Identification of candidate biomarkers of the exposure to PCBs in contaminated cattle: A gene expression- and proteomic-based approach

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Abbreviations: ACN, acetonitrile; AhR, aryl hydrocarbon receptor; APO, apolipoprotein; CNRQ, calibrated normalized relative quantity; DL, dioxin-like; ESI-Q, electrospray ionization quadrupole; GPX, glutathione peroxidase; HP, haptoglobin; IL-2, interleukin 2; LIT, linear ion trap; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; NDL, non dioxin-like; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-p-dioxin; PCDF, polychlorinated dibenzofuran; q-PCR, quantitative RT-PCR; SOD, superoxide dismutase; TCDD, 2,3,7,8 tetrachloro-dibenzo-p-dioxin; TEQ, toxic equivalent; TFA, trifluoroacetic acid; 2-DE, two-dimensional gel electrophoresis; TNF- α, tumor necrosis factor-α; TTR, transthyretin.

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ABSTRACT

Dioxins and polychlorinated biphenyls (PCBs) are widespread and persistent contaminants. Through a combined gene expression/proteomic-based approach, candidate biomarkers of the exposure to such environmental pollutants in cattle subjected to a real eco-contamination event were identified. Animals were removed from the polluted area and fed a standard ration for 6 months. The decontamination was monitored by evaluating dioxin and PCB levels in pericaudal fat two weeks after the removal from the contaminated area (day 0) and then bimonthly for six months (days 59, 125 and 188). Gene expression measurements demonstrated that CYP1B1 expression was significantly higher in blood lymphocytes collected in contaminated animals (day 0), and decreased over time during decontamination. mRNA levels of interleukin 2 showed an opposite quantitative trend. MALDI-TOF-MS polypeptide profiling of serum samples ascertained a progressive decrease (from day 0 to 188) of serum levels of fibrinogen β-chain and serpin A3-7-like fragments, apolipoprotein (APO) C-II and serum amyloid A-4 protein, along with an augmented representation of transthyretin isoforms, as well as APOC-III and APOA-II proteins during decontamination. When differentially represented species were combined with serum antioxidant, acute phase and proinflammatory protein levels already ascertained in the same animals (Cigliano et al., 2016), bioinformatics unveiled an interaction network linking together almost all components. This suggests the occurrence of a complex PCB-responsive mechanism associated with animal contamination/decontamination, including a cohort of protein/polypeptide species involved in blood redox homeostasis, inflammation and lipid transport. All together, these results suggest the use in combination of such biomarkers for identifying PCB-contaminated animals, and for monitoring the restoring of their healthy condition following a decontamination process.

Keywords: Cattle; Polychlorinated biphenyls; Biomarkers; AhR genes; MALDI-TOF-MS serum profiling; TEQ

1. Introduction

Polychloro-dibenzo-*p*-dioxins (PCDDs), polychloro-dibenzo-furans (PCDFs), and polychlorinated biphenyls (PCBs) are hazardous and highly persistent environmental pollutants of anthropogenic origin. A large array of adverse effects have been reported in animals and humans as the result of the prolonged exposure to such contaminants, including tumour promotion and teratogenicity, and perturbation of the immune, endocrine and nervous systems (Tavakoly Sany et al., 2015). For the so-called "dioxin-like" (DL) compounds, it is believed that most of the above effects are mediated by the interaction with the aryl hydrocarbon receptor (AhR). This triggers the transcription of the "AhR gene battery", which comprises genes encoding for a number of biotransformation enzymes (e.g. CYP1A1, CYP1B1, UGT1A, and GSTA1), as well as proteins involved in the regulation of proliferation and differentiation, and in the homeostasis of the immune system (e.g. interleukin 2, IL-2) (Bock and Koehle, 2009). PCBs not able to bind to the AhR receptor are defined as non-DL (NDL), and are mainly involved in neurotoxicity, immune suppression and endocrine disruption (Hamers et al., 2011).

Due to their high lipophilicity, PCDDs, PCDFs and PCBs are reported to accumulate along the food chain and to contaminate animal products (meat, milk and dairy products), which represent by far the most important non-occupational source of contamination for humans (Malisch and Kotz, 2014). Analytical methods for screening and confirmation of DL-compounds in animal products are rather expensive and time consuming, thereby limiting the number of tests to be carried out in the frame of the National Control Plans on food and feed. Thus, there is a growing interest in the development of faster and cost effective complementary screening methods able to identify the contaminated animals. A promising approach is the search for biomarkers resulting from biological events ensuing the binding of DL-compounds to AhR in target cells or tissues that should be easily collectable in living organisms. In this respect, different expression profiles between waste incineration workers and controls were reported upon the gene microarray analysis of peripheral blood mononuclear cells (Kim et al., 2004). Accordingly, circulating lymphocytes from individuals

exposed to 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD) during the Seveso disaster displayed significant differences in AhR-dependent gene expression and inducibility compared to unexposed subjects (Landi et al., 2003). In line with the findings in humans, we have recently demonstrated that the gene expression of CYP1B1 in lymphocytes is significantly up-regulated in dairy cows naturally exposed to DL-compound contaminated feedstuffs, as assessed by bulk milk Toxic Equivalent (TEQ) values (Girolami et al., 2013).

Another readily available biological matrix is serum or plasma, which contains a complex array of proteins that may be correlated to biological events occurring in the entire organism (Villanueva et al., 2004), including the exposure to DL-compounds (Joo et al., 2003). Proteomic methods based on two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and/or electrospray ionization quadrupole (ESI-Q)-TOF mass spectrometry (MS) have been applied to plasma samples to identify differentially represented proteins in workers at municipal incinerators (Kang et al., 2005; Kim et al., 2004), and in rats experimentally treated with TCDD (Son et al., 2003). More recently, the serum protein profiling of TCDD-exposed rats through magnetic bead separation and MALDI-TOF mass spectrometry showed several peaks (i.e. peptides) having differential representation between treated and control animals (Chen et al., 2012). A similar approach has already been employed in the bovine species for the detection of the treatment with illegal growth promoting agents in veal calves (Della Donna et al., 2009); however, no data are available about the use of serum protein profiling for the study of DL-compound contamination in food producing animals.

The present study has been carried out in Limousine heifers accidentally exposed to a forage contaminated by the emissions of an industrial plant specialized in PCB treatment. Subsequently, the animals underwent a decontamination procedure based on the removal from the polluted area and the feeding of a controlled diet for 6 months. We have already described the changes of specific indices of blood redox homeostasis and inflammation associated with the decontamination

(Cigliano et al., 2016). Here we report on gene expression and protein changes in corresponding lymphocytes and serum as determined by transcript and protein profiling procedures, respectively.

2. Materials and methods

2.1 Animals and sampling

All the information regarding the animals included in the study and the sampling program, together with the source of contamination, the decontamination procedure (i.e. location and diet) and the evaluation of the contamination extent were detailed elsewhere (Cigliano et al., 2016). Briefly, eight 1-year old PCB contaminated heifers were removed from the contaminated area and housed in an experimental facility located far away this area for 6 months. The animals were weighed, blood sampled and submitted to a pericaudal biopsy to get fat samples 2 weeks after their arrival in the experimental facility (day 0, sampling A), and then bimonthly during the decontamination period (59, 125 and 188 days after starting the decontamination; samplings B, C, and D, respectively). Fat samples were used for the measurement of TEQ values (DL-PCB + PCDD/F), and of NDL-PCB concentrations. Blood samples were divided in dry or in EDTA tubes for serum and lymphocytes separation, respectively. After clotting, serum was separated by centrifugation at 1272 *g* for 15 min at 25 °C, divided into aliquots and stored at -80 °C pending MALDI-TOF-MS protein profiling. Circulating lymphocytes were isolated by Histopaque-1077 gradient centrifugation (Spalenza et al., 2011), and stored at -80 °C until gene expression analysis.

2.2 DL-compound and NDL-PCB determinations

Quantitative determination of PCDD/F,DL-PCB and NDL-PCB in pericaudal fat biopsies was reported in Cigliano et al. (2016). Samples were analyzed by GC-HR-MS, according to the requirements of the quality assurance parameters of the Commission Directive 2002/69/EC and 2002/70/EC of July 2002 laying down the sampling methods and the methods of analysis for the

determination of PCBs in foodstuffs and feeding stuffs, respectively. Analytical methods were accredited to the ISO 17025 standard.

2.3 Quantitative RT-PCR on blood lymphocytes

Total RNA was isolated using QIAzol Lysis Reagent (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA purity and quantity were evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Scientific); the ratio of the optical densities measured at 260 and 280 nm were >1.9 for all RNA samples. RNA integrity was assessed using an automated electrophoresis station (Experion Instrument, Bio-Rad). All the samples had a RNA Integrity Number (RIN) > 7. Total RNA (1 µg) was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad), according to manufacturer's instructions. Sufficient cDNA was prepared in a single run to perform q-PCR experiments for all selected genes. Primers for AhR, ARNT and CYP1B1 were from Girolami et al., (2011), whereas primers for IL-2 (Fw: AGTCATTGCTGCTGGATTTACAG; Rev: CAATTCTGTAGCGTTAACCTTGG) were designed on bovine GenBank sequences using Primer 3 Software v. 0.4.0 (Rozen and Skaletsky, 2000). The specificity of primers was tested using a BLAST analysis against the genomic NCBI database. q-PCR product sizes were all between 118 and 203 bp. Each primer set efficiency was evaluated through the dilution method using a pool of all RNA samples, and it was comprised between 95% and 100%.

q-PCR reactions were performed on 100-500 ng of cDNA, in a final volume of 20 µl consisting of the iTaq SYBR Green Supermix with ROX (Bio-Rad) and an optimized concentration of each primer set (300-900 nM range). PCR amplification was run on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) in 96-well optical plates, under the following conditions: 30s at 95 °C for polymerase activation, and 40 cycles of 15 s at 95 °C and of 60 s at 60 °C. Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA.

Relative gene expression was calculated as described by Hellemans et al. (2007), using S24 and PPIA as reference genes (Spalenza et al., 2011) and RNA from liver (for AhR and ARNT), from BME-UV cells (for CYP1B1) and from cultured lymphocytes (for IL-2) as inter-run calibrators. Data were expressed as calibrated normalized relative quantity (CNRQ).

2.4 MALDI-TOF-MS serum protein profiling

Serum aliquots were thawed and diluted 1:40 in 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). Polypeptide species were extracted with C4 ZipTip (Millipore, Darmstadt, Germany) devices, eluted in 3 µl of matrix solution (10 mg/ml sinapinic acid in 50% acetonitrile - ACN and 50% TFA 0.1%), and then added with 3 µl of 0.1% TFA. Each diluted sample was spotted in triplicate (1 µl per spot) on a MSP 96 target ground steel plate (Bruker Daltonics, Bremen, Germany) and allowed to dry for 10 min, at 25 °C. Spectral profiles were acquired using a MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics) operating in positive linear ion mode within the *m/z* range 2000-20000, under the control of the FlexControl (v. 3.0) software (Bruker Daltonics). The mass spectrometer was calibrated after every sixth replicate using an external standard (Protein Standard and Peptide Standard kits, Bruker Daltonics). Data analysis was performed by mean of FlexAnalysis (v. 3.0, Bruker Daltonics) and ClinProTools (v. 2.2, Bruker Daltonics) softwares, and statistically processed applying Kruskall-Wallis ANOVA test.

2.5 Polypeptide marker identification

Serum samples (50 µl) were diluted 1:1 with 0.1% TFA, resolved on a C18 column (4.6 × 250 mm, 5 µm) (Vydac/Grace Davison, USA) with an Agilent 1100 chromatographer (Agilent, Santa Clara, CA) and monitored at 220 nm. Separation was carried out with a gradient of solvent B (0.1% TFA in ACN) in solvent A (0.1% TFA in water), at a flow rate of 1 mL/min. After 5 min of isocratic condition, solvent B ramped from 5% to 30% over 21 min, from 30% to 60% over 42 min,

and from 60% to 95% over 22 min. Chromatographic fractions were collected and concentrated to 10 µL.

Fractions were first analysed by linear MALDI-TOF-MS to reveal those presenting signals already identified as differentially represented during MALDI-TOF-MS profiling of serum samples; measurements were performed with an UltrafleXtreme mass spectrometer (Bruker Daltonics), equipped with FlexControl (v. 3.4) software. Each sample (0.5 µL) was separately mixed with 0.5 µL of a solution of matrix, *i.e.* α-cyano-4-hydroxycinnamic acid or sinapinic acid (Bruker Daltonics) prepared as previously reported (Sassi et al., 2015), loaded onto a Ground Steel Target (Bruker Daltonics) and allowed to dry at 25 °C. Depending on the matrix, spectra were acquired in positive linear mode in the *m/z* range 600-8,000 or 5,000-20,000 using instrument parameters already described (Sassi et al., 2015), accumulating 2,000 shots per spectrum, and externally calibrated with polypeptide standards. Acquired spectra were processed with FlexAnalysis software (v. 3.4, Bruker Daltonics).

After collection of chromatographic fractions bearing discriminant polypeptides, MALDI-TOF-TOF-MS and/or nanoLC-ESI-linear ion trap (LIT)-MS/MS analysis was carried out for their identification. In the first case, fragmentation of selected precursor ions was obtained in LIFT mode, without collision gas, using instrument parameters already described (Sassi et al., 2015). In the second case, polypeptides were analyzed with a LTQ XL mass spectrometer (Thermo, USA) equipped with a Proxeon nanospray source connected to an Easy-nLC (Thermo, USA) (Renzone et al., 2015) with or without previous sequential treatment with dithiothreitol, iodoacetamide and trypsin (Esposito et al., 2015). Intact polypeptides and their tryptic digests were resolved on an Easy C18 column (100 x 0.075 mm, 3 µm) (Thermo, USA) (Renzone et al., 2015). Spectra were acquired in the range *m/z* 400-2000. Instrument acquisition parameters were already reported (Renzone et al., 2015).

MASCOT search engine (version 2.2.06, Matrix Science, UK) was used to identify polypeptides of interest by searching raw data from MALDI-TOF-TOF-MS and/or nanoLC-ESI-LIT-MS/MS

against an updated bovine UniProtKB database (June 2015). For intact polypeptide species, searching parameters were: mass tolerance value of 1.8 and 0.8 Da for precursor ion and MS/MS fragments, respectively, no proteolytic enzyme, and Met oxidation, pyroglutamate formation (at N-terminal Gln), sulphoTyr, and phosphorylation (Ser, Thr and Tyr) as variable modifications. For tryptic digests, searching parameters were as those reported above, except Cys carbamidomethylation as fixed modification, trypsin as proteolytic enzyme, and a missed cleavages maximum value of 2. In both cases, other MASCOT parameters were kept as default. Candidates with at least two repetitions of 1 assigned peptide with a peptide expectation value < 0.05 (corresponding to a confidence level for peptide identification > 95%) were considered confidently identified. Definitive peptide assignment was associated with manual spectra verification.

2.6 Western blot analysis

Sera from samplings A and D were desalted with Zeba™ Micro Spin Desalting Columns and depleted of albumin with Pierce™ Albumin Depletion Kit (Thermo Scientific) using the appropriate buffer for bovine serum. Equal amounts of protein (100 µg/lane) were resolved with SDS-PAGE on 15% polyacrylamide gels under reducing conditions, and transferred onto a nitrocellulose membrane (Bio-rad, Valencia, CA, USA). Following incubation for 2 h with primary antibodies, *i.e.* goat anti-human apolipoprotein (APO) CIII (Novus Biologicals, Littleton, CO, USA) and sheep anti-bovine transferrin (Bethyl Laboratories, Montgomery, TX, USA) at 25°C, proteins were visualized using appropriate HRP-conjugated secondary antibodies and enhanced chemiluminescence substrate (Clarity™ Western ECL Blotting Substrate, Bio-Rad). Immunoblot bands were visualized by means of the ChemiDoc MP System (Bio-Rad) and protein expression was quantified by densitometry using Imagelab software version 4.1 (Bio-Rad). Relative density of APO-CIII band was normalized to that of transferrin.

2.6 Statistical analysis

Values were expressed as mean ± SEM. Significance of statistical differences was evaluated by the Kruskall-Wallis ANOVA test followed by the Dunn's multiple comparisons test, for comparing gene expression data of all samplings, or by the Wilcoxon matched-pairs signed-ranks test, for comparing protein expression data from sampling A and sampling D. Differences were considered statistically significant when the two-sided *P* value was less than 0.05. Analyses were performed with the GraphPad Prism 7.03 software (Graph Pad Software, San Diego, CA, USA).

2.7 Bioinformatics

Protein interaction network was obtained with STRING (*http://string-db.org*), using the bovine database, the differentially represented proteins detected in this study, those showing a contamination-dependent activity (Cigliano et al., 2016) and default setting parameters.

3. Results

Details on the PCDD/F, DL-PCB, and NDL-PCB contamination profile of the investigated heifers, along with the decontamination kinetics during the experimental period, are reported by Cigliano et al. (2016). In summary, the total TEQ value in pericaudal fat at sampling A (day 0; two weeks after the removal of the animals from the contaminated area and their arrival to the uncontaminated experimental farm) was approximately 6-fold higher (25.34 ± 1.405 pg/g fat) than that legally permitted, i.e. 4 pg/g fat (EC Regulation 1259/2011). Due to the source of animal exposure (ingestion of forages contaminated by the emissions of an industrial plant specialized in PCB treatment), DL-PCB provided by far the highest contribution to the TEQ value (about 96%). The initial value rapidly decreased during the first two months of the decontamination period (8.33 ± 0.35 pg/g fat at sampling B, day 59), and then progressively declined (4.87 ± 0.22 pg/g fat at sampling C, day 125), reaching the threshold value for PCDD/F and DL-PCB by the end of the experimental period (3.13 ± 0.18 pg/g fat at sampling D, day 188). Likewise, NDL-PCB levels were

approximately 4-folds above the legal limit (40 ng/g fat, EC Regulation 1259/2011) at sampling A, and then rapidly declined to reach normal values at sampling C.

3.1 Gene expression analysis on blood lymphocytes

In order to investigate the gene expression modulation by DL-PCB contamination, the mRNA levels of selected AhR-target genes (i.e. AhR, ARNT, CYP1B1, and IL-2) were measured in circulating lymphocytes from the examined animals at each sampling point (from A to D). Owing to a high erythrocyte contamination during lymphocyte isolation, RNA samples from all individuals (n = 8) were available only for samplings A and C; sampling B and D consisted of 5 and 7 samples, respectively. All the analysed genes were detectable in all samples, with Ct levels comprised between 21.5 (AhR) and 30.5 (CYP1B1). The relative gene expression of AhR and ARNT did not vary among the 4 samplings (data not shown), whereas CYP1B1 expression was significantly higher in lymphocytes collected at sampling A ($P < 0.05$), which corresponds to the time-point displaying the highest TEQ value, compared to all the other samplings (Fig. 1A). On the other hand, the mRNA levels of IL-2 increased over time during the decontamination procedure, leading to a statistically significant difference of sampling A compared to samplings C ($P < 0.05$) and D ($P < 0.01$) (Fig. 1B). No appreciable changes in the same parameters were detected in unexposed heifers of a different breed fed with a similar diet (data not shown).

3.2 MALDI-TOF-MS serum protein profiling

To examine the effects of DL- and NDL-PCB exposure on protein profiling, collected sera from the investigated animals (n = 8) at each sampling time (A, B, C, D) were subjected to C4 reversed-phase extraction in triplicate and analysed by MALDI-TOF MS profiling (Della Donna et al., 2009); assay performance was assessed across runs using an external calibrator. The acquired spectra were normalised by importing raw data to Flex Analysis, and any signal to noise ratio intensity beyond 50:1 was considered as a peak. Average spectra from each sampling were

generated with ClinProTools and a list of 68 peaks was obtained. Statistical analysis identified 48 peaks, ranging from *m/z* 2143.76 to 15274.44, which were significantly different among groups ($P < 0.05$). Then, a visual inspection of all significant peaks was performed, selecting those displaying a consistent increasing or decreasing intensity trend from sampling A to sampling D (Table 1). A total of 10 peaks emerged, 5 of which with growing intensity values (*m/z* 5693.9, 6779.0, 6839.6, 8550.9 and 8873.4), and 5 of which with declining intensity values (*m/z* 2193.0, 2427.7, 4102.5, 7945.1 and 12784.2). Comparing the first and last samplings (A and D), the degree of peak intensity modulation was from 133.11% to 395.68% for the over-represented peaks, and from -37.30% to -83.07% for the down-represented ones, respectively (Table 1). Fig. 2 depicts exemplificative ClinProTools clustering for signals at *m/z* 5693.9, 7945.1, 8873.4 and 12784.2. In all cases, a good discrimination was achieved when comparing A and D samplings, as deduced by MALDI-TOF-MS analysis of corresponding serum samples. No appreciable changes in the same parameters were detected in unexposed heifers of a different breed fed with a similar diet (data not shown).

3.3 Identification and validation of differentially represented proteins

To identify polypeptide marker candidates defined by the MALDI-TOF-MS serum profiling, we used an integrated strategy based on LC-MALDI-TOF-MS detection of the compounds of interest in the fractions obtained by chromatographic separation of sera, followed by their subsequent identification by tandem mass spectrometry analysis. The latter was carried out either by direct fragmentation (by MALDI-TOF-TOF-MS or nanoLC-ESI-LIT-MS/MS experiments) of the molecular species detected in the corresponding chromatographic fractions, or by nanoLC-ESI-LIT-MS/MS analysis of their tryptic digests. In all cases, mass spectrometry data were used for database searching using MASCOT software. An approach based on direct MS-based measurement of mass value and MS/MS-based detection of mass fragments (and consequently of peptide sequence) thus allowed the identification of the molecular species, namely fibrinogen β-chain (1-20) and (1-21), serpin A3-7-like (143-178), serum amyloid A-4 protein and APOC-II (29-101), A-II (24-99) and C-

III (24-96) (Table 2). Although detected in the corresponding chromatographic fraction, the compound with a MH^+ signal at *m/z* 5693.9 was not assigned, while the signals at *m/z* 6779.0 and 6839.6 corresponded to the doubly-charged ions of the counterparts at *m/z* 13557.3 and 13676.6. The latter, which were also detected as variably represented in the spectra, were assigned to transthyretin (TTR) and its adduct with cysteine, based on molecular mass values and previous observations during MALDI-TOF-MS profiling of bovine serum (Della Donna et al., 2009). Among the differentially represented polypeptides, the one showing the highest degree of variation during the decontamination period (from A to D sampling), namely APOC-III, was selected for further validation. To this aim, equal amount of serum proteins from the investigated heifers (n = 8) at samplings A and D, corresponding to the time-points of the highest and lowest TEQ levels, respectively, were subjected to Western blot analysis. In accordance with the results of the linear MALDI-TOF-MS serum profiling, APOC-III showed a lower expression at sampling A compared to sampling D ($P < 0.01$) in every investigated animal (Fig. 3).

4. Discussion

This study was carried out in a group of Limousine heifers that were accidentally exposed to DL- and NDL-PCBs by the ingestion of forages contaminated by the emissions of an industrial plant. The animals underwent a decontamination protocol through the feeding of a control diet for 6 months, and the extent of contamination at different time points was monitored on individual fat samples. Our goal was to evaluate the changes in AhR-target gene expression in circulating lymphocytes, and in the serum polypeptide representation during the decontamination process.

The search for biomarkers of exposure to DL-compounds has been attempted with different approaches and in different species with promising results. Taking into account the well-characterized mechanisms of toxicity of such substances, changes in AhR-dependent gene expression and/or inducibility in easily accessible matrices (i.e. blood cells) have been explored. In the above cited paper, Landi et al. (2003) reported for the first time differences in AhR, ARNT, and

CYP1B1 mRNA levels in circulating lymphocytes from individuals accidentally exposed to TCDD. Likewise, we demonstrated the constitutive expression and the ligand-mediated modulation of the AhR pathway in bovine cultured blood lymphocytes (Girolami et al., 2011), as well as the positive correlation between the field exposure to DL-compound contaminated feedstuffs and the CYP1B1 expression in blood lymphocytes from dairy cows (Girolami et al., 2013). Accordingly, CYP1B1 was the only gene induced in leukocytes from workers occupationally exposed at waste incinerators (Toide et al., 2003). Actually, although CYP1A1 is considered the most inducible AhR-gene, it was not taken into account in the present study due to its lack of expression in uncultured blood lymphocytes (Girolami et al., 2013; Landi et al., 2003). In line with the above-mentioned results, the mRNA level of CYP1B1 in lymphocytes from heifers investigated in the present study was significantly higher at the beginning of the decontamination procedure, when the TEQ values in fat were more than 6-fold over the legal permitted limit. Subsequently, CYP1B1 expression dropped rapidly in the first two months (approximately - 96%) along with the decline of the contamination extent by more than 3 fold, and remained almost unchanged during all the rest of the experimental period, when the TEQ values gradually decreased (from 8.33 to 3.13 pg/g fat in 129 days). Such results suggest that the AhR-mediated modulation of CYP1B1 is most likely associated only with high levels of contamination. Indeed, there was no direct correlation between the TEQ values and CYP1B1 mRNA levels in all samplings. Moreover, in line with the data of our previous work (Girolami et al., 2013), blood samples corresponding to the most contaminated time-point (A) were characterized by a high degree of variability in CYP1B1 expression, which was greatly lowered in the subsequent samplings, supporting the hypothesis of significant individual differences in response to DL-compound exposure. Interestingly, individual variation in the dioxin-mediated induction of CYP1B1 mRNA was also noticed in human leukocytes (Toide et al., 2003), in accordance with the polymorphic inducibility reported *in vitro* (Spencer et al., 1999).

Although several phase I and phase II xenobiotic-metabolizing enzymes are considered among the prototypical targets of the AhR pathway, factors involved in the modulation of the immune

system are also affected. TCDD is a potent immunosuppressive agent able to depress both humoral and cellular immunity, primarily through the alteration of the adaptive immune response driven by $CD4^+$ T helper cells (Kerkvliet, 2009). Such cells are the main producer of IL-2, a cytokine playing a key role in the regulation of T cell biology (Boyman and Sprent, 2012). Murine IL-2 promoter contains distal Dioxin Response Elements that can be addressed by the AhR (Jeon and Esser, 2000), and modulation of IL-2 by AhR ligands has been reported both *in vivo* and *in vitro* in mouse cells. In thymocytes from non-immunized mice injected intraperitoneally with 50 µg TCDD/kg bw, a IL-2 mRNA induction was observed (Jeon and Esser, 2000). However, such effect was dependent on cell maturation stage, since IL-2 induction by TCDD was not detected in spleen, a source of fully mature T cells. On the other hand, cultured splenocytes from mice immunized with ovalbumin and then treated with TCDD, displayed a reduced production of IL-2 as measured by ELISA (Chen et al., 2013; Nohara et al., 2002). In accordance with the reported down-regulation of IL-2 by TCDD in mature T cells, we observed an inverse correlation between IL-2 mRNA expression in peripheral lymphocytes and the level of PCB contamination. This is the first report of IL-2 down-regulation by DL-compounds in blood cells, suggesting, if confirmed at protein level, the possible role of serum IL-2 as a candidate biomarker of exposure.

Some interesting findings, were also detected in the serum polypeptide profile of the investigated heifers. In this case, contrary to the AhR-mediated-gene expression variations described above, the causal contribution of NDL-PCBs or of other pollutants (*e.g.* heavy metals) not assayed in this study cannot be excluded. As regards apolipoproteins, the most significant changes were displayed by APOC-III, *i.e.* a progressive increase during the decontamination period, consistent with a protein down-representation triggered by DL- and NDL-PCB exposure. This variation was further validated by Western blotting. Moreover, APOA-II and C-II were also found to be modulated. APOA-II representation resembled that of APOC-III, although with a slightly lower degree of variation (4.3 *vs* 4.9 fold change between sampling A and sampling D, respectively). In contrast, APOC-II representation was halved after the first 2 months of decontamination, and then remained

unchanged. To the best of our knowledge, direct evidence of the modulation of these serum apolipoproteins by PCBs has not been reported so far. Although such complex changes are difficult to interpret, it is worth nothing that TCDD and PCBs have been long associated with alterations of lipid metabolism, such as hypercholesterolemia and hypertriglyceridemia (Brewster et al., 1988; Singh and Chan, 2018). Even if the mechanisms underlying such effects have not been completely elucidated, it appears that changes in the amount of apolipoproteins responsible for lipid transport could play a significant role. Indeed, some studies describe the modulation of APOA-I, the major apolipoprotein responsible for cholesterol blood transportation along with APOA-II, and APOA-IV upon TCDD treatment in different species and tissues. For example, a single oral dose of TCDD (from 10 to 100 µg/kg) in rats was reported to increase liver either APOA-I (Pastorelli et al., 2006) or APOA-IV (Lee et al., 2005), as assessed by 2-DE coupled to nanoLC-ESI-MS/MS. On the other hand, Zhang et al. (2013) described a reduction of the same apolipoproteins in the heart of adult zebrafish after the exposure for 1 h to 1 ng/ml TCDD in waterborne conditions. Likewise, mice administered a single oral dose of TCDD (30 µg/kg) displayed a decrease in hepatic APOA-I gene expression, accompanied by altered serum lipid profiles (Angrish et al., 2013). Finally, PCDDs, PCDFs and PCBs may influence not only apolipoprotein expression but also their function. Due to their nonpolar nature, it is reasonable to expect these compounds to have a high affinity for blood apolipoproteins. Indeed, it has been demonstrated that TCDD, but not DL-PCBs, binds to human APOC-II inducing significant changes in the protein structure, which highly affect its properties in carrying lipids (Arehart et al., 2004).

In this study, further serum polypeptides were significantly modulated by the decontamination procedure, and namely fibrinogen β-chain fragments (1-20) and (1-21), serpin A3-7-like (α1-antichymotrypsin) fragment (143-178) and serum amyloid A-4, which all displayed a reduced representation (from 37% to 83%) at sampling D. These results were consistent with an over-representation of these species elicited by the exposure to DL- and NDL-PCBs. Besides fibrinopeptides, α1-antichymotrypsin is also involved in the coagulation process with a prevalent

pro-coagulant effect (Banfi et al., 2010). TCDD has been associated with the occurrence of disseminated intravascular coagulation in rats affected by pneumonia (Calkosinski et al., 2013), suggesting a role for DL-compounds in the hemostasis activation. In line with such findings and the results of our study, fibrinogen γ-chain was up-regulated in plasma from waste incineration workers exposed to TCDD (Kang et al., 2005), as well as in the liver of one-day-old chicks after a single dioxin *in ovo* injection (Bruggeman et al., 2006). To the best of our knowledge, no data are available about DL-compound or NDL-PCB mediated changes in α1-antichymotrypsin. The only published report describes the increased expression of another member of the serpin family (serpin B2) in human keratinocytes treated with 10 nM TCCD (Hu et al., 2013). Similarly, our data show for the first time the modulation of serum amyloid A-4 upon PCB exposure. A rather comparable effect (*i.e.* the increased level of serum amyloid A) has been reported in healthy humans experimentally exposed to wood smoke containing polycyclic aromatic hydrocarbons able to bind the AhR, even if the presence of PCDDs, PCDFs or PCBs in the air has not been demonstrated (Barregard et al., 2006). No data about NDL-PCB effects on the identified proteins have been published so far.

Differentially represented species reported in Fig. 1 and Table 2 were combined with other serum proteins previously demonstrated to be modulated in the same contaminated heifers (Cigliano et al., 2016), and namely antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX), acute phase protein haptoglobin (HP) and proinflammatory tumor necrosis factor-α (TNF-α). Upon STRING analysis, an interaction network linking together most (11 out of 13) components was observed (Fig. 4). Within this network, three subnetworks were recognized: i) the one linking together apolipoproteins, fibrinogens, TTR and HP; ii) the one bridging various proteins involved in signaling processes (including IL-2 and TNF-α); iii) the one linking together various antioxidant enzymes and CYP1B1. Central nodes in this network are HP, GSTA5 and SOD2, suggesting their prominent functional role in the animal response to PCB-associated

contamination, and confirming the primary importance of redox biology processes in regulating reaction and adaptation to xenobiotic-induced stresses (Klotz and Steinbrenner, 2017).

These findings suggests the occurrence of a complex DL- and NDL-PCB-mediated mechanism associated with the contamination/decontamination process, which includes protein/polypeptide species involved in blood redox homeostasis, inflammation and lipid transport. In this context, in addition to the experimental evidences reported above for CYP1B1, IL2, APOC-III, APOC-II, APOA-II and fibrinogen β-chain, it is worth noting that SOD, GPX and/or TTR were already reported to change their quantitative levels in a number of instances, as assessed by 2-DE-based proteomics. This was the case for liver of mice injected intraperitoneally with 10 µg 2,3,7,8-tetrabromodibenzo-p-dioxin/kg bw (Nguyen et al., 2017), in rat hepatoma cells treated with 1 nM TCDD for 8 h (Sarioglu et al., 2006), in plasma of rats after short-term or long-term oral exposure to TCDD (30 µg/kg) (Son et al., 2003) or of waste incinerating workers exposed to TCDD (Kim et al., 2004). Since a number of components reported in Fig. 4 returned to values comparable to those of unexposed animals at the end of the decontamination period (Cigliano et al., 2016 and data not shown), such molecules might be used in (partial) combination for identifying (in principle) PCB-contaminated animals, with the potential for monitoring the restoring of their healthy condition following a decontamination process.

In conclusion, changes in selected AhR-mediated gene expression and protein representation were detected in heifers accidentally exposed mainly to a mixture of DL- and NDL-PCBs and offered a non-contaminated diet for 6 months. Further experiments on a cohort of animals specifically treated with different doses of these contaminants are needed to fully understand the molecular basis underlying the phenomenon here ascertained in cattle subjected to a real eco-contamination event. In light of the results discussed above, future investigations are also encouraged in order to screen bovine populations living in geographic areas that are in close proximity to PCB-contamination sources.

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References

Angrish MM, Dominici CY, Zacharewski TR. TCDD-elicited effects on liver, serum, and adipose lipid composition in C57BL/6 mice. Toxicol Sci 2013; 131: 108-115.

Arehart E, Giasson G, Walsh MT, Patterson H. Dioxin alters the human low-density and very low-density lipoprotein structure with evidence for specific quenching of Trp-48 in apolipoprotein C-II. Biochemistry 2004; 43: 8503-8509.

Banfi C, Parolari A, Brioschi M, Barcella S, Loardi C, Centenaro C, et al. Proteomic analysis of plasma from patients undergoing coronary artery bypass grafting reveals a protease/antiprotease imbalance in favor of the serpin alpha1-antichymotrypsin. J Proteome Res 2010; 9: 2347-2357.

Barregard L, Sällsten G, Gustafson P, Andersson L, Johansson L, Basu S, et al. Experimental exposure to wood-smoke particles in healthy humans: effects on markers of inflammation, coagulation, and lipid peroxidation. Inhal Toxicol 2006; 18: 845-853.

Bock KW, Koehle C. The mammalian aryl hydrocarbon (Ah) receptor: from mediator of dioxin toxicity toward physiological functions in skin and liver. Biol Chem 2009; 390: 1225-1235.

Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 2012; 12: 180-190.

Brewster DW, Bombick DW, Matsumura F. Rabbit serum hypertriglyceridemia after administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). J Toxicol Environ Health 1988; 25: 495-507.

Bruggeman V, Van den Bergh G, Clerens S, Dumez L, Onagbesan O, Arckens L, et al. Effect of a single in ovo injection of 2,3,7,8-tetrachlorodibenzo-p-dioxin on protein expression in liver and ovary of the one-day-old chick analyzed by fluorescent two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 2006; 6: 2576-2585.

Calkosinski I, Rosinczuk-Tonderys J, Dobrzynski M, Palka L, Bazan J. Occurrence of disseminated intravascular coagulation in 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced pneumonia in the rat. Adv Exp Med Biol 2013; 788: 283-292.

Chen HM, Lee YH, Chen RJ, Chiu HW, Wang BJ, Wang YJ. The immunotoxic effects of dual exposure to PCP and TCDD. Chem Biol Interact 2013; 206: 166-174.

Chen X, Ma S-w, Ma X-m, Xu Y-j, Tang N-j. Changes in fibrinopeptide A peptides in the sera of rats chronically exposed to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Environ Toxicol Pharmacol 2012; 33: 191-196.

Cigliano L, Nebbia C, Rychen G, Feidt C, Girolami F, Rossetti C, et al. Evaluation of serum markers of blood redox homeostasis and inflammation in PCB naturally contaminated heifers undergoing decontamination. Sci Total Environ 2016; 542: 653-664.

Della Donna L, Ronci M, Sacchetta P, Di Ilio C, Biolatti B, Federici G, et al. A food safety control low mass-range proteomics platform for the detection of illicit treatments in veal calves by MALDI-TOF-MS serum profiling. Biotechnol J 2009; 4: 1596-1609.

Esposito G, Schiattarella GG, Perrino C, Cattaneo F, Pironti G, Franzone A, et al. Dermcidin: a skeletal muscle myokine modulating cardiomyocyte survival and infarct size after coronary artery ligation. Cardiovasc Res 2015; 107: 431-441.

Girolami F, Spalenza V, Carletti M, Perona G, Sacchi P, Rasero R, et al. Gene expression and inducibility of the aryl hydrocarbon receptor-dependent pathway in cultured bovine blood lymphocytes. Toxicol Lett 2011; 206: 204-209.

Girolami F, Spalenza V, Carletti M, Sacchi P, Rasero R, Nebbia C. Modulation of aryl hydrocarbon receptor target genes in circulating lymphocytes from dairy cows bred in a dioxin-like PCB contaminated area. Sci Total Environ 2013; 450: 7-12.

Hamers T, Kamstra JH, Cenijn PH, Pencikova K, Palkova L, Simeckova P, Vondracek J, Andersson PL, Stenberg M, Machala M. In vitro toxicity profiling of ultrapure non-dioxin-like polychlorinated biphenyl congeners and their relative toxic contribution to PCB mixtures in humans. Toxicol Sciences 2011; 121:88-100.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007; 8.

Hu Q, Rice RH, Qin Q, Phinney BS, Eigenheer RA, Bao W, et al. Proteomic analysis of human keratinocyte response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure. J Proteome Res 2013; 12: 5340-5347.

Jeon MS, Esser C. The murine IL-2 promoter contains distal regulatory elements responsive to the Ah receptor, a member of the evolutionarily conserved bHLH-PAS transcription factor family. J Immunol 2000; 165: 6975-6983.

Joo WA, Kang MJ, Son WK, Lee HJ, Lee DY, Lee E, et al. Monitoring protein expression by proteomics: human plasma exposed to benzene. Proteomics 2003; 3: 2402-2411.

Kang MJ, Lee DY, Joo WA, Kim CW. Plasma protein level changes in waste incineration workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Proteome Res 2005; 4: 1248-1255.

Kerkvliet NI. AHR-mediated immunomodulation: The role of altered gene transcription. Biochem Pharmacol 2009; 77: 746-760.

Kim MK, Oh S, Lee JH, Im H, Ryu YM, Oh E, et al. Evaluation of biological monitoring markers using genomic and proteomic analysis for automobile emission inspectors and waste incinerating workers exposed to polycyclic aromatic hydrocarbons or 2,3,7,8,-tetracholrodedibenzo-p-dioxins. Exp Mol Med 2004; 36: 396-410.

Klotz LO, Steinbrenner H. Cellular adaptation to xenobiotics: Interplay between xenosensors, reactive oxygen species and FOXO transcription factors. Redox Biol 2017;13: 646-654.

Landi MT, Bertazzi PA, Baccarelli A, Consonni D, Masten S, Lucier G, et al. TCDD-mediated alterations in the AhR-dependent pathway in Seveso, Italy, 20 years after the accident. Carcinogenesis 2003; 24: 673-680.

Lee SH, Lee DY, Son WK, Joo WA, Kim CW. Proteomic characterization of rat liver exposed to 2,3,7,8-tetrachlorobenzo-p-dioxin. J Proteome Res 2005; 4: 335-343.

Malisch R, Kotz A. Dioxins and PCBs in feed and food - Review from European perspective. Sci Total Environ 2014; 491: 2-10.

Nguyen HT, Tsuchiya MC, Yoo J, Iida M, Agusa T, Hirano M, et al. Strain differences in the proteome of dioxin-sensitive and dioxin-resistant mice treated with 2,3,7,8-tetrabromodibenzo-p-dioxin. Arch Toxicol 2017; 91: 1763-1782.

Nohara K, Fujimaki H, Tsukumo S, Inouye K, Sone H, Tohyama C. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. Toxicology 2002; 172: 49-58.

Pastorelli R, Carpi D, Campagna R, Airoldi L, Pohjanvirta R, Viluksela M, et al. Differential expression profiling of the hepatic proteome in a rat model of dioxin resistance: correlation with genomic and transcriptomic analyses. Mol Cell Proteomics 2006; 5: 882-894.
Renzone G, Arena S, Scaloni A. Proteomic characterization of intermediate and advanced glycation end-products in commercial milk samples. J Proteomics 2015; 117: 12-23.
Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000; 132: 365-386.
Sarioglu H, Brandner S, Jacobsen C, Meindl T, Schmidt A, Kellermann J, et al. Quantitative analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced proteome alterations in 5L rat hepatoma cells using isotope-coded protein labels. Proteomics 2006; 6: 2407-2421.
Sassi M, Arena S, Scaloni A. MALDI-TOF-MS Platform for Integrated Proteomic and Peptidomic Profiling of Milk Samples Allows Rapid Detection of Food Adulterations. J Agric Food Chem 2015; 63: 6157-6171.
Singh K, Chan HM. Association of blood polychlorinated biphenyls and cholesterol levels among Canadian Inuit. Environ Res 2018; 160: 298-305.
Son WK, Lee DY, Lee SH, Joo WA, Kim CW. Analysis of proteins expressed in rat plasma exposed to dioxin using 2-dimensional gel electrophoresis. Proteomics 2003; 3: 2393-2401.
Spalenza V, Girolami F, Bevilacqua C, Riondato F, Rasero R, Nebbia C, et al. Identification of internal control genes for quantitative expression analysis by real-time PCR in bovine peripheral lymphocytes. Vet J 2011; 189: 278-283.
Spencer DL, Masten SA, Lanier KM, Yang XP, Grassman JA, Miller CR, et al. Quantitative analysis of constitutive and 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced cytochrome P450 1B1 expression in human lymphocytes. Cancer Epidemiol Biomarkers Prev 1999; 8: 139-146.
Tavakoly Sany SB, Hashim R, Salleh A, Rezayi M, Karlen DJ, Razavizadeh BB, et al. Dioxin risk assessment: mechanisms of action and possible toxicity in human health. Environ Sci Pollut Res Int 2015; 22: 19434-19450.
Toide K, Yamazaki H, Nagashima R, Itoh K, Iwano S, Takahashi Y, et al. Aryl hydrocarbon hydroxylase represents CYP1B1, and not CYP1A1, in human freshly isolated white cells: trimodal distribution of Japanese population according to induction of CYP1B1 mRNA by environmental dioxins. Cancer Epidemiol Biomarkers Prev 2003; 12: 219-222.
Villanueva J, Philip J, Entenberg D, Chaparro CA, Tanwar MK, Holland EC, et al. Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. Anal Chem 2004; 76: 1560-1570.
Zhang J, Lanham KA, Heideman W, Peterson RE, Li L. Statistically enhanced spectral counting approach to TCDD cardiac toxicity in the adult zebrafish heart. J Proteome Res 2013; 12: 3093-3103.

Table 1

Mass signals and corresponding intensity values (mean ± SEM) of compounds detected in bovine serum that showed a consistent increasing or decreasing trend among the different samplings, as deduced by MALDI-TOF-MS profiling procedures. Fold and percent changes of significant peak intensity between sampling A and sampling D are also reported.

MH^+ (*m/z*)	Kruskall-Wallis *P* value	A	B	C	D	Fold change (D *vs*. A)	% change	Outcome
2193.0	0.00144	210.78 ± 0.45	155.82 ± 0.66	106.92 ± 0.56	132.15 ± 0.72	0.63	-37.30%	Decrease
2427.7	0.0115	11.34 ± 0.60	2.69 ± 0.12	1.84 ± 0.11	1.92 ± 0.08	0.17	-83.07%	Decrease
4102.5	0.00417	5.35 ± 0.15	3.48 ± 0.10	4.22 ± 0.19	2.58 ± 0.10	0.48	-51.78%	Decrease
5693.9	0.00267	2.22 ± 0.12	2.73 ± 0.12	2.01 ± 0.12	7.95 ± 0.17	3.58	+258.11%	Increase
6779.0	0.00252	3.61 ± 0.14	6.09 ± 0.14	7.02 ± 0.24	9.77 ± 0.15	2.71	+170.64%	Increase
6839.6	0.00144	1.51 ± 0.08	2.36 ± 0.05	2.84 ± 0.13	3.52 ± 0.08	2.33	+133.11%	Increase
7945.1	0.00144	2.25 ± 0.07	1.34 ± 0.05	1.23 ± 0.04	1.21 ± 0.05	0.54	-46.22%	Decrease
8550.9	0.00927	13.38 ± 0.24	38.82 ± 0.54	34.92 ± 0.70	57.71 ± 0.51	4.31	+331.32%	Increase
8873.4	0.00144	1.85 ± 0.09	5.48 ± 0.19	6.50 ± 0.28	9.17 ± 0.18	4.96	+395.68%	Increase
12784.2	0.00144	2.02 ± 0.11	0.65 ± 0.05	0.78 ± 0.06	0.78 ± 0.05	0.39	-61.39%	Decrease

Table 2

List of the polypeptide species identified by mass spectrometry procedures. Experimental and theoretical mass value, protein name, UniProt/NCBI accession, protein fragment details, amino acid sequence, modification, modified residue and MS method for protein identification are reported.

Exp. MH^+ (m/z)	Theor. MH^+ (m/z)	Description	UniProt/NCBI accession	Protein fragment	Sequence	Modification	MS method
2193.0	2192.3	Fibrinogen β chain [#]	P02676/1346006	1-20	qFPTDYDEGQDDRPKVGLGA	q1-pGlu	MALDI
2427.7	2428.6	Fibrinogen β chain [#]	P02676/1346006	1-21	qFPTDyDEGQDDRPKVGLGAR	q1-pGlu, y6- sulphoTyr	MALDI
4102.5	4103.9	Serpin A3-7-like [# @]	555999676	143-178	LHPLNVSFNRPFLLSIFCKETQSIIF LGKVTNPKEA		ESI
7945.1	7946.0	Apolipoprotein C-II [@]	P19034/166897678	29-101	DEASSPALLTQVQESLLGYWDTA KAAAQKLYKKTYLPAVDEKIRDI YSKSTAAVTTYAGIITDQVFSVLS GKD		ESI
8850.9	8550.6	Apolipoprotein A-II [@]	P81644/109940051	24-99	qAEESNLQSLVSQYFQTVADYGK DLVEKAKGSELQTQAKAYFEKTQ EELTPFFKKAGTDLLNFLSSFIDPK KQPAT	q24-pGlu	ESI
8873.4	8874.6	Apolipoprotein C-III [@]	P19035/528976847	24-96	EEGSLLDKMQGYVKEATKTAKD ALSSVQESQVAQQARDWMTESFS SLKDYWSSFKGKFTDFWESATSPt QSPP	t92-HexNAc-Hex-NeuAc*	ESI
12784.2	12783.1	Serum amyloid A-4 protein [@]	Q32L76/122138722	19-129	DSWYSFFKEAVQGASDLWRAYW DMRDANVQNSGRYFRARGNYEA AQRGPGGVWAAKIISNVGEYLQG FLYQIYLGDSYGLEDQVSNRRAEE WGRSGQDPDHFRPAGLPKKY		ESI

[#] Identification obtained by direct MS/MS analysis of the differentially represented species. [@] Identification obtained by MS/MS analysis of the differentially represented species after tryptic digestion. * Modification inferred from what reported for the human counterpart (P02656 - APOC3_HUMAN). MALDI, MALDI-TOF/TOF-MS; ESI, nanoLC-ESI-LIT-MS/MS. Modified amino acids are reported in small letters. pGlu, pyroglutamate

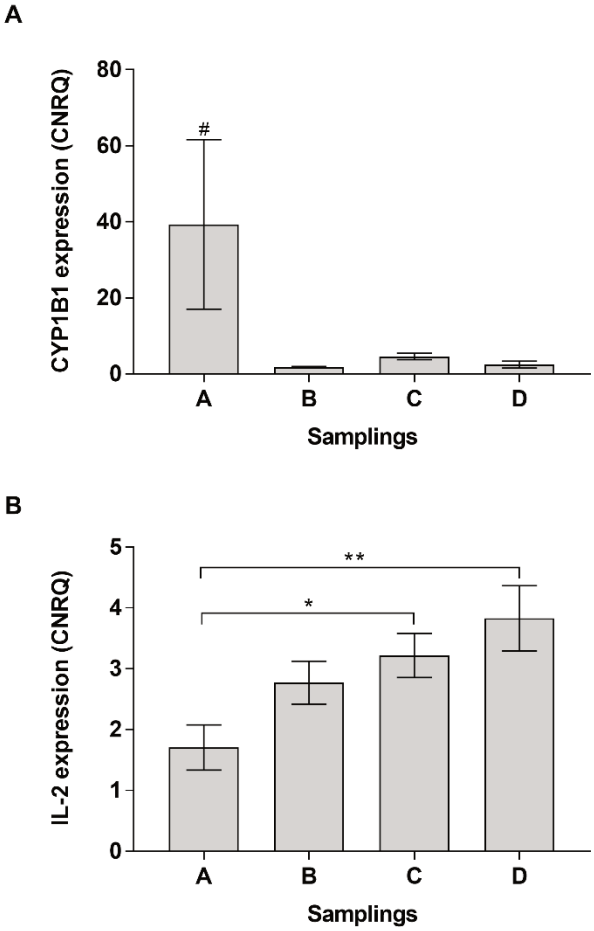

Fig. 1. Relative gene expression of CYP1B1 (A) and IL-2 (B) in blood lymphocytes depicted as mean ± SEM. # $P < 0.05$ compared to all other samplings; * $P < 0.05$ compared to sampling A; ** $P < 0.01$ compared to sampling A.

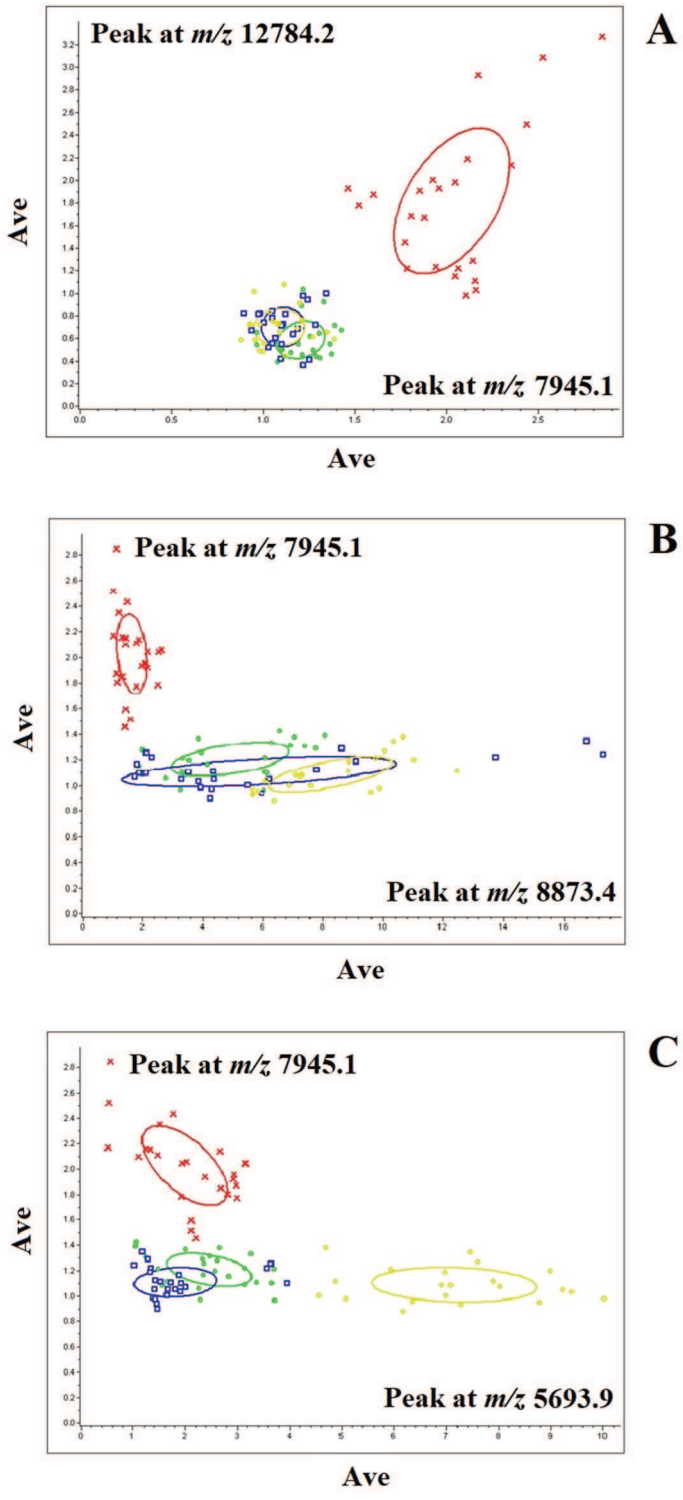

Fig. 2. ClinProt statistic views for bovine serum at sampling A, B, C and D as resulting from MALDI-TOF-MS analysis. Data referring to sampling A, B, C and D are shown in red, green, blue and yellow, respectively. Considered signals are reported at corresponding axes; they were: *m/z* 12784.2 and 7945.1 (panel A); *m/z* 7945.1 and 8873.4 (panel B); *m/z* 7945.1 and 5693.9 (panel C).

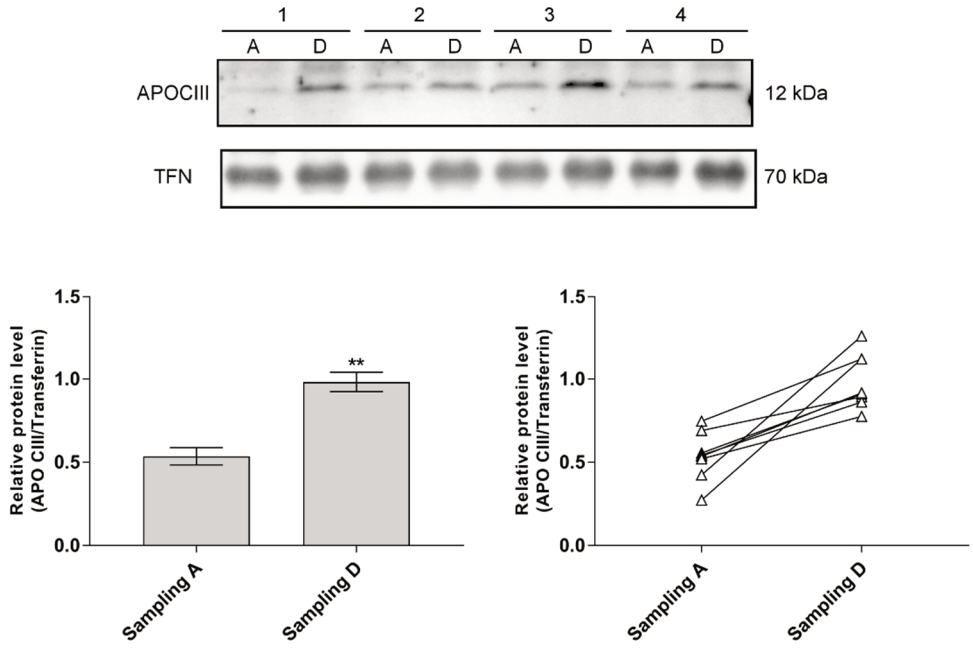

Fig. 3. Protein expression level of apolipoprotein C3 (APO-CIII) in serum from samplings A and D (n = 8). Data are expressed as relative protein level using transferrin (TFN) as loading control (mean ± SEM). The gel is representative of 4 animals at each time-point. ** $P < 0.01$.

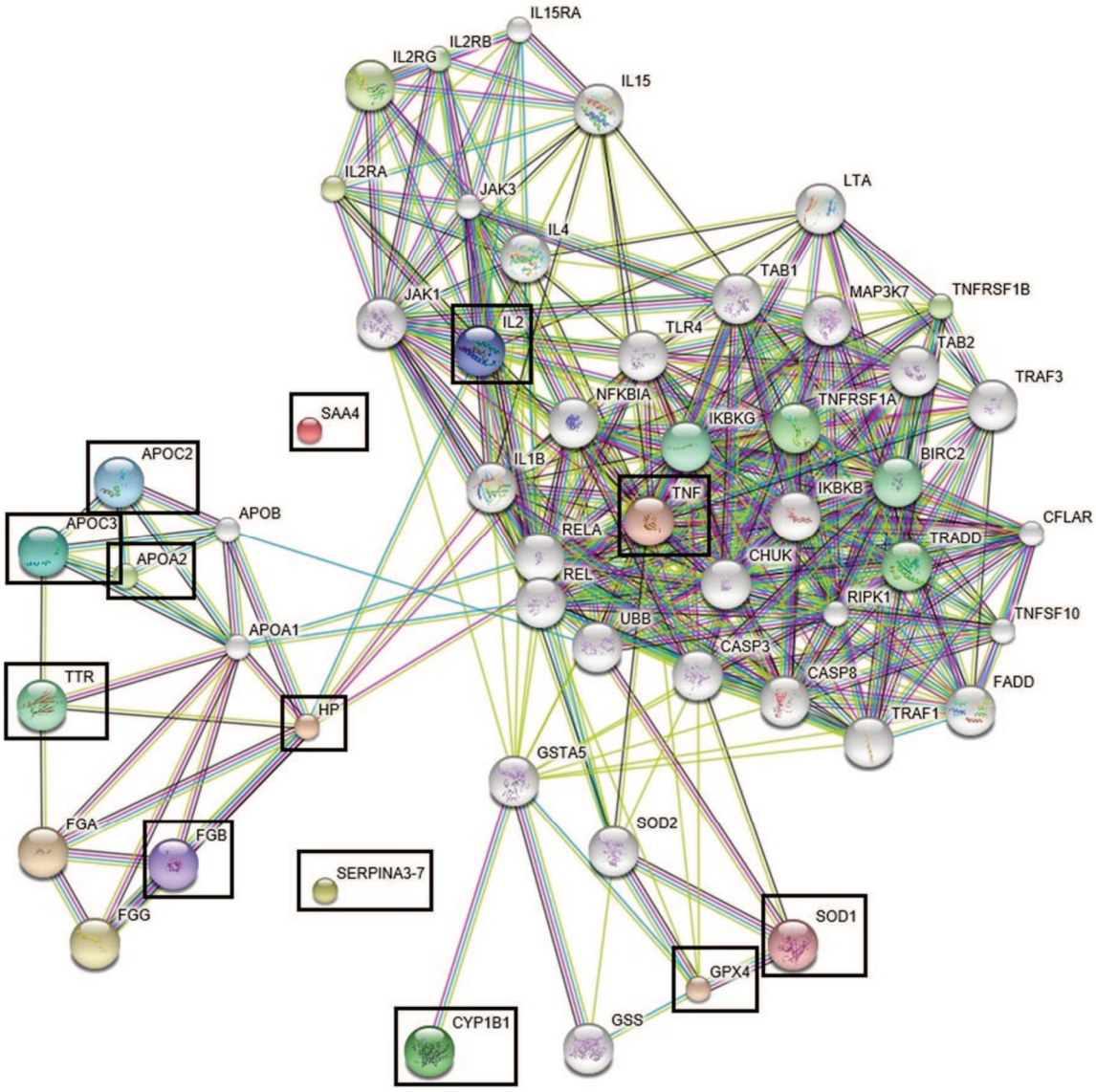

Fig. 4. STRING analysis of differentially represented proteins in bovine serum from samplings A to D, as obtained using default software setting parameters. Boxed are the differentially represented polypeptides/proteins detected in this study as well as those showing contamination-dependent changes (Cigliano et al., 2016). Abbreviations are reported in the manuscript text.