers (Table 1). This evidence is consistent with the functional conceptual model describing the relationships between these various lysosomal parameters in the context of cellular health and oxidative environmental stress developed by Moore (2008).

Recent developments in many research fields are leading to the discovery of prognostic biomarkers that may be suitable for use as risk indicators of various pathologies (Moore et al., 2006; Jenkins et al., 2011; Ortiz et al., 2011; Berghella et al., 2014). Many biomarkers probably only exhibit a response in a part of the "health status space" (Depledge et al., 1993; Allen and Moore, 2004; Moore et al., 2006), where they will indicate whether a reaction has taken place and may even indicate health status within a narrow range, or what has induced the response, but they do not generally indicate the health status of the animal for the whole range from healthy to irreversible damage (Köhler et al., 2002). In terms of environmental prognostics, the first step is to relate biomarker responses to the health status of individual organisms by mapping the said responses

against an integrated "health status" indicator (Köhler et al., 2002; Allen and Moore, 2004; Moore et al., 2004, 2006).

Lysosomes have attracted a great deal of interest in the field of ecotoxicology, as they are the frequent target of a wide range of contaminants (Allison and Mallucci, 1964; Moore et al., 2004; 2007, 2008; 2009; Sforzini et al., 2014; Viarengo et al., 1985, 2007) and they are present in all nucleated cells. The evidence is steadily accumulating that LMS is a generic indicator of cellular health in eukaryotic cells, as is indicated from studies on protozoans, coelenterates, annelids, crustaceans, molluscs, fish and mammals (Lin et al., 2010; Moore et al., 2006, 2007, 2012; Sohaebuddin and Tang, 2013). This parameter, LMS, is now considered a highly sensitive biomarker that allows one to follow the evolution of the stress syndrome from its early phase to the development of pathological conditions (Moore, 1988; Moore et al., 2004). LMS has previously been used in the liver cells of the flatfish flounder (*Platichthys flesus*) to predict the degree of liver degeneration as a result of PAH and organochlorine exposure (Köhler et al., 2002). Furthermore, LMS in digestive cells of mussels is directly and mechanistically related to scope for growth; and also, in the digestive cells of oysters (*Crassostrea virginica*) to larval viability (Allen and Moore, 2004; Moore et al., 2004, 2006; Ringwood et al., 2004).

Lysosomal membrane stability (LMS) is considered to be prognostic, as it constitutes a cellular injury likely to lead to further pathological changes. Although ROS increases may occur first, they do not in themselves constitute cell injury to the functional organisation of the cell, and can be the result of adaptive responses (Guzy et al., 2005). An increase in ROS would have to result in measurable cellular damage (e.g., a decrease in LMS) in order to be considered prognostic for pathology: they can be indicative, but are not in themselves prognostic. ROS inhibits mTOR (mTORC1), which will destabilise the lysosomal membrane as phosphorylated mTOR is necessary to maintain normal lysosomal membrane permeability (Cang et al., 2013; Li et al., 2013; Scherz-Shouval & Elazar, 2011). ROS mediated inhibition of mTOR also induces augmented autophagy,

however, measurement of ROS on their own is not necessarily prognostic for cell injury as antioxidant defences could counter this effect (Scherz-Shouval & Elazar, 2011).

Another factor worthy of consideration, in the light of the measured DNA damage, is that autophagy of parts of the cell nucleus (particularly proteins that regulate gene function associated with the inner nuclear membrane) is now believed to help protect the cell from becoming neoplastic following exposure to carcinogens (Dou et al., 2015; Luo et al., 2016).

Principal Component Analysis (PCA) is an effective method for integrating biomarker data into a "health status space" reducing the multi-dimensionality of the problem to a simple two dimensional representation (Chatfield and Collins, 1980; Allen and Moore, 2004). PCA is commonly used as a cluster analysis tool and effectively captures the variance in a dataset in terms of principle components. PCA has facilitated modelling the integrated responses of multiple biomarkers in the context of "health status space" (Allen and Moore, 2004; Moore et al., 2006). These models clearly show that there is a strong relationship between LMS, as an indicator of cellular health, and the other combined biomarker responses (Moore et al., 2006; Sforzini et al., 2015). There is also a strong relationship between the exposure concentrations of Cr(VI) and the integrated biomarker responses.

PCA and the associated statistical tests have shown that lysosomal biomarkers (e.g., LMS) combined with either of the COMET assays (BIO-ENV routine: $Rs = 0.860$, $P = 0.001$ for COMET standard; and $Rs = 0.866$, $P < 0.001$ for COMET-fpg) provides an effective integrated assessment of adverse effects on physiological function and genetic integrity (genotoxicity). These findings support previous investigations by Sforzini et al. (2015) that PCA can aid interpretation of multiple biomarker responses and pathological reactions to environmental contaminants.

Finally, multivariate analysis is the first stage in developing numerical and network models for environmental impact on the health of sentinel animals such as earthworms (Allen and Moore, 2004; Moore, 2010).

5. Conclusions

Overall, the various parameters evaluated in this study were able to reveal multiple adverse effects at cellular/subcellular level in amoeboid leukocytes of *E. andrei* exposed to different sublethal concentrations of Cr(VI). In particular, the analysis of the results shows that Cr(VI) triggered severe negative reactions; the first events were an increase of intracellular ROS levels, generating in the cells oxidative stress conditions leading to membrane lipid peroxidation and oxidative DNA damage. Lysosomes showed relevant changes such as a strong membrane destabilization which was accompanied by an increased catabolism of cytoplasmic proteins and accumulation of lipofuscin. With the increase of the dose and/or time of exposure, the physiological status of intracellular organelles (such as lysosomes, nucleus and mitochondria) showed a further impairment and amoebocytic immune functions were affected, as shown by the decrease of the phagocytic activity. A strong correlation among the different biological parameters was found; and, of all the biomarkers used, LMS was the most strongly correlated with the other parameters. By mapping the responses of the different parameters evaluated, diagnostic of (oxidative) stress events, against LMS, a "health status" indicator (able to describe the stress syndrome from its early phase to pathology), we have shown that this biomarker is suitable as prognostic for health of earthworms. This is viewed as a crucial step toward the derivation of explanatory frameworks for prediction of pollutant impact on animal health; and will facilitate the development of a conceptual mechanistic model linking lysosomal damage and autophagic dysfunction with injury to cells, tissues, and the whole animal (McVeigh et al., 2004; Moore, 2002, 2004, 2008; Moore et al., 2004, 2015; Moore, in preparation).

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872

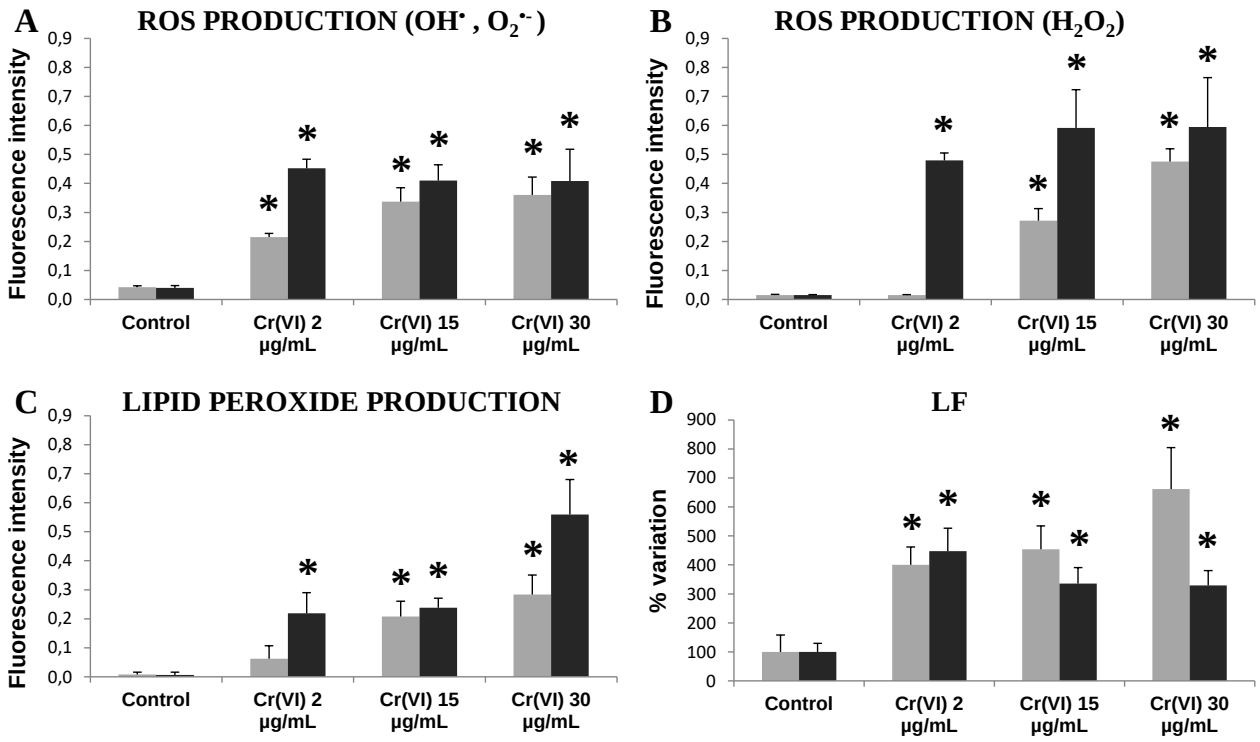
873 Fig. 1. Oxidative stress biomarker responses in amoeboid immunocytes of *E. andrei* after exposure
874 of worms for 1 d (grey columns) and 3 d (black columns) to different Cr(VI) concentrations. ROS
875 production evaluated using the probes A) TEMPO-9-AC and B) DHR 123; C) lipid peroxide
876 production; D) lysosomal accumulation of lipofuscin (LF). Data represent the mean±SD of at least
877 five replicates. * $p < 0.05$ (Mann-Whitney *U*-test). **Pictures: (deleted)** Representative images of *i)*
878 cells labelled with the probe BODIPY® 581/591 C11 (C1: control; C2: earthworms exposed to 30
879 µg mL^{-1} Cr(VI) for 3 d), specific for lipid peroxides; *ii)* and reacted for LF accumulation (D1:
880 control; D2: earthworms exposed to 30 µg mL^{-1} Cr(VI) for 1 d). (See **Fig. … in Supplementary**
881 **information** for the **images** of A and B).
882
883 Fig. 2. Effects of Cr(VI) (2, 15, 30 µg mL^{-1}) on DNA damage (St.) and oxidative DNA damage (by
884 the use of the Fpg enzyme) in amoeboid immunocytes of *E. andrei* exposed for 1 d (grey columns)
885 and 3 d (black columns). Data, expressed as %DNA content in the tail, represent the mean±SD of at
886 least five replicates. * $p < 0.05$ (Mann-Whitney *U*-test). A) control; B): earthworms exposed to 30
887 µg mL^{-1} Cr(VI) for 3 d.
888
889 Fig. 3. Cell function related biomarker responses in amoeboid immunocytes of *E. andrei* after
890 exposure of worms for 1 d (grey columns) and 3 d (black columns) to different Cr(VI)
891 concentrations. A) mitochondrial mass; B) lysosomal membrane stability (LMS); C) lysosomal
892 autophagy of *in situ* FITC-labeled intracellular proteins; **D) lactate dehydrogenase (LDH) leakage**
893 **(deleted);** E) phagocytic activity. Data represent the mean±SD of at least five replicates. * $p < 0.05$
894 (Mann-Whitney *U*-test). **Pictures: (deleted)** Representative images of *i)* NR-derived fluorescent
895 staining of the lysosomes in cells of control (B1) and Cr(VI)-exposed earthworms (B2, 30 µg mL^{-1}
896 for 1 d); *ii)* FITC-derived fluorescent staining in cells of control (C1) and Cr(VI)-exposed

earthworms (C2, 30 µg mL^{-1} for 1 d). (See **Fig. … in Supplementary information** for the **pictures** of A and **D).**

Fig. 4. Principal Component Analysis (PCA) with superimposed Cluster Analysis. A) All biomarkers; and B) lysosomally-related biomarkers only (i.e., LMS, lipofuscin & autophagy - FITC diacetate).

Fig. 5. Multiple regressions (Spearman's Correlation) for all biomarkers and regressions for biomarkers with lysosomal membrane stability (LMS) are shown highlighted, as these showed the strongest correlations with the other biomarkers. (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS - not significant).

Fig. 6. Lysosomal membrane stability (LMS) as an integrated indicator of health plotted against the first principal component scores (PC 1) for all the remaining cellular biomarkers; and LMS plotted against the first principal component scores (PC 1) for the other lysosomally-related biomarkers (i.e., Lipofuscin & Autophagy - FITC-diacetate). The data has been log transformed and normalised.



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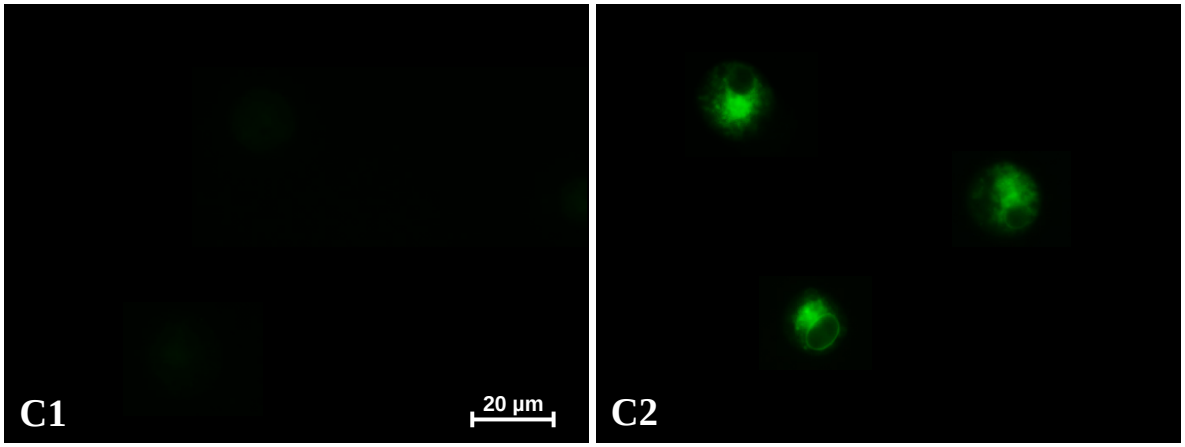

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919 ….

920 Fig. 1.

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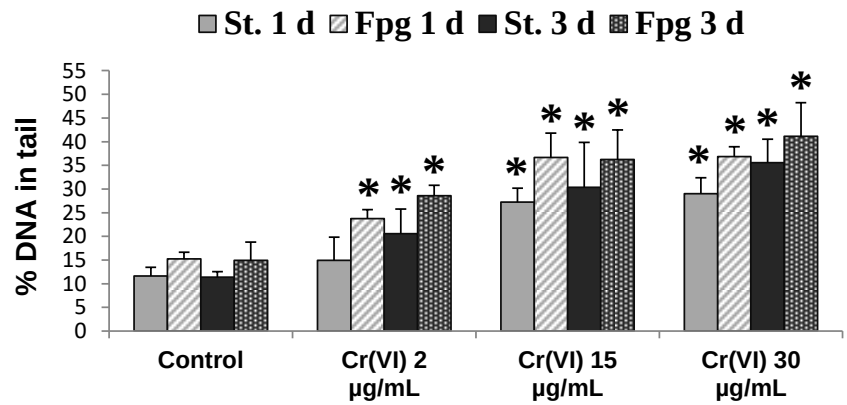

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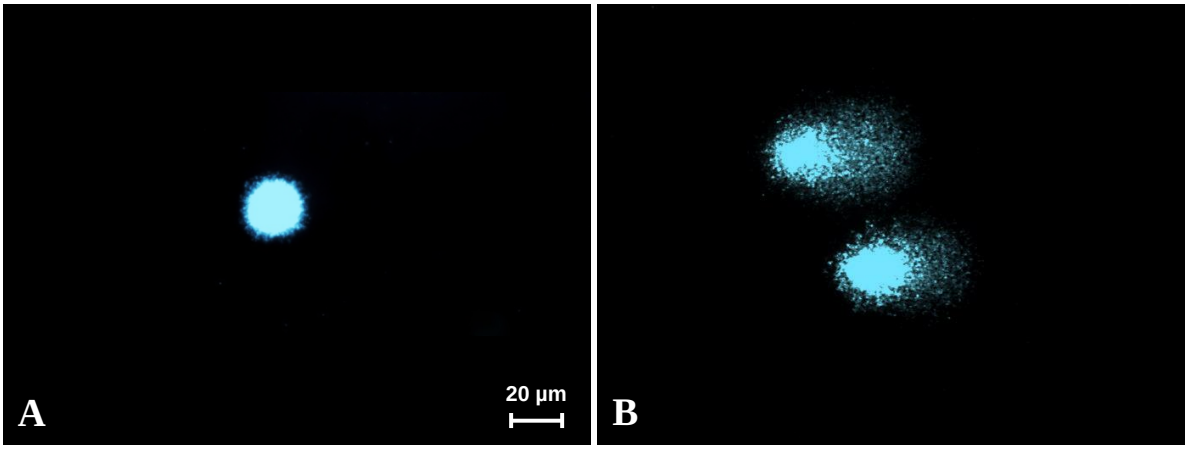

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924 Fig. 2.

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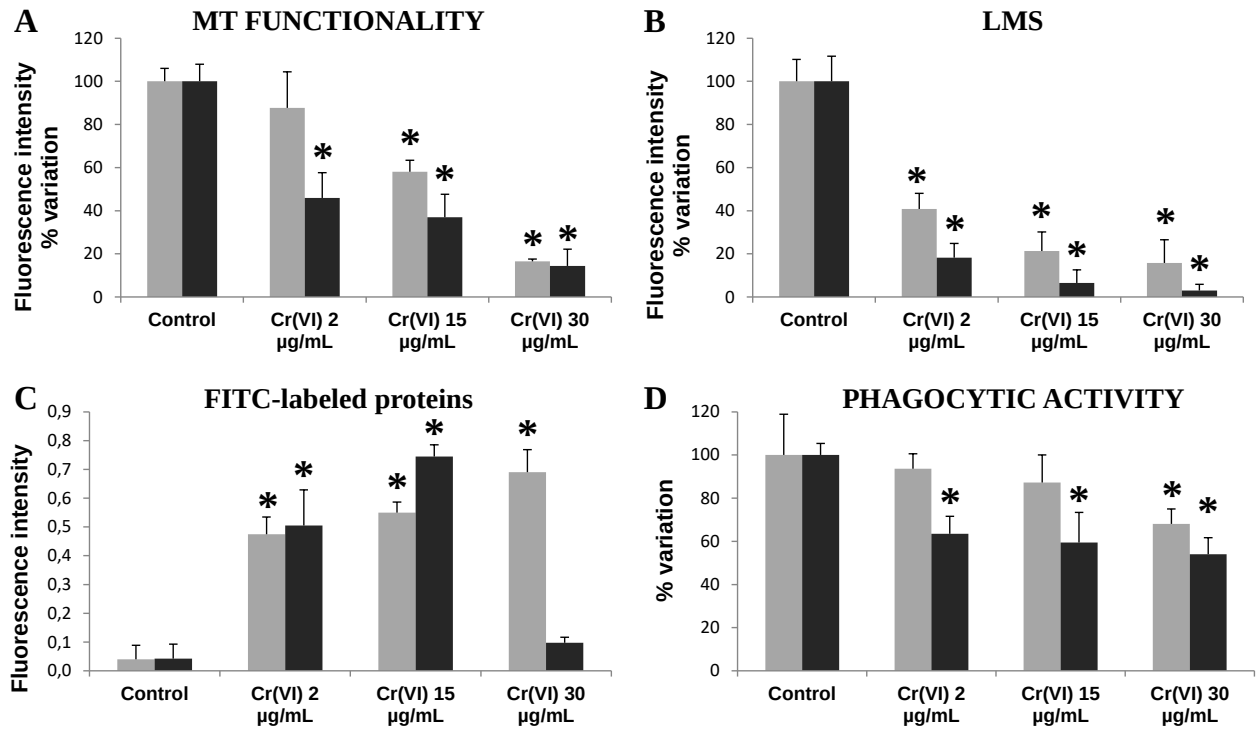

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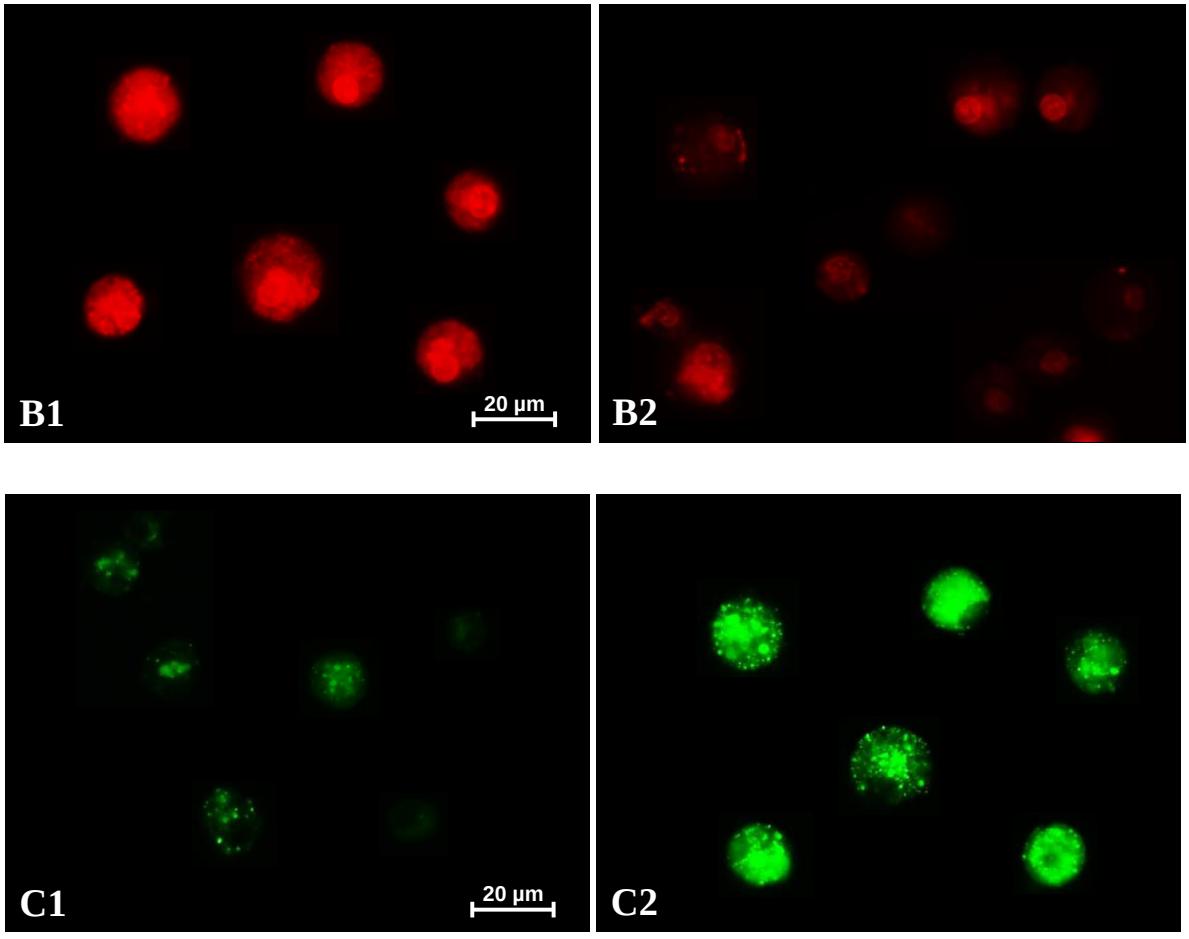

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929 Fig. 3.

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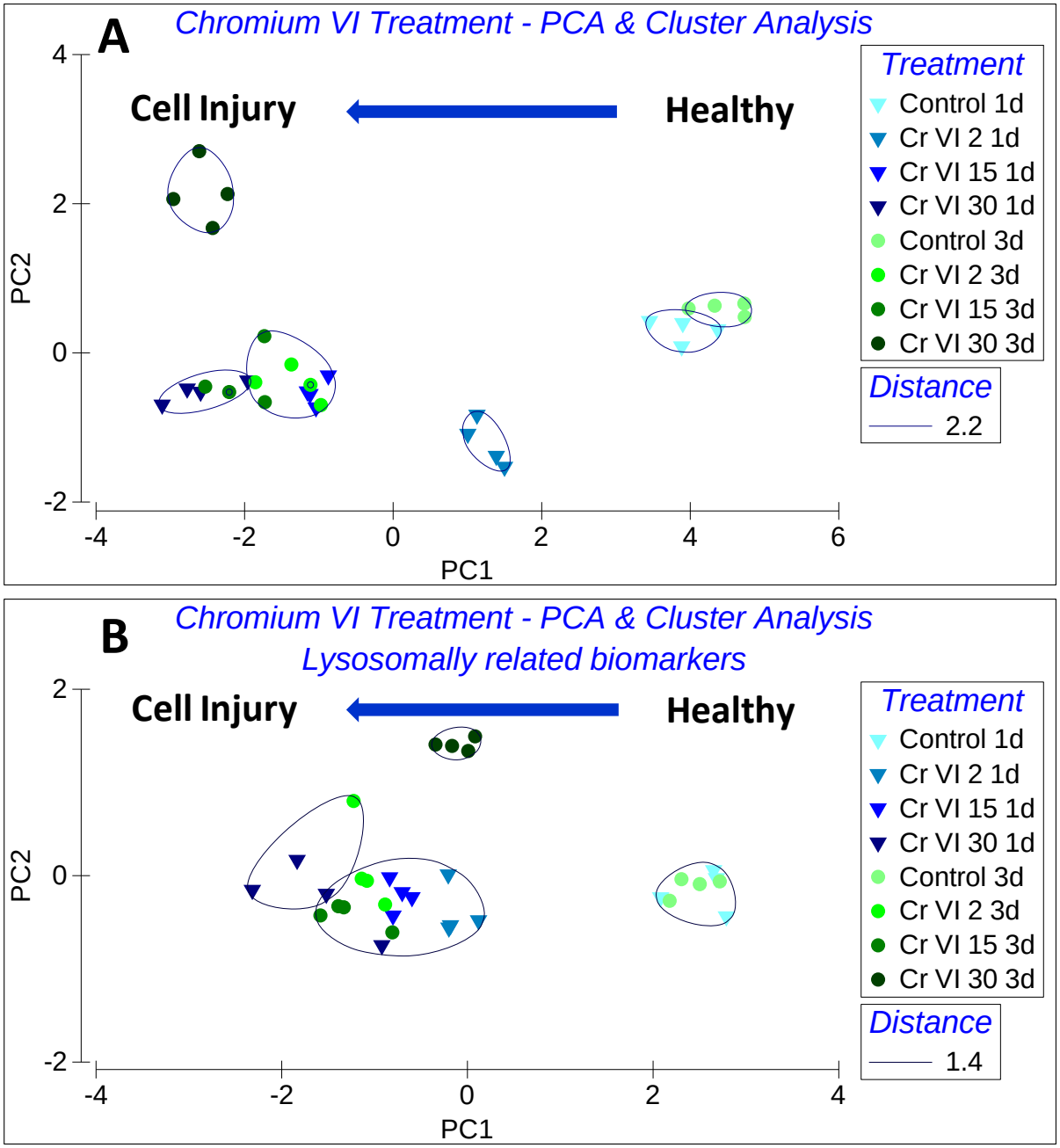

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932 Fig. 4.

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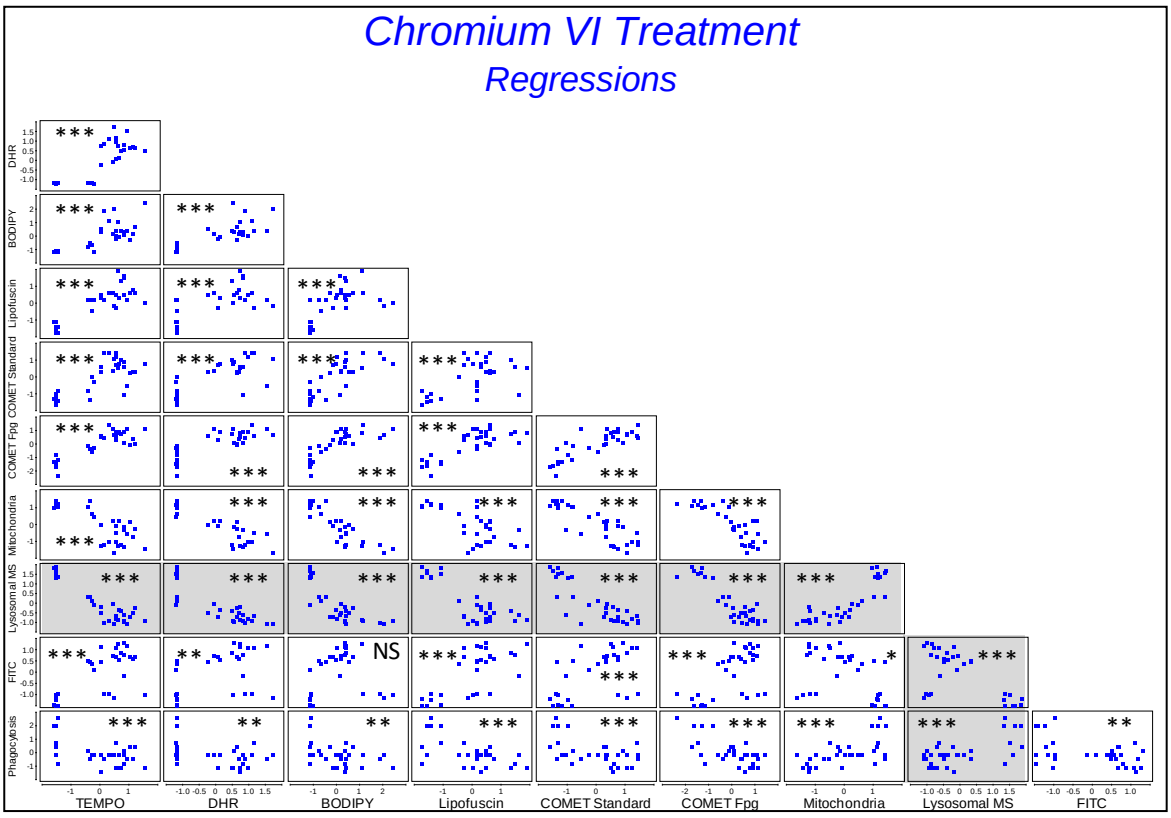

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935 Fig. 5.

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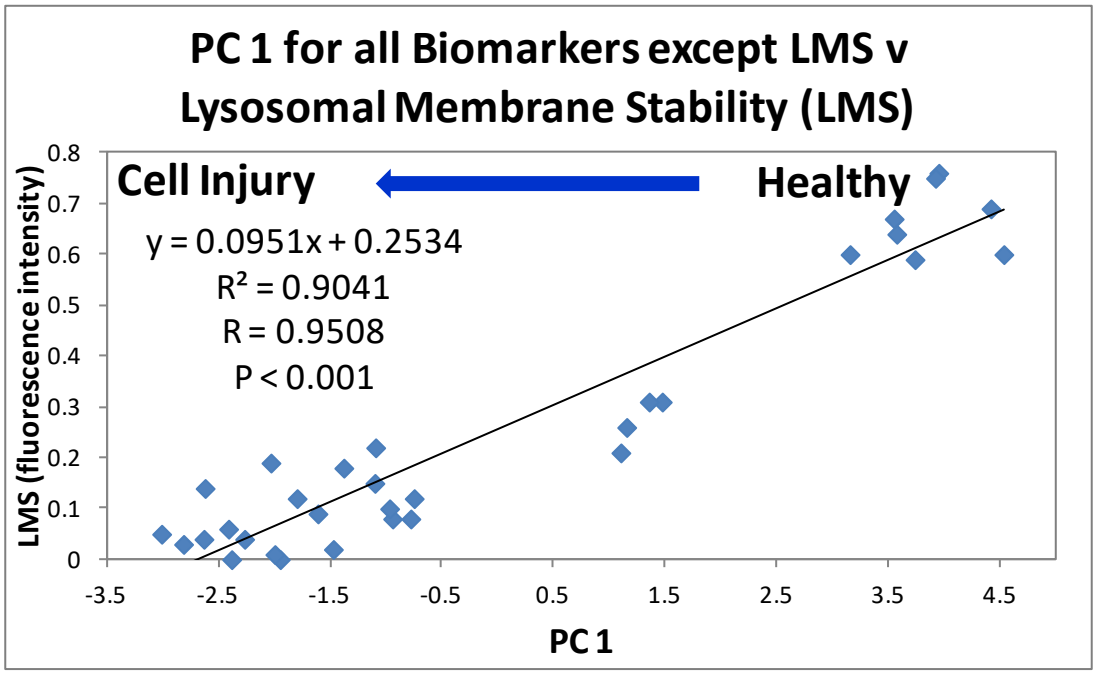

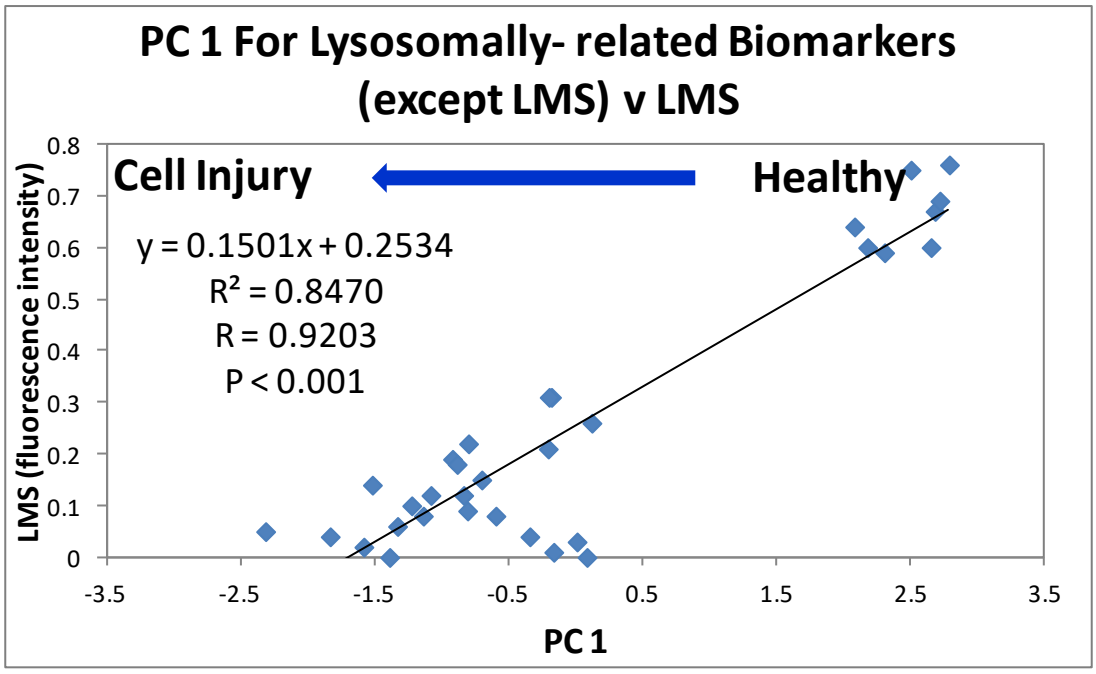

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938 Fig. 6.

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