Gene expression profiling of thymus in beef cattle treated with prednisolone

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A B S T R A C T

Glucocorticoids (GCs) are extensively used in livestock production, not only for their anti-inflammatory properties but also to improve the quality and quantity of meat in veal and beef production. In Italy, an increase in GC-positive cases has been observed in cattle since 2008, particularly prednisolone (PDN). Recent studies clearly demonstrate that both histopathological analysis and high-performance liquid chromatography tandem mass spectrometry (HPLC/MS-MS) were unable to detect PDN treatments. The aim of this study was to identify transcriptomic signatures of PDN administration in the thymus of experimentally treated animals by comparison with untreated controls, in order to identify gene expression changes or pathways alteration induced by the corticosteroid treatment. Microarray data analysis showed substantial modifications in thymus gene expression profiles after PDN treatment. Several of the 388 differentially expressed genes encoded pro-inflammatory and anti-inflammatory mediators or immune regulators which showed that PDN might have a role in the regulation of immunologic homeostasis, act on both innate and acquired components of the immunity and mainly induce the activation of immune tolerance and anti-inflammatory pathways. Thus, this study allowed to deepen the effects of PDN on the immune system and showed the potentiality of gene expression profiling by DNA-microarray as a powerful tool to complement the existing methods against the illegal use of growth promoting hormones, especially when working on samples collected after slaughtering.

Keywords:
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1. Introduction

The illegal use of growth promoters in food-producing animals constitutes a potential risk for human health. In this context, synthetic glucocorticoids (GCs) possess hormone-like activity, which may influence the hypothalamic-pituitary-adrenal and -gonadal axis through a negative feedback mechanism (Sapolsky, 1985; Cunha et al., 2004; Krasner, 2009). To protect consumers against the illegal use of drugs in food-producing animals, the EU has defined appropriate maximal residue limits (MRLs) in tissues and milk intended for human consumption (Council of the European Communities 1990; Council Regulation 90/2377/EEC). To prevent the accumulation of residues in animal products, withdrawal periods of up to several weeks have been suggested, depending on the specific formulation of GCs. Although their administration in animals is primarily therapeutic, GCs are also illegally utilised in some European countries as growth promoters, either alone or in association with anabolic steroids, to improve the quality and quantity of meat in veal calves and beef production (Tarantola et al., 2004; Gottardo et al., 2008). In Italy, the Italian national program for residue surveillance (PNR) in meat and meat derivatives has observed a marked increase in the number of GC-positive cases of cattle since 2008, including a specific increase in the use of prednisolone (PDN) (PNR, 2010). PDN is a synthetic GC that is extensively used as a therapeutic agent in veterinary practice for the treatment of a wide range of metabolic diseases, shock, stress and inflammatory disorders in farm animals (Leclere et al., 2010; Kovalik et al., 2012a,b). The use of PDN is permitted in cattle, and the MRLs are 4 lg/kg in muscle and fat, 10 lg/kg in liver and kidney and 6 lg/ kg in milk (EEC Commission Regulation N° 37/2010). Because of their steroidal structure, GCs are included in group A3 (substances with an anabolic effect and unauthorised substances/steroids) of the PNR of some member states (Italy, the Netherlands and Denmark), whereas they are assigned to the B2f group (other pharmacologically active substances) in other states (EU, 2010). The continuous evolution of illicit treatments makes the analytic detection of residues in food increasingly difficult. For instance, the standard method of using LC/MS, although specific and sensitive, is time consuming, expensive and requires trained personnel. Therefore, the availability of a reliable screening test would be highly useful. Rapid and easy analytical techniques or novel screening tools that target GC substances or their residues need to be developed for use in routine analyses. In this respect, a histo-pathological test is already routinely employed in the Netherlands to evaluate the illegal use of hormones, and it was officially introduced in the Italian PNR for screening purposes in 2008. Anatomo-histopathological examination of the thymus allows the detection of illegal treatment with GCs, even if residues are not found in the urine (Cannizzo et al., 2011; Bozzetta et al., 2011; Vascellari et al., 2012). However, a recent study clearly demonstrated that, contrary to the effect of dexamethasone (DEX) administration to beef cattle, the long-term administration of low doses of PDN does not cause thymus atrophy in the same category of animals (Cannizzo et al., 2011). Consequently, gross and histopathological investigations do not appear to be useful in detecting illegal treatment with PDN at anabolic dosages in beef cattle. The same study reported that HPLC/MS-MS techniques were unable to detect PDN residues in urine and liver samples taken from treated animals at the slaughterhouse. Innovative methods that can help to identify animals treated with anabolic agents include the so-called omics techniques (transcriptomics, proteomics and metabolomics) and are based on the simultaneous detection of biomarkers predictive of the administration of specific substances (Toffolatti et al., 2006; Reiter et al., 2007; Courant et al., 2009; Carraro et al., 2009; Becker et al., 2011; Dervilly-Pinel et al., 2012; McGrath et al., 2013). In recent years, several research groups investigated these indirect methods to identify biomarkers indicative of the illegal use of growth promoters. These novel testing methods are not based on the direct detection of specific drug residues but rather on the indirect evidence of ‘‘perturbations to physiological activity’’ resulting from the administration of growth promoters. Numerous scientific papers have been published using transcriptomic (Riedmaier et al., 2009; Carraro et al., 2009; Divari et al., 2011; Lopparelli et al., 2012), proteomic (Draisci et al., 2007; Della Donna et al., 2009; Stella et al., 2011) and metabolomic (Dervilly-Pinel et al., 2011, 2012) approaches. The results of these studies have encouraged continued investigation in this direction (Nebbia et al., 2011). These technologies are promising and may lead to the development of a panel of biomarkers that would show the biological effects induced by illicit drugs in specific target organs, thereby overcoming the limits of traditional methods. The aim of this study was to identify transcriptomic signatures of PDN administration in the thymus of experimentally treated animals by comparison with untreated controls, in order to identify gene expression changes or pathways alteration induced by the corticosteroid treatment.

2. Materials and methods

2.1. Animals

Fifteen male Italian Friesian beef cattle (7-15 month-old, about 470 kg weight) were bought from local breeders, allowed to acclimatize for 2 months. When beef cattle were about 10-18 month-old were randomly divided into two groups: group P (n = 8) was administered prednisolone acetate (PA, Novosterol, Ceva Vetem spa, Italy) 30 mgday"[1] per os for 35 days, from day 51 day 85, while group K (n = 7) served as a control. The beef were slaughtered 6 days after drug withdrawal. Before the distribution of the feed each morning, the animals were tied to the feeding trough, and two trained technicians used a drenching gun to administer one capsule containing the compound to each animal. Controls were treated with a placebo. The animals were housed for about 90 days in ventilated stables, and the experiment was carried out according to the European Union animal welfare legislation. All groups of experimental animals were kept in separate 10 x 15 m boxes and fed a diet consisting of corn silage, corn, hay and a commercial protein supplement; water was supplied ad libitum. The beef cattle were weighed monthly for 4 times: T0 (day 0), T1 (day 36), T2 (day 61), T3 (day 92) (Fig. 1). The average daily gain (ADG) was calculated as the difference between two subsequent body weights (BWs). The beef health status was monitored daily by recording all individual pathological events and medical treatments. The experiment was authorized by the Italian Ministry of Health and the Ethics Committee of the University of Turin. Carcasses of treated animals were appropriately destroyed (2003/74/ CE - DL 16 March 2006, ns. 158).

2.2.Sample collection and RNA extraction

Thymus samples were collected from each animal after slaughter. A portion of the gland (approximately 100 mg) was immediately fixed in RNAlater® (Ambion®, Life Technologies, Carlsbad, CA, USA) and stored at —80 °C for the gene expression analysis. Total RNA from the thymus was extracted using TRIzol® Reagent (Ambion®, Life Technologies) according to the manufacturer's protocols.

DNAse digestion was performed on the extracted RNA using the QIAGEN RNase-Free DNase Set (Qiagen, Hilden, Germany) to remove DNA contamination. The RNA was then purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. The concentrations of the RNA samples were measured using a UV-Vis spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, USA), and RNA integrity was determined by running each sample on an RNA-chip in an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA).

2.3.RNA amplification, labelling and hybridisation

The labelling and hybridisation of samples were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Briefly, for each sample, 200 ng of total RNA was linearly amplified and labelled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labelling to monitor the microarray analysis work-flow. Labelled cRNA was purified with the RNeasy Mini Kit (Qiagen), and Cy3 incorporation (pmol Cy3/lg cRNA) was measured in the NanoDrop ND-1000 spectrophotometer. A total of 1.650 ng of labelled cRNA was prepared for fragmentation by adding 11 lL of lOx Blocking Agent and 2.2 lL of 25 x Fragmentation Buffer, heated at 60 °C for 30 min, and finally diluted by the addition of 55 lL 2x GE Hybridization buffer. A total of 100 lL of hybridisation solution was then dispersed onto the gasket slide, which was assembled into the microarray slide (each slide contained four arrays). Bovine-specific oligo arrays (Agilent Bovine-Four-Plex G2519F) were used. For most of the transcripts represented on this array, two identical probes were synthesised at two distinct positions on the slide; therefore, the average value of the intensities of the two replicate probes was used. The slides were incubated for 17 h at 65 °C in an Agilent Hybridisation Oven, subsequently removed from the hybridisation chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GE Wash Buffer 1 for approximately 1 min, followed by one additional wash in pre-warmed (37 °C) GE Wash Buffer 2. The hybridized slides were scanned at 5 lm resolution using an Agilent G2565BA DNA microarray scanner. The default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The microarray data have been deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002) and are accessible through the GEO Series accession number GSE40884.

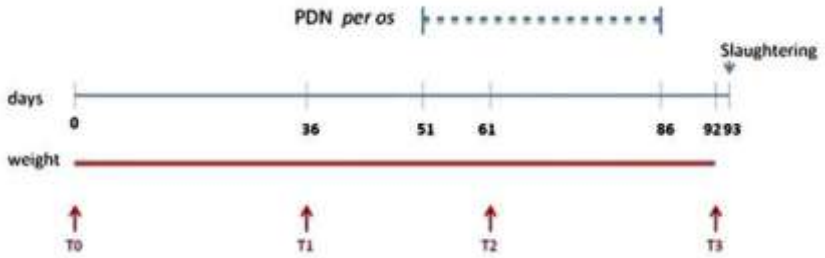

Fig. 1. Treatment protocol and weight detection times. Beef cattle were divided into two experimental groups: Group P administered prednisolone acetate (PA, Novosterol, Ceva Vetem spa, Italy) 30 mg day"[1] per os for 35 days; Group K was used as control. The animals were slaughtered 6 days after drug withdrawal. The beef cattle were weighed monthly for 4 times: T0 (day 0), T1 (day 36), T2 (day 61), T3 (day 92).

2.4.Normalisation of microarray data

The two linked images generated from the scanned slide were analyzed together, data were extracted, and the background was subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) software, version 9.5.1. The Feature Extraction software returns a series of spot quality measures to evaluate the quality and reliability of the spot intensity estimates. To avoid possible bias due to sample processing and slide variation, spike-in viral RNAs were added to each sample at the beginning of the microarray experiment to provide an internal quality control. Each spike-in RNA had a different known concentration based on a dilution series, and there were 32 replicate probes for each spike-in RNA on the array. Spike-in control intensities were used to identify the best normalization procedure for each dataset. All control features except for spike-in (spike-in viral RNAs) data were excluded from subsequent analyses. After normalization, the spike intensities were expected to be uniform across all experiments in a given dataset.

Probes with intensity values <11 in at least 70% of the samples were removed from the dataset (this intensity threshold value was selected on the basis of the mean intensity of the second lowest spike-in concentration). Filtering and normalisation were performed using the R statistical software available at http://www.r-project.org/.

2.5. Quantitative RT-PCR

A set of 8 genes (Table 1) was used for the external validation of microarray data by real-time RT-PCR. For each selected target gene and for the reference gene (RS5), a quantitative (q)RT-PCR assay was designed. Gene-specific primers that encompassed one intron were designed for each transcript using the program Primer Express version 2. To design intron-spanning primers, putative intron-exon boundaries were deduced from the Genome Browser Database (http://genome.ucsc.edu/cgibin/hgGateway?hgsid= 105081852 &clade=vertebrate&org=Cow&db=0). One microgram of the total RNA for each sample was reverse transcribed to cDNA using Superscript II (Invitrogen). An aliquot (2.5 lL) of diluted (1:50 or 1:100) cDNA template was amplified in a final volume of 10 lL containing 5 lL of KAPA SYBR® FAST Universal 2x qPCR Master Mix (Kapa Biosystems, Inc., Woburn, USA) and 0.25 lL of each gene-specific primer (10 lM). The amplification protocol included an initial step of 2 min at 95 °C, followed by 45 cycles of 3 s at 95 °C and 40 s at 60 °C. All experiments were performed in a LightCycler 480 (Roche Diagnostics, Milan, Italy). To evaluate the efficiency of each assay, standard curves were obtained by amplifying a twofold serial dilution of the same cDNA, which was used as a calibrator. For each sample, the crossing point (Cp) was used to determine the relative amount of the target gene; each measurement was performed in duplicate and normalized to the reference gene RS5, which was also measured in duplicate.

Table 1
Genes and primers used in the Real-time PCR validation assays.

Gene name	Probe name	Forward primer	Reverse primer	Length[b]
CALCOCO1	A_73_109980	AGCTTGAGAAGGCTCTGGCA	GGGAAAGCCCCTTATACTGCTC	72
LCP1	A_73_105903	GCTGCCAGAACTGTTGCCTAA	CTCCAGCATTTCTTCGTCGG	71
LSMD1	A_73_107680	CGACCGTGACTGCAACGTTA	CGGCAGAGAAGGAATCCGAC	71
MRPS7	A_73_100812	GGTTATTAAAGCTGCCCCAGC	TGGTGAATTTACTGATCACTGGGT	71
PFDN1	A_73_103325	TGGAGTTGAAGAAGGCTTTCACA	TGCAAGCTTCACCTTCTGTTGAG	71
RAG1AP1	A_73_114081	CACCACGGATGTCAACAACCT	CGATGATTAGCGTCCAGTTTCC	71
ROBLD3	A_73_101314	AAGGCTTTGACCCAGGTGCTA	TCGTTATTCAGCAGCAGGGTG	71
TIA1	A_73_106456	AAAAGACATGGCGACAGGAAAG	TTTCGGCATCCCATTTGTTG	71
RS5[a]	A_73_109595	CATCAAGACCATTGCCGAGTG	CGTAGGAATTGGAGGAGCCCT	71

[a] Reference gene.
[b] Amplicon lenght.

Table 3
Average daily gain of the control and treated bulls during the experimental period.

	Group P (n = 8)	) Group K (n = 7)	P
Average daily gain kg/day			
T0-T1	1.89	0.0	0.0001
T1-T2	1.21	1.59	0.028
T2-T3	1.54	1.03	0.0002
T0-T3	1.59	1.18	0.007

2.6. Statistical analyses

A principal component analysis (PCA) of normalized and filtered data was carried out using the TMEV suite (Saeed et al., 2003, 2006). The t-test statistics were performed using Microsoft Excel® 2010 (Redmond, Washington, USA) to test for differential expression, and the differentially regulated genes were selected using a p-value of <0.05 and a fold change (FC) of 1.5 (the value assigned to the control samples was 1). The Spearman rank correlation tests were carried out using SPSS software (IBM, Armonk, New York, USA).

2.7. Functional annotation

Enrichment analysis on differentially up- and down-regulated genes was performed using the Functional Annotation tool available in the DAVID Database (http://david.abcc.ncifcrf.gov/). GO terms and KEGG pathways included in the DAVID knowledgebase were considered. For KEGG terms, the following parameters were used: gene count 4, ease 0.05. For GO Biological Process and Molecular Function BP_FAT, MF_FAT respectively, with gene count 4, ease 0.05 was used.

3. Results

3.1. Animal health status and growth performance

The health status of the beef cattle was good during the entire experimental period. None of the beef cattle received specific medical treatment. At T0, the animals showed no significant differences in weight (Table 2). In comparison with the control group, overall weight gains were statistically larger in group P (27%) (P = 0.0047) indicating that growing cattle will respond to exogenous PDN administration (Table 2). ADG was higher in the animals in group P compared to those in the control group (1.587 vs. 1.178 kg/day; P = 0.007) (Table 3).

3.2. Quality assessment of DNA microarray data

In DNA microarray experiment data, quality is essential. An RNA integrity number (RIN) was used as a measure to standardize the interpretation of RNA quality. In the present study, a conservative threshold was enforced to reduce experimental biases due to poor RNA quality. Only RNA samples with RIN numbers P7.0 were included in the analysis.

Based on the uniformity of the spike-in intensities across the samples, the cyclic lowess normalization procedure was applied. The comparison of normalized, averaged spike-in signals across the different experimental replicates provided a strong indication of the robustness of the process, as the spike-in variation across the samples was minimal. All of the samples (negative and treated samples) were normalized in a single run to avoid potential biases.

After normalization, a further quality control step was performed by removing all of the probes with intensity values lower than the second lowest spike-in concentration because this value was considered to be too close to the limit of detection. The filtering process resulted in the removal of 4445 unique transcripts.

Finally, gene-specific quantitative (q) RT-PCR assays were developed and used to quantify the relative expression of 8 genes in the entire set of samples (Table 1) to provide independent evidence of the data quality. The quantitative changes observed with the DNA microarray were confirmed in the selected genes by RT-PCR, and a positive correlation in the expression values between the two methods was found, confirming the experimental reliability (Table 4).

3.3. Microarray data analyses

After data extraction, normalization, and filtering, processed signals for 17,030 unique transcripts in 15 thymus samples were analyzed using PCA as an unsupervised method to identify differences in gene expression profiles of controls and treated animals. Fig. 2 shows the first two components, which represent 61% of the total variance. No clear separation could be observed between controls and PDN-treated animals, with a substantial dispersion of samples along the x-axis, suggesting the influence of additional factors other than treatment on gene expression profiles. Neither animal age nor body weight showed significant correlation with sample scores on the first component (age, Spearman rho = —0.007, p = 0.979; body weight, Spearman rho =-0.048, p = 0.864) or the second one (age: r = 0.001, p = 0.994; body weight: Spearman rho =-0.075, p = 0.793). It is therefore likely that other, unknown factors underlie the observed individual variation.

Microarray gene expression data from P and K animal groups were compared using T-test statistics with p-value <0.05

and FC of 1.5. A total of 388 transcripts were differentially expressed, of these 154 were up-regulated and 234 were down-regulated (Supplementary Tables 1 and 2). A first evaluation of GO terms and KEGG pathways that are significantly enriched among regulated genes was performed using DAVID and the results are reported in Table 5.
Based on the obtained evidence, PDN treatment seems to affect ion transport and in particular to regulate calcium signaling pathways. Regulation of RNA metabolic process and signal transduction linked to cell surface receptors were also found to be enriched, with 17 and 18 genes respectively included in these terms being significantly altered.
In addition to functional enrichment analysis, a detailed evaluation of gene expression profiles for pro-inflammatory and anti-inflammatory mediators or immune regulators was carried out, in consideration of the target organ and the known effects of GCs on the immune system. Obtained results are reported in Table 6.

4. Discussion

DNA microarray analysis showed a relatively large set of differentially expressed genes between controls and PDN-treated animals. Enrichment analysis pointed at ion transport activity regulation, and in particular calcium influx. It is known that Ca2+ entry is critical for several responses in immune cells, including proliferation and cytokine production by T cells, cytokine production by mast cells and natural killer (NK) cells, differentiation of B cells into plasma cells, and differentiation of naïve T cells into Th1, Th2, and Th17 effectors subtypes. However, increase in intracellular calcium in T lymphocytes is also reported to induce energy (Schwartz, 1997) and apoptosis (King and Ashwell, 1993). Calcium plays important roles in thymocytes as well, controlling differentiation, proliferation, and apoptosis, depending on the intensity and the duration of signaling events (Puthier et al., 2004). In the present work, up-regulation of the calcium channel, voltage -dependent, T type, alpha 1H subunit (CACNA1H) was observed, which could increase intracellular calcium concentration. A similar effect might have the 1.8 up-regulation of cholecystokinin A receptor (CCKAR), which is known to elicit inositol trisphosphate (IP3)-induced calcium release from endoplasmic reticulum (Staljanssens et al., 2012). On the other hand, up-regulation of the solute carrier protein 8A1 (SLC8A1), which functions as Na+/Ca2+ exchanger from the cell, might increase Ca2+ efflux. Down-regulation of the Ca2+/ CaM-dependent protein kinase 2B, which is known to directly activate CACNA1H was observed. Therefore, it is not clear which might be the final effect of the observed transcriptomic changes on Ca2+ balance in thymocytes.

Several genes (17) involved in the regulation of RNA metabolic process were also differentially expressed. Among these, the prion protein (PRNP) transcript was 1.6-fold up-regulated. Gene expression microarrays have revealed murine PRNP to be up-regulated in certain types of regulatory T cell (Huehn et al., 2004), via a Stat6-dependent mechanism during interleukin (IL)-4 driven Th0 to Th2 differentiation (Chen et al., 2003) and in CD8+ memory T cells (Goldrath et al., 2004).

Synovial sarcoma X chromosome breakpoint 5 (SSX5) was 1.8-fold down-regulated. SSX5 is a member of the class of proteins known as cancer-testis antigens (CTAs), which are expressed in lymphoid tissues such as medullar cells of the thymus during T-cell selection. To date, only a few CTAs have been shown to elicit both humoral and cell-mediated immune responses in humans, including SSX5, but their function is still not fully understood (Smith and McNeel, 2010).

Significant enrichment was observed also for the Biological Process ‘‘regulation of cell surface receptor-linked signal transduction’’, with many differentially expressed genes.

Several genes (9) encoding olfactory receptors (ORs) were down-regulated after PDN treatment. ORs are expressed in several tissues, but their function outside the olfactory epithelium is largely unknown. However, OR genes are found right next to the MHC region in both human and mouse genome. The traditional definition of the MHC and its partition into three regions (class II, class III and class I) was actually extended to the flanking regions that contain genes in tight linkage with MHC loci. It was thus supposed that MHC-linked OR genes could have a role in the detection of MHC diversity (Amadou et al., 2003). However, other putative and still not clear functions of OR genes in immune mechanisms cannot be excluded.

Since GCs are known to act on the immune system by both suppressing and stimulating a large number of pro-inflammatory or anti-inflammatory mediators, particular attention was paid to the regulation of immune-related genes (Table 6) and to their putative role in thymocytes. The most interesting findings are discussed below.
Several cytokines are broadly down-regulated by GCs and similarly secretion of C-C and CXC chemokine is suppressed. On the opposite, GCs were proved to up-regulate anti-inflammatory cytokines such as IL-10 or to some

extent IL-1RA, which promote development of Th2 cells (Ramírez et al., 1996). In the present study, IL-19 and IL-1a were up-regulated with a FC > 1.5. IL-19 is a IL-10 family member expressed in monocytes, T and B lymphocytes, but its biological role is still unclear (Kunz et al., 2006). It has been evidenced that IL-19 affects the Th1/Th2 balance by inhibiting IFN-c (Th1 cytokine) and increasing Th2 cytokines production (Oral et al., 2006). These effects, may suggest a putative role of PDN in the differentiation of naïve Th cells into Th2 cells. In addition, since IL-19 does share many important characteristics with IL-10 which was also evidenced to be a stimulatory factor for mast cells, B cells, and thymocytes (Liao et al., 2004), similar effects for IL1 9 cannot be excluded.

On the other hand, there is growing evidence for intracellular functions of IL-1a, including promotion of senescence, cell growth and differentiation and regulation of gene expression (Werman et al., 2004). In particular, IL1-alpha seemed to enhance the proliferation of CD4-CD8- double negative (DN) thymocytes which are the immature precursor cells (Suda et al., 1990) but its role in mature thymocytes is still not known. It was also shown that T-cell receptor-mediated Th2 cell proliferation is dependent on endogenous production of IL-l alpha (Zubiaga et al., 1991) supporting the possible effect of PDN on T-helper differentiation into Th2 lineage.

IL17F was down-regulated by 1.7-fold. Recently, the pro-inflammatory cytokines IL1 7 and IL17F were shown to be produced by a new line of T helper cells (Th17) suggesting that they may have a regulatory role in inflammatory processes. Thymocytes were identified as a powerful source of IL1 7 and an altered IL1 7 production by thymocytes might be a key regulator in the education of adaptive immunity (Hofstetter et al., 2006).

Regulatory T cells (Tregs) and Th1 7 cells seemed to provide an effective balance between tolerance and immunity with their opposing actions (Korn et al., 2009). In concordance with previous findings, neuritin 1 (NRN1) gene was 3.2-fold up-regulated. Neuritin has been identified as being critical for the maintenance of Tregs homeostasis. It is a surface GPI-anchored molecule found to be specifically expressed by natural Tregs and anergized T cells (Barbi et al., 2010). Therefore, PDN may divert differentiation of naïve T cell from the Th1 7 lineage and on the other hand may induce Tregs differentiation in the thymus, thus confirming an anti-inflammatory rather than pro-inflammatory action.

These putative effects seemed to be confirmed also by the regulation of CD30 and of the major histocompatibility (MHC) II molecules, CD80 and CD70 observed in PDN-treated animals.In particular, CD80 was 1.6- fold down-regulated. CD28-CD80/ 86 interaction was discovered as critical to the activation of mature T cells and also have significant effects on T cell development and repertoire selection. CD86 and CD80 are responsible, at least in part, for the ability of B-1 cells to induce Th17 cell differentiation, activated B-2 cells may acquire this ability as well (Wang and Rothstein, 2012; Williams et al., 2010).

CD30 transcript was also down-regulated 1.8-fold. CD30 ligand (L)/CD30 was recently proved to play a critical role in Th17 differentiation in mice (Sun et al., 2010).

On the other hand, CD70 transcript was up-regulated in PDN-treated animals. Expression of CD70 is highly restricted and activation-dependent, as it is only trasnsiently expressed on activated T cell, B cells, and dendritic cells. CD70 expression in human lymphocytes can be enhanced by cytokines such as IL-1a, which was up-regulated in PDN-treated animals. In addition, at the cellular level, T cells chronically stimulated via CD70 produce the anti-inflammatory cytokine IL-10 (van Gisbergen et al., 2009). The CD70/CD27 axis seemed to be also involved in vitro development of Tregs as a consequence of CD27 co-stimulation, although it is still contro-versial whether CD27 plays an important role for Tregs in vivo (Nolte et al., 2009).

Based on the observed interaction between networks that include cytokines, differential co-stimulatory molecules expression and cell activities (Tables 5 and 6), microarray data showed that PDN might have a role in the regulation of immunologic homeostasis and to act on both innate and acquired components of the immunity. In particular, the results of the present study showed that PDN may direct naïve T cell differentiation mainly towards Th2 and Tregs subpopulations, which could result in the activation of immune tolerance and anti-inflammatory pathways.

While several signaling pathways were found to be represented in the list of differentially expressed genes, there was no evidence for activation of apoptotic processes at the transcriptome level. This is in agreement with results of histology, which did not evidence apoptotic bodies and ‘‘starry sky’’ appearance in the thymus of PDN-treated animals (Cannizzo et al., 2011), at variance with what is usually observed after DEX treatment (Cannizzo et al., 2010; Vascellari et al., 2012).

5. Conclusions

The present study showed that the effect of PDN on thymus gene transcription appeared relatively weak in terms of FC, with no transcript being markedly over- or under-expressed. Such marginal FC values might hinder a routine use of gene biomarkers for detection of illicit PDN treatment at least using thymus as target organ. Nevertheless, the results obtained represent a step forward compared to the lack of sensitivity reported when using standard histology. While several studies confirmed the usefulness of histological analyses as a screening tool to detect illegal DEX treatments in cattle (Biolatti et al., 2005; Cannizzo et al., 2010; Vascellari et al., 2012), the same approach was not able to identify PDN-treated animals as this compound apparently does not induce any alteration in thymus morphology, at least at low concentration (Cannizzo et al., 2011). In the same study, no PDN residues were found in the urine of treated animals confirming that when PDN is administered at low dosage, its rapid metabolism and excretion make determination of residues difficult even by LC-MS methods, especially several days after the suspension of the treatment. This observation is in contrast with the evidence from growth performance. In the present study, the administration of PDN showed a significant positive effect on final live weight and growth performances of treated animals confirming the potential of this hormone purpose a growth promoter. Its effectiveness at low doses appears to be linked to improved feed intake, increased live weight gain, reduced feed conversion ratio, reduced nitrogen retention and increased water retention. Illicit administration of PDN therefore might be extremely attractive for dishonest producers, especially in consideration of the marked increases in economic gain and the reduced risks due to the low dose and the limited sensitivity of screening methods. The transcriptional profiles of thymus, identified a number of immune pathways and functions that were modulated after PDN treatment. The principal pathways affected suggest deregulation of calcium influx, cell metabolism and T cells differentiation. We postulated that profiling genetic alterations in thymus would help identify the major immune pathways that were compromised by PDN exposure, generate valuable information regarding potential mechanisms of action, and elucidate transcriptional profiles that could contribute to identify animals illegally treated with this drug. The full mechanism of action of a chemical is multifactorial and dependent upon the tissue and conditions of exposure. However, the genomic analysis can be a useful tool for evaluating the most significant perturbations of the immune system induced by these substances and indirectly identify their use which represents a serious risk to human health.

Table 4
Spearman's rho for the set of selected genes used for qRT-PCR validation.

		Group P (n = 8)	Group K (n = 7)	P
Live weight	kg			
T0		486 ± 96	469 ± 64	ns
T1		554 ± 88	499 ± 55	ns
T2		582 ± 81	544 ± 55	ns
T3		632 ± 76	575 ± 55	ns
T3–T0		146 ± 26	106 ± 21	0.0047

ns: not significant.

Gene name	FC qRT-PCR	FC array	Spearman's rho
CALCOCO1	−1.1	−2.4	0.071
LCP1	−1.1	−1.2	0.346
LSMD1	0.9	0.8	0.175
MRPS7	−0.8	−2.2	0.059
PFDN1	1	0.9	0.382
RAG1AP1	1	0.4	0.454
ROBLD3	0.9	0.9	0.214
TIA1	1	0.8	0.121

Fold Change (FC) was calculated comparing Group P vs. Group K animals.

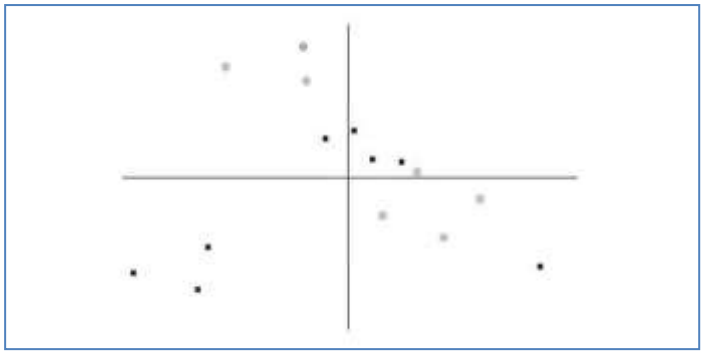

Fig. 2. PCA of the bovine thymus gene expression profiles. A PCA plot shows the two principal components of greatest variation, covering 35.8% (x-axis) and 25.1% (y-axis) of the total variance. The grey circles represent the control animals, and the black rectangles represent the PDN-treated animals.

Table 5

GO Biological Process, GO Molecular functions and KEGG pathways analysis of differentially regulated genes. DAVID functional annotation of the complete list of differentially regulated genes between group P and group K.

Category	Term	Count	*P* Value	FE	FDR
GO_BP_FAT	Ion transport	16	0.002	2.5	2.5
GO_BP_FAT	Monovalent inorganic cation transport	9	0.006	3.2	8.7
GO_BP_FAT	Cation transport	11	0.013	2.4	18.2
GO_BP_FAT	Regulation of RNA metabolic process	17	0.023	1.8	28.9
GO_BP_FAT	Metal ion transport	9	0.025	2.5	31.5
GO_BP_FAT	Adult behavior	4	0.028	6.0	34.3
GO_BP_FAT	Nucleoside triphosphate biosynthetic process	5	0.035	4.0	41.1
GO_BP_FAT	Cell surface receptor linked signal transduction	18	0.036	1.7	41.9
GO_BP_FAT	Regulation of transcription, DNA-dependent	16	0.037	1.7	43.4
GO_BP_FAT	Potassium ion transport	5	0.041	3.8	46.4
GO_MF_FAT	Ion channel activity	10	0.003	3.2	4.3
GO_MF_FAT	Substrate specific channel activity	10	0.004	3.2	4.7
GO_MF_FAT	Passive transmembrane transporter activity	10	0.004	3.1	5.4
GO_MF_FAT	Channel activity	10	0.004	3.1	5.4
GO_MF_FAT	Solute:sodium symporter activity	4	0.011	8.5	12.9
GO_MF_FAT	Solute:cation symporter activity	4	0.022	6.6	24.6
GO_MF_FAT	Ligand-gated channel activity	5	0.025	4.5	27.4
GO_MF_FAT	Ligand-gated ion channel activity	5	0.025	4.5	27.4
GO_MF_FAT	Metal ion transmembrane transporter activity	7	0.026	3.0	28.9
GO_MF_FAT	Gated channel activity	7	0.038	2.8	38.8
GO_MF_FAT	Cation channel activity	6	0.042	3.1	42.4
KEGG	Glycosaminoglycan degradation	4	0.006	10.4	6.3
KEGG	Neuroactive ligand-receptor interaction	9	0.034	2.3	31.9
KEGG	Calcium signaling pathway	7	0.037	2.8	34.4

GO, gene ontology; BP: biological process; MF, molecular function; CC, cellular component; *P* value: modified Fisher exact *P* value calculated by DAVID software; FE, fold enrichment defined as the ratio of the two proportions: input genes involved in a biological process and the background information; FDR, false discovery rates.

Table 6
List of the pro-inflammatory and anti-inflammatory mediators and immune system regulators differentially expressed between PDN-treated animals and the control ones.

Gene name	Accession number	FC
Neuritin 1 (NRN1)	NM_001046438	3.2
Rho GTPase activating protein 23 (ARHGAP23)	XM_601322	2.5
Interleukin 19 (IL19)	XM_606029	1.9
Interleukin 1 alpha (IL1alpha)	NM_174092	1.8
Na^+/K^+-ATPase beta 1polypeptide (ATP1B1)	NM_001035334	1.7
Cluster of differentiation 70 (CD70)	XM_600347	1.5
C1q and tumor necrosis factor related protein 6 (CTRP6)	XM_602976	−3.6
FC receptor-like B (FCRLB or FREB2 or FCRL2)	NM_001192565	−2.2
C1q and tumor necrosis factor related protein 8 (CTRP8)	NM_001101872	−1.9
Tumor necrosis factor receptor 8 (TNFR8 or CD30)	XM_002694054	−1.9
Guanine nucleotide binding protein (G protein), gamma 12 (GNG12)	BC142068	−1.8
Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP)	NM_001039962	−1.8
Interleukin 17F (IL17F)	NM_001192082	−1.7
Suppressor of cytokine signaling 3 (SOCS3)	NM_174466	−1.6
Cluster of differentiation 80 (CD80)	NM_001206439	−1.6
Interferon, beta 1, fibroblast (IFNB1)	NM_174350	−1.6
Myosin, light chain 7, regulatory (MYL7)	XM_585011	−1.6
Mannan-binding lectin serine peptidase 2 (MASP2)	XM_582170	−1.6

Fold change (FC) was calculated comparing Group P vs. Group K animals.

6. Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rvsc.2013.03.025.

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