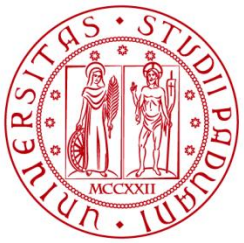

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di

BIOMEDICINA COMPARATA E ALIMENTAZIONE

Corso di dottorato di ricerca in

SCIENZE VETERINARIE

Ciclo XXIX

TRANSCRIPTOMIC APPROACHES TO STUDY THE EFFECTS OF XENOBIOTICS IN RUMINANTS

Tesi redatta con il contributo finanziario della Commissione Europea – Programma europeo ERASMUS MUNDUS Action 2 (AL FIHRI)

Coordinatore:

Ch.mo Prof. (**Alessandro ZOTTI**)

Supervisore:

Ch.mo Prof. (**Mauro DACASTO**)

Dottorando: **RAMY ELGENDY**

2017

To my parents, sister and brother..

Even if you don't fully understand what I am doing,
and probably won't read this thesis ever,
Thank you for being always proud of me.
شكراً لكل ما فعلتموه من أجلي

To all the obstacles and hardships..

You have made me stronger

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor – and friend – Prof. **Mauro Dacasto** for the continuous support of my PhD study and related research, for his guidance and motivation. His encouragement and oppeness allowed me to grow both scientifically and professionally. I couldn't have imagined having a better advisor.

Besides my advisor, I would like to thank Dr. **Mery Giantin** who taught me many things from the scratch. Without her knowledge and precious support it would not be possible to conduct this research. She has been a caring and supportive big sister to me.

I would also like to thank both Prof. **Juan Loor** and Dr. **Massimo Bionaz** for acting as external evaluators of my thesis and for providing me with valuable comments and observations.

This thesis represents not only my work in the lab or at the computer's keyboard, it is a collective representation of more than three years of team work at the Pharmacogenetics and Toxicogenomics laboratory of Prof Dacasto. That's why I want to thank my fellow labmates for everything we encountered together. Thanks to **Vanessa** who helped me in the lab during my first year of the PhD. Thanks to **Rosa**, **Eleonora**, **Roberta**, and **Silvia** for the long days (and evenings) we were working together, and for all the fun we have had in the last three years.

Also, I thank all my friends here in Agripolis. Thanks to **Federico**, **Marianna**, **Maria Elena**, **Max**, **Enrico**, **Rosella**, **Davide**,**Valentina**, **Alessandro**, **Lisa**, **Cincia**, **Giulia**, **Roberta**, **Jessica**, **Maria Laura**, **Massimo**, **Serena**, **Matteo**, and **Morgan** for all the smiles and coffees we shared together. Thanks to **Francesco** - my friend, flatmate and colleague - for all the days and nights we cycled together to and from work, and for all the nice adventures we encountered together.

I would like to thank my friends **Joy**, **Ibrahim**, **Eslam**, and **Basma** for accepting nothing less than excellence from me. Thanks to **Biljana** for believing in me and for being supportive and caring – definitely life is more colorful now.

Last but not the least, I would like to thank my **Family**; my parents, my brother and sister for supporting me spiritually throughout my life.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS iii
SUMMARY iv
1. BACKGROUND 1
1.1. Growth promoters in food-producing animals 3
1.2. Transcriptomics 7
1.3. Nutrigenomics 10
2. AIMS 13
3. REFERENCES 14
I. Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone 22
Abstract 23
Introduction 23
Materials and methods 25
Animals and experimental design 25
Sample collection and RNA extraction 26
RNA amplification, labeling and hybridization 27
Normalization of the microarray data 28
Quantitative Real time PCR 29
Statistics and Principal Component Analysis (PCA) 32
Results 33
Animal health status and growth performance 33
Confirmatory qPCR analysis 35
Clustering and principal component analyses 38
Discussion 42
Conclusions 47
Supplementary materials 47
References 48
II. The transcriptome of muscle and liver is responding differently to a combined trenbolone acetate and estradiol implant in cattle 55
Abstract 56
Introduction 56
Materials and methods 58
Animals and experimental design 58
Sample collection and RNA extraction 59
RNA amplification, labeling and hybridization 59
Normalization of microarray data and identification of DE genes 60
Functional enrichment analysis 60
Quantitative Real time RT-PCR (qPCR) validation 60
Statistics and Principal Component Analysis (PCA) 62
Results 63
Phenotypic measures 63
Microarray quality assessment and data analyses 63

Revalor-XS® modulated common genes, but no functional GO terms or pathways, in both muscle and liver 67
Differentially expressed genes can differentiate treated muscle samples, but not liver, from the control ones 69
Discussion 69
Conclusions 74
Supplementary materials 74
References 75
III. Transcriptomic Characterization Of Bovine Primary Cultured Hepatocytes and Madin-Darby Bovine Kidney (MDBK) Cell Line 79
Abstract 80
Introduction 80
Materials and methods 82
Primary hepatocytes isolation, cell cultures and liver tissue samples 82
Cell Harvesting and RNA Isolation 83
Microarray Hybridization And Data Analysis 84
Cross-Validation by Quantitative Real-Time PCR (qPCR) 85
Functional Annotation and Statistical Analysis 87
Results 88
Microarray Analysis 88
qPCR cross-validation 91
Functional Annotation and Pathway analysis 93
Discussion 99
Conclusions 102
Supplementary materials 102
References 103
IV. Transcriptomic signature of high dietary organic selenium supplementation in sheep 107
Abstract 108
Introduction 109
Materials and methods 110
Animals and Study Design 110
Blood Collection and Se Analysis 111
Microarray Custom Design and Annotation 112
RNA Isolation and Microarray Analysis 112
Quantitative Real-Time PCR 114
Functional Annotation and Gene Set Enrichment Analysis 117
Statistical Analysis 117
Results 118
Discussion 128
Conclusions 132
Supplementary materials 132
References 133
4. GENERAL CONCLUSIONS AND PERSPECTIVES 139
SCIENTIFIC COMMUNICATION 141
BIOSKETCH 143

LIST OF ABBREVIATIONS

ADF Acid Detergent Fiber
ADG Average daily gain
ADL Acid detergent lignin
AR Adrenergic receptor
BP Biological process
bp Base pair
BW Body weight
CAR Constitutive androstane receptor
CC Cellular process
cDNA Complementary DNA
CHs Cultures hepatocytes
CLEN Clenbuterol
CP Crude protein
cRNA Complementary RNA
Cy3 Cyanine 3
CYPs Cytochrome P450
d day/days
DAVID Database for Annotation, Visualization and Integrated Discovery
DEGs Differentially expressed genes
DEX Dexamethasone
DIM Days in milk
DM Dry matter
DTs Drug transporters
E2 Estradiol
ES Enrichment score
EST Estimated sequence tag
EU European union
FC Fold change
FCI Feed conversion index
FCS Fetal calf serum
FDA Food and drug administration
FDR False discovery rate
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GEO Gene expression omnibus
GO Gene ontology
GPs Growth promoters
GPX Glutathione peroxidase
GSEA Gene set enrichment analysis
ICP-MS Ionized coupled plasma mass spectrometer
IPA Ingenuity pathway analysis
KEGG Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS Liquid chromatography–mass spectrometry
LXR Liver X receptor
MDBK Madin-Darby bovine kidney
MF Molecular function
MTs Methyl transferases
NDF Neutral detergent fiber
NE_g Net energy for gain
NRs Nuclear receptors
PCA Principal component analysis
PPAR Peroxisome proliferator-activated receptor
PXR Pregnane X receptor
RIN RNA integrity number
RPLP0 Ribosomal protein lateral stalk subunit P0
RQ Relative quantification
RXR Retinoid X receptor
SAM Significance analysis of microarray
Se Selenium
SLCs Solute carriers
STs Sulfotransferases
TBA Trenbolone acetate
TBP TATA-box binding protein
MEV Multiple Experiment Viewer
UPL Universal probeLibrary

SUMMARY

This thesis is concerned with the study and characterization of the xenobiotics-induced transcriptomic signature in some ruminants. Based on the different studies presented in this thesis, the microarray-based transcriptomics approach was able to provide a holistic view on the global gene expression in diverse types of tissues – namely, skeletal muscle, liver, whole blood, primary hepatocytes- and kidney-derived cell lines. The pre-designed commercial bovine microarray enabled the discovery of many biomarkers with which the differentiation between illicitly-treated and untreated veal calves was possible. It also demonstrated the transcriptomic signature dissimilarity between 2 tissues (i.e. skeletal muscle and liver) exposed to the same treatment (i.e. anabolic steroids). Also, the same approach revealed the presence of some transcriptomic landscape convergence between the hepatocytes primary cultures and the Madin-Darby bovine kidney (MDBK) cell line, which in turn spots the light on the MDBK cells as a possible surrogate *in vitro* tool for some liver-based functional studies. Finally, a custom-designed whole-transcriptome sheep (*Ovis aries*) microarray revealed the immune-system-induction and the transcriptional-modulation capacity of organic selenium in sheep. Collectively, the transcriptomics approach overcame the shortcoming of focusing on changes in expression of a *priori* list of selected genes – instead, it looks at the bigger picture within the protein-coding part of the genome. It is important to mention that using an alternative functional analysis tools [i.e. Gene set enrichment analysis (GSEA)] was useful to cross-validate the output of the conventional overrepresentation tools like the Database for Annotation, Visualization and Integrated Discovery (DAVID). The collective body of work represented here shows the adequacy of using microarray, commercial and custom-designed, to depict a holistic picture about the global gene expression profile of a given tissue. Still, there are some challenges in data analysis, interpretation and integration with the output of other alternative omic techniques – those challenges are highlighted and discussed across the different chapters of this thesis.

1. BACKGROUND

Agricultural science and technology is one of the world's fastest growing and exciting sectors within the global marketplace. This is because the world food economy is being increasingly driven by the shift of diets towards animal-based products such as meat, milk and dairy (Webb and Buratini, 2016). To fulfill the high demand on these animal proteins, millions of livestock are being produced every year (**Table 1**). To maintain a healthy status for this big number of animals – thus ensuring that food is harmless- and to protect such a strong and profit-oriented industry, it is quite practically impossible to keep those animals in a pharmaceuticals-free environment. In another word, using drugs or feed supplements in the livestock is mostly inevitable - they are considered by the food-animal producers as fundamental to the animal's health and well-being and for the economy of the agribusiness.

Mostly all the pharmacologically-active compounds that are administered to animals are generically being called 'xenobiotics'. Food-producing animals - representing a readily available source of food for humans- are exposed to xenobiotics in multiple ways which could be present in their products. Xenobiotics in an organism go through a series of stages, including absorption, distribution, metabolism and excretion, forming part of the pharmacokinetics or toxicokinetics according to the effects produced by a particular substance (pharmacological or toxicological). Xenobiotics enter a food-producing animal organism and, according to its kinetics, reach the tissues which will become food for human beings. Collectively, all the pharmaceuticals (both of natural origin or chemically synthesized) administered to food animals could be considered as xenobiotics.

Table 1. Live animal production (ruminants) in 2013 - thousand heads;

	2000	2013
Cattle	**1 302 895**	**1 494 349**
Sheep	**1 059 082**	**1 172 833**
Goats	**751 632**	**1 005 603**
Buffaloes	**164 114**	**199 784**

Food and Agriculture Organization (FAO). Statistical Pocketbook. Rome: FAO, 2015. Available on: www.fao.org/3/a-i4691e.pdf [Retrieved: October 24, 2016]

The reported benefits of using pharmaceuticals (i.e. xenobiotics) in farm animals are mainly derived from keeping the animals in a good health, thereby reducing the possibility of a disease becoming transmitted from animals to humans, and furthermore ensuring a high quality food product (Lozano and Trujillo, 2012). Still, with these benefits come some challenges that need to be addressed and questions to be answered, such as (1) What are the possible direct and indirect effects of hundreds of pharmaceutical compounds on both the animal and human health?, (2) What regulates – and ensures- the correct usage of those compounds?, (3) Are the conventional methods for veterinary drug monitoring and screening still valid or other methods should be implemented?

In principle, all pharmaceutical preparations administered to food-producing animals – after achieving their desirable pharmacological effect- can give rise to residues in edible tissue, milk or eggs, and therefore reach humans (McEvoy, 2002). In spite of most drugs representing a relatively low risk to the general public, when used responsibly and in line with instructions approved by the laboratories making veterinary drugs, adverse reactions have been frequently reported for some compounds; these would include antibacterial drugs and growth promoters (Lozano and Trujillo, 2012). Another concern is that not all of these pharmaceuticals are being administered to animals for their original pharmacological purposes, therefore some of them fall under the category of 'illicit' drugs.

Any discussion regarding illicit drugs can become confused by the ambiguity as to what exactly defines an illicit drug. The confusion stems from the fact that illicit drugs are not limited exclusively to illegal drugs – that is, drugs with no medical use. Illicit drugs can include active ingredients from *bona fid*e registered pharmaceuticals having valuable therapeutic uses – like some antibacterials. They can also include active ingredients that are banned from all use under various international conventions or national law, as they are deemed as having a public health concern – like growth promoters in the European Union (EU). Whether a drug is illicit (or illegal) can be dictated by a number of different characteristics, including the chemical structure of the active ingredient or the way in which the drug is manufactured, formulated, labeled, distributed, acquired, or used (Daughton, 2011).

To cope with the advancements in the food animals' industry, and to address the public health concerns about the exposure of animals to various pharmaceuticals, a huge amount

of scientific contribution has been made, which in turn have led to different legislations, regulations and laws. On the one hand, a number of both *in vitro* and *in vivo* experimental models were established to examine the kinetics of the different xenobiotics administered to food-producing animals. On the other hand, several screening techniques were developed to detect the presence (or the absence) of certain xenobiotics in the tissues (usually liver or muscle) or the biological fluids (i.e. blood, urine or milk) of treated animals.

There is a big variety of methods for identifying, confirming and quantifying drug traces (i.e. analytes) which could be used individually or coupled to each other in a suitable way. Some of them are 'immunochemical assays' such as the enzyme-linked immunosorbent assay (ELISA), direct and indirect competitive enzyme linked immunosorbent assays, immunoaffinity chromatography (IAC), radioimmunoassay (RIA), the enzyme-monitored immunotest (EMIT), the fluorescent immunoassay (FIA) and the chemiluminescence immunoassay. Alternatively, they can be 'physico-chemical assays' like gas chromatography (GC), high performance liquid chromatography (HPLC), ionic chromatography (IC), mass spectrometry (MS), mass spectrometry in tandem (MS/MS), or a coupled HPLC-MS (Mastovska, 2011; Lozano and Trujillo, 2012).

1.1. Growth promoters in food-producing animals

Growth promoters are substances which produce improvements in growth rate when added to animal feed in sub-therapeutic dosages over an extended period of time. A wide spectrum of pharmaceuticals is known or suspected of being used in livestock as growth promoters, and an extensive literature exists on this subject, but the practice differs greatly among countries (Daughton, 2011). Some of these drugs are also abused by humans, so they can serve as another source contributing to environmental residue levels; others are unique to veterinary practice. Among the drugs in use, many may be registered for veterinary use but not for the purposes actually employed. Others may not be approved for any purpose. Included are hormones (corticosteroids, anabolic steroids, and thyreostats such as the thiouracils), β-agonists (e.g., clenbuterol), and some antibacterials (Courtheyn et al., 2002; Stolker et al., 2007).

Using these compounds, either natural or synthetic, as growth promoters in food-producing animals is not allowed in the EU, due to potential adverse effects to human health, unlike in the United States where some anabolic hormonal-type growth promoters

are still permitted. That's why foods of animal origin are being monitored for the presence of veterinary drug residues in the EU. The European Food Safety Authority (EFSA) – likewise, the US Department of Agriculture's (USDA) in the USA- is the keystone of the EU's risk assessment regarding food and animal feed safety. Based on the scientific opinion of EFSA, the EU took some measures to ban the use of growth promoters (mainly of hormonal origin) in food-producing animals. In 1981, with Directive 81/602/EEC (European Economic Community, 1981), the EU prohibited the use of substances having a hormonal action for growth promotion in farm animals. Examples of these kind of growth promoters are oestradiol 17ß, testosterone, progesterone, zeranol, trenbolone acetate and melengestrol acetate. This prohibition applies to Member States and imports from third countries alike. The legal instrument in force is Directive 96/22/EC (European Commission, 1996) which has been amended later by the Directive 2003/74/EC (European Commission, 2003) to broaden the scope of this ban to also include any substance that has a hormonal or thyrostatic action, as well as beta-agonists.

Due to the continuation to use some of the banned items by some of the food animal farmers – with a growing 'black market' for these growth promoters, as well as the emergence of new growth promoter cocktails (i.e. new combinations with lower dosages), the scientific community had to find new alternatives to the conventional screening methods. Consequently, research groups started to investigate the presence of the illicit drug traces in other tissues away from the conventional ones (i.e. blood, urine and milk), which in turn led to the implementation of the molecular 'biomarkers' approach.

The identification of molecular biomarkers to distinguish physiological conditions or clinical stages is an emerging research field that has grown substantially during the last years. The main fields in which molecular biomarker research is performed are clinical diagnostics, risk assessment, and therapeutic areas, but also in other fields like food safety, where the request for molecular biomarkers has come into focus (Riedmaier et al., 2009; Divari et al., 2011; Riedmaier and Pfaffl, 2013). Molecular biomarkers can be identified on different molecular levels, namely the genome, the epigenome, the transcriptome, the proteome, the metabolome and the lipidome. With special "omic" technologies these molecular biomarkers can be identified and quantitatively measured (Riedmaier and Pfaffl, 2013).

The corticosteroid dexamethasone (DEX) is licensed for therapy in veterinary medicine, and is frequently used in livestock production. Although its original purpose is to be used as an anti-inflammatory and immunosuppressive agent, it was found to be illegally used as a growth promoter in animal husbandry - usually at low dosages either alone or in cocktails containing different anabolic agents (Gottardo et al., 2008). Because of its public health concern, the EU has established a maximum residue limit for DEX – and other fellow corticosteroids - in several biological matrices, such as muscle, kidney, liver and milk from different species. A substantial number of research work has been performed in that field. The thymus was one of the alternative tissue matrices used to control the illegal use of corticosteroids in veal calves and beef cattle, and it has been the subject to several research works (Biolatti et al., 2005; Cannizzo et al., 2008; Cannizzo et al., 2010; Cannizzo et al., 2011; Vascellari et al., 2012). Others have investigated the changes in gene expression in the testis (Lopparelli et al., 2010; Lopparelli et al., 2011), neutrophils and lymphocytes (Lopparelli et al., 2012), liver (Giantin et al., 2010), or skeletal muscle (Carraro et al., 2009) of DEX-treated cattle. More recently, proteomic (Stella et al., 2011; Guglielmetti et al., 2014; Stella et al., 2016) and transcriptomic (Pegolo et al., 2012) biomarkers were proposed to depict a molecular signature behind the illicit use of DEX in food animals.

Another banned growth promoter in the EU is the beta-agonist clenbuterol (CLEN). This β_2-agonist compound elicits its growth-promoting effect by enhancing protein synthesis and cell hypertrophy, which is known as the 'repartitioning effect' (Baker et al., 1984). These effects may result in a reduction of carcass fat by up to 40% and an increase of carcass protein content by up to 40%, yielding a consistent advantage for the meat producers (Courtheyn et al., 2002). Because of a possible synergetic effect when combined at lower doses with other molecules such as corticosteroids, the cocktail DEX-CLEN has gained the attention of many research groups. This attention was combined with a controversy regarding whether to accept or prohibit using CLEN in animal production. It was not considered as potentially oncogenic or mutagenic – still, it was embryotoxic in large doses whilst its adverse effects on consumers became evident when recommended withdrawal times were not respected and when excessive doses were used (Brambilla et al., 2007). That was quite enough for the EU to develop programs and mechanisms for the monitoring of CLEN and its fellow growth promoters. In this context, Odore and colleagues (Odore et al., 2006, 2007) have investigated the effects induced by

a treatment with CLEN, 17*β*-oestradiol and DEX, similar to those illegally performed in the field, on the regulation of glucocorticoid, beta- and androgenic receptors in veal calves. Also, Cantiello and colleagues have examined the effect of CLEN-containing cocktails on the serum immunoglobulins and lymphocytes proliferation (Cantiello et al., 2007), the cytochrome P450 inhibition (Cantiello et al., 2008) - as well as its expression profile (Cantiello et al., 2009) - in veal calves. Recently, a transcriptomic biomarker signature was proposed to detect the abuse of growth promoters in the liver of veal calves (Riedmaier et al., 2014).

The use of anabolic steroids began in the beef cattle industry over 50 years ago to improve efficiency, and approximately 90% of all feedlot cattle receive at least 1 implant throughout their lives (APHIS, 2013). Currently, more than 30 implants are approved for use in beef cattle in the United States (FDA, 2013). In the EU it is quite different from that in the US since all the anabolic steroids have been banned in animal production for over 25 years. At the time of the initial imposition of the ban, there were only five licensed hormonal growth promoters (the naturally occurring 17*β*-estradiol, testosterone and progesterone, and two synthetic hormones, trenbolone and zeranol). These hormones were all administered by means of pellets, which were injected into the ears of recipient animals (Mooney et al., 2009). On a monitoring-and-drug-abuse-discovery basis, some studies have been performed for either the direct or the indirect detection of anabolic steroids in cattle in Europe, in which investigations on transcriptomic profiling of thymus (Cannizzo et al., 2013), uterine endometrium (Becker et al., 2011), liver (Becker et al., 2010), vaginal smears (Riedmaier et al., 2011) and muscle (De Jager et al., 2011; Pegolo et al., 2014) have been conducted. In the same context, other groups have investigated the use of alternative ‘‘omic” techniques, such as proteomics (McGrath et al., 2013; Kinkead et al., 2015; Stella et al., 2015) and metabolomics (Graham et al., 2012; Jacob et al., 2014; Kouassi Nzoughet et al., 2015).

The full procedure and the methodologies for confirmatory analysis are costly in time, equipments and chemicals. In addition, they require trained personnel with high expertise. Control laboratories must face a large number of samples, with a variety of analytes, to be analyzed in relatively short periods of time. Thus, there is a need for screening methods that allow the analysis of such a large number of samples in short periods of time. This means that high-throughput methods with low cost must be available (Toldrá and Reig, 2006; Reig and Toldrá, 2008).

The fact that the traces of a drug molecule are frequently present in very low concentrations, and that some practices use ‘‘cocktails’’ (mixtures of low amounts of several substances that exert a synergistic effect) that are difficult to quantify, represents an important challenge for the conventional analytical detection (Reig and Toldrá, 2008; Cannizzo et al., 2013). Accordingly, it was necessary to implement other methods that can either overcome the shortcomings of the analytical methods or simply change the strategy from a ‘physico-chemical’ towards a more holistic ‘systems biology’ approach. Providentially, the advent and the integration of the high-throughput ‘omic’ technologies (e.g., genomics, transcriptomics, proteomics, metabolomics and lipidomics) has revolutionized the way biology is done, allowing the systems biology of an organisms to be elucidated (Cantacessi et al., 2012). These technologies started to be widely implemented in the veterinary science.

1.2. Transcriptomics

The “transcriptome” is defined as the complete set of mRNA molecules (or transcripts, which represents a template for protein synthesis) present in a cell or tissue at a given time or condition. It represents the link between the genotype and the phenotype, and it is essential to understand how a biological system works in both a physiological or a pathological context (Wang et al., 2009). High-throughput genomic technologies, such as high-density microarrays and sequencing-based tools [i.e. massively parallel mRNA sequencing (mRNA-seq)], provide whole genome approaches to address biological questions (Rinaldi et al., 2010). The advent of microarrays as a tool for a global gene expression analysis (Schena et al., 1995) has paved the way for the transcriptomics era to prevail. More than 10 years later, this advancement was followed by the revolutionary RNA sequencing (RNA-seq) (Wang et al., 2009) that is becoming now the gold-standard for transcriptomic studies. Although the so called ‘the death of microarrays’ – because of the advent of the high-throughput gene sequencing technologies - was questioned some years ago (Ledford, 2008), the microarray- and the RNA-seq-based studies are both growing at the same pace **(Figure 1).**

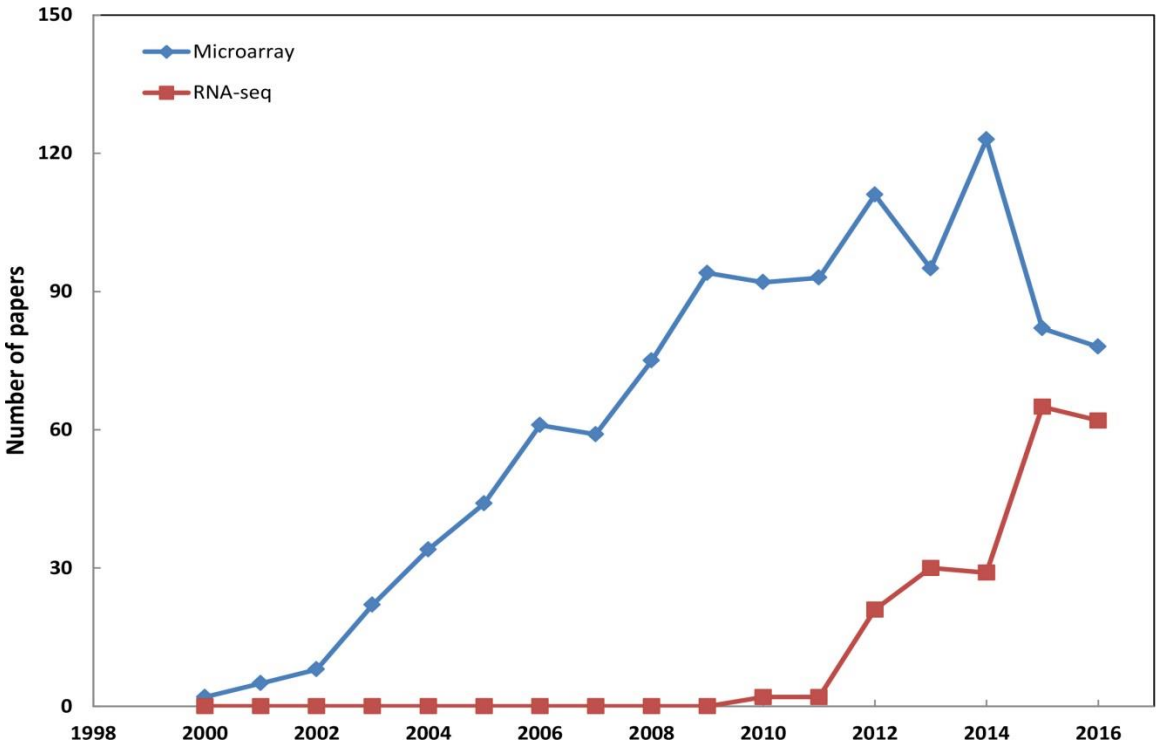

Figure 1: *Number of scientific papers found in PubMed using microarray or RNA-seq approaches to study the bovine and ovine species. The search was performed using the key words "microarray [Title/abstract] AND (bovine or cow or ovine or sheep) NOT review"; and "(RNAseq [Title/abstract] OR RNA-seq [Title/abstract]) AND (bovine or cow or ovine or sheep) NOT review." The search was performed on October 24, 2016. The idea of the figure is inspired by Loor et al. (2013).*

A DNA microarray, sometimes called DNA chip, is a technology that involves the immobilization of DNA fragments or oligonucleotides of known sequences (probes) on a solid support – usually a spotted (arrayed) glass slide - allowing the profiling of thousands of genes or interactions in one single experiment. The word 'micro' is usually used to, for practical reasons, distinguish, for practical reasons, between microarrays and macro-arrays, whereas typical spot sizes are 'more than 300' and 'less than 200' microns for the macro- and microarray, respectively (Bier et al., 2008). The arrayed probes can represent either the whole transcriptome or a selected (customized) panel of target genes or coding regions. The "target" is the free nucleic acid sample, usually labelled by a fluorescent dye during the preparation process, which interacts with the probe by hybridization (Bier et al., 2008).

Large-scale bovine microarrays started with ~3,800 cDNA probes (Band et al., 2002), then it scaled up to contain ~7,000 (Everts et al., 2005) and 13,000 oligonucleotide sequences (Loor et al., 2007). Later on, the commercial microarrays such as the

'Affymetrix Bovine GeneChip' and the 'Agilent Bovine Gene Expression Microarray' provided a 'somehow complete' coverage of the bovine transcriptome, with more than 23,000 transcripts covered.

A typical microarray experiment produces lots of data. That's why a good experimental design that allows unambiguous interpretation of results and reporting of data for a genome-wide comparability is required (Spielbauer and Stahl, 2005). In 2001, the Functional Genomics Data Society (FGED) created for this purpose the Minimum Information About a Microarray Experiment (MIAME) guidelines, which provide all the necessary information required to potentially reproduce a robust microarray experiment (Brazma et al., 2001; Brazma, 2009). Following the compliance with the MIAME guidelines, and to allow reproducibility, the microarray data has to be deposited into a publicly accessible repository, such as the Gene Expression Omnibus (GEO; Edgar et al., 2002) and the ArrayExpress archive (Parkinson et al., 2010). The MicroArray Quality Control (MAQC) project - now is known as the MicroArray/Sequencing Quality Control (MAQC/SEQC) - was initiated to address the concerns about studies with dissimilar or altogether contradictory results, obtained using different microarray platforms to analyze identical RNA samples (Shi et al., 2006). This project demonstrated the intra-platform consistency across test sites as well as a high level of inter-platform concordance in terms of genes identified as differentially expressed.

Microarray still has some shortcomings such as the hybridization-based limitations associated with background noise and saturation or probe set issues such as incorrect annotation and isoform coverage (Zhao et al., 2014). On the other hand, the more recent RNA-seq technology overcomes the challenges posed by microarray. It is superior in detecting low abundance transcripts, differentiating biologically critical isoforms, allowing the identification of genetic variants, and having a broader dynamic range than microarray (Zhao et al., 2014). It also allowed the understanding of transcription initiation sites, the cataloguing of sense and antisense transcripts, improving the detection of alternative splicing events, and detection of gene fusion (reviewed in Loor et al., 2015). Still, there is a considerable amount of microarray-based scientific literature that is keep growing (see **Figure 1**). One could say that microarrays remain useful and accurate tools for measuring expression levels, and RNA-seq complements and extends microarray measurements (Malone and Oliver, 2011).

An the present time it is not the matter of generating the omics data but how these data will be handled, analyzed and interpreted at the end. A big challenge is to interpret the findings of an omics-based study in a more biologically meaningful fashion and eventually communicate biological knowledge systematically (Loor et al., 2013). If data are not properly processed and analyzed, the existence of massive data sets certainly is not a guarantee that useful information in a given system will be obtained (Loor et al., 2015). Another challenge is that the use of any one of the omics tools alone cannot provide a full picture of the biological system and thus can be misleading when interpreting biological outcomes (Loor et al., 2013). That's why omics data integration (e.g. integrative analysis of transcriptomics plus proteomics data) is being now considered fundamental in both human and animal (Vogel and Marcotte, 2012; Haider and Pal, 2013; Shahzad et al., 2014; Loor et al., 2015; Ritchie et al., 2015)

1.3. Nutrigenomics

Modern research aims to understand the genome-wide influence not only for pharmaceuticals but also for nutrients. Studying how nutrients can alter the expression and/or the structure of an individual's genetic makeup is known as nutritional genomics or more simply 'nutrigenomics' (Kaput and Rodriguez, 2004; reviewed in Bionaz et al., 2015). Still, nutrigenomics should not be understood as the effect of nutrients on the sequence of DNA; rather, it should be interpreted as the nutrient–gene interactions through the intermediate action of the transcriptional regulatory or the epigenetic factors in the short to medium and medium to long term, respectively (Bionaz et al., 2015). Nutrigenomics is actually addressed to clarify the molecular activity of bioactive and non-nutrient compounds affecting transcription factors (transcriptomics), protein expression (proteomics), metabolite production (metabolomics) and even DNA structure (epigenomics) (De Godoy and Swanson, 2013). Nutrigenomics aims (1) to understand the cellular functions of nutrients and other bioactive components and how they affect homeostasis in specific tissues or in the whole organism, (2) to relate the different phenotypes to differences in the cellular and/or genetic response of the biological system, (3) to identify genes that are involved in the onset of the disease, and therefore, molecular biomarkers and, consequently, (4) to prevent diet-related diseases (Fenech et al., 2011).

According to the major activity of nutrients on transcription factors, the analysis of changes in mRNA expression is often the first step to study the flow of molecular information from the genome and one of the main goals of nutrigenomics research. For years, gene expression has been determined by quantification of mRNA with northern blotting but, gradually, it has been replaced by more sensitive techniques such as real-time PCR. Both techniques, however, can only analyze a limited number of candidate genes at a time. This is an important limitation for their application in nutrigenomics research since the analysis of a reduced number of genes may not provide a true insight about relationship between the bioactive food constituent and its biological effect (García-Cañas et al., 2010). The high-throughput technologies such as NGS and microarray overcome this limitation by their ability to profile the whole-transcriptome of a given biological matrix at once, that's why they are widely adopted now in nutrigenomics.

In ruminants, nutrigenomics is a recent field of research that holds great potential to improve animals' health and productivity (Bionaz et al., 2015). In the last decade, microarray technology has been extensively utilized in livestock species as a nutrigenomic research tool to improve food production, quality and their safety in dairy and meat industries (Zduńczyk and Pareek, 2009; Bionaz et al., 2015). The initial data from nutrigenomics studies in ruminants strongly indicate that this branch of science will play a critical role in future strategies to better feed livestock such as the dairy cattle (Bionaz et al., 2015). An overview of the microarray technology applications in nutrigenomics is depicted in **Figure 2**.

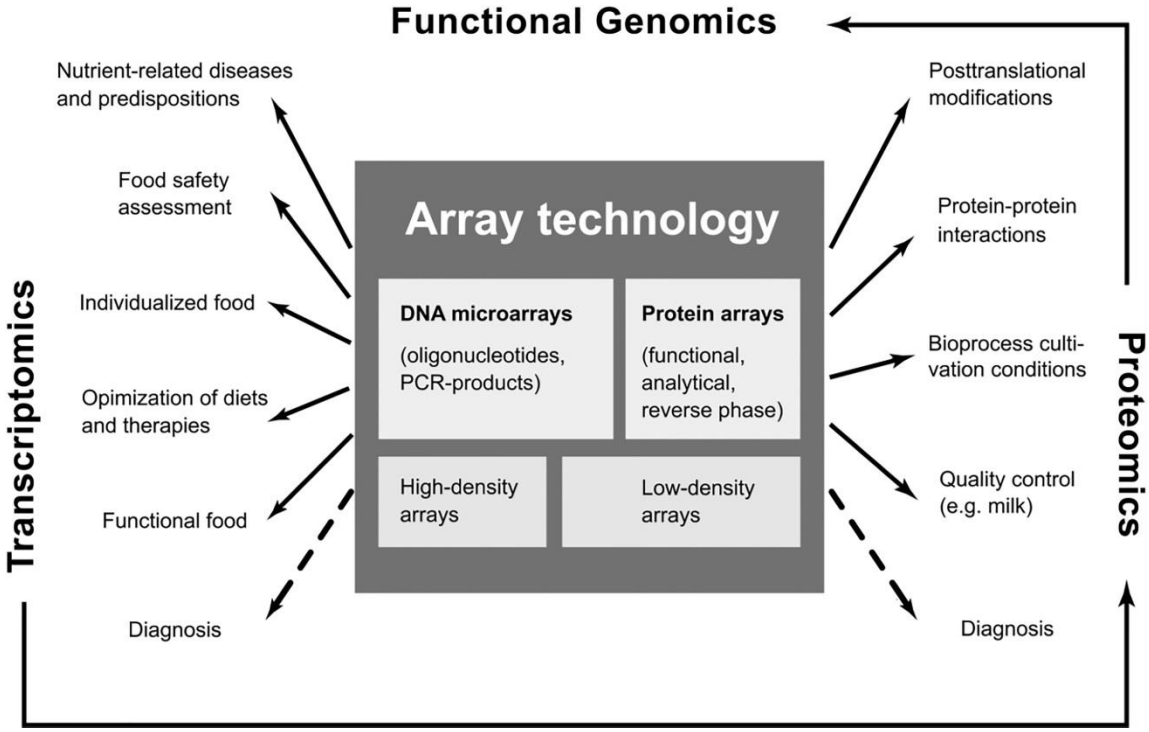

Figure 2: *Schematic overview of potential applications of biochips in nutrition and food research. The array technology plays a key role in the scientific analysis of the interactions between nutrients and genes. The influence of nutrition on the global gene expression profile can be analyzed by using high-density, whole-genome DNA microarrays. Figure from Spielbauer and Stahl (2005).*

2. AIMS

The main objective of this thesis was to study and characterize the transcriptomic profile of some ruminants – bovine and ovine species – following their exposure to different xenobiotics , in the form of illegal schedule or regular feed supplementation. More in-depth, the specific objectives were to:

1- Examine the changes in the skeletal muscle's (i.e. meat) transcriptome of beef cattle following the experimental exposure to an illicit protocol of either dexamethasone alone, or in combination with clenbuterol. Then, to identify a set of differentially expressed genes that could be used as indirect biomarkers for growth promoters treatment abuse. And, finally, to compare the results with transcriptomics data from dexamethasone-positive field monitoring samples as well as proteomics data previously obtained from the same set of samples.

2- Study the effect of a combined trenbolone acetate (TBA) and estradiol (E2) implant on the transcriptome of muscle (target tissue for anabolic steroids) and liver (main biotransformation site). A specific objective was to point out the main differences and similarities (if any) between both tissue matrices, and to list out the main differentially expressed genes that can be used as a ''pool" for anabolic steroids biomarker discovery in the future.

3- Characterize the transcriptome of the Madin-Darby bovine kidney (MDBK) cell line and compare it with that of the cattle hepatocyte primary cultures and the *ex vivo* hepatic tissue.

4- Investigate the effect of dietary Selenium on the whole-transcriptome of sheep (*Ovis aries*), and to test whether or not a high dietary selenium supplementation would induce genes of the immune system of sheep and leave a noticeable molecular signature behind.

3. REFERENCES

Animal and Plant Health Inspection Service (APHIS). 2013. The use of growth-promoting implants in U.S. feedlots. USDA-APHIS Veterinary Services, Fort Collins, CO.

Baker, P. K., R. H. Dalrymple, D. L. Ingle, and C. A. Ricks. 1984. Use of a-adrenergic agonist to alter muscle and fat deposition in lambs. J. Anim. Sci. 59:1256–1261.

Band, M. R., C. Olmstead, R. E. Everts, Z. L. Liu, and H. A. Lewin. 2002. A 3800 gene microarray for cattle functional genomics: comparison of gene expression in spleen, placenta, and brain. Anim. Biotechnol. 13:163–172.

Becker, C., I. Riedmaier, M. Reiter, A. Tichopad, M. W. Pfaffl, and H. H. Meyer. 2010. Effect of trenbolone acetate plus estradiol on transcriptional regulation of metabolism pathways in bovine liver. Horm Mol Biol Clin Investig 2:257–265. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25961197

Becker, C., I. Riedmaier, M. Reiter, a. Tichopad, M. J. Groot, a. a M. Stolker, M. W. Pfaffl, M. F. W. Nielen, and H. H. D. Meyer. 2011. Influence of anabolic combinations of an androgen plus an estrogen on biochemical pathways in bovine uterine endometrium and ovary. J. Steroid Biochem. Mol. Biol. 125:192–201. Available from: http://dx.doi.org/10.1016/j.jsbmb.2011.01.007

Bier, F. F., M. von Nickisch-Rosenegk, E. Ehrentreich-Förster, E. Reiss, J. Henkel, R. Strehlow, and D. Andresen. 2008. DNA microarrays. Adv. Biochem. Eng. Biotechnol. 109:433–453. Available from: papers2://publication/doi/10.1007/10_2007_087

Biolatti, B., E. Bollo, F. T. Cannizzo, G. Zancanaro, M. Tarantola, M. Dacasto, M. Cantiello, M. Carletti, P. G. Biolatti, and G. Barbarino. 2005. Effects of Low-dose Dexamethasone on Thymus Morphology and Immunological Parameters in Veal Calves. J. Vet. Med. Ser. A 52:202–208.

Bionaz, M., J. Osorio, and J. J. Loor. 2015. Triennial Lactation Symposium: Nutrigenomics in dairy cows: nutrients, transcription factors, and techniques. J. Anim. Sci. 93:5531–5553.

Brambilla, G., S. di Bez, D. Pietraforte, M. Minetti, L. Campanella, and A. Loizzo. 2007. Ex vivo formation of gastric metabolites of clenbuterol: Preliminary characterisation of their chemical structure. Anal. Chim. Acta 586:426–431.

Brazma, A., P. Hingamp, J. Quackenbush, G. Sherlock, P. Spellman, C. Stoeckert, J. Aach, W. Ansorge, C. A. Ball, and H. C. Causton. 2001. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nat. Genet. 29:365–371.

Brazma, A. 2009. Minimum information about a microarray experiment (MIAME)–successes, failures, challenges. Sci. World J. 9:420–423.

Cannizzo, F. T., B. Miniscalco, F. Riondato, E. Bollo, G. Barbarino, P. Giorgi, C. Mazzini, and B. Biolatti. 2008. Effeds of anabolic and therapeutic doses of dexamethasone on thymus morphology and apoptosis in veal calves. Vet. Rec. J. Br. Vet. Assoc. 163.

Cannizzo, F. T., F. Spada, R. Benevelli, C. Nebbia, P. Giorgi, N. Brina, E. Bollo, and B. Biolatti. 2010. Thymus atrophy and regeneration following dexamethasone administration to beef cattle. Vet. Rec. 167:338–343.

Cannizzo, F. T., P. Capra, S. Divari, V. Ciccotelli, B. Biolatti, and M. Vincenti. 2011. Effects of low-dose dexamethasone and prednisolone long term administration in beef calf: Chemical and morphological investigation. Anal. Chim. Acta 700:95–104.

Cannizzo, F. T., S. Pegolo, L. Starvaggi Cucuzza, L. Bargelloni, S. Divari, R. Franch, M. Castagnaro, and B. Biolatti. 2013. Gene expression profiling of thymus in beef cattle treated with prednisolone. Res. Vet. Sci. 95:540–547. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23642483

Cantacessi, C., B. E. Campbell, and R. B. Gasser. 2012. Key strongylid nematodes of animals - Impact of next-generation transcriptomics on systems biology and biotechnology. Biotechnol. Adv. 30:469–488. Available from: http://dx.doi.org/10.1016/j.biotechadv.2011.08.016

Cantiello, M., M. Carletti, F. T. Cannizzo, C. Nebbia, C. Bellino, S. Pié, I. P. Oswald, E. Bollo, and M. Dacasto. 2007. Effects of an illicit cocktail on serum immunoglobulins, lymphocyte proliferation and cytokine gene expression in the veal calf. Toxicology 242:39–51.

Cantiello, M., M. Carletti, M. Dacasto, P. G. P. Martin, T. Pineau, F. Capolongo, G. Gardini, and C. Nebbia. 2008. Cytochrome P450 inhibition profile in liver of veal calves administered a combination of 17beta-estradiol, clenbuterol, and dexamethasone for growth-promoting purposes. Food Chem. Toxicol. 46:2849–55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18602204

Cantiello, M., M. Giantin, M. Carletti, R. M. Lopparelli, F. Capolongo, F. Lasserre, E. Bollo, C. Nebbia, P. G. P. Martin, T. Pineau, and M. Dacasto. 2009. Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. Biochem. Pharmacol. 77:451–463. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19022227

Carraro, L., S. Ferraresso, B. Cardazzo, C. Romualdi, C. Montesissa, F. Gottardo, T. Patarnello, M. Castagnaro, and L. Bargelloni. 2009. Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters. Physiol. Genomics 38:138–148.

Courtheyn, D., B. Le Bizec, G. Brambilla, H. F. De Brabander, E. Cobbaert, M. Van de Wiele, J. Vercammen, and K. De Wasch. 2002. Recent developments in the use and abuse of growth promoters. Pap. Present. 4th Int. Symp. Horm. Vet. Drug Residue Anal. Antwerp,Belgium,4-7 June 2002. Part I 473:71–82. Available from: http://www.sciencedirect.com/science/article/pii/S0003267002007535

Daughton, C. G. 2011. Illicit drugs: contaminants in the environment and utility in forensic epidemiology. In: Reviews of Environmental Contamination and Toxicology Volume 210. Springer. p. 59–110.

De Godoy, M. R. C., and K. S. Swanson. 2013. COMPANION ANIMALS SYMPOSIUM: Nutrigenomics: Using gene expression and molecular biology data to understand pet obesity. J. Anim. Sci. 91:2949–2964.

De Jager, N., N. J. Hudson, A. Reverter, Y.-H. Wang, S. H. Nagaraj, L. M. Cafe, P. L. Greenwood, R. T. Barnard, K. P. Kongsuwan, and B. P. Dalrymple. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fiftyfold increase in circulating oxytocin in cattle. Physiol. Genomics 43:467–478.

Divari, S., F. T. Cannizzo, F. Uslenghi, P. Pregel, C. Mulasso, F. Spada, R. De Maria, and B. Biolatti. 2011. Corticosteroid hormone receptors and prereceptors as new biomarkers of the illegal use of glucocorticoids in meat production. J. Agric. Food Chem. 59:2120–2125.

Edgar, R., M. Domrachev, and A. E. Lash. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30:207–210.

European Commission (EC). 1996. Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of ß-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC.
http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:01996L0022-20081218

European Commission (EC). 2003. Directive 2003/74/EC of the European Parliament and of the Council of 22 September 2003 amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists.

http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32003L0074

European Economic Community (EEC). 1981. Council Directive 81/602/EEC of 31 July 1981 concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action.

http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:31981L0602

Everts, R. E., M. R. Band, Z. L. Liu, C. G. Kumar, L. Liu, J. J. Loor, R. Oliveira, and H. A. Lewin. 2005. A 7872 cDNA microarray and its use in bovine functional genomics. Vet. Immunol. Immunopathol. 105:235–245.

Fenech, M., A. El-Sohemy, L. Cahill, L. R. Ferguson, T.-A. French, E. S. Tai, J. Milner, W.-P. Koh, L. Xie, and M. Zucker. 2011. Nutrigenetics and nutrigenomics: viewpoints on the current status and applications in nutrition research and practice. J. Nutrigenet. Nutrigenomics 4:69–89.

García-Cañas, V., C. Simó, C. León, and A. Cifuentes. 2010. Advances in Nutrigenomics research: novel and future analytical approaches to investigate the biological activity of natural compounds and food functions. J. Pharm. Biomed. Anal. 51:290–304.

Giantin, M., R. M. Lopparelli, V. Zancanella, P. G. Martin, A. Polizzi, G. Gallina, F. Gottardo, C. Montesissa, L. Ravarotto, T. Pineau, and M. Dacasto. 2010. Effects of illicit dexamethasone upon hepatic drug metabolizing enzymes and related transcription factors mRNAs and their potential use as biomarkers in cattle. J. Agric. Food Chem. 58:1342–1349. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20041653

Gottardo, F., M. Brscic, G. Pozza, C. Ossensi, B. Contiero, A. Marin, and G. Cozzi. 2008. Administration of dexamethasone per os in finishing bulls. I. Effects on productive traits, meat quality and cattle behaviour as indicator of welfare.

Graham, S. F., A. Ruiz-Aracama, A. Lommen, F. T. Cannizzo, B. Biolatti, C. T. Elliott, and M. H. Mooney. 2012. Use of NMR metabolomic plasma profiling methodologies to identify illicit growth-promoting administrations. Anal. Bioanal. Chem. 403:573–582.

Guglielmetti, C., M. Mazza, M. Pagano, S. Carrella, S. Sciuto, S. Nodari, M. Pezzolato, G. B. Richelmi, E. Baioni, M. Caramelli, P. L. Acutis, and E. Bozzetta. 2014. Identification by a proteomic approach of a plasma protein as a possible biomarker of illicit dexamethasone treatment in veal calves. Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess. 31:833–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24588558

Haider, S., and R. Pal. 2013. Integrated analysis of transcriptomic and proteomic data. Curr. Genomics 14:91–110.

Jacob, C. C., G. Dervilly-Pinel, G. Biancotto, F. Monteau, and B. Le Bizec. 2014. Global urine fingerprinting by LC-ESI(+)-HRMS for better characterization of metabolic pathway disruption upon anabolic practices in bovine. Metabolomics 11:184–197. Available from: http://link.springer.com/10.1007/s11306-014-0685-3

Kaput, J., and R. L. Rodriguez. 2004. Nutritional genomics: the next frontier in the postgenomic era. Physiol. Genomics 16:166–177.

Kinkead, R. A., C. T. Elliott, F. T. Cannizzo, B. Biolatti, and M. H. Mooney. 2015. Proteomic identification of plasma proteins as markers of growth promoter abuse in cattle. Anal. Bioanal. Chem. 407:4495–4507.

Kouassi Nzoughet, J. J., G. Dervilly-Pinel, S. Chéreau, G. Biancotto, F. Monteau, C. T. Elliott, and B. Le Bizec. 2015. First insights into serum metabolomics of trenbolone/estradiol implanted bovines; screening model to predict hormone-treated and control animals' status. Metabolomics. Available from: http://link.springer.com/10.1007/s11306-015-0775-x

Ledford, H. 2008. The death of microarrays? Nature 455:847.

Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L.

Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol. Genomics 32:105–116.

Loor, J. J., M. Bionaz, and J. K. Drackley. 2013. Systems physiology in dairy cattle: nutritional genomics and beyond. Annu. Rev. Anim. Biosci. 1:365–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25387024

Loor, J. J., M. Vailati-Riboni, J. C. McCann, Z. Zhou, and M. Bionaz. 2015. TRIENNIAL LACTATION SYMPOSIUM: Nutrigenomics in livestock: Systems biology meets nutrition. J. Anim. Sci. 93:5554–5574.

Lopparelli, R., M. Giantin, and G. Pozza. 2012. Target gene expression signatures in neutrophils and lymphocytes from cattle administered with dexamethasone at growth promoting purposes. Res. Vet. … 93:226–233. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21807391

Lopparelli, R. M., V. Zancanella, M. Giantin, L. Ravarotto, G. Cozzi, C. Montesissa, and M. Dacasto. 2010. Constitutive expression of drug metabolizing enzymes and related transcription factors in cattle testis and their modulation by illicit steroids. Xenobiotica. 40:670–680.

Lopparelli, R. M., V. Zancanella, M. Giantin, L. Ravarotto, G. Pozza, C. Montesissa, and M. Dacasto. 2011. Steroidogenic enzyme gene expression profiles in the testis of cattle treated with illicit growth promoters. Steroids 76:508–516. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21295601

Lozano, M. C., and M. Trujillo. 2012. Chemical residues in animal food products: An issue of public health. Public Heal. Environ. Syst. Issues, InTech DOI 10:163–188.

Malone, J. H., and B. Oliver. 2011. Microarrays, deep sequencing and the true measure of the transcriptome. BMC Biol. 9:34.

Mastovska, K. 2011. Multiresidue analysis of antibiotics in food of animal origin using liquid chromatography–mass spectrometry. Mass Spectrom. food Saf. Methods Protoc.:267–307.

McEvoy, J. D. G. 2002. Contamination of animal feedingstuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. Anal. Chim. Acta 473:3–26.

McGrath, T. F., J. A. van Meeuwen, A.-C. Massart, E. de Pauw, P. Delahaut, J. Buijs, A. A. Bergwerff, C. T. Elliott, and M. H. Mooney. 2013. Effect-based proteomic detection of growth promoter abuse. Anal. Bioanal. Chem. 405:1171–1179.

Mooney, M. H., C. T. Elliott, and B. Le Bizec. 2009. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. TrAC - Trends Anal. Chem. 28:665–675. Available from: http://dx.doi.org/10.1016/j.trac.2009.03.011

Odore, R., P. Badino, S. Pagliasso, C. Nebbia, B. Cuniberti, R. Barbero, and G. Re. 2006.

Changes in lymphocyte glucocorticoid and beta-adrenergic receptors in veal calves treated with clenbuterol and steroid hormones for growth-promoting purposes. J. Vet. Pharmacol. Ther. 29:91–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16515662

Odore, R., P. Badino, and R. Barbero. 2007. Regulation of tissue β-adrenergic, glucocorticoid and androgen receptors induced by repeated exposure to growth promoters in male veal calves. Res. Vet. … 83:227–233. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17307208

Parkinson, H., U. Sarkans, N. Kolesnikov, N. Abeygunawardena, T. Burdett, M. Dylag, I. Emam, A. Farne, E. Hastings, and E. Holloway. 2010. ArrayExpress update—an archive of microarray and high-throughput sequencing-based functional genomics experiments. Nucleic Acids Res.:gkq1040.

Pegolo, S., G. Gallina, C. Montesissa, F. Capolongo, S. Ferraresso, C. Pellizzari, L. Poppi, M. Castagnaro, and L. Bargelloni. 2012. Transcriptomic markers meet the real world: finding diagnostic signatures of corticosteroid treatment in commercial beef samples. BMC Vet. Res. 8:205.

Pegolo, S., F. T. Cannizzo, B. Biolatti, M. Castagnaro, and L. Bargelloni. 2014. Transcriptomic profiling as a screening tool to detect trenbolone treatment in beef cattle. Res. Vet. Sci. 96:472–481. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24746288

Reig, M., and F. Toldrá. 2008. Veterinary drug residues in meat: Concerns and rapid methods for detection. Meat Sci. 78:60–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22062096

Riedmaier, I., C. Becker, M. W. Pfaffl, and H. H. D. Meyer. 2009. The use of omic technologies for biomarker development to trace functions of anabolic agents. J. Chromatogr. A 1216:8192–9.
Available from: http://www.ncbi.nlm.nih.gov/pubmed/19233374

Riedmaier, I., M. Reiter, A. Tichopad, M. W. Pfaffl, and H. H. D. Meyer. 2011. The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents. Exp. Clin. Endocrinol. Diabetes 119:86–94.

Riedmaier, I., and M. W. Pfaffl. 2013. Transcriptional biomarkers--high throughput screening, quantitative verification, and bioinformatical validation methods. Methods 59:3–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22967906

Riedmaier, I., M. Spornraft, and M. W. Pfaffl. 2014. Identification of a potential gene expression biomarker signature in bovine liver to detect the abuse of growth promoters. Food Addit. Contam. A, Chem. Anal. Control. Expo. risk Assess. 31:641–649. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24559206

Rinaldi, M., R. W. Li, and A. V Capuco. 2010. Mastitis associated transcriptomic disruptions in cattle. Vet. Immunol. Immunopathol. 138:267–279.

Ritchie, M. D., E. R. Holzinger, R. Li, S. A. Pendergrass, and D. Kim. 2015. Methods of integrating data to uncover genotype-phenotype interactions. Nat. Rev. Genet.

16:85–97.

Schena, M., D. Shalon, R. W. Davis, and P. O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science (80-.). 270:467.

Shahzad, K., M. Bionaz, E. Trevisi, G. Bertoni, S. L. Rodriguez-Zas, and J. J. Loor. 2014. Integrative analyses of hepatic differentially expressed genes and blood biomarkers during the peripartal period between dairy cows overfed or restricted-fed energy prepartum. PLoS One 9:e99757. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4051754&tool=pmcentrez&rendertype=abstract

Shi, L., L. H. Reid, W. D. Jones, R. Shippy, J. A. Warrington, S. C. Baker, P. J. Collins,….., R. G. Perkins, et al. 2006. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat. Biotechnol. 24:1151–1161.

Spielbauer, B., and F. Stahl. 2005. Impact of microarray technology in nutrition and food research. Mol. Nutr. Food Res. 49:908–917.

Stella, R., G. Biancotto, M. Krogh, R. Angeletti, G. Pozza, M. C. Sorgato, P. James, and I. Andrighetto. 2011. Protein expression changes in skeletal muscle in response to growth promoter abuse in beef cattle. J. Proteome Res. 10:2744–2757. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21425879

Stella, R., G. Biancotto, G. Arrigoni, F. Barrucci, and P. James. 2015. Proteomics for the detection of indirect markers of steroids treatment in bovine muscle. :1–21.

Stella, R., F. Barrucci, R. Angeletti, P. James, C. Montesissa, and G. Biancotto. 2016. Targeted proteomics for the indirect detection of dexamethasone treatment in bovines. Anal. Bioanal. Chem.:1–11.

Stolker, A. A. M., T. Zuidema, and M. W. F. Nielen. 2007. Residue analysis of veterinary drugs and growth-promoting agents. TrAC Trends Anal. Chem. 26:967–979.

Toldrá, F., and M. Reig. 2006. Methods for rapid detection of chemical and veterinary drug residues in animal foods. Trends Food Sci. Technol. 17:482–489.

U.S. Food and Drug Administration (FDA). 2013. Approved animal drug products (green book). Accessed Oct. 24, 2016.

http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/ucm226606.htm

Vascellari, M., C. Katia, S. Annalisa, B. Giancarlo, M. Letizia, S. Roberto, P. Giandomenico, and M. Franco. 2012. Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. BMC Vet. Res. 8:129.

Vogel, C., and E. M. Marcotte. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. 13:227–232.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10:57–63.

Webb, R., and J. Buratini. 2016. Global Challenges for the 21st Century: the Role and Strategy of the Agri-Food Sector. Anim. Reprod. 13:133–142. Available from: http://www.cbra.org.br/pages/publicacoes/animalreproduction/issues/download/v13/v13n3/p133-142(AR882).pdf

Zduńczyk, Z., and C. S. Pareek. 2009. Application of nutrigenomics tools in animal feeding and nutritional research. J. Anim. Feed Sci.:3–16.

Zhao, S., W.-P. Fung-Leung, A. Bittner, K. Ngo, and X. Liu. 2014. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One 9:e78644.

I. Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone;

Identification of new candidate biomarkers and their validation using samples from a field monitoring trial[1]

[1] Adapted- and reproduced in the current layout- from: **Elgendy, R.**, Giantin, M., Montesissa, C., & Dacasto, M. (**2015**). **Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone: identification of new candidate biomarkers and their validation using samples from a field monitoring trial.** Food Additives & Contaminants: Part A, 32(9), 1448-1463.
http://www.tandfonline.com/doi/full/10.1080/19440049.2015.1070307

Abstract

Growth promoters (GPs) such as the glucocorticoid dexamethasone (DEX) and the β-adrenergic agonist clenbuterol (CLEN), are still abusively used in beef cattle production. Transcriptomic markers for indirect detection of such GPs have been discussed either in experimentally treated animals or commercial samples separately. In the present study, we examined the transcriptomic signature of DEX alone or in combination with CLEN in skeletal muscle of experimentally treated beef cattle, and, furthermore, comparing them to previously screened commercial samples from a field-monitoring study, as well as to a proteomics data representing the same set of samples. Using a DNA microarray technology, the transcriptomic profiling was performed on 12 samples representing 3 groups of animals: DEX (0.75 mg/animal/day, n = 4), a combination of DEX (0.66 mg/animal/day) and CLEN (from 2 to 6 mg/animal/day, n = 4) and a control group (n = 4). The analyses evidenced the differential expression of 198 and 39 transcripts in DEX and DEX-CLEN groups, respectively. Both groups had no common modulated genes in-between, neither with the proteomics data. Sixteen candidate genes were validated via qPCR. They showed high correlation with the corresponding microarray data. Principal Component Analysis (PCA) on both the qPCR and normalized microarray data resulted in the separation of treated animals from the untreated ones. Interestingly, all the PCA plots grouped the DEX positive samples (experimental or commercial) apart from each other. In brief, our study provided some interesting glucocorticoid-responsive biomarkers whose expression was contradictory to what is reported in human studies. Additionally, our study pointed out the transcriptomic signature dissimilarity between commercial and experimentally treated animals.

Introduction

The misuse of growth promoters (GPs) such as anabolic hormones, corticosteroids and β_2-agonists in cattle is a major concern in food safety, because of its public health interest. The synthetic glucocorticoid dexamethasone (DEX) is known to be illicitly used in meat cattle either alone or in combination with other active principles, such as the β_2-agonist clenbuterol (CLEN). It is known that low doses of DEX interfere with endogenous cortisol synthesis and metabolism; therefore, it result in improved feed intake, increased weight gain, reduced feed conversion ratio, reduced nitrogen retention and increased

water retention and fat content (Möstl et al. 1999; Courtheyn et al. 2002; König et al. 2006; Cannizzo et al. 2013). As regards β_2-agonists, they elicit their growth-promoting effect enhancing protein synthesis and cell hypertrophy, by inhibition of proteolysis in the muscle tissue and induction of lipolysis in the adipose tissue, which is known as the "repartitioning effect". These effects may result in a reduction of carcass fat up to 40% and an increase of carcass protein content up to 40%, yielding a consistent advantage for the meat industry (Courtheyn et al. 2002; Leporati et al. 2014). Phramacologically, the repartitioning and induced muscle growth of CLEN are mediated via interaction with *β*-adrenergic receptors (*β*-AR; Rothwell and Stock 1987), however, this effect soon becomes attenuated due to decreased density (Huang et al. 2000) or desensitization of the *β*-AR in skeletal muscle upon long-term exposure to β_2-agonists (Badino et al. 2005). On the other hand, glucocorticoids are thought to reverse the homologous down-regulation of the *β*-AR number and mRNA expresseion (Mak et al. 1995), and hence are considered as part of the strategy to enhance the anabolic effects of *β*-adrenergic agonists (Huang et al. 2000). The effect of continuous administration of low doses of DEX and CLEN on animals' muscle mass and performance has been illustrated earlier (Odore et al. 2006; Biancotto et al. 2013).

The use of various illicit schedules, such as newly designed drugs or cocktails containing lower GP concentrations, makes the mission of control authorities more difficult, especially when these low GP concentrations bypass the threshold limits of current official detection methods (Cantiello et al. 2007; Riedmaier et al. 2014). For that reason, looking for alternative detection methods was inevitable. In recent years, some pilot studies investigated the effect of DEX, alone or in combination with CLEN, on different biological parameters, in order to highlight direct or indirect markers that could be used for screening purposes. In this context, regulation of tissue beta-adrenergic and glucocorticoid receptors, in veal calves, has been studied following repeated exposure to DEX or CLEN (Odore et al. 2007). Moreover, effect of DEX/CLEN-containing cocktail on serum immunoglobulins, lymphocyte proliferation and cytokine gene expression in veal calves has also been evaluated (Cantiello et al. 2007). Others have used protein expression changes (Stella et al. 2011), thymus morphology and serum cortisol concentration (Vascellari et al. 2012), or corticosteroid profiling of urine using coupled LC-MS/MS (Biancotto et al. 2013), as indirect markers to detect illegally administered GPs. On the other hand, commercial beef samples were also used to look for diagnostic

signatures for DEX treatment in a field monitoring study (Pegolo et al. 2012). Collectively, high-throughput-omic methodologies, such as epigenomics, genomics, transcriptomics (e.g. the whole-transcriptome expression profile using DNA microarrays), proteomics and metabolomics, have recently been incorporated in this field of research, similar to other biological sciences (Riedmaier & Pfaffl 2013).

The purpose of this study was threefold: (1) to measure the changes in cattle skeletal muscle transcriptome induced by treatment with DEX alone, or in combination with CLEN, similar to those illegally performed in the field; (2) to identify a set of differentially expressed genes (DEGs) that could be used as an indirect biological marker for illicit treatment abuse; (3) to understand, through the comparison with transcriptomic data from field monitoring samples (Pegolo et al. 2012) and proteomic results (Stella et al. 2011), if the single approach – strategy (i.e., proteomics, transcriptomics or analytical chemistry) could be enough for defining a universal panel of biomarkers, or a multi-approach strategy is needed. Finally, another question of this study, was to discover whether or not experimentally treated animals respond differently from those present in the field.

Materials and methods

Animals and experimental design

Twenty-four clinically healthy Charolais bulls (18- to 20-month-old) were used in this study. Animals were weighed, housed in ventilated stables and all the experimental procedures were carried out according to the European Union animal welfare legislation. The experiment began after 3 weeks of acclimatization. The animals were randomly divided into three groups of 8 animals each. The first one was used as a control (CTR); the second group was treated with DEX, administered via feed 0.75 mg per capita for 42 days (group DEX); the third one (DEX-CLEN) was administered DEX via feed (0.66 mg per capita for 21 days) in combination with an increasing dose of CLEN, e.g., 2 mg per capita during the first week, 4 mg per capita on the second week, and 6 mg per capita during the third and the fourth weeks (28 days in total, see **Figure 1**). The products to be administered were dissolved in water, and the desired dosage was achieved by mixing 15 ml of water containing an appropriate concentration of each drug with 100 g of feed. This

feed was carefully offered to each animal, ensuring that no significant residue remained in the feeder. In addition, feed conversion index (FCI) and body weight were recorded all over the study.

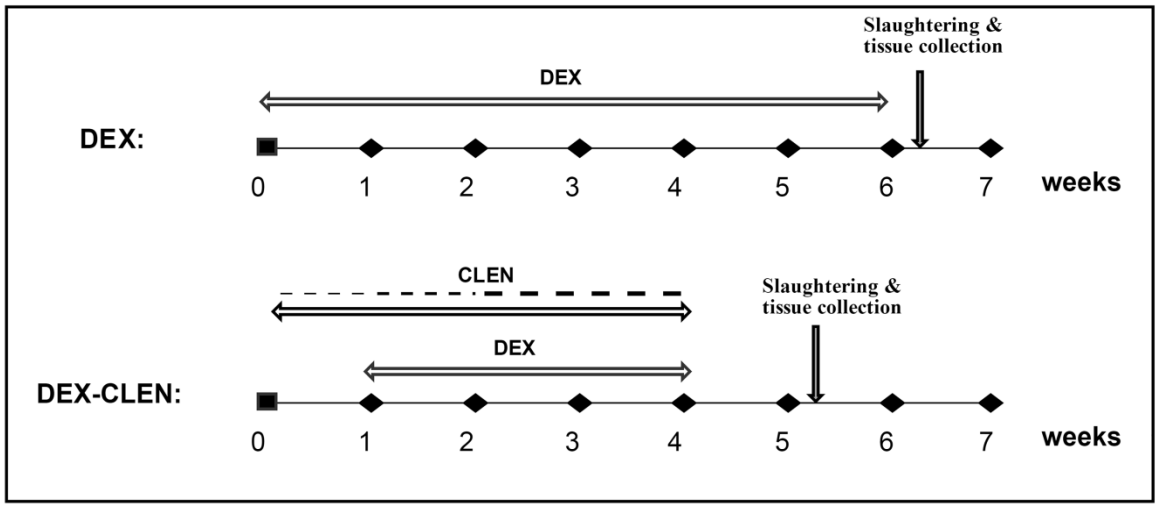

Figure 1: *Experimental design (treatment). Charolais beef cattle were treated via feed with dexamethasone (DEX) alone or in combination with clenbuterol (CLEN). Animals were administered DEX at a dose rate of 0.75 mg per capita for 6 weeks. One group (DEX-CLEN, n=8), besides DEX, was administered with CLEN increasing dosages, e.g. 2 mg per capita during the first week, 4 mg per capita during the second week, and 6 mg per capita during the third and the fourth weeks (4 weeks in total). A third group served as control (CTR).*

Sample collection and RNA extraction

At the slaughterhouse, small *biceps brachii* muscle tissue specimens were sampled from all the animals. Muscle samples were immediately frozen in vessels containing liquid nitrogen (within 1 min of removal) and stored at -80ºC prior to subsequent analyses. Total RNA was isolated by TRIzol® reagent (Life Technologies, USA) and subsequently purified using the RNeasy Mini kit (Qiagen, Italy), according to the manufacturer's instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Italy) was performed. Total RNA concentration was determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDropTechnologies, USA), and its quality was measured by the 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, USA). High quality input (hybridized RNA) is essential to have an unbiased and reproducible output in terms of gene expression data; therefore, the isolated RNAs were tested for proper concentration and

integrity. The best 4 samples of each group (total number = 12) were selected for the microarray analyses, using a specific RNA quality parameter as a criterion, e.g. RNA concentration ≥ 40 ng/µl and RNA integrity number (RIN) ≥ 6.5. The mean RIN value of these 12 samples was 6.93 ± 0.51, indicating intact RNA. Additionally, tissue specimens of 4 illicitly DEX-treated animals (were classified as positive by LC-MS and thymus histological analyses in the study performed by Pegolo et al., 2012) were provided and fresh RNA was isolated from them, then tested for quantity and quality as before. On the bases of having positively DEX treated samples from the field, those 4 samples have been analyzed by qPCR, then implemented in further statistical analyses (see statistics and principal component analyses) along with our experimental samples.

RNA amplification, labeling and hybridization

Sample amplification, labeling and hybridization were performed following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Briefly, for each individual sample 50 ng of total RNA were linearly amplified and labeled with Cy3-dCTP using Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, USA). A mixture of 10 different viral polyadenylated RNAs (Spike-In Mix, Agilent Technologies, USA) were added to each RNA sample before amplification and labeling. A purification step was applied to the labeled cRNA using the RNeasy Mini Kit (Qiagen, Italy), and sample concentration and specific activity (pmol Cy3/µg cRNA) were measured. A total of 1.65 µg of labeled cRNA was fragmented by using the Gene Expression Hybridization kit (Agilent, USA) according to the manufacturer's instructions, and finally diluted by the addition of 55 µL of 2X GE Hybridization buffer. A volume of 100 µL of hybridization solution was then dispensed in the gasket slide and assembled to the microarray slide, with each slide containing four arrays. Bovine-specific oligo-arrays (Bovine V1, 4x44k G2519F, Design ID 015354, Agilent Technologies, USA) were used. Slides were firstly incubated for 17 h at 65ºC in a Hybridization Oven (Agilent Technologies, USA), then washed using wash buffer 1 and 2 according to the manufacturer's instructions. Hybridized slides were scanned at 5 µm resolution using a G2565BA DNA microarray scanner (Agilent Technologies, USA). Default settings were modified in order to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). A general workflow of the microarray experiment is reported in **Figure 2**. Microarray data

have been deposited in the NCBI's Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE61934.

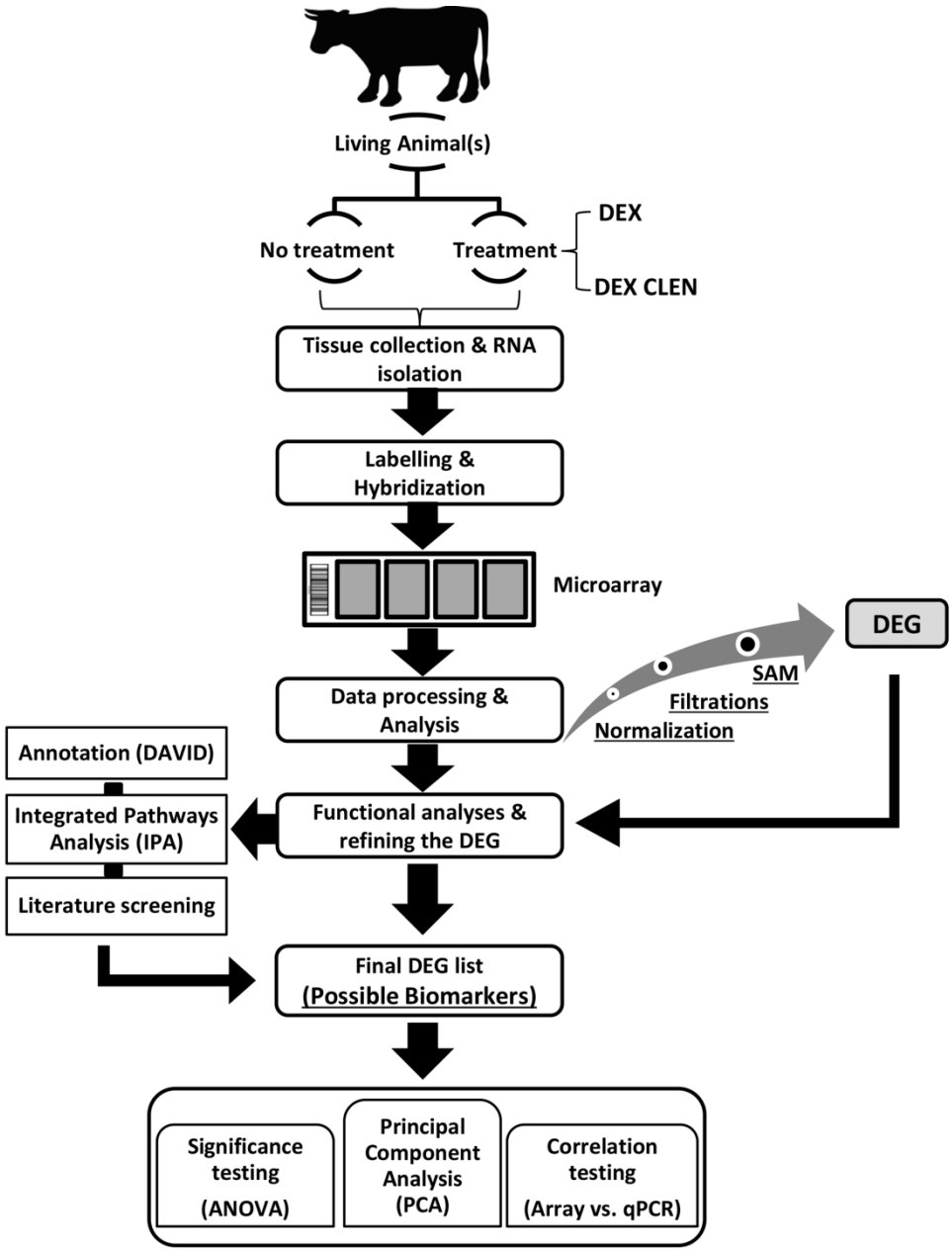

***Figure 2**. General workflow of the microarray experiment.*

Normalization of the microarray data

Data were extracted and the background was subtracted using the default settings of the Agilent's Feature Extraction Software version 9.5.1 (Agilent Technologies, USA). Extracted data were normalized and processed as previously described in Giantin et al.,

(2014). A further filtering step was carried out by removing probes that reported missing values or no reactivity (flag equal to 0) in at least 50% of samples. Missing values (probes with Feature Extraction flag equal to 0) were imputed by using the microarray data analysis tool TIGR Multiple Array Viewer (TMEV; Saeed et al., 2003). In addition, raw microarray data (30 samples) from a monitoring study were downloaded from GEO (GSE26318: Pegolo et al., 2012). Experimental and monitoring samples (n = 42) were grouped, normalized and filtered together in one run, to avoid any possible analytical bias, and then used in subsequent analyses. The functional analysis of DEGs list was performed using the Ingenuity Pathway Analysis (IPA) online platform (http://www.ingenuity.com, Qiagen, USA).

Quantitative Real time PCR

Sixteen target genes, and three housekeeping (HK) genes (RPLP0, GAPDH and TBP) were chosen for the external validation of microarray findings by qPCR. To increase the number of data points in the statistical and correlation analyses between DNA microarray and qPCR, samples from all the 24 animals of our study were included in the qPCR analysis. Gene-specific primers (**Table** 1), encompassing one intron, and the most appropriate Universal Probe Library (UPL) probe were designed by using the UPL Assay Design Centre web service (Roche Applied Science, USA). First-strand cDNA was synthesized from 0.15 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) according to the manufacturer's protocol and stored at -20ºC until further use. Overall, qPCR reactions (10 µL final volume) consisted of 1X LightCycler 480 Probe Master (Roche Applied Science, USA), 300 or 600 nM forward and reverse primers (Integrated DNA Technology, Italy) according to the assay set-up, 200 nM human UPL probe (final concentrations) and 2.5 µL of 1:7.5 diluted cDNA (15 ng/µL). Each qPCR analysis was performed, in duplicate, in a LightCycler 480 Instrument (Roche Applied Science, USA) using the standard PCR conditions (an activation step at 95ºC for 10 minutes; forty-five cycles at 95 ºC for 10 seconds and at 60ºC for 30 seconds; a cooling step at 40ºC for 30 seconds) and LightCycler 480 clear plates (Roche Applied Science, USA). To determine the efficiency of each qPCR assay, non-template and no-reverse transcription controls were included on each plate. Moreover, standard curves obtained by amplifying eight threefold serial dilution of the same cDNA pool were used. Data were analyzed with the LightCycler480 software

release 1.5.0 (Roche Applied Science, USA) using the second derivative or fit point method. Messenger RNA relative quantification was performed by the ΔΔCq method (Livak & Schmittgen 2001).

Table 1: Oligonucleotide sequences for qPCR.

Gene acronym	Gene name	NCBI RefSeq	Primer sequence 5' >>> 3'	Amplicon size (bp)[a]
RPLP0[b]	Ribosomal protein, large, P0	NM_001012682	F: CAA CCC TGA AGT GCT TGA CAT [c] R: AGG CAG ATG GAT CAG CCA[c]	227
GAPDH[b]	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	F: ACA CCC TCA AGA TTG TCA GCA A R: TCA TAA GTC CCT CCA CGA TGC	82
TBP[b]	TATA box binding protein	NM_001075742	F: ACA ACA GCC TCC CAC CCT ATG C R: GTG GAG TCA GTC CTG TGC CGT AA	111
C7	Complement component 7	NM_001045966	F: GGA CGG TGC TGA TGA AGA CA R: TGT AAC CAC GTC CGG TAA GC	101
CCDC80	Coiled-coil domain containing 80	NM_001098982	F: GTC ACT GGA AAA CTT CCT AT CCA R: CAT CAT TAG GGG CCG AGA T	71
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2	NM_001100299	F: AGT CAG AGA GAA ACG GCG TG R: GTG GTG TAG CAG TCC AGG TC	100
FKBP5	FK506 binding protein 5	NM_001192862	F: GTG GAG TGC TGC GAC AAG R: CTT GGC TGA CTC GAA CTC GT	105
LIPG (LOC509808)	Endothelial lipase-like	XM_002697766	F: CGC CTA TTG TGG CTT TGC R: GCG GAG GTT AAA TCT CAC AGG	127
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	NM_174745	F: CGA TGC TGT GTA CGA AGA CC R: CTG GCT GAA TAG ACC CAG TAT TC	78
MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response	NM_174118	F: CAG CAG CTC TCA GGA CGT G R: GTC CAT GTT CTC CAA ATT CCA	67
RASD1	RAS, dexamethasone-induced 1	NM_001206261	F: CAC CGC AAG TTC TAC TGC AT R: GCT GAA CAC CAG GAT GAA CA	132
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, 1	NM_177521	F: CAC GGC TCC TCA AGA CAC ACT R: GGG CGA TGT AGA TCA CCT TG	84
CYP1A1	Cytochrome P450, subfamily I, polypeptide 1	XM_002696635	F: GGC CTT TAT CCT GGA GAC CT R: AAG CCG TTC AGA TTG CTG TC	90
CCL24	Chemokine (C-C motif) ligand 24	NM_001046596	F: GCT ACC AGC TTA CCA ACA GGA R: GAA CTT CTG GCC CTT CTG G	74
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	NM_001192835	F: CGG GAT CTG TCC TAC ATC AAG R: CAC GCG GTT CAC GAC ATA	60
C1QA	Complement component 1, q subcomponent, A chain	NM_001014945	F: CCT GGA AAC CCA GGC AGA AT R: TGG CTG GTC CTT GAT GTT CC	114
MEDAG	Mesenteric estrogen-dependent adipogenesis	NM_001083660	F: TCC AGA AAA GAA GGA GAC CATT R: TGC AAT TAA AAA CTT CAT CTA TTG AAC	131
FGL2	Fibrinogen-like 2	NM_001046097	F: GGCAAATGTTCATCTAAGTGTCC R: ACTGCTTCTTTTGCCTATTGTGT	114
HSPA8	Heat shock 70kDa protein 8	NM_174345	F: AAC CAA GTC GCA ATG AAT CC R: GCA TCA TCA AAT CTT CGT CCA	74

[a] Base Pairs; [b] Reference gene; [c] Robinson et al., 2007.

Statistics and Principal Component Analysis (PCA)

To identify DEGs, a two-class unpaired test was implemented in the program SAM (Significance Analysis of Microarrays) release 4.0 (Tusher et al. 2001), enforcing a False Discovery Rate (FDR) of 5% with a fold change (FC) threshold of 2. All other statistical tests (linear regression, non-parametric Spearman correlation analysis and Mann-Whitney test) were carried out by the GraphPad Prism 5 software (San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Using TMEV, a PCA analysis was carried out on our microarray processed samples (n = 12), in order to test the efficiency of our identified DEGs in differentiating treated from untreated samples. Hence, only normalized and filtered microarray intensities of the previously obtained DEGs (198 transcripts of DEX vs. CTR and 39 of DEX-CLEN vs. CTR) were used.

To visualize the multivariate response of the selected classifiers to the treatment and see if the chosen set of gene markers could result in the best separation of treated and untreated animals, another dynamic PCA approach, based on qPCR results (relative quantification values; RQ), was performed. The objective was to test if our proposed set of the 16 genes could differentiate DEX-treated samples (experimental or commercial) from the untreated ones. For that purpose, the later PCA was performed using RQ values corresponding to the CTR (n = 8), and DEX (n = 8) groups, along with the 4 samples claimed to be positive for DEX from the monitoring study. The PCA on qPCR RQ values was executed by the GenEx v.5 software (Bergkvist et al. 2010), adopting the following settings: mean center scaling, Ward's algorithm and Manhattan distance. Finally, another PCA analysis was carried out on our microarray processed (n = 12) and all the monitoring samples (n = 30) or the DEX-positive ones (n = 4), in order to test if they could group into different clusters based on their DNA microarray raw data. For each PCA, samples were grouped together in one set, and a blind PCA was carried out.

Results

Animal health status and growth performance

The health status of all the experimental animals was satisfactory all throughout the experiment. Except for DEX and/or CLEN, no other drugs have been administered during the experimental procedure. Information about the animals' performance and feed conversion index have been presented in details elsewhere (Biancotto et al. 2013).

Microarray quality control and data analyses

The comparison of the DEX group with the CTR one (DEX vs. CTR) resulted in a list of 198 down-regulated transcripts, representing 123 characterized transcripts and 75 estimated sequence tags (ESTs: see *Supplementary Table 1*). Estimated sequence tag transcripts were excluded from further analyses due to limited or not available information on annotation. Among the 123 down-regulated genes, 29 ones had a FC > 4, and the highest FC (-14.77-fold) was noticed for the LOC509808 (LIPG) gene.

Following the comparison between DEX-CLEN and CTR groups (DEX-CLEN vs. CTR), the analysis with SAM resulted in a much shorter list of DEGs. A total of 39 DEGs, representing 21 characterized transcripts and 18 ESTs (thereby excluded from further analyses), was identified. Among the 21 genes, 16 and 5 were respectively up- and down-regulated (*Supplementary Table 2*). There were no overlapping DEGs between the 2 comparisons.

To better describe the transcriptome functional modifications and to mine through the obtained DEG lists, IPA was used to find out and explore pathways, networks and bio-functions somewhat modulated by the used GPs. Data analysis identified some networks and bio-functions, e.g. molecular and cellular functions, physiological system development and canonical pathways (see *Supplementary Table 3* for a detailed IPA report). Moreover, glucocorticoid receptor and xenobiotic metabolism signaling pathways were highlighted among the canonical pathways output following the IPA core analysis performed on our DEGs (**Figure 3**).

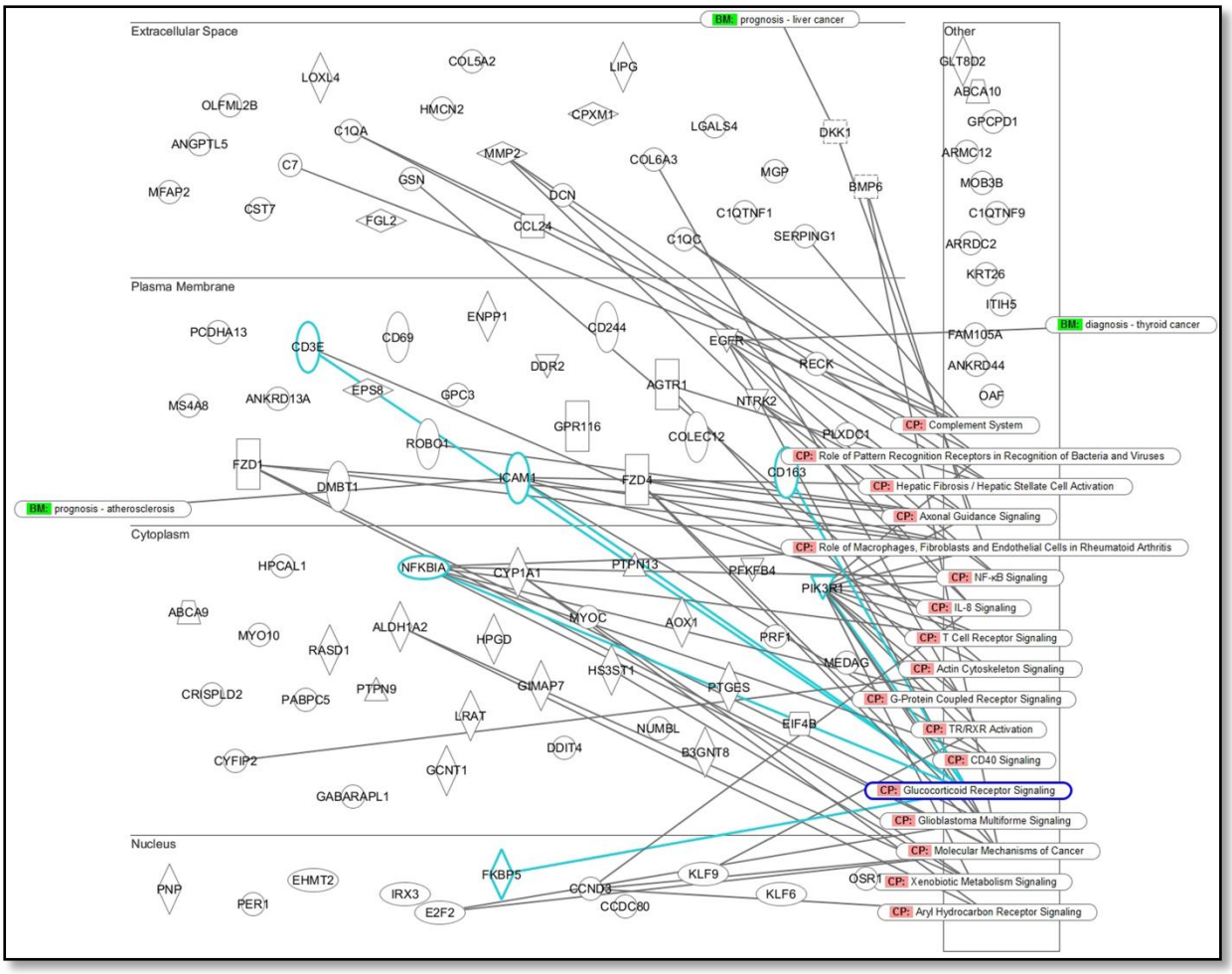

Figure 3: *Ingenuity Pathways Analysis (IPA). Canonical pathways tree following a core analysis by IPA and using human, mouse and rat databases as a background. The glucocorticoid receptor signaling pathway is highlighted.*

To reduce the complexity of a long list of significant genes, an additional approach was to focus on those transcripts that seemed more relevant to our study. Through an extensive literature screening, using NCBI database and gene summaries present in GeneCards of the human gene database (http://www.genecards.org), together with some predictions suggested by IPA, we collected information about the direct function of each gene and the corresponding target tissue (when available). This strategy helped us in refining our DEG lists and grouping genes into 3 main categories, e.g. glucocorticoids responsive, skeletal muscle related and coagulation cascade related genes (*Supplementary Table 4*). A set of 16 genes shown to be modulated in DEX (15 genes: CYP1A1, FKBP5, CCL24, RASD1, MYOC, PFKFB4, MEDAG, SULT1A1, LIPG, CRISPLD2, C1QA, FGL2, CCDC80, C7, and MMP2), and DEX-CLEN groups (1 gene: HSPA8) were considered for the final validation analysis by qPCR.

Confirmatory qPCR analysis

To cross-validate our platform performance and identify some potential biomarkers, a relative quantification approach by using qPCR was a must. All the aforementioned 16 genes were found to be significantly regulated in the muscle tissue, comparing treated with control animals (**Figure 4**).

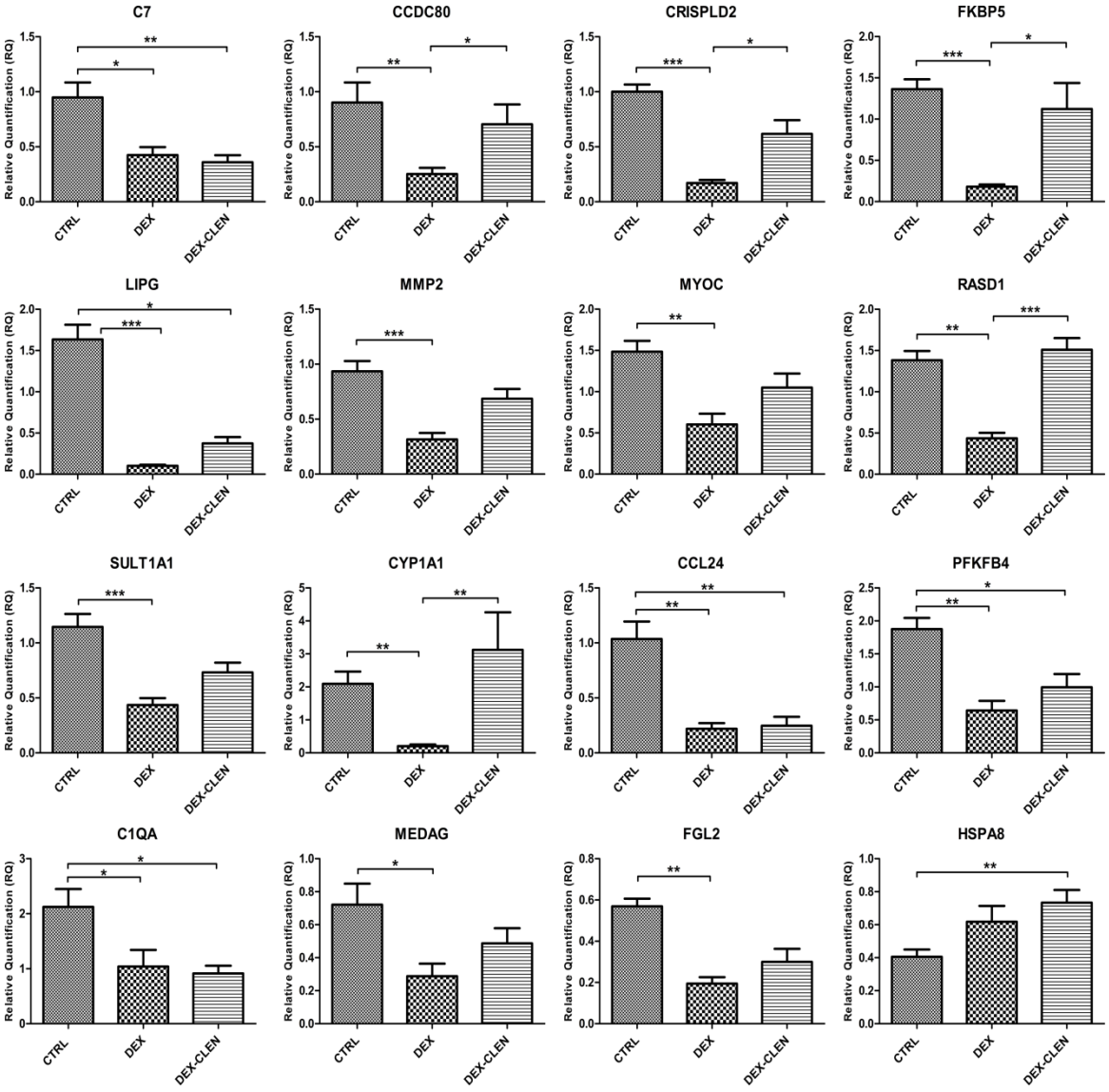

Figure 4: *Validation of 16 differentially expressed target genes. Expression levels (Relative Quantification; RQ values) of genes, relative to non-treated (CTR) samples determined by qPCR. Significance was tested by using the one-way ANOVA (Kruskal-Wallis test) followed by Dunn's post-hoc test. Multiple bars show means ± SE.*

Statistically significant differences of DEX vs. CTR values were calculated at P < 0.05, 0.01 & 0.001 (, ** & ***), respectively.*

A Spearman rank correlation test was then performed for each target gene, comparing qPCR RQ values with the corresponding microarray's intensities (**Figure 5**).

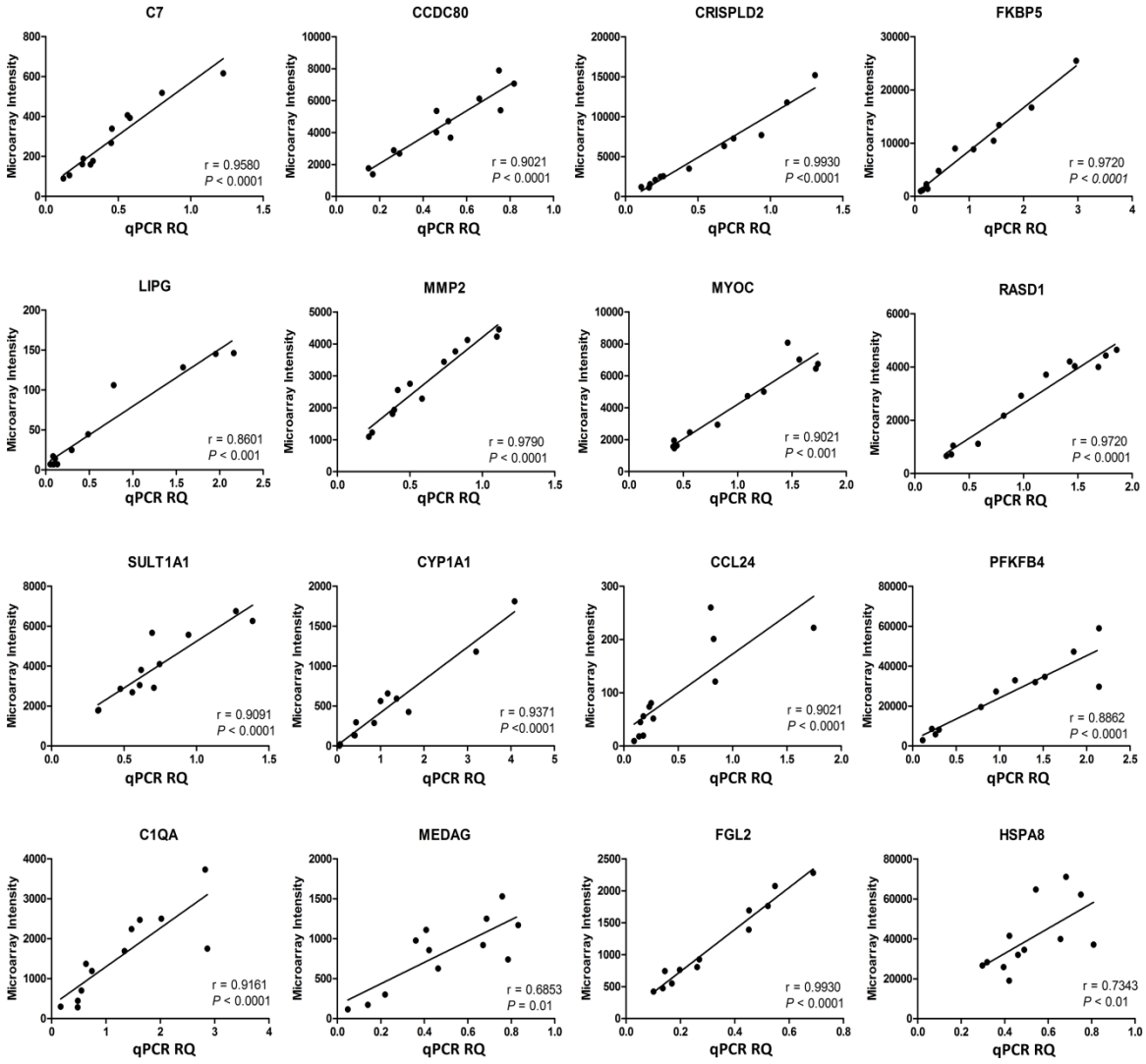

Figure 5: *Plots of qPCR RQ values versus corresponding normalized-microarray probe intensities for individual target genes. Each solid dot represents a sample from the twelve samples (4/group) included in the microarray analysis as mentioned before. Both the Pearson's rho (r), representing the correlation coefficient, and the obtained p-value is shown within each plot.*

At DEX group, 14 out of 15 genes showed high correlation coefficients (Spearman rho > 0.8; $P < 0.001$), while only one gene (MEDAG) was significantly correlated, but with a lower correlation coefficient (rho = 0.685; $P = 0.01$). At DEX-CLEN group, a significant relationship was only found for HSPA8 (rho = 0.734; $P < 0.01$: **Table 2**).

Table 2: Spearman's rho for the selected genes used for qPCR validation.

Gene acronym	FC qPCR	FC array	Spearman's rho†
C7	-2.12	-2.54	0.9580
CCDC80	-3.44	-2.53	0.9021
CRISPLD2	-5.65	-7.07	0.9930
FKBP5	-7.14	-8.66	0.9720
LIPG	-15.32	-14.77	0.8601
MMP2	-2.83	-2.58	0.9790
MYOC	-2.36	-4.44	0.9021
RASD1	-3.05	-4.75	0.9720
SULT1A1	-2.49	-2.54	0.9091
CYP1A1	-10.39	-10.41	0.9371
CCL24	-4.84	-5.14	0.9021
PFKFB4	-3.17	-4.38	0.8862
C1QA	-1.69	-3.61	0.9161
MEDAG	-2.50	-3.28	0.6853
FGL2	-2.82	-3.16	0.9930
HSPA8	1.52	2.11	0.7343

Fold-change (FC) was calculated comparing group DEX vs. group CTR or group DEX-CLEN vs. group CTR

†Spearman's rho (r) calculated at $P < 0.01$.

Furtheremore, the correlation made on the average FC obtained with DNA microarray and qPCR technologies showed a high and significant correlation coefficient (Spearman's r = 0.754, $p < 0.0001$), with a slope of linear regression equal to 0.94 (see **Figure 6**).

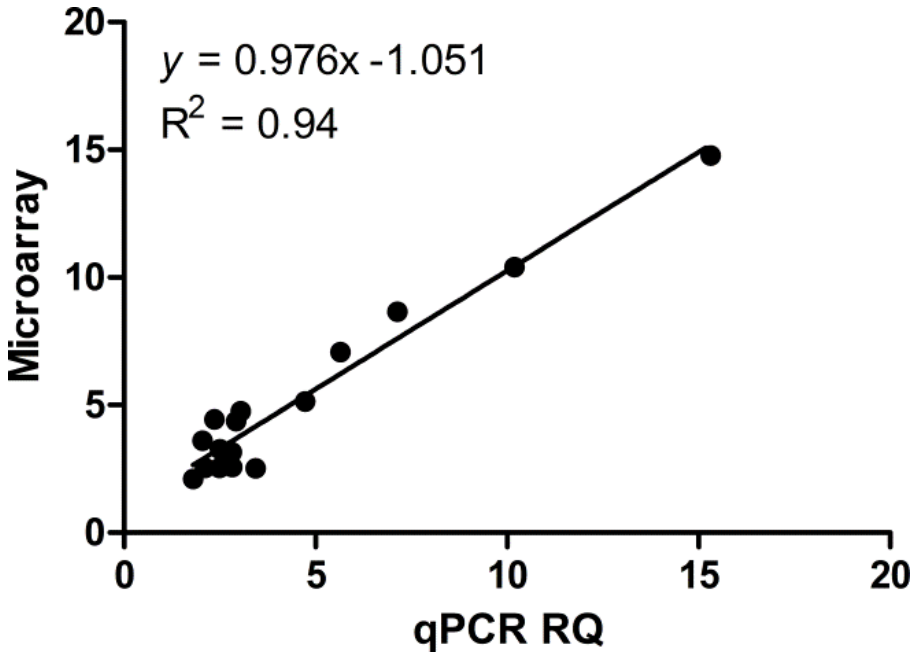

Figure 6: *Overall comparison between microarray and qPCR data, expressed as estimated fold-changes, referring to the final 16 candidate transcript biomarkers. $P < 0.001$*

Clustering and principal component analyses

The first two components, which accounted for 81.124 % of the total variance, clearly identified and distinguished DEX from CTR samples, while DEX-CLEN samples were distributed along the x and y axis with no clear grouping trend (**Figure 7A**). By using the RQ values of the proposed 16 gene markers, the PCA was able to distinguish 3 groups of samples, e.g. CTR (n = 8), DEX-treated animals (n = 8) and the four DEX-positive samples from the monitoring study. The first two principal components of greatest variation covered the 94.60% of the total variance. Quite unexpectedly, samples from DEX group and DEX-positive samples from the monitoring (MO) study did not group together on the PCA plot (**Figure 7B**). Likewise, samples clustered independently from each other also on the relative dendrogram (**Figure 8**).

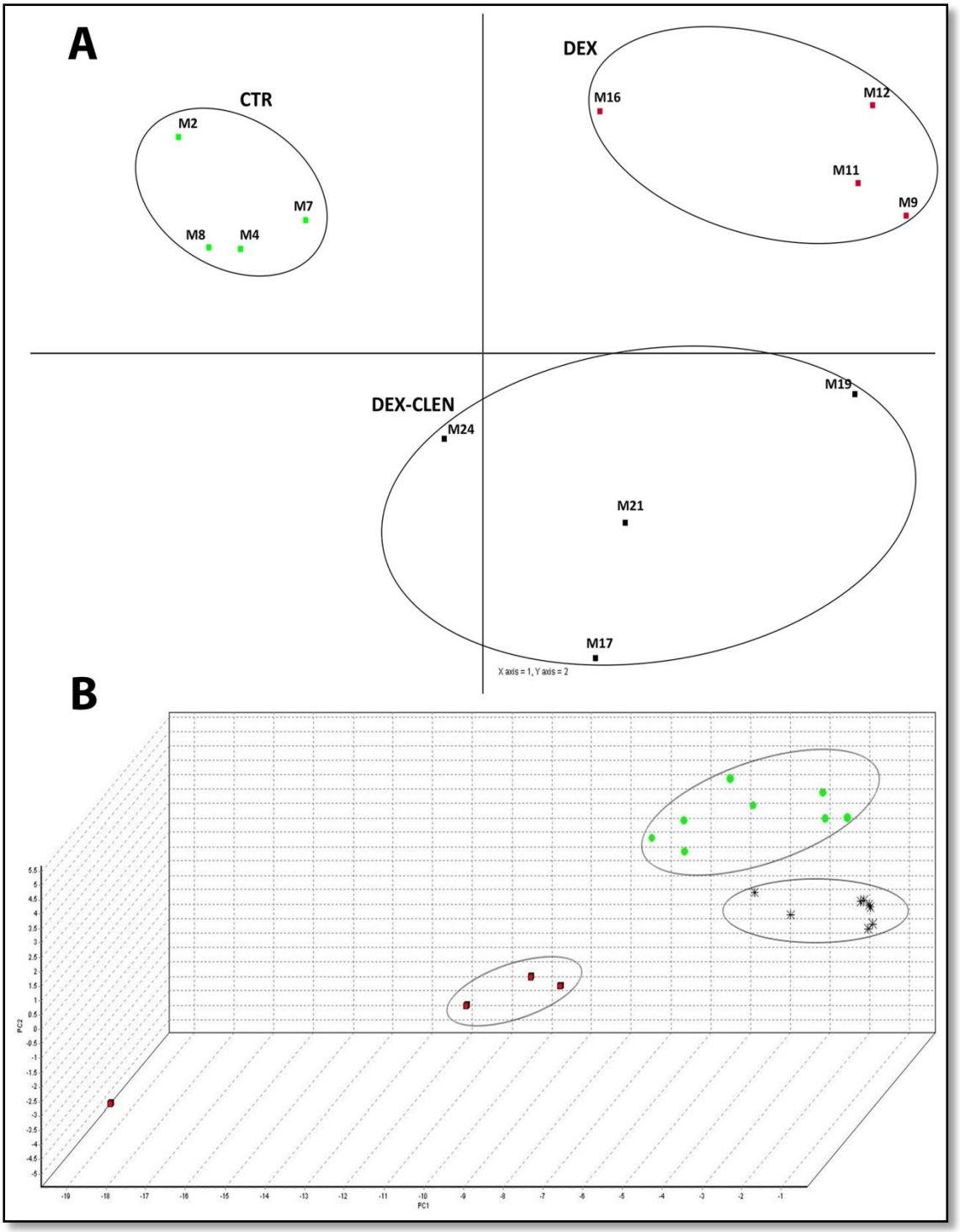

Figure 7: *Principal component analysis (PCA).* ***(A)*** *PCA plot of the 12 samples of the current study, showing normalized microarray data of all the differentially expressed genes (198 and 39 in DEX vs. CTR and DEX-CLEN vs. CTR, respectively). The PCA shows the two principal components of greatest variation, accounting for 67.8% (x-axis) and 13.3% (y-axis) of the total variance.* ***(B)*** *Plot showing the three principal components of variance for the three experimental groups. Animals of the control group (CTR) are represented by green circles; dexamethasone-treated group (DEX) are represented by black stars, while animals of the monitoring study (4 DEX positive samples) are shown by red squares. Ellipses distinguish different treatment groups. Eigenvalues for PC1, PC2 and PC3 were 75.955%, 18.651% and 2.915%, respectively.*

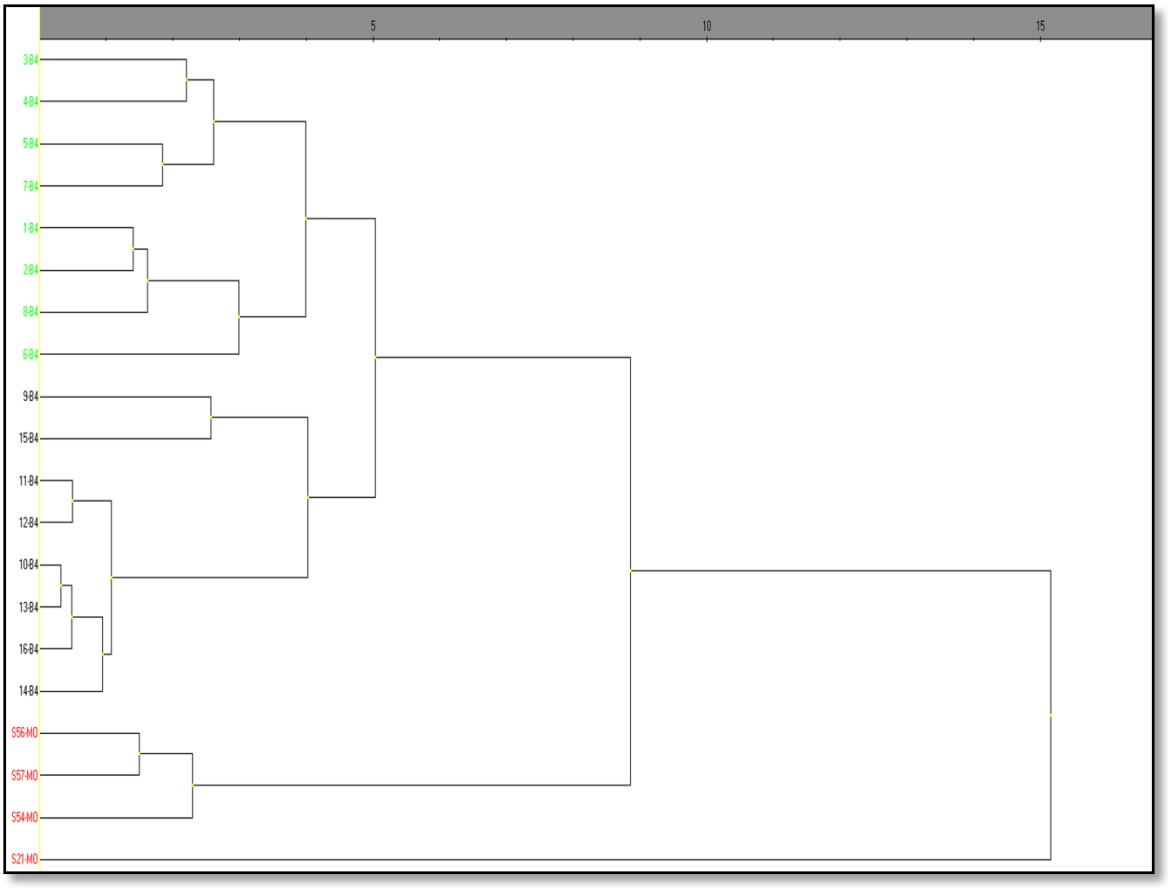

Figure 8: *Dendrogram showing the hierarchical clustering of the three experimental groups; controls (CTR, B4:1-B4:8), animals administered with dexamethasone (DEX, B4:9-B4:16) and monitoring DEX positive cattle (MO:S21, S54, S56 and S57). This tree represents the similarity between genes and/or samples, based on the gene expression profiles (RQ values) measured by qPCR assays. Average linkage and Euclidean distance were used as a clustering method and distance measure, respectively.*

To better understand why DEX-treated animals clustered apart from each other, and particularly to verify whether this behavior was a treatment-specific response or a technical bias, a broad dynamic PCA, using the raw microarray data of all the samples (present study: n = 12, field monitoring: n = 30), was performed (**Figure 9A**). In this case, the first two components, in compliance with Pegolo et al. (2012), accounted for 66.5% of the total variance and separated the monitoring samples (MO-1 and MO-2) from our experimental samples (EXP). The y-axis, representing 24.3% of the total variance, separated our 12 samples from group MO-2, including the four DEX-positive samples and other unknown samples. To further confirm these findings and to avoid any possible distortion in the PCA plot due to many unknown samples in the monitoring study, the PCA was repeated between our 12 samples (EXP) and the 4 DEX-positive samples (MO-POS), using the differentially regulated gene list (11,484 unique transcripts) following a

one-class SAM analysis, and enforcing an FDR of 0%. Again, we found that our samples and the monitoring ones were grouped apart from each other's (**Figure 9B**).

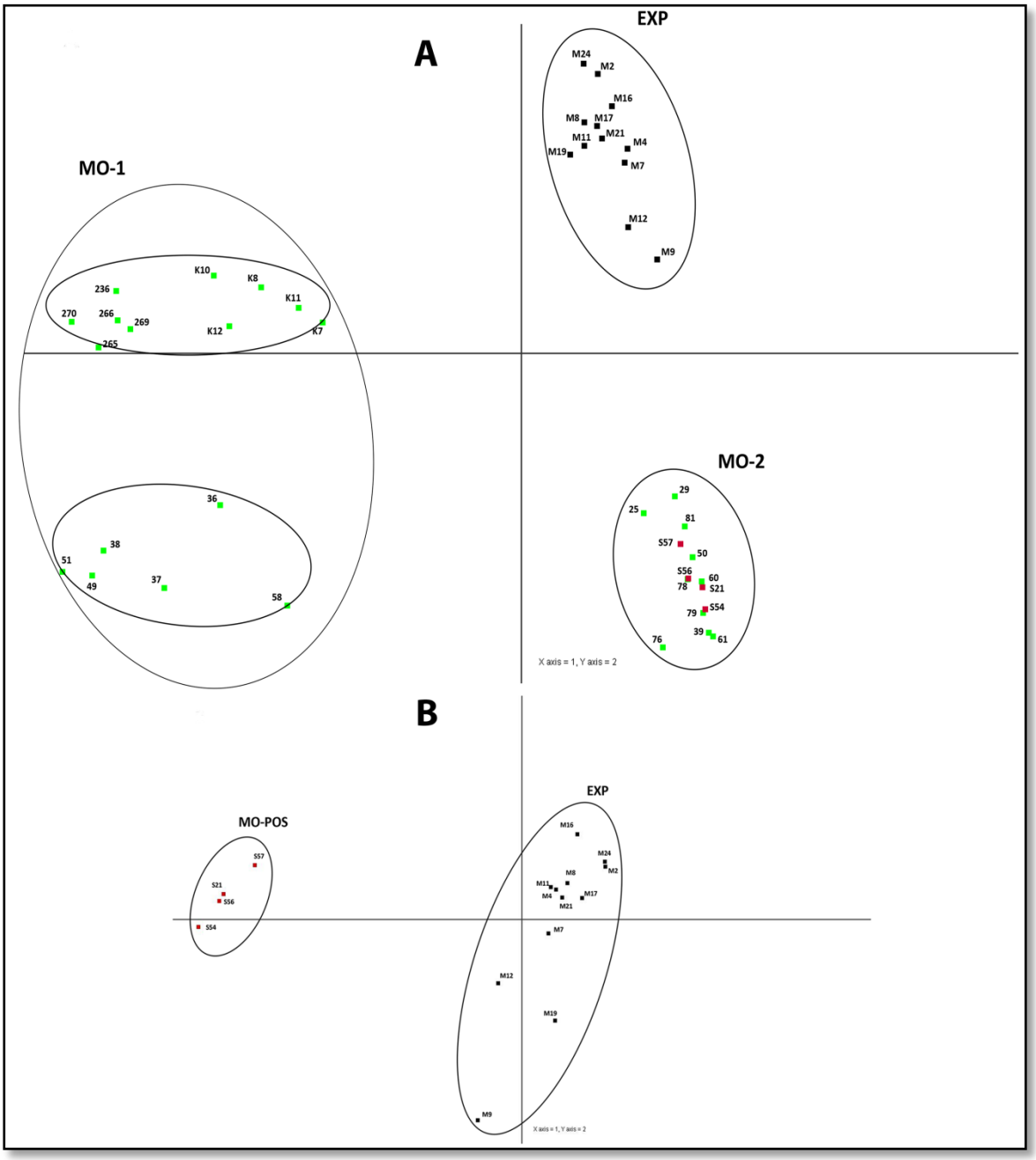

Figure 9. *PCA of the bovine skeletal muscle gene expression profiles.* ***(A)*** *PCA plot showing the grouping of the 12 experimental samples distinctively away from commercial field monitoring samples (MO-1 and MO-2). The plot was created following a one-class SAM analysis (via TMEV) on normalized microarray intensities, by using a differentially regulated gene list (11,484 unique transcripts) and a false discovery rate (FDR) of 0%. The first two components accounted for 42.2% and 24.3%, respectively.* ***(B)*** *PCA plot, excluding all the monitoring study samples except for the 4 dexamethasone (DEX) -positive ones; the x-axis and y-axis cover 60.8% and 8.8% of the total variance, respectively. The ellipse on the right side represent the 12 experimental samples, while the 4 samples on the left side of the plot are the 4 DEX-positive ones from the monitoring study.*

Discussion

Although the development of additional detection methods for GPs' abuse in beef cattle has already been faced by different "omic" disciplines, such as genomics, transcriptomics, proteomics and metabolomics, the "gold standard" detection technology or the perfect biomarkers panel is seemingly out of reach for the moment. In the present study, we investigated the effect of some GPs on the muscle's transcriptome of beef cattle. The foremost objective was to detect possible changes in gene expression and compare these transcriptional effects with those obtained applying a proteomic approach (Stella et al. 2011) and MS-based analytical investigations (Biancotto et al. 2013). Moreover, another goal was to test our suggested transcriptional biomarkers against pre-validated DEX-positive samples (Pegolo et al., 2012). Shortly, a set of potential transcriptional biomarkers was identified and cross-validated with an independent method. However, the comparison of our DEGs list with that of the proteomics study revealed no common gene-coded protein or pathway(s) in-between. Finally, after plotting our samples on PCA against DEX-positive samples coming from a monitoring study, our proposed target genes did not group the experimental and monitoring samples together; on the contrary, they were clustered separately. These distinct results are hereby discussed more in depth.

Indirect biological markers have a comparable cost, higher output and high sensitivity compared with other methods (Balizs & Hewitt 2003; Carraro et al. 2009). Based on results here obtained, the use of illicit schedules containing DEX alone could be reliably identified, with high confidence, by using fifteen genes; on the other hand, the use of a DEX-CLEN combination was eventually identified by the HSPA8 gene only. The identification of 198 DEGs in the DEX-treated group and only 39 ones in the DEX-CLEN group could suggest a CLEN-masking effect upon DEX. Counteraction between DEX and CLEN, where the latter caused mild attenuation of the effects of DEX on some physiological parameters, has already been reported (Huang et al. 2000). Several genes were broadly down-regulated in the DEX-treated group, and we will shortly discuss only the ones showing the highest response in terms of fold-changes and/or relevance to the purpose of the study.

The most down-regulated gene (-15.32-fold) is the endothelial lipase (LIPG) gene. Endothelial lipase is a member of the triglycerides (TG) lipase gene family, showing a significant phospholipase activity on high-density lipoprotein (HDL) particles (Goldberg 1996; Jaye et al. 1999; Griffon et al. 2006); furthermore, LIPG gene inactivation has been

shown to increase HDL levels (Qiu et al. 2007). Although the relationship between LIPG and lipid metabolism is well documented, there is not much information in the literature regarding any relationship between this gene and glucocorticoids. However DEX has been shown to decrease LIPG activity in the liver of rats (Peinado-Onsurbe et al. 1991). Moreover, a recent study reported that DEX administration in horses decreased the expression of genes involved in hormone signaling, cholesterol synthesis and steroidogenesis (Ing et al. 2014). The well-studied and evident effect of DEX on lipid metabolism (Zhou & Cidlowski 2005; Xu et al. 2009; Martin et al. 2009; Campbell et al. 2011) could be a reason to hypothesize a relationship between LIPG and DEX treatment.

The cytochrome P450 1A1 gene was down-regulated by 10.39-fold. This gene is involved in oxidative drug metabolism (Beresford 1993) and it depends from the aryl hydrocarbon receptor (AhR) for its regulation. It has been reported that CYP1A1 expression in adult human hepatocytes was negatively regulated by DEX at the protein level, but no effects were noticed upon mRNA (Monostory et al. 2005). On the other hand, DEX was shown to have no inhibitory potency on the CYP1A1 level either in human hepatocytes (Vrzal et al. 2009) or in rainbow trout (Burkina et al. 2013). In the same context, DEX was recently proved to suppress CYP1A1 transactivation in gene reporter assays (Stejskalova et al. 2013).

FKBP5 (also known as FKBP51) can act as an important determinant of the responses to steroids, especially to glucocorticoids in stress and mood disorders in humans (Kang et al. 2008; Jaaskelainen et al. 2011). Up-regulation of FKBP5 in response to corticosteroid use has been consistently demonstrated in many studies (Franchimont et al. 2002; Almon et al. 2005; Tissing et al. 2007), and has been associated with the loss of efficacy of corticosteroids (Fisher et al. 2005). Surprisingly, in the present study FKBP5 was down-regulated (-7.14-fold). Most of the information about FKBP5 are closely related to human, and very little information is available about this gene in animals, if any. However, it has been recently reported that FKBP5 is down-regulated following a 21 days' treatment with the corticosteroid prednisolone in a collagen-induced arthritis mouse model (Ellero-Simatos et al. 2014). It should be emphasized that illicit schedules in cattle substantially differ, in terms of dosage and duration of administration, from the ones used in human, and it is also worth mentioning that the glucocorticoid signaling system is highly stochastic, and differ greatly from one tissue to another (Kino 2007).

Dexamethasone-induced Ras-related protein 1 (RASD1) is a member of the Ras family of proteins that is usually activated following the administration of corticosteroids (Kemppainen & Behrend 1998; Tu & Wu 1999). Surprisingly, we observed a down-regulation of this gene (-3.05-fold). Up to our knowledge, there is no available information about RASD1 gene expression in cattle. In humans, RASD1 mRNA is constitutively expressed in many tissues such as brain, heart, liver and kidney (Kemppainen & Behrend 1998; Tu & Wu 1999; Fang et al. 2000), while no records are available about skeletal muscle. The inactivation of RASD1 and its correlation with resistance to DEX, as a consequence of methylation, was recently discussed by Nojima et al. (2009). A thorough explanation of these contradictory findings needs further investigations and comparative studies to be performed, in order to understand if there are species- and/or tissue- related variations.

The chemokine (C-C motif) ligand 24 (CCL24) is predominantly involved in chemotaxis of T cells compatible with an anti-inflammatory effect of glucocorticoids (Ehrchen et al. 2007). In few studies performed on cattle, CCL24 was shown to be normally down-regulated in the gestation period of female cows (Oliveira et al. 2010; Laporta et al. 2014). However, data from human and experimental animal models, in accordance with our findings, showed that CCL24 was down-regulated following DEX treatment (Goleva et al. 2008; Luesink & Jansen 2010; Louten et al. 2012). Overall, these findings are in concordance with the down-regulation (-4.84-fold) we observed following DEX treatment.

The myocilin, trabecular meshwork inducible glucocorticoid response (MYOC) gene was shown to be moderately down-regulated (-2.36-fold). This result is apparently in contrast with other studies, where MYOC was up-regulated upon DEX treatment in both human and cattle anterior segments of the eye, namely the trabecular meshwork cells (Taniguchi et al. 2000; Ishibashi et al. 2002; Rozsa et al. 2006; Mao et al. 2011) Nevertheless, it was recently reported (Kumar et al. 2013) that MYOC showed decreased expression in the trabecular meshwork cells isolated from eyes of mice treated with steroids. Furthermore, the effect of DEX upon MYOC gene expression varies greatly from one tissue matrix to another (Morgan et al. 2014).

One of the other interesting results was the down-regulation (-3.44-fold) of the cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2) gene. This transcript has

only recently been considered as a glucocorticoid-responsive gene (Himes et al. 2014). What is interesting here is that CRISPLD2 showed an over expression pattern in some primary human cell lines following a 24-48 hours of DEX treatment (Masuno et al. 2011; Greer et al. 2013; Himes et al. 2014), while it was down-regulated in our study. Very little information is actually available for this gene in the literature, except for humans. The present contradictory result might be due to the different illicit protocol (GP combination) and/or the DEX dosages here used.

Likewise to CRISPLD2, the complement component 7 (C7), a key component of the adaptive immunity (Actor et al. 2001; Mashruwala et al. 2011), has been recently considered as a glucocorticoid-responsive gene (Himes et al. 2014). In our experimental conditions, C7 was found to be down-regulated (-2.12-fold). This could be explained by the fact that glucocorticoids are known to repress the expression of adaptive immune-related genes (Galon et al. 2002; Franchimont 2004; Azuma 2010). The only up-regulated gene in our validated set of genes was the heat shock proteins A8 (HSPA8) which has been examined as a glucocorticoid-induced gene in other model systems (Smoak & Cidlowski 2006). However, no information relating this gene to CLEN is present in the literature so far.

A second purpose of the present work was the comparison between our DEG lists (transcriptomics data) with the one referring to differentially expressed proteins (e.g., proteomics data), for which the same muscle samples from the same animal were used (Stella et al. 2011). Interestingly, the comparison resulted into no common differentially regulated genes/protein in-between. In cattle, the proteomic approach has been explored as a tool for detection of protein expression patterns in skeletal muscles (Keady et al. 2013; Stella et al. 2014), body fluids (Draisci et al. 2007; Della Donna et al. 2009; Guglielmetti et al. 2014) and some target organs such as liver (Gardini et al. 2006). Until recently, there was an implicit assumption in the systems biology literature of the existence of proportional relationship between mRNA and protein expressions measured from a tissue. However, analysis of mRNA and protein expression data from the same cells under similar conditions have failed to show a high correlation between the two domains in multiple studies (Pascal et al. 2008; Ghazalpour et al. 2011). Moreover, small non coding RNAs (miRNA) and post-translational modifications such as phosphorylation, SUMOylation and ubiquitination have been shown to modulate the expression, regulation, stability and function of glucocorticoid molecular targets and pathways (Duma

et al. 2006; De Iudicibus et al. 2013). Therefore, finding no single gene overlap was somehow surprising, but further confirm that the transcriptomic and proteomic approaches are independent from each other, even if the target tissue is the same. This agrees with Timperio et al. (2009), who reported that proteomics and transcriptomics data seldom overlapped when compared upon analysis of liver samples from two different *Bos taurus* breeds. Hence, the need for an integrated approach against GPs' misuse in cattle seems to be required to improve the effectiveness of the indirect biomarker approach for screening purposes.

On the microarray level, the defined DEGs were able to distinguish our experimental groups, one from another. This has an effect on the efficiency of the analyses and the robustness of the obtained data. In addition, the validated and proposed set of biomarkers (16 genes, whose level of expression was measured by qPCR) grouped the field monitoring DEX-positive samples away from our experimental DEX group on the PCA; furthermore, they were also clustered in different batches on a dendrogram. This unexpected behavior of the monitoring samples prompted us to decide to use the whole microarray raw data set of all the monitoring samples along with ours, to check whether monitored commercial animals still behave differently from the experimentally treated ones or not. Interestingly, all of our samples grouped together and distinctively away from the other monitoring samples, that were represented on the PCA as previously reported in Pegolo et al. (2012). Moreover, this result was confirmed after repeating the PCA using our 12 samples against only the 4 DEX-positive samples. Despite the efforts made to decrease any possible outliers affecting samples' distribution on the PCA, we again had the 2 groups apart. Once again, variables such as breed (maybe), diet and breeding conditions (more probable), the use of different DEX-containing illicit protocols and/or new cocktails containing different GPs, could be the reason(s) for this conflict. Indeed, intraspecies transcriptomic comparison is needed to reveal the differences in drug response between animals under field and experimental conditions.

Conclusions

A qPCR-validated panel of 16 genes was able to distinguish the DEX-treated (both experimental and field-monitoring) from the untreated animals. This can render those genes – or a subset of it - applicable in the screening purposes for DEX-illicitly-treated animals. Still, more DEX-positive samples from the field are needed to validate this panel because different DEX 'cocktails' are expected to have different impact on the treated animals. Further, the present work showed that despite the attractions of comparative profiling of transcripts and proteins on a global 'omic' scale, there are obvious biological and technical differences preventing transcriptomics and proteomics from having a convergence. Moreover, the idea of having a "universal set of biomarkers" that can be applied to all the illicitly treated cattle still seems elusive at the moment. Indeed, one 'stand-alone' technology does not suffice for gaining a comprehensive understanding of the biological system complexity. An approach that incorporates the various 'omic' platforms and their data would be the key to solve this puzzle.

Supplementary materials

https://drive.google.com/drive/folders/0BxmPKazb5UxsdW4wUFh1WjdnWnM?usp=sharing

References

Actor JK, Breij E, Wetsel RA, Hoffmann H, Hunter RLJ, Jagannath C. 2001. A role for complement C5 in organism containment and granulomatous response during murine tuberculosis. Scand J Immunol. 53:464–474.

Almon RR, Dubois DC, Jin JY, Jusko WJ. 2005. Temporal profiling of the transcriptional basis for the development of corticosteroid-induced insulin resistance in rat muscle. J Endocrinol. 184:219–232.

Azuma M. 2010. Role of the glucocorticoid-induced TNFR-related protein (GITR)-GITR ligand pathway in innate and adaptive immunity. Crit Rev Immunol. 30:547–557.

Badino P, Odore R, Re G. 2005. Are so many adrenergic receptor subtypes really present in domestic animal tissues? A pharmacological perspective. Vet J. 170:163–174.

Balizs G, Hewitt A. 2003. Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry. Appl Liq Chromatogr coupled to Mass Spectrom Pharmacol. 492:105–131.

Beresford AP. 1993. CYP1A1: friend or foe? Drug Metab Rev. 25:503–517.

Bergkvist A, Rusnakova V, Sindelka R, Garda JM, Sjogreen B, Lindh D, Forootan A, Kubista M. 2010. Gene expression profiling--Clusters of possibilities. Methods. 50:323–335.

Biancotto G, Stella R, Pozza G, Stefani A, Lega F, Angeletti R. 2013. Sub-therapeutic treatments of bulls with dexamethasone: direct and indirect markers of treatment. Food Addit Contam A, Chem Anal Control Expo risk Assess. 30:430–442.

Burkina V, Zlabek V, Zamaratskaia G. 2013. Clotrimazole, but not dexamethasone, is a potent in vitro inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout. Chemosphere. 92:1099–1104.

Campbell JE, Peckett AJ, D'souza AM, Hawke TJ, Riddell MC. 2011. Adipogenic and lipolytic effects of chronic glucocorticoid exposure. Am J Physiol Cell Physiol. 300:C198–209.

Cannizzo FT, Pegolo S, Starvaggi Cucuzza L, Bargelloni L, Divari S, Franch R, Castagnaro M, Biolatti B. 2013. Gene expression profiling of thymus in beef cattle treated with prednisolone. Res Vet Sci. 95:540–547.

Cantiello M, Carletti M, Cannizzo FT, Nebbia C, Bellino C, Pie S, Oswald IP, Bollo E, Dacasto M, Pié S, et al. 2007. Effects of an illicit cocktail on serum immunoglobulins, lymphocyte proliferation and cytokine gene expression in the veal calf. Toxicology. 242:39–51.

Carraro L, Ferraresso S, Cardazzo B, Romualdi C, Montesissa C, Gottardo F, Patarnello T, Castagnaro M, Bargelloni L. 2009. Expression profiling of skeletal muscle in

young bulls treated with steroidal growth promoters. Physiol Genomics. 38:138–148.

Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiele M, Vercammen J, De Wasch K. 2002. Recent developments in the use and abuse of growth promoters. Pap Present 4th Int Symp Horm Vet Drug Residue Anal Antwerp,Belgium,4-7 June 2002 Part I. 473:71–82.

De Iudicibus S, Lucafo M, Martelossi S, Pierobon C, Ventura A, Decorti G. 2013. MicroRNAs as tools to predict glucocorticoid response in inflammatory bowel diseases. World J Gastroenterol. 19:7947–7954.

Della Donna L, Ronci M, Sacchetta P, Di Ilio C, Biolatti B, Federici G, Nebbia C, Urbani A. 2009. 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. 4:1596–1609.

Draisci R, Montesissa C, Santamaria B, D'Ambrosio C, Ferretti G, Merlanti R, Ferranti C, De Liguoro M, Cartoni C, Pistarino E, et al. 2007. Integrated analytical approach in veal calves administered the anabolic androgenic steroids boldenone and boldione: urine and plasma kinetic profile and changes in plasma protein expression. Proteomics. 7:3184–3193.

Duma D, Jewell CM, Cidlowski JA. 2006. Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J Steroid Biochem Mol Biol. 102:11–21.

Ehrchen J, Steinmuller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, Nordhues U, Sorg C, Sunderkotter C, Roth J. 2007. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. Blood. 109:1265–1274.

Ellero-Simatos S, Fleuren WW, Bauerschmidt S, Dokter WH, Toonen EJ. 2014. Identification of gene signatures for prednisolone-induced metabolic dysfunction in collagen-induced arthritic mice. Pharmacogenomics. 15:629–641.

Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH. 2000. Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron. 28:183–193.

Fisher I, Abraham D, Bouri K, Hoffman EP, Muntoni F, Morgan J. 2005. Prednisolone-induced changes in dystrophic skeletal muscle. FASEB J. 19:834–836.

Franchimont D, Galon J, Vacchio MS, Fan S, Visconti R, Frucht DM, Geenen V, Chrousos GP, Ashwell JD, O'Shea JJ. 2002. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. J Immunol (Baltimore, Md 1950). 168:2212–2218.

Franchimont D. 2004. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Ann N Y Acad Sci. 1024:124–137.

Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, O'Shea JJ, Chrousos GP, Bornstein SR. 2002. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J Off Publ Fed Am Soc Exp Biol. 16:61–71.

Gardini G, Del Boccio P, Colombatto S, Testore G, Corpillo D, Di Ilio C, Urbani A, Nebbia C. 2006. Proteomic investigation in the detection of the illicit treatment of calves with growth-promoting agents. Proteomics. 6:2813–2822.

Ghazalpour A, Bennett B, Petyuk VA, Orozco L, Hagopian R, Mungrue IN, Farber CR, Sinsheimer J, Kang HM, Furlotte N, et al. 2011. Comparative analysis of proteome and transcriptome variation in mouse. PLoS Genet. 7:e1001393.

Giantin M, Granato A, Baratto C, Marconato L, Vascellari M, Morello EM, Vercelli A, Mutinelli F, Dacasto M. 2014. Global gene expression analysis of canine cutaneous mast cell tumor: could molecular profiling be useful for subtype classification and prognostication? PLoS One. 9:e95481.

Goldberg IJ. 1996. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res. 37:693–707.

Goleva E, Hauk PJ, Hall CF, Liu AH, Riches DW, Martin RJ, Leung DY. 2008. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. J Allergy Clin Immunol. 122:550–9.e3.

Greer S, Page CW, Joshi T, Yan D, Newton R, Giembycz MA. 2013. Concurrent agonism of adenosine A2B and glucocorticoid receptors in human airway epithelial cells cooperatively induces genes with anti-inflammatory potential: a novel approach to treat chronic obstructive pulmonary disease. J Pharmacol Exp Ther. 346:473–485.

Griffon N, Budreck EC, Long CJ, Broedl UC, Marchadier DHL, Glick JM, Rader DJ. 2006. Substrate specificity of lipoprotein lipase and endothelial lipase: studies of lid chimeras. J Lipid Res. 47:1803–1811.

Guglielmetti C, Mazza M, Pagano M, Carrella S, Sciuto S, Nodari S, Pezzolato M, Richelmi GB, Baioni E, Caramelli M, et al. 2014. Identification by a proteomic approach of a plasma protein as a possible biomarker of illicit dexamethasone treatment in veal calves. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 31:833–8.

Himes BE, Jiang X, Wagner P, Hu R, Wang Q, Klanderman B, Whitaker RM, Duan Q, Lasky-Su J, Nikolos C, et al. 2014. RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells. PLoS One. 9:e99625.

Huang H, Gazzola C, Pegg GG, Sillence MN. 2000. Differential effects of dexamethasone and clenbuterol on rat growth and on beta2-adrenoceptors in lung and skeletal muscle. J Anim Sci. 78:604–608.

Ing NH, Forrest DW, Riggs PK, Loux S, Love CC, Brinsko SP, Varner DD, Welsh Jr TH. 2014. Dexamethasone acutely down-regulates genes involved in steroidogenesis in stallion testes. J Steroid Biochem Mol Biol. 143:451–459.

Ishibashi T, Takagi Y, Mori K, Naruse S, Nishino H, Yue BY, Kinoshita S. 2002. cDNA microarray analysis of gene expression changes induced by dexamethasone in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci. 43:3691–3697.

Jaaskelainen T, Makkonen H, Palvimo JJ. 2011. Steroid up-regulation of FKBP51 and its role in hormone signaling. Curr Opin Pharmacol. 11:326–331.

Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ. 1999. A novel endothelial-derived lipase that modulates HDL metabolism. Nat Genet. 21:424–428.

Kang CB, Hong Y, Dhe-Paganon S, Yoon HS. 2008. FKBP family proteins: immunophilins with versatile biological functions. Neurosignals. 16:318–325.

Keady SM, Kenny DA, Ohlendieck K, Doyle S, Keane MG, Waters SM. 2013. Proteomic profiling of bovine M. longissimus lumborum from Crossbred Aberdeen Angus and Belgian Blue sired steers varying in genetic merit for carcass weight. J Anim Sci. 91:654–665.

Kemppainen RJ, Behrend EN. 1998. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. J Biol Chem. 273:3129–3131.

Kino T. 2007. Tissue glucocorticoid sensitivity: beyond stochastic regulation on the diverse actions of glucocorticoids. Horm Metab Res. 39:420–424.

König T, Schuberth HJ, Leibold W, Zerbe H. 2006. Dexamethasone depresses the expression of L-selectin but not the in vivo migration of bovine neutrophils into the uterus. Theriogenology. 65:1227–1241.

Kumar S, Shah S, Deutsch ER, Tang HM, Danias J. 2013. Triamcinolone acetonide decreases outflow facility in C57BL/6 mouse eyes. Invest Ophthalmol Vis Sci. 54:1280–1287.

Laporta J, Rosa GJ, Naya H, Carriquiry M. 2014. Liver functional genomics in beef cows on grazing systems: novel genes and pathways revealed. Physiol Genomics. 46:138–147.

Leporati M, Bergoglio M, Capra P, Bozzetta E, Abete MC, Vincenti M. 2014. Development, validation and application to real samples of a multiresidue LC-MS/MS method for determination of beta2 -agonists and anabolic steroids in bovine hair. J Mass Spectrom. 49:936–946.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–8.

Louten J, Mattson JD, Malinao MC, Li Y, Emson C, Vega F, Wardle RL, Van Scott MR, Fick RB, McClanahan TK, et al. 2012. Biomarkers of disease and treatment in murine and cynomolgus models of chronic asthma. Biomark Insights. 7:87–104.

Luesink M, Jansen JH. 2010. Advances in understanding the pulmonary infiltration in acute promyelocytic leukaemia. Br J Haematol. 151:209–220.

Mak JC, Nishikawa M, Shirasaki H, Miyayasu K, Barnes PJ. 1995. Protective effects of a glucocorticoid on downregulation of pulmonary beta 2-adrenergic receptors in vivo. J Clin Invest. 96:99–106.

Mao W, Tovar-Vidales T, Yorio T, Wordinger RJ, Clark AF. 2011. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. Invest Ophthalmol Vis Sci. 52:8068–8075.

Martin S, Okano S, Kistler C, Fernandez-Rojo MA, Hill MM, Parton RG. 2009. Spatiotemporal regulation of early lipolytic signaling in adipocytes. J Biol Chem. 284:32097–32107.

Mashruwala MA, Smith AK, Lindsey DR, Moczygemba M, Wetsel RA, Klein JR, Actor JK, Jagannath C. 2011. A defect in the synthesis of Interferon-gamma by the T cells of Complement-C5 deficient mice leads to enhanced susceptibility for tuberculosis. Tuberculosis (Edinb). 91 Suppl 1:S82–9.

Masuno K, Haldar SM, Jeyaraj D, Mailloux CM, Huang X, Panettieri RAJ, Jain MK, Gerber AN. 2011. Expression profiling identifies Klf15 as a glucocorticoid target that regulates airway hyperresponsiveness. Am J Respir Cell Mol Biol. 45:642–649.

Monostory K, Kohalmy K, Prough RA, Kobori L, Vereczkey L. 2005. The effect of synthetic glucocorticoid, dexamethasone on CYP1A1 inducibility in adult rat and human hepatocytes. FEBS Lett. 579:229–235.

Morgan JT, Wood JA, Walker NJ, Raghunathan VK, Borjesson DL, Murphy CJ, Russell P. 2014. Human trabecular meshwork cells exhibit several characteristics of, but are distinct from, adipose-derived mesenchymal stem cells. J Ocul Pharmacol Ther. 30:254–266.

Möstl E, Messmann S, Bagu E, Robia C, Palme R. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Zentralbl Veterinarmed A. 46:621–631.

Nojima M, Maruyama R, Yasui H, Suzuki H, Maruyama Y, Tarasawa I, Sasaki Y, Asaoku H, Sakai H, Hayashi T, et al. 2009. Genomic screening for genes silenced by DNA methylation revealed an association between RASD1 inactivation and dexamethasone resistance in multiple myeloma. Clin Cancer Res. 15:4356–4364.

Odore R, Badino P, Barbero R. 2007. Regulation of tissue β-adrenergic, glucocorticoid and androgen receptors induced by repeated exposure to growth promoters in male veal calves. Res Vet. 83:227–233.

Odore R, Badino P, Pagliasso S, Nebbia C, Cuniberti B, Barbero R, Re G. 2006. Changes in lymphocyte glucocorticoid and beta-adrenergic receptors in veal calves treated with clenbuterol and steroid hormones for growth-promoting purposes. J Vet Pharmacol Ther. 29:91–97.

Oliveira LJ, McClellan S, Hansen PJ. 2010. Differentiation of the endometrial macrophage during pregnancy in the cow. PLoS One. 5:e13213.

Pascal LE, True LD, Campbell DS, Deutsch EW, Risk M, Coleman IM, Eichner LJ, Nelson PS, Liu AY. 2008. Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. BMC Genomics. 9:246.

Pegolo S, Gallina G, Montesissa C, Capolongo F, Ferraresso S, Pellizzari C, Poppi L, Castagnaro M, Bargelloni L. 2012. Transcriptomic markers meet the real world: finding diagnostic signatures of corticosteroid treatment in commercial beef samples. BMC Vet Res. 8:205.

Peinado-Onsurbe J, Soler C, Galan X, Poveda B, Soley M, Llobera M, Ramirez I. 1991. Involvement of catecholamines in the effect of fasting on hepatic endothelial lipase activity in the rat. Endocrinology. 129:2599–2606.

Qiu G, Ho AC, Yu W, Hill JS. 2007. Suppression of endothelial or lipoprotein lipase in THP-1 macrophages attenuates proinflammatory cytokine secretion. J Lipid Res. 48:385–394.

Riedmaier I, Pfaffl MW. 2013. Transcriptional biomarkers--high throughput screening, quantitative verification, and bioinformatical validation methods. Methods. 59:3–9.

Riedmaier I, Spornraft M, Pfaffl MW. 2014. Identification of a potential gene expression biomarker signature in bovine liver to detect the abuse of growth promoters. Food Addit Contam A, Chem Anal Control Expo risk Assess. 31:641–649.

Rothwell NJ, Stock MJ. 1987. Effect of a selective beta 2-adrenergic agonist (clenbuterol) on energy balance and body composition in normal and protein deficient rats. Biosci Rep. 7:933–940.

Rozsa FW, Reed DM, Scott KM, Pawar H, Moroi SE, Kijek TG, Krafchak CM, Othman MI, Vollrath D, Elner VM, Richards JE. 2006. Gene expression profile of human trabecular meshwork cells in response to long-term dexamethasone exposure. Mol Vis. 12:125–141.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34:374–378.

Smoak K, Cidlowski JA. 2006. Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol Cell Biol. 26:9126–9135.

Stejskalova L, Rulcova A, Vrzal R, Dvorak Z, Pavek P. 2013. Dexamethasone accelerates degradation of aryl hydrocarbon receptor (AHR) and suppresses CYP1A1 induction in placental JEG-3 cell line. Toxicol Lett. 223:183–191.

Stella R, Arrigoni G, Biancotto G, Krogh M, Vascellari M, Lega F, Pozza G, Angeletti R, Andrighetto I, James P. 2014. Confirmation of protein biomarkers of corticosteroids treatment in veal calves sampled under field conditions. J Proteome Res. 13:1794–1799.

Stella R, Biancotto G, Krogh M, Angeletti R, Pozza G, Sorgato MC, James P, Andrighetto I. 2011. Protein expression changes in skeletal muscle in response to growth promoter abuse in beef cattle. J Proteome Res. 10:2744–2757.

Timperio AM, D'Alessandro A, Pariset L, D'Amici GM, Valentini A, Zolla L. 2009. Comparative proteomics and transcriptomics analyses of livers from two different Bos taurus breeds: "Chianina and Holstein Friesian." J Proteomics. 73:309–322.

Tissing WJ, den Boer ML, Meijerink JP, Menezes RX, Swagemakers S, van der Spek PJ, Sallan SE, Armstrong SA, Pieters R. 2007. Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood. 109:3929–3935.

Tu Y, Wu C. 1999. Cloning, expression and characterization of a novel human Ras-related protein that is regulated by glucocorticoid hormone. Biochim Biophys Acta. 1489:452–456.

Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 98:5116–5121.

Vascellari M, Katia C, Annalisa S, Giancarlo B, Letizia M, Roberto S, Giandomenico P, Franco M. 2012. Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. BMC Vet Res. 8:129.

Vrzal R, Stejskalova L, Monostory K, Maurel P, Bachleda P, Pavek P, Dvorak Z. 2009. Dexamethasone controls aryl hydrocarbon receptor (AhR)-mediated CYP1A1 and CYP1A2 expression and activity in primary cultures of human hepatocytes. Chem Biol Interact. 179:288–296.

Xu C, He J, Jiang H, Zu L, Zhai W, Pu S, Xu G. 2009. Direct effect of glucocorticoids on lipolysis in adipocytes. Mol Endocrinol. 23:1161–1170.

Zhou J, Cidlowski JA. 2005. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids. 70:407–417.

II. The transcriptome of muscle and liver is responding differently to a combined trenbolone acetate and estradiol implant in cattle[2]

[2] Adapted- and reproduced in the current layout- from: **Elgendy, R.,** Giantin, M., Montesissa, C., & Dacasto, M. **(2016). The transcriptome of muscle and liver is responding differently to a combined trenbolone acetate and estradiol implant in cattle. Steroids, 106, 1-8.**
http://www.sciencedirect.com/science/article/pii/S0039128X15002809

Abstract

We investigated the transcriptomic signature of some anabolic steroids in cattle. Our main objective was to evaluate the effect of a combined trenbolone acetate (TBA, 200 mg) and estradiol-17β (E2, 40 mg) implant (Revalor-XS®, REV) on the transcriptome of muscle (target tissue for anabolic steroids) and liver (main biotransformation site). Transcriptomic profiling was performed on 60 samples (30 per tissue) representing 2 groups of animals: REV (sustained release implant for 71 days, n = 15), and a control group (CTR, n = 15). The analyses (REV vs. CTR) evidenced the differential expression of 431 (down-regulated) and 503 transcripts (268 up-regulated and 235 down-regulated) in muscle and liver tissues, respectively. Functional annotation showed the enrichment of several ion transport systems (cation, metal ion and potassium ion transport) in muscle, while revealing the enrichment of carbohydrate, protein and glycoprotein metabolism and biosynthesis mechanisms in the liver. Both tissues had 20 genes commonly expressed in-between. Seven randomly-selected genes showed positive correlation with their corresponding microarray data upon a qPCR cross-validation step. In muscle, but not the liver, Principal Component Analysis (PCA) on the microarray data resulted in the separation of treated animals from the untreated ones (first 2 components = 97.87%.). Overall, the identification of different genes, pathways and biological processes has illustrated the distinctive transcriptomic profile of muscle and liver in response to anabolic steroids. Moreover, it is becoming more clear that anabolic steroids are working through a complex interaction of numerous pathways and processes incorporating different tissues.

Introduction

Beef cattle production is a strong animal industry worldwide. The economic challenges and the profit-oriented strategies in this business are, logically, favoring the cost-reduction procedures. Growth promoters (GPs), such as anabolic steroids, are used to increase animal productivity and feed conversion rates; hence, reducing costs. Although they are legally and widely used in the USA, anabolic steroids are prohibited by law in the European Union (European Union 1996 & 2002) because of their public health concerns. Despite this ban, GPs are still illicitly employed in bovine meat production due to their economic benefits resulting from the improved animal growth (Courtheyn et al.

2002; White et al. 2003; De Brabander et al. 2007; Mooney et al. 2009). Subcutaneous implantation of androgenic and estrogenic compounds, such as trenbolone acetate (TBA) and β-estradiol (E2), administered alone or in combination, is one of the most common ways of anabolic steroid applications for cattle, due to its sustained release that can last up to 200 days (Food and Drug Administration; FDA 2007).

On a monitoring-and-drug-abuse-discovery basis, some studies have been performed for the indirect detection of TBA and E2 in cattle in Europe, in which investigations on transcriptomic profiling of thymus (Cannizzo et al. 2013), uterine endometrium (Becker et al. 2011), vaginal smears (Riedmaier et al. 2011) and muscle (De Jager et al. 2011; Pegolo et al. 2014) have been conducted. In the same context, other groups have investigated the use of alternative "omic" techniques, such as proteomics (Stella et al. 2015) and metabolomics (Jacob et al. 2014; Kouassi Nzoughet et al. 2015).

Being the main target organ of such anabolic steroids, muscle has been given the main attention in the few "omic" studies that investigated the effect of TBA and/or E2 on muscle either in a physiological (De Jager et al. 2011) or detection and monitoring context (Becker et al. 2011; Parr et al. 2011; Cannizzo et al. 2013; Pegolo et al. 2014; Stella et al. 2015). However, despite the well-documented effectiveness of anabolic steroid implants in increasing bovine muscle growth, information on the mechanism of action in nonmuscle tissues (e.g. Liver) have not been extensively studied (Dayton & White 2014). Moreover, the liver has never been considered in a global gene expression microarray analysis following TBA or E2 administration, and was only considered either in direct residue detection studies (MacNeil et al. 2003; MacNeil et al. 2008; Wang et al. 2009) or mRNA expression of a set of pre-defined genes (White et al. 2003; Reiter et al. 2007; Giantin et al. 2010). The fact that anabolic steroids can cause a number of serious side effects, including immunity and liver dysfunction (Brenu et al. 2011) was a further reason for the inclusion of liver in our study.

In the present study, we conducted a comparative investigation on bulls treated by a combination of anabolic compounds (sexual steroids) forbidden in the European Union, and untreated bulls. We used a commercially available ear implant containing 200 mg of TBA and 40 mg of E2 (Revalor-XS®, Merck Animal Health, USA) that is approved in the United States in bovine breeding. To the best of our knowledge, this was the first comparative global gene expression profiling study to be performed on both muscle and

liver of cattle implanted with Revalor-XS®. The primary aim of this study was to compare, via transcriptomic approach, the response of both muscle and liver to anabolic steroids, and to point out the main differences and similarities between both matrices. In addition, a second aim was to list out the main differentially expressed (DE) genes that can be used as a "pool" for biomarker investigations in the future.

Materials and methods

Animals and experimental design

Animals in this study were part of a transcriptomic-proteomics comparative trial, where 30 Charolais cattle, all males, aged 10–14 months, were randomly divided into two groups of 15 animals each. The first group received no treatment and served as a control (CTR), while the second was exposed for 71 days to the steroid hormone subcutaneous implant Revalor-XS® (group REV). The beef cattle were weighed at the beginning and the end of the treatment. The average daily gain (ADG) was calculated as the difference between two subsequent body weights. The procedure was checked and approved by the Animal Experimentation Ethics Committee of the University of Bologna on January 31, 2011 (PROT: 8134-X/10 and 4783-X/10). Health status was monitored daily by recording all individual pathological events and medical treatments. The experimental workflow is shown in **Figure 1**.

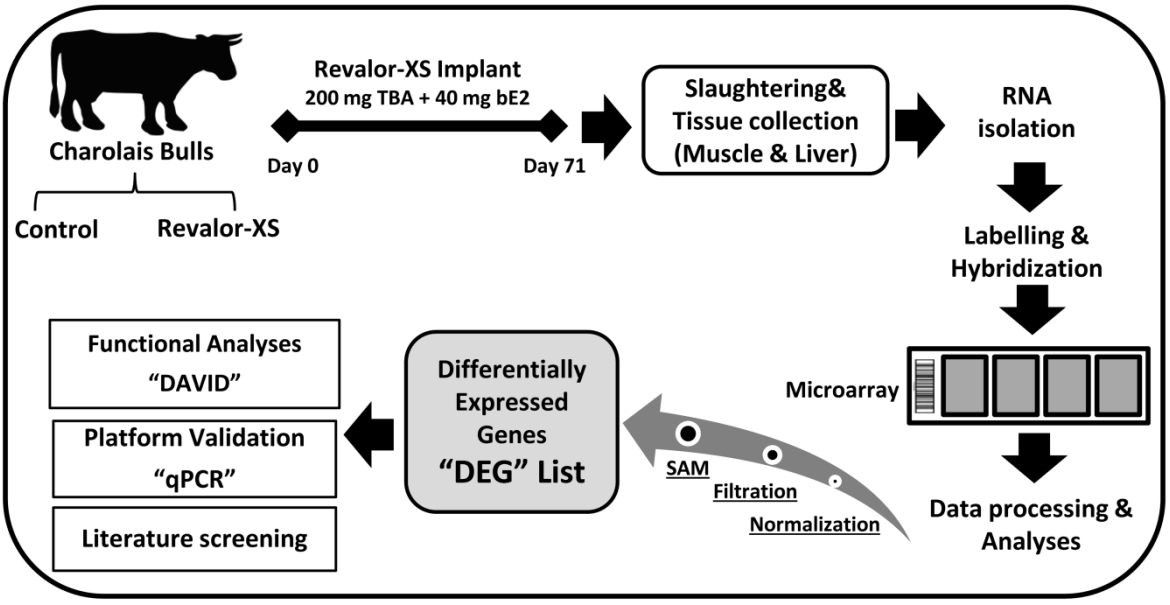

Figure 1: *General workflow of the experiment.*

Sample collection and RNA extraction

At the slaughterhouse, small biceps brachii muscle and liver specimens were sampled from all the animals. Both muscle and liver samples were immediately stored in RNAlater solution (Life Technologies, USA), then stored at -80ºC until analyses. Total RNA was isolated by TRIzol® reagent (Life Technologies, USA) and subsequently purified using the RNeasy Mini kit (Qiagen, Italy), according to the manufacturer's instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Italy) was performed. Total RNA concentration was determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDropTechnologies, USA), and its quality was measured by using the 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, USA). The isolated RNAs were tested for proper concentration and integrity. All the 60 samples (30 from muscle and 30 from liver) passed the RNA quality criteria [i.e: RNA concentration ≥ 40 ng/µl and RNA integrity number (RIN) ≥ 7]. Hence, they were all considered for the subsequent microarray analyses.

RNA amplification, labeling and hybridization

Sample amplification, labeling and hybridization were performed following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Briefly, each of the 60 RNA samples was labeled with Cy3 (green) fluorescent dye label using Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, USA). Ten different viral polyadenylated RNAs were used as reference "spikes" (Spike-In Mix, Agilent Technologies, USA). A purification step was applied to the labeled cRNA using the RNeasy Mini kit (Qiagen, Italy), and sample concentration and specific activity (pmol Cy3/µg cRNA) were measured. A total of 1.65 µg of labeled cRNA was fragmented using the Gene Expression Hybridization kit (Agilent, USA) according to the manufacturer's instructions, and finally diluted by the addition of 55 µL of 2X GE Hybridization buffer. A volume of 100 µL of hybridization solution was then dispensed in the gasket slide and assembled to the microarray slide, with each slide containing four arrays. Bovine-specific oligo-arrays (Bovine V1, 4x44k G2519F, Design ID 015354, Agilent Technologies, USA) were used. The slides were firstly incubated for 17 h at 65ºC in a hybridization oven (Agilent Technologies, USA), then washed using wash buffer 1 and 2 according to the manufacturer's instructions. The hybridized slides were scanned at 5 µm resolution using a G2565BA DNA microarray scanner (Agilent Technologies, USA). The default settings were modified in order to scan the same slide twice at two different sensitivity

levels (XDR Hi 100% and XDR Lo 10%). The total RNA of 4 samples was labeled twice and hybridized separately in different slides to generate technical replicates. The entire set of expression data corresponding to the 60 hybridizations was deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE62002.

Normalization of microarray data and identification of DE genes

Microarray data of both muscle and liver samples (n = 30, each) were processed, normalized and analyzed independently from each other. The data were extracted and the background subtracted by using the default settings of the Agilent Feature Extraction Software version 9.5.1 (Agilent Technologies, USA). The extracted data were normalized and processed as previously described (Giantin et al. 2014). A further filtering step was carried out by removing probes that reported missing values or no reactivity (flag equal to 0) in at least 50% of the samples. Missing values (probes with Feature Extraction flag equal to 0) were imputed by using the microarray data analysis tool TIGR Multiple Array Viewer [TMEV: (Saeed et al. 2003)].

Functional enrichment analysis

Enrichment analysis of the differentially up- and down-regulated genes was performed using the Functional Annotation tool available in the DAVID Database (http://david.abcc.ncifcrf.gov/). Only the probes present in the microarray chip were used as a 'gene reference background'. All GO terms and KEGG pathways included in the DAVID knowledge base were considered. For KEGG terms, the following parameters were used: gene count 5, EASE 0.05. For GO, Biological Process and Molecular Function (BP_FAT and MF_FAT, respectively), gene count 5 and EASE 0.05 were used.

Quantitative Real time RT-PCR (qPCR) validation

Seven randomly selected genes (2 for muscle and 5 for liver datasets), along with other three internal control ones (RPLP0, GAPDH and TBP) were chosen for the external validation of microarray findings by qPCR. For each candidate gene, a short description, the accession number, primer sequences, the corresponding Universal Probe Library (UPL) probe, and amplicon length are provided in **Table 1**.

Table 1. Genes and primers used for the qPCR validation assays

Gene	Description	Accession	Forward primer (5' >> 3')	Reverse primer (5' >> 3')	UPL Probe	Amplico -n (bp)
NR4A2	Nuclear receptor subfamily 4, group A, member 2	NM_001076208	CGGTCTCAAGGAACCCAAG	CGGTTCAAACCCCCATTATT	25	96
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	NM_001166499	GTGATTGTTGGTTCGCCTTT	TGCCCTCATGACCATTGTAG	50	70
ANGPTL4	Angiopoietin 4	NM_001046043	TGGCTCCGTGGACTTTAACC	GATGGGGAACTGCAAGGACT	135	178
GK	Glycerol kinase	NM_001075236	CTCGGCAGAGATAAACCCGT	ATAAGGTGCATACAGCCCCG	46	192
HAMP	Hepcidin antimicrobial peptide	NM_001114508	AGACACGACAGCTCACAGAC	ATGGGAAAGTGGGTGTCTCG	50	103
MT2A	Metallothionein 2A	NM_001075140	CAAAGATTGCAAGTGCGCCTC	CAGCTGCACTTGTCCGAAGC	104	114
MAP3K14	Mitogen-activated protein kinase kinase kinase 14	NM_001192178	CCGGTGGATTATGAGTACCG	CTGTGGACCTCTCCAAAGGA	113	86
RPLP0[a]	Ribosomal protein, large, P0	NM_001012682	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	145	227
GAPDH[a]	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	ACACCCTCAAGATTGTCAGCAA	TCATAAGTCCCTCCACGATGC	119	82
TBP[a]	TATA box binding protein	NM_001075742	ACAACAGCCTCCCACCCTATGC	GTGGAGTCAGTCCTGTGCCGTAA	81	111

[a] *Internal control gene*

Where possible, primers were designed across exon/intron boundaries to avoid genomic DNA amplification. First-strand cDNA was synthesized from 0.5 µg of total RNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies, USA) according to the manufacturer's protocol and stored at -20ºC until further use. The primer specificity was evaluated at first in silico by means of the Primer-BLAST tool, then by Power SYBR Green I (Life Technologies, USA) amplification and melting curve analysis. Overall, qPCR reactions (10 µL final volume) consisted of 1X LightCycler 480 Probe Master (Roche Applied Science, USA), 300 or 600 nM forward and reverse primers (Integrated DNA Technology, Italy) according to the assay set-up, 200 nM human UPL probe (final concentrations) and 2.5 µL of 1:25 diluted cDNA (50 ng/µL). Each qPCR analysis was performed, in duplicate, in a LightCycler 480 instrument (Roche Applied Science, USA) using the standard PCR conditions (an activation step at 95ºC for 10 minutes; forty-five cycles at 95ºC for 10 seconds and at 60ºC for 30 seconds; a cooling step at 40ºC for 30 seconds) and LightCycler 480 clear plates (Roche Applied Science, USA). To determine the efficiency of each qPCR assay, non-template and no-reverse transcription controls were included in each plate. Moreover, standard curves obtained by amplifying eight-threefold serial dilution of the same cDNA pool were used. Data were analyzed with the LightCycler 480 software release 1.5.0 (Roche Applied Science, USA) using either the second derivative or the fit point method. Messenger RNA relative quantification was performed according to the ΔΔCt method (Livak & Schmittgen 2001).

Statistics and Principal Component Analysis (PCA)

To identify the DE genes, a two-class unpaired test was implemented in the program SAM (Significance Analysis of Microarrays) release 4.0 (Tusher et al. 2001), enforcing a False Discovery Rate (FDR) of less than 5% with a 1.5-fold change (FC) threshold. All other statistical tests (non-parametric Spearman correlation analysis and Mann-Whitney test) were carried out by the GraphPad Prism 5 software (San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Principal components analysis is a technique used to reduce multidimensional data sets to lower dimensions for analysis. This statistical method was used to determine whether there was a clustering, based on DE genes, between control and treatment groups. Following SAM, the corresponding normalized microarray intensities of all the DE

transcripts were extracted from both datasets (muscle and liver), and used to generate separate PCA using TMEV.

Results

Phenotypic measures

The health status of all the experimental animals was satisfactory all throughout the experiment. No drugs (other than Revalor-XS®) have been administered during the experimental procedure. Detailed information about body weight gain and feed conversion ratio have been presented elsewhere (Stella et al. 2015).

Microarray quality assessment and data analyses

A mean RIN values of 7.76 ± 0.40 and 7.90 ± 0.37 for muscle and liver tissues, respectively, were enough to guarantee all the 60 samples access to the microarray labelling and hybridization process. The used Agilent microarray platform contains, by default, a total number of 44,407 features (spots), of which 42,990 features consist of 60-mer probes that correspond to 21,475 unique transcripts. All the non-biological control probes (n = 1097) and the first two spikes that were out of range in the log relative concentration plot (n = 64), were excluded from the dataset before the quantile normalization was applied. Due to the relatively robust number of samples in each dataset (30 per tissue), only 3 and 2 probe intensities were excluded from muscle and liver datasets, respectively, as they did not meet the criteria for inclusion. The processed signals for 42,987 and 42,988 probes in muscle and liver, respectively, were finally analyzed by SAM to identify the DE genes between REV and CTR groups. Finally, to cross-validate our platform performance, a relative quantification using qPCR was performed. The quantitative changes observed in the DNA microarray for the selected genes were confirmed by qPCR, and a positive correlation in the expression values between the two methods was found, confirming the experimental reliability (**Table 2**).

Table 2. qPCR cross-validation and correlation coefficient between qPCR and Microarray.

Gene	FC* qPCR	FC array	Spearman's rho
NR4A2	-2.08	-2.08	0.922
ITAG2	-1.17	-2.37	0.127
ANGPTL4	3.89	2.67	0.935
GK	2.11	2.34	0.946
HAMP	1.89	2.09	0.919
MT2-A	1.8	2.01	0.933
MAP3K14	1.16	2.29	0.368

** Fold Change (FC) was calculated comparing REV vs. CTR group.*

Transcriptomic signature of Revalor-XS®-71 days- treatment in muscle and liver tissues

In muscle, the comparison between REV and CTR groups (REV vs. CTR) resulted in a list of 431 down-regulated transcripts. Following a careful re-annotation process and genome database screening, a total of 84 transcripts (corresponding to 84 unique probes ID on the Agilent bovine V.1 microarray slide) appeared to be withdrawn or discontinued records from the gene bank database, and hence they were excluded from our final DE list. Moreover, among the DE transcripts there were 64 estimated sequence tags (ESTs) that we also did not include in the final DE list. The remaining 283 characterized and fully annotated transcripts were considered in the functional analyses. A full list of all the 431 DE transcripts in the muscle tissue is provided in *Supplementary Table 1*. The enrichment analysis using the Functional Annotation tool in DAVID identified several GO terms and KEGG pathways that were significantly enriched in the REV group of muscle samples. Noteworthy, there were several genes involved in the transmembrane transport (17 genes) and ion transport (19 genes). Significant enrichment of other biological transport systems (i.e. cation, metal ion and potassium ion transport) were also observed. In addition, cytokine receptor interaction and Jak-STAT signaling pathways were also enriched (**Table 3**).

Table 3. GO Biological Process, GO Molecular functions and KEGG pathway analysis of DE genes. DAVID functional annotation of the complete list of differentially regulated genes between CTR and REV groups (REV vs. CTR) in muscle tissue.

Category and GO term	**Count**	**Genes**	***P*-value**	**FE**
BP: Transmembrane transport	17	KCND3, SLC26A2, SLC9A3, TRPC2, CACNA1E, SLC5A9 ,KCNQ3, SLC17A2, LOC522784, SLC25A45, KCNG2, RHCG, ATP4A, KCNS1, SLC2A5, CACNA1G, KCNG3	0.0002	2.95
BP: Ion transport	19	KCNJ6, KCND3, SLC26A2, SLC9A3, TRPC2, CACNA1E, SCNN1B, KCNQ3, ATP1A3, SLC17A2, KCNG2, GABRE, RHCG, ATP4A, KCNS1, HTR3B,, CACNA1G, KCNG3, FXYD7	0.0005	2.51
BP: Cation transport	15	KCNJ6, KCND3, SLC9A3, TRPC2, CACNA1E, SCNN1B, KCNQ3, ATP1A3, SLC17A2, KCNG2, RHCG, ATP4A, KCNS1, CACNA1G, KCNG3	0.0007	2.84
BP: Monovalent inorganic cation transport	11	KCNJ6, KCNG2, KCND3, SLC9A3, SCNN1B, ATP4A, KCNQ3, KCNS1, ATP1A3, SLC17A2, KCNG3	0.0009	3.57
BP: Metal ion transport	12	KCNJ6, KCNG2, KCND3, SLC9A3, CACNA1E, TRPC2, SCNN1B, KCNQ3,, KCNS1, SLC17A2, CACNA1G, KCNG3	0.0031	2.85
BP: Potassium ion transport	6	KCNJ6, KCNG2, KCND3, KCNQ3, KCNS1, KCNG3	0.0169	3.98
MF: Voltage-gated cation channel activity	8	KCNJ6, KCNG2, KCND3, CACNA1E, KCNQ3, KCNS1, CACNA1G, KCNG3	0.0004	5.81
MF: Ion channel activity	13	KCNJ6, GABRE, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, HTR3B, CACNA1G, KCNG3, FXYD7	0.0005	3.35
MF: Substrate specific channel activity	13	KCNJ6, GABRE, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, HTR3B, CACNA1G, KCNG3, FXYD7	0.0005	3.31
MF: Channel activity	13	KCNJ6, GABRE, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, HTR3B, CACNA1G, KCNG3, FXYD7	0.0006	3.26
MF: Passive transmembrane transporter activity	13	KCNJ6, GABRE, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, HTR3B, CACNA1G, KCNG3, FXYD7	0.0006	3.26
MF: Cation channel activity	10	KCNJ6, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, CACNA1G, KCNG3	0.0006	4.18
MF: Gated channel activity	11	KCNJ6, GABRE, KCNG2, KCND3, CACNA1E, SCNN1B, KCNQ3, KCNS1, HTR3B, CACNA1G, KCNG3	0.0007	3.72
MF: Voltage-gated channel activity	8	KCNJ6, KCNG2, KCND3, CACNA1E, KCNQ3, KCNS1, CACNA1G, KCNG3	0.0016	4.64

MF: Voltage-gated ion channel activity	8	KCNJ6, KCNG2, KCND3, CACNA1E, KCNQ3, KCNS1, CACNA1G, KCNG3	0.0016	4.64
MF: Metal ion transmembrane transporter activity	10	KCNJ6, KCNG2, KCND3, CACNA1E, TRPC2, SCNN1B, KCNQ3, KCNS1, CACNA1G, KCNG3	0.0019	3.56
MF: Iron ion binding	11	CYP4F2, RSAD2, SCD, CYP4A11, CYP26C1, HPX, HBE1, TBXAS1, TPO, NOS2, MIOX	0.0019	3.26
MF: Heme binding	7	CYP4F2, CYP4A11, CYP26C1, HBE1, TBXAS1, TPO, NOS2	0.0034	4.74
MF: Voltage-gated potassium channel activity	6	KCNJ6, KCNG2, KCND3, KCNQ3, KCNS1, KCNG3	0.0037	5.75
MF: Tetrapyrrole binding	7	CYP4F2, CYP4A11, CYP26C1, HBE1, TBXAS1, TPO, NOS2	0.0044	4.52
MF: Potassium channel activity	6	KCNJ6, KCNG2, KCND3, KCNQ3, KCNS1, KCNG3	0.0103	4.50
KEGG pathway:Cytokine-cytokine receptor interaction	10	CRLF2, TNFRSF8, FLT4, IFNAC, IL2RG, IL15RA, OSM, TPO, CXCR4, CCL4	0.0011	3.74
KEGG pathway: Neuroactive ligand-receptor interaction	10	GABBR2, GABRE, P2RY2, GIPR, GRPR, SSTR1, NTSR1, APLNR, F2RL2, GALR2	0.0061	2.91
KEGG pathway: Jak-STAT signaling pathway	6	CRLF2, IFNAC, IL2RG, IL15RA, OSM, TPO	0.0427	3.07

GO: gene ontology; BP: biological process; MF: molecular function; 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.

Regarding the liver tissue, 503 transcripts were found to be differentially expressed in the REV group when compared to the CTR one. Among these ones, 268 and 235 were up- and down-regulated, respectively (*Supplementary Table 2*). Following the same strategy of re-checking the status of those transcripts in the GenBank database, 57 and 104 transcripts were excluded from the final list, as they were either withdrawn or EST sequences, respectively. A final list of 342 fully annotated DE transcripts was analyzed using DAVID. Functional annotation of liver DE transcripts (n = 342) evidenced the enrichment of many GO terms which were mainly biological processes incorporating carbohydrate, protein and glycoprotein metabolism and biosynthesis mechanisms. In addition, MAPK signaling pathways was the only KEGG pathway significantly enriched in this list (**Table 4**).

Revalor-XS® modulated common genes, but no functional GO terms or pathways, in both muscle and liver

Twenty genes were found to be commonly expressed between muscle and liver data sets (*Supplementary Table 3*). Among these genes, some steroid-related genes such as the gamma-aminobutyric acid B receptor 2 (GABBR2) and the glutamate decarboxylase 1 (GAD1) were represented. All the genes were down-regulated in both tissues, except for fatty acid synthase (FASN) gene, which was down-regulated in muscle (FDR 0%) and up-regulated in liver (FDR 3.43%).

Table 4. GO Biological Process, GO Molecular functions and KEGG pathway analysis of DE genes. DAVID functional annotation of the complete list of differentially regulated genes between CTR and REV groups (REV vs. CTR) in liver tissue.

Category and GO term	Count	Genes	*P*-value	FE
BP: Carbohydrate biosynthetic process	7	CHST10, PMM2, ATF4, ATF3, B4GALT1, CHST12, G6PC	0.0008	6.24
BP: ncRNA metabolic process	9	METTL1, TBL3, MOCS3, FTSJ1, WARS2, FARSA, WDR4, DIMT1, CARS	0.0038	3.53
BP: Glycoprotein biosynthetic process	7	ST3GAL1, PHLDA1, MGAT1, B4GALT1, B3GNT6, CHST12, ST6GALNAC2	0.0039	4.60
BP: RNA modification	5	METTL1, MOCS3, FTSJ1, WDR4, DIMT1	0.0050	7.11
BP: Cellular carbohydrate biosynthetic process	5	PMM2, ATF4, ATF3, B4GALT1, G6PC	0.0072	6.42
BP: Glycoprotein metabolic process	7	ST3GAL1, PHLDA1, MGAT1, B4GALT1, B3GNT6, CHST12, ST6GALNAC2	0.0100	3.80
BP: Ribosome biogenesis	6	TSR1, TBL3, BYSL, FTSJ1, MRTO4, DIMT1	0.0102	4.51
BP: Hexose metabolic process	7	PMM2, RPIA, ATF4, ATF3, B4GALT1, ENO2, G6PC	0.0192	3.29
BP: tRNA metabolic process	6	METTL1, MOCS3, WARS2, FARSA, WDR4, CARS	0.0211	3.76
BP: Ribonucleoprotein complex biogenesis	6	TSR1, TBL3, BYSL, FTSJ1, MRTO4, DIMT1	0.0221	3.71
BP: Monosaccharide metabolic process	7	PMM2, RPIA, ATF4, ATF3, B4GALT1, ENO2, G6PC	0.0299	2.97
BP: Protein amino acid glycosylation	5	ST3GAL1, MGAT1, B4GALT1, B3GNT6, ST6GALNAC2	0.0412	3.81
BP: Biopolymer glycosylation	5	ST3GAL1, MGAT1, B4GALT1, B3GNT6, ST6GALNAC2	0.0412	3.81
BP: Glycosylation	5	ST3GAL1, MGAT1, B4GALT1, B3GNT6, ST6GALNAC2	0.0412	3.81
BP: ncRNA processing	6	METTL1, TBL3, MOCS3, FTSJ1, WDR4, DIMT1	0.0422	3.13
MF: Sequence-specific DNA binding	15	GSX1, NFIL3, MAFB, CEBPD, ATF3, CREB3L3, ONECUT3, GATAD2A, LHX1, ZHX3, ATF4, XBP1, CRX, ESRRB, SIX1	0.0012	2.70
MF: Transcription factor activity	17	NFIL3, MAFB, CEBPD, NFIX, MYC, ATF3, CREB3L3, ONECUT3, E2F4, GATAD2A, LHX1, ZHX3, ATF4, XBP1, CRX, ESRRB, SIX1	0.0057	2.13
MF: Protein dimerization activity	9	NFIL3, GABPB2, CEBPD, XBP1, ATF4, ATF3, B4GALT1, CREB3L3, CD3D	0.0374	2.35
KEGG pathway: MAPK signaling pathway	11	CACNG6, FGFR4, GADD45B, PLA2G5,, GADD45A, MAP3K14, MYC, ATF4, MAP3K8, IL1A, TAOK1	0.0249	2.20

GO: gene ontology; BP: biological process; MF: molecular function; 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.

Differentially expressed genes can differentiate treated muscle samples, but not liver, from the control ones

An PCA analysis was carried out separately on each dataset. To avoid any possible distortion in the PCA and to test the ability of the obtained DE transcripts to distinguish treated from control animals, only normalized and filtered microarray intensities of the previously obtained DE transcripts (431 and 503 transcripts for muscle and liver, respectively) were used. In the PCA on muscle samples (**Figure 2**), the first two components accounted for 97.87% of the total variance. On the other hand, in the PCA on liver samples (**Figure 2**) the first two components accounted for 72.57% of the total variance. A clear separation could be observed between CTR and REV-treated animals in the case of muscle samples, while, in the liver, grouping of treated from untreated samples couldn't be clearly seen.

Discussion

On the phenotypic level, chronic exposure (71 days) to the TBA+E2, resulted in a significant increase in the ADG, but not in the final weight, of treated animals compared to the control ones. The main metabolites of TBA and E2 (α, β-trenbolone and α, β-estradiol, respectively) were significantly present in the urine of animals, confirming the dissemination of the Revalor-XS® implant into the circulation of the treated animals (Stella et al. 2015). It should be noted that the approach used in the current study was fairly conservative, as the implant was applied only for 71 and not 200 days (the full treatment schedule). Moreover, in a steady-state study like ours, a dynamic response might be expected in the early or even later period of exposure. However, the presence of the main metabolites of TBA and E2 in the urine of the treated animals, along with the large sample size (30 samples per tissue), assures the robustness of the provided results.

A number of DE genes were identified in skeletal muscle and liver of steroid-treated cattle, with 20 genes commonly regulated between both tissues. Functional analyses of the resulted DE gene lists pointed out the enrichment of many biological processes, molecular functions and biochemical pathways.

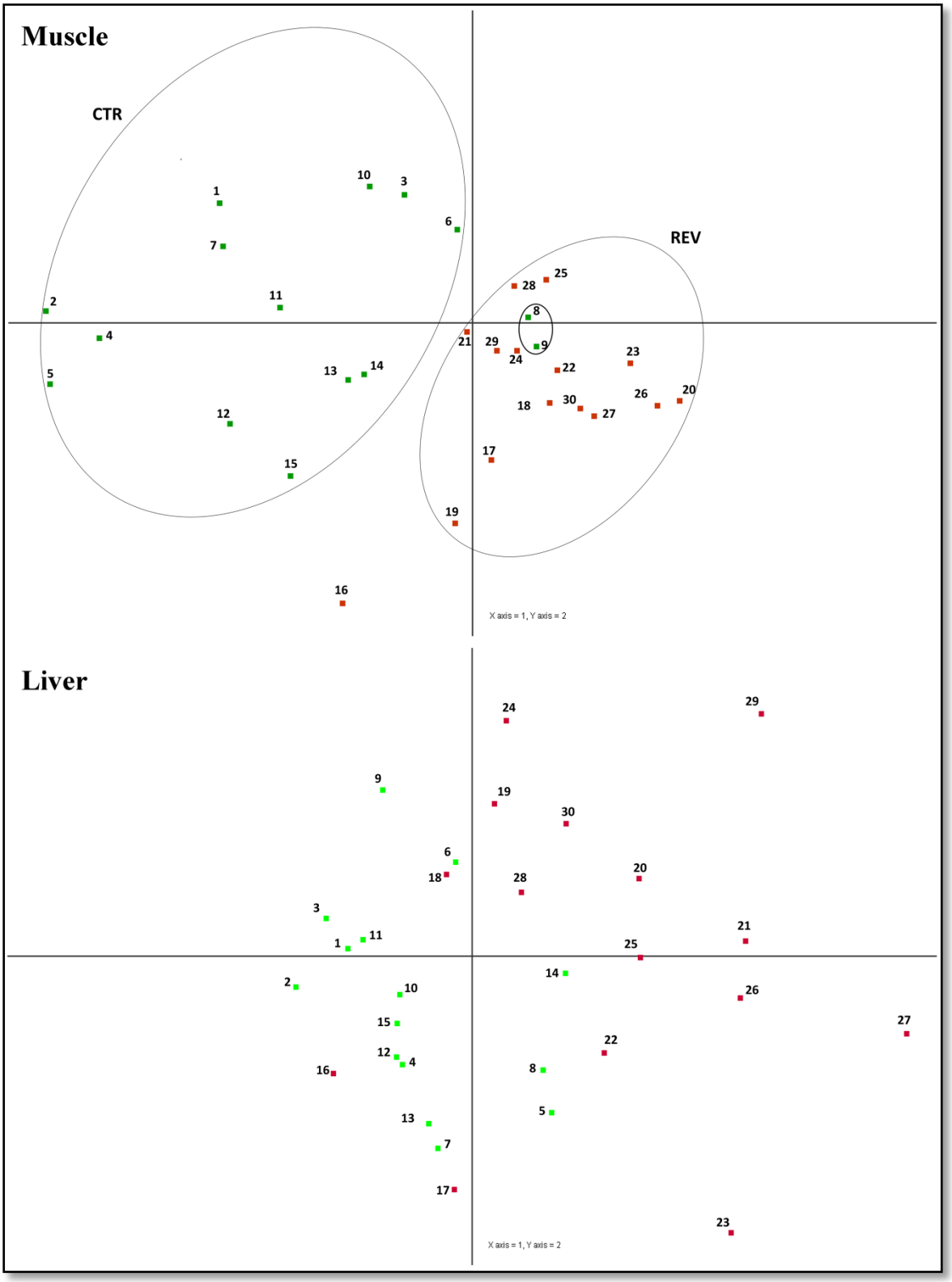

Figure 2: *PCA of the gene expression profiles.* ***Top****: PCA plot for the muscle samples shows the two principal components of greatest variation, covering 75.24% (x-axis) and 22.63% (y-axis) of the total variance. Ellipses distinguish different treatment groups, where the ellipse on the right illustrates the REV-treated samples from the CTR ones (left ellipse).* ***Bottom****: PCA plot for the liver samples, where the first two components of greatest variation cover 72.58%. The "green squares" denote the CTR samples (numbers 1-15), while the REV-treated samples (numbers 16-30) are denoted by "red squares".*

In muscle, several genes involved in the cytokine-cytokine receptor interaction pathway were significantly down-regulated, which still support the fact of steroids being immune-suppressive agents (Morell 1995; Verthelyi 2001; Eisenberg et al. 2008). Among these genes, the chemokine C-X-C motif receptor 4 (CXCR4), the tumor necrosis factor receptor superfamily member 8 (TNFR-SF8) and the chemokine C-C motif ligand 4 (CCL4), were down-regulated by 2.3-, 1.8- and 1.8-fold changes, respectively. In agreement with our results, the suppressive effect of steroids on CXCR4 (Ruiz et al. 2010), TNFR (Riedmaier et al. 2009; Massart et al. 2015) and CCL4 (Sánchez et al. 2014), were previously discussed. In addition, the Jak-STAT signaling pathway, which is a key pathway controlling myoblast proliferation and differentiation (Sun et al. 2007), was also enriched in our data set.

A significant enrichment was also observed for the biological processes "transmembrane transport", "ion transport", "metal ion transport" and "potassium ion transport", with many significantly down-regulated genes involved. The growth-promoting potency of TBA is based on both anabolic activity as an androgen and anti-catabolic activity as an anti-glucocorticoid (Thomas & Rodway 1983; Sillence & Rodway 1990; Jones et al. 1991; Meyer 2001; Hunter 2010). Moreover, an earlier report by Danhaive and Rousseau (Danhaive & Rousseau 1988) suggested that anabolic steroids act via muscle glucocorticoid receptors, rather than via muscle androgen receptors, thereby antagonizing the catabolic activity of endogenous glucocorticoids. Taking into consideration that glucocorticoids are a key player in the ion transport machinery, and that steroids are potent absorbagogues (Sellin & DeSoignie 1985), an explanation for the enrichment of transmembrane and ion transport biological processes in our data set can be depicted.

It is worth mentioning that we expected to see molecular signals in the steroid-treated muscle related to one or more of 1) the classical anabolic steroid-related genes AR, IGF1 and GH and 2) the canonical promyogenic transcription factors MYOD1 and MYOG. Surprisingly, of these regulators, only the IGF1-like (LOC512788) was down-regulated by 1.6-fold. This result was in contrast to the IGF1 up-regulation reported in De Jager et al. (De Jager et al. 2011), but agreed with Pegolo et al. (Pegolo et al. 2014) who reported a limited under-expression of IGF1.

In the liver, the combination of TBA and E2 (REV treatment) seemed to evoke a response putative of a transcriptional regulation. The modulation of genes involved in the mitogen-

activated protein kinase (MAPK) pathway (11 genes) and the transcription factor (TFs) activity (17 genes), was observed in the liver following REV treatment. Among the modulated TFs, nuclear factor, interleukin 3 (NFIL3), activating transcription factor 3 (ATF3), activating transcription factor 4 (ATF4) and E2F transcription factor 4 (E2F4) were all up-regulated by 2.15-, 1.75-, 1.5- and 1.84-fold, respectively. The classical estrogen signaling is known to occur through either a direct binding of estrogen receptor (ER) dimers to estrogen responsive elements (EREs), or via a second mechanism in which ERs interact with other transcription factors. Moreover, estrogen may also elicit effects through non-genomic mechanisms, which involve the activation of downstream signaling cascades such as MAPK (Faulds et al. 2012). In our study, as no differential regulation has been observed in the ER, we postulated the prevalence of the non-genomic mechanism of E2 in exerting its effect on the liver.

The enrichment of several carbohydrate and protein biosynthesis and metabolic processes was evident in the liver and not observed in the muscle. Several genes involved in the carbohydrate biosynthesis process were differentially expressed. Among these, the carbohydrate sulfotransferase 10 (CHST10) and 12 (CHST12) were 1.65- and 1.9-fold down-regulated, respectively. Moreover, the transcription factors ATF3 and ATF4 were also among the involved genes. In the liver, glucuronidation converts steroid hormones into more water-soluble products, facilitating removal from the circulation. In addition to glucuronidation, sulfation (through sulfotransferases) also plays a role in counteracting the presence of steroid hormones (Raftogianis et al. 2000; Suzuki-Anekoji et al. 2013). Recently, CHTS10 has been suggested to regulate estrogen in vivo, and its loss, blocked the down-regulation of steroids (especially estrogen) in the mouse (Suzuki-Anekoji et al. 2013). These findings point out, to some extent, the dominating signature of E2 over TBA in the liver. The down-regulation of some genes encoding fast fiber-type subunits (mezzanine 3, MYOZ3; myosin 16, MYO16; troponin C type 2, TNNC2) was interestingly observed in the liver and not in the muscle tissue. Although these findings do not support the slow fibre phenotype shift suggested by De Jager et al. (De Jager et al. 2011), they still come to an agreement with data published by Pegolo et al. (Pegolo et al. 2014).

Despite the distinct transcriptomic profile, it was interesting to find 20 commonly regulated genes between muscle and liver following REV treatment. While all the

common genes showed a similar down-regulation trend, FASN was the only gene appeared to be down-regulated in muscle and up-regulated in the liver tissue. This gene codes for one of the key lipogenic enzymes catalyzing the de novo synthesis of fatty acids (Jensen-Urstad et al. 2012). Also, Chung et al. (2011) reported that the implant with a combination of TBA and E2 decreased the adipogenic gene expression in finishing steers. Moreover, FASN has been recently reported to be among the down-regulated lipogenic genes in the adipocytes of steroid-implanted steers (Duckett and Pratt 2014). However, FASN up-regulation in the liver shifted the argument from decreased to increased lipogenesis machinery, which might be resulting from a fatty liver (a characteristic feature of high producing cattle) or to a dominating effect of E2, which is believed to cause increased hepatic lipid accumulation (Murondoti et al. 2014). Although not being the main objective of this study, those commonly regulated genes could be further addressed in a targeted gene biomarker assays.

From the PCA results, we could observe the consistency of the DE gene list of muscle over that of the liver. The one of the muscle was able to separate mostly all the treated from the untreated animals, while the liver one was less powerful in discriminating the 2 animal groups. This could be, logically, addressed to the muscle being the main target tissue of anabolic steroids, and that the machinery of muscle fiber hypertrophy (main effect of anabolic steroids) is taking place solely in muscle. Accordingly, this could still favor the use of muscle tissue over the non-muscle one to screen beef cattle for anabolic steroid fraud.

Conclusions

The present study showed that the effect of TBA and E2 combination on muscle and liver's gene transcription appeared relatively weak in terms of FC, with no transcripts being markedly over- or under-expressed. However, a robust number of DE genes were identified in both tissues following the treatment with REV, that resulted in a better understanding of the transcriptomic signature an exogenous administration of anabolic hormones could leave behind. It is becoming clear that anabolic steroid-enhanced bovine muscle growth involves a complex interaction of numerous pathways and receptors. The muscle's transcriptome response was directed toward mineral and ion transport processes, while the liver was expressing more a carbohydrate and protein biosynthesis patterns. Additionally, although TBA and E2 were not administered separately, and were combined within a sustained release implant, we postulated that the transcriptomic changes in the liver tissue were driven mainly by E2. Moreover, treated animals were better distinguished from the untreated ones based on the DE genes identified in muscle and not in the liver. Consequently, additional in vivo and in vitro studies are necessary to understand the mechanisms involved in this complex process, and the role of crosstalk between various receptors and pathways in anabolic steroid-enhanced animal growth needs to be depicted.

Supplementary materials

https://drive.google.com/drive/folders/0BxmPKazb5UxsanBLdkp1U0g4NjQ?usp=sharing

References

Becker C, Riedmaier I, Reiter M, Tichopad a., Groot MJ, Stolker a. a M, Pfaffl MW, Nielen MFW, Meyer HHD. 2011. Influence of anabolic combinations of an androgen plus an estrogen on biochemical pathways in bovine uterine endometrium and ovary. J Steroid Biochem Mol Biol. 125:192–201.

De Brabander HF, Le Bizec B, Pinel G, Antignac J-P, Verheyden K, Mortier V, Courtheyn D, Noppe H. 2007. Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals. J Mass Spectrom. 42:983–998.

Brenu EW, McNaughton L, Marshall-Gradisnik SM. 2011. Is there a potential immune dysfunction with anabolic androgenic steroid use?: A review. Mini Rev Med Chem. 11:438–445.

Cannizzo FT, Pegolo S, Starvaggi Cucuzza L, Bargelloni L, Divari S, Franch R, Castagnaro M, Biolatti B. 2013. Gene expression profiling of thymus in beef cattle treated with prednisolone. Res Vet Sci; 95:540–547.

Chung KY, Baxa TJ, Parr SL, Luque LD, Johnson BJ. 2011. Administration of estradiol (E2), trenbolone acetate (TBA), and TBA/E2 implants alters adipogenic and myogenic gene expression in bovine skeletal muscle. J Anim Sci.

Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiele M, Vercammen J, De Wasch K. 2002. Recent developments in the use and abuse of growth promoters. Pap Present 4th Int Symp Horm Vet Drug Residue Anal. 473:71–82.

Danhaive PA, Rousseau GG. 1988. Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat. J Steroid Biochem. 29:575–581.

Dayton WR, White ME. 2014. Meat science and muscle biology symposium--role of satellite cells in anabolic steroid-induced muscle growth in feedlot steers. J Anim Sci. 92:30–38.

Duckett SK, Pratt SL. 2014. Meat science and muscle biology symposium--anabolic implants and meat quality. J Anim Sci. 92:3–9.

Eisenberg SWF, Cacciatore G, Klarenbeek S, Bergwerff AA, Koets AP. 2008. Influence of 17beta-oestradiol, nortestosterone and dexamethasone on the adaptive immune response in veal calves. Res Vet Sci. 84:199–205.

European Union. 1996. Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC, OJ EC L 125 (1996) 10–31.

European Union. 2002. Decision 2002/657/EC of 12 August 2002 on the implementing

Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. Eur. Communities L 221 (2002) 8–36.

Faulds MH, Zhao C, Dahlman-Wright K, Gustafsson J-A. 2012. The diversity of sex steroid action: regulation of metabolism by estrogen signaling. J Endocrinol. 212:3–12.

Food and Drug Adminsitration, FDA. 2007. Freedom of Information Summary. Original New Animal Drug Application NADA 141–269. Revalor-XS (TBA and Estradiol). Implant (pellets) for Cattle (Steers Fed in Confinement for Slaughter).

Giantin M, Granato A, Baratto C, Marconato L, Vascellari M, Morello EM, Vercelli A, Mutinelli F, Dacasto M. 2014. Global gene expression analysis of canine cutaneous mast cell tumor: could molecular profiling be useful for subtype classification and prognostication? PLoS One. 9:e95481.

Giantin M, Lopparelli RM, Zancanella V, Martin PG, Polizzi A, Gallina G, Gottardo F, Montesissa C, Ravarotto L, Pineau T, Dacasto M. 2010. Effects of illicit dexamethasone upon hepatic drug metabolizing enzymes and related transcription factors mRNAs and their potential use as biomarkers in cattle. J Agric Food Chem; 58:1342–1349.

Hunter R a. 2010. Hormonal growth promotant use in the Australian beef industry. Anim Prod Sci. 50:637–659.

Jacob CC, Dervilly-Pinel G, Biancotto G, Monteau F, Le Bizec B. 2014. Global urine fingerprinting by LC-ESI(+)-HRMS for better characterization of metabolic pathway disruption upon anabolic practices in bovine. Metabolomics. 11:184–197.

De Jager N, Hudson NJ, Reverter A, Wang Y-H, Nagaraj SH, Cafe LM, Greenwood PL, Barnard RT, Kongsuwan KP, Dalrymple BP. 2011. Chronic exposure to anabolic steroids induces the muscle expression of oxytocin and a more than fiftyfold increase in circulating oxytocin in cattle. Physiol Genomics. 43:467–478.

Jensen-Urstad APL, Semenkovich CF. 2012. Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger? Biochim Biophys Acta. 1821:747–753.

Jones SJ, Johnson RD, Calkins CR, Dikeman ME. 1991. Effects of trenbolone acetate on carcass characteristics and serum testosterone and cortisol concentrations in bulls and steers on different management and implant schemes. J Anim Sci. 69:1363–1369.

Kouassi Nzoughet JJ, Dervilly-Pinel G, Chéreau S, Biancotto G, Monteau F, Elliott CT, Le Bizec B. 2015. First insights into serum metabolomics of trenbolone/estradiol implanted bovines; screening model to predict hormone-treated and control animals' status. Metabolomics.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–8.

MacNeil JD, Reid J, Fedeniuk RW. 2008. Distribution of trenbolone residues in liver and various muscle groups of heifers that received multiple implants at the

recommended site of application. J AOAC Int. 91:670–674.

MacNeil JD, Reid J, Neiser CD, Fesser ACE. 2003. Single-laboratory validation of a modified liquid chromatographic method with UV detection for determination of trenbolone residues in bovine liver and muscle. J AOAC Int. 86:916–924.

Massart S, Redivo B, Flamion E, Mandiki SNM, Falisse E, Milla S, Kestemont P. 2015. The trenbolone acetate affects the immune system in rainbow trout, Oncorhynchus mykiss. Aquat Toxicol. 163:109–120.

Meyer HH. 2001. Biochemistry and physiology of anabolic hormones used for improvement of meat production. APMIS. 109:1–8.

Mooney MH, Elliott CT, Le Bizec B. 2009. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. TrAC - Trends Anal Chem. 28:665–675.

Morell V. 1995. Zeroing in on how hormones affect the immune system. Science. 269:773–775.

Murondoti A, Jorritsma R, Beynen AC, Wensing T, Geelen MJH. 2004. Unrestricted feed intake during the dry period impairs the postpartum oxidation and synthesis of fatty acids in the liver of dairy cows. J Dairy Sci. 87:672–679.

Parr SL, Chung KY, Hutcheson JP, Nichols WT, Yates DA, Streeter MN, Swingle RS, Galyean ML, Johnson BJ. 2011. Dose and release pattern of anabolic implants affects growth of finishing beef steers across days on feed. J Anim Sci. 89:863–873.

Pegolo S, Cannizzo FT, Biolatti B, Castagnaro M, Bargelloni L. 2014. Transcriptomic profiling as a screening tool to detect trenbolone treatment in beef cattle. Res Vet Sci; 96:472–481.

Raftogianis R, Creveling C, Weinshilboum R, Weisz J. 2000. Estrogen metabolism by conjugation. J Natl Cancer Inst Monogr.:113–124.

Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HHD. 2007. Modification of mRNA expression after treatment with anabolic agents and the usefulness for gene expression-biomarkers. Anal Chim Acta. 586:73–81.

Riedmaier I, Reiter M, Tichopad A, Pfaffl MW, Meyer HHD. 2011. The potential of bovine vaginal smear for biomarker development to trace the misuse of anabolic agents. Exp Clin Endocrinol Diabetes. 119:86–94.

Riedmaier I, Tichopad A, Reiter M, Pfaffl MW, Meyer HHD. 2009. Influence of testosterone and a novel SARM on gene expression in whole blood of Macaca fascicularis. J Steroid Biochem Mol Biol. 114:167–173.

Ruiz A, Salvo VA, Ruiz LA, Baez P, Garcia M, Flores I. 2010. Basal and steroid hormone-regulated expression of CXCR4 in human endometrium and endometriosis. Reprod Sci. 17:894–903.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34:374–378.

Sánchez J, Bonet ML, Keijer J, Van Schothorst EM, Möllller I, Chetrit C, Martinez-Puig D, Palou A. 2014. Blood cells transcriptomics as source of potential biomarkers of articular health improvement: Effects of oral intake of a rooster combs extract rich in hyaluronic acid. Genes Nutr. 9.

Sellin JH, DeSoignie RC. 1985. Steroids alter ion transport and absorptive capacity in proximal and distal colon. Am J Physiol. 249:G113–9.

Sillence MN, Rodway RG. 1990. Effects of trenbolone acetate and testosterone on growth and on plasma concentrations of corticosterone and ACTH in rats. J Endocrinol. 126:461–466.

Stella R, Biancotto G, Arrigoni G, Barrucci F, James P. 2015. Proteomics for the detection of indirect markers of steroids treatment in bovine muscle. :1–21.

Sun L, Ma K, Wang H, Xiao F, Gao Y, Zhang W, Wang K, Gao X, Ip N, Wu Z. 2007. JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. J Cell Biol. 179:129–138.

Suzuki-Anekoji M, Suzuki A, Wu S-W, Angata K, Murai KK, Sugihara K, Akama TO, Khoo K-H, Nakayama J, Fukuda MN, Fukuda M. 2013. In vivo regulation of steroid hormones by the Chst10 sulfotransferase in mouse. J Biol Chem. 288:5007–5016.

Thomas KM, Rodway RG. 1983. Effects of trenbolone acetate on adrenal function and hepatic enzyme activities in female rats. J Endocrinol. 98:121–127.

Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 98:5116–5121.

Verthelyi D. 2001. Sex hormones as immunomodulators in health and disease. Int Immunopharmacol. 1:983–993.

Wang H, Wang Z, Shao B, Liu Z, Liu S. 2009. Determination of trenbolone residual in bovine liver by liquid chromatography-mass spectrometry. Bull Environ Contam Toxicol. 82:415–418.

White ME, Johnson BJ, Hathaway MR, Dayton WR. 2003. Growth factor messenger RNA levels in muscle and liver of steroid-implanted and nonimplanted steers. J Anim Sci. 81:965–972.

III. Transcriptomic Characterization Of Bovine Primary Cultured Hepatocytes and Madin-Darby Bovine Kidney (MDBK) Cell Line;

Which One Resembles More The Bovine Liver?[3]

[3] Adapted- and reproduced in the current layout- from: **Elgendy, R.,** Giantin, M., & Dacasto, M. **(2016). Transcriptomic Characterization Of Bovine Primary Cultured Hepatocytes and Madin-Darby Bovine Kidney (MDBK) Cell Line; Which One Resembles More The Bovine Liver?.** *(Submitted manuscript)*

Abstract

Bovine primary cultured hepatocytes (CHs) are widely used *in vitro* models for liver toxicity testing. However, little is known about their whole-transcriptome profile and its resembelence to the normal liver tissue. In the present study, we profiled – by microarray - the whole-transcriptome of bovine CHs (n = 4) and compared it with the transcriptomic landscape of control liver samples (n = 8), as well the Madin-Darby bovine kidney (MDBK) cell line (n = 4). Compared with liver tissue, the bovine CHs expressed (fold-change; FC > 2, $P < 0.05$) about 2,155 and 2,073 transcripts at a relative lower or higher abundance, respectively. Of those expressed at a lower abundance, many were drug biotransformation enzyme-coding transcripts (i.e. genes), such as the the cytochrome P450 family (CYPs), sulfotransferases, methyltransferases, and glutathione peroxidases. Also, many drug transporters and solute carriers were expressed at a lower abundance in bovine CHs. 'Drug metabolism', 'PPAR signaling', and 'metabolism of xenobiotics by CYPs' were among the most negatively-enriched pathways in bovine CHs compared with liver. A qPCR cross-validation using 8 selected genes evidenced a high correlation (r = 0.95, $P = 0.001$) with the corresponding microarray results. Although from a kidney origin, and albeit to a lower extent compared with bovine CHs, the MDBK cells showed a basal expression of a wide array of CYP-coding transcripts. Our study provide a whole-transcriptome-based evidence for the bovine CHs and hepatic tissue resembelance. Overall, the bovine CHs' transcriptomic profile renders it unreliable as an *in vitro* model to study drug metabolism.

Introduction

In spite of the fact that in vitro experimental models can not fully resemble the complexity of a living organism, their simplicity provides the ability to manipulate and analyze specific parameters (Jia and Liu, 2007). Several hepatic *in vitro* systems are currently available, ranging from hepatocyte subcellular fractions to whole isolated perfused livers (Godoy et al., 2013; Vinken et al., 2014). Among those, primary cultured hepatocytes (CHs), especially in human medicine, are generally considered as the gold standard in the field of liver-based *in vitro* modeling (Fraczek et al., 2013; Godoy et al., 2013).

Primary cultured hepatocytes are cells isolated from liver and maintained in culture, and indeed they have some intrinsic advantages that result in the closest representation of the

hepatic *in vivo* situation (Benet et al., 2015). They retain active uptake and excretion mechanisms as well as metabolism to a level comparable to hepatocytes in the liver (Jigorel et al., 2005). However, the fact that cultured hepatocytes are kept in an artificial environment differing from that of the liver might result in relevant phenotypic changes. Thus, it was argued that the drug metabolism activities of cultured hepatocytes may significantly differ from *in vivo* and this would throw uncertainty on the value and relevance of the *in vitro* data (Hewitt et al., 2007). Moreover, primary CHs provide a good reflection of the hepatic *in vivo* situation only for some days when properly cultured, hence their cultures can only be used for short-term purposes (Vinken and Rogiers, 2015).

As long-term cultivation of primary hepatocytes is to a great extent hindered by the dynamic loss of the hepatocyte-particular phenotype both at the morphological and at the functional level (Vinken and Rogiers, 2015), there is a strong need for robust long-term *in vitro* screening models, the use of which ensure a well-maintained phenotypic and functional characteristics, as well as a reduced usage of animals in drug development studies. For instance, hepatoma cell lines offer some advantages for *in vitro* studies, such as high availability, easy handling, nearly unlimited life-span, and stable phenotype that does not depend on donor characteristics (Donato et al., 2015). Still, there are no available bovine hepatoma cell lines and the currently available human hepatoma cell lines (as HepG2 cells) are not a good alternative to cultured hepatocytes, as they show an extensively dedifferentiated hepatic phenotype (Benet et al., 2015) as well as limited expression of drug metabolizing enzymes and transporters (Guo et al., 2011; Hilgendorf et al., 2007).

The absence of an immortalized bovine hepatocytes cell line and the generally difficult-to-transfect primary CHs (Ourlin et al., 1997; Tur-Kaspa et al., 1986) has inspired the usage of other non-hepatic cell lineages. For example, the Madin-Darby bovine kidney (MDBK) cells- an immortalized bovine kidney distal tubule cell line (Madin and Darby, 1958)- are easy to transfect (Nadeau, 2012) and have been widely used as a transient or stably-transfected *in vitro* model. In specific, MDBK cells have been used as a model for studying some functional pathways (mainly lipid metabolism) in cows (Bionaz et al., 2008b; Thering et al., 2009; White et al., 2012), following the earlier report by Bionaz et

al., (2008a) that showed a similar gene expression profile between MDBK cells and periparturient bovine liver tissue.

As a primary *in vitro* hepatic model, more attention was, logically, given to study primary hepatocytes. Several studies have investigated the transcriptomic profile of human primary cultured hepatocytes in many toxicological and pharmacological investigation (reviewed in: Fasinu et al., 2012; Godoy et al., 2013; Vinken and Rogiers, 2015). To date, there is no evidence on the global gene expression (whole-transcriptome) of the popular liver-derived *in vitro* model in bovine's toxico-pharmacological studies; the bovine primary CHs. Therefore, the objectives of this study were; (1) to charachterize the transcriptomic profile (basal transcript abundance) of bovine primary CHs and to compare it with that of the bovine liver tissue, and (2) to characterize the global gene expression of MDBK cells in order to determine if this cell line can be a suitable model to replace primary hepatocytes in long-term toxicological and pharmacological studies in the bovine species.

Materials and methods

Primary hepatocytes isolation, cell cultures and liver tissue samples

The bovine primary CHs were isolated from the liver caudate lobe of two healthy Charolais heifers (15-18 months old, ~400 kg body weight) as previously reported in Giantin et al. (2012). Briefly, at the slaughterhouse, liver specimens (100-150 g) were excised from the caudate lobe then rinsed immediately with ice-cold Eurocollins buffer (15 mM KH2PO4, 42 mM K2 HPO4, 15 mM KCl, 1 mM NaHCO3 and 0.2 M glucose, pH 7.4) supplemented with 1 mM ethylene glycol-bis(2-aminoethylether)- N,N,N0,N0-tetraacetic acid (EGTA). The specimens were transported to the laboratory where the hepatocytes were isolated according to the method of Klooster et al. (1992), with few modifications (Giantin et al., 2012). Isolated hepatocytes were washed three times with William's E medium (WEm), and the final pellet was weighed to estimate the appropriate amount of culture medium to be added (6 mL of medium per each 1 g of hepatocytes). The total number of isolated hepatocytes and the percentage of viable cells were determined by the trypan blue dye exclusion test. The isolated cells were suspended in WEm with 10% fetal bovine serum (FBS), 1.67 mM glutamine, 26.6 µM NaHCO3, 50 µg mL-1 gentamicin sulfate, 1 µM hydrocortisone 21-hemisuccinate and 1 µM insulin) to a

final density of 106 cells mL-1. Then, 6 mL of the cell suspensions (total of 6 x 106 cells) was seeded in a 9 cm plastic petri dish coated with rat-tail type-I-collagen (150 µL of a 1 mg mL-1 collagen solution). Cells were then incubated in a humidified atmosphere (O2/CO2 95:5) at 37°C and left to attach for 4 h. The medium was then replaced with the same WEm mentioned above but without FCS. One replicate of primary cultured hepatocytes from the liver of each heifer (a total of 4 samples) were chosen for the microarray hybridizations.

MDBK cells (American Type Culture Collection; ATCC, Catalog CCL-22) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% FBS, 1% penicillin-streptomycin (PS), and L-glutamine. Cells were grown in 25-cm2 tissue culture flasks (at 37°C and 5% CO2 atmosphere), and routinely sub-cultivated when attained 80% confluence, according to the product information supplied by ATCC. Two replicates of untreated cells (a total of 4 samples) were used for the microarray experiments.

With respect to liver tissue, we have used a set of 8 raw microarray data (correspond to 8 liver samples) that have been previously published by our group (Elgendy et al., 2015). The original data was a set of 15 untreated (control) liver samples that have been deposited into the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE62002. In the present study, we have used the raw microarray data that correspond to 8 liver control samples and re-analyzed them with the microarray data of the primary cultured hepatocytes. The accession numbers of the eight liver control samples are GSM1518119, GSM1518120, GSM1518123, GSM1518126, GSM1518127, GSM1518129, GSM1518130 and GSM1518131, respectively.
(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62002).

Cell Harvesting and RNA Isolation

Total RNA from primary cattle hepatocyte cultures and MDBK cells was isolated by TRIzol (Life Technologies, USA) and subsequently purified using the RNeasy Mini kit (Qiagen, Italy) according to the manufacturer's instructions. Briefly, the medium of two replicate Petri dishes for each cell culture was aspirated and 2 mL of ice-cold 0.1 mM phosphate buffer solution (PBS, pH 7.4) was added to each dish; monolayer were then scraped off. Cells were then pelleted by centrifugation (1500g, 5 min at 4 °C), the buffer

was discarded and the remaining pellet was processed for RNA isolation. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Italy) was performed during the RNA purification steps. Total RNA concentration was determined using the NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDropTechnologies, USA), and its quality was measured by the 2100 Bioanalyzer (Agilent Technologies, USA). RNA aliquots (stored at -80°C) that have been previously isolated from the control liver samples (n = 8) were quantified and tested for RNA integrity again. All the 16 RNA samples (8 liver tissue; 4 primary cultured hepatocytes; 4 MDBK cells) passed the RNA quality criteria (i.e., RNA concentration ≥ 40 ng/µL and RNA integrity number (RIN) ≥ 8). Hence, they were all considered for the subsequent microarray hybridizations and/or qPCR assays.

Microarray Hybridization And Data Analysis

Bovine-specific 4x44K oligo-microarray (AMADID-015354, Agilent Technologies, USA) with a possible 22,000 unique probes (correspond to more than 18,000 unique transcripts) was used in the present study. Sample amplification, labeling and hybridization were performed following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol as previously described (Elgendy et al., 2015). Briefly, RNA samples were labeled with Cy3 (green) fluorescent dye label using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, USA). A purification step was applied to the labeled cRNA using the RNeasy Mini kit (Qiagen, Italy), and sample concentration and specific activity (pmol Cy3/µg cRNA) were measured. A total of 1.65 µg of labeled cRNA was fragmented using the Gene Expression Hybridization kit (Agilent Technologies, USA) according to the manufacturer's instructions, and then diluted by the addition of 55 µL of 2X GE hybridization buffer. A final volume of 100 µL of the hybridization sample mixture was dispensed on the gasket slide and then the active side of the microarray slide was placed on the top of it to form a ''sandwich slide pair". The slides were firstly incubated for 17 h at 65 °C in a hybridization oven (Agilent Technologies, USA), then washed using wash buffer 1 and 2 according to the manufacturer's instructions. The hybridized slides were scanned at 5 µm resolution using the G2565BA DNA microarray scanner (Agilent Technologies, USA) where the default settings were modified in order to scan the same slide twice to produce images at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The array images were

analyzed by the Agilent's Feature Extraction Software (version 9.5.1), using the GE2-v5_95_Feb07 FE extraction protocol.

The microarray data files from each sample were imported into GeneSpring 13.0 (Agilent Technologies, USA) to perform the normalization and the differential expression analysis. Two independent analyses have been conducted by GeneSpring. The first analysis was a comparison between CHs and the liver tissue samples (CHs vs. liver tissue), and the second was between MDBK cells and CHs (MDBK vs. CHs). The raw data were normalized using the percentile algorithm (lower cutoff: 20th percentile and upper cutoff: 100th percentile). Probes that were flagged as marginal or absent were excluded, specifying that at least 50% of the samples in both experimental groups must possess flags to be employed in the differential expression analyses. The differentially expressed genes were then identified through fold change (FC), and P values were calculated using the Mann-Whitney unpaired test followed by a Benjamini-Hochberg multiple testing correction. The threshold for up- and down-regulated genes was set as a FC $\geq$ 2.0 and P values and FDR values < 0.05. The microarray data were deposited in the ArrayExpress public database (https://www.ebi.ac.uk/arrayexpress/) at the European Bioinformatics Institute and are accessible through the accessions E-MTAB-4424 and E-MTAB-4430.

Cross-Validation by Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed to confirm the expression pattern of 8 candidate genes of interest. Those genes were chosen from the list of down-regulated genes in the primary cultured hepatocytes as compared to the liver tissue. The GenBank accession number and the qPCR primer oligonucleotide sequences used for the quantification of the genes encoding cytochromes P450 (CYPs) 1A1, 2C19 and 3A5, solute carrier (SLC) 10A1 and O2B1, complement 7 (C7), and tyrosine aminotransferase (TAT) are reported in **Table 1**.

Table 1. Oligonucleotide sequences used for the qPCR cross-validation assays.

Gene	GenBank accession	Primer sequence (5' >> 3')	Amplicon size (bp)	Reference
CYP1A1	XM_588298	F: GACCTGAATCAGAGGTTCTACGTCT R: CCGCATGTGACCCTTCTCAA	81	Giantin et al., 2008
CYP1A2	XM_010817139	F: ACCATGACCCGAAGCTGTG R: CAATGGTGGTGCCATCAGAC	78	Giantin et al., 2008
CYP2C19	NM_001109792	F: TGACCTTGTCCCCAGCAGTA R: CTGCCCTGGGTTGGAAAACT	142	*Ex novo*
CYP3A5	NM_174531	F: CCAGAGACGTGGTCTACTTTGA R: CCCTACTCACCAGCAAGTACAGT	76	Zancanella et al., 2014
SLC10A1	NM_001046339	F: GCTTCTCCTTGTTGCCATCTTTAG R: AGGTCATTTTTGTGTCATCTCTGG	71	Zancanella et al., 2013
SLCO2B1	NM_174843	F: GTGTGGAATACATCACGCCCT R: TTGGTGTAGAAGACCTGGCTTTT	88	Zancanella et al., 2013
C7	NM_001045966	F: GGACGGTGCTGATGAAGACA R: TGTAACCACGTCCGGTAAGC	101	Elgendy et al., 2015
TAT	NM_001034590	F: CTGAAGTTACCCAAGCAATGAAAG R: CCTCCCGACTGGATAAGTAGCC	90	Cantiello et al., 2009
TBP[a]	NM_001075742	F: ACAACAGCCTCCCACCCTATGC R: GTGGAGTCAGTCCTGTGCCGTAA	111	Lisowski et al., 2008
B2M[a]	NM_173893	F: TCGTGGCCTTGGTCCTTCT R: AATCTTTGGAGGACGCTGGAT	71	Zancanella et al., 2013

B2M: beta-2-microglobulin; bp: base pairs; C7: complement component 7; CYP1A1: cytochrome P450 1A1; CYP1A2: cytochrome P450 1A2; CYP2C19: cytochrome P450 2C19; CYP3A5: cytochrome P450 3A5; SLC10A1: Solute carrier family 10, member 1; SLCO2B1: Solute carrier organic anion transporter family, member 2B1; TAT: tyrosine aminotransferase; and TBP: TATA box binding protein.

The beta-2-microglobulin (B2M) and the TATA box binding protein (TBP) genes were used as an internal control (reference) genes. To ensure a uniform workflow, the RNA samples used for qPCR were the same used for the microarray hybridizations. First-strand cDNA was synthesized from 0.5 µg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and stored at -20ºC until further use. Quantitative RT-PCR was performed using Power SYBR Green I (Thermo Fisher Scientific Inc, Waltham, MA, USA). Briefly, the qPCR reactions (10 µL final volume) consisted of a SYBR Green master mix, 300 or 600 nM forward and reverse primers (Integrated DNA Technology; TEMA ricerca, Bologna, Italy), and 2.5 µL of 50 ng/µL cDNA. Assays were performed in duplicates by the Stratagene MX3000P real-time PCR system (Stratagene, CA, USA) using the standard PCR conditions (an activation step at 95 ºC for 10 min; 45 cycles at 95 ºC for 10 s and at 60 ºC for 30 s, and a cooling step at 40 ºC for 30 s). No template and no-reverse-transcription controls were used in each assay to ensure a specific and uncontaminated reaction. For each qPCR assay, 8 standard curves were generated using duplicate 3-fold serial dilutions of cDNA pool. Messenger RNA relative quantification (RQ) was calculated by the ΔΔCt method (Livak and Schmittgen, 2001), and normalized using the average RQ values of the 2 internal control genes (B2M and TBP). The selected reference genes showed an unchanged expression pattern between the experimental groups after a quality control qPCR step, hence they were used for the data normalization.

Functional Annotation and Statistical Analysis

Functional analysis of the DE genes was performed by the analysis of the enrichment for specific Gene Ontology (GO) terms using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Dennis et al., 2003; Huang da et al., 2009) as implemented in the online platform (http://david.abcc.ncifcrf.gov/). The probes present in the microarray were used as reference background, and the default options were modified to identify enriched GO terms at a minimum gene count of '10' and an EASE score (a modified Fisher Exact *P*-value) ≤ 0.01.

Statistical tests were carried out by the GraphPad Prism 5 software (San Diego, CA, USA). The RQ values of the qPCR-analyzed genes were expressed as FC, and the significance of gene expression between experimental groups (primary hepatocytes versus liver tissue) was determined using the nonparametric Mann-Whitney test (Mann

and Whitney, 1947). The correlation significance between qPCR and microarray data on the level of either individual genes (qPCR-derived RQ value of a specific gene compared to its corresponding microarray intensity) or the level of quantification technology (qPCR versus microarray; using FC of the respective genes) was carried out by the Spearman's nonparametric correlation test. Statistical significance for all analyses was declared at $P \leq 0.05$.

Results

Microarray Analysis

Bovine Primary Cultured Hepatocytes (CHs) versus Liver

Out of the 21,535 unique probes -automatically identified by GeneSpring for each raw data file-, a total number of 14,399 probes (66.9 %) appeared to have flags (i.e. above-threshold intensities) in at least 50% of the 2 experimental groups (CHs and liver). Of those flagged probes, 11,832 (~82%) passed the 'filtering by expression' step (percentile 20-100%) and hence considered in the differential expression analysis. A number of 4,327 probes showed differential expression (FC $\geq$ 2.0, $P \leq 0.05$) in CHs when compared with liver tissue, of which 2,125 and 2,202 probes –corresponded to 2,073 and 2,155 unique transcripts– were over- and under-expressed, respectively. A hierarchical clustering heatmap of CHs (n = 4) and liver tissue samples (n = 8) is shown in **Figure 1A.** The detailed list of the up- and down- regulated probes, their corresponding gene symbol (if any), and a description of each probe is reported in **Supplementary Table 1 & 2**, respectively.

Primary cultured hepatocytes showed a low abundance of many biontransformation-related genes compared to the liver tissue **(Supplementary Table 2)**. Many phase I biotransformation genes, such as cytochrome P450s (CYPs; CYP1A1, CYP1A2, CYP3A5, CYP2C19, CYP214, CYP4V2 and CYP4A22), flavin-containing monooxygenases (FMOs; FMO3, FMO5 and FMO2), and alcohol dehydrogenases (ADHs; ADH4, ADH1C, ADH6 and ADHFE) were down-regulated in the CHs compared to the liver tissue samples (CHs vs. liver). Other phase I biotransformation enzymes (NADPH-reducatases, esterases, amidases and hydrolases) were also expressed at lower amounts in CHs. Some major phase II biotransformation enzymes such as methyltransferases (MTs; AMT, BHMT, SHMT, COMT and GAMT), sulfotransferases

(STs; CHST, CHST13 and HS3ST3B1), glutathione peroxidases (GST; GSTA2, GSTA4, GST2, GSTT and GSTA3), and glucuronsyltransferases (UGTs; UGT2B1, UGT2A, UGT2A3, UGT1A6 and UGT31) were also under-expressed in CHs. Many ATP-binding cassette (ABC) transporters and solute carriers (SLCs) were also expressed at lower levels in CHs, compared to the liver tissue.

Madin-Darby Bovine Kidney (MDBK) cells versus Bovine Primary Cultured Hepatocytes (CHs)

The MDBK cells –in comparison with the CHs– expressed 13,803 probes (~64% of the total probes count) in at least 50% of the samples (n = 4, each). The 'filtering by expression' retained a final number of 13,030 transcripts (94.4% of the flagged probes) which we have used to check for the DE gene between groups (MDBK vs. CHs). Finally, a total of 5,306 probes showed differential expression (FC $\geq$ 2.0, $P \leq 0.05$) in MDBK cells when compared with CHs, of which 2,601 and 2,705 probes –corresponded to 2,536 and 2,640 unique transcripts– were over- **(Supplementary Table 3)** and under-expressed **(Supplementary Table 4)**, respectively. A hierarchical clustering heatmap of MDBK (n = 4) and CHs (n = 4) is shown in **Figure 1B.**

On the level of biotransformation-related genes, MDBK cells –compared with the bovine CHs– had a lower transcript abundance of many CYPs (CYP1A1, 1A2, 3A5, 2C18, 2C19, 2C87, 2D14, 4A22, 4F2, 4V2, 11A1, 17A1, 26A1, 27A1 AND 39A1). Although those genes were not abundant in MDBK cells, they were still detected **(Supplementary Table 4)**. Other genes involved in biotransformation (i.e. STs, MTs, UGTs, ABC transporters) were expressed in MDBK cells with no distinct pattern of abundance (neither all were over- nor down-expressed) in comparison with the bovine CHs.

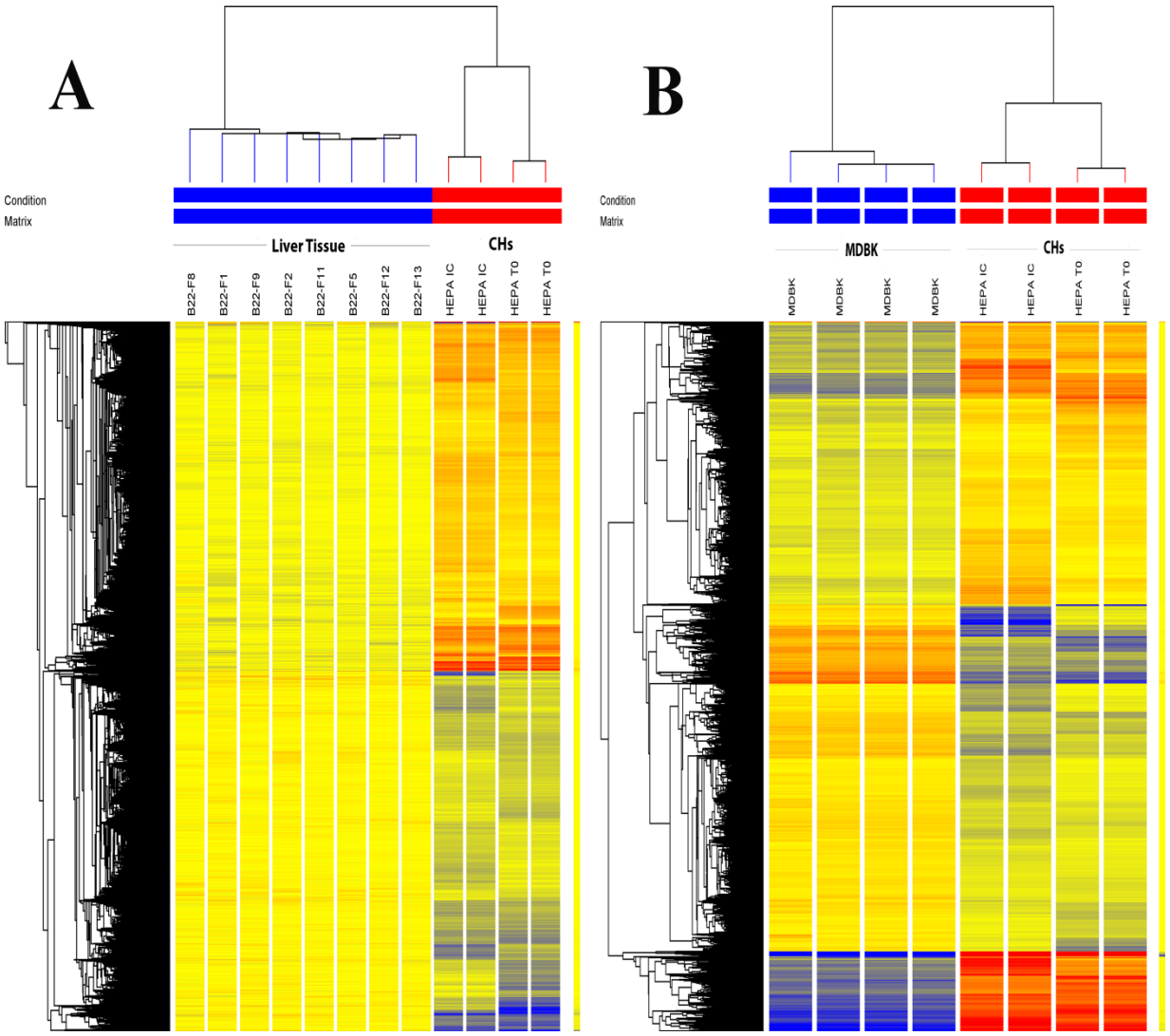

Figure 1: Cluster dendrogram of the differentially expressed (DE) genes in both comparisons (CHs vs. Liver and MDBK vs. CHs). *A heatmap was generated from the DE genes (> 2-fold) in both comparisons; CHs vs. Liver (A), and MDBK vs. CHs (B). The hierarchical clustering (Euclidean distance clustering algorithm) option in the GeneSpring software v.13.1 (http://genespringsupport.com/) was used. In the heatmap, red indicates an expression level higher than the mean across all subjects, and blue denotes expression level lower than the mean.*

qPCR cross-validation

There were strong correlations between the expression ratios (expressed in FC) of the microarray and qPCR results ($P < 0.01$; **Table 2**) across all the tested 8 genes. The correlation plots of all the individual qPCR-tested genes are shown in **Figure 2**.

Table 2. qPCR cross-validation analysis. Nine differentially expressed (DE) genes between bovine primary cultured hepatocytes (CHs) and liver were tested by qPCR, then correlated to their corresponding microarray data. The difference in expression is reported as a fold-change (FC).

Gene name	FC[1] qPCR	FC array	Spearman's rho[†]
CYP1A1*	-61.5	-79.8	0.9441
CYP1A2	-26.7	-51.6	0.7972
CYP2C19	-9.2	-9.5	0.7622
CYP3A5	-11.8	-10.7	0.7692
SLC10A1	-33.4	-29	0.8021
SLCO2B1	-4.8	-6.9	0.7902
C7	-149	-121.6	0.9231
TAT	-52.2	-121.5	0.9091

** CYP1A1 was expressed in MDBK cells in a lower abundance compared with CHs (- 4.8-fold) and liver (- 147.9-fold, while the other 8 genes were detected at very high Ct (>35) and the qPCR assays were inefficient.*

[1]The mRNA relative quantification (RQ) was calculated by the ΔΔCt method, and normalized using the average of the RQ values of 2 internal control (reference) genes (TBP and B2M). For the microarray, the FC was calculated using the Mann-Whitney test in the GeneSpring software, comparing primary cultured hepatocytes (CHs) versus the liver tissue samples (CHs vs. Liver)

[†]Correlations were calculated using the RQ values of the genes analyzed by qPCR and their corresponding microarray intensities (normalized raw data). All correlations were significant at $P \leq 0.01$.

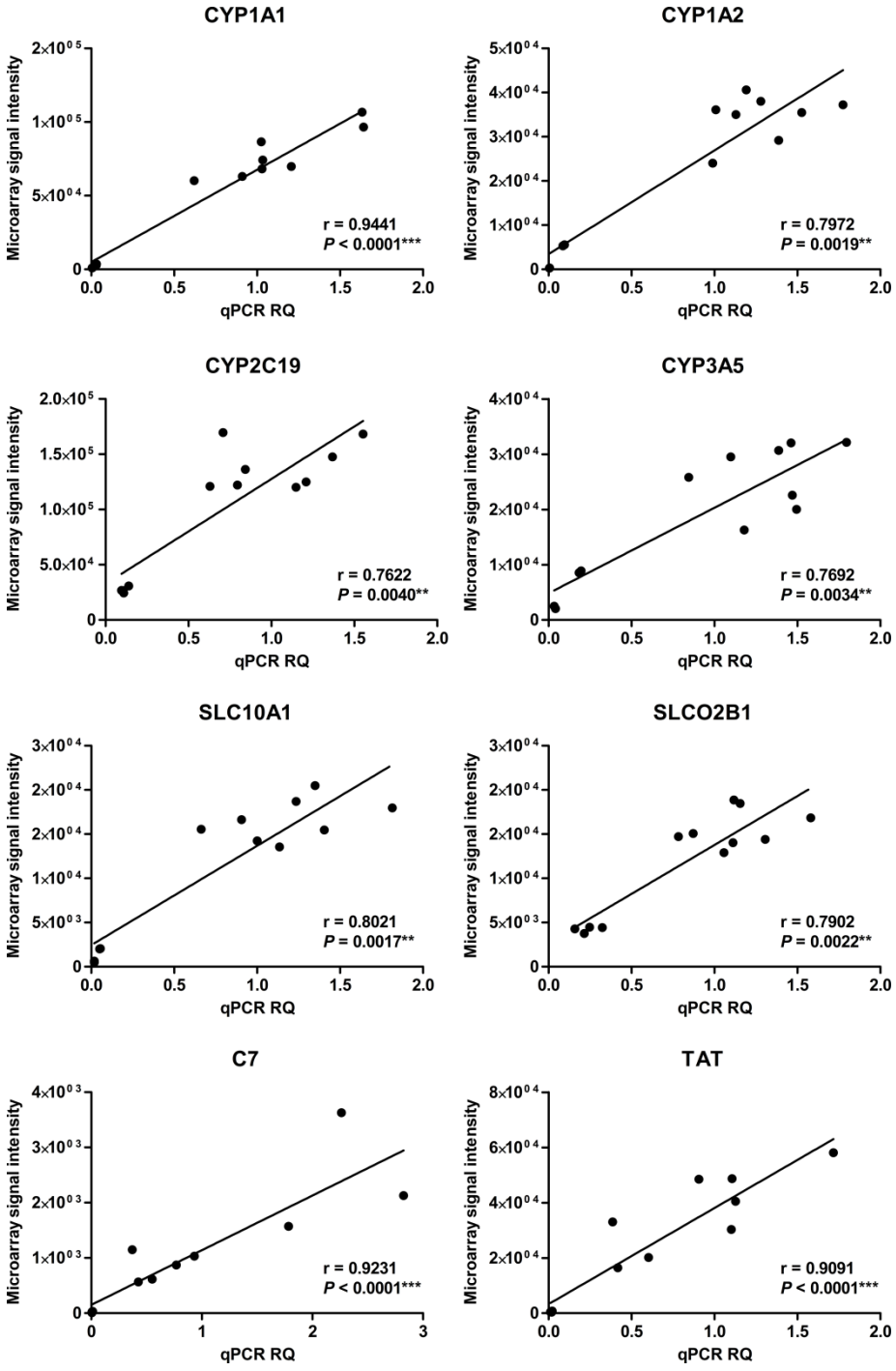

Figure 2: qPCR cross-validation of 8 differentially expressed (DE) genes in bovine primary cultured hepatocytes (CHs) when compared with normal liver tissue (CHs vs. Liver). *The plots represent the qPCR RQ values of the qPCR-analyzed genes versus their corresponding normalized-microarray probe intensities. Each solid dot represents a sample from twelve samples (CHs, n = 4; liver tissue, n = 8). Both the Pearson's rho (r), representing the correlation coefficient, and the obtained p-value are shown within each plot.*

In addition, both technologies (i.e. microarray vs. qPCR) showed a strong correlation (Spearman's r = 0.95, *P* = 0.0011) in-between, in terms of the calculated FC values (**Figure 3**). In MDBK cells, CYP1A1 was the only quantifiable gene with an efficient qPCR assay, while the other 7 genes (CYP1A2, CYP2C19, CYP3A5, SLC10A1, SLCO2B1, C7, and TAT) were poorly quantified (*Ct* values > 35, and non-efficient qPCR assays). The CYP1A1 gene was expressed in a lower abundance compared with both the bovine CHs (- 4.8-fold) and liver tissue (- 147.9-fold).

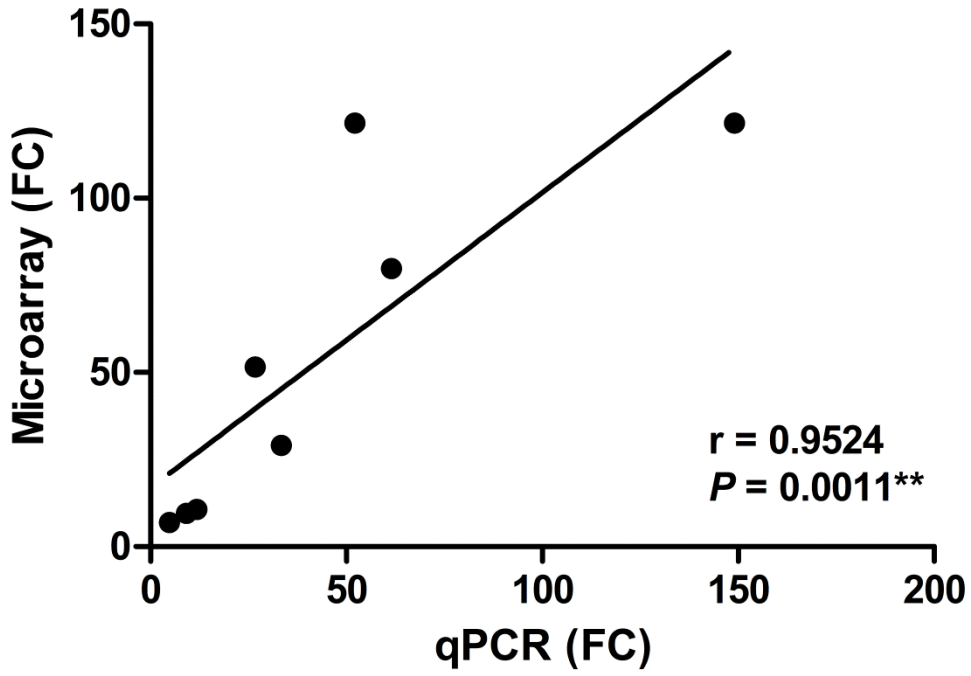

Figure 3: Overall correlation between the microarray and qPCR data. *Using the calculated fold-change (FC) of selected 8 genes, both technologies (i.e. microarray vs. qPCR) showed a strong correlation (Spearman's r = 0.95, P = 0.0011) in-between.*

Functional Annotation and Pathway analysis

The functional annotation highlighted the most relevant GO terms and signaling pathways associated with each list of the DE genes and, accordingly, their corresponding biological condition (i.e. CHs, liver tissue or MDBK cells). The most enriched KEGG pathways (n = 21) in CHs –compared with liver tissue– and the direction of the enrichment is presented in **Table 3**. In CHs, the data indicated that pathways related to 'metabolism' were the most represented, with a larger inhibition of main metabolic pathways, such as drug

metabolism, retinol metabolism, metabolism of xenobiotics by CYPs, fatty acid metabolism, tyrosine and tryptophan metabolism. The data also indicated a higher inhibition of pathways related to amino acid and lipid metabolism in CHs. Among those the 'PPAR signaling' and the 'fatty acid metabolism' pathways were highly impacted **(Table 3).** Finally, nucleotide metabolism and cell death related pathways were overall induced in CHs, with 'proteasome' (the protein-destroying apparatus) being the most induced pathway (**Table 3**).

Table 3. Pathway analysis of the differentially regulated genes between primary culture hepatocytes (CHs) and the liver tissue (CHs vs. Liver).

Direction	KEGG Pathway	Count	Genes	FE*	P Value†
–	Bta00280:Valine, leucine and isoleucine degradation	18	HSD17B10, ALDH6A1, ACAA2, ACADM, EHHADH, BCKDHB, HIBADH, DBT, MUT, ALDH7A1, HMGCS2, MCCC1, AOX1, ALDH2, ACAD8, PCCB, HMGCL, ACAA1	5.77	2.19E-09
–	Bta04610:Complement and coagulation cascades	22	C7, MBL2, C4A, MASP1, C3, F13A1, C6, C5, F9, SERPING1, C4BPB, C4BPA, PLG, PROC, C1QA, C8B, C1QB, F5, CFI, SERPIND1, CFD, CPB2	4.37	7.79E-09
–	Bta00982:Drug metabolism	18	CYP3A5, GSTA3, GSTA4, CYP2C18, CYP2D14, MAOB, GSTT1, CYP1A2, UGT1A6, FMO5, LOC511498, ADH4, FMO2, MGC152010, FMO3, AOX1, UGT2A3, MGST2	5.18	1.49E-08
–	Bta03320:PPAR signaling pathway	19	ACOX2, CPT1B, PPARA, CPT2, ACADM, EHHADH, ACADL, PCK1, APOA2, CYP4A22, APOA5, FABP1, SCD5, SCP2, SLC27A2, PLTP, SLC27A5, ACAA1, NR1H3	4.01	4.73E-07
–	Bta00830:Retinol metabolism	15	CYP3A5, CYP1A1, CYP2C18, CYP1A2, RDH5, ALDH1A1, UGT1A6, ALDH1A2, CYP4A22, LRAT, DHRS4, LOC511498, ADH4, MGC152010, UGT2A3	5.04	5.10E-07
–	Bta00260:Glycine, serine and threonine metabolism	13	GLYCTK, SHMT1, ALAS1, GATM, AMT, MAOB, BHMT, GCAT, GAMT, AGXT2, PSAT1, PIPOX, CBS	5.56	1.17E-06
–	Bta00980:Metabolism of xenobiotics by cytochrome P450	13	CYP3A5, GSTA3, GSTA4, CYP1A1, CYP2C18, GSTT1, CYP1A2, UGT1A6, LOC511498, ADH4, MGC152010, UGT2A3, MGST2	4.37	2.04E-05
–	Bta00071:Fatty acid metabolism	12	CPT1B, GCDH, ACAA2, ALDH7A1, CYP4A22, CPT2, ACADM, EHHADH, ADH4, ALDH2, ACADL, ACAA1	4.46	3.96E-05
–	Bta00380:Tryptophan metabolism	12	DDC, GCDH, ALDH7A1, TDO2, CYP1A1, EHHADH, AOX1, MAOB, ALDH2, CAT, CYP1A2, AFMID	4.23	6.70E-05
–	Bta00350:Tyrosine metabolism	10	DCT, DDC, GOT1, ADH4, AOX1, MAOB, HGD, COMT, TAT, FAH	4.28	3.33E-04
–	Bta00330:Arginine and proline metabolism	12	GLS2, ALDH7A1, GOT1, ASS1, GATM, CKMT2, OTC, MAOB, ALDH2, GAMT, CPS1, ASL	3.26	8.11E-04
+	Bta03050:Proteasome	16	PSMA7, PSMB8, PSMB9, PSMB5, PSMA1, PSMB4, PSMD14, PSME1, PSMD12, PSMA5, PSMA4, PSMB2, PSMD1, PSMC1, PSMD2, PSMD6	4.02	3.36E-06
+	Bta00240:Pyrimidine metabolism	24	DCTD, POLR2G, NUDT2, CTPS, DTYMK, UPP1, POLR1B, POLR2D, TK1, POLD3, UMPS, ITPA, POLE4, UPRT, NT5M, POLD1, NT5C2, RRM1, PRIM2, ENTPD8, CDA, TXNRD1, UCK2, DUT	2.91	3.41E-06
+	Bta03030:DNA replication	13	MCM3, MCM4, MCM5, POLD3, RPA1, RFC3, POLE4, RFC4, RFC2, POLD1, PRIM2, PCNA, FEN1	4.23	2.19E-05

+	Bta00970:Aminoacyl-tRNA biosynthesis	13	YARS, CARS, DARS, PSTK, SARS, AARS, GARS, GTF2H4, KARS, WARS, LARS, HARS, MARS	3.59	1.35E-04
+	Bta04210:Apoptosis	18	CFLAR, IRAK1, DFFA, TP53, FADD, ENDOD1, BAD, BCL2L1, BIRC3, CAPN2, CASP6, IRAK3, TNFRSF1A, CASP3, CASP7, RIPK1, CASP8, FAS	2.43	8.33E-04
+	Bta04110:Cell cycle	23	CDC7, ANAPC1, E2F4, DBF4, TP53, SKP2, SMAD3, CDK7, MCM3, CDK4, CDC26, CDC27, MCM4, MCM5, CDK2, TGFB2, CDC25B, YWHAH, CCND3, CDKN2B, GSK3B, PCNA, BUB3	2.07	0.001276
+	Bta03420:Nucleotide excision repair	11	RPA1, POLD3, RFC3, POLE4, RFC4, RFC2, POLD1, PCNA, GTF2H4, CDK7, ERCC1	2.97	0.002809
+	Bta04620:Toll-like receptor signaling pathway	18	IRAK1, MAP2K1, MAP2K3, FADD, TLR4, CD40, IFNAR1, IKBKE, MAPK1, STAT4, MAPK13, JUN, RIPK1, CASP8, MAPK3, TICAM1, SPP1, TRAF3	2.12	0.003924
+	Bta05200:Pathways in cancer	41	TRAF1, PTGS2, PML, CDH1, NFKB2, BCL2L1, ITGB1, MMP1, FLT3LG, TGFB2, CASP3, STAT4, CDKN2B, ITGAV, CASP8, SLC2A1, FAS, TRAF4, AXIN1, TRAF3, EGFR, MSH6, MAP2K1, TGFBR1, SKP2, TP53, SMAD3, ITGA3, FZD3, FADD, BAD, BIRC3, CDK4, CDK2, JUP, MAPK1, NRAS, ITGA6, GSK3B, JUN, MAPK3	1.50	0.008156
+	Bta05213:Endometrial cancer	11	EGFR, NRAS, MAPK1, MAP2K1, GSK3B, ILK, MAPK3, TP53, CDH1, BAD, AXIN1	2.53	0.009253

**FE = Fold Enrichment*

[†]*The significance of enrichments was set at P < 0.01, a minimum gene count of 10, and an EASE score of 0.01.*

The identified biological process (BP) and molecular function (MF) GO terms are described in **Table 4** and **Supplementary Table 5 (Appendix 5)**. Oxidation reduction and lipid-metabolism-related processes were strongly represented as inhibited BP terms in CHs. Several binding-related molecular functions **(Table 4)** were also inhibited in CHs, with the 'cofactor' and 'coenzyme' binding being the most enriched (lowest *P* values) terms, and 'ion binding' being the one with the highest gene count (190 genes). Lastly, the functional annotation evidenced the induction of several biological processes, of which the 'programmed cell death' was the most induced. As it was not the objective of the present study, and because of the different tissue nature and the expected transcriptomics divergence, no functional analysis was conducted on the DE gene list of the MDBK cells comparison with CHs (MDBK vs. CHs).

Table 4. Functional annotation of the differentially expressed genes in the primary culture hepatocytes (CHs) samples_compared to the liver tissue (CHs vs. Liver). Minimum gene count was set to 20 and an EASE score of 0.01 was applied. Counts represent the number of genes from the list associated with a given GO term. Negatively and positively enriched GO terms are indicated by '−' and '+', respectively. The detailed gene list for each GO term is reported in ***Supplementary Table 5***.

Direction	Category and GO term	Count	FE	*P* value
−	BP;GO:0055114~oxidation reduction	86	2.687	9.88E-18
−	MF;GO:0048037~cofactor binding	42	3.625	5.43E-13
−	MF;GO:0050662~coenzyme binding	30	3.767	7.33E-10
−	BP;GO:0016054~organic acid catabolic process	20	5.343	1.68E-09
−	BP;GO:0046395~carboxylic acid catabolic process	20	5.343	1.68E-09
−	MF;GO:0005509~calcium ion binding	61	1.914	1.04E-06
−	MF;GO:0005506~iron ion binding	34	2.418	3.44E-06
−	MF;GO:0009055~electron carrier activity	25	2.918	3.58E-06
−	MF;GO:0046906~tetrapyrrole binding	20	3.098	1.79E-05
−	BP;GO:0006631~fatty acid metabolic process	21	2.953	2.11E-05
−	MF;GO:0030246~carbohydrate binding	24	2.687	2.36E-05
−	MF;GO:0043167~ion binding	190	1.291	2.77E-05
−	MF;GO:0043169~cation binding	187	1.283	4.86E-05
−	MF;GO:0046872~metal ion binding	183	1.270	1.14E-04
−	BP;GO:0009611~response to wounding	23	2.106	0.001229
−	MF;GO:0000287~magnesium ion binding	21	1.989	0.004218
−	MF;GO:0008289~lipid binding	23	1.798	0.008696
−	BP;GO:0008610~lipid biosynthetic process	20	1.897	0.008745
−	BP;GO:0048878~chemical homeostasis	24	1.764	0.00892
+	BP;GO:0012501~programmed cell death	39	2.457	2.73E-07

+	BP;GO:0006915~apoptosis	38	2.445	4.54E-07
+	BP;GO:0008219~cell death	40	2.283	1.35E-06
+	BP;GO:0016265~death	40	2.230	2.46E-06
+	BP;GO:0008283~cell proliferation	25	2.725	9.16E-06
+	BP;GO:0006260~DNA replication	21	2.954	1.63E-05
+	BP;GO:0006259~DNA metabolic process	43	1.985	1.99E-05
+	BP;GO:0034660~ncRNA metabolic process	27	2.438	3.01E-05
+	BP;GO:0042981~regulation of apoptosis	46	1.886	3.69E-05
+	BP;GO:0043067~regulation of programmed cell death	46	1.867	4.76E-05
+	BP;GO:0045321~leukocyte activation	23	2.601	4.88E-05
+	BP;GO:0010941~regulation of cell death	46	1.861	5.11E-05
+	BP;GO:0006396~RNA processing	42	1.910	6.33E-05
+	BP;GO:0007049~cell cycle	40	1.854	1.86E-04
+	BP;GO:0001775~cell activation	24	2.323	1.98E-04
+	BP;GO:0043065~positive regulation of apoptosis	23	2.319	2.85E-04
+	BP;GO:0043068~positive regulation of programmed cell	23	2.300	3.22E-04
+	BP;GO:0010942~positive regulation of cell death	23	2.281	3.63E-04
+	BP;GO:0051726~regulation of cell cycle	22	2.335	3.64E-04
+	BP;GO:0010033~response to organic substance	31	1.974	3.93E-04
+	BP;GO:0044265~cellular macromolecule catabolic	41	1.710	8.37E-04
+	BP;GO:0010605~negative regulation of macromolecule	34	1.781	0.001262
+	BP;GO:0033554~cellular response to stress	34	1.758	0.001572
+	BP;GO:0006397~mRNA processing	23	2.016	0.001986
+	BP;GO:0016071~mRNA metabolic process	25	1.939	0.002082
+	BP;GO:0044093~positive regulation of molecular function	27	1.877	0.002118
+	BP;GO:0009057~macromolecule catabolic process	42	1.554	0.004429
+	BP;GO:0001944~vasculature development	20	2.000	0.004634
+	BP;GO:0065003~macromolecular complex assembly	38	1.580	0.005323
+	BP;GO:0042127~regulation of cell proliferation	36	1.584	0.00652
+	BP;GO:0070271~protein complex biogenesis	26	1.747	0.006738
+	BP;GO:0006461~protein complex assembly	26	1.747	0.006738
+	BP;GO:0043933~macromolecular complex subunit	39	1.532	0.007832
+	BP;GO:0043066~negative regulation of apoptosis	22	1.823	0.008415
+	BP;GO:0060548~negative regulation of cell death	22	1.798	0.00979
+	BP;GO:0043069~negative regulation of programmed cell	22	1.798	0.00979

GO: gene ontology; BP: biological process; MF: molecular function; FE: fold enrichment (defined as the ratio of the two proportions: input genes involved in a biological process and the background information); P value: modified Fisher exact P value calculated by DAVID software.

Discussion

The main focus of the present study was the comparison between bovine CHs and liver tissue, in terms of global gene expression. The transcriptomic profiling of the MDBK cells was performed just to examine whether they share any common genetic features (individual genes or gene batteries) with the bovine CHs that could render them alternative *in vitro* model for hepatic-based pharmaco-toxicological studies. The present study illustrates -in details- the transcriptomic landscape of bovine CHs in comparison with the normal liver tissue, and furthermore shows the ability of MDBK cells, albeit to a lower extent compared to CHs, to express some of the key hepatic drug metabolizing enzyme-coding genes.

Each *in vitro* model has its own pros and cons. A major drawback of the use of isolated hepatocytes and their cultures is their limited life span and progressive loss of liver-specific functions (Guillouzo et al., 1990), especially those controlled by the CYPs family (Elaut et al., 2006; López-García, 1998; Wang et al., 2002), rendering them an inadequate model for the study of xenobiotic biotransformation and toxicity. The CYP enzymes were reported to be downregulated with time in rat hepatocytes culture (Baker et al., 2001; Sidhu and Omiecinski, 1995). In agreement, the bovine CHs in the present study were shown to have a low basal abundance of most of the key CYPs-coding genes, especially the CYP1A1, CYP1A2 and CYP3A5 which are main players in the metabolism of steroids, fatty acids, and xenobiotics. The expression levels of many CYPs are known to decrease tremendously in rat cultured hepatocytes (Boess et al., 2003). Some studies have previously used bovine CHs to assess the CYP-mediated metabolism and cytotoxicity of aflatoxins (Kuilman et al., 2000) and the CYP-induced activity to some environmental contaminants such as the dioxin-like compounds (Guruge et al., 2009). CYPs play a pivotal role in the toxication of some compounds (Vignati et al., 2005), thus the low enzyme levels in CHs might result in an underestimation of the toxicity of some compounds. Therefore, attention should be given when using bovine CHs to study biotransformation- and CYPs-based studies, as their basal expression is very low and doesn't resemble their corresponding liver tissue status.

Many phase II biotranformation enzymes-coding genes (MTs, STs and GSTs) were present at a lower abundance in CHs as compared with the liver tissue. In agreement, a marked downregulation of MTs (Baker et al., 2001) and STs (Baker et al., 2001; Boess et

al., 2003) was previously reported in human primary hepatocytes. In earlier studies, some GSTs have shown either unchanged (Baker et al., 2001) or down-regulation (Lee and Boyer, 1993) pattern in rat hepatocytes. Although present in the MDBK cells, phase II drug metabolizing enzymes showed no distinct pattern of abundance in comparison with CHs. The aim of profiling the transcriptome of MDBK cells was more quantitative than qualitative (i.e. the presence of a specific gene rather than its quantity), therefore the ability of MDBK cells to show basal expression of some drug metabolism-related genes was based only on the microarray data. Thus, more specific prospective studies might be needed to better characterize the capacity of MDBK cell line to express (or foster) certain gene(s).

In the comparison between MDBK cells and bovine primary CHs (MDBK vs. CHs), the relatively high number of detected (i.e. expressed) probes (~64% of all the unique probes) in both groups together reflects a quite large resemblance in their transcriptomic profile. The MDBK cells were previously shown (Bionaz et al., 2008a) to have a large similarity in mRNA abundance with the bovine liver tissue of periparturient cows, suggesting MDBK cells could be a suitable model to study transcription regulation in bovine liver. Although the transcriptome in general is thought to be tissue-specific (Su et al., 2004), different tissues from the same species share common transcriptomic features (Wang et al., 2008); that's why the shared transcriptomic profile between MDBK cells and the bovine primary CHs was not surprising. In the present study, MDBK cells were shown to have less abundance of the key CYP-coding genes compared with the CHs, which render the bovine CHs more suitable than MDBK cells when it comes to CYP-related studies. Still, liver tissue was superior in its basal level of CYPs and the other biotransformation-related genes over the CHs and the MDBK cells, respectively (i.e. Liver > CHs > MDBK). The fact that MDBK cells are stably transfected and easy to handle would encourage the idea of inducing their basal level of drug metabolizing enzymes (using certain ligands) or inserting the gene(s) of interest by transfection.

The peroxisome proliferator-activated receptor gamma (PPARγ) was expressed at higher abundance (< 20-fold) in MDBK cells compared with the CHs. Interestingly, the lipoprotein lipase (LPL), which is one of the PPARγ-responsive genes, was expressed at a very low abundance (- 151-fold) in MDBK cells compared with the CHs. In partial agreement, Bionaz et al. (2008b) reported that LPL was almost undetectable in untreated

MDBK cells, while it showed an increased expression only in the rosiglitazone (specific PPARγ agonist)-treated MDBK cells. In the present study, it was still interesting that LPL exhibited this very low abundance while PPARγ was expressed at a higher amount. A possible explanation is that the exhibited higher abundance of PPARγ in our MDBK cells has been just a relative basal-expression difference between MDBK cells and CHs and not a real activation of the gene.

Logically, when the expression of a closely related group of genes (e.g. CYPs) changes, their dependent metabolic or signaling pathways will be subsequently modified (see Fraczek et al., 2013; Gómez-Lechón et al., 2004). In the present study, the marked inhibition in pathways such as the 'drug metabolism', 'Retinol signaling', and 'metabolism of xenobiotics by CYP' was a further confirmation of the CYP-suppressed status in the bovine CHs compared with the liver tissue.

In both comparisons [(CHs vs. liver tissue) or (MDBK vs. CHs)], we expected to see abundance difference related to one or more of the nuclear receptors (NRs)-coding genes, such as the retinoid X receptor (RXR), the constitutive androstane receptor (CAR), the liver X receptor (LXR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor (PPAR). Surprisingly, of these NRs, only the PPAR showed a difference in abundance in CHs compared with liver tissue (PPARα; - 2-fold) and in MDBK cells compared with CHs (PPARγ; 20-fold). Getting back to the original microarray platform, we found that only CAR and PPAR were present in the annotated platform while the others (RXR, LXR and PXR) were not; hence, a full picture on those NRs could not be depicted by the microarray analysis. Still, that was not the scope or the objective of the study. It should also be noted that the CHs and the control liver tissue samples were from different animal individuals. Still, they represented the same cattle breed and the same tissue matrix (i.e. Liver).

Conclusions

In conclusion, the present study showed that although the transcritomic makeup of bovine primary CHs largely resembles its parental tissue; i.e. the liver, they still have their own shortcomings when it comes to the drug metabolizing enzymes-coding genes. The bovine primary CHs seems to have most of the biotransformation-related gene batteries; however, their basal level is much less than the *in vivo* status. Thus, attention should be paid not to draw firm conclusions on pharmaco-toxicological studies performed with bovine CHs. Furtheremore, the transcriptomic profile of the MDBK cells evidenced a good resemblance with the bovine primary CHs; hence, presumably, with their parental tissue (i.e. the liver). This might shed light on the stably-transfected cell line; MDBK cells, to be considered as a probable surrogate *in vitro* model that can spontaneously or inductively express most of the hepatic genes upon a putative successful transfection.

Supplementary materials

https://drive.google.com/drive/folders/0BxmPKazb5Uxsbk8xR3ZFaHZRT3c?usp=sharing

References

Baker, T.K., Carfagna, M.A., Gao, H., Dow, E.R., Li, Q., Searfoss, G.H., Ryan, T.P., 2001. Temporal gene expression analysis of monolayer cultured rat hepatocytes. Chem. Res. Toxicol. 14, 1218–1231.

Benet, M., Jover, R., Bort, R., 2015. Transfection of Primary Hepatocytes with Liver-Enriched Transcription Factors Using Adenoviral Vectors. Methods Mol. Biol. 1250, 213–221. doi:10.1007/978-1-4939-2074-7_15

Bionaz, M., Baumrucker, C.R., Shirk, E., Vanden Heuvel, J.P., Block, E., Varga, G.A., 2008. Short Communication: Characterization of Madin-Darby Bovine KLabelney Cell Line for Peroxisome Proliferator-Activated Receptors: Temporal Response and Sensitivity to Fatty AcLabels. J. Dairy Sci. 91, 2808–2813. doi:10.3168/jds.2007-0789

Bionaz, M., Everts, R.E., Lewin, H.A., Drackley, J.K., Loor, J.J., 2008. Bovine kidney (MDBK) cells and liver tissue of periparturient cows share remarkable similarity in gene expression profiles 2.

Boess, F., Kamber, M., Romer, S., Gasser, R., Muller, D., Albertini, S., Suter, L., 2003. Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: possible implications for toxicogenomics use of in vitro systems. Toxicol. Sci. 73, 386–402.

Cantiello, M., Giantin, M., Carletti, M., Lopparelli, R.M., Capolongo, F., Lasserre, F., Bollo, E., Nebbia, C., Martin, P.G.P., Pineau, T., Dacasto, M., 2009. Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. Biochem. Pharmacol. 77, 451–463. doi:10.1016/j.bcp.2008.10.025 [doi]

Dennis, G.J., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., Lempicki, R.A., 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 4, P3.

Donato, M.T., Tolosa, L., Gomez-Lechon, M.J., 2015. Culture and Functional Characterization of Human Hepatoma HepG2 Cells. Methods Mol. Biol. 1250, 77–93. doi:10.1007/978-1-4939-2074-7_5

Elaut, G., Henkens, T., Papeleu, P., Snykers, S., Vinken, M., Vanhaecke, T., Rogiers, V., 2006. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. Curr. Drug Metab. 7, 629–660.

Elgendy, R., Giantin, M., Montesissa, C., Dacasto, M., 2015. Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone: identification of new candidate biomarkers and their validation using samples from a field monitoring trial. Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess. 1–16. doi:10.1080/19440049.2015.1070307

Fasinu, P., Bouic, P.J., Rosenkranz, B., 2012. Liver-based in vitro technologies for drug

biotransformation studies - a review. Curr. Drug Metab. 13, 215–224.

Fraczek, J., Bolleyn, J., Vanhaecke, T., Rogiers, V., Vinken, M., 2013. Primary hepatocyte cultures for pharmaco-toxicological studies: at the busy crossroad of various anti-dedifferentiation strategies. Arch. Toxicol. 87, 577–610. doi:10.1007/s00204-012-0983-3

Giantin, M., Carletti, M., Capolongo, F., Pegolo, S., Lopparelli, R.M., Gusson, F., Nebbia, C., Cantiello, M., Martin, P., Pineau, T., 2008. Effect of breed upon cytochromes P450 and phase II enzyme expression in cattle liver. Drug Metab. Dispos. 36, 885–893.

Giantin, M., Gallina, G., Pegolo, S., Lopparelli, R.M., Sandron, C., Zancanella, V., Nebbia, C., Favretto, D., Capolongo, F., Montesissa, C., Dacasto, M., 2012. Primary hepatocytes as an useful bioassay to characterize metabolism and bioactivity of illicit steroids in cattle. Toxicol. In Vitro 26, 1224–1232. doi:10.1016/j.tiv.2012.06.003 [doi]

Godoy, P., Hewitt, N.J., Albrecht, U., Andersen, M.E., Ansari, N., Bhattacharya, S., Bode, J.G., et al., 2013. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch. Toxicol. 87, 1315–1530. doi:10.1007/s00204-013-1078-5

Gómez-Lechón, M.J., Donato, M.T., Castell, J. V, Jover, R., 2004. Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. Curr. Drug Metab. 5, 443–62. doi:10.2174/1389200043335414

Guillouzo, A., Morel, F., Ratanasavanh, D., Chesne, C., Guguen-Guillouzo, C., 1990. Long-term culture of functional hepatocytes. Toxicol. Vitr. 4, 415–427.

Guo, L., Dial, S., Shi, L., Branham, W., Liu, J., Fang, J.-L., Green, B., Deng, H., Kaput, J., Ning, B., 2011. Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab. Dispos. 39, 528–538. doi:10.1124/dmd.110.035873

Guruge, K.S., Yamanaka, N., Hasegawa, J., Miyazaki, S., 2009. Differential induction of cytochrome P450 1A1 and 1B1 mRNA in primary cultured bovine hepatocytes treated with TCDD, PBDD/Fs and feed ingredients. Toxicol. Lett. 185, 193–196. doi:10.1016/j.toxlet.2009.01.002

Hewitt, N.J., Lechón, M.J.G., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J.G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A.P., LeCluyse, E., Groothuis, G.M.M., Hengstler, J.G., 2007. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev. 39, 159–234. doi:10.1080/03602530601093489

Hilgendorf, C., Ahlin, G., Seithel, A., Artursson, P., Ungell, A.-L., Karlsson, J., 2007. Expression of thirty-six drug transporter genes in human intestine, liver, kidney,

and organotypic cell lines. Drug Metab. Dispos. 35, 1333–1340. doi:10.1124/dmd.107.014902

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57. doi:10.1038/nprot.2008.211 [doi]

Jia, L., Liu, X., 2007. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr. Drug Metab. 8, 822–829.

Jigorel, E., Le Vee, M., Boursier-Neyret, C., Bertrand, M., Fardel, O., 2005. Functional expression of sinusoidal drug transporters in primary human and rat hepatocytes. Drug Metab. Dispos. 33, 1418–1422. doi:10.1124/dmd.105.004762

Kuilman, M.E.M., Maas, R.F.M., Fink-Gremmels, J., 2000. Cytochrome P450-mediated metabolism and cytotoxicity of aflatoxin B 1 in bovine hepatocytes. Toxicol. Vitr. 14, 321–327.

Lee, S.J., Boyer, T.D., 1993. The effect of hepatic regeneration on the expression of the glutathione S-transferases. Biochem. J. 293, 137–142.

Lisowski, P., Pierzchała, M., Gościk, J., Pareek, C.S., Zwierzchowski, L., 2008. Evaluation of reference genes for studies of gene expression in the bovine liver, kidney, pituitary, and thyroid. J. Appl. Genet. 49, 367–372.

López-García, M.P., 1998. Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes. FEBS Lett. 438, 145–149.

Madin, S.H., Darby, N.B., 1958. Established Kidney Cell Lines of Normal Adult Bovine and Ovine Origin. Exp. Biol. Med. 98, 574–576. doi:10.3181/00379727-98-24111

Mann, H.B., Whitney, D.R., 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Stat. 50–60.

Nadeau, J., 2012. EXPERIMENTAl Biological Methods for Physical Scientists.

Ourlin, J.C., Vilarem, M.J., Daujat, M., Harricane, M.C., Domergue, J., Joyeux, H., Baulieux, J., Maurel, P., 1997. Lipid-mediated transfection of normal adult human hepatocytes in primary culture. Anal. Biochem. 247, 34–44. doi:10.1006/abio.1997.2025

Sidhu, J.S., Omiecinski, C.J., 1995. Modulation of xenobiotic-inducible cytochrome P450 gene expression by dexamethasone in primary rat hepatocytes. Pharmacogenet. Genomics 5, 24–36.

Su, A.I., Wiltshire, T., Batalov, S., Lapp, H., Ching, K.A., Block, D., Zhang, J., Soden, R., Hayakawa, M., Kreiman, G., 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc. Natl. Acad. Sci. U. S. A. 101, 6062–6067.

Thering, B.J., Bionaz, M., Loor, J.J., 2009. Long-chain fatty acid effects on peroxisome proliferator-activated receptor-alpha-regulated genes in Madin-Darby bovine kidney cells: optimization of culture conditions using palmitate. J Dairy Sci 92,

2027–2037. doi:10.3168/jds.2008-1749

Tur-Kaspa, R., Teicher, L., Levine, B.J., Skoultchi, A.I., Shafritz, D.A., 1986. Use of electroporation to introduce biologically active foreign genes into primary rat hepatocytes. Mol. Cell. Biol. 6, 716–718.

Vignati, L., Turlizzi, E., Monaci, S., Grossi, P., De Kanter, R., Monshouwer, M., 2005. An in vitro approach to detect metabolite toxicity due to CYP3A4-dependent bioactivation of xenobiotics. Toxicology 216, 154–167.

Vinken, M., Maes, M., Oliveira, A.G., Cogliati, B., Marques, P.E., Menezes, G.B., Dagli, M.L.Z., Vanhaecke, T., Rogiers, V., 2014. Primary hepatocytes and their cultures in liver apoptosis research. Arch. Toxicol. 88, 199–212. doi:10.1007/s00204-013-1123-4

Vinken, M., Rogiers, V., 2015. Protocols in In Vitro Hepatocyte. doi:10.1007/978-1-4939-2074-7

Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P., Burge, C.B., 2008. Alternative isoform regulation in human tissue transcriptomes. Nature 456, 470–476.

Wang, K., Shindoh, H., Inoue, T., Horii, I., 2002. Advantages of in vitro cytotoxicity testing by using primary rat hepatocytes in comparison with established cell lines. J. Toxicol. Sci. 27, 229–237.

White, H.M., Koser, S.L., Donkin, S.S., 2012. Gluconeogenic enzymes are differentially regulated by fatty acid cocktails in Madin-Darby bovine kidney cells. J. Dairy Sci. 95, 1249–56. doi:10.3168/jds.2011-4644

Zancanella, V., Giantin, M., Dacasto, M., 2014. Absolute quantification and modulation of cytochrome P450 3A isoforms in cattle liver. Vet. J. 202, 106–111. doi:10.1016/j.tvjl.2014.07.028

Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C., Dacasto, M., 2013. Tissue distribution and phenobarbital induction of target SLC- and ABC-transporters in cattle. J. Vet. Pharmacol. Ther. 36, 358–369. doi:DOI 10.1111/j.1365-2885.2012.01427.x

IV. Transcriptomic signature of high dietary organic selenium supplementation in sheep;

A nutrigenomic insight using a custom microarray platform and gene set enrichment analysis[4]

[4] Adapted- and reproduced in the current layout- from: **Elgendy, R.,** Giantin, M., Castellani, F., Grotta, L., Palazzo, F., Dacasto, M., & Martino, M. **(2016). Transcriptomic signature of high dietary organic selenium supplementation in sheep: A nutrigenomic insight using a custom microarray platform and gene set enrichment analysis.** Journal of Animal Science: 94 (8), 3169-3184.
https://dl.sciencesocieties.org/publications/jas/abstracts/94/8/3169

Abstract

The objective of this study was to investigate the effect of a high dietary selenium (Se) supplementation on the whole-transcriptome of sheep. A custom-sheep whole-transcriptome microarray, with more than 23,000 unique transcripts, was designed, and then used to profile the global gene expression of sheep after a high dietary supplementation of organic Se. Lactating cross-bred ewes (N = 10, 3 to 4 y of age; 55 to 65 kg BW) at their late lactation [100 ± 8 d in milk (DIM)] were acclimated to indoor individual pen feeding of a basal control diet (0.40 mg Se/d, Na-selenite) for 4 wk. Sheep were then kept on a diet with an extra (high) supplementation of organic Se (1.45 mg Se/d as Sel-Plex, Alltech, Australia) for 40 d. Whole blood (2.5 ml) was collected at 2 time-points [last day of the acclimatization period (T0), and after 40 d of the high Se supplementation (T40)], then total RNA was isolated and labeled for the subsequent microarray analysis. Significant analysis of microarray (SAM), using a paired *t*-test, of the microarray data (T40 versus T0) evidenced the up- and down-regulation of 942 and 244 transcripts (FDR < 0.05), respectively. Seven genes showed the same trend of expression (up- or down-regulation) when tested by qPCR in a cross-validation step. The microarray evidenced the up-regulation of some selenoproteins at T40, such as the selenium binding protein 1 (SELENBP1), selenoprotein W1 (SEPW1), glutathione peroxidase 3 (GPX3), and septin 8 (SEPT8), where the expression trend for SEPW1 and SEPT8 has been additionally validated using qPCR. Functional annotation of the differentially expressed (DE) genes showed the enrichment of several immune system-related biological processes (lymphocyte activation, cytokine binding, leukocyte activation, T cell differentiation, B cell activation) and pathways (cytokine and interleukin signaling). Moreover, gene set enrichment analysis (GSEA) evidenced the enrichment of B and T cell receptors signaling pathways with an enrichment score (ES) of 0.63 and 0.59, respectively. Overall, these results provide- on a global gene expression (whole-transcriptome) scale- the main genes, biological processes and pathways regulated by a high Se supplementation in sheep, which mainly reflect an immune-system and transcription-modulation-induced transcriptomic signature. Moreover, the study delivers a custom whole transcriptomic microarray platform that can be used in further global gene expression studies in the ovine species.

Introduction

In some of the geographical areas where the soil (and hence the produced forages) is selenium (Se)-deficient (Oldfield, 2002), supplementing the animals' diet with an external source of Se is a common practice (Tinggi, 2003). Selenium is authorized in the European Union (EU) as a nutritional additive for all species, and the current European commission regulations (EU No. 427/2013; EU No. 489/2015) allow the organic form of Se to be added to all of the food producing animals' diet (including sheep) not to exceed 0.5 mg/kg of diet (as fed). The U.S. Food and Drug Administration (FDA) upper limit of dietary Se supplementation in ruminants is 0.3 mg Se/kg DM (FDA, 2012). Therefore, a Se-level between 0.3 and 0.5 mg/kg DM while within limits in the EU is considered supranutritional Se supplementation according to the FDA. High dietary Se supplementation has been associated with variable improvements in animal performance and immune status of ewes and lambs (Rooke et al., 2004). Recent studies on sheep (Hall et al., 2011; Meyer et al., 2011; Stewart et al., 2012; Hall et al., 2013) and cattle (Stockdale et al., 2011; Hall et al., 2014a; Hall et al., 2014b) have focused on the effect of high dietary (supranutritional) Se on the animals' overall performance and immune system. From a genomic point of view few (Hall et al., 2011; Hugejiletu et al., 2013; Chauhan et al., 2014; Chauhan et al., 2015) have investigated the effect of high dietary Se on the mRNA expression of a pre-selected set of genes in sheep. However, to date, the effect of Se on the global gene expression of sheep (using high-throughput technologies) has not been investigated. Our hypothesis was that high dietary Se would induce the immune system of sheep and leave a noticeable molecular signature behind. Therefore, a whole-genome microarray platform has been custom designed for the present study, and used to examine the effect of high dietary Se on the global gene expression (transcriptome) of sheep.

Materials and methods

All procedures conducted in the present study were approved by the Teramo University Institutional Animal Care and Use Committee. No animals have been sacrificed in this study, and only blood samples were collected at different time points, using the least invasive approach to avoid animals' distress. Animals were managed according to the Directive 86/609/EEC regarding the protection of animals used for experimentation or other scientific purposes, enforced by the Italian D. Lgs n. 116 of January 27, 1992 and by the Directive 63/2010.

Animals and Study Design

Ten lactating cross-bred ewes (3 to 4 y of age; 55 to 65 kg BW) at their late lactation period [100 ± 8 d in milk (DIM)] were used in this study, where a "before-and-after" experimental design was implemented. The trial was conducted within a farm in the region of Abruzzo (Italy), where the tradition of sheep farming is well-established. Sheep had a 4 wk acclimatization period, in which they were kept on a basal diet, which consisted of mainly hay *ad libitum* [2.7 kg/(animal · d); ~4.5% of BW] plus 0.5 kg/(animal · d) of a custom-formulated concentrate (**Table 1**) that has been offered to the animals in 2 parts during the day (0.3 kg in the morning and 0.2 kg in the afternoon). The basal diet was formulated to meet the ewes' requirements for maintenance and milk production (NRC, 2007), with an adequate level of Se [0.40 mg Se/d, equals ~0.13 mg Se/kg DM of the complete feed (3.2 kg/(animal · d)] in the form Na-selenite. The acclimatization period was then followed by a 40 d high dietary Se supplementation phase, where the same sheep received an organic Se [Sel-Plex (2,000 mg Se/kg DM), Alltech, Australia] supplementation of 1.45 mg Se/d [equivalent to 0.45 mg Se/kg DM of the complete feed (3.2 kg/(animal · d)], that has been added to the concentrate as a replacement of the Na-selenite. The organic Se source used in this study (Sel-Plex) has a guaranteed amount of 2 g organically bound Se [63 % as selenomethionine (SeMet), and 34-36 % as low molecular weight selenocomponents] per kg supplement, as described by the manufacturer (EFSA, 2011). Selenium supplementation in this study has been set not to exceed the maximum level of organic Se (0.5 mg/kg DM) permitted in the EU (EU No. 427/2013; EU No. 489/2015). Sheep had continuous access to water, and a specialized staff monitored the correct and complete ingestion of the formulated concentrate which

was offered separately to each animal using individual troughs. Body temperature, feed intake and milk production were recorded daily for all sheep.

Table 1. Ingredients and chemical composition of the formulated basal diet.

Item	Dietary content
Ingredients of the total diet, % of dietary DM	
Hay	83.00
Concentrate	17.00
Ingredients of the concentrate, % of dietary DM	
Barley	59.55
Soybean flour 44%	22.75
Corn	15.00
Calcium carbonate	2.00
Dicalcium phosphate 17%	0.50
Mineral-vitamin mix[1]	0.20
Nutrient composition, DM basis	
NE_g, Mcal/kg	1.02
CP, %	19.59
NDF, %	17.90
ADF, %	6.01
ADL, %	1.0
Se (Na-selenite), mg/kg	0.80

[1]*Mineral and vitamin premix contained (per kg) 32.00 mega-IU vitamin A, 3.20 mega-IU vitamin D3, 48 mg vitamin E, 166 mg Fe as FeCO3, 62 mg Mn as MnO, 6.2 mg of I as Ca(IO3)2 and 198 mg Zn as ZnO.*

Blood Collection and Se Analysis

To determine the level of Se in blood, jugular venous blood samples were collected at 0, 30, 35 and 40 d of the high dietary Se supplementation. Blood was collected into evacuated tubes with EDTA (2 mL; final EDTA concentration, 2 g/l) and stored on ice until it could be frozen at −20 °C for a whole-blood (WB) Se assay. Selenium concentrations were determined using an Agilent 7500ce ionized coupled plasma mass spectrometer (ICP-MS; Agilent 7500ce, Agilent Technologies, Santa Clara, CA, USA) with modifications as previously described (Tinggi et al., 2004). For the molecular assays (microarray and qPCR), 2.5 mL of WB were collected at T0 (before high dietary Se supplementation) and T40 (after 40 d of high dietary Se supplementation). Blood was collected in each of 2 PAXgene tubes (Qiagen SpA, Milan, MI, Italy) according to the manufacturer's instructions for subsequent RNA isolation. The PAXgene tubes were first stored at room temperature overnight and then at −20 °C until RNA isolation.

Microarray Custom Design and Annotation

A custom whole-transcriptome 4x44K microarray was designed for this study. Briefly, we used the complete *Ovis aries* cDNA library (23,112 mRNA sequences; genome assembly Oar_V3.1) available from the ENSEMBL online repository (ftp://ftp.ensembl.org/pub/release-80/fasta/ovis_aries/). In addition, a library of sheep's Estimated Sequence Tag (EST) sequences (338,827 sequences) has been retrieved from the GenBank repositories (http://www.ncbi.nlm.nih.gov/genbank/dbest/dbest_access/), and the EST sequences have been blasted (using BLASTX on a local Unix server) against themselves and against the ENSEMBL cDNA library to check for any non-overlapping entries. We obtained 1,662 EST non-overlapping sequences that were added to the mRNA library (23,112 sequences) FASTA file. The mRNA and the ESTs (total 24,774 sequences) were then uploaded to the eARRAY online portal hosted by Agilent for array design (http://earray.chem.agilent.com/earray), and 2 oligo probes (60-mer each) per sequence have been designed. Using the quality scores assigned for each probe by the Agilent's probe design algorithm (denoted as BC score, with a range from 1 - high quality to 4 - low quality), we excluded all probes with BC scores of 2 and above. Moreover, all the probes showing a possible non-self perfect match (NSPM) have been excluded as well. Finally, the designed platform contained 43,803 oligo probes (average of 1.84 probes per sequence) of which 500 probes (50 probes repeated 10 times) were assigned as biological probes control group, and 1,417 probes were Agilent internal technical control probes (45,220 features in total). All arrays were printed using Sureprint technology (Agilent Technologies, USA). This custom microarray platform was deposited in the Gene Expression Omnibus (GEO) online repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number **GPL20576**.

RNA Isolation and Microarray Analysis

Total RNA was isolated in accordance with the protocol of the PAXgene blood RNA kit (PreAnalytics/Qiagen, Milan, Italy). A DNase treatment was performed on the column for quality assurance before RNA was eluted from the filter and stored at −80°C until processed. Total RNA concentration was determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA), and its quality was measured by using the 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). All the 20 samples have passed the RNA quality

criteria, i.e., RNA concentration ≥ 40 ng/µL and RNA integrity number (RIN) ≥ 8, hence; they were all considered for the subsequent microarray analysis.

Preparation of the labeled cDNA probes and subsequent Genechip hybridizations were performed in accordance with the Agilent Technologies One-Color Microarray gene expression analysis guidelines as previously described (Elgendy et al., 2015). Briefly, total RNA (100 ng) from each animal was labeled individually with Cy3 using the Low RNA Input Linear Amplification Kit, One-Color (Agilent Technologies, USA). A mixture of 10 Agilent's different viral polyadenylated RNAs (Spike-In Mix) was added to each RNA sample before amplification and labelling. A purification step was applied to the labelled cRNA using the RNeasy Mini Kit (Qiagen, Milan, Italy), and sample concentration and specific activity (Cy3 pmol/µg cRNA) were measured. Each single one-color labeled sample was hybridized (65°C, 17 h) in Agilent's SureHyb Hybridization Chambers containing 100 µL (corresponding to 1.65 µg of Cy3-labeled cRNA) of the final hybridization mixture. Microarrays were scanned at 5 µm/pixel resolution by the Agilent DNA Microarray Scanner G2505B (Agilent Technologies, USA), and the images were analyzed by the Agilent's Feature Extraction Software (version 9.5.1), using the GE2-v5_95_Feb07 FE extraction protocol [default settings were modified to scan the same slide twice at 2 different resolution levels (XDR Hi 100% and XDR Lo 10%)]. The extracted data were normalized and processed as previously described in Elgendy et al. (2015). Briefly, The 20 samples have been normalized together in a single analysis to ensure a uniform normalization and to check for any possible inter-array outliers. Data Normalization was performed by the statistical software "R" (http://www.r-project.org). The intensities of the polyadenylated Spike-In control probes were used to identify the best normalization procedure. Quantile normalization using the "*limma"* package yielded the best results, thus quantile-normalized data were used in all subsequent analyses. After normalization, all control features and Spike-In probes were filtered out. A further filtering step was carried out by removing probes that reported missing values or no reactivity (probes with Feature Extraction flag equal to 0) in at least 50% of samples. Numerical values corresponding to the missing microarray intensities were imputed by the MultiExperiment Viewer (MeV; http://www.tm4.org/) application as described by Saeed et al. (2003). The microarray data have been deposited in GEO and are accessible through the accession number **GSE69997**.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was performed to confirm the expression pattern of 7 genes. The PCR primer sequences used for the quantification of the genes encoding ankyrin repeat domain 10 (ANKRD10), selenoprotein W1 (SEPW1), septin 8 (SEPT8), adenylate cyclase 6 (ADCY6), G protein-coupled estrogen receptor 1 (GPER1), Sec61-alpha-1-subunit (SEC61A1) and SLX4 interacting protein (SLX4IP) are reported in **Table 2**.

Table 2. Genes and their corresponding oligonucleotide sequences for qPCR validation

Gene	Description	Ensembl Gene ID	Forward primer (5' >> 3')	Reverse primer (5' >> 3')	UPL Probe	Amplicon (bp)
ANKRD10	Ankyrin repeat domain 10	ENSOARG00000007048	TCGACCTGAGAAATGCCAGT	GGTGCACTCTCGGAAACC	74	71
SEPW1	Selenoprotein W, 1	ENSOARG00000011453	GGTCGTCGTCCGAGTTGTTT	CCAAACGGCTAGGGAACTCA	117	98
SEPT8	Septin 8	ENSOARG00000014705	AGAAACTGGACAGCAAGGTGA	CTCGTCATCCGTGGGAAACT	56	149
ADCY6	Adenylate cyclase 6	ENSOARG00000019182	AACCGTGGTGATGCCTTC	GTGTGCAGATCCCAATGACA	150	85
GPER1	G protein-coupled estrogen receptor 1	ENSOARG00000018024	TTCAACCTGGACGAGCAGTA	GAGGAAGAAGACGCTGCTGTA	4	96
SEC61A1	Sec61 alpha 1 subunit (S. cerevisiae)	ENSOARG00000005237	CTTCAACGGAGCCCAAAAG	ATCCCCGTCATCACGTACAC	9	75
SLX4IP	SLX4 interacting protein	ENSOARG00000010370	GGTGTGTGGAACAACTACTTCCT	TCTCAGTTCTGTTCACAATTTCTTG	117	78
GAPDH[a]	Glyceraldehyde-3-phosphate dehydrogenase	ENSOARG00000007894	GGTCGGAGTGAACGGATTTG	ACCATGTAGTGAAGGTCAATGAAG	147	117
SDHA[a]	Succinate dehydrogenase complex, subunit A	ENSOARG00000015619	CCACCAGGTCCCATACTGTC	TCACGGTGTCGTAGAAGTGC	25	99
YWHAZ[a]	Tyrosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein, zeta	ENSOARG00000018661	GCAAAAGACGGAAGGTGCTG	AAAAGCTTCTTGGTATGCTTGC	102	262
G6PD[a]	Glucose-6-phosphate dehydrogenase	ENSOARG00000004237	TATCATCATGGGTGCATCGGG	TGTAGGTGTCTTCGGGCAAA	83	98

[a] *Internal control gene*

Five genes (ADCY6, ANKRD10, GPER1, SEC61A1, and SLX4) were randomly selected from the ranked list of the differentially expressed (DE) genes according to their fold-change (FC) and false discovery rate (FDR) values. The ADCY6 and ANKRD10 genes were selected from the up-regulated DE genes, while GPER1, SEC61A1 and SLX4IP were selected from the down-regulated ones. Genes with high FC and low FDR were favored in the selection. The other 2 genes (SEPW1 and SEPT8) were intentionally selected to check for any Se-induced molecular signature (in the form of induced selenoproteins). To ensure a uniform workflow, the RNA samples used for qPCR were the same used for the microarray analysis. Where possible primers were designed across exon/intron boundaries to avoid genomic DNA amplification. To ensure the qPCR product specificity, consideration has been taken to design (*de novo*) all primers using the mRNA sequences of the corresponding transcripts in the list of the DE genes (each transcript has its unique ID in the custom-microarray platform). Additionally, an *in-silico* analysis using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was further performed to ensure the specificity of the possible amplified qPCR product. First-strand cDNA was synthesized from 0.7 µg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and stored at -20ºC until further use. Quantitative RT-PCR was performed using Power SYBR Green I (Thermo Fisher Scientific Inc, Waltham, MA, USA) and Roche universal probe library (UPL; Roche Diagnostics, Indianapolis, IN, USA) assays as previously described (Elgendy et al., 2015). Briefly, the qPCR reactions (10 µL final volume) consisted of 1X LightCycler 480 Probe Master (Roche Applied Science, Indianapolis, IN, USA), 300 or 600 nM forward and reverse primers (Integrated DNA Technology; TEMA ricerca, Bologna, Italy), 200 nM human UPL probe (final concentrations) and 2.5 µL of 50 ng/µL cDNA. Assays were performed in duplicates by the LightCycler 480 instrument (Roche Applied Science, USA) using the standard PCR conditions (an activation step at 95 ºC for 10 min; 45 cycles at 95 ºC for 10 s and at 60 ºC for 30 s, and a cooling step at 40 ºC for 30 s). No template and no-reverse-transcription controls were used in each assay to ensure a specific and uncontaminated reaction. For each qPCR assay, 8 standard curves were generated using duplicate 3-fold serial dilutions of control sheep cDNA pool.

Data were analyzed with the LightCycler 480 software release 1.5.0 (Roche Applied Science, USA) using either the second derivative or the fit point method. Messenger RNA

relative quantification (RQ) was calculated by the ΔΔ*Ct* method (Livak and Schmittgen, 2001), and normalized using the average RQ values of 4 internal control (reference) genes (GAPDH, SDHA, YWHAZ and G6PD). The internal control genes were selected after the study by Vorachek et al. (2013) on reference gene selection for qPCR studies in sheep neutrophils. The selected reference genes showed an unchanged expression pattern between the experimental groups (T40 versus T0) after a quality control qPCR step, hence they were used for the data normalization.

Functional Annotation and Gene Set Enrichment Analysis

The functional analysis of transcript lists of interest was performed by the analysis of the enrichment for specific Gene Ontology (GO) terms using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Dennis et al., 2003; Huang da et al., 2009) as implemented in the online platform (http://david.abcc.ncifcrf.gov/). The annotated probes for the list of the DE genes were matched to their bovine (*Bos taurus*) orthologs, using the Ensembl genome browser data-mining tool (BioMart; http://www.ensembl.org/biomart/martview/), and analyzed against the DAVID's built-in *Bos taurus* genome. Analyses were performed using the default GO_FAT terms and KEGG pathways included in the DAVID knowledgebase, implementing a gene count of 5 and an EASE score of 0.05. The annotation clusters in the present study were selected using an enrichment score (ES) of 1.17 or more. Gene Set Enrichment Analysis (GSEA; http://www.broadinstitute.org/gsea/index.jsp) was also used to examine the significantly-enriched pathways by comparing the normalized data of the entire gene transcripts from the T40 group to 880 curated canonical pathway gene sets in the GSEA Molecular Signatures Database (MsigDB; Subramanian et al., 2005). All the annotated transcripts (33,413 features in total) with expression values were uploaded to the software and compared with catalog C2 (4725 curated gene sets).

Statistical Analysis

Differentially expressed genes were identified using the two-class paired *t*-test in the Significance of Microarray Analysis (SAM) software v.4.0 (Tusher et al., 2001), enforcing an FDR ≤ 0.05 and an FC threshold ≥ 1.5. All other statistical tests (Spearman correlation analysis, one-way ANOVA and Student's *t*-test) were carried out by the GraphPad Prism 5 software (San Diego, CA, USA). In evaluating the effect of Se supplementation on WB-Se concentration, data were analyzed by one-way ANOVA

(Kruskal-Wallis test) followed by Dunn's Multiple Comparison Test. The RQ values of the qPCR-analyzed genes were expressed as FC, and the significance of gene expression between groups (T0 and T40) was determined using the Student's *t*-test. Data are reported as means ± SD, and statistical significance was declared at $P \leq 0.05$.

Results

The health status of all the experimental animals was satisfactory throughout the experiment. Ewes produced an average daily amount of 600 ± 95 mL of milk, and none of them showed any clinical signs of Se toxicity at any time during the study. The effect of high dietary Se supplementation on WB-Se concentrations are shown in **Figure 1**. At d 0 (baseline), WB-Se concentration was 112.5 ± 14.63 ng/mL. High dietary Se supplementation increased (all $P < 0.0001$) WB-Se concentrations at d 30 to 283 ± 18.75 ng/mL, and the Se-concentration remained stable at 35 (287.5 ± 21.32 ng/mL) and 40 d (287.5 ± 21.68 ng/mL). Therefore, after a stable WB-Se concentration (from d 30 to d 40), the blood collected at T40 has been used to evaluate the effect of this supplementation on the transcriptome of sheep.

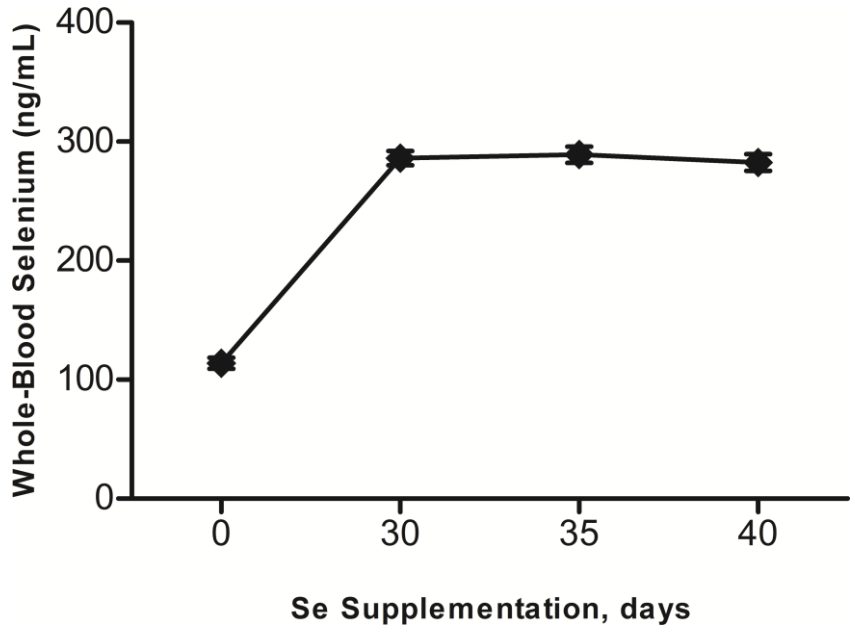

Figure 1: The effect of high dietary organic selenium (Se) on whole-blood (WB)- Se concentration in sheep. *Whole-blood (WB)- Se concentrations were measured after 0, 30, 35, and 40 d of organic Se supplementation (0.45 mg Se/kg DM; ~1.40 mg Se/d). Whole-blood Se concentration for each time point are shown as separate bars. At baseline (d 0), WB-Se concentration was 112.5±14.63 ng/mL. High organic Se supplementation increased (P < 0.0001) WB-Se concentrations at 30 d to 283±18.75 ng/mL, where the Se concentration remained stable at 35 (287.5±21.32 ng/mL) and 40 d (287.5±21.68 ng/mL).*

After screening more than 23,000 unique transcripts in the microarray platform, a total of 1,186 unique transcripts displayed significant differences in expression of 1.5-fold or more between the T40 and T0 samples (T40 versus T0). Of those transcripts, 942 and 244 were up- and down-regulated, respectively (*Supplementary Table 1*). Those identified transcripts were able to discriminate the 2 experimental groups, on a heatmap scale, upon a hierarchical clustering analysis (**Figure 2**) as well as after a principal component analysis (PCA; **Figure 3**).

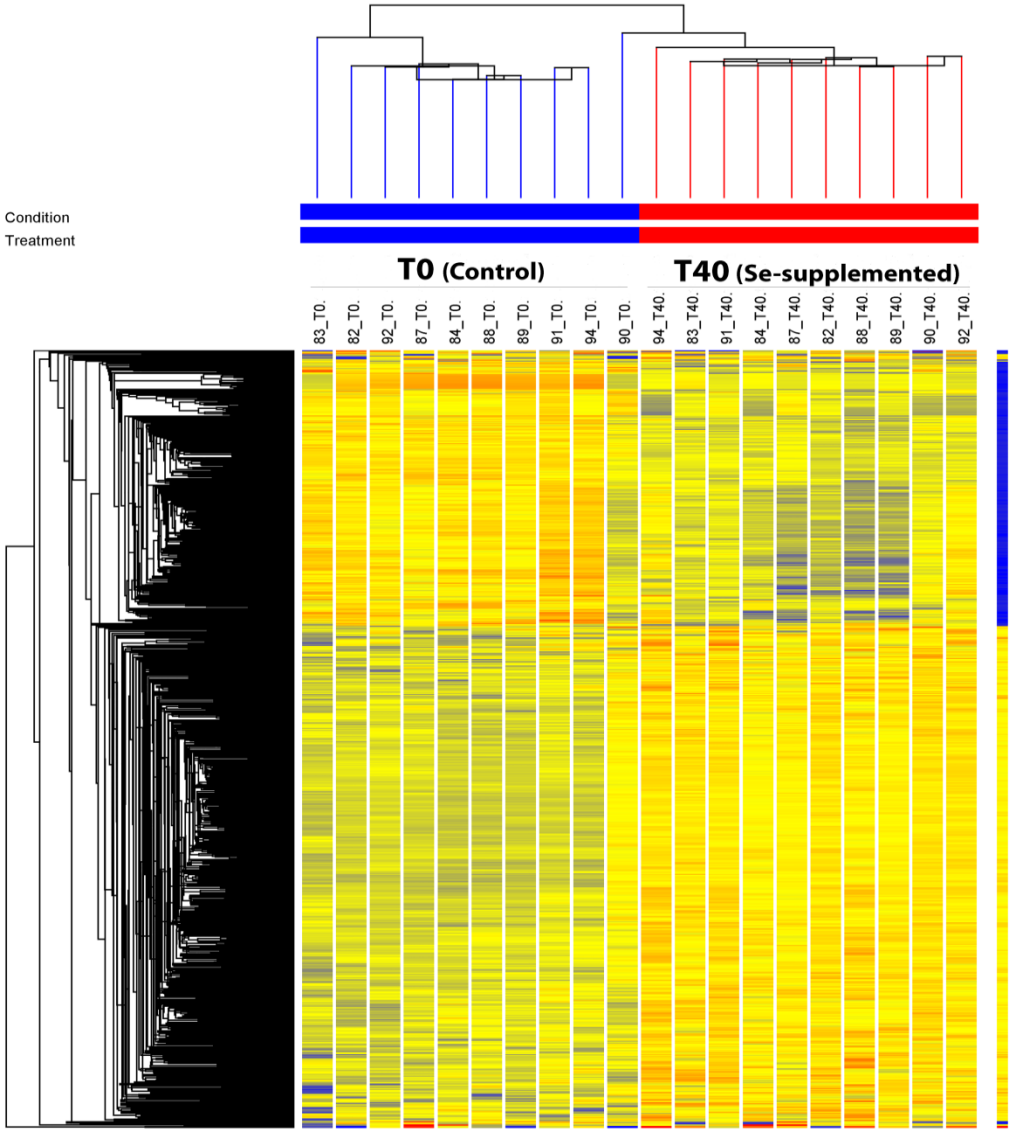

Figure 2: Cluster dendrogram of the differentially expressed (DE) genes at T0 [before high dietary selenium (Se) supplementation] and T40 (after 40 d high dietary Se). *A heatmap was generated from the differentially expressed (DE) transcripts (≥ 1.5-fold with respect to controls) using the hierarchical clustering (Euclidean distance clustering algorithm) option in the GeneSpring software v.13.1 (http://genespring-support.com/). In the heatmap, red indicates an expression level higher than the mean across all subjects, and blue denotes expression level lower than the mean.*

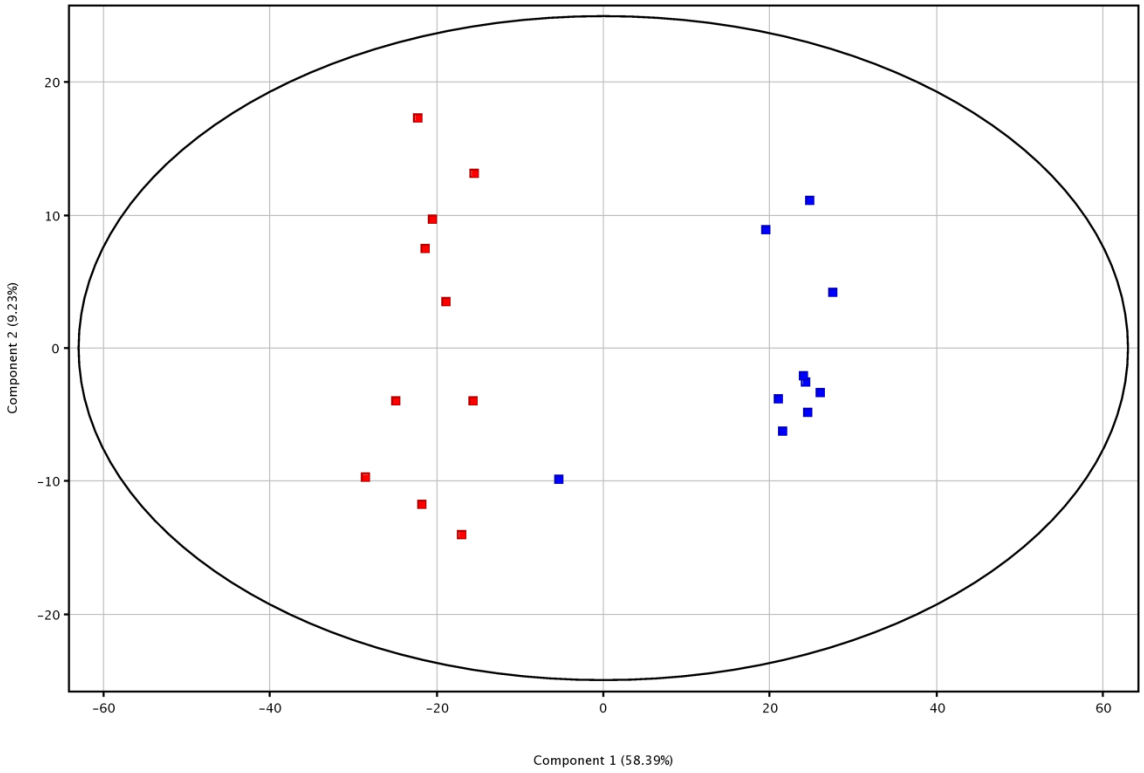

Figure 3: *PCA plot of the Se-supplemented sheep at two time points (T40 and T0) shows the two principal components of greatest variation, covering 58.39% (x-axis) and 9.23% (y-axis) of the total variance. Squares distinguish the different time points, where the red-colored squares (left) represents the Se-supplemented animals after 40-d of Se supplementation (T40), while the blue-colored squares (right) depict the same animals before the supplementation (T0).*

While the genome of *Ovis aries* is relatively poorly annotated, the corresponding genes of all the DE transcripts in this study (some transcripts encoded the same gene) have been matched to their bovine (*Bos taurus*) orthologs, which resulted in retaining 772 and 192 genes out of the total 942 and 244 up- and down-regulated transcripts, respectively (*Supplementary Table 2*). Those filtered *Bos taurus* ortholog genes were used in the subsequent functional annotation analyses.

To cross-validate the microarray platform, the relative expression of 7 genes (ANKRD10, SEPW1, SEPT8, ADCY6, GPER1, SEC61A1 and SLX4IP) was analyzed by qPCR. Four genes (ANKRD10, SEPW1, SEPT8 and GPER1) revealed statistically significant differences between T40 and T0 (**Table 3**), while the other 3 genes (ADCY6, SEC61A1 and SLX4IP), however not statistically significant when compared to the T0 group, they showed the same trend of expression (up- or down-regulation) compared to their

corresponding microarray FC values (**Figure 4**). The correlations between the microarray and the qPCR output are reported in **Table 3**.

Table 3. Spearman's rho for the set of selected genes used for the qPCR cross-validation.

Gene name	FC[1] qPCR	FC microarray	Spearman's rho[†]
ANKRD10	1.71[*]	2.04	0.6301
SEPW1	1.88[*]	1.95	0.9068
SEPT8	1.41[*]	1.69	0.3714
ADCY6	2.72	3.3	0.4571
GPER1	-1.26[*]	-1.8	0.2977
SEC61A1	-1.1	-1.91	0.3684
SLX4IP	-1.1	-1.97	0.4045

[1]FC = Fold Change. For the qPCR, the mRNA relative quantification (RQ) was calculated by the ΔΔCt method, and normalized using the average of the RQ values of 4 internal control (reference) genes (GAPDH, SDHA, YWHAZ and G6PD). For the microarray, the FC was calculated using the paired t-test in the SAM (Significance of Microarray) software, comparing T40 (40 d high organic Se supplementation) versus T0 (control) values.
[*]Genes that were significantly (P ≤ 0.05) regulated between groups (T0 and T40) by qPCR analysis.
[†]Correlations were calculated using the RQ values of the genes analyzed by qPCR and their corresponding microarray intensities (normalized raw data).

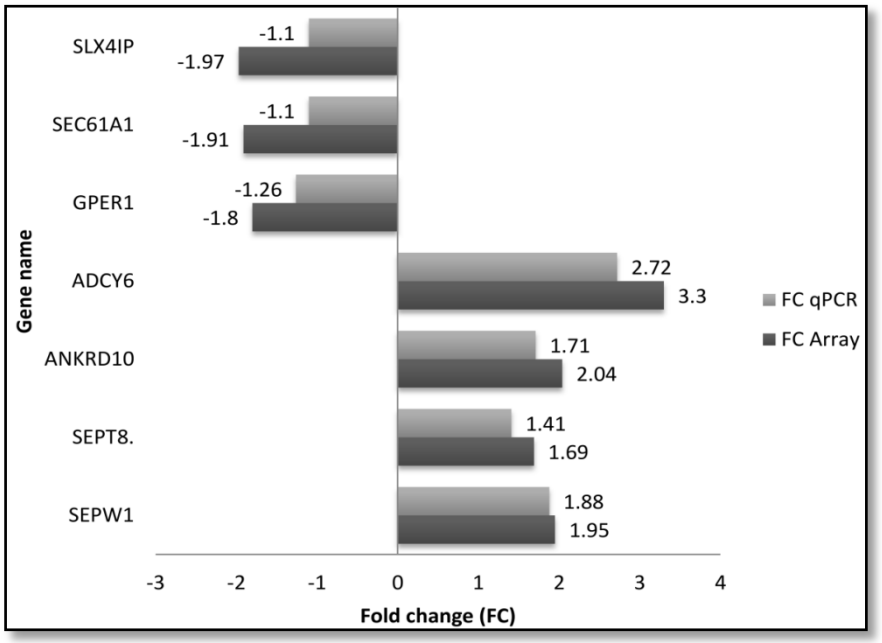

Figure 4: Microarray data validation on a subset of 7 differentially expressed genes by qPCR. *Fold changes are expressed as T40 (40 d high dietary Se supplementation) versus T0 (control), in which negative fold changes indicate down-regulation while positive fold changes indicate up-regulation of expression due to high dietary organic Se supplementation. FC = Fold change.*

The functional GO terms with the highest *P* values (all $P \leq 0.05$) in the enrichment analysis, along with the involved genes in each term are shown in **Table 4**. The significantly enriched terms included genes involved in lipid binding ($P = 0.0009$), lymphocyte and leukocyte activation, positive regulation of T and lymphocyte cell differentiation, leukocyte and lymphocyte proliferation, and cytokine binding.

Table 4. Functional annotation of the differentially expressed genes (FDR ≤ 0.05) in the T40 (40 d high dietary Se) samples compared to the T0 (control) ones. Functional annotation was performed using the default settings in DAVID, an EASE score of 0.05 and GO term categories. Counts represent the number of genes from the list associated with a given GO term. Genes that were up-regulated are indicated by '+' while those down-regulated are indicated by '−'.

Direction	Category and term	Count	Genes	Fold enrich-ment	P Value
+	MF:GO:0008289~lipid binding	18	PRKCD, APOA5, HNF4A, RXRB, BPIFB3, ITPR3, BPIFA1, ENSOART00000006166, DEF8, SNX20, ENSOART00000019129, SNX5, ARHGAP32, SNX27, FABP6, KIF16B, APOD, ANXA9	2.49	0.0009
+	MF:GO:0005088~Ras guanyl-nucleotide exchange factor activity	8	RANBP10, ITPR3, PREX1, FGD3, ARHGEF11, ITSN2, ARHGEF12, ABR	4.11	0.0030
+	BP:GO:0046649~lymphocyte activation	10	PRKCD, CCND3, ERCC1, RAG2, FOXP3, LAX1, CXCR4, CD40, FAS, ENSOART00000017658	3.2	0.0038
+	BP:GO:0045321~leukocyte activation	11	PRKCD, CCND3, ERCC1, TLR4, RAG2, FOXP3, LAX1, CXCR4, CD40, FAS, ENSOART00000017658	2.93	0.0041
+	BP:GO:0045582~positive regulation of T cell differentiation	5	FOXP3, VNN1, HLX, IL2RG, AP3D1	7.13	0.0045
+	BP:GO:0045619~regulation of lymphocyte differentiation	6	FOXP3, FAS, VNN1, HLX, IL2RG, AP3D1	5.35	0.0047
+	BP:GO:0070661~leukocyte proliferation	5	PRKCD, CCND3, RAG2, CXCR4, ENSOART00000017658	6.79	0.0055
+	BP:GO:0046651~lymphocyte proliferation	5	PRKCD, CCND3, RAG2, CXCR4, ENSOART00000017658	6.79	0.0055
+	BP:GO:0032943~mononuclear cell proliferation	5	PRKCD, CCND3, RAG2, CXCR4, ENSOART00000017658	6.79	0.0055
+	MF:GO:0019955~cytokine binding	8	CXCR4, CCR9, IL10RA, ENSOART00000021229, IL1RL1, CXCR6, IL2RG, TNFRSF1A	3.66	0.0058
+	MF:GO:0035091~phosphoinositide binding	7	ITPR3, ARHGAP32, SNX27, SNX20, ENSOART00000019129, KIF16B, SNX5	4.18	0.0061
+	BP:GO:0006631~fatty acid metabolic process	11	PLP1, SCD5, ALOX5, ALOX12, CPT2, ALOX15, FADS2, ADIPOR1, PPARA, TYRP1, TNFRSF1A	2.75	0.0064
+	BP:GO:0045621~positive regulation of lymphocyte differentiation	5	FOXP3, VNN1, HLX, IL2RG, AP3D1	6.48	0.0065
+	MF:GO:0005543~phospholipid binding	9	ITPR3, ENSOART00000006166, SNX20, ENSOART00000019129, SNX5, ARHGAP32, SNX27, KIF16B, ANXA9	3.21	0.0066
+	BP:GO:0042127~regulation of cell proliferation	19	FLT4, KDR, DHCR7, MYD88, CDH5, FGFR4, ALOX12, ALOX15, FOXP3, CD40, DPT, GRN, DNMT1, GHRL, HLX, CTNNA1, IFI30, NOS2, EPO	1.97	0.0076
+	MF:GO:0005089~Rho guanyl-nucleotide exchange factor activity	7	FGD3, ARHGEF11, ITPR3, PREX1, ITSN2, ABR, ARHGEF12	3.87	0.0089
+	MF:GO:0005085~guanyl-nucleotide exchange factor activity	10	RANBP10, FGD3, ARHGEF11, ITPR3, PREX1, ITSN2, ABR, ARHGEF12, RAB3IL1, DOCK6	2.79	0.0094

+	CC:GO:0005886~plasma membrane	55	RHOH, KDR, ADRB3, LAPTM5, CDH5, NECAP1, PCDH19, GNB1, CALB2, RAB11B, FLOT2, PNPLA2, ADRA1A, TMPRSS11F, CISH, LIPE, AQP3, ITPR3, KCNA1, CD40, KCNMA1, TLR4, ALOX12, ALPL, TF, ARCN1, LAX1, CXCR4, PIGR, LAMP1, CCR9, SLC30A3, ENSOART00000022569, CTNNA1, IL2RG, DGKD, AP3D1, ENSOART00000021229, SNX20, SYN1, HRH1, LPAR5, CXCR6, ITGA5, FAS, MALL, RAB19, MS4A1, VNN1, TNS1, JUP, GJA3, CHRND,	1.36	0.0114
+	BP:GO:0001775~cell activation	11	PRKCD,CCND3,ERCC1,TLR4,RAG2,FOXP3,LAX1,CXCR4,CD40,FAS,ENSOART00000017658,	2.51	0.0120
+	BP:GO:0050865~regulation of cell activation	9	TLR4,FOXP3,LAX1,CD40,FAS,VNN1,HLX,IL2RG,AP3D1	2.88	0.0123
+	MF:GO:0019899~enzyme binding	13	PRKCD,CDH5,SQSTM1,RANBP10,CCND3,EPAS1,CUL4A,CD40,LAX1,DOCK6,HINFP,RTKN,JUP	2.24	0.0133
+	CC:GO:0005829~cytosol	17	ALOX5,RILPL2,PNPLA2,ARF1,ALOX12,POTEJ,ENSOART00000010183,ALOX15,LIPE,PDE9A,SNX27,NXN,SELENBP1,JUP,NOS2,ENSOART00000017658,ANXA9	1.9	0.0135
+	BP:GO:0035023~regulation of Rho protein signal transduction	7	FGD3, ARHGEF11, ITPR3, PREX1, ITSN2, ABR, ARHGEF12	3.44	0.0153
+	BP:GO:0021700~developmental maturation	6	PLP1, KDR, CDH5, EPAS1, VSX1, EPO	3.98	0.0164
+	BP:GO:0051249~regulation of lymphocyte activation	8	FOXP3, LAX1, CD40, FAS, VNN1, HLX, IL2RG, AP3D1	3	0.0166
+	BP:GO:0045580~regulation of T cell differentiation	5	FOXP3, VNN1, HLX, IL2RG, AP3D1	4.92	0.0174
+	BP:GO:0048469~cell maturation	5	PLP1, KDR, VSX1, EPAS1, EPO	4.6	0.0219
+	BP:GO:0006350~transcription	27	ZNF677, USP22, HNF4A, ENSOART00000003469, RXRB, EPAS1, VSX1, MAML2, ZNF574, LIPE, RBM14, RFX2, NR2F2, IRF1, PPARA, PHTF1, ZNF554, POLR1B, ENSOART00000020476, DNMT1, HINFP, HLX, ELF5, ZSCAN2, BANP, OVOL1, ACTL6B	1.57	0.0227
+	BP:GO:0043122~regulation of I-kappaB kinase/NF-kappaB cascade	6	RHOH, MYD88, SQSTM1, TLR4, CD40, TNFRSF1A	3.64	0.0233
+	BP:GO:0006357~regulation of transcription from RNA polymerase II promoter	16	ID1, HNF4A, ENSOART00000004159, RXRB, EPAS1, MAML2, NR2F2, FOXP3, RBM14, CRYM, PPARA, ENSOART00000020476, HINFP, OVOL1, EPO, TNFRSF1A	1.85	0.0259
+	MF:GO:0030695~GTPase regulator activity	14	NRK,RANBP10,FGD3,ADAP1,ARHGEF11,ITPR3,PREX1,ITSN2,ABR,ARHGDIG,RTKN,ARHGEF12,RAB3IL1,DOCK6	1.95	0.0272
+	MF:GO:0005083~small GTPase regulator activity	11	NRK, RANBP10, FGD3, ADAP1, ARHGEF11, ITPR3, PREX1, ITSN2, ABR, ARHGDIG, ARHGEF12	2.21	0.0273
+	BP:GO:0002694~regulation of leukocyte activation	8	FOXP3, LAX1, CD40, FAS, VNN1, HLX, L2RG, AP3D1	2.68	0.0287
+	BP:GO:0050867~positive regulation of cell activation	7	TLR4, FOXP3, CD40, VNN1, HLX, IL2RG, AP3D1	2.94	0.0310
+	MF:GO:0003777~microtubule motor activity	6	DNAH11, KIF24, KLC3, KIF3C, KIF16B, DNAH2	3.38	0.0313
+	BP:GO:0042113~B cell activation	5	PRKCD, ERCC1, RAG2, LAX1, CD40	4.07	0.0327
+	CC:GO:0016023~cytoplasmic membrane-bounded vesicle	14	NECAP1, CSPG5, SYT4, SLC1A5, TF, ARCN1, ENSOART00000010183, LAMP1, MALL, ENSOART00000016099, DGKD, SYN1, TYRP1, SYN2	1.89	0.0336
+	MF:GO:0060589~nucleoside-triphosphatase regulator activity	14	NRK, RANBP10, FGD3, ADAP1, ARHGEF11, ITPR3, PREX1, ITSN2, ABR, ARHGDIG, RTKN, ARHGEF12, RAB3IL1, DOCK6	1.89	0.0344
+	CC:GO:0031410~cytoplasmic vesicle	16	NECAP1, CSPG5, SYT4, SLC1A5, TF, ARCN1, ENSOART00000010183, ITPR3, LAMP1, SLC30A3, MALL, ENSOART00000016099, DGKD, SYN1, TYRP1, SYN2	1.77	0.0350

+	BP:GO:0002252~immune effector process	7	PRKCD, MYD88, ERCC1, FOXP3, LAX1, FAS, SAMHD1	2.85	0.0351
+	CC:GO:0031988~membrane-bounded vesicle	14	NECAP1, CSPG5, SYT4, SLC1A5, TF, ARCN1, ENSOART00000010183, LAMP1, MALL, ENSOART00000016099, DGKD, SYN1, TYRP1, SYN2	1.85	0.0396
+	BP:GO:0008283~cell proliferation	9	PRKCD, GNB1, CCND3, ERCC1, RAG2, RXRB, CXCR4, ENSOART00000017136, ENSOART00000017658	2.31	0.0402
+	BP:GO:0042592~homeostatic process	20	PLP1, KDR, ADRB3, AIPL1, SLC9A1, IKBKB, EPAS1, KCNMA1, TF,FOXP3, ENSOART00000022569, SEPW1, ENSOART00000017658, NCDN, FAS, NXN, GHRL, QSOX1, SLC9A2, EPO	1.62	0.0403
+	BP:GO:0002684~positive regulation of immune system process	10	MYD88, TLR4, FOXP3, LAX1, AQP3, CD40, VNN1, HLX, IL2RG, AP3D1	2.16	0.0415
+	CC:GO:0031982~vesicle	16	NECAP1, CSPG5, SYT4, SLC1A5, TF, ARCN1, ENSOART00000010183, ITPR3, LAMP1, SLC30A3, MALL, ENSOART00000016099, DGKD, SYN1, TYRP1, SYN2,	1.72	0.0437
+	MF:GO:0000166~nucleotide binding	67	ENSOART00000000072, PRKCD, FLT4, RHOH, KDR, NRK, PLK3, ENSOART00000002476, MKNK1, ENSOART00000002789, WRNIP1 ,ARL10, ENSOART00000003168, TAOK2, PGS1, ENSOART00000004309, IRAK2, PANK3, RAB11B, STK3, DIRAS2, FGFR4, RPS6KA2, IKBKB, DNAH11, POTEJ, ENSOART00000009416, RAVER2, KIF24, ARF1, GTPBP2, RBM14, UCK1, PDE2A, DDX27, RBM12, ATP2A3, CHD3, ARL16, PFKFB4, SYN1, ACTL6B, KIF3C, CLCN7, ABCB6, MYH14, DNAH2, SRSF3, RALYL, SNRNP70, NOS2, MAP3K8, SYN2, PGK1, DDX23, CRYM, CSNK1E, HNRNPA1, BLK, RBM23, HK2, DOCK6, POR, RAB19, SART3, KIF16B, SEPT8	1.23	0.0438
+	BP:GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	5	PRKCD, MYD88, ERCC1, FOXP3, FAS	3.57	0.0499
+	BP:GO:0002449~lymphocyte mediated immunity	5	PRKCD, MYD88, ERCC1, FOXP3, FAS	3.57	0.0499
+	BP:GO:0048871~multicellular organismal homeostasis	5	KDR, ADRB3, AIPL1, EPAS1, NCDN	3.57	0.0499
+	BP:GO:0002250~adaptive immune response	5	PRKCD, MYD88, ERCC1, FOXP3, FAS	3.57	0.0499
+	KEGG PATHWAY:bta03320:PPAR signaling pathway	9	SCD5, APOA5, RXRB, CPT2, ENSOART00000017136, FADS2, FABP6, PPARA, SLC27A2	3.46	0.0040
+	KEGG PATHWAY:bta04920:Adipocytokine signaling pathway	7	IKBKB, RXRB, ENSOART00000017136, ADIPOR1, PPARA, RELA, TNFRSF1A	2.86	0.0338
-	CC:GO:0044456~synapse part	5	CHRNB2, PLCB4, GABRA1, SYT17, SYT10	5.52	0.0118
-	CC:GO:0045202~synapse	5	CHRNB2, PLCB4, GABRA1, SYT17, SYT10	4.07	0.0321
-	CC:GO:0005856~cytoskeleton	10	ENSOART00000000099, ENSOART00000000870, LYST, DYNLRB2, PLCB4, FRMPD4, VIL1, KRT75, MYO1B, H1F0	2.05	0.0466
-	BP:GO:0006955~immune response	6	TLR5, LYST, CCL8, VTN, CD28, IL36RN	2.9	0.0521
-	KEGG PATHWAY:bta04080:Neuroactive ligand-receptor interaction	5	MC2R, RXFP1, NMUR2,GABRA1, SSTR1	3.33	0.0545

GO: gene ontology; BP: biological process; MF: molecular function; 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.

In addition, some of the annotated genes were grouped in 3 signaling pathways: Peroxisome proliferator-activated receptor (PPAR) and adipocytokine signaling pathways (for the genes expressed at greater levels at T40), and neuroactive ligand-receptor interaction pathways (for the genes expressed at lower levels at T40). Furthermore, the molecular function (MF) "nucleotide binding" and the biological process (BP) "transcription" were among the significant GO terms of the highest gene count with 67 and 27 up-regulated genes, respectively (**Table 4**). Cluster enrichment scores are geometrical means of *P*-values for each GO term included in the cluster and expressed on a negative logarithmic scale. In the present study, the clustering analysis by DAVID uncovered 5 positively-enriched clusters of functionally relevant genes in the T40 group with ES of 1.17 or more (*Supplementary Table 2*). The first cluster had an ES of 2.48 and was represented mainly by the MF "lipid" and "phospholipid binding", while the second cluster (ES = 1.65) consisted of MF terms related to guanyl-nucleotide exchange factor activity, GTPase regulator activity and Ras protein signal transduction. The third (ES = 1.5) and the fourth cluster (ES = 1.4) were dominated by immune-system-related (lymphocyte and leukocyte activation, adaptive immune response, cytokine production, positive regulation of T and B cell differentiation) BP terms. The least positively-enriched cluster (ES = 1.17) represented membrane trafficking-related (cytoplasmic, and membrane-bounded vesicle) CC terms.

To further consider the biological significance of our data, we used GSEA to identify pathways that correlate with the higher dietary Se supplementation. GSEA is a computational method that identifies shared differential gene expression of predefined, functionally related gene sets representing biological pathways. This is quantified by using a different type of ES, a weighted Kolmogorov-Smirnov-like statistic that evaluates if the members of the pathway are randomly distributed or found at the extremes (top or bottom) of the list (Subramanian et al., 2005). The GSEA analysis revealed that most of the core-enriched genes contributing to each individual gene set significantly enriched at T40 (positive ES) fell into an immunity related pathway, such as B and T cell receptor signaling pathways, adipocytokine signaling pathway and Toll-like receptor signaling pathway (**Figure 5**). On the other hand, some other pathways, such as oxidative phosphorylation, steroid hormone biosynthesis, basal transcription factors and selenoamino acid metabolism were among the top pathways less enriched (negative ES) in the T0 group compared to the T40 one (data not shown). A full list of the rank ordered genes participating in the top positively enriched pathways are reported in *Supplementary Table 4*.

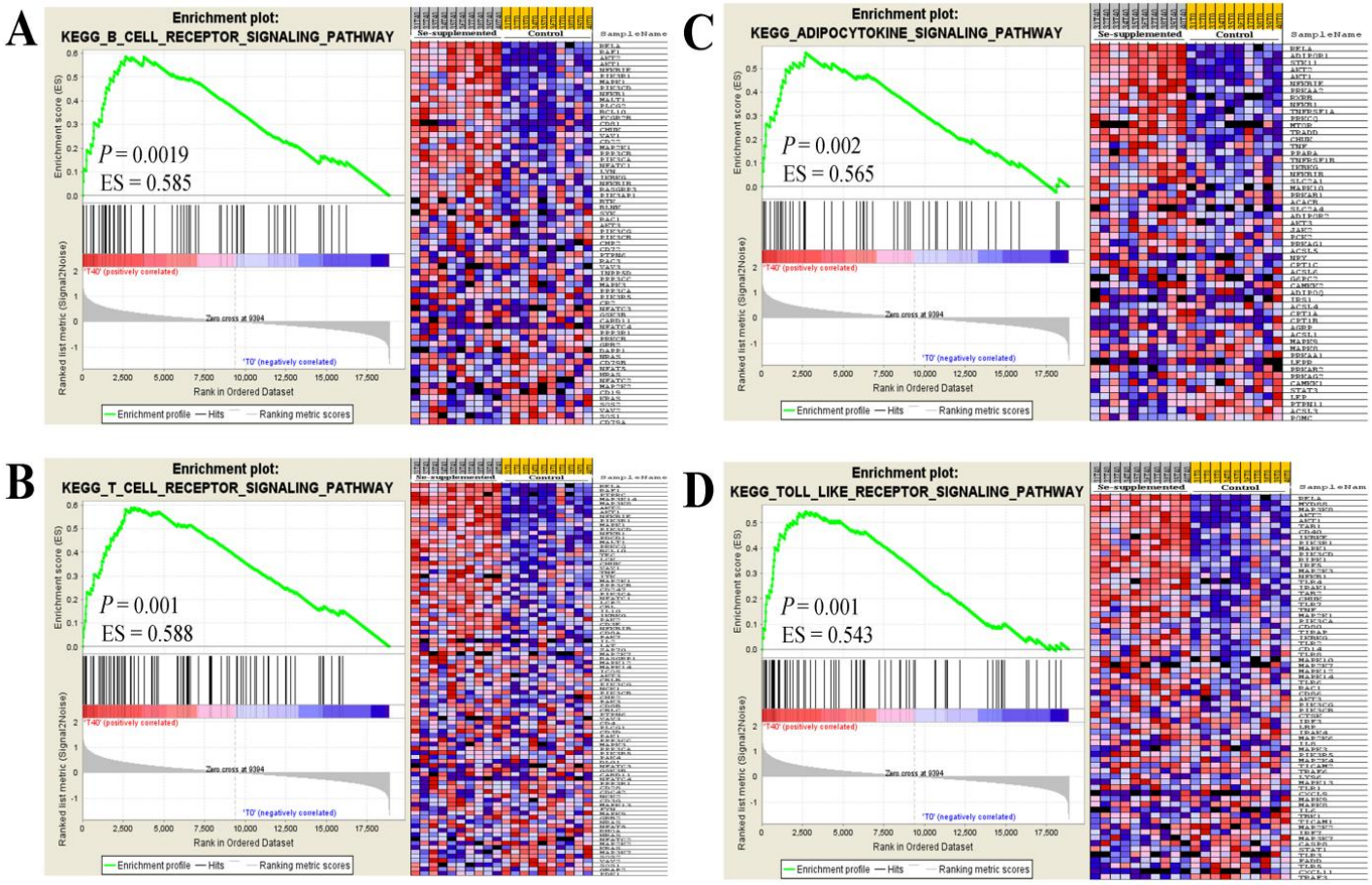

Figure 5: GSEA Enrichment plot (score curves). *Gene set enrichment analysis (GSEA) was performed with the canonical pathway gene sets in GSEA Molecular Signatures Database (880 sets). "Signal-to-Noise" ratio (SNR) statistic was used to rank the genes according to their correlation with either the T40 (40 d high dietary Se supplementation) phenotype (red) or the T0 (control) phenotype (blue). The heatmap on the right side of each panel visualizes the ranked, ordered, non-redundant list of genes contributing to the enriched pathway (for the detailed list see* ***Supplementary Table 5****). On each panel, the vertical black lines indicate the position of each of the genes of the studied gene set in the ordered, non-redundant data set (Supplementary Table 5). The green curve corresponds to the ES (enrichment score) curve, which is the running sum of the weighted enrichment score obtained from GSEA software. A, B, C and D denote the most enriched (significant) pathways (i.e. Gene sets); B cell receptor signaling, T cell receptor signaling, Adipocytokine signaling and Toll-like receptor signaling pathway, respectively.*

Discussion

Microarray technology can simultaneously measure the differential expression of thousands of genes in a given tissue. The resulted information can be used to examine a certain condition from the perspective of the biological processes or molecular functions involved (functional profiling), rather than from the expression levels of individual genes (Tarca et al., 2006). The objective of this study was to examine the transcriptomic-signature of a high dietary Se supplementation in sheep, and to evaluate weather this signature reflects an induced immune-system. The major finding from the present study was that a high dietary level of Se can modulate the expression of a large number of genes in sheep and leave a noticeable molecular-signature that mainly reflects an immune-system activation and transcription-regulation patterns.

Appropriate dietary Se supplementation in sheep and goats varies from 0.1 to 0.3 mg Se/kg DM in the total diet (Ullery et al., 1978; Smith and Sherman, 2011). While blood concentrations of Se are usually derived from a corresponding dietary Se intake, blood Se levels less than 50 ng/mL are considered as Se-deficient while levels between 50 and 100 are marginal, and greater than 100 are adequate (Koller and Exon, 1986). Ewes in the present study were fed on a basal diet that contained an appropriate level of Se (0.13 mg Se/kg DM of the complete feed) that resulted in an adequate blood Se concentration (112.5 ng/mL) after 4 wk of acclimatization. Thus, the Se status of those ewes at the first sampling time (T0) could be considered as Se-adequate or as Se-nondeficient, and the blood Se concentration at T40 (287.5 ng/mL), and the subsequent results, would be interpreted as the added effect of high dietary Se supplementation in sheep. The fact that Blood Se concentrations respond to supplemental Se in proportion to the magnitude of supplementation is much straightforward in subjects of deficient to low Se status (Xia et al., 2005). However, for Se-nondeficient subjects the relationship of Se intake and blood Se level depends mainly on the form of Se consumed (Combs, Jr., 2015), where organic Se, represented by SeMet, is thought to increase blood Se in adequate and high Se status (Broome et al., 2004; Burk et al., 2006; Combs et al., 2012). The organic source of Se (Se-yeast) used in the present study consisted mainly of SeMet (63%), which partly explains the duplication in blood Se level seen after at 30 to 40 d of Se supplementation to a group of Se-nondeficient sheep.

The importance of Se is commonly ascribed to its wide array of biological roles in the immune system (Mckenzie et al., 2002; Arthur et al., 2003) and the antioxidant machinery (Burk, 2002), where dietary Se can modify the readout of the genetic code thourghout the procees of being transcriptionally-incorporated into selenoproteins (Howard et al., 2013; Duntas and Benvenga, 2014). In the present study, the microarray analysis identified 1,186 transcripts that exhibited changes in expression of 1.5-fold or more after a high dietary Se supplementation. This indicates that Se, even in short-term supplementation (40 d), has a traceable effect on the transcriptome of sheep that could be attributed to it capacity of being a transcriptional modulator. Although this effect appeared relatively moderate in terms of FC, with no transcripts being markedly over- or under-expressed, it is quite expected of nutritional studies not to result in large differences in gene expression (Blanchard et al., 2001; Afman and Muller, 2006; Pagmantidis et al., 2008). High levels of Se in the present study resulted in changes in expression of a small number of the selenoprotein-related genes; namely, SEPW1, selenium binding protein 1 (SELENBP1), glutathione peroxidase 3 (GPX3) and SEPT8. While GPX3 and SEPW1 are classified as nonessential selenoproteins (McCann and Ames, 2011), it was quite unexpected not to find some of the other classical (essential) selenoproteins, e.g., GPX4, SEPP1, SEPS1 or TXNRDs among the affected genes. It was reported by Howard et al. (2013) that the "hierarchy" of differential selenoprotein expression in response to selenium availability is likely accounted for the diversity of health effects associated with dietary Se intake. In support; after a supranutritional Se supplementation, Hugejiletu et al. (2013) reported a significant increase in the expression of GPX4 and SEPS1 (anti-inflammatory selenoproteins) in footrot-affected sheep, while the expression of SEPW1 and GPX1 (antioxidant selenoproteins) showed non-significant changes. In the former study, it could be understood that high dietary Se supplementation resulted in a selective induction of some of the anti-inflammatory selenoproteins, on the expense of other ones, i.e., the antioxidant selenoproteins, as a result of the associated health condition (oxidative stress caused by footrot). On the other hand, sheep in the present study were in normal physiological status with no induced- or spontaneously-occurring pathological stress, which we believe affects the sensitivity of gene expression levels of selenoproteins to the status of Se in the animal's body. Additionally, it is worth mentioning that the findings of the present study represent the difference between high and adequate-Se supplementation rather than high and no-Se supplementation, which might have had more impact on the number and type of regulated selenoproteins.

Selenium and the immune response have been always mentioned together, and Se deficiency is known to contribute to a higher prevalence and severity of diseases present in animal populations (reviewed in Arthur et al., 2003). Supranutritional Se induced both the innate and humoral immune functions in footrot-affected sheep (Hall et al., 2011; Hall et al., 2013; Hugejiletu et al., 2013) and Se-replete dairy cows (Hall et al., 2014b). Furtheremore, Se supplementation improved the growth rate, antioxidant status and humoral immune response in lambs (Kumar et al., 2009). In agreement, the functional analysis in the present study revealed that most of the significantly-regulated genes were mainly involved in multiple immune system-related biological processes. Several genes involved in the lymphocyte and leukocyte activation were significantly up-regulated, and this still supports the fact of Se being an immunostimulant agent (Arthur et al., 2003). Of those genes was the PRKCD gene, which is one of the Protein kinase C (PKC) family that is believed to has a crucial and diverse role in multiple signaling pathways utilized by adaptive and innate immune cells (Altman and Kong, 2014), and the cyclin D3 (CCND3) which is one of the D-type Cyclins fundamental for immature T lymphocytes normal expansion (Sicinska et al., 2003). Moreover, the forkhead box P3 (FOXP3) gene, which is also up-regulated in our data set, is one of the potential targets of dietary Se supplementation (Schomburg, 2012; Duntas and Benvenga, 2014).

Selenium is known to elicit its antioxidative action through selenoamino acid incorporation into selenoproteins, which in turn influences the lipid metabolism in both physiologic and stress conditions (Holben and Smith, 1999; Kohrl et al., 2000). Our functional analysis using DAVID revealed that the MF "lipid binding" was the most significantly-affected GO term at T40, with a count of 18 up-regulated genes. Of those genes, the protein kinase C, delta (PRKCD), the apolipoprotein A-V (APOA5) and the fatty acid binding protein 6 (FABP6) were up-regulated by 1.95-, 1.5- and 2.05-fold, respectively. The PRKCD gene is considered one of the oxidative stress responsive-genes (Nitti et al., 2008; Vazquez et al., 2011), while the APOA5 and FABP genes are among those genes that modify the association of Se and lipid levels (Mutakin et al., 2013; Galan-Chilet et al., 2015). Additionally, some of the lipid-trafficking-endosomal-sorting-nexin (SNX) genes (Cullen, 2008) such as SNX20, SNX27 and SNX5 were also up-regulated. It is also worth mentioning that the BP "fatty acid metabolic process" and the MF "phospholipid binding" were among the significantly enriched GO terms in our data

set. These findings partly confirm, on the transcriptomic level, the association between Se and lipid metabolic processes.

Pathway analysis has been reported to be critical for identification of expression pattern changes after nutritional interventions (Hesketh, 2008). DAVID analysis showed that PPAR and adipocytokine signaling pathways were the most represented KEGG pathways in the set of the up-regulated genes at T40. Likewise, The Se-induced PPAR activation as a part of the peroxisomes role in oxidative stress has been discussed in human (Schrader and Fahimi, 2006; Arnaud et al., 2009), and Se has been also shown to be linked to adipocytokine regulation in humans (Kim and Song, 2014). The functional annotation tool DAVID works via testing a set of a priori selected, significantly DE genes, for overrepresentation in annotated gene sets such as GO or KEGG, using standard statistical tests for enrichment (Huang da et al., 2009). However, this approach does not account either for genes with small changes in expression that might be biologically relevant (Mootha et al., 2003) or for the multivariate nature of the expression changes (Glazko and Emmert-Streib, 2009; Emmert-Streib and Glazko, 2011). As genes do not work in isolation (Pagmantidis et al., 2008), GSEA could be considered as an alternative technique that considers differential expression of gene sets and not just priori selected genes, instead it treats a gene set as a unit of expression (Ackermann and Strimmer, 2009; Dinu et al., 2009; Rahmatallah et al., 2015). Because we had small changes in expression (in terms of FC), we assumed that using the microarray intensities of all the probes instead of only a selected range of genes (represented by symbols or accession IDs) would give GSEA an advantage over DAVID in better describing the biological significance of the obtained data, and also to check whether both analyses would have a convergence. The top signaling pathways (B and T cells, adipocytokines, and Toll-like receptor-signaling pathways) identified by GSEA, partly confirms the complex effect of Se-supplementation on the immune system, which mainly reflects an induced adaptive rather than innate immunity trend, and furthermore shows a partial convergence (in terms or results) between both methods.

In the present study, the relatively high number of up-regulated genes involved in “nucleotide-binding” and “transcription” GO terms suggests a Se-induced transcription-modulation effect. For Se to induce its effects it needs to be specifically incorporated into selenoproteins as selenocysteine (SeCys) residues, which requires translational recoding

(Heider et al., 1992; Howard et al., 2013). However, the predominant form of Se in the supplemental source (Sel-Plex) used in the present study is SeMet and not SeCys, which logically raises the question "what was the possible source (if any) of SeCys?". Firstly, Se is known to be retained largly by tissues in protein-bound forms, SeCys being specifically incorporated into selenoproteins and SeMet being nonspecifically incorporated into all proteins (reviewed in Combs, Jr., 2015). Moreover, SeMet enters the methionine pool and a variable proportion goes where methionine rather than Se is required, but partial conversion to SeCys via a lyase and adenosylmethionine is possible (NRC, 2005). Also, some transfer of Se from SeMet to SeCys have been suggested to occur when SeMet enters the Se-transport (selenoprotein P, SEPP) pool to be incorporated into functional proteins such as GPXs (Suttle, 2010). A hypothesis could be that higher dietary levels of SeMet used in the present study resulted in high levels of Se to be recycled and made available for incorporation into SeCys. Because in our DE genes' list we did not recognize a SeCys-specific gene regulation pattern, i.e., no DE of SEPP, GPX1, GPX4 or thioredoxin reductases (TRs) genes, this hypothesis couldn't be fully supported. As selenoproteins and their downstream targets are the functional outcome of dietary Se intake (Hesketh, 2008), the fact that the sheep in the present study were Se-nondeficient could support a less Se-induced effect in terms of regulated selenoproteins.

Conclusions

In conclusion, the present study has shown that it is feasible to use microarrays, combined with biological processes and signaling pathway identification, to identify differential gene expression patterns in sheep supplemented with Se. The findings suggest that, in sheep, high dietary Se supplementation leads to change of expression of several genes involved in the immune-system machineries (both adaptive and innate), and provide further insights on the transcriptional-modulation capacity of Se. In addition, this study provides a custom-designed whole-transcriptome microarray platform which can be used in future studies on the ovine species.

Supplementary materials

https://drive.google.com/drive/folders/0BxmPKazb5UxsdmdlWVBHZ2JvRms?usp=sharing

References

Ackermann, M., and K. Strimmer. 2009. A general modular framework for gene set enrichment analysis. BMC Bioinformatics. 10:47.

Afman, L., and M. Muller. 2006. Nutrigenomics: from molecular nutrition to prevention of disease. J. Am. Diet. Assoc. 106:569–576.

Altman, A., and K.-F. Kong. 2014. Protein kinase C inhibitors for immune disorders. Drug Discov. Today 19:1217–1221.

Arnaud, J., T. N. Akbaraly, I. Hininger-Favier, C. Berr, and A.-M. Roussel. 2009. Fibrates but not statins increase plasma selenium in dyslipidemic aged patients--the EVA study. J. Trace Elem. Med. Biol. 23:21–28.

Arthur, J. R., R. C. McKenzie, and G. J. Beckett. 2003. Selenium in the immune system. J. Nutr. 133:1457S–9S.

Blanchard, R. K., J. B. Moore, C. L. Green, and R. J. Cousins. 2001. Modulation of intestinal gene expression by dietary zinc status: effectiveness of cDNA arrays for expression profiling of a single nutrient deficiency. Proc. Natl. Acad. Sci. U. S. A. 98:13507–13513.

Broome, C. S., F. McArdle, J. A. M. Kyle, F. Andrews, N. M. Lowe, C. A. Hart, J. R. Arthur, and M. J. Jackson. 2004. An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status. Am. J. Clin. Nutr. 80:154–162.

Burk, R. F. 2002. Selenium, an antioxidant nutrient. Nutr. Clin. Care. 5:75–79.

Burk, R. F., B. K. Norsworthy, K. E. Hill, A. K. Motley, and D. W. Byrne. 2006. Effects of chemical form of selenium on plasma biomarkers in a high-dose human supplementation trial. Cancer Epidemiol. Biomarkers Prev. 15:804–810.

Chauhan, S. S., P. Celi, F. T. Fahri, B. J. Leury, and F. R. Dunshea. 2014. Dietary antioxidants at supranutritional doses modulate skeletal muscle heat shock protein and inflammatory gene expression in sheep exposed to heat stress. J. Anim. Sci. 92:4897–4908.

Chauhan, S. S., P. Celi, B. J. Leury, and F. R. Dunshea. 2015. High dietary selenium and vitamin E supplementation ameliorates the impacts of heat load on oxidative status and acid-base balance in sheep. J. Anim. Sci. 93:3342–3354.

Combs, G. F. J., M. I. Jackson, J. C. Watts, L. K. Johnson, H. Zeng, J. Idso, L. Schomburg, A. Hoeg, C. S. Hoefig, E. C. Chiang, D. J. Waters, C. D. Davis, and J. A. Milner. 2012. Differential responses to selenomethionine supplementation by sex and genotype in healthy adults. Br. J. Nutr. 107:1514–1525.

Combs, Jr., G. 2015. Biomarkers of Selenium Status. Nutrients. 7:2209–2236.

Cullen, P. J. 2008. Endosomal sorting and signalling: an emerging role for sorting nexins.

Nat. Rev. Mol. Cell Biol. 9:574–582.

Dennis, G. J., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 4:P3.

Dinu, I., J. D. Potter, T. Mueller, Q. Liu, A. J. Adewale, G. S. Jhangri, G. Einecke, K. S. Famulski, P. Halloran, and Y. Yasui. 2009. Gene-set analysis and reduction. Brief. Bioinform. 10:24–34.

Duntas, L. H., and S. Benvenga. 2014. Selenium: an element for life. Endocrine. 48:756–775.

Efsa. 2011. Scientific Opinion on Safety and efficacy of Sel-Plex ® (organic form of selenium produced by Saccharomyces cerevisiae CNCM I-3060) for all animal species. The EFSA Journal. 9:1–52.

Elgendy, R., M. Giantin, C. Montesissa, and M. Dacasto. 2015. Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone: identification of new candidate biomarkers and their validation using samples from a field monitoring trial. Food Addit. Contam. Part A. 32:1448–1463

Emmert-Streib, F., and G. V Glazko. 2011. Pathway analysis of expression data: deciphering functional building blocks of complex diseases. PLoS Comput. Biol. 7:e1002053.

European Commission. 2013. Commission Implementing Regulation (EU) No 427/2013 of 8 May 2013 concerning the authorisation of selenomethionine produced by Saccharomyces cerevisiae NCYC R646 as a feed additive for all animal species and amending Regulations (EC) No 1750/2006, (EC) No 634/2007 and (EC) No 900/2009 as regards the maximum supplementation with selenised yeast.

European Commission. 2015.Commission Implementing Regulation (EU) No 489/2015 of 23 March 2015 concerning the authorisation of selenomethionine produced by Saccharomyces cerevisiae NCYC R645 as a feed additive for all animal species.

FDA (US Food and Drug Administration). 2012. Code of federal regulations title 21—food and drugs chapter 1—Food and Drug Administration, Department of Health and Human Services Subchapter E—animal drugs, feeds, and related products Part 573—Food additive permitted in feed and drinking water of animals Subpart B—Food Additive Listing Section 573920—Selenium. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/

Galan-Chilet, I., E. Guallar, J. C. Martin-Escudero, G. De Marco, A. Dominguez-Lucas, I. Gonzalez-Manzano, R. Lopez-Izquierdo, J. Redon, F. J. Chaves, and M. Tellez-Plaza. 2015. Do genes modify the association of selenium and lipid levels? Antioxid. Redox Signal. 22:1352–1362.

Glazko, G. V, and F. Emmert-Streib. 2009. Unite and conquer: univariate and multivariate approaches for finding differentially expressed gene sets. Bioinformatics. 25:2348–2354.

Hall, J. a, R. L. Sendek, R. M. Chinn, D. P. Bailey, K. N. Thonstad, Y. Wang, N. E. Forsberg, W. R. Vorachek, B. V Stang, R. J. Van Saun, G. Bobe, and R. J. Van Saun. 2011. Higher whole-blood selenium is associated with improved immune responses in footrot-affected sheep. Vet. Res. 42:99.

Hall, J. a., W. R. Vorachek, W. C. Stewart, M. E. Gorman, W. D. Mosher, G. J. Pirelli, and G. Bobe. 2013. Selenium supplementation restores innate and humoral immune responses in footrot-affected sheep. PLoS One. 8:e82572.

Hall, J. A., G. Bobe, W. R. Vorachek, C. T. Estill, W. D. Mosher, G. J. Pirelli, and M. Gamroth. 2014a. Effect of supranutritional maternal and colostral selenium supplementation on passive absorption of immunoglobulin G in selenium-replete dairy calves. J. Dairy Sci. 97:4379–4391.

Hall, J. A., G. Bobe, W. R. Vorachek, K. Kasper, M. G. Traber, W. D. Mosher, G. J. Pirelli, and M. Gamroth. 2014b. Effect of Supranutritional Organic Selenium Supplementation on Postpartum Blood Micronutrients, Antioxidants, Metabolites, and Inflammation Biomarkers in Selenium-Replete Dairy Cows. Biol. Trace Elem. Res. 161:272–287.

Heider, J., C. Baron, and A. Böck. 1992. Coding from a distance: dissection of the mRNA determinants required for the incorporation of selenocysteine into protein. EMBO J. 11:3759.

Hesketh, J. 2008. Nutrigenomics and selenium: gene expression patterns, physiological targets, and genetics. Annu. Rev. Nutr. 28:157–177.

Holben, D. H., and A. M. Smith. 1999. The diverse role of selenium within selenoproteins: a review. J. Am. Diet. Assoc. 99:836–843.

Howard, M. T., B. A. Carlson, C. B. Anderson, and D. L. Hatfield. 2013. Translational redefinition of UGA codons is regulated by selenium availability. J. Biol. Chem. 288:19401–19413.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44–57.

Hugejiletu, H., G. Bobe, W. R. Vorachek, M. E. Gorman, W. D. Mosher, G. J. Pirelli, and J. a. Hall. 2013. Selenium supplementation alters gene expression profiles associated with innate immunity in whole-blood neutrophils of sheep. Biol. Trace Elem. Res. 154:28–44.

Kim, H.-N., and S.-W. Song. 2014. Concentrations of chromium, selenium, and copper in the hair of viscerally obese adults are associated with insulin resistance. Biol. Trace Elem. Res. 158:152–157.

Kohrl, J., R. Brigelius-Flohe, A. Bock, R. Gartner, O. Meyer, and L. Flohe. 2000. Selenium in biology: facts and medical perspectives. Biol. Chem. 381:849–864.

Koller, L. D., and J. H. Exon. 1986. The two faces of selenium-deficiency and toxicity--are similar in animals and man. Can. J. Vet. Res. 50:297–306.

Kumar, N., a. K. Garg, R. S. Dass, V. K. Chaturvedi, V. Mudgal, and V. P. Varshney. 2009. Selenium supplementation influences growth performance, antioxidant status and immune response in lambs. Anim. Feed Sci. Technol. 153:77–87.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–8.

McCann, J. C., and B. N. Ames. 2011. Adaptive dysfunction of selenoproteins from the perspective of the triage theory: why modest selenium deficiency may increase risk of diseases of aging. FASEB J. 25:1793–1814.

Mckenzie, R. C., J. R. Arthur, S. M. Miller, T. S. Rafferty, and G. J. Beckett. 2002. 12 Selenium and the Immune System. Nutr. immune Funct. 229.

Meyer, a M., J. J. Reed, T. L. Neville, J. F. Thorson, K. R. Maddock-Carlin, J. B. Taylor, L. P. Reynolds, D. a Redmer, J. S. Luther, C. J. Hammer, K. a Vonnahme, and J. S. Caton. 2011. Nutritional plane and selenium supply during gestation affect yield and nutrient composition of colostrum and milk in primiparous ewes. J. Anim. Sci. 89:1627–39.

Mootha, V. K., C. M. Lindgren, K.-F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstrale, E. Laurila, N. Houstis, M. J. Daly, N. Patterson, J. P. Mesirov, T. R. Golub, P. Tamayo, B. Spiegelman, E. S. Lander, J. N. Hirschhorn, D. Altshuler, and L. C. Groop. 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34:267–273.

Mutakin, A. Meiliana, A. Wijaya, K. Kobayashi, C. Yamazaki, S. Kameo, M. Nakazawa, and H. Koyama. 2013. Association between selenium nutritional status and metabolic risk factors in men with visceral obesity. J. Trace Elem. Med. Biol. 27:112–116.

Nitti, M., M. A. Pronzato, U. M. Marinari, and C. Domenicotti. 2008. PKC signaling in oxidative hepatic damage. Mol. Aspects Med. 29:36–42.

NRC. 2005. Mineral Tolerances of Animals. 2nd rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 2007. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. Natl. Acad. Press, Washington, DC.

Oldfield, J.E. 2002. Selenium world atlas, 2nd Edition. Selenium-tellurium development Association (STDA), Grimbergen, Belgium.

Pagmantidis, V., C. Meplan, E. M. van Schothorst, J. Keijer, and J. E. Hesketh. 2008. Supplementation of healthy volunteers with nutritionally relevant amounts of selenium increases the expression of lymphocyte protein biosynthesis genes. Am. J. Clin. Nutr. 87:181–189.

Rahmatallah, Y., F. Emmert-Streib, and G. Glazko. 2015. Gene set analysis approaches for RNA-seq data: performance evaluation and application guideline. Brief.

Bioinform. 4:bbv069

Rooke, J. A., J. J. Robinson, and J. R. Arthur. 2004. Effects of vitamin E and selenium on the performance and immune status of ewes and lambs. J. Agric. Sci. 142:253–262.

Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34:374–378.

Schomburg, L. 2012. Selenium, selenoproteins and the thyroid gland: interactions in health and disease. Nat. Rev. Endocrinol. 8:160–171.

Schrader, M., and H. D. Fahimi. 2006. Peroxisomes and oxidative stress. Biochim. Biophys. Acta. 1763:1755–1766.

Sicinska, E., I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A. A. Ferrando, S. D. Levin, Y. Geng, H. von Boehmer, and P. Sicinski. 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell. 4:451–461.

Smith, M. C., and D. M. Sherman. 2011. Goat medicine. John Wiley & Sons, Hoboken, New Jersey.

Stewart, W. C., G. Bobe, G. J. Pirelli, W. D. Mosher, and J. a Hall. 2012. Organic and inorganic selenium: III. ewe and progeny performance. J. Anim. Sci. 90:4536–4543.

Stockdale, C. R., P. M. Shields, A. McKenna, G. P. Walker, F. R. Dunshea, and P. T. Doyle. 2011. Selenium levels in cows fed pasture and concentrates or a total mixed ration and supplemented with selenized yeast to produce milk with supra-nutritional selenium concentrations. J. Dairy Sci. 94:262–272.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. 102:15545–15550.

Suttle, N. F. 2010. Mineral Nutrition of Livestock, 4th Ed. CABI International, Wallingford, UK.

Tarca, A. L., R. Romero, and S. Draghici. 2006. Analysis of microarray experiments of gene expression profiling. Am. J. Obstet. Gynecol. 195:373–388.

Tinggi, U. 2003. Essentiality and toxicity of selenium and its status in Australia: a review. Toxicol. Lett.. 137:103–110.

Tinggi, U., T. Gianduzzo, R. Francis, D. Nicol, M. Shahin, and P. Scheelings. 2004. Determination of selenium in red blood cells by inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion. J. Radioanal. Nucl. Chem.

259:469–472.

Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. 98:5116–5121.

Ullery, D. E., M. R. Light, P. S. Brady, P. A. Whetter, J. E. Tilton, H. A. Henneman, and W. T. Magee. 1978. Selenium supplementations in salt for sheep. J. Anim. Sci. 46:561–562.

Vazquez, M. C., T. del Pozo, F. A. Robledo, G. Carrasco, L. Pavez, F. Olivares, M. Gonzalez, and S. Zanlungo. 2011. Alteration of gene expression profile in Niemann-Pick type C mice correlates with tissue damage and oxidative stress. PLoS One. 6:e28777.

Vorachek, W. R., Hugejiletu, G. Bobe, and J. a. Hall. 2013. Reference gene selection for quantitative PCR studies in sheep neutrophils. Int. J. Mol. Sci. 14:11484–11495.

Xia, Y., K. E. Hill, D. W. Byrne, J. Xu, and R. F. Burk. 2005. Effectiveness of selenium supplements in a low-selenium area of China. Am. J. Clin. Nutr. 81:829–834.

4. GENERAL CONCLUSIONS AND PERSPECTIVES

In 2015, agriculture in the EU (all 28 member states; EU-28) generated around EUR 164 billion added value to the whole economy - of which around 41 % came from the food-producing animal sector (Eurostat, 2016). The EU regulates this industry with the aim to protect the animal species kept for the production of food (i.e. meat and milk), wool, or for other farming purposes, as well as to protect the consumers from any food-related harm. The enforcement of such regulations indeed requires a strong – and updated - scientific background on the effect of xenobiotics on both the food-producing animals and humans (i.e. consumers). The 'omics' technologies are becoming more accessible and affordable to a greater number of livestock scientists. Currently, these tools are considered fundamental when it comes to study the effect of different xenobiotics and nutritional additives on the overall performance of livestock.

Based on the different studies presented in this thesis, the microarray-based transcriptomics approach was able to provide a holistic view on the global gene expression in diverse types of tissues – namely, skeletal muscle, liver, whole blood, primary hepatocytes- and kidney-derived cell lines. The pre-designed commercial bovine microarray enabled the discovery of many biomarkers with which the differentiation between illicitly-treated and untreated veal calves was possible. It also demonstrated the transcriptomic signature dissimilarity between two tissues (i.e. skeletal muscle and liver) exposed to the same treatment (i.e. anabolic steroids). Also, the same approach revealed the presence of some transcriptomic landscape convergence between the hepatocytes primary cultures and the MDBK cell line, which in turn spots the light on the MDBK cells as a possible surrogate *in vitro* tool for some liver-based functional studies. Finally, a custom-designed whole-transcriptome sheep (*Ovis aries*) microarray revealed the immune-system-induction and the transcriptional-modulation capacity of organic selenium in sheep. Collectively, the transcriptomics approach overcame the shortcoming of focusing on changes in expression of a *priori* list of selected genes – instead, it looks at the bigger picture within the protein-coding part of the genome. It is important to mention that using an alternative functional analysis tools [i.e. Gene set enrichment analysis (GSEA)] was useful to cross-validate the output of the conventional overrepresentation tools like DAVID.

Although the transcriptomics-based approaches provide an extensive view on the differentially regulated genes in a given tissue/living organism, it is evident it can't fill all the gaps in understanding a complex biological system. The present work showed a no overlap between the transcriptomics and proteomics data obtained from the same cohort of samples. Although puzzling, this showed the necessity to change the strategy from comparing two different omics data sets (i.e. transcriptomics vs. proteomics) to try integrating them in a way that one can be used to fill the gaps in the other. From a systems biology approach this could be more interesting. Loor et al. (2015) discussed the fact that researchers should focus on those cases where the expected correlations between the transcriptome and the proteome are absent as this could reveal the possibly hidden regulatory information lacking from the original knowledge base of the biological system. Altogether, the transcriptomics approach is a very powerful data generation tool, but the data integration at various levels (genomics, transcriptomics, proteomics, metabolomics) is necessary to arrive at a holistic view of how animals or biological systems function.

SCIENTIFIC COMMUNICATION

Original Publications

Ramy Elgendy, Mery Giantin, Federica Castellani, Lisa Grotta, Fiorentina Palazzo, Mauro Dacasto, Giuseppe Martino

Transcriptomic signature of high dietary organic selenium supplementation in sheep: A nutrigenomic insight using a custom microarray platform and gene set enrichment analysis.

Journal of Animal Science 04/2016; 94(8). DOI:10.2527/jas.2016-0363

Ramy Elgendy, Mery Giantin, Clara Montesissa, Mauro Dacasto

The transcriptome of muscle and liver is responding differently to a combined trenbolone acetate and estradiol implant in cattle.

Steroids 11/2015; DOI:10.1016/j.steroids.2015.11.002

Ramy Elgendy, Mery Giantin, Clara Montesissa, Mauro Dacasto

Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone: identification of new candidate biomarkers and their validation using samples from a field monitoring trial.

Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 07/2015; 32(9). DOI:10.1080/19440049.2015.1070307

Ramy Elgendy, Mery Giantin, Mauro Dacasto

Transcriptomic Characterization Of Bovine Primary Cultured Hepatocytes and Madin-Darby Bovine Kidney (MDBK) Cell Line; Which One Resembles More The Bovine Liver?

Submitted manuscript

Oral presentations:

Ramy Elgendy, Vanessa Zancanella, Mery Giantin, Mauro Dacasto

Transcriptomic Characterization Of Madin-Darby Bovine Kidney (MDBK) Cell Line And Its Comparison With Cattle Primary Hepatocytes And Liver Tissue.

The 13th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT 2015), Nantes, France; 07/2015

Ramy Elgendy, Mery Giantin, Clara Montesissa, Mauro Dacasto

Transcriptomic analysis of skeletal muscle from beef cattle exposed to illicit schedules containing dexamethasone: identification of new candidates and their comparison with proteomics data and samples from field monitoring.

The 13th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT 2015), Nantes, France; 07/2015

Posters:

Fiorentina Palazzo, Ramy Elgendy, Federica Castellani, Lisa Grotta, Sonia Marchetti, Mery Giantin, Mauro Dacasto, Giuseppe Martino.

Transcriptomic signature of selenium versus zinc and iodine supplementation in sheep.

The 67th Annual Meeting of the European Association for Animal Production, At Belfast, UK; 09/2016. DOI: 10.13140/RG.2.2.30455.42409

BIOSKETCH

Ramy Elgendy was born in Egypt on the 10th of August, 1986. He currently (at the time of writing this thesis; January 2017) is a PhD candidate in Veterinary Sciences (Pharmacology and Toxicology) at the department of Comparative Biomedicine and Food Science at the University of Padua in Italy. Prior to Padua, Ramy obtained his DVM degree in 2008, as well as his M.Sc. in Veterinary Pharmacology in 2012, from the Suez Canal University School of Veterinary Medicine in Egypt -where he also worked as a teaching and research assistant for 4 years. Early 2013, Ramy travelled to the USA for a 9-month fellowship training at the Tuskegee University Cancer Research Center where he worked on a project with a main focus on the chemoprevention of colon cancer in humans. In November 2013, Ramy moved to Italy to pursue his PhD at the university of Padua. He currently studies the 'Genomic/Transcriptomic Signature' induced by the administration of some xenobiotics in farm animals. He uses the high-throughput technologies such as microarrays and next generation sequencing (NGS) to profile the transcriptome of different tissue matrices, and to understand how tissues respond differently to pharmacological compounds. Through transcriptomic profiling, Ramy tries to discover some 'biomarkers' that can help in the indirect detection of illicit drugs administered to farm animals for body-building or growth-enhancement objectives. He also works on nutrigenomics where he examines the effect of some nutrients on the transcriptomic landscape of some ruminants. Ramy also collaborates in 2 comparative oncology projects, using animal models, in which he is responsible about studying the transcriptomic makeup of the cancer tissue before and after a specific treatment. Beginning 2017 – and for at least 3 years – Ramy will start his residency program at the European College of Veterinary Pharmacology and Toxicology (ECVPT). Ramy's main research interests are Pharmacogenomics, Food Safety, Comparative Oncology, and transcriptomic profiling by microarray and RNAseq-based technologies. When he is not glued to a lab bench or a computer screen, he spends his time riding his road bike, learning Italian and trying hard to cook his own food.